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Peptide-MHC Class I Tetrameric Complexes Display Exquisite Ligand Specificity

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The production of synthetic MHC-peptide tetramers has revolutionized cellular immunology by revealing enormous CD8⁺ T cell expansions specific for peptides from various pathogens. A feature of these reagents, essential for their staining function, is that they bind T cells with relatively high avidity. This could, theoretically, promote cross-reactivity with irrelevant T cells leading to overestimates of epitope-specific T cell numbers. Therefore, we have investigated the fine specificity of CTL staining with these reagents for comparison with functional data. Using a panel of CTL clones with distinct fine specificity patterns for analogs of an HLA-B8-binding EBV epitope, together with B8 tetramers incorporating these peptides, we show a very good correlation between tetramer staining and peptide activity in cytolysis assays. Significant staining only occurred with tetramers that incorporate strong stimulatory agonist peptides and not weak agonists that are unlikely to induce full T cell activation at physiological levels of presentation. In almost every case where a peptide analog had >10-fold less activity than the optimal EBV peptide in cytotoxicity assays, the corresponding tetramer stained with >10-fold less intensity than the EBV epitope tetramer. Furthermore, by examining an EBV-specific clonotypic T cell expansion in EBV-exposed individuals, we show similar fine specificity in tetramer staining of fresh peripheral T cells. Collectively, our data demonstrate the exquisite specificity of class I MHC-peptide tetramers, underlining their accuracy in quantifying only those T cells capable of recognizing the low levels of cell surface peptide presented after endogenous Ag processing. The Journal of Immunology, 2000, 165: 6229–6234.

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ligands with higher avidity and slower dissociation rates than individual MHC-peptide complexes (3), the present investigation aimed to assess whether T cell specificity for peptide presented in MHC class I tetramers is equivalent to that for peptide presented on the cell surface at levels roughly analogous to those presented naturally after endogenous processing. Using a panel of HLA B8 tetramers incorporating a highly immunogenic peptide from EBV (FLRGRAYGL) (19), or single amino acid variants of this peptide, together with a series of CTL clones with a range of fine specificities for these peptides, we now demonstrate that tetramer staining faithfully reflects these fine peptide specificities and is therefore a very good surrogate marker for T cell recognition of physiological levels of presented peptide.

**Materials and Methods**

*Establishment and maintenance of cell lines*

Lymphoblastoid cell lines (LCLs) were established by exogenous transformation of peripheral B cells with EBV derived either from the QIMR-Wil or the Ag876 cell lines. The QIMR-Wil EBV strain encodes the CTL epitope sequence FLRGRAYGL, which binds to HLA B8, whereas the Ag876 EBV strain encodes a variant of this sequence (LLRGRAYQG), which does not bind to HLA B8 and is therefore not a target for CTL recognition (19, 20). CTL clones were generated by agar cloning as previously described (14) following initial stimulation with γ-irradiated (8000 rad) autologous LCLs transformed with the QIMR-Wil EBV strain, using a responder-stimulator ratio of 50:1. Clones were amplified in culture with bi-weekly stimulation with γ-irradiated autologous LCLs transformed with QIMR-Wil EBV. CTL clones LC13, CF34, CF4, RL42, CF8, and WY6 have been described previously (14, 17).

**Cytotoxicity assay**

CTL clones were tested in duplicate for cytotoxicity in the standard 5-h chromium release assay (E:T ratio of 2:1). To test synthetic peptides for activity as agonists, facilitating CTL lysis, each peptide was added directly to 32P-labeled HLA B8 LCLs transformed with the Ag876 strain of EBV, incubated for 1 h before CTL addition, and remained present throughout the assay. To test peptides as antagonists of CTL lysis, the synthetic peptides were added to HLA B8+ LCLs transformed with the QIMR-Wil strain of EBV, incubated for 1 h before CTL addition, and remained present throughout the assay. Toxicity testing of all peptides was performed by adding peptide to 32P-labeled LCLs in the absence of CTL effectors. A Topcount Microplate β scintillation counter (Packard, Meriden, CT) was used to measure 32P levels in assay supernatant samples. The mean spontaneous lysis for target cells in culture medium was <20%, and the variation about the mean specific lysis was <10%. Peptides were made by Mimotopes (Clayton, Victoria, Australia).

