Targeted deletion of T-cell clones using alpha-emitting suicide MHC tetramers

Rui Rong Yuan, Phillip Wong, Michael R. McDevitt, Ekaterina Doubrovina, Ingrid Leiner, William Bornmann, Richard O’Reilly, Eric G. Pamer, and David A. Scheinberg

Immunosuppressive agents in current use are nonspecific. The capacity to delete specific CD8+ T-cell clones of unique specificity could prove to be a powerful tool for dissecting the precise role of CD8+ T cells in human disease and could form the basis for a safe, highly selective therapy of autoimmune disorders. Major histocompatibility complex (MHC) tetramers (multimeric complexes capable of binding to specific CD8+ T-cell clones) were conjugated to 225Ac (an alpha-emitting atomic nanogenerator, capable of single-hit killing from the cell surface) to create an agent for CD8+ T-cell clonal deletion. The “suicide” tetramers specifically bound to, killed, and reduced the function of their cognate CD8+ T cells (either human anti–Epstein-Barr virus (EBV) or mouse anti–Listeria in 2 model systems) while leaving the nonspecific control CD8+ T-cell populations unharmed. Such an approach may allow a pathway to selective ablation of pathogenic T-cell clones ex vivo or in vivo without disturbing general immune function. (Blood. 2004;104:2397-2402)

Introduction

Immune recognition by CD8+ T cells is determined by binding of αβ T-cell receptors (TCRs) to target cell antigen-derived peptides displayed in the target’s major histocompatibility complex (MHC) class I molecule.1-4 These antigenic peptides can be nonnative peptide fragments derived from foreign viral or bacterial proteins, or derived from normal or mutated self proteins.5,6 Soluble tetrameric forms of peptide–MHC class I complexes can bind stably to the TCRs on a given specific CD8+ T-cell clone.7-9 Such specific tetramers, which are fluorescently tagged, have been used to identify or isolate antigen-specific T cells from peripheral blood and other tissues.10,11

High linear energy transfer (LET) alpha-particle emitters are of unique interest as cytototoxic agents because they need not be internalized to kill cells and are potent enough to selectively kill individual cells within a short range with a single decay. Bi-213 and At-211 alpha-emitting antibody constructs are in human cancer trials.12 Actinium-225 (225Ac) is an alpha-emitting atomic nanogenerator that yields 4 net alpha particle emissions.13-15 Monoclonal antibodies were conjugated to 225Ac for cancer therapy in animal models and have shown that small doses (nCi) of 225Ac-antibody conjugates are capable of eradicating tumors without significant toxicity.16 The characteristics of the alpha generators suggest that they also have unique interest as cytotoxic agents because they need not be internalized to kill cells and are potent enough to selectively kill specific T-cell clones.

In this report, we describe new techniques for making and using armed alpha-emitting tetramers capable of targeting specific peptide–MHC class I-restricted CD8+ T-cell clones. We used well-characterized CD8+ T-cell lines of human or murine origin to demonstrate that small doses of 225Ac-radiolabeled “suicide” tetramers were capable of killing targeted CD8+ T cells while leaving the nonspecific control CD8+ T-cell populations unharmed. This is the first description of a method to kill specific CD8+ T-cell clones.

Materials and methods

Human and mouse CD8+ T-cell lines

Human Epstein-Barr virus (EBV) or influenza virus peptide-specific CD8+ T-cell lines, and the mouse Listeria monocytogenes–specific CD8+ T-cell lines chosen for this study were established as described previously.16,18 The cell lines specific for the human EBV latency membrane protein 1 peptide (LMP1; YLLEMLWRL) and the influenza peptide (Flu58-66; GILGFVFTL) were generated from the HLA-A2 healthy donors. In brief, for the purpose of conducting the assays, T cells were isolated from peripheral blood by using Ficoll-