Correlation between CD8 Dependency and
Determinant Density Using Peptide-induced,
L^d-restricted Cytotoxic T Lymphocytes

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Summary

We have taken advantage of some unique properties of H-2L^d to investigate the determinant
density requirements for cytotoxic T lymphocyte (CTL) priming versus effector function and
to correlate the determinant density requirements with CD8 dependency. In a previous study
(Lie, W.-R., N. B. Myers, J. Gorka, R. J. Rubocki, J. M. Connolly, and T. H. Hansen. 1990.
Nature [Lond.], 344:439), we demonstrated that culturing normal cells with peptides known to
be restricted by H-2L^d led to a two- to fourfold increase in surface L^d expression. In the present
study, we demonstrate the generation of L^d-restricted, peptide-specific in vitro primary CTL
by culturing spleen cells with murine cytomegalovirus or tum^-peptide at concentrations
previously shown to result in maximum induction of L^d expression. Target cells can be sensitized
for recognition by these CTL with lower doses of peptide than are required for the primary
sensitization. This demonstrates differences in the determinant density requirements for priming
versus effector function. The in vitro primary CTL generated with peptide can weakly lyse target
cells that express the determinant endogenously, and CTL lines and clones capable of strong
lysis of endogenous expressors are easily obtained. In both cases, target cells treated with exogenous
peptide are lysed better than target cells expressing antigen endogenously. This suggested that
there are differences in the determinant density of peptide-fed versus endogenous targets. This
interpretation was substantiated when it was observed that the level of lysis of target cells expressing
endogenous determinants correlated inversely with the amount of peptide required to sensitize
targets for recognition by various tum^-specific CTL clones. Furthermore, simultaneous titration
of both the peptide used to treat target cells and the antibody to CD8 revealed that the various
CTL clones analyzed displayed widely disparate CD8 dependencies. In each case, the CD8 de-
pendency correlated inversely with the determinant density requirement. Therefore, CD8 dependency
of CTL is relative, but shows an absolute and quantitative correlation with their dependency
on determinant density. These findings suggest that under physiologic conditions, where only
low determinant densities are likely to be encountered, all CTL clones will show at least partial
CD8 dependency.

CTL responses restricted by MHC class I molecules in-
volve recognition of processed antigenic peptides con-
tained within a groove formed by the alpha helices of the two
NH2-terminal domains of the MHC glycoprotein (1). Al-
though short synthetic peptides have been shown to serve
as target antigen for CTL primed with whole virus (2, 3),
peptide fragments (4, 5), or xenoantigen (6), peptide alone
could not easily prime specific CTL in vivo (4) or in vitro
(7). This in vitro defect was overcome at high cell density,
but the resulting CTL were in some cases MHC crossreac-
tive and none were capable of lysing target cells expressing
endogenous determinants (7). In vivo priming with peptide
has been achieved only by chemical modification of the pep-
tides (8, 9). A possible explanation for the inability to prime
with peptide is that a higher density of antigenic determinants
is required for priming and this is difficult to achieve with
peptide alone. We were able to test this hypothesis by taking
advantage of a unique feature of H-2L^d. We recently demon-
strated that culturing normal cells with peptides known to
be restricted by H-2L^d led to a dramatic increase in the ex-
pression of L^d, suggesting that from 60% to 75% of the
surface L^d molecules contained the fed peptide (10). We
therefore reasoned that peptide-fed cells would express suf-
cient antigenic determinants to serve as stimulators for CTL.
Another attractive feature of this system is that the L^d-
restricted peptides selected for this analysis have been well
characterized by others as CTL recognition determinants. One
of the peptides is derived from the murine cytomegalovirus
(MCMV)\(^1\) immediate early protein (iel) and has been shown

