

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 dependency 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 involve recognition of processed antigenic peptides contained within a groove formed by the α helices of the two NH₂-terminal domains of the MHC glycoprotein (1). Although 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 crossreactive 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 peptides (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 demonstrated that culturing normal cells with peptides known to be restricted by H-2L^d led to a dramatic increase in the expression 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 sufficient 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.

by Koszinowski and associates (11, 12) to be a major antigenic determinant recognized by L^d-restricted CTL from MCMV-immune mice. The other peptide (tum⁻) is derived from a mutant tumor cell line and has been shown by Boon and associates (13) to contain the only antigenic determinant recognized by L^d-restricted CTL from mice immunized with the mutant tumor cell, P91A. Cell lines endogenously expressing the determinants defined by the MCMV, and tum⁻ peptides were made available to us by Drs. U. Koszinowski (University of Ulm, FRG) and T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). Since induction of L^d with peptide results in expression of a high density of the L^d/peptide complex and these same peptides at low concentrations have been shown to sensitize target cells for recognition by CTL clones derived from animals immunized with virus or mutant cells (11, 13), it was possible to analyze a broad range of determinant densities by titrating the peptide concentration.

In this study, we report the generation of L^d-restricted, peptide-specific *in vitro* primary CTL using low numbers of peptide-fed cells or peptide alone as immunogen and standard numbers of responding cells. In contrast to earlier reports (7–9), lysis was obtained on both peptide-fed targets and on targets expressing endogenous determinants. In addition, we have been able to maintain long-term, L^d-restricted, peptide-specific CTL clones and lines using lower doses of peptide. Clones were obtained that differed in their ability to lyse endogenous expressors, in the amount of exogenous peptide required to sensitize target cells and/or in the requirement for CD8. The CTL clones were therefore used to assess the determinant density requirement for target cell recognition and to correlate determinant density with the requirement for CD8. This report is the first demonstration of class I-restricted, peptide-specific primary CTL induced *in vitro* that are not crossreactive with other MHC haplotypes and that can lyse target cells that express the antigenic determinant endogenously. Furthermore, using this system, peptide-specific CTL clones were generated that possessed widely disparate determinant density requirements, thus allowing us to quantitatively assess the relationship between determinant density and CD8 dependency.

Materials and Methods

Mice and Antibodies. BALB/c Kh (H-2^d) mice were bred in the animal facility of Dr. Donald C. Shreffler, Washington University School of Medicine, St. Louis, MO. mAbs 30-5-7 and 28-14-8 are both mouse IgG2a isotypes that have previously been shown to react with the $\alpha 1/\alpha 2$ and $\alpha 3$ domains of H-2L^d, respectively (14, 15). mAb 53-6.72 is a rat IgG2 that reacts with CD8 of all mouse strains (16). Culture supernatants of the mAbs were used for flow cytometry analysis as described below. An ascites of 53-6.72, produced in nude mice, was used for blocking CTL function as described below.

Peptides. The amino acid sequence of the MCMV peptide corresponds to residues 168–176 (YPHFMPNTL) of the MCMV immediate early protein pp89 (11). The amino acid sequence of the tum⁻ peptide corresponds to residues 12–24 (ISTQNHRA-LPLVA) of the mutant protein P91A⁻ (exon 4) from the tum⁻ P815 variant (13).

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*- π -Benzyloxymethyl-L-histidine supplied by Bachem Inc. (Torrance, 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 sulfide 10:1:1 (vol/vol/vol) for 50 min at 0°C. The cleaved peptide was washed with diethylether 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 ($\geq 90\%$) by reverse phase HPLC and subjected to purity assessment techniques as previously described (18).

