Quantitation of Lysozyme Peptides Bound to Class II MHC Molecules Indicates Very Large Differences in Levels of Presentation

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Quantitation of Lysozyme Peptides Bound to Class II MHC Molecules Indicates Very Large Differences in Levels of Presentation

Carlos Velazquez, Richard DiPaolo, and Emil R. Unanue

Knowing the abundance of peptides presented by MHC molecules is a crucial aspect for understanding T cell activation and tolerance. In this report we determined the relative abundance of four distinct peptide families after the processing of the model Ag hen egg-white lysozyme. The development of a sensitive immunochemical approach reported here made it possible to directly quantitate the abundance of these four epitopes presented by APCs, both in vitro and in vivo. We observed a wide range of presentation among these four different epitopes presented on the surface of APCs, with 250-fold differences or more between the most abundant epitope (48–63) and the least abundant epitopes. Importantly, we observe similar ratios of presentation from APCs in vitro as well as from APCs from the spleens and thymi of hen egg-white lysozyme transgenic mice. We discuss the relationship between the amount of peptide presented and their binding to I-Ak molecules, immunogenicity, and tolerogenicity. The Journal of Immunology, 2001, 166: 5488–5494.

A

lthough peptides have been isolated from class I and II MHC molecules, their precise quantitation has been difficult. Estimations of peptide abundance using mass spectrometry are possible by placing known standards as references (1–3). However, the estimation is subject to experimental variables that are difficult to control. Moreover, evaluation of different peptides in one isolation is a laborious procedure. We report a new approach to quantitate peptides associated with the class II MHC molecule I-Ak using the hen egg-white lysozyme (HEL) protein as the Ag. We are examining the HEL peptides selected by I-Ak and isolating and characterizing the major families of peptides (1, 4–7). Four HEL peptide families have been identified, made up of the 18–33, 31–47, 48–62, and 115–29 HEL peptides. About 90% of the HEL-specific T cell response (I-Ak restricted) is directed to these epitopes (7, 8). Peptides are selected as families centered on a core sequence that occupies the binding site of the I-Ak molecule. At this point we have gathered information on the biochemical basis for the selection of the peptide families during processing of HEL, as well as on the T cell responses to them (1, 4–7).

The purpose of this paper was to report on four issues. First, we report details of the method to estimate the peptide content from extracts of I-Ak molecules and on our first results using HEL as an Ag. We generated a panel of mAbs that react with the core sequence of the peptides. These Abs were selected on the basis of their reactivity with free peptide in solution. Using these mAbs, we developed sensitive ELISA methods that allowed for quantitation of the HEL peptides from class II molecules of APC. Second, we indicate that there are marked differences (as much as 250-fold) in the presentation of various peptide families on APC. Third, we report studies in vivo in which the levels of peptide-MHC complexes can also be estimated and related to immunologic responses. Lastly, we comment on peptide selection and immunogenicity based on these results.

Materials and Methods

Cell lines

We used the murine B cell lymphoma lines M12.C3.F6 (M12-Ak) (9) expressing class II I-Ak molecules and the M12.C3.F6 cell line transfected with a membrane form of HEL (M12-Ak mHEL) (10). Both cell lines were cultured in DMEM supplemented with 5% heat-inactivated normal calf serum.

Mice

To produce the mAbs, we used CAF.1/J or B10.BR mice (obtained from The Jackson Laboratory, Bar Harbor, ME) or CBA/J mice (purchased from the National Cancer Institute, Bethesda, MD). The transgenic mHEL mice had been generated previously using a cDNA gene for a chimeric HEL-Ld fusion protein under the I-Eα promoter (7, 11). The mHEL mice express HEL tethered to the membrane of class II-positive cells in both thymus and peripheral lymphoid organs. The ML-5 HEL transgenic mice expressing HEL under the metallothionine promoter were obtained from Christopher Goodnow (John Curtin School of Medical Research, Canberra, Australia) (12, 13). All mice were maintained at Washington University small animal facility.

Peptides

The peptides used in this study were synthesized by F-moc chemistry (model 432A, Applied BioSystems, Foster City, CA) and purified by reverse phase HPLC. To synthesize the biotinylated peptides, biotinamidocaproate N-hydroxysuccinimide ester (Sigma, St. Louis, MO) was used to bind to the amino terminus. The sequences and purity of each synthetic peptide were checked by mass spectrometry.

