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*J Immunol* 1998; 161:90-96; 
http://www.jimmunol.org/content/161/1/90
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Most CTL responses to epitopes from influenza virus are restricted by MHC class Ia molecules. However, a synthetic peptide corresponding to residues 173 to 190 of influenza A/JAP/305/57 hemagglutinin (HA) can induce, in vitro, a CTL response to peptide presented by a mouse class Ib molecule encoded by a gene telomeric to H2-Q. Here, we identify the molecule as H2-M3 and show that the last five residues of HA173–190, MLI1W, is the minimal epitope for CTL recognition. Cells that express M3⁴, from C57BL/6 or BALB/c mice, are sensitized by both MLI1W and the longer peptide HA173–190, whereas cells that express M3, from ACA or B10.M mice, are sensitized only by MLI1W; a single amino acid change at residue 31 (V→M) of M3 accounts for this difference. Although M3-restricted CTLs preferably recognize N-formylated epitopes, i.e., those of mitochondrial or prokaryotic origin, our findings show that M3-restricted primary CTL responses can be generated in vitro against nonformylated epitopes. The Journal of Immunology, 1998, 161: 90–96.

MHC (Mhc) class I molecules mediate the elimination of virally infected cells by presenting viral epitopes to CTLs. In mice, the class I molecules involved in these responses typically are expressed from the H2-K, D, or L (class Ia) loci, which represent only a fraction of the >50 class I genes in the mouse Mhc. The remaining genes, encoded in the H2-Q, T, and M regions, are nonclassical class I, or class Ib, genes, and their roles in antiviral responses are uncertain.

In 1991, Milligan et al. reported evidence for class Ib presentation of an epitope from influenza virus (1). By stimulating naive mouse spleen cells in vitro, they demonstrated that a peptide corresponding to residues 173 to 190 of influenza (A/JAP/305/57) hemagglutinin (HA)³ induces a cytotoxic response by CD8⁺ T cells with αβ receptors. These CTLs are restricted by a molecule encoded telomeric to the H2-Q region of the mouse Mhc. The molecule associates with β₂-microglobulin (β₂m) and has limited polymorphism, as lymphoblast target cells of H2 haplotypes b, d, k, s, and a, but not f, are sensitized to lysis by HA173–190. Interestingly, antipeptide CTLs were unable to lyse virus-infected cells.

Over 40 class I genes are mapped to the H2-T and M regions. Only four products from these regions are known to be recognized by T cells, and none appears to be a good candidate for presenting the HA peptide. Least likely is T10/T22, a ligand for 66 T cells that does not require peptide for stable surface expression (2–4). A second candidate, TL (encoded by duplicate genes T3 and T18), is capable of binding peptides and being recognized by αβ T cells when expressed as a transgene (5, 6), but normal expression is limited to the gut epithelium and the thymus (7, 8); moreover, both C57BL/6 and CB17, the strains used for characterizing the HA173–190 response, carry the b allele, but their CTLs are capable of recognizing peptide-sensitized lymphoblasts encoding either a-, b-, or c-type TL molecules (1). The other two candidates, Qa1 (encoded by T23) and M3, are ubiquitously expressed and recognized by αβ CTLs in peptide-specific responses. But Qal seems unlikely for the same reason as TL: anti-HA CTLs lyse both Qa1α-positive and Qa1β-positive lymphoblasts equally well, whereas CTLs to Qal typically distinguish allelic forms (9, 10). M3 is unlikely because it preferentially binds peptides containing N-terminal formylmethionine (fMet), and all M3-restricted CTLs known are directed to fMet peptides (11–15); HA173–190 begins with a nonformylated valine and the only methionine is located 12 residues inward and 5 residues from the C terminus. Thus, with hopes of identifying a new class Ib molecule, we characterized the anti-HA173–190 response.

Materials and Methods

Mice

C57BL6/J (B6), C57BL/10J (B10), BALB/c, and ACA mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.BR-mfl0/M, B10.CAS3/Kfl, B10.CAS3(R1)/Kfl (B10.R1), B10.CAS4(R2)/Kfl (B10.R2-R1), B6.CAS3(R4-N,Fy)/Kfl (B6.R4-R1), B6.CAS3(R10)/Kfl (B6.R10-R1), B10.SH1(R27)/Kfl (B10.R27), and B10.CAS4(R34)/Kfl (B10.R34) were bred and maintained in our mouse colony. (For review of these strains, see Ref. 15.)