\(^1\) Abbreviations used in this paper: MCMV, murine cytomegalovirus; RCAS, rat Con A supernatant.
Peptides were synthesized using Merrifield's solid phase method (17) on a peptide synthesizer (model 431A; Applied Biosystems, Inc., [ABI], Foster City, CA). All peptide synthesis reagents were of high purity (>99%) and supplied by ABI. The resin used for peptide synthesis was phenylacetamidomethyl and was pre-loaded with 0.5 mM of required amino acid. All amino acids used were t-Boc protected at the NH₂ terminus, and their reactive side chains were protected with the standard groups recommended by ABI for t-Boc synthesis, with the exception of histidine. The histidine derivative used was N-α-Boc-N-ω-Benzoxymethyl-L-histidine supplied by Bachem Inc. (Borrance, CA). The Boc amino acids were coupled using carbodiimide-hydroxybenzotriazole coupling cycles as recommended by the manufacturer. The peptides were simultaneously deprotected and cleaved from the resin by treatment with anhydrous hydrogen fluoride/anisole/dimethyl sulfoxide 10:1:1 (vol/vol/vol) for 50 min at 0°C. The cleaved peptide was washed with diethyl ether to remove organic byproducts generated during HF cleavage. The peptide was then extracted from the resin with 30% acetic acid. The acetic acid was removed by rotary evaporation, and the remaining aqueous peptide solution was diluted fourfold with H₂O, shell frozen, and lyophilized. Peptides were purified (≥90%) by reverse phase HPLC and subjected to purity assessment techniques as previously described (18).

**Cell Lines and Peptide Induction.** R1.1-L₄, R1.1-D₄, R1.1-L₄, and R1.1-D₄ were generated by introducing the L₄, D₄, L₅, and D₅ genes, respectively, into the R1.1 (H₂K) thymoma cell line by electroporation and selected in medium containing G418 antibiotic. P815 is a H₂-K mouse mastocytoma cell line and P911 (from Dr. T. Boon) is a mutant cell line derived from P815 that expresses the tum⁻ antigen, P91A⁻ (13). L₄ was generated by introducing the L₄ gene into thymidine kinase-deficient mouse L cell fibroblasts (H₂-K) by calcium phosphate coprecipitation. L/iel/L₉ (from Dr. U. Koszinowski) is a mouse L cell fibroblast transfected with both the L₄ gene and the gene encoding MCMV immediate early protein pp89 (12). The cell lines were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with penicillin, streptomycin, glutamine, and 10% FCS (HyClone Laboratories, Logan, UT). For peptide induction, 5 x 10⁵ cells/ml in RPMI-FCS were cultured overnight (16–18 h) in the presence of 50–100 µM peptide. The cells were washed and used as stimulators, targets, or analyzed by flow cytometry.

**Flow Cytometry.** For fluorescence analysis, cells were washed and resuspended in HBSS lacking phenol red but containing 0.2% BSA and 0.1% sodium azide. 4 x 10⁵ cells/well were incubated for 30 min at 4°C in the presence of a saturating concentration of mAb or medium alone in a 96-well plate. The cells were washed and incubated in a saturating concentration of fluorescein-conjugated, affinity-purified F(ab')₂ fragment of goat anti-mouse IgG, Fc-specific, or of goat anti-rat IgG (Cooper Biomedical, Inc., Malvern, PA) for 30 min at 4°C. The cells were then washed and resuspended in medium containing 10 µg/ml propidium iodide, used to exclude dead cells from analysis.

Cells were analyzed on a FACS IV® (Becton Dickinson & Co., Mountain View, CA) equipped with an argon ion laser tuned to 488 nm and operating at 300 mW of power. Fluorescence histograms were generated with logarithmic amplification of fluorescence emitted by single viable cells. Each sample analyzed comprised a minimum of 5 x 10⁶ cells. Cells labeled with only the fluorescein-conjugated antibody were always included as controls.

**Generation of In Vitro Primary Peptide-specific CTL.** 5 x 10⁴ or 7.5 x 10⁵ responding BALB/c splenocytes were cocultured with 10⁶ stimulating peptide-fed P815 cells (10,000 rad) or with various concentrations (10⁻⁵ to 10⁻⁴ M) of MCMV or tum⁻ peptide in
24-well Linbro trays (Flow Laboratories, ICN Biomedicals, Horsham, PA) containing 2 ml RPMI 1640 supplemented with t-glutamine, sodium pyruvate, nonessential amino acids, penicillin, streptomycin, 5 × 10^{-5} M β-mercaptoethanol, and 10% FCS. After 5 d at 37°C in the presence of 5% CO2, effector cells were harvested, washed, and resuspended in RPMI 1640 supplemented with 10% FCS (RPMI-FCS).

