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In vitro stimulation of mouse splenocytes with hemagglutinin (HA) 173–190, a peptide derived from influenza virus hemagglutinin (A/JAP/305/57, H2N2), induces CTLs that are directed to the MHC class I b molecule, H2-M3. M3 preferably binds peptides bearing an N-terminal formylmethionine. In this study, we show that several related nonformylated peptides can induce anti-HA CTLs in vitro: MLIIW (the minimal epitope), derived from HA186–190 at the C-terminal end of HA173–190; MLIIWG; MLIIWGV; and MLIIWGI, as well as formylated MLIIW. The heptamer peptides correspond to a polymorphism of HA192 in H2 strains of influenza; they have the highest relative affinities for M3 of the nonformylated peptides and higher affinities than some formylated mitochondrial peptides. Depending on the affinity of the peptide, a range of concentrations can be used to induce CTLs. One nanomolar of the high affinity f-MLIIW peptide can induce anti-HA CTLs, whereas 100-fold more of the lower affinity MLIIW peptide is needed. Lines induced with high concentrations (1 μM or greater) of f-MLIIW recognize Ag poorly, and the most efficient CTLs are induced with the lowest concentrations of peptide. Analysis with a panel of anti-TCRVβ Abs shows that different T cells respond to high vs low peptide; the repertoire of cells responding to higher concentrations is more diverse, consistent with the expansion of more, but less efficient, clones. Thus, peptide affinity and concentration should be considered together for generating efficient antipeptide CTLs in vitro. The Journal of Immunology, 1999, 163: 3022–3028.
When the anti-HA response was first identified, Milligan et al. (24) reported that anti-HA CTLs were unable to lyse cells infected with influenza. We have suggested that this was due to the inability of the nonformylated HA epitope to compete with the endogenous formylated mitochondrial peptides for binding to M3 (13). However, it is also possible that their CTLs were inefficient at recognizing Ag, having been stimulated with high concentrations of peptide. Now that the minimal epitope for recognition by anti-HA CTLs has been identified, it is important to determine whether peptides shorter than HA173–190 can induce effective CTLs. In this study, we show that CTLs can be induced with shorter HA peptides, and we use the unique qualities of M3 presentation to show the linked effects of peptide affinity and concentration on the generation of anti-HA CTLs in vitro.

Materials and Methods

**Peptides**

HA173–190 (VAKGSYNNTSGEMLIIW), HA186–190 (MLIIW), HA186–191 (MLIIWGH), HA186–192V (MLIIWGV), HA186–192I (MLIIWGI), and the formylated mitochondrial peptides f-ND1-6V (f-MFFINVTL) and f-COI-3I (f-MFINRWLFS) were synthesized on a Rainin Symphony peptide synthesizer, using standard F-moc chemistry, as previously described (13, 19). Lyophilized peptides were dissolved in DMSO for 1–2 mM stock solutions.

**CTL lines**

Anti-HA CTLs were generated in vitro, as described previously (13, 24). Briefly, cell suspensions were prepared from the spleens of immunized female C57BL/6J (B6) mice (6–12 wk old) obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in our conventional (not specific pathogen-free) facility. For lines generated against 5′-irradiated (3000 rad) and preincubated with medium and were used as target cells in standard 51 Cr release assays, as described previously (13). CTLs were more specific for the peptide used for induction, e.g., the peptide used for induction was recognized just above background, and MLIIWG and MLIIWGV were the most potent. Recognition of the longer peptide, as judged by their ability to sensitize target cells for lysis. Because M3 can bind peptides longer than five amino acids, we sought to determine whether hexamer and heptamer peptides beginning at HA186 could bind M3 any better. A database search identified two peptides, MLIIWGV and MLIIWGI, derived from residues 186–192 of influenza A type 2 hemagglutinin that contain the minimal MLIIW epitope. These peptides differ at position 192, with a valine for A/JAP HA and an isoleucine for some avian strains of influenza.

Nonformylated MLIIW, MLIIWGV, and MLIIWGI peptides were synthesized and used to generate CTL lines from the spleen cells of immunized B6 mice. All peptides induced CTLs that lysed Pc11198 cells loaded with peptide, but not untreated target cells (Fig. 1). (Pc11198 cells are ideal for measuring M3-restricted CTL responses, because they are H2* and eliminate the chance for H2-restricted or Qa-restricted killing by the B6 CTLs (13).

