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Independent Selection by I-A\textsuperscript{k} Molecules of Two Epitopes Found in Tandem in an Extended Polypeptide Antigen\textsuperscript{1}

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The protein hen egg white lysozyme (HEL) contains two segments, in tandem, from which two families of peptides are selected by the class II molecule I-A\textsuperscript{k}, during processing. These encompass peptides primarily from residues 31–47 and 48–63. Mutant HEL proteins were created with changes in residues 52 and 55, resulting in a lack of binding and selection of the 48–63 peptides to I-A\textsuperscript{k} molecules. Such mutant HEL proteins donated the same amount of 31–47 peptide as did the unmodified protein. Other mutant HEL molecules containing proline residues at residue 46, 47, or 48 resulted in extensions of the selected 31–47 or 48–62 families to their overlapping regions (in the carboxyl or amino termini, respectively). However, the amount of each family of peptide selected was not changed. We conclude that the presence or absence of the major peptide from HEL does not influence the selection of other epitopes, and that these two families are selected independently of each other. The Journal of Immunology, 2000, 165: 3206–3213.

We are examining the biochemical basis of peptide selection by class II MHC molecules that result from the intracellular processing of a protein. Our studies are focused on the model Ag hen egg-white lysozyme (HEL).\textsuperscript{5} Processing of HEL, when given exogenously, or when expressed as a membrane protein, gives rise to a dominant family of peptides associated with the class II I-A\textsuperscript{k} proteins and centered on the core residues 52–61. Most of the peptides start at residue 48, ending at 61, 62, or 63 (1–4). A family is represented by a core segment of nine residues that fits in the combining site, with some of its amino acid side chains interacting with the PI, P4, P6, P7, and P9 pocket sites, and flanking residues of variable lengths at the amino and carboxyl ends (4). The 48–63 HEL family of peptides binds to the I-A\textsuperscript{k} with high affinity and can occupy up to 20% of all surface class II molecules on APC (5, 6). Among all HEL peptides that we have studied, the 48–63 family has the highest affinity for I-A\textsuperscript{k}. Recently, we developed a peptide immunoaffinity capture technique to isolate and quantify minor HEL determinants (7). We were successful in identifying another family of peptides, this time encompassing primarily residues 31–47 (AAKFESNFQTAT NRNT). This epitope has a 20-fold weaker binding affinity to the class II I-A\textsuperscript{k} than the 48–63 peptides and occupies only 0.3% of class II I-A\textsuperscript{k} molecules. One explanation for this lower occupancy is that the stronger binding 48–63 peptides compete with the adjacent 31–47 family for class II presentation. Competition among peptides in a protein or between peptides from different proteins has been thought to play a role in peptide selection. Thus, in this study we isolated and quantitated the selection of the HEL determinant 31–47 in B lymphoma lines expressing mutated forms of membrane HEL. Intramolecular epitope competition did not play a major role in the selection process. The generation of dominant and minor determinants took place independently.

Materials and Methods

Antibodies

The mAb 40F (8), which recognizes the class II I-A\textsuperscript{k} molecule, was used in flow cytometry, cell binding studies, and immunoaffinity chromatography. The mAb anti-HEL Ab, F10.6.6 (9), and a rabbit anti HEL antisera were used in flow cytometry and cell binding experiments. F10.6.6 is a conformation-dependent mAb that recognizes an epitope around the Arg\textsuperscript{68} residue (10). The AW3 mAb, generated in this laboratory (6), was used in cell binding studies for quantitation of the HEL\textsuperscript{48–63} peptide-class II I-A\textsuperscript{k} complex. The mAbs VAL-3 and 151.48-61 were used in the peptide immunoaffinity capture technique. VAL-3 recognizes the HEL fragment 31–47, specifically its FESNF sequence (7). The 151.48-61 Ab is specific for the HEL\textsuperscript{48–63} peptide. This Ab was generated by repeated s.i. injections of female CBA mice with 200 μg of the 48–61 peptide coupled to BSA by bromoacetyl succinimide (Sigma, St. Louis, MO). mAbs were purified from ascites by using protein A-Sepharose (Sigma). The ascites was generated by the injection of hybridoma cells into pristane (Sigma)-treated SCID mice. Anti-HEL rabbit polyclera was purified by DEAE chromatography.