**Generation of Peptide-specific CTL Lines and Clones.** CTL lines were established from primary cultures by incubating primary CTL for one additional week at 3 × 10^6/well in 2 ml of sensitization medium containing various concentrations of peptide. Subsequently, lines were maintained by weekly restimulation of 2 × 10^6 cells/well in the presence of 10^5 irradiated (2,000 rad) BALB/c spleen cells and 0.3-1.2 × 10^{-5} M peptide in 2 ml sensitization medium containing 10% rat Con A supernatant (RCAS). In some cases, clones were established from lines by limiting dilution. For cloning directly from primary cultures, 40 ml at 5 × 10^6 cells/ml were cultured in a 75-cm^2 flask for 6 d in the presence of 10^{-5} M peptide. The cells were washed and plated in round-bottomed microtiter plates at five different cell concentrations in replicates of 96. In addition, the wells contained 10^5 irradiated BALB/c spleen cells in 0.2 ml of sensitization medium containing RCAS and 10^{-5} M peptide. The clones were restimulated weekly by replacing 100 μl of medium with fresh medium containing 10^5 irradiated BALB/c spleen cells, RCAS, and 10^{-5} M peptide. After 3 wk, the clones were tested in a ^{51}Cr release assay for activity against P815 with and without peptide. Positive wells were selected for expansion based on the likelihood of clonality according to Poisson statistics (19). Selected clones were maintained by weekly restimulation in 24-well plates as described above.

**^{51}Cr Release Assay.** 10^6 target cells were labeled with 150-300 μCi of ^{51}Cr (Na^{51}CrO_{4}, 10-25 mCi/ml; Amersham Corp., Arlington Heights, IL) in 100 μl of RPMI-FCS with or without peptide for 1 h at 37°C in 5% CO2. In some experiments, cells were cultured overnight with peptide before labeling. For experiments in which the peptide concentration was titrated, target cells were pre-incubated with ^{3}HCr and 2.5 × 10^6 labeled cells in 500 μl of RPMI-FCS were added to several different tubes with the appropriate concentration of peptide. After 1 h, the cells were washed twice and 5 × 10^5 cells were added to the wells of round-bottomed microtiter plates. For antibody blocking, 50 μl of antibody was preincubated with 100 μl of effector cells for 15 min at 37°C. 50 μl of target cells was added and the plates were spun at 50 g for 1 min and incubated for 4 h at 37°C, 5% CO2. 100 μl of supernatant was counted in an ISOMEDIC gamma counter (ICN Biomedicals). The mean of triplicate samples was calculated and percent ^{51}Cr release was determined according to the following equation: percent ^{51}Cr release = 100 × ([experimental ^{51}Cr release - control ^{51}Cr release]/ [maximum ^{51}Cr release - control ^{51}Cr release]); where experimental ^{51}Cr release represents counts from target cells mixed with effector cells, control ^{51}Cr release represents counts from target cells incubated in medium alone (spontaneous release), and maximum ^{51}Cr release represents counts from target cells exposed to 5% Triton-X 100. For the data presented in this paper, the SEM percent specific lysis was <5% of the value of the mean.

**Results**

**Generation of In Vitro Primary L^d-restricted Peptide-specific CTL.** To obtain maximum induction of L^d expression, P815 cells were cultured overnight with 10^{-4} M MCMV peptide (10), previously shown to use L^d as the restricting element (14). The cells were washed and used as stimulators for BALB/c responder spleen cells. As seen in Fig. 1 A, CTL were generated that were capable of lysing peptide-induced (10^{-4} M, overnight) R11-L^d target cells but not R11-L^d without peptide treatment (not shown). In addition, including peptide alone (3 × 10^{-5} M) with the BALB/c spleen cell cultures was even more efficient at generating peptide-specific CTL. This is independent of the responder cell concentrations tested, which are in the range normally used for in vitro primary CTL cultures (Fig. 1 B). However, there is a direct correlation between the amount of peptide required to generate peptide-specific CTL in vitro (Fig. 1 B) and that required to induce increased expression of L^d (10). Even at relatively low peptide concentrations and low responder cell concentrations, peptide-specific CTL were obtained. In addition, this peptide-specific primary CTL response was found to be strongly CD8 dependent (Fig. 1).