Cell Lines and Peptide Induction. R1.1-L^d, R1.1-D^d, R1.1-L^q, and R1.1-D^q were generated by introducing the L^d, D^d, L^q, and D^q genes, respectively, into the R1.1 (H2^k) thymoma cell line by electroporation and selected in medium containing G418 antibiotic. P815 is an H-2^d 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). L-L^d was generated by introducing the L^d gene into thymidine kinase-deficient mouse L cell fibroblasts (H-2^k) by calcium phosphate coprecipitation. L/iel/L^d (from Dr. U. Koszinowski) is a mouse L cell fibroblast transfected with both the L^d 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, 5×10^5 cells/ml in RPMI-FCS were cultured overnight (16–18 h) in the presence of 50–100 μ M 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. 4×10^5 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 μ g/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 5×10^4 cells. Cells labeled with only the fluorescein-conjugated antibody were always included as controls.

Generation of *In Vitro* Primary Peptide-specific CTL. 5×10^6 or 7.5×10^6 responding BALB/c spleen cells were cocultured with 10^5 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 L-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 2×10^5 cells/well in the presence of 10^5 irradiated (2,000 rad) BALB/c spleen cells and $0.3\text{--}1.2 \times 10^{-5}$ 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-cm² flask for 6 d in the presence of 10^{-4} 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 10^6 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 μ l of medium with fresh medium containing 10^6 irradiated BALB/c spleen cells, RCAS, and 10^{-5} M peptide. After 3 wk, the clones were tested in a ⁵¹Cr 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.

⁵¹Cr Release Assay. 10^6 target cells were labeled with 150–300 μ Ci of ⁵¹Cr (Na⁵¹CrO₄, 10–25 mCi/ml; Amersham Corp., Arlington Heights, IL) in 100 μ l 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 ⁵¹Cr, and 2.5×10^5 labeled cells in 500 μ l of RPMI-FCS were added to several different tubes with the appropriate concentration of peptide. After 1 h, the cells were washed twice and 5×10^3 cells were added to the wells of round-bottomed microtiter plates. For antibody blocking, 50 μ l of antibody was preincubated with 100 μ l of effector cells for 15 min at 37°C. 50 μ l 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 μ l of supernatant was counted in an ISOMEDIC gamma counter (ICN Biomedicals). The mean of triplicate samples was calculated and percent ⁵¹Cr release was determined according to the following equation: percent ⁵¹Cr release = $100 \times [(\text{experimental } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}) / (\text{maximum } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release})]$; where experimental ⁵¹Cr release represents counts from target cells mixed with effector cells, control ⁵¹Cr release represents counts from target cells incubated in medium alone (spontaneous release), and maximum ⁵¹Cr 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

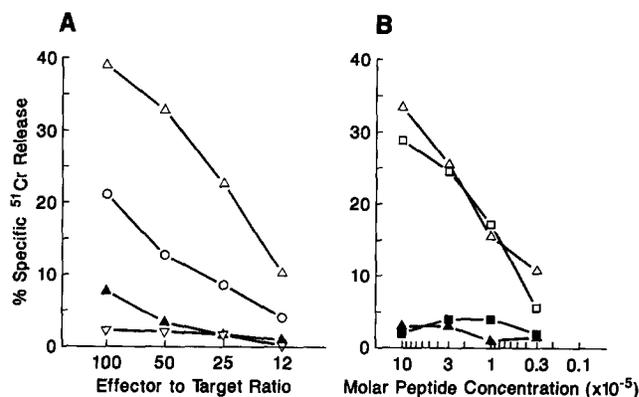


Figure 1. (A) MCMV peptide-specific primary CTL induced with peptide-fed P815 or peptide alone. 7.5×10^6 responding BALB/c spleen cells were cocultured for 5 d with 10^5 peptide-fed P815 (10,000 rad) or with 3×10^{-5} M MCMV peptide alone. The CTL were washed and assayed on R1.1-L^d target cells that had been induced overnight with 10^{-4} M MCMV peptide. (Δ) BALB/c anti-MCMV peptide; (O) BALB/c anti-MCMV peptide-fed P815; (▲) BALB/c anti-MCMV peptide + 1:500 anti-CD8 ascites during assay; (▽) BALB/c alone. (B) MCMV peptide dose-response for peptide-induced primary CTL. Either 5×10^6 (□) or 7.5×10^6 (Δ) responding BALB/c spleen cells were cultured for 5 d in the presence of various concentrations of MCMV peptide. Target cells were R1.1-L^d induced overnight with 10^{-4} MCMV peptide before the assay. Shaded symbols (▲, ■) represent CTL assayed in the presence of 1:500 anti-CD8 ascites.