Generation of anti-HEL peptide mAbs

We generated a panel of mAbs to the core sequences of the different I-Ak-restricted HEL epitopes. The mAbs used in this study were 15B7-1B5 mAb (IgG1, anti-HEL18–33 peptide), VAL-3 (IgG1, anti-HEL31–47 peptide) (1), 48.151.2 (IgG1, anti-HEL48–61 peptide), and 115.171 (IgG1, anti-...
HEL\textsubscript{115–129} Peptide) (Table I). These mAbs recognize the free HEL peptide in solution or bound to an ELISA plate. The anti-HEL peptide mAbs were generated by repeated immunizations of mice with synthetic HEL peptides coupled to carrier protein using bromoacetyl succinimidide (Sigma). Specifically, the synthetic peptide HEL\textsubscript{115–125} (DNYGRYSGLNWVSAAK) and HEL\textsubscript{118–127} (TDVQAWIRGC) were coupled to OVA, the HEL\textsubscript{34–45} peptide was coupled to the carrier protein keyhole limpet hemocyanin (1), and the HEL\textsubscript{48–61} peptide was coupled to BSA. (There was no particular reason for the use of different carrier proteins or mice; the mAbs were developed at different times, involving more than one investigator.) In all peptides except 118–127, a cysteine was added at the carboxyl terminus to facilitate coupling to the carrier. In the 18–33 peptide, a serine was substituted for cysteine at residue 30. Mice were first injected s.c. with 200–400 μg of HEL peptide-carrying protein in CFA (Sigma), followed by three i.p. injections in IFA (200–400 μg of peptide-carrier protein immunization) at 2- to 4-wk intervals. Finally, 3 days before B cell fusion, the mice were boosted i.v. with 100 μg of HEL peptide-carrying protein in sterile saline solution. Spleen cells from immunized mice were fused with the myeloma fusion partner P3 \times 63Ag8 using polyethylene glycol 1500 (Roche, Indianapolis, IN) by standard procedures. The B cell hybridomas were selected by testing supernatants in ELISA to plates coated with the peptides. Positive supernatants were immediately screened by the binding to free peptide in the same ELISA procedure (see below).

The mAb (all IgG1) were purified from ascites of pristane-treated CB.17 SCID mice using protein A (Sigma). These mAbs recognized the core sequences of the HEL epitopes. The most abundant members of the HEL\textsubscript{33–61} peptide family are HEL\textsubscript{48–61} (DGSTDYGILQINSR), HEL\textsubscript{51–62} (DGSTDYGIL QINSRW), and HEL\textsubscript{61–72} (DGSTDYGILQINSRW). The anti-HEL\textsubscript{33–61} peptide mAb 48.151.2 recognizes the HEL\textsubscript{48–62} peptide better than the HEL\textsubscript{61–72} peptide (≈10-fold better) or than the HEL\textsubscript{41–61} peptide (≈300-fold better). The same pattern of peptide recognition was observed with other mAbs, suggesting that these HEL peptides in solution adopt different conformations. Based on this observation, we used a mix of the HEL peptides 48–61, 48–62, and 48–63 as standard peptide in the HEL\textsubscript{41–61} ELISA. The molar ratio of these synthetic peptides in the mix was such so as to obtain the naturally processed forms of these peptides on the B lymphoma cell line (M12–A\texttextsuperscript{4} nHEL) and other APC (4, 5, 10). The ratios established were 48–61/62/63 = 10/45/45. The 15B7-1B5 (anti-HEL\textsubscript{18–33} peptide) and the VAL-3 (anti-HEL\textsubscript{31–47} Peptide) mAbs did not show any preferential recognition for any member of the HEL\textsubscript{18–33} or HEL\textsubscript{31–47} peptide families, respectively. Our chemical studies of the 115–129 epitope are in progress. Thus, we used the synthetic HEL\textsubscript{18–33}, HEL\textsubscript{31–47}, and HEL\textsubscript{115–129} as standard peptides to generate calibration curves in the ELISAs.

Isolation of I-A\textsuperscript{a}-bound HEL peptides

Class II I-A\textsuperscript{a} molecules were isolated from 4 \times 10\textsuperscript{9} to 10\textsuperscript{10} B cells (M12-A\texttextsuperscript{4} nHEL) or control B cells (M12-A\texttextsuperscript{4}; 20–1 of cell culture) and from the spleens and thyms of 132 mHEL or ML-5 transgenic mice (7) by immunoaffinity chromatography as previously described (1), but with some modifications to improve the peptide isolation method. (Although we present results using 6 \times 10\textsuperscript{6} cells, the method for quantitation can easily detect peptides in 10\textsuperscript{5} cells.)