MCMV-specific primary CTL, obtained by direct addition of peptide to the cultures, were tested on a panel of R11 transfectant cell lines, expressing the class I molecules, L^d, D^d, L^3, or D^3, that were cultured overnight with 10^{-4} MCMV peptide. As shown in Fig. 2 A, only cells bearing L^d were lysed, confirming that these CTL are indeed L^d restricted. In addition, primary, peptide-specific CTL were obtained using a different peptide, turn-, also previously shown to use L^d as the restricting element (13). The CTL generated to the turn- peptide lysed only targets bearing L^d plus turn-, and CTL generated to MCMV peptide lysed only targets bearing L^d plus MCMV peptide, demonstrating...
the peptide specificity of primary CTL generated with peptide alone (Fig. 2, B and C).

Recognition by Peptide-induced Primary CTL Is Accomplished at Low Target Cell Determinant Density. For the experiments described above, the target cells were treated overnight with peptide to induce maximum levels of expression of the L<sup>d</sup>/peptide complex. The peptide-specific CTL were generated with high doses of peptide (10<sup>-4</sup> M) and thus resulted from stimulation with cells expressing high determinant density. Since these CTL were also strongly CD8 dependent, we wanted to determine if these primary CTL would then only recognize target cells expressing high determinant density. We treated target cells with MCMV or turn<sup>-</sup> peptide under conditions that give maximum induction of L<sup>d</sup> expression (10<sup>-4</sup> M, overnight), low but detectable induction of L<sup>d</sup> expression (10<sup>-4</sup> M, 1 h), or no detectable induction of L<sup>d</sup> expression (10<sup>-5</sup> to 10<sup>-10</sup> M, 1 h). These lower doses have been shown to result in lysis by specific clones derived from MCMV-infected mice (11) and mice immunized with P911, the cell line expressing the turn<sup>-</sup> determinant endogenously (13). As seen in Table 1, near maximum levels of lysis are obtained when target cells are treated with peptide for only 1 h as compared with overnight incubation. At 10<sup>-4</sup> M for 1 h, 90-95% of the lysis obtained at 10<sup>-4</sup> M overnight is seen. When target cells are treated with peptide concentrations as low as 10<sup>-10</sup> M, 70-80% of the lysis is still obtained. These data demonstrate that a significant proportion of the peptide-induced primary CTL are capable of lysing target cells expressing low determinant density. These data also demonstrate a difference in the determinant density requirements for sensitization vs. effector cell function. Although a high determinant density was required to induce primary CTL in vitro, these same CTL were capable of recognizing target cells with relatively low determinant density.

Peptide-induced Primary CTL Can Lysie Target Cells Endogenously Expressing Antigen. In previous studies by Carbone et al. (7), CTL generated in vitro with peptide were unable to lyse targets expressing the determinant endogenously. The most plausible explanation for this observation was that the endogenous determinant was expressed at too low a den-

Table 1. Effect of Target Cell Determinant Density on Recognition by Primary Peptide-induced CTL

| Exp. | Peptide<sup>*</sup> | 10<sup>-4</sup> M (overnight) | 10<sup>-4</sup> M (1 h) | 10<sup>-5</sup> M (1 h) | 10<sup>-4</sup> M (1 h) | 10<sup>-8</sup> M (1 h) | 10<sup>-10</sup> M (1 h) |
|------|----------------|----------------------------|----------------|----------------|----------------|----------------|----------------|
| 1    | MCMV          | 80                         | 76             | 66             | 67             | ND             | ND             |
| 2    | MCMV          | 53                         | ND             | ND             | 45             | 35             | 36             |
| 3    | turn<sup>-</sup> | 87                         | 79             | ND             | 79             | 66             | 67             |