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 R1.1 transfectant cell lines, expressing the class I molecules, L^d, D^d, L^q, or D^q, 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, tum⁻, also previously shown to use L^d as the restricting element (13). The CTL generated to the tum⁻ peptide lysed only targets bearing L^d plus tum⁻, and CTL generated to MCMV peptide lysed only targets bearing L^d plus MCMV peptide, demonstrating

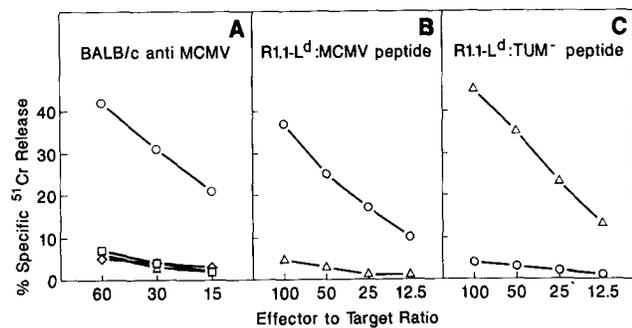


Figure 2. Peptide-induced primary CTL are L^d restricted and peptide specific. (A) BALB/c anti-MCMV primary CTL were assayed on a panel of R1.1 target cells transfected with the L^d (O), D^d (\square), L^q (Δ), or D^q (\diamond) gene. The target cells were cultured overnight with 10^{-4} M MCMV peptide and washed before the assay. (B and C) Lysis of R1.1- L^d cultured overnight with 10^{-4} M MCMV peptide (B) or 10^{-4} M tum^- peptide (C) by BALB/c anti-MCMV primary CTL (O) or BALB/c anti- tum^- primary CTL (Δ).

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^d /peptide complex. The peptide-specific CTL were generated with high doses of peptide (10^{-4} 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 tum^- peptide under conditions that give maximum induction of L^d expression (10^{-4} M, overnight), low but detectable induction of L^d expression (10^{-4} M, 1 h), or no detectable induction of L^d expression (10^{-5} to 10^{-10} 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 tum^- 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.

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

Exp.	Peptide*	Percent specific ^{51}Cr release from P815 † treated with peptide at:					
		10^{-4} M (overnight)	10^{-4} M (1 h)	10^{-5} M (1 h)	10^{-6} M (1 h)	10^{-8} M (1 h)	10^{-10} M (1 h)
1	MCMV	80	76	66	67	ND	ND
2	MCMV	53	ND	ND	45	35	36
3	tum^-	87	79	ND	79	66	67

* Primary CTL were induced with peptide at 10^{-4} M for 5 d.

† An E/T ratio of 80:1 is shown. Additional ratios were run at each point with similar results.

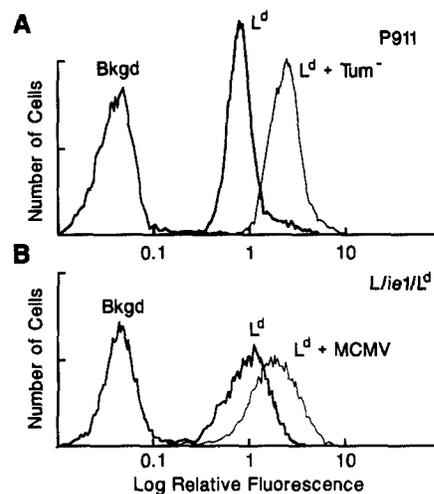


Figure 3. Surface expression of L^d on peptide-induced and -uninduced cell lines that express endogenous, L^d -restricted antigenic determinants. P911 was cultured overnight with or without 10^{-4} M tum^- peptide, and L/iel/ L^d was cultured overnight with or without 10^{-4} M MCMV peptide. L^d expression was measured with mAbs 30-5-7 and 28-14-8, and FITC-conjugated, F(ab') $_2$ goat anti-mouse IgG, Fc-specific antibody was used as developing reagent. Background (Bkgd) represents fluorescence emitted from cells incubated with the developing reagent alone.