**Generation of HLA-B8-peptide tetramers**

Tetrameric HLA-B8-peptide complexes were prepared essentially as previously described (3). Briefly, recombinant HLA B8 and human β2 microglobulin, produced in Escherichia coli, were solubilized in urea and injected together with each synthetic peptide into a refolding buffer consisting of 100 mM Tris (pH 8.0), 400 mM arginine, 2 mM EDTA, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione. Refolded complexed were purified by anion exchange chromatography using DE52 resin (Whatman). Tetrameric complexes were then purified by gel filtration through a Superdex 75 column (Amersham Pharmacia Biotech, Piscataway, NJ). The refolded HLA-B8-peptide complexes were biotinylated by incubation for 16 h at 30°C with the BirA enzyme (Avidity, Denver, CO). Tetrameric HLA-B8-peptide complexes were produced by the stepwise addition of extravidin-conjugated PE (Sigma, St. Louis, MO) to achieve a 1:4 molar ratio (extravidin-PE:biotinylated class I). Tetramer stock concentrations were ~200 ng/µl.

**Cell staining and FACS analysis**

CTL clones or PBMCs (2–5 × 106) were incubated for 50 min at 4°C or 37°C with each tetramer (1:100 diluted) in 100 µl of 10% FCS/RPMI 1640 and then washed twice in PBS containing 1% FCS. The PBMCs were then incubated at 4°C with TriColor anti-human CD8 Ab (1:100 diluted; Caltag, Burlingame, CA). Stained cells were analyzed on a FACScalibur (Becton Dickinson, Mountain View, CA) using CellQuest software. Logical gating on forward and side scatter was selected for activated T cells with minimal autofluorescence and cell death.

**Results and Discussion**

**CTL clones raised against an EBV epitope display distinct fine specificity patterns**

Eight EBV-specific CTL clones that recognize the HLA-B8-binding peptide FLRGRAYGL, from the nuclear Ag EBNA3A, were raised from five unrelated individuals and used in this study. The α and β TCR chains of six of these CTL clones (LC13, CF34, CF4, RL42, CF8, and WY6) were sequenced at the junctional regions in a previous investigation and are known to be very different for each clone (14, 17). The two other CTL clones (BK10 and CF19) that have not been described previously were raised in the same way as the other clones from healthy HLA-B8+ EBV-sero+ individuals against autologous cells presenting the EBV epitope. Each CTL clone was tested in a chromium-release assay for lysis of target cells that had been preincubated with various concentrations of peptide FLRGRAYGL. These target cells were HLA-B8+ LCL that had been transformed with the Ag876 strain of EBV, which are not recognized by these CTL clones in the absence of exogenous peptide because this EBV strain encodes a variant of the CTL epitope that does not bind to HLA B8 (19). As shown in Fig. 1A, the different CTL clones recognized the EBV peptide with similar efficiencies, with half-maximum lysis values of between 30 nM (for LC13 and CF4) and 150 nM (for WY6) of exogenously added peptide.

Previous crystallization studies have suggested that positions 1, 4, 7, and 8 are the likely TCR-accessible residues within peptides bound to HLA B8 (21). Therefore, the CTL clones were also tested for recognition of varying concentrations of four peptide analogs of FLRGRAYGL that had single amino acid substitutions at these positions (position 1, Phe to Thr; position 4, Gly to Ser; position 7, Tyr to Phe; or position 8, Gly to Val) (Fig. 1A). Each CTL clone displayed a distinct pattern of fine specificity for the panel of closely related peptides. For example, comparing data for CTL clones LC13 and CF34, the relative impact of the different amino acid substitutions on the activation of each clone was inverse, i.e., the most tolerated substitutions for LC13 were the least well tolerated by CF34.

The eight CTL clones were also tested in cytotoxicity assays against a target panel of four HLA B8+ (Fig. 1B) and four HLA B8− (Fig. 1C) LCLs that were transformed with the QIMR-Wil strain of EBV which is known to encode the FLRGRAYGL epitope. As has been shown in previous studies using CTL clones (22, 23), lysis levels of LCL target cells presenting endogenously processed EBV epitopes are rarely as high as are seen with saturating amounts of exogenously added peptide (Fig. 1B). EBV-latent Ag peptides are presumably presented at relatively low density on LCLs after endogenous processing, and CTLs are unable to achieve the very high avidity of interaction that is possible with target cells preincubated with synthetic peptide. This experiment was also performed at higher E:T ratios (up to 20:1), but LCL lysis levels were never reached those observed with synthetic peptide (data not shown). Although lysis levels of HLA B8− LCLs were relatively low, they were certainly significant compared with the negligible lysis of HLA B8− LCLs (Fig. 1C).