°Received for publication September 8, 1997. Accepted for publication February 27, 1998.

¹This work was supported by National Institutes of Health Grant AI37818 and the Medical Scientist Training Program (D.E.B.).

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³Abbreviations used in this paper: HA, hemagglutinin; β₂m, β₂-microglobulin; fMet, formylmethionine; anti-HA173 CTL, cytotoxic T lymphocyte lines induced by stimulation with HA173–190; cas, costimulant; ER, endoplasmic reticulum; TAP, transporters associated with antigen processing; SPF, specific pathogen-free.
HA173–190 peptide-reactive CTL lines were generated in vitro as described by Milligan et al. (1), or with some modifications according to Alsheikhly (19). Briefly, 7 to 10 × 10^6 cells, prepared from fresh spleens of 6- to 12-wk-old C57BL/6J mice, were incubated at 37°C in the presence of 5 μM peptide (from stock solutions in 10 ml RP10, in upright 25-cm² tissue culture flasks (430168; Coming, Corning, NY)). For lines generated according to Alsheikhly, splenocytes were incubated with 5 or 25 μM peptide in 4 ml of RP10 for 4 h, then diluted to 20 ml with RP10. After 7 to 10 days, 1 to 2 × 10^6 cells from primary cultures were restimulated with 6 to 8 × 10^6 γ-irradiated (1500 rads) syngeneic splenocytes, 1 to 5 μM HA173–190, and mouse IL-2 in 12-well tissue culture plates (3 ml RP10 per well). Restimulations were continued weekly thereafter to generate the long-term lines used in cytotoxicity assays. IL-2 for restimulations was partly purified from the supernatant of EL4.H2 cells (American Type Culture Collection, Manassas, VA; no. TIB 181), assayed, and used as described in (20). The alloreactive anti-M3Mo CTLs used as controls for some experiments are described elsewhere (16, 21).

Cytotoxic T lymphocytes

HA173–190 is the minimal epitope for recognition by anti-HA173 CTL lines. 51Cr-labeled Pc11198 cells (H2d) were mixed with the indicated concentration of either HA173–190 (YAKGSYNNTSGEQMLIIW) or HA186–190 (MLII), and tested their abilities to sensitize Pc11198 cells. In Figure 1, MLIIW sensitized target cells as well as HA173–190, losing potency in the 100 nM range, but the tetramer, which lacks the C-terminal tryptophan, was not recognized at all, even at 10 μM. Because HA186–190 is the last five residues of HA173–190 and has similar potency, if not greater, we concluded that MLIIW represents the minimal epitope for the anti-HA173 response and used it for target cell sensitization in subsequent experiments.

The CTL response maps to the H2-M region

Lysis by anti-HA173 CTLs is restricted by a β-m-associated MHC class I molecule encoded in the Mhc distal to the H2-Q region (1). To more precisely define the restriction element, we tested a panel of Con A lymphoblasts derived from congenic and Mhc recombinant mice (Table I). Experiment 1 showed that, in the presence of HA186–190, anti-HA173 CTLs recognized H2b (B6) and H2d2 (B10.BR-mBOM) lymphoblasts but not those carrying H2au (B10.CAS3) genes. This allowed us to use lymphoblasts from cas taneus (cas) recombinants to determine the region encoding the restriction element. Consistent with the previous results, K, D, and Q regions were not involved, as B10.R1 lymphoblasts, which are cas proximal to the T region, were lysed. However, the CTL response was not restricted by an element from the T region, as B10.R2-I lymphoblasts, which are cas in the T region, were lysed and B6.R4-I lymphoblasts, which are k in the T region, were not. Therefore, the response must be directed to an element expressed from the M region, as was most clearly shown in experiment 3, with B10.R2-I lymphoblasts being lysed and B6.R10-I not lysed by anti-HA173 CTLs. In all experiments, lysis required the HA186–190 peptide: a formylated ND1 peptide, which binds to H2-M3Mo, was not recognized. Lymphoblasts sensitized with formylated ND1 were recognized by M3-restricted, ND1-specific clones and verified restriction to the M region. Thus, these findings confirmed our prediction that the anti-HA173 response was not directed to a T region molecule, such as T10/T22, TL, or Qa1.