Cell lines and flow cytometric analysis

The murine B cell lymphoma line M12.C3.F6 expressing class II I-A\textsuperscript{k} (10) was transfected with a gene encoding a membrane form of HEL, as previously described (3). Two lines were examined containing I-A\textsuperscript{k}: M12-A\textsuperscript{k} mHEL-20 (referred to as mHEL-20) and M12-A\textsuperscript{k} mHEL-242 (referred to as mHEL-242; see below). The fusion protein was constructed by joining in-frame the entire HEL gene to a segment of the MHC class I Ld gene. To generate mutated HEL genes, site-directed mutagenesis was performed using the HEL\textsuperscript{48–63} peptide. This Ab was generated by repeated s.i. injections of female CBA mice with 200 μg of the 48–61 peptide coupled to BSA by bromoacetyl succinimide (Sigma, St. Louis, MO). mAbs were purified from ascites by using protein A-Sepharose (Sigma). The ascites was generated by the injection of hybridoma cells into pristane (Sigma)-treated SCID mice. Anti-HEL rabbit polyclera was purified by DEAE chromatography.

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The transfected cell lines were sorted for high class II I-A\(^d\) and membrane HEL expression. Cells were stained with 40F conjugated to FITC and biotinylated F10.6.6 (Molecular Probes, Eugene, OR) and incubated at 4°C for 40 min. The cells were subsequently washed twice and resuspended in medium. At least 20,000 cells were collected using the Becton Dickinson cell sorter (Mountain View, CA). Unstable cell lines were in turn, subcloned into a 96-well flat-bottom plate at a frequency of one cell per well. Single colonies were screened for high surface expression using the FACScalibur (Becton Dickinson). Binding studies on B cell lymphoma lines were performed as previously described (6). Using the conformation-dependent Ab F10.6.6, we found that the mHEL-242 and mHEL(K52; K55) had the same amount of reactive material, calculated to be about 6,000 molecules/cell. The mHEL-20 contained about 4-fold more (21,000 sites/cell). The polyvalent rabbit anti-HEL recognized about 10,000 molecules/cell in mHEL-242 and mHEL(K52; K55) and about twice that amount in mHEL-20.

**T cell assays**

B cell lymphoma cells expressing wild-type or mutated forms of membrane HEL were titrated at 2-fold dilutions starting from 10\(^5\) cells/well. The T cell hybridomas were added at a constant cell number of 10\(^5\)/well and incubated for 20 h. Subsequently, each well was assayed for levels of IL-2 by ELISA. The T cell hybridomas used were 3A9, A6A2, NH18, and NH115, which recognize peptides 48–62, 31–47, 18–33, and 115–129, respectively.

**Peptide binding reactions**

Peptides were synthesized by F-moc chemistry (model 432A; Applied Biosystems, Foster City, CA) using a Synergy 432A peptide synthesizer (Applied Biosystems) and were purified by conventional reverse phase HPLC (Waters 600E; Millipore, Bedford, MA). The purity and sequence specificity of each peptide were analyzed using matrix-assisted laser desorption ionization mass spectrometry, and the peptides were found to be \(~ 95\%\) pure.

Peptide binding reactions were performed with detergent-solubilized I-A\(^d\) molecules, purified from the T2-A\(^d\) cell line (provided by Dr. P. Cresswell, Yale University School of Medicine, New Haven, CT). Purified I-A\(^d\) protein (12.5 pmol) was incubated with 0.25 pmol of the radiolabeled reference peptide (\(^{125}\)I-YE-DYGILQINSR), which binds to I-A\(^d\) with high affinity (5). The reference peptide was iodinated by the chloramine-T method at a specific activity of 0.5 mCi/1.5 nmol of peptide. For each binding reaction, known amounts of cold test peptide were added to the mixture and incubated for 72 h at room temperature. The peptide-A\(^d\) complex was separated from the free peptides by centrifuging the material through a Bio-Spin P6 gel filtration column (Bio-Rad, Hercules, CA). The excluded material was counted with a gamma counter (Wallac, Turku, Finland). A measure of binding strength is the ability of the test peptide to inhibit 50\% binding of the radiolabeled reference peptide.

**FIGURE 1.** APC expressing mutant mHEL(K52; K55) do not present 48–63, but present three other HEL epitopes. C3.F6 cells expressing the wild-type (\(\bullet\); mHEL-242) or mutated form of membrane HEL (\(\bullet\); mHEL(K52; K55)) were cultured with the HEL 31–47 peptide ELISA. The VAL-3 mAb was preincubated with known quantities of synthetic HEL 31–47 peptide or different dilutions of isolated peptide extracts. The concentration of standard peptide inhibited by 50% was \(~ 0.3\) pmol. The arrows indicate the inhibition of the OD reading induced for the different peptide extracts (1/40 of the total sample).