<sup>*</sup> Primary CTL were induced with peptide at 10<sup>-4</sup> M for 5 d.
<sup>†</sup> An E/T ratio of 80:1 is shown. Additional ratios were run at each point with similar results.
Peptide-induced primary CTL are capable of lysing target cells expressing endogenous determinants. (A) BALB/c spleen cells cultured for 5 d with 10^{-4} M tum^− peptide were assayed for lysis of P815 (□), P911 (O), or P815 + tum^− peptide (Δ). BALB/c spleen cells cultured for 5 d with 10^{-4} M MCMV peptide were assayed for lysis of L-L^d (□), L/ie1/L^d (O), or L-L^d + MCMV peptide (Δ).

Peptide-induced primary CTL are capable of lysing target cells expressing endogenous determinants at a density much lower than obtained with peptide-fed P815, suggesting that P911 cells express the determinant at a density much lower than obtained with peptide feeding. In addition, tum^− peptide-fed P911 were lysed at levels equivalent to tum peptide-fed P815 (not shown) excluding differences in target cell lysability. However, in the case of L/ie1/L^d, peptide feeding L-L^d target cells (or L/ie1/L^d, not shown) results in a modest increase in lysis compared with L/ie1/L^d, suggesting that L/ie1/L^d may express endogenous MCMV determinants at levels greater than P911 expresses endogenous tum^− determinants. This is consistent with L/ie1/L^d being a transfected cell line with multiple gene copies, thus leading to overproduction of the gene products. Also, since L cells are poor targets for primary CTL, possibly due to low basal expression of surface intercellular adhesion molecule 1 (21), additional factors may contribute to lysability differences. We therefore wanted to examine the effect of determinant density on CTL recognition more thoroughly. Because the targets expressing endogenous antigen are lysed weakly by peptide-induced primary CTL, and because L cells are poor targets for primary CTL, we decided to generate CTL lines and clones that would be useful for this analysis, and to rely primarily on P911 and peptide-fed P815 as targets.

Analysis of Determinant Density Requirements Using Peptide-induced CTL Clones. CTL lines and clones were established from MCMV peptide and tum^− peptide-induced primary CTL cultures using lower doses of peptide than required for the generation of in vitro primary peptide-specific CTL. As shown in Fig. 5, highly efficient MCMV-specific CTL were obtained after successive restimulation with peptide. A possible explanation for the inverse correlation observed between the response of the CTL and the peptide concentration used to restimulate (Fig. 5 A) is that the responding cell concentration was sufficiently high to result in competition for nutrients. These CTL maintained their CD8 dependency for...
several additional months in culture at the lowest dose used (3 μM). Several additional MCMV- and tum-specific lines and clones were obtained using 6–10 μM peptide for weekly restimulation. These lines and clones possessed various but stable CD8 dependencies, suggesting that the amount of peptide used did not select for high or low affinity CTL.

When the tum peptide was used, the majority of clones obtained were able to lyse P911 to various degrees. Some of the clones lysed P911 and peptide-fed P815 equally well, whereas other clones displayed better lysis of peptide-fed targets. For the data shown in Fig. 6, three representative clones were selected based on the differences in their ability to lyse P911 as compared with tum peptide-fed P815. When the tum peptide used to sensitize targets was titrated from 10^-4 to 10^-13 M, a direct correlation was observed between the level of lysis on P911 and the amount of peptide required to sensitize P815. Clone 5.4, which does not lyse P911 at all, even at higher E/T ratios than shown here, requires a high concentration of peptide to obtain maximum lysis of P815. Clone 7H5, which shows the strongest lysis of P911, requires the least amount of peptide to sensitize P815, and clone 7E5 has a phenotype intermediate between the two.

These data demonstrate an inverse correlation between the amount of exogenous peptide required to sensitize the target cell for recognition by a given clone and the degree to which that clone is able to lyse the target expressing the antigen endogenously. In addition, peptide alone can be used to induce specific CTL with vastly different antigen density requirements.