At 10^{-4} M for 1 h, 90–95% of the lysis obtained at 10^{-4} M overnight is seen. When target cells are treated with peptide concentrations as low as 10^{-10} 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 Lyse 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-

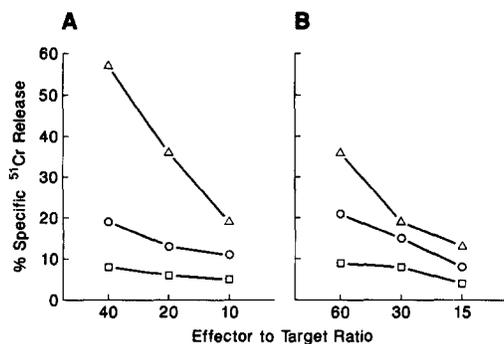


Figure 4. Peptide-induced primary CTL are capable of lysing target cells expressing endogenous determinants. (A) BALB/c spleen cells cultured for 5 d with 10^{-4} M tum⁻ peptide were assayed for lysis of P815 (□), P911 (○), or P815 + tum⁻ peptide (Δ). (B) BALB/c spleen cells cultured for 5 d with 10^{-4} M MCMV peptide were assayed for lysis of L-L^d (□), L/iel/L^d (○), or L-L^d + MCMV peptide (Δ).

sity. Since L^d-restricted, peptide-specific CTL were more readily obtained during in vitro primary culture, in comparison with previous attempts, and because they could lyse targets treated with a low concentration of peptide, we wanted to determine if they were capable of lysing target cells endogenously expressing the tum⁻ or MCMV antigens. The target cells used for this analysis are P911, a mutant derived from P815 that endogenously expresses the tum⁻ antigen, and L/iel/L^d, an L cell transfected with both the L^d gene and the gene coding for the immediate early protein (pp89) of MCMV that endogenously expresses the MCMV antigen. Both of these cell lines have been previously shown to be recognized by L^d-restricted CTL clones generated, respectively, by in vivo immunization with P911 or MCMV viral infection (12, 13, 20). The peptides used in our studies have been shown by Lurquin et al. (tum⁻) (13) and Reddehase et al. (MCMV) (11) to serve as the optimum target determinant for recognition by these CTL clones. The surface expression of L^d on P911 is lower than K^d and D^d (not shown), just as it is on the parent line, P815 (10). As shown in Fig. 3, surface L^d expression is induced when P911 is cultured overnight with 10^{-4} M tum⁻ peptide and also when L/iel/L^d is cultured overnight with 10^{-4} M MCMV peptide. The level of induction of L^d is comparable with that obtained with tum⁻ peptide-fed P815 and MCMV peptide-fed L-L^d, as previously reported (10). This demonstrates that the endogenous tum⁻ or MCMV peptide is not produced in amounts sufficient to significantly increase the expression of L^d. These cell lines were used as targets for primary, L^d-restricted, peptide-specific CTL. P815 and L-L^d incubated with exogenous peptide for 1 h were used as control targets. Several interesting points can be made from the results of this analysis shown in Fig. 4. First, in contrast to the earlier reports (7, 8), peptide-induced, L^d-restricted primary CTL are capable of lysing target cells that express antigen endogenously. Second, the lysis is weak on P911 in comparison 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