**FIGURE 2.** Quantitation of the HEL\(^{31–47}\) peptide on B lymphoma lines expressing mutated forms of membrane HEL by an ELISA inhibition assay.

Class II I-A\(^d\) molecules were isolated from the different B lymphoma cell lines analyzed: mHEL-242, mHEL(K52;K55), and Pro\(^{47}\) mHEL (~7 \times 10\(^5\) cells/cell line). The class II MHC-bound peptides were released by 0.1% TFA, and the peptide extracts were tested in a competitive HEL\(^{31–47}\) peptide ELISA as described previously (8). A. Calibration plot of the HEL\(^{31–47}\) peptide ELISA. The VAL-3 mAb was preincubated with known quantities of synthetic HEL\(^{31–47}\) peptide or different dilutions of isolated peptide extracts. The concentration of standard peptide inhibited by 50% was \(~ 0.3\) pmol. The arrows indicate the inhibition of the OD reading induced for the different peptide extracts (1/40 of the total sample).

Arrows: A, mHEL-242 (242 in B); B, Pro\(^{47}\) mHEL: C, mHEL(K52; K55) (KK in B). B. Abundance of the HEL\(^{31–47}\) peptide on the three different APCs analyzed. The content of 31–47 peptide in the peptide extract was calculated using the calibration curve shown in A. The amounts of I-A\(^d\) molecule isolated \(~4 \times 10^6\) APCs from 1.7–1.9 mg. In the graph, the amounts are plotted per milligram of I-A\(^d\) molecule. (In this same experiment we also estimated the amounts of the 18–35 family of peptide using anti-peptide Ab just developed in Ref. 8. The amounts were 3.0, 3.7, and 2.5 pmol for mHEL-242, mHEL(K52, K55), and Pro\(^{47}\) lines, respectively.)

The Journal of Immunology
Isolation of I-A<sup>+</sup>-associated HEL peptides

Naturally processed peptides of HEL were isolated by immunofluorescence chromatography as previously described (7) but with minor modifications that improved the isolation strategy. B lymphoma cells (2×10<sup>8</sup>) expressing wild-type or mutated forms of membrane HEL were solubilized with 20 mM each of MEGA 8 and MEGA 9 detergents, in the presence of protease inhibitors (500 mM PMSF, 10 mM iodoacetamide, and 20 μM leupeptin). The class II I-A<sup>k</sup> molecules were recovered from the cell lysate by cyanogen bromide-activated Sepharose. From a 50% (v/v) slurry, 500 ml of distilled water. The HEL peptides were then eluted from each column with 0.1% TFA and captured in presiliconized microfuge tubes. The samples were dried and examined immediately or were stored at −80°C.

Third, we examined a number of autologous peptides. Their isolation involved the purification of class II I-A<sup>+</sup> molecules from 2×10<sup>9</sup> B lymphoma cells as described above. The 40F-Sepharose was loaded into the 14-cm Polyrep disposable chromatography column and washed with 40 ml of wash buffer, 60 ml of PBS, 60 ml of 0.01 M Tris (pH 7.4), and 10 ml of distilled water. The peptide extract was eluted with 0.1% TFA and then passed through a Centrifor-10 concentrator. The final peptide extract was dried and stored until mass spectrometric analysis.

Analysis by mass spectrometry

Analysis of the class II I-A<sup>+</sup> peptides was performed with a Finnigan liquid chromatography quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA). Briefly, the peptides were reconstituted in 40 μl of 2% acetonitrile/0.6% acetic acid solution, and 15 μl of this sample was injected into the capillary reverse phase HPLC (a Zorbax C<sub>18</sub> 0.3-mm × 25-cm column; Micro Tech, Sunnyvale, CA) connected on-line to the electrospray mass spectrometer. The total column effluent, which was maintained at a 4.5 μl/min flow rate, was admitted to the mass spectrometer. Data acquisition was started 10 min from the time of sample injection. The peptides were identified by mass (MS mode) in the scan range of m/z 600-1300 in the profile mode in which every three microscans were averaged to give one scan. In the tandem (MS/MS) mode, sequence information was derived by collision-induced dissociation. Sequence analysis was performed using an automated protein database sequencing program (SEQUEST) on an ICIS workstation (Finnigan), which compares the experimental mass of each peptide against the naturally processed HEL peptides identified previously (7). Each standard peptide was weighed using a microbalance (ATI CAHN) with a mass accuracy of ±2 μg. The peptides were weighed in the range of 200–400 μg and dissolved in 1 ml of a 50% acetonitrile solution. Inoculating known amounts of standard peptide (500, 100, 50, 20, and 10 fmol) into the reverse phase HPLC columns on-line to the electrospray mass spectrometer allowed establishment of a total ion chromatogram. Calibration curves were constructed by calculating the area under the total ion
chromatogram (y-axis) for each amount of peptide injected. For the native peptides for which there was no sequence-specific calibration curves, their recovery was quantified using as standards the synthetic peptides that closely resembled the native ones.