CD8 Dependency of Peptide-induced CTL Clones Correlates with Determinant Density Requirement. It has been suggested that the CD8 dependency of CTL correlates directly with the target cell antigen density required for recognition and inversely with the affinity of the CTL (22). We have previously defined CD8 dependency based on the ability of a high concentration of mAb to CD8 (1:500 ascites) to block recognition of target cells expressing high determinant density.

We wanted to determine the relationship between the target cell determinant density requirement and the CD8 dependency for peptide-induced CTL clones. The clones described above are ideal for this analysis because they vary greatly in the minimum amount of peptide required to sensitize P815 for recognition and therefore also in their ability to lyse P911.

Antibody to CD8 strongly inhibited lysis of P911 by all of the clones tested (18 total), two of which are shown in Fig. 7. However, when anti-CD8 was used to inhibit lysis of peptide-fed P815, one of the clones, 7E5, was completely...
inhibited, whereas the other, 7H5, was not inhibited at all. The activity of clone 5.4, which only lysed peptide-fed P815, was also strongly inhibited by anti-CD8 (not shown).

Therefore, clone 7H5, which required the least amount of exogenous peptide and lysed P911 at levels nearly equivalent to lysis of peptide-fed P815, was the least dependent on CD8. Since this clone was CD8 dependent when tested on P911, it seemed reasonable to assume that reducing the amount of exogenous peptide added to the target cells would alter the CD8 dependency of the clone. The data shown in Fig. 8 indicate that this is indeed the case. CD8-dependent and CD8-independent turn- and MCMV-specific CTL clones were compared over a broad range of target cell determinant densities by titrating the amount of peptide used to sensitize the target cells. Anti-CD8 antibody was titrated at each peptide concentration. Those clones, which require a higher antigen density for optimum lysis (7E5 and 2.9C1), are dependent on CD8 at all peptide concentrations tested. The clones that require low target antigen density (7H5 and 6.15A8) function independently of CD8 at higher peptide concentrations. However, when the antigen density decreases to a threshold level (which may be different for each clone), the CD8-independent clones become increasingly dependent on CD8. Importantly, there are peptide concentrations that result in maximum or near maximum levels of lysis of the target cell (10⁻⁸ to 10⁻¹⁰ M in Fig. 8), yet all of the clones tested are CD8 dependent. These data demonstrate an inverse correlation between the antigen density of a target cell and the requirement for CD8 by the effector cell. Also, CD8 dependency is not absolute for a given clone since the CD8 dependency can change as the antigen density changes.

The CTL clones shown in Fig. 8 were analyzed for surface expression of the CD8 receptor and were all found to express CD8 at high levels. Although there were slight differences between clones, there was no correlation between the level of surface expression of CD8 and the sensitivity to inhibition by antibody to CD8 (Fig. 9).

Discussion

In this report we demonstrate, quantitatively, the effect of determinant density on the priming of, recognition by, and CD8 dependency of L^d-restricted, peptide-specific CTL. A significant finding reported here is that MHC-restricted, peptide-specific CTL are easily generated in vitro with peptide alone, and that these CTL are capable of lysing target cells expressing endogenous antigen. The concentrations of peptide required to induce detectable activity during primary in vitro culture are the same as those required to increase the expression of L^d on cell lines such as P815 and L-L^d. Since overnight culture of these cell lines with specific peptide ligands results in a 2.5-4-fold increase in the level of expression of L^d, 60-75% of the surface L^d molecules contain the fed peptide, resulting in a high density of the specific MHC-peptide complex. It is likely that it is this increased determinant density that accounts for the ability to generate primary L^d-restricted CTL in vitro with peptide. Indeed, the strength of the response correlates precisely with the amount of peptide and therefore the level of induction of L^d.