To determine the potency of the longer peptides, we tested the ability of CTLs to recognize cells incubated with 1 μM or 1 nM concentrations of each peptide. As shown in Fig. 1, 1 μM sensitized cells to lysis by all CTLs; at 1 nM, MLIIWGV was the most potent for sensitizing target cells. Of the other peptides, MLIIW was recognized just above background, and MLIIWGV and MLIIWGI were the most potent. Recognition of the longer peptides was the same in serum-free conditions and was not due to proteolytic trimming by dipetidases in the medium (31). Some CTLs were more specific for the peptide used for induction, e.g., anti-MLIIW, and similar results have been obtained with other lines stimulated with MLIIWGV. In general, peptide-specific recognition is observed after prolonged restimulation; the anti-MLIIWGV preference in Fig. 1 was seen after 12 wk of restimulation, whereas the specificity of the line was not apparent at 4 wk (data not shown).

To better determine the relative affinities of the nonformylated epitopes, we tested the ability of the most potent HA peptides to compete for binding to M3 in an inhibition assay using a CTL line, B6 α anti-γ, specific for the f-ND1-6V (f-MFFINVTL) peptide presented by M3 (26). Target cells were incubated with 1 nM of f-ND1-6V peptide and increasing concentrations of MLIIW, MLIIWGV, or MLIIWGI or another high affinity mitochondrial peptide, f-COI-3I (32), and then tested for recognition by B6 α

**Flow cytometry analysis**

CTLs were harvested 5 to 6 days after restimulation, washed, and resuspended to 4 × 10⁶ cells/ml in FACS staining buffer (PBS containing 1% BSA and 0.1% sodium azide). Fifty microliters of cells (2 × 10⁶) were added per well to a 96-well microtiter plate and doubly stained with PE-labeled anti-CD8 and 1 of 13 different FITC-labeled anti-mouse TCRβ Abs (2, 4, 5, 1/2, 6, 7, 8, 1/2, 8.3, 9, 10, 11, 12, 13, and 14 (all obtained from PharMingen, San Diego, CA)) for 20 min at 4°C. Free Ab was washed away, and cells were resuspended in cold staining buffer and analyzed on a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA). Vβ expression of CD8⁺ lymphocytes was determined with CelQuest software (Becton Dickinson) by counting the percentage of cells that stained higher than background (no anti-TCRβ).

**Results**

Penta-, hexa-, and hepta-mer peptides can induce anti-HA CTLs

The peptide-binding cleft of H2-M3 is shorter than those of other class I molecules; the A pocket is blocked and the N-terminal formylmethionine side chain is buried in the B pocket instead (29). Therefore, peptides that are presented by M3 are typically five to seven amino acids long (18, 30). Previously, we showed that CTLs induced with HA173–190 recognized a minimal epitope, MLIIW, presented by M3 (13). This nonformylated epitope has at least 100-fold less affinity for M3 than the formylated form of the peptide, as judged by their ability to sensitize target cells for lysis. Because M3 can bind peptides longer than five amino acids, we sought to determine whether hexamer and heptamer peptides beginning at HA186 could bind M3 any better. A database search identified two peptides, MLIIWGV and MLIIWGI, derived from residues 186–192 of influenza A type 2 hemagglutinin that contain the minimal MLIIW epitope. These peptides differ at position 192, with a valine for A/JAP HA and an isoleucine for some avian strains of influenza.

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anti-g CTLs. We chose 1 nM f-ND1-6V, because this concentration submaximally sensitizes Pc11198 cells to lysis and, therefore, is optimal for measuring inhibition of binding of other peptides to M3. As shown in Fig. 2, the relative affinities of the peptides correlated with the relative potency observed in Fig. 1. MLIIWGI had the highest relative affinity of the three HA peptides tested. Still, none of the peptides inhibited recognition to the level of f-COI-3I, because they lack the critical N-formyl group. Indeed, similar but separate experiments have shown that formylated MLIIW binds to M3 with an affinity equal to f-ND1-6A and slightly better than f-COI-3I (data not shown). Together, Figs. 1 and 2 indicate that the nonformylated 7-mer peptides bind to M3 better than the minimal epitope, MLIIW.