Results

Characterization of the mutant HEL(K52; K55) B lymphoma line

We previously reported that the immunodominant HEL 48–63 epitope binds to I-A^k at a 20-fold higher abundance than the minor HEL 31–47 determinant, and that this difference correlated with the stronger binding affinity for the class II I-A^k (7). To examine the interrelationship between these two determinants, we mutagenized the HEL cDNA to abrogate the selection of the 48–63 epitope (DGSTDYGILQINSRWW). This was performed by substituting the aspartic acid at position 52 and the isoleucine at position 55 with lysine residues. Asp^{52} is the main anchor residue of the 48–63 family, whereas Ile^{55} contributes little to the binding, but substitution of it with a lysine hinders binding. The 48–61 mutant peptide DGSTKYGKLQINSR failed to bind to detergent-solubilized class II I-A^k molecules. For example, while the 48–61 peptide (DGSTDYGILQINSR) inhibited the standard binding reaction with an IC_{50} of 73.9 nM, neither of the two mutant peptides
mHEL-20

59% 27% 14%

A A K F E R N K R Q A K D T G S T

mHEL (K52; K55)

60% 21% 13%

A A K F E R N K R Q A K D T G S T

Pro-46

54% 23% 23%

A A K F E R N K R Q A K D T G S T

Pro-47

46% 24% 30%

A A K F E R N K R Q A K D T G S T

Pro-48

45% 20% 35%

A A K F E R N K R Q A K D T G S T

FIGURE 4. Summary of the distribution of the 31–47 family of peptides from mutant mHEL B lymphoma lines compared with the peptide profile in mHEL-242. The relative yield of each peptide was determined by calculating the area for each reconstructed ion chromatogram and divided by the total area for all peptides in the family. The arrows indicate the relative yields for peptides commencing or ending at a particular residue.

Identification and quantitation of the HEL31–47 family of peptides from the mutant mHEL(K52; K55) line

Class II I-A<sup>k</sup> molecules were purified from B lymphoma cell lysates, and the self-peptides were released by addition of 0.1% TFA. We quantitated the amounts of the 31–45 family of peptides by a new approach that uses an ELISA inhibition assay. Fig. 2 indicates that there were no differences in the amounts of the 31–47 family of peptides between the line expressing the wild-type and that expressing the mutant HEL molecule. A second experiment gave identical results with equal amounts of peptides (not shown). (We are now studying the selection of the 18–33 family of HEL peptides. An initial estimate in the cell lines gave identical contents (see Fig. 2).)

Using the Ab VAL-3, we recovered the family of HEL31–47 peptides and injected the mixture onto the reverse phase HPLC column that was on-line to the mass spectrometer. We could identify up to 19 doubly charged ions from the mHEL-20 and mHEL-242 lines specific for the family of HEL31–47 peptides (Table I and Fig. 3). Seven of these peptides started at residue 31 (Ala), six at residues 32 (Ala), and six at residue 33 (Lys). There was greater variation on the carboxyl terminus where the peptides ended at residues 45, 46, 47, 48, 49, 51, and 52 (Table I shows the major species identified). The peptide profile obtained from the mutant mHEL(K52; K55) line was not significantly different. About 60% of peptides commenced at residue 31 in both mutant and wild-type cells, whereas 70% of peptides ended at 46 or 47 at the carboxyl terminus. Interestingly, in the mutant mHEL(K52; K55) line, the few peptides ending at residue 51 were preferred over those few peptides ending at 52.
The recovery of the 31–47 family of HEL peptides was determined from each cell line using the calibration curves that were determined with the sequence-specific synthetic HEL peptides. There was no significant difference in the amounts displayed between the mHEL-242 and mHEL(K52; K55) lines. The higher peptide recovery (3800 fmol) from the mHEL-20 cells was due to the 4-fold increase in surface HEL expression. From the mHEL-242 and mutant mHEL(K52; K55) cells, the total recoveries of peptide were 960 and 2110 fmol, respectively. We do not consider significant the 2-fold differences found using this approach. Thus, the biochemical results are consistent with those presented in Fig. 1, where we did not find an elevated presentation of 31–47 peptides in the mutant mHEL line (K52; K55) in the absence of the immunodominant 48–62 HEL peptide.