Although high peptide concentrations are required for in vitro stimulation, target cell sensitization is accomplished with much lower doses of peptide. Initially, target cells were sensitized with a peptide concentration known to induce maximum expression of L^d to ensure optimum lysis by primary CTL. However, these primary, peptide-induced CTL can efficiently lyse target cells sensitized with peptide under conditions known to result in little or no detectable increase in L^d surface expression, demonstrating a significant difference in the determinant density requirement for in vitro priming of precursor CTL as compared with effector cell recognition. Indeed, these peptide-induced primary CTL are capable of lysing target cells expressing the determinant endogenously. In a report by Carbone et al. (7), cells expressing endogenous determinants were not lysed by primary CTL generated with either OVA peptides or an influenza peptide, even after long-term culture. The authors argued that the assumed higher surface density during priming in their system would only allow T cells with low receptor affinity to respond, thus accounting for the inability to lyse cells expressing endogenous determinants. In one case, restimulation of a long-term influenza peptide-specific CTL line with virus-infected cells allowed CTL to develop that were capable of lysing endogenous targets. This suggested that higher affinity clones were present in very low numbers. The authors also recognized the possibility that the OVA peptide they used for in vitro priming was not presented as an endogenous determinant by the target cell. Our system has the advantage that the peptides selected were previously shown to include a major antigenic determinant for MCMV-specific CTL generated in vivo (23) and the only antigenic determinant for P91A^- (turn^-)-specific CTL generated in vivo (13). Although the optimal size of the synthetic peptide in vitro may not be identical.
to the size of the processed fragments, the peptide-induced CTL can nonetheless recognize the processed fragments on both P911 and L/iel/Ld. Analysis of the determinant density requirements of several clonal populations of tum^peptide-specific CTL demonstrates that a correlation exists between the amount of peptide required to sensitize target cells for recognition and the ability to lyse P911. This suggests that the poor recognition of P911 by primary CTL results from low determinant density. In agreement with this quantitative interpretation, Chen et al. (24) recently reported that an HLA-DR11.1-restricted, HLA-B7 peptide-specific CTL line was capable of lysing targets homozygous but not heterozygous for both B7 and DR11.1.

In previous reports, we demonstrated that the CD8/class I interaction is required for priming responses to alloantigens and that CD8-independent CTL cannot develop in the absence of a CD8-dependent response (25, 26). In the studies reported here, when the level of the Ld/peptide complex is increased to approximate the level of expression of alloantigens, a peptide-specific in vitro primary response is obtained. This primary response is strongly CD8 dependent, as is the in vitro primary response to alloantigens, emphasizing the significance of the participation of CD8 for priming both allogeneic and MHC-restricted responses. From the data presented here, we would conclude that the CD8/class I interaction is achieved more efficiently in the presence of a high determinant density on the stimulator cells.

We (26) and others (27) demonstrated that the TCR and the CD8 molecule on the CTL must interact with the same class I molecule. Therefore, titering the peptide during target cell sensitization results in the simultaneous titration of both the TCR ligand and the CD8 ligand. Using this approach, we were able to dramatically and quantitatively demonstrate an inverse correlation between determinant density on the target cell and the CD8 dependency of peptide-specific CTL clones. More antibody was necessary to block target cell recognition by clones that required lower determinant density on the target cell. For example, all of the tum^-specific clones isolated required some participation of CD8 to lyse P911, even the highest affinity clones isolated, such as 7H5. However, more antibody was required to block the recognition of P911 by 7H5 than was required to block recognition of P911 by 7E5. In addition, CD8 dependency is independent of the level of expression of CD8 by the CTL, since the clones analyzed express CD8 at comparably high levels.

What is clearly and quantitatively demonstrated here is that the degree to which a given clone is dependent on CD8 is a function of the determinant density of the target cell. Therefore, CD8 dependency is not absolute for a given clone, nor are there two distinct classes of CTL: CD8 dependent and CD8 independent. CD8 dependency is generally defined by the ability of saturating concentrations of antibody to CD8 to block CTL recognition of targets with fairly high determinant density. This is convenient for relative comparisons between populations of CTL, since dramatic differences in CD8 dependency do exist among clones. However, the predicted CD8 dependency defined by in vitro analysis may not reflect the actual CD8 dependency under in vivo conditions. In vivo, a CTL is more likely to encounter target determinants expressed at low density and will therefore require the participation of CD8 to achieve effective interaction. This effect of determinant density has important biological consequences. For example, it has been proposed that tumor-specific peptides may bind class I molecules leading to destruction of the tumor cell by host CTL (28). Indeed, tumor cells that downregulate class I expression avoid immune recognition (29-33), and when converted to a high class I antigen expressing phenotype through gene transfer, become less malignant when injected into the host (34-37). This implies that the tumor cells with higher determinant density are now recognized by the immune system.