The dashed lines in Fig. 1A correspond to the mean level of lysis by each clone of the four HLA B8+ LCLs transformed with the QIMR-Wil EBV strain, calculated from Fig. 1B, and the concentration of exogenously added peptide FLRGRAYGL required to induce this level of lysis in HLA B8− LCLs transformed with the Ag876 strain of EBV. For most of the clones, this peptide concentration is close to the point of inflection on the titration curve.

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1 Abbreviation used in this paper: LCL, lymphoblastoid cell line.
where a small drop in peptide concentration results in a large fall in activity. Therefore, it seems unlikely that analogs of FLRGRAYGL that require over 10 times this peptide concentration to promote T cell lysis will be activating ligands if presented naturally on LCLs (assuming the single amino acid changes would not improve processing efficiency significantly). For example, CTL clone LC13 lysed the four HLA B8+ LCLs transformed with the QIMR-Wil EBV strain at an average of 33%, which is roughly equivalent to the level of lysis observed after adding 30 nM of synthetic FLRGRAYGL peptide to target cells transformed with the Ag876 EBV strain (Fig. 1A, dashed line on LC13 graph). Extrapolating from this data, analogs of this EBV peptide that do not facilitate lysis by clone LC13 when added at 30 nM are unlikely to be agonists for this CTL clone if presented at physiological levels. Thus, peptide FLRSRAYGL, which is a weak activating ligand for LC13 (Fig. 1A), is unlikely to facilitate CTL lysis if processed endogenously and presented naturally on an HLA B8+ LCL infected with a hypothetical EBV variant encoding a Gly to Ser mutation at this position. Therefore, these data have defined the fine specificity of these eight CTL clones for five closely related peptides over a range of concentrations, including an estimated physiologically relevant concentration, for comparison with subsequent tetramer staining experiments.

**FIGURE 1.** Eight CTL clones, raised against the EBV epitope FLRGRAYGL, display distinct fine specificity patterns in cytotoxicity assays. Chromium-release assay data for CTL clones LC13, CF34, CF4, BK10, RL42, CF8, CF19, and WY6 used as effectors against the following target cells: HLA B8+ LCLs transformed with the Ag876 strain of EBV and treated with various concentrations of the peptides indicated in the legend (A); HLA B8+ LCLs from donors L.C., S.C., M.B., and R.M. transformed with the QIMR-Wil EBV strain (B); and HLA B8+ LCLs from donors J.C., G.H., A.H., and C.J. transformed with the QIMR-Wil EBV strain (C). The QIMR-Wil strain of EBV encodes the EBV epitope, FLRGRAYGL, whereas the Ag876 strain encodes a variant sequence that is not recognized by the CTLs. The E:T ratio was 2:1. The dashed lines in A correspond to the mean level of lysis for each clone of the four HLA B8+ LCLs transformed with the Ag876 strain, calculated from B, and the concentration of exogenously added peptide FLRGRAYGL required to induce this level of lysis in the HLA B8+ LCL transformed with the Ag876 EBV strain.

**Tetramer staining accurately reflects the fine specificity of CTL clones for peptides presented at physiological levels on target cells**

HLA B8 tetramers were produced that incorporated either the FLRGRAYGL epitope or one of the four analogs described above. CTL clone LC13 was stained with the tetramers, and the most intense staining was observed with the FLRGRAYGL tetramer (Fig. 2). The only other tetramer that bound significantly was that made with the 7LRGRAYGL peptide, which stained with slightly less intensity than the FLRGRAYGL tetramer. This result was consistent with our earlier cytotoxicity data showing the 7LRGRAYGL peptide to be a very strong agonist for CTL clone LC13 (Fig. 1A). Also consistent with the chromium release assay data was the lack of staining with the FLRGRAYGL tetramer because this peptide was a null ligand for this clone. Surprisingly, tetramers made with peptides FLRGRAFGL and FLRSRAYGL, which were recognized by clone LC13 in cytotoxicity assays 8- and 100-fold, respectively, less efficiently than the parent viral peptide, did not stain the clone above background levels. As an additional specificity control for this experiment, a CTL clone with known specificity for another HLA B8-binding EBV epitope (QAKWRLQTL) did not stain with the tetramers (data not shown).