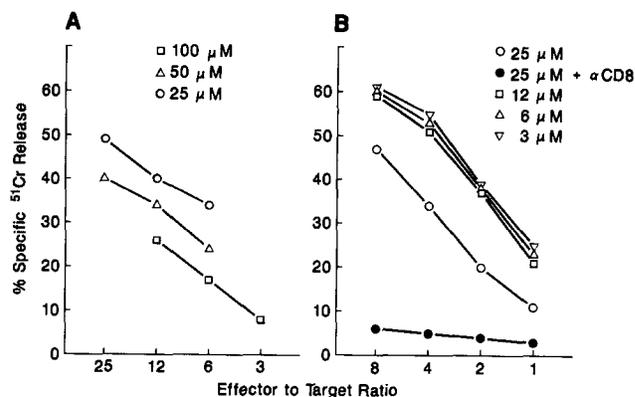


Figure 5. Re-stimulation of peptide-induced primary CTL with lower doses of peptide. (A) Cells obtained from the MCMV peptide-induced primary CTL culture shown in Fig. 2 A were restimulated for one additional week at 3×10^6 cells/ml with 100, 50, and 25 μ M MCMV peptide and assayed against 10^{-4} M R1.1-L^d + MCMV peptide, overnight. (B) The cells from the 25- and 50- μ M peptide groups in A were pooled and restimulated for an additional week at 2×10^5 /ml in the presence of RCAS and irradiated BALB/c spleen cells (see Materials and Methods) with 25, 12, 6, and 3 μ M MCMV peptide and assayed against 10^{-4} M R1.1-L^d + MCMV peptide, overnight. 25 μ M MCMV stimulated CTL plus 1:500 anti-CD8 ascites during assay (●).

at levels equivalent to tum peptide-fed P815 (not shown) excluding differences in target cell lysability. However, in the case of L/iel/L^d, peptide feeding L-L^d target cells (or L/iel/L^d, not shown) results in a modest increase in lysis compared with L/iel/L^d, suggesting that L/iel/L^d may express endogenous MCMV determinants at levels greater than P911 expresses endogenous tum⁻ determinants. This is consistent with L/iel/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

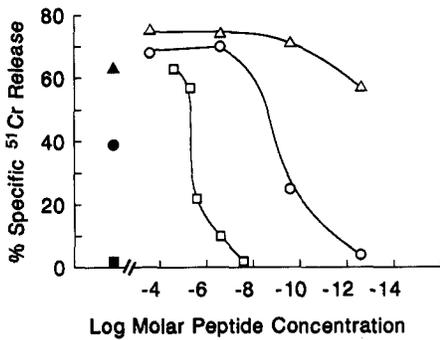


Figure 6. Correlation between lysis of P911 and the amount of tum⁻ peptide required to sensitize P815 for lysis by tum⁻-specific CTL clones. The closed symbols represent lysis of P911 by clones 5.40 (▲), 7E5 (●), and 7H5 (■). The open symbols represent lysis of P815 treated for 1 h with various concentrations of tum⁻ peptide by the same clones, 5.40 (□), 7E5 (○), and 7H5 (△). The E/T ratio was 10:1 for 5.40 and 7.5:1 for 7E5 and 7H5.

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.

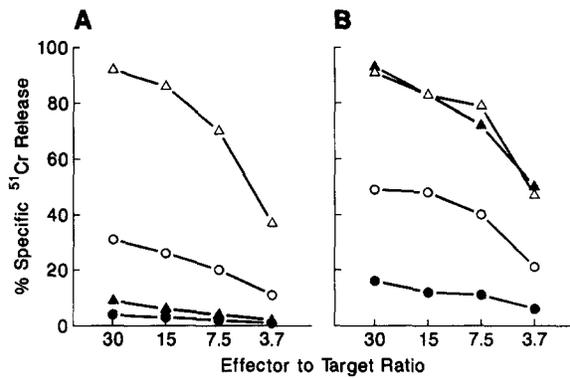


Figure 7. Comparison of CD8 dependency of clones 7E5 (A) and 7H5 (B) tested on P911 and tum⁻ peptide-fed P815. Lysis of P911 (○) and 10⁻⁴ M P815 + tum⁻ peptide, for 1 h (△). Closed symbols (●, ▲) represent lysis in the presence of 1:500 anti-CD8 ascites during the assay.