The APCs were lysed with 40 mM MEGA8/MEGA9 detergent (Sigma) for the core sequences of the main I-A\textsuperscript{a}-restricted HEL epitopes, HEL\textsubscript{18–33}, HEL\textsubscript{31–47}, HEL\textsubscript{48–61}, and HEL\textsubscript{115–129} (Table I) (7). These Abs were selected for their ability to recognize peptides both in solution and bound to an ELISA plate. The Abs remove most of the T cell reactive peptides from solution (1, 22) (our unpublished observations). Comparing the amount of inhibition by peptide extract from I-A\textsuperscript{a} to the standard inhibition with synthetic peptides allowed quantitation of the epitopes. Fig. 1 shows the calibration curves for the four peptides in a competitive ELISA. The amount of the standard synthetic peptide required to inhibit 30–50% of the maximum OD reading was 0.3 pmol for both HEL\textsubscript{18–33} and HEL\textsubscript{31–47}, 1.0 pmol for HEL\textsubscript{48–61}, and about 25 pmol for HEL\textsubscript{115–129} assay (Table I). The contents of HEL peptides in the extract isolated from APCs were calculated using these calibration curves.

HEL peptide quantitation

We developed four ELISAs to estimate the HEL peptide contents extracted from I-A\textsuperscript{a} molecules. The Nunc Maxisorp ELISA plate (Nunc, Roskilde, Denmark) was precoated with 100 μl of 1 μg/ml streptavidin (Sigma) in 0.1 M sodium bicarbonate buffer (pH 8.8) overnight at 4°C. The plate wells were washed with PBS-0.05% Tween 20 and incubated with 100 μl of 0.1–0.4 μM biotinylated HEL peptide in 0.1 M sodium bicarbonate buffer, pH 8.8, for 75 min at room temperature. The plate was blocked with PBS-1% BSA for 1 h at room temperature and washed. In an additional 96-well U-bottom plate (Costar, Corning, NY), 100 μl of 0.025 μg/ml anti-HEL peptide mAb in PBS-1% BSA solution was added per well and incubated for 75 min at room temperature with known quantities of synthetic HEL peptide or several dilutions of the peptide extract from the APC (the total volume in the well was 110 μl). After this incubation time, 100 μl from each well was transferred into an ELISA plate well, and the incubation was continued 75 min longer. The ELISA plate was washed, and 100 μl of secondary Ab (goat anti-mouse IgG-peroxidase; Roche) in PBS-1% BSA was added per well. The ELISAs were developed with 1 mM 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in citrate buffer with 0.03% H\textsubscript{2}O\textsubscript{2} (Roche), and the absorbency was measured at 415 nm. The total amount of HEL peptides contained in the samples was determined by referring to the calibration curves made with synthetic peptides. The recovery of peptides was determined using radiolabeled peptides, quantitating their amounts through the different steps of the procedure (Fig. 3).

Binding to I-A\textsuperscript{a} molecules

Binding to I-A\textsuperscript{a} molecules was performed as described by Latek et al. (15, 16), but using a baculovirus-produced preparation of I-A\textsuperscript{a} molecules. Indicated in Table III is the concentration of unlabeled peptide required to inhibit by 50% the binding of a standard peptide (sequence YEDYGIL QINSWR). Binding was performed at optimal pH of 5.5 for each peptide.

Results

Development of a sensitive ELISA to quantify the class II MHC-bound peptides

An important aspect in the analysis of peptides selected and bound to class II MHC molecules during protein processing by APC is their quantitation. The standard procedure consists of fractionating the peptide extract by reverse phase HPLC, analyzing every peptide fraction in a T cell assay, and then sequencing the peptides that stimulate the T cell using mass spectrometry analysis (1–3, 17–21). For the class II MHC-bound peptides, this strategy introduces serious problems, particularly with regard to those peptides displayed in low abundance on APC. There is peptide loss during all the steps of peptide isolation and also during the chemical analysis. Another limitation is that the T cell assay skews against low affinity binding peptides, which are required to be at a high molar concentration and are competed by other peptides present in the same HPLC fraction. These problems can be resolved by the immunochromatographic approach described in this paper, which does not depend on a T cell readout and which avoids losses during the peptide isolation protocols and HPLC analysis.