A correlation between determinant density and CD8 dependency has been suggested by other investigators. In one case, papain treatment of target cells reduces H-2 density and increases susceptibility to blocking of CTL killing by anti-CD8 antibody (38). However, enzyme treatment could affect other surface molecules in an unknown manner. Others have used class I-bearing artificial membranes to stimulate cells from primed animals (39). Although their data suggest a reciprocal relationship between antigen density and susceptibility to blocking with anti-CD8, in agreement with us and others (22), they conclude that the antigen density requirements are identical for activation of precursor CTL and for effector function. However, their conclusion is based on results with in vitro secondary responses. In another study, the ability of CTL clones to lyse target cells expressing low class I versus the same target cells expressing high class I after IFN-γ induction was correlated with inhibition by anti-CD8 antibody (22). These authors concluded that CTL clones that are less dependent on antigen density are more resistant to inhibition by anti-CD8.

The advantage of our system is that, using peptide alone, we were able to generate Ld-restricted peptide-specific primary CTL and CTL clones with vastly different determinant density requirements. By titering the amount of target cell peptide over a broad range of peptide concentrations, we were able to quantitatively correlate CD8 dependency with determinant density. A significant observation is that even a very high affinity clone, one that appears to be CD8 independent, requires the participation of CD8 when faced with a low determinant density expressing target cell. Since low determinant density is likely to be encountered in vivo, the participation of CD8 during effector function may be more significant than estimated by in vitro analysis.

There is, however, one intriguing aspect of the Ld-restricted CTL response reported here that is not easily explained in the context of the earlier report by Carbone et al. (7) supporting a determinant density model. Their model would predict that the extraordinary accessibility of the Ld ligand-binding site to peptide would result in stimulators with a high determinant density capable of preferentially eliciting CTL with lower affinities. However, we report here that peptide-induced, Ld-restricted CTL can lyse target cells that express low determinant density, including those that express
the determinant endogenously. This property of the Ld peptide response appears to be unique, thus distinguishing it from previous reports in which peptide-induced CTL could not lyse endogenous expressors or in some cases showed broad MHC crossreactivity (7). There are examples of peptide either coupled to a known mitogenic lipid moiety (8) or incor-
ported into the adipant glycoside Quil A (9) that in-
duced CTL in vivo capable of lysing endogenous targets, even though noncoupled peptide failed to do so. In addition, an HLA class II–restricted CTL line specific for an endogenous HLA class I peptide was obtained only after long-term stim-
ulation with peptide (24). It is therefore intriguing why Ld ligands can so easily stimulate CTL capable of recognizing targets with a low determinant density. Perhaps this is a reflection of the TCR repertoire to Ld/peptide. Previous reports have demonstrated that Ld molecules clearly have a unique relationship with peptide ligand (10, 40). Perhaps Ld molecules have a smaller pool of endogenous peptide ligand, com-
pared with other class I molecules such as Dd or Kd, or the structure of Ld renders them more selective or dependent upon peptide to maintain structure (40). Given the recent demonstration suggesting the involvement of endogenous pep-
tide in thymic education (41), the TCR repertoire for Ld could be quantitatively different from those for other class I molecules. This feature of Ld could contribute to the ability to elicit Ld-restricted CTL with peptide alone, which can lyse targets with low determinant density. In any case, this putative uniqueness of Ld does not preclude its well-
documented in vivo function as a restriction element (12, 13).

Therefore, the data presented here clearly establish this as an ideal system to investigate the cellular consequences of the interaction of peptide with class I.

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