The effect of concentration on generating anti-HA CTLs

CTLs generated against HA173–190 as well as MLIIW, MLIIWG, or MLIIWGV/I consistently recognize target cells sensitized with either nonformylated or formylated MLIIW. However, CTLs generated against 5 μM of the high affinity f-MLIIW peptide (5 μM is the concentration originally used for generating anti-HA CTLs (24)) lysed target cells incubated with 1 μM f-MLIIW, but not MLIIW (Table I, Expt. 1). Furthermore, these anti-f-MLIIW lines were more difficult to generate than the anti-HA173–190 lines and often died within a few weeks of induction. Recently, a number of reports have shown that the concentration of peptide used for generating CTLs in vitro can profoundly affect the sensitivity of the line generated (4, 12, 15). Our results in Table I show the same is true for the anti-HA response. In experiment 2, six lines from the same pool of spleen cells were stimulated with 1, 0.1, or 0.01 μM of MLIIW and f-MLIIW peptides and tested for recognition of target cells loaded with 1 μM of the respective peptides. Again, although background lysis was high, the line induced with the high concentration of f-MLIIW failed to distinguish cells incubated

Table I. Lysis by CTLs induced with different concentrations of MLIIW and f-MLIIW peptides

<table>
<thead>
<tr>
<th>CTLs</th>
<th>E:T</th>
<th>% Lysis PeC11198 + 1 μM Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Expt. 1 (5 μM) a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA173-190</td>
<td>0:4:1</td>
<td>4</td>
</tr>
<tr>
<td>f-MLIIW</td>
<td>18:1</td>
<td>-1</td>
</tr>
<tr>
<td>Expt. 2 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLIIW</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>7:1</td>
<td>7</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>6:1</td>
<td>3</td>
</tr>
<tr>
<td>0.01 μM</td>
<td>12:1</td>
<td>1</td>
</tr>
<tr>
<td>f-MLIIW</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>6:1</td>
<td>23</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>8:1</td>
<td>4</td>
</tr>
<tr>
<td>0.01 μM</td>
<td>5:1</td>
<td>-1</td>
</tr>
</tbody>
</table>

a Expt. 1 CTLs were induced with 5 μM peptide and derived from different mice and tested 5 wk (HA173-190) and 4 months (f-MLIIW) after induction.

a Expt. 2 CTLs were induced with stimulator cells pulsed with peptide at the concentrations indicated (see Materials and Methods). CTLs were derived from the same pool of spleen cells from two mice and were tested 3 wk after induction.
with the nonformylated epitope from untreated cells, but did recognize those incubated with f-MLIIW. By contrast, lines induced with 0.1 and 0.01 μM f-MLIIW recognized both peptides equally; by decreasing the concentration of f-MLIIW, CTLs can be induced that recognize both f-MLIIW and MLIIW peptides. With the lower affinity MLIIW peptide, 1 and 0.1 μM induced lines that recognized both peptides, yet 0.01 μM failed to induce any CTL corresponding with the lower affinity of the nonformylated peptide.

The results in Table I show lysis of target cells incubated with 1 μM peptide, but f-MLIIW is at least 100-fold more potent than the low affinity MLIIW peptide for binding to M3 (13). Therefore, to determine the sensitivity of the CTLs induced with the high and low affinity peptides, target cells were incubated with various concentrations of f-MLIIW and MLIIW, and lysis was measured. Fig. 3 shows that the sensitivity of the CTLs was inversely correlated with the concentration of peptide used to induce the lines. For example, the 0.01 μM f-MLIIW line lysed target cells incubated with 1 μM f-MLIIW, whereas the 1 μM f-MLIIW line failed to lyse target cells incubated with 10,000-fold more f-MLIIW. The 0.1 μM f-MLIIW line was an intermediate between the two. Similarly, CTLs induced against 0.1 μM MLIIW were more sensitive than those induced with 10-fold more of the same peptide. Titration of the two peptides thus shows that the CTLs induced from the same spleen cell pool were vastly different in their ability to recognize Ag.

None of the CTLs appeared to be specific for the peptide used for induction, as lines induced with f-MLIIW and MLIIW recognized both peptides similarly, except that 100-fold more nonformylated peptide was needed to sensitize lysis to an equivalent level. The inability of the 1 μM line to recognize the low affinity MLIIW peptide is most likely due to its poor ability to recognize Ag, although an f-MLIIW-specific component to recognition may exist. At least 1 μM of the high affinity f-MLIIW peptide was needed to sensitize target cells to lysis (Table I), so 100 μM MLIIW would be needed to sensitize at an equivalent level. However, incubating target cells with 100 μM MLIIW would require them to be exposed to 10% DMSO, which causes too high 51Cr release backgrounds. Whether or not the 1 μM f-MLIIW line is specific for the formylated peptide, its sensitivity to Ag is extremely poor. Together, the results in Table I and Fig. 3 demonstrate the importance of using the lowest concentration of peptide still capable of inducing CTLs to generate efficient lines in vitro.