We developed a sensitive and specific ELISA to quantitate the HEL peptides isolated from I-A\textsuperscript{a} molecules using mAbs specific for the core sequences of the main I-A\textsuperscript{a}-restricted HEL epitopes, HEL\textsubscript{18–33}, HEL\textsubscript{31–47}, HEL\textsubscript{48–61}, and HEL\textsubscript{115–129} (Table I) (7). These Abs were selected for their ability to recognize peptides both in solution and bound to an ELISA plate. The Abs remove most of the T cell reactive peptides from solution (1, 22) (our unpublished observations). Comparing the amount of inhibition by peptide extract from I-A\textsuperscript{a} to the standard inhibition with synthetic peptides allowed quantitation of the epitopes. Fig. 1 shows the calibration curves for the four peptides in a competitive ELISA. The amount of the standard synthetic peptide required to inhibit 30–50% of the maximum OD reading was 0.3 pmol for both HEL\textsubscript{18–33} and HEL\textsubscript{31–47}, 1.0 pmol for HEL\textsubscript{48–61}, and about 25 pmol for HEL\textsubscript{115–129} assay (Table I). The contents of HEL peptides in the extract isolated from APCs were calculated using these calibration curves.

Fig. 2 shows a representative result that illustrates the procedure used to detect HEL\textsubscript{31–47}. We previously reported the isolation and quantitation of 31–47 peptide using electrospray tandem mass
spectrometry and the same Ab as a device to capture the peptide for mass spectrometry analysis (1, 22). Quantitating the peptides with both approaches gave roughly comparable results. For example, we estimated using the current ELISA method in which the amount of peptide eluted from I-Ak was 10.2 pmol/10^9 cells, while by mass spectrometry we first reported 11.9 pmol/10^9 cells (1). (The amounts recovered were corrected for losses, which were estimated to be 69% for the ELISA approach and 35% for the mass spectrometry approach.) However, quantitation by mass spectrometry has varied as much as 100% in subsequent trials (22), while the ELISA method described here is highly reproducible (Fig. 3).

In brief, the anti-peptide mAb serves two functions: precise quantitation of the peptides of the MHC eluate, and isolation of the peptide for the mass spectrometry analysis (1). Thus, when the peptide sequence is known or suspected, the use of anti-peptide mAbs represents a useful addition to the chemical identification.

HEL peptides from B lymphoma cells line expressing HEL

We conducted five experiments quantitating the four families of HEL peptides from a B lymphoma line (M12-Ak. mHEL) that expresses a membrane form of HEL. We found very marked differences in the amounts of HEL peptides selected by the I-Ak class II molecule (Fig. 3). From five different experiments a mean of 2260 pmol of the 48–61 family of peptides was isolated from 5.6 \times 10^9 APCs (Table II). The 31–47 set of peptides was the second most abundant peptide represented in I-Ak, with a mean of 38.8 pmol, amounts ~58-fold less than that for 48–61. Peptide 18–33 was represented 251-fold less than 48–61 at 9 pmol. The actual characterization of this family of peptides is now in progress; the natural epitope is actually 20–35, in which the core sequence encompasses residues 24–32 (C. Velazquez, I. Vidavsky, K. van der Drift, M. Gross, and E. R. Unanue, manuscript in preparation). Finally, peptide 115–129 could not be detected, suggesting that the abundance of this epitope is below the limit of detection of the ELISA, which is 25 pmol.

The binding strength of these peptides for I-Ak is indicated in Table II. The 48–61 peptides bind with the highest affinity (4, 5, 10, 15). The 20–35 peptide and 31–47 bind 7- and 13-fold less, respectively, than 48–61. Our studies with 115–129 are not completed, but this epitope appears to be the weakest binding peptide for I-Ak molecules.