Expression of M3 is required for lysis

Transcripts of three class I genes from the M region, M2, M3, and M10, have been identified by Northern hybridization or reverse transcription-PCR; of these, M3 is the only gene for which a functional class I molecule has been described (15). Although M3 binds nonformylated epitopes poorly (16), it was an easily tested

FIGURE 1. HA186–190 is the minimal epitope for recognition by anti-HA173 CTL lines. 51Cr-labeled Pc11198 cells (H2d) were mixed with the indicated concentration of either HA173–190 (YAKGSYNNTSGEQMLIIW) or HA186–190 (MLII), and tested their abilities to sensitize Pc11198 cells. In Figure 1, MLIIW sensitized target cells as well as HA173–190, losing potency in the 100 nM range, but the tetramer, which lacks the C-terminal tryptophan, was not recognized at all, even at 10 μM. Because HA186–190 is the last five residues of HA173–190 and has similar potency, if not greater, we concluded that MLIIW represents the minimal epitope for the anti-HA173 response and used it for target cell sensitization in subsequent experiments.

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candidate for presentation of the HA186–190 epitope. In Table I, we observed that anti-HA173 CTLs did not recognize lymphoblasts that express the Cas alleles in the M region. Thus, to determine whether M3 presented HA186–190, we used CM3, a B10.CAS2 fibroblast cell line transfected with an M3 wt cDNA; B10.CAS2 cells express M3 cas, a phenotypic null allele of M3, and are not lysed by M3 wt-restricted CTLs (16). To our surprise, anti-HA173 CTLs lysed CM3 cells sensitized with peptide (Fig. 2). Lysis of the fibroblasts was peptide specific, as CM3 cells alone were not lysed, and required M3 wt expression, as B10.CAS2 cells were not recognized. These findings strongly suggested that the nonformylated epitope binds to M3 for presentation to anti-HA173 CTLs.

HA186–190 recognition is inhibited by some fMet peptides

To verify presentation by M3, we tested the ability of HA186–190 to compete with various formylated peptides representing the amino termini of mitochondrially encoded proteins. If M3 wt presents the HA186–190 epitope, addition of formylated peptides known to bind M3 with high affinity should inhibit the ability of HA186–190 to sensitize target cells (11, 23). Pc11198 cells were mixed with equimolar concentrations (1 μM) of HA186–190 and the individual formylated peptides, and lysis by anti-HA173 CTLs, and lysis by anti-HA173 CTLs was assayed (Fig. 3). CTLs lysed targets sensitized with HA186–190 in the presence of formylated peptides ND5, ATPase6, ATPase8, and COIII, but not ND1, COI, or ND4. These results indicate that formylated ND1, COI, and ND4 competed away HA186–190 binding to M3, whereas formylated ND5, ATPase6, ATPase8, and COIII did not. Similar experiments have shown that ND1, COI, and ND4 have the highest affinity for M3, and the other peptides bind with low to negligible affinity (15, 21). Thus, peptides with high affinity for M3 outcompete HA186–190, whereas lower affinity peptides compete less well. Together, the results in Figures 2 and 3 indicate that the B6 anti-HA173 CTL response is restricted by M3 wt.

Table I. Killing by anti-HA173 CTLs is restricted by the H2-M region

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Straina</th>
<th>H2 Regions</th>
<th>% Lysis of Con A Blasts Treated with Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C57BL/6J</td>
<td>K D Q T M Tpx1b f-ND1c</td>
<td>HA186–190 f-ND1c</td>
</tr>
<tr>
<td>2</td>
<td>B10.R1</td>
<td>K k k k c c c b b</td>
<td>24.9</td>
</tr>
<tr>
<td>3</td>
<td>B10.R2-l</td>
<td>K c c c c c c c b b</td>
<td>16.4</td>
</tr>
</tbody>
</table>

a See Methods and Materials for complete strain names. E:T ratio varied from 3:1 to 20:1 between experiments.
b Tpx1 is a marker for the region just distal to H2-M.
c f-ND1 sequence: fMFFINVLTL.
d H2 k-derived alleles of recombinant strains are from C3H and not B10.BR.
e H2 bc is from C3H.SW.