Vβ usage differs between the lines induced with high vs low peptide

When T cell clones are strongly stimulated via their receptors by high concentrations of peptide on APC, by anti-CD3 treatment, or by superantigen treatment, TCRs are internalized, rendering the T cells less responsive to Ag (16). These results suggest that our anti-HA CTLs induced with high concentrations of peptide could be less responsive to Ag due to decreased numbers of surface TCRs. Alternatively, poor recognition of Ag could be the result of differences in the affinity of TCRs for binding Ag.

To determine the reason for poor recognition by the CTLs induced with high peptide, we analyzed receptor expression by flow cytometry, using a panel of Abs to mouse TCRVβ-chains. Recently, a similar panel was used by Busch et al. to characterize the T cell repertoire in mice that respond to different L. monocytogenes epitopes (4); this panel covers >90% of the naive CD8+ T cell repertoire in B6 mice (unpublished data and (4)). Table II shows the Vβ expression of the lines, from Table I and Fig. 3, stimulated with the MLIIW and f-MLIIW peptides. The 1 μM f-MLIIW line expressed Vβ14 (>99%), while the others largely expressed Vβ4 (73% for 0.1 μM f-MLIIW, 96% for 0.01 μM f-MLIIW, 83% for 0.01 μM MLIIW, and >99% for 0.1 μM MLIIW). Because each line was stimulated from the same pool of spleen cells, these results show that differences in the concentration of peptide used for stimulation affect which T cells are amplified in vitro. The more efficient lines were Vβ4+, whereas the inefficient 1 μM f-MLIIW line was Vβ14+, yet each line began with the same distribution of Vβ4+ and Vβ14+ precursors.

To get a better picture of the T cell response to high and low concentrations of peptide, we stimulated six lines from three individual B6 mice using 5 and 0.1 μM of the MLIIW peptide and examined Vβ expression after 5 wk (Fig. 4). All lines were directed to the HA peptide (data not shown), yet the T cell repertoire was diverse. For each mouse, the TCR usage differed in the CTLs induced with 5 μM vs 0.1 μM. Moreover, Vβ usage in the lines stimulated with the high concentration of peptide was more diverse than in the lines stimulated with the low concentration (although...
we cannot be certain for the 0.1 μM line from mouse B, because "other" includes all Vβ-chains not recognized by the Abs). Similar results have been observed in lines induced with other HA peptides as well (Table II and unpublished results).

TCR expression level, as measured by anti-CD3ε staining by flow cytometry, was not strongly correlated with the responsiveness of the line (data not shown). Others have made similar observations (15). This is probably because the lines use different TCRs with different affinities for peptide/MHC.

Discussion

Presentation of 5-, 6-, and 7-mer HA peptides

The M3-restricted anti-HA response was first described based on the ability of an 18-mer peptide, HA173–190, to induce a CTL response in vitro. In this study, we have shown that the minimal epitope, MLIIW, as well as MLIIWG and MLIIWGV/I are capable of inducing CTLs (Fig. 1). CTLs induced with these peptides are not necessarily specific for the very peptide used for induction, although, with continued restimulation of the lines, some examples of specific recognition have been observed.

The heptamer peptides have the highest affinity for M3, and 10-fold less MLIIWG is needed to inhibit recognition of f-ND1-6V, compared with MLIIW (Fig. 2). With a similar inhibition assay, we have compared the relative affinities of the N-formylated peptides derived from the 13 proteins encoded in the mitochondria (26). Formylated ND1 and f-COI have the highest affinities, followed by nine peptides of medium to low affinity and two peptides that do not inhibit recognition of f-ND1-6V at all. When the 13 peptides were tested for recognition by M3-alloreactive bulk cultures, the high affinity and some of the medium affinity peptides sensitized target cells to lysis. Our results show that MLIIW, MLIIWG, and MLIIWGI can inhibit recognition of f-ND1-6V better than some formylated mitochondrial peptides. Moreover, with ~10-fold less relative affinity than the f-COI-3I peptide, MLIIWGI could certainly be classified as a medium affinity peptide. These results suggest that the longer peptides could be naturally presented by M3 on cells infected with influenza virus.