We compared the results of M12-Ak.mHEL with those of M12-Ak cultured with exogenous HEL. More HEL peptides were recovered, but the relative proportions were identical with the line expressing the mHEL (Fig. 3B and Tables II and III). As expected, analysis of peptide extract from control APCs (M12-Ak) did not

<table>
<thead>
<tr>
<th>mAb</th>
<th>Peptide Family</th>
<th>Peptide Sequence</th>
<th>Sensitivity* of ELISA (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15B7-1B5</td>
<td>HEL18–33</td>
<td>DNYRGYSLGNVCAAK</td>
<td>0.3</td>
</tr>
<tr>
<td>VAL-3</td>
<td>HEL31–47</td>
<td>AAKFESNFNTQATNRT</td>
<td>0.3</td>
</tr>
<tr>
<td>48.151.2</td>
<td>HEL48–61</td>
<td>DGSTDYGILQINSR</td>
<td>1.0</td>
</tr>
<tr>
<td>115.171</td>
<td>HEL115–129</td>
<td>CKGTVQAWIRGCRL</td>
<td>25.0</td>
</tr>
</tbody>
</table>

* The limit of peptide detection in this assay was defined as the amount of standard peptide required to inhibit 30–50% of the maximum OD reading in the ELISA. The four mAbs are of the IgG1 isotype.

![FIGURE 1](http://www.jimmunol.org/) The ELISA method for the quantitation of HEL peptide bound to class II MHC molecules. Calibration curves using the synthetic HEL peptides HEL18–33, HEL31–47, HEL48–61, and HEL115–129 were made as described in Materials and Methods. The abundance of HEL peptide on different APCs shown in Fig. 3A and Table II was derived from these calibration curves. The limit of peptide detection (the amount of the standard peptide required to inhibit 30–50% of the maximum OD reading) for the different ELISAs was 0.3 pmol of HEL18–33 and HEL31–47 peptides, 1.0 pmol of HEL48–61 peptide, and 25 pmol of HEL115–129 peptide. Δ, OD reading in the presence of irrelevant peptide.
detect peptide in the different ELISAs (Fig. 2). Thus, the processing of the vesicular bound protein and that of the exogenous protein are identical.

HEL epitope quantitation on APCs from spleens and thymi of HEL transgenic mice

To determine whether the large differential in peptide distribution also applies to physiological APC, particularly in an in vivo situation, we examined the class II-bound peptide from the spleens and thymi of 132 mHEL transgenic mice. These mice express a membrane-bound form of HEL on all APCs (under a class II promoter) (7). The relative abundance among HEL peptides on both tissues was comparable to that observed in the B cell line, i.e., HEL\textsubscript{48–61}.

**FIGURE 2.** Quantitation of a HEL peptide using a competitive ELISA. This figure shows a representative experiment of the quantitation method used to evaluate HEL peptide content in the peptide extracts from APCs. Class II I-A\textsuperscript{k} molecules were purified by immunoaffinity from a lysate of M12-A\textsuperscript{k} cells or M12-A\textsuperscript{k} mHEL (\(\sim 7 \times 10^9\) cells). The peptides associated with I-A\textsuperscript{k} were released by acid treatment and dried. The peptide extracts were reconstituted in 1% BSA in PBS, and HEL\textsubscript{31–47} peptide content was determined by a competitive ELISA. A, A calibration curve for the HEL\textsubscript{31–47} peptide was developed by using different known amounts of synthetic HEL\textsubscript{31–47} peptide (0–2.5 pmol) as described in Materials and Methods. The arrows indicate the inhibition of the OD reading observed in the presence of the peptide extract from M12-A\textsuperscript{k} cells (control APC line) or M12-A\textsuperscript{k} mHEL. B, A linear calibration curve was generated from the linear part of the calibration curve shown in A. The peptide extracts were diluted (2-fold serial dilutions) and tested in the ELISA. The OD readings within the linear range of the calibration curve were selected to calculate the amount of HEL\textsubscript{31–47} peptide in the sample. The arrow indicates the inhibition of the OD reading observed in the presence of 1/530th part of the peptide extract from M12-A\textsuperscript{k} mHEL cells. By using the equation of the linear calibration curve, the peptide content in the sample was calculated. C, The total amount of HEL\textsubscript{31–47} peptide in the peptide extract from M12-A\textsuperscript{k} mHEL cells was 48.9 pmol. As expected, analysis of peptide extract from control APCs (M12-A\textsuperscript{k}) showed no detectable peptide.