FIGURE 2. H2-M3 wt expression by target cells is required for peptide-sensitized lysis by anti-HA173 CTL lines. H2-M3 wt-transfected CM3 (circles) and untransfected B10.CAS2 (H2-M3 cas) (squares) fibroblast cell lines were mixed with 4 μM HA186–190 peptide (filled symbols) or DMSO (open symbols) and used as targets in a 51 Cr release assay. M3 wt expression was verified with an M3 wt anti-M3 wt CTL clone (data not shown).

FIGURE 3. Peptide competition assay using B6 anti-HA173 CTL lines. Light bars represent the specific lysis of 51 Cr-labeled Pc11198 targets mixed with 1 μM HA186–190 and 1 μM of the formylated peptide indicated on the left. Black bars represent the lysis of Pc11198 targets sensitized with the formylated peptides alone; these values were near zero, excluding cross-reactivity, and are therefore hidden by the baseline for some peptides, e.g., f-ND1. E:T ratio was 10:1.
Role of fMet in recognition of HA186–190

The presence of a formyl group is not required for T cell recognition but is important for anchoring peptides in M3 for high affinity binding (16, 24). Some M3-restricted CTLs are capable of recognizing nonformylated peptides, when 100- to 10,000-fold more peptide is used for sensitization (14, 16), and this was consistent with the anti-HA173 response as well. As shown in Figure 4, anti-HA173 CTLs recognize the formylated HA186–190 as well as the nonformylated form with the same level of maximal lysis, but, as predicted, the formylated peptide was more potent: approximately 100-fold less formyl-peptide was needed for sensitization. The formyl group on MLIIW did not appear to alter the recognition of peptide bound to M3, but it did increase the affinity of the peptide for M3 (as measured by the concentration needed for half-maximal lysis).

Presentation by M3 f

M3 f (of H2 f mice) differs from M3 wt (of most other laboratory strains of mice) at three residues, 31(Val→Met), 219(Lys→Arg), and 236(Ser→Ala), and is not recognized by some CTLs specific for mitochondrial ND1 or Listerial Fr38 (21, 25), although alloreactive M3 cas anti-M3 f CTLs capably recognize M3 f (21). Similarly, Milligan et al. (1) showed that anti-HA173–190 CTLs do not lyse H2 f lymphoblast targets sensitized with HA173–190 and even used this difference for mapping the gene encoding the restriction element. However, we noticed that our antipeptide CTLs lysed both A.CA and B10.M (H2 f) lymphoblasts sensitized with the short peptide, HA186–190. To resolve this conflict, lymphoblasts from B10, BALB/c, B10.M, and a recombinant strain, B10.R27 (Table II), were sensitized with 10 μM concentrations of either HA186–190, HA173–190, or HA183–190 peptides and then tested for recognition by anti-HA173 CTLs. (a 10 μM peptide concentration corresponds to the concentration (10 μM/ml) used by Milligan et al. (1)) All three peptides sensitized M3 f, as expected, but only the short peptide sensitized M3 f (Fig. 5). The longer peptides, including HA183–190, which has only three residues before the MLIIW epitope, did not sensitize M3 f.

Our choice of mouse strains verified that the inability of peptides with extended N termini to sensitize targets was based on allelic differences in M3: the effect was Mhc linked, as B10.M and B10.R27 differ from B10 only in the Mhc, and was not the result of differences in the class II or III regions (e.g., transporters associated with Ag processing (TAP)1/2, LMP2, LMP7, or Tapasin) as B10.R27 and B10 share these regions.

To determine how well HA186–190 sensitizes M3 f, we titrated the pentamer on A.CA (H2 f) and BALB/c (H2 d) blasts. Figure 6 shows that 10 μM was the minimal concentration capable of sensitizing A.CA, whereas BALB/c could be equally sensitized with 100-fold less peptide. Together, Figures 5 and 6 indicate that anti-HA173 CTLs recognize M3 f, but M3 f presents the HA epitope poorly; HA186–190 sensitizes only at high concentrations and the longer peptides do not sensitize at all.