CTL clones CF34 and WY6, which had quite distinct fine specificity patterns from LC13 and from each other, were then stained with the tetramers. Again, the most efficient peptides in activating CTL lysis stained with the highest intensity when incorporated into HLA B8 tetramers (Figs. 2A and 2). Weak agonist peptides that are unlikely to be activating ligands for the CTL clones if presented at physiological levels on target cells did not stain at high intensity when incorporated into B8 tetramers.

The other five CTL clones were then stained with the five tetramers, and this data is presented in Fig. 3, B and C, as median fluorescence intensity, together with data from clones LC13, CF34, and WY6. A recent study indicated that the specificity of CTL interaction with peptide-MHC class I tetrameric complexes is temperature dependent and that much more specific staining is obtained by incubating tetramers with T cells at 37°C rather than 4°C (24). Therefore, we stained all the clones using either a 4°C (Fig. 3B) or a 37°C (Fig. 3C) incubation. Also presented in Fig. 3A for
comparison with the tetramer staining data are values of the exogenous peptide concentration required by each clone for half-maximal lysis of HLA B8\(^+\) LCLs transformed with the Ag876 EBV strain, as determined from the chromium release assay data shown in Fig. 1A.

Although the 37°C tetramer incubation generally resulted in higher intensity staining compared with the 4°C incubation, there was only a slight increase in the specificity of tetramer staining at 37°C. Regardless of the staining temperature, there was generally a good correlation between the functional cellular response elicited by the peptide variants and the intensity of staining by tetramers incorporating these peptides (Fig. 3). Considering data from all the clones, in the 19 cases where a peptide had >10-fold less activity than the wild-type EBV peptide in cytotoxicity assays, 17 (using 4°C) or 18 (using 37°C) of the corresponding tetramers stained with >10-fold less intensity compared with the EBV epitope tetramer. These data suggest that MHC class I peptide tetramers bind T cells with an avidity sufficiently low to discriminate between strong stimulatory agonists and weak agonists that may not induce full T cell activation at physiological levels of presentation. In additional specificity controls for these experiments, none of these CTL clones stained with an HLA B8 tetramer incorporating another EBV epitope, RAKFKQLL (data not shown).

Peptides with close homology to activating TCR ligands can sometimes act as antagonists of T cell effector function (25). Therefore, we considered the possibility that tetramer staining may, in some cases, be reflecting such peptide interactions. The four peptide analogs of the FLRGRAYGL EBV epitope were each screened at a range of concentrations (from 1 nM to 100 \(\mu\)M) for their ability to reduce lysis of an HLA B8\(^+\) LCL, transformed with the QIMR-Wil EBV strain, in chromium release assays using the eight CTL clones (data not shown). There were just two peptide-CTL combinations where antagonism was evident. Lysis by CTL clone RL42 was reduced from 20 to 1% by peptide FLRSGAYGL at 10 \(\mu\)M, and lysis by CTL clone WY6 was reduced from 15 to 1% by peptide FLRGRAFGL at 1 \(\mu\)M. Interestingly, these peptide-CTL combinations were the only ones where a peptide that had no detectable activity as an agonist was staining as a tetramer with a median fluorescence intensity above 10 (Fig. 3). However, the relatively low tetramer staining intensity observed here suggests that...
HLA B8 molecules presenting these antagonist peptides bind their TCR ligands with much lower avidity compared with peptides that promote CTL lysis.

Interestingly, there were two examples (CTL clones CF4 and CF19) where a peptide (FLRGRAYVL) had little activity as either an agonist or an antagonist in the functional assays, and yet quite strong staining with a tetramer incorporating this peptide was observed. This demonstrates that a peptide-MHC complex that binds the Ag receptor of a T cell with quite high avidity can be a null ligand for that T cell. Perhaps such ligands simultaneously trigger both the activation and the antagonism pathway, thereby negating any detectable T cell function. Supporting this notion, it is known that many T cell antagonist peptides can act as agonists of T cell activation at high concentrations (25).