Identification of the 31–47 family of HEL peptides from proline mutant lines

The abundance of proline residues at the penultimate position of many naturally processed peptides has been well documented (11–

![Mass spectra of the HEL peptides recovered from the proline mutant B lymphoma lines.](image)

**FIGURE 5.** Mass spectra of the HEL peptides recovered from the proline mutant B lymphoma lines. Class II I-A^k^ molecules were purified from 2 × 10^9^ cells and the peptides released with 0.1% TFA. To recover the 31–47 family of peptides, the peptide extract was first incubated with the VAL-3 Ab. The samples were reconstituted with 2% acetonitrile/0.6% acetic acid and injected into the reverse phase HPLC on-line to the mass spectrometer.

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*Autologous peptides associated with the class II I-A^k^ molecules*. The I-A^k^ molecules were purified from B lymphoma cells (Fig. 6).
FIGURE 6. Total ion chromatogram of autologous peptides associated with the class II I-Ak. The I-Ak molecules were purified from M12.C3.F6 (A) and mHEL-20 cells (B). Indicated in larger number is the peak containing the HEL48–62 species. The pool of self-peptides was released with 0.1% TFA and separated from the I-Ak molecules by a Centricon-10 concentrator. The peptides were injected into the reverse phase HPLC on-line to the mass spectrometer, and mass spectra was acquired after a 10-min delay.

13). Prolines stop exopeptidases involved in peptide trimming. Recent studies in our laboratory demonstrated that the presence of proline residues at the amino-terminal flanks of the 48–63 peptides resulted in the selection of longer peptides extended on the amino side, whereas the total amount of 48–63 peptides presented was the same (3). We are interested in these proline mutant lines because the major epitopes that were selected iminged on the carboxyl terminus of the selected minor determinant 31–47. The B lymphoma cells were transfected with the HEL cDNA consisting of single amino acid substitutions for a proline residue at positions 46, 47, and 48. Class II I-Ak molecules were purified from cell lysates of each B cell lymphoma line, and the HEL peptides were recovered by the anti-HEL-31–47 peptide Ab VAL-3 and the anti-HEL48–61 peptide Ab 151.48-61.

The peptide species isolated from these proline mutant lines commenced at residue 31, 32, or 33 in similar proportion to that of the peptides isolated from the wild-type mHEL line. Peptides isolated from the Pro46 mHEL line ended predominantly at residues 46 and 47 (63%; Figs. 4 and 5). The peptides ending at residue 47 (41%) were preferred over those that ended at 46. Interestingly, a significant number of peptides extended to residue 52 (17%). In the Pro47 mHEL line, 92% of peptides end at residues 48 (Asp) or 47 (63%; Figs. 4 and 5). The peptides ending at residue 47 were few (Fig. 4). Of the peptides extracted from the Pro48 mHEL line, 73% ended at residue 46, and none at 47. Thus, the effect of proline in extending the peptides bound with Pro47 was not observed in the Pro48 line.

In our previous study (3) we extensively characterized the peptide family bearing the 52–61 core of these proline mutants. The 52–61 family of peptides presented by these proline lines was extended by one residue on the amino terminus in the case of Pro46, 99% of the peptides started at residue 47. With Pro47, 96% started at residue 46. In the case of the Pro48 peptide, 100% of the 31–47 peptides ending in residue 48 or 49 overlapped with the 48–62 family of peptides, most of which started at residue 46. In the case of Pro49, 99% started at residue 46 (3).

Under these circumstances, was the selection of the immuno-dominant determinant favored over that of the minor determinant? We confirmed the results of our previous report, that the Pro47 line expressed the same number of complexes of the 48–63 family as the controls; binding with labeled AW Ab on Pro47 resulted in 9.4 ng of Ab/10⁶ cells, in contrast to the standard m242 cells that exhibited 9.3. As shown in Fig. 2, the amount of the 31–46 family of peptides was the same in the Pro47 line. This result indicates that these two epitopes are selected independently from separate HEL molecules (i.e., do not originate from a single protein molecule).