Effect of residue 31

In M3 f, residue 31 is in the α1 domain and points away from the peptide binding groove, making no direct contact with peptide (24). Residues 219 and 236 are in the α3 domain, even farther

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### Table II. Haplotypes of lymphoblast targets used in Figure 5

<table>
<thead>
<tr>
<th>Strain</th>
<th>K</th>
<th>D</th>
<th>Q</th>
<th>T</th>
<th>M</th>
<th>M3 Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>wt</td>
</tr>
<tr>
<td>BALB/c</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>wt</td>
</tr>
<tr>
<td>B10.M</td>
<td>f</td>
<td>f</td>
<td>f</td>
<td>f</td>
<td>f</td>
<td>f</td>
</tr>
<tr>
<td>B10.R27</td>
<td>b</td>
<td>b</td>
<td>?</td>
<td>sh1</td>
<td>sh1</td>
<td>f</td>
</tr>
</tbody>
</table>

*sh1 haplotype originated from the SUB-SHH mouse stock and encodes an M3 allele identical to f.
transfected with an M3 cDNA encoding Met31 instead of a Val31. Hypothesis, we used a B10.CAS2 cell line, M31, which has been shown to be capable of sensitizing target cells to lysis. These findings support that the key role of the formyl group in M3\textsuperscript{wt}-restricted responses is for binding peptides to the groove.

**Binding by long peptides**

We are uncertain how, and if, the long peptides HA173–190 and HA183–190 bind to M3\textsuperscript{f}. Untransfected M3\textsuperscript{f} (B10.CAS2) fibroblast cells or cells that had been transfected with M3\textsuperscript{f} (CM3) or M3 with a Met\textsuperscript{31} (M3\textsuperscript{31M}) were incubated overnight with 10 \textmu M HA186–190 (CM3), HA173–190 (\textbullet{}), or no peptide (\texttimes{}) and used as targets in a 3.5-h \textsuperscript{3}Cr-release assay with anti-HA173 CTLs. All CM3 and M31 targets were lysed with similar efficiency by alloreactive anti-M3\textsuperscript{wt} CTLs in parallel experiments.

**Recognition of nonformylated epitopes**

M3-restricted CTLs directed to iMet peptides can recognize analogous nonformylated peptides, but the formylated ones are 100- to 10,000-fold more potent than the nonformylated ones (14, 16). Equally, anti-HA173 CTLs, which are induced against a nonformylated peptide, recognize the formylated peptide as well; again, the formylated analogue is 100-fold more potent than the nonformylated peptide used for inducing the response (Fig. 4). In all cases, the formyl group does not appear to alter T cell recognition, as peptide-specific CTLs lyse cells displaying either form of the peptides at the same maximal level, but it does affect the potency of the peptide for sensitizing target cells to lysis. These findings suggest that the key role of the formyl group in M3\textsuperscript{wt}-restricted responses is for binding peptides to the groove.

**Discussion**

Our results show that the in vitro-generated CTL response to a synthetic influenza HA peptide is directed to the class Ib molecule, H2-M3\textsuperscript{wt}. Current dogma maintains that anti-M3 CTLs naturally respond only to peptides containing a fMet at the N-terminus, i.e., those of mitochondrial or prokaryotic origin, but here we demonstrated that M3-restricted CTLs can be induced against a nonformylated epitope that exists at the C-terminal end of a larger peptide.

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of HA173–190 in our experiments to induce the same CTL response as Milligan et al. (1).

Presentation by M3

Like other class Ib molecules, M3 is not very polymorphic. Five alleles have been described that together encode proteins differing at 10 residues total; the spritus allele alone accounts for five of these changes (15). Furthermore, all molecules but M3 are recognized less well by some peptide-specific CTLs, e.g., anti-ND1 (25), anti-ND1 (21), and anti-HA (Fig. 5).

Poor recognition of M3 by anti-HA CTLs could be due to low surface expression or a structural change that affects peptide binding and interaction with TCRs. We believe that M3 and M3G are expressed similarly based on the equal recognition by some allospecific CTL clones, but we lack the M3-specific Abs to measure surface expression more directly. Our results with transfected cell lines indicate that poor recognition of M3 is largely due to a structural change caused by the Met31 (Fig. 7). The structure of M3G shows that Val31 points away from the groove and makes a van der Waals contact with the Tyr209 in the α3 domain (24). The bulkier methionine substitution must alter the groove of M3 such that peptides bind poorly and recognition by peptide-specific CTLs is abrogated (Fig. 6). In hindsight, earlier studies in our laboratory using M31 cells support this: allospecific CTLs recognized M3G and M31 specifically, whereas M31 was recognized less well by peptide-specific CTLs (18).