**Fresh peripheral blood T cells stain with MHC class I tetramers that incorporate strong, but not weak, agonist peptides**

Previous studies have shown that the FLRGRAYGL EBV epitope often induces a strong and exceptionally restricted memory response in healthy EBV-sero + HLA B8 + individuals (14, 26, 27). For reasons that are not yet clear, a single public αβ TCR dominates this response in some people. This dominant TCR is the same as that expressed by the LC13 CTL clone (26). This unusual feature of the memory response to the FLRGRAYGL epitope provided an opportunity to examine whether the fine specificity of tetramer staining, evident using CTL clones, is also observed with fresh T cells. PBMCs from a healthy EBV-sero + HLA B8 + donor who was known to carry a high frequency of FLRGRAYGL-reactive T cells that express this conserved TCR (14, 26, 27) were stained with each of the tetramers, followed by an anti-CD8 Ab. The tetramer incubations were performed at 37°C or 4°C, but only data using 37°C is shown (Fig. 4) because the frequencies of tetramer-positive cells did not vary significantly between the two protocols. PBMCs from an HLA B8 - donor were also used as a control and were found not to stain with the tetramers above the background level (data not shown).

The proportion of CD8 + T cells that stained with tetramer incorporating the wild-type EBV peptide was 2.66%. Consistent with the restricted usage of a TCR like that of LC13 in the anti-FLRGRAYGL response in this individual, similar frequencies were observed using the 7LRGRAYGL tetramer (2.56% of CD8 + cells). As was observed with the LC13 CTL clone, staining of fresh T cells with the 7LRGRAYGL tetramer was somewhat less intense compared with the viral epitope tetramer. The proportions of CD8 + cells staining with the tetramers incorporating the FLRGRAYVL (0.12%), FLRGRAFGL (0.17%), or FLRSRAYGL (0.05%) peptides were not significantly greater than background levels, which is also consistent with the dominance of a single T cell clonotype in this donor and the use of the same TCR as LC13. Similar data were obtained with PBMCs from a second healthy EBV-sero + HLA B8 + donor who was known to carry a high frequency of FLRGRAYGL-reactive T cells that express the LC13 TCR (data not shown). Thus, tetramer staining of fresh peripheral T cells appears to be just as specific as the staining of cultured T cells.

This report has addressed any lingering doubts about the Ag specificity of the huge T cell expansions that have been detected using MHC-peptide tetramers. Although these past studies have included quite convincing specificity controls of lymphocytes from individuals unexposed to the relevant Ag, the numbers of these negative controls for each study have been quite limited. If MHC-peptide tetramers stained with broad specificity, chance cross-reactivity with irrelevant T cells leading to overestimates of epitope-specific T cell numbers may only have been expected in a proportion of individuals, particularly in adult humans, where large clonal CD8 + T cell expansions of unknown origin are often observed in the absence of acute infections (28). Such concerns, which prompted our detailed investigation into tetramer specificity, are certainly alleviated by the data presented herein.

The issue of tetramer fine specificity/degeneracy will be particularly critical if using this technology to analyze T cell responses to genetically unstable pathogens, such as HIV-1 or hepatitis C, in which immune-pressure-driven point mutations often arise within viral antigenic determinants during infection of a single host (24). The results of this study suggest that, regardless of the incubation temperature used, class I MHC-peptide tetramers should have the capacity to distinguish CTLs specific for a variant viral strain from those specific for the original infecting strain.

The recent report from Whelan et al. (24) has raised some doubts about the interpretation of previously published tetramer data by suggesting that staining of T cells with tetramers at 4°C, but not 37°C, leads to a degree of promiscuity in binding. HIV-specific CTL clones, which failed to recognize certain variants of their HIV target epitopes in cytotoxicity assays even at very high synthetic peptide concentrations, were shown to strongly stain with tetramers incorporating these variants when a 4°C incubation was used. In contrast, our EBV-reactive T cells failed to stain strongly with tetramers incorporating nonactivating peptides, regardless of whether tetramer incubations were performed at 4°C or 37°C. The basis for the inconsistency between these two studies is unclear.

This report should encourage the broad future use of class I MHC-peptide tetramers to quantify or isolate peptide-specific CTLs ex vivo in the laboratory and in the clinic. These reagents were specifically designed to bind T cells with far greater avidity than the sum of the individual monomeric affinities. Fortuitously...
and fortunately, this avidity level appears to be optimal for accurately reflecting the fine specificity of T cells for peptides presented on the cell surface at physiological levels.

References