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

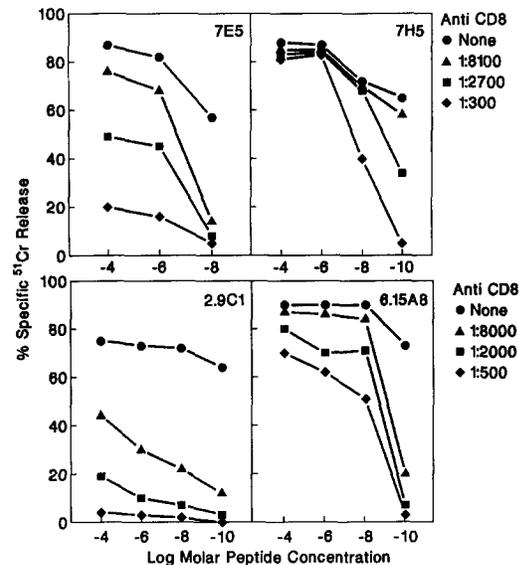


Figure 8. Correlation between CD8 dependency of CTL clones and target cell determinant density. Tum⁻-specific, CD8-dependent (7E5) and CD8-independent (7H5); and MCMV-specific, CD8-dependent (2.9C1) and CD8-independent (6.15A8) CTL clones were assayed against P815 treated for 1 h with various concentrations of either tum⁻ or MCMV peptide. Several dilutions of anti-CD8 antibody were included during the 4-h assay for each target cell peptide concentration. 7E5 and 7H5 were assayed against tum⁻-treated P815 at an E/T ratio of 7.5:1, and 2.9C1 and 6.15A8 were assayed against MCMV-treated P815 at an E/T ratio of 2:1. All of the clones showed no lysis of P815 without peptide.

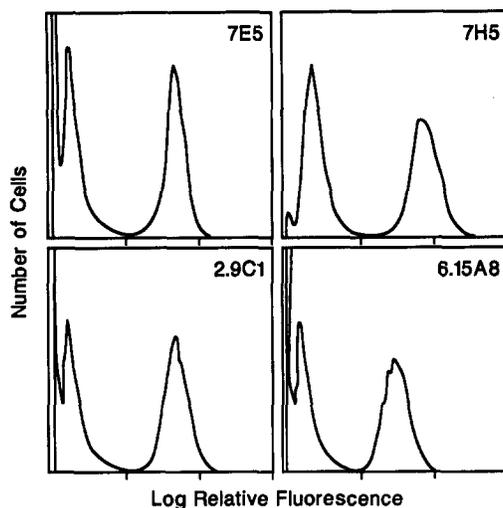


Figure 9. Surface expression of CD8 by various CTL clones. Cells were incubated with anti-CD8 culture supernatant and developed with an FITC-conjugated, goat anti-rat IgG. Mean fluorescence above background was 122 for 7E5, 127 for 7H5, 129 for 2.9C1, and 111 for 6.15A8.

inhibited, whereas the other, 7H5, was not inhibited at all. The activity of clone 5.4, which only lyses 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 tum⁻ and MCMV-specific CTL clones were compared over a broad range of target cell determinant densities by titering 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⁻ (tum⁻)-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^d/L^d. 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 L^d/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 cells 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 L^d-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 L^d-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 L^d 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, L^d-restricted CTL can lyse target cells that express low determinant density, including those that express

the determinant endogenously. This property of the L^d 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 incorporated into the adjuvant glycoside Quil A (9) that induced 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 stimulation with peptide (24). It is therefore intriguing why L^d 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 L^d/peptide. Previous reports have demonstrated that L^d molecules clearly have a unique

relationship with peptide ligand (10, 40). Perhaps L^d molecules have a smaller pool of endogenous peptide ligand, compared with other class I molecules such as D^d or K^d, or the structure of L^d renders them more selective or dependent upon peptide to maintain structure (40). Given the recent demonstration suggesting the involvement of endogenous peptide in thymic education (41), the TCR repertoire for L^d could be quantitatively different from those for other class I molecules. This feature of L^d could contribute to the ability to elicit L^d-restricted CTL with peptide alone, which can lyse targets with low determinant density. In any case, this putative uniqueness of L^d 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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