**FIGURE 3.** Quantitation of HEL peptides on a B cell lymphoma line (M12-A\textsuperscript{k} mHEL) shows very large differences in the amounts of peptide presented. Peptides bound to I-A\textsuperscript{k} class II molecules were isolated from 3.5 to 7.8 \(\times 10^9\) M12-A\textsuperscript{k} mHEL cells, and the contents of I-A\textsuperscript{k}-restricted HEL peptides in the extract were evaluated using different HEL peptide ELISAs. A, The recovered amount of HEL peptides 18–33, 31–47, and 48–61 from five independent experiments. The HEL\textsubscript{115–129} peptide could not be detected by the specific ELISA, suggesting that the abundance of this epitope is below the limit of detection of this assay. The total recoveries of HEL peptides 18–33, 31–47, and 48–61 were estimated to be 58.4, 68.5, and 60.4%, respectively. B, Molar ratio among the HEL epitopes on different APC (B cell lymphoma line, M12-A\textsuperscript{k} + HEL (○), M12-A\textsuperscript{k} mHEL (■), and APCs from spleen (■) and thymi (●) of mHEL transgenic mice). The relative abundances of the HEL peptides were similar in the different APC analyzed.
peptide > HEL-31–47 peptide > HEL-18–33 peptide > HEL-115–129, which was not detected (Table III and Fig. 3B). The molar ratio between the most abundant peptide (HEL-48–61) and the least abundant (HEL-18–33) detectable peptide on APCs from spleen was 217-fold (Fig. 3B). The amount of HEL peptides isolated from the spleens was about 10-fold higher than the amount of HEL peptides recovered from thymi (Table II).

We also examined the content of HEL peptides from the ML-5 HEL transgenic mice developed in the Goodnow laboratory; these mice released soluble HEL at ~10–20 ng/ml of blood (12, 13). Although the tissue APC from these mice expressed considerably less HEL, 48–61 was the chemically dominant peptide family.

Experiments in progress with isolated dendritic cells or macrophages indicate the same ratios in selected peptides. Thus, our conclusion is that although APC vary in their efficiency in yielding total peptides, the processing of HEL and the selection of peptides result in the same relative quantitative display, with some peptides in higher abundance than others.

**Discussion**

The chemical assay described here is sensitive and reproducible and allowed us for the first time to identify the large differences in peptide represented by class II MHC molecules in APC. Of course, the procedure depends on some prior information on the peptides being presented, as in our study with HEL. Based on the results, we comment on the relationships between peptide display and binding strength of the peptide for I-A<sup>+</sup>, and peptide display and T cell response.

Peptides of the 48–61 family are clearly chemically dominant and, likewise, have a higher binding strength for the I-A<sup>+</sup> molecule. The differences in the binding strength of the 48–61 family compared with those of the other HEL peptides is quite notable. In experiments in progress we have found that reducing the binding strength of the 48–61 segment by changing the Asp<sup>52</sup> residue, the main residue that accounts for binding (23, 24), results in a marked loss in the amount selected and presented (experiments to be published with Robert Latke). Likewise, the 115–129 peptide has the weakest binding affinity for I-A<sup>+</sup> and is the least represented (biochemical experiments on this epitope are in progress with Ravi Veraswamy). Thus, for these two families of peptides a relationship exists between degree of binding and selection. However, when examining and comparing the other two peptides, a strict one-to-one relationship between binding and display is not found. The 20–35 peptide family binds to I-A<sup>+</sup> slightly better than 31–47, yet the 31–47 peptide is expressed in severalfold higher amounts. Other factors need to be taken into account in explaining the presence of a peptide bound to MHC molecules from the vesicles in which processing takes place, to the roles of auxiliary molecules in peptide editing, to their protease sensitivity and half-life in the APC of the peptide-MHC complex (5, 25–29).

The second issue is the lack of relationship between the relative abundance of peptide presentation and the number of T cells responding to each epitope after immunization. We previously reported that the number of T cells responding to each of these four epitopes was essentially the same after immunizing mice with 10 nmol of HEL protein in CFA (7). Although the HEL-18–33 peptides are presented at close to 250-fold lower amounts than the

<table>
<thead>
<tr>
<th>Peptide extractions (pmol)</th>
<th>48–63</th>
<th>31–47</th>
<th>18–33</th>
<th>115–125</th>
</tr>
</thead>
<tbody>
<tr>
<td>M12-A&lt;sup&gt;+&lt;/sup&gt; HEL (per 10&lt;sup&gt;9&lt;/sup&gt;)</td>
<td>403.6</td>
<td>6.9</td>
<td>1.6</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>M12-A&lt;sup&gt;+&lt;/sup&gt; + HEL (per 10&lt;sup&gt;9&lt;/sup&gt;)</td>
<td>2149.3</td>
<td>54.2</td>
<td>11.0</td>
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<tr>
<td>Thymi, HEL mice (n = 132):</td>
<td>413.5</td>
<td>5.3</td>
<td>1.9</td>
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<td>Thymi - HEL mice (n = 132):</td>
<td>3.4</td>
<td>&lt;0.4</td>
<td>&lt;1.4</td>
<td></td>
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<tr>
<td>Binding to I-A&lt;sub&gt;k&lt;/sub&gt; IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>0.065</td>
<td>0.867</td>
<td>0.448</td>
<td>2.083</td>
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</table>