Selection of autologous peptides from class II I-Ak

Because the dominant 48–62 peptide does not influence the expression of the 31–47 peptide family, we examined whether HEL could influence the expression of autologous epitopes. During the processing of HEL, up to 20% of the class II molecules can be occupied by the 48–63 peptides among a spectrum of some several hundred self-peptides processed and presented at steady state (6). Analysis of class II I-Ak self-peptides in the M12-Ak and mHEL-20 lines revealed an array of self-peptides at different retention times (Fig. 6). None of the self-peptides was presented to the same extent as the HEL48–62 peptide, and this was most obvious at 25 min. On the basis of collision-induced dissociations (MS/MS mode), the complete sequences and sources of prominent self-peptides were determined using the SEQUEST database search. In the mHEL-20 line, the relative recovery of the seven self-peptides analyzed was about the same as that observed in the M12.C3.F6 line (Table II). In the absence of HEL selection, we did not observe an increased presentation of other self-peptides; in particular, the Aβ peptide with a binding strength similar to that of the HEL48–62 peptide (5) was not preferentially selected. The processing of HEL did not impede presentation of the other self-peptides.

Discussion

The peptides in the 31–47 family are selected and presented by I-Ak independently of the chemically dominant family of 48–63. Specifically, the amounts of the family of 31–47 HEL peptides are the same for the mutant mHEL (K52, K55) cells as those for the wild-type mHEL-242. Despite the absence of the 48–63 determinant, the amounts of the 31–47 family of peptides are the same for these two cell lines, expressing similar levels of surface I-Ak molecules and HEL. Second, peptides isolated from the Pro47 mutant line give rise to adjacent determinants, which totally overlapped at their amino and carboxyl termini. The amount of 31–47 peptides recovered was the same in Pro47 as in wild-type lines. Thus, these two determinants are generated from separate HEL molecules.
The scenario that we favor for HEL processing is that the protein, which in its native state is relatively resistant to proteolysis, needs first to be reduced (14, 15). After reduction and/or partial unfolding, the polypeptide is available to bind to I-A<sup>B</sup> through the segments that contain a satisfactory binding core sequence, such as 52–61. This binding protects the peptide segment and the MHC molecule from proteolysis (16–18), but the portion of the polypeptide that extends beyond the binding site will be trimmed by amino and carboxyl peptidases. The results of our previous and present experiments, in which prolines were substituted on the amino-terminal flanks of 48–62 and caused longer peptides to be presented, support this interpretation. In agreement, some of the long natural peptides isolated from class II MHC molecules have been shown to contain prolines (11, 12). Our present findings indicate that this effect of proline also applies to the carboxyl terminus of the selected 31–47 segment. This and our previous biochemical analysis of the trimming of the peptide-containing segments indicate that the trimming takes place up to the edge of the I-A<sup>B</sup> molecule (19). When the distance between the proline residue and I-A<sup>B</sup> increases, the effect of proline disappears, and the segment can again be trimmed to its original length (i.e., note the results with the Pro<sup>48</sup> mutant). Prolyl dipeptidase and endopeptidase may be responsible for such trimming.

Despite the large predominance of 48–63 peptides compared with 31–49, binding of the former did not exclude the selection of the latter. There are two, not mutually exclusive, possibilities to explain these results. First, the lack of influence may be due to the low binding affinity of 31–47 for I-A<sup>A</sup>. The lack of a 48–63 segment in HEL should not influence the ultimate concentration of HEL in vesicles, a parameter that should be critical for selection; for a low affinity binding segment such as 31–47, this amount of HEL may be limiting and too low to allow optimal binding affinity. Aside from this, other self peptides may have higher affinity for I-A<sup>B</sup> and should preferentially bind. Second, the 31–47 family may be selected from a minor set of HEL molecules at a cellular site considered as described above. Regardless, these results do tell us that it is difficult, if not impossible, to compete for presentation of autologous peptides even by a high affinity peptide, an issue that has been argued, without any chemical data, to support peptide therapy for the control of autoimmunity.

Finally, the issues of intramolecular competition of a protein for Ag processing has been raised as an explanation for the differences in T cell responses to peptides (22, 23). Without questioning such results, our findings and those of others (24) suggest that such phenomena need to be substantiated by chemical approaches independent of T cell readouts.

References