Competition with mitochondrial fMet peptides

CTL recognition of virus-infected cells requires endogenous synthesis of viral proteins, cytosolic proteolysis to short peptides, and TAP-transport of peptides into the endoplasmic reticulum (ER), where they are loaded onto class I molecules and subsequently conveyed to the cell surface. In their original report, Milligan et al. demonstrated that anti-HA173–190 CTLs failed to lyse flu-infected cells or cells infected with an HA173-190 minigene and suggested that the undefined class Ib molecule may acquire peptides through a unique pathway not used for processing viral Ags (1). However, M3 acquires peptides through the classical pathway: anti-ND1 CTLs are TAP dependent, because neither RMA-S (TAP-2 negative) cells nor TAP-1- lymphoblasts are lysed by M3-restricted T cells (15). These results indicate that ND1 must be transported into the ER to bind M3 for surface expression.

Influenza HA is a transmembrane protein that is cotranslationally translocated into the ER. Although antigen processing of transmembrane proteins is poorly understood, CTL responses to the HA of influenza A/JAP/305/57 have been well characterized. One strongly immunodominant region, HA202-221, which is close to HA186–190, contains two overlapping H2-Kd-restricted epitopes, HA202-212 and HA211-221 (26). Presentation of these epitopes is proteasome dependent and requires translocation into the ER (27, 28). Thus, HA epitopes are loaded onto class I molecules in the ER, where M3 acquires peptides as well. So why did Milligan et al.’s anti-HA173–190 CTLs not lyse cells expressing the epitope? It is possible that HA186–190 is proteolytically cleaved and not available for binding M3, but our most compelling explanation is provided in Figure 3. Formylated ND1, COI, and ND4 have very high affinities for M3: at equimolar concentration, they prevent HA186–190 from binding and, because they are formylated, are at least 100-fold more potent than the nonformylated HA186–190. Because most naturally presented peptides have a high affinity and slow off-rate for class I molecules (29, 30), the nonformylated epitope may be unable to compete against the high affinity mitochondrial peptides for binding to M3, and it is therefore not presented on the cell surface.

Historically, the generation of M3-restricted CTLs has required immunization of mice with either spleen cells or bacteria to prime the response, but here we show that stimulation in vitro with nonformylated peptide epitopes can induce M3-restricted responses as well. The response is not unique to HA173–190, as a nonformylated Bla-z (MFVLNKFF) peptide (the formylated form is known to bind well to M3 (23)) can also induce specific, M3-restricted CTLs. Lenz and Bevan have shown recently that the two formylated epitopes from Listeria monocytogenes, f-LemA and f-MIVIL, similarly can induce primary responses in vitro (31). However, in contrast to their finding that antipeptide CTLs are generated only from mice raised in conventional, not specific pathogen-free (SPF) conditions (or from SPF mice immunized with Listeria), our preliminary data indicate, at least for C57BL/6J, that spleen cells from SPF mice respond to HA173–190 just like cells from conventional mice do (data not shown). It is not clear whether there is a real discrepancy between these results, as we are comparing responses of different strains of mice (C57BL/6J vs BALB/c) against separate epitopes (HA186–190 vs f-LemA and f-MIVIL).

We still doubt whether M3 can naturally present nonformylated peptides: at least 10 nM peptide is required for half-maximal response to HA186–190 (Fig. 4), whereas naturally presented peptides are potent in the picomolar range. But the ease with which all these responses can be generated ex vivo suggests that a portion of the T cell repertoire in mice may be devoted to recognizing similar peptides presented by M3.

Acknowledgments

Special thanks to Vik Dabhi for providing mitochondrial peptides, M3-restricted CTL clones, and words of wisdom about keeping CTLs happy. We thank Annie Eubanks, Margaretan Stolfo, and Henry Taylor for their excellent upkeep of and assistance with our mouse facility and Steve Madden, Lynn Mayfield, and Clive Slaughter of the Howard Hughes Biopolymers Facility at the University of Texas Southwestern Medical Center for peptide syntheses and helpful discussion.

References