**Table III. Comparison of peptide display, IC<sub>50</sub>, and T cell response**

<table>
<thead>
<tr>
<th>Content of Peptides</th>
<th>M12-A&lt;sup&gt;+&lt;/sup&gt; HEL</th>
<th>M12-A&lt;sup&gt;+&lt;/sup&gt; HEL</th>
<th>Spleen</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>T Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratios</td>
<td>48–61/31–47</td>
<td>58</td>
<td>40</td>
<td>78</td>
<td>0.08 (13x)</td>
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<tr>
<td></td>
<td>48–61/18–33</td>
<td>252</td>
<td>195</td>
<td>218</td>
<td>0.15 (7x)</td>
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<tr>
<td></td>
<td>31–47/18–33</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>1.93</td>
</tr>
</tbody>
</table>

<sup>a</sup> The results of Table II are plotted here as ratios, based on the figures shown in Table II. The IC<sub>50</sub> figures in parenthesis for 48–61/31–47 and 48–61/18–33 indicate the extent by which 48–61 binds better to I-A<sup>+</sup>.
HEL_{48–62} peptides, similar numbers of T cells were generated after immunizing mice with HEL protein. That is, there is not a direct correlation between the levels of presentation among these four epitopes and the numbers of T cells that are primed after immunizing with 10 nmol of HEL in CFA. We are currently investigating factors that may explain how similar numbers of T cells can be primed to epitopes that are presented at such different levels. Clearly the conditions used to activate T cells in the previous studies led to high levels of presentation of all epitopes under optimal priming conditions (CFA). We speculate that under such conditions the response becomes independent of the vast differences in the levels of presentation shown here. The dependence of the T cell response to these different epitopes on the amount of Ag and on the expression of costimulatory molecules is currently under investigation by testing lesser amounts of HEL, including costimulator-deficient mice.

Finally, we had established that tolerance in mHEL or ML-5 mice was profound. When transgenic mice expressing a membrane form of HEL in all their APC were immunized with 10 nmol of HEL in CFA, we could not detect any T cells to HEL, including the minor epitopes now quantitated in this analysis. Transgenic mice with soluble HEL at 10–20 μg/ml of blood (ML-5) responded with 2 or 3 per APC (30).

<table>
<thead>
<tr>
<th>Transgenic Lines</th>
<th>mHEL</th>
<th>ML-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of thymi</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>Total amount of I-A(^{k})</td>
<td>770 μg</td>
<td>720 μg</td>
</tr>
<tr>
<td>12.8 nmol</td>
<td>12 nmol</td>
<td></td>
</tr>
<tr>
<td>7.7 × 10(^{5}) molecules</td>
<td>7.2 × 10(^{5}) molecules</td>
<td></td>
</tr>
<tr>
<td>Peptide 48–61 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total recovered</td>
<td>34.2 pmol</td>
<td>3.4 pmol</td>
</tr>
<tr>
<td>2.06 × 10(^{3}) molecules</td>
<td>2.05 × 10(^{3}) molecules</td>
<td></td>
</tr>
<tr>
<td>Peptide per 10(^{6}) I-A(^{k}) molecules</td>
<td>2600</td>
<td>284</td>
</tr>
<tr>
<td>Peptide 31–47 per 10(^{6}) I-A(^{k})</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>Peptide 20–35 per 10(^{6}) I-A(^{k})</td>
<td>13</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^{a}\) Detailed here are the calculations made on the amounts of peptide recovered from the thymi of the two transgenic lines. The ratio of peptide of 48–61 to 31–47 was estimated on both to be 57 based on the results with the mHEL mice. The ratio of 48–61 to 18–33 was estimated at 200. Results are not corrected for peptide recovery, which is estimated at 50%.

References

the type I diabetes-associated MHC class II molecule of NOD mice. **Immunity** 12:699.