Efficient CTLs

CTLs can be highly sensitive to Ag. For some clones, fewer than 10 peptide/MHC complexes are needed to activate lysis of target cells (33); other CTLs require 104-fold more complexes for activation (34). TCR number and affinity for peptide/MHC as well as coreceptor interactions all contribute to the sensitivity of a T cell to Ag. In this study, we took advantage of the unique qualities of peptide presentation by M3 to show the linked effects of peptide concentration and affinity for inducing sensitive CTLs in vitro. Alexander-Miller et al. first showed that the concentration of peptide used to induce T cells in vitro affects the sensitivity of the resulting line (15), and this is true for M3-restricted anti-HA CTLs as well.

CTLs induced with 0.1 μM MLIIW are more sensitive than those generated with 1 μM. Even more dramatic, 0.01 μM
f-MLI IW induces CTLs that require at least 10^3-fold less peptide for recognition than those generated with 1 μM f-MLI IW. Sensitivity is dose dependent: the less peptide used for induction, the more sensitive the resulting CTL line. However, the optimal concentration depends on the affinity of the peptide for MHC. By adding a formyl group to the MLI IW epitope, a peptide is created that is 100-fold more potent for sensitizing target cells to lysis, yet confers no discernible specificity for the formyl group by the resulting CTL line (Fig. 3). This difference in affinity correlates with the ability of peptide to induce CTLs in vitro, as summarized in Table III. One hundred nanomolar of the MLI IW peptide is the minimal concentration capable of inducing CTL, whereas 100-fold less f-MLI IW is needed. Consistent with this result, 10-fold less medium affinity MLI IW G I peptide can induce CTLs as well. Our results show that efficient CTLs, i.e., those capable of recognizing the nonformylated peptide, are generated by a narrow range of concentrations: too low, and the CTLs do not expand; too high, and the CTLs are inefficient.

So, what is the optimal concentration of peptide to use for inducing CTLs in vitro? For high affinity peptides that sensitize lysis in the picomolar range, data suggest 0.1–10 nM. The best anti-H A CTLs were generated with 1–10 nM f-MLI IW (Fig. 3); high avidity CTL lines against the I10 epitope derived from HIV-1 were generated with 0.1 nM (15); and two immunodominant epitopes from L. monocytogenes, LLO91–99 and pep6217–225, presented by K^d, require 1 and 10 nM concentrations, respectively (4). By contrast, the lower affinity nonformylated HA epitopes require ~100 nM (Table III), similar to two subdominant epitopes derived from L. monocytogenes (4).

Reper toire analysis

Each T cell expresses a single TCRβ-chain, so Vβ expression is a marker for detecting differences among the T cells that are amplified following peptide stimulation. All the lines in Table II arose from the same pool of precursor T cells. However, the line induced with 1 μM f-MLI IW expressed Vβ4, whereas the other four lines mainly expressed Vβ4. When stimulated with high concentrations of peptide, high avidity T cells are overstimulated and die by apoptosis (35). If Vβ4 were a marker for high avidity anti-H A T cells in these lines, it is probable that 1 μM f-MLI IW overstimulated the Vβ4 population and these cells died, allowing the less efficient Vβ14^+ T cells to proliferate. Fig. 4 supports the point that stimulation with higher concentrations of Ag induces lower avidity T cells. In all three mice tested, the response to 5 μM peptide was more polyclonal than the response to 0.1 μM peptide. The cells responding to the low concentration of peptide were primarily composed of a single Vβ (with the possible exception of mouse B) and, in two of three mice (A and B), that Vβ was not observed in the line stimulated with the higher concentration. Less peptide induces a narrower repertoire of cells, probably because fewer T cells can respond to the lower concentrations of peptide.

In summary, we propose the following model. A number of different anti-HA/M3 receptors exist in the precursor T cell pool, and each T cell responds to peptide within an ideal concentration range. High concentrations of peptide overstimulate the high avidity T cells, which die by activation-induced cell death or apoptosis, allowing the more diverse low avidity T cells to proliferate. Stimulation with lower concentrations of peptide induces only the rarer, high avidity T cells to proliferate. The high avidity cells will be more adept at recognizing naturally presented Ag. As for anti-H A CTLs, these higher avidity cells will be useful for determining the ability of M3 to naturally present the nonformylated HA epitope on flu-infected cells.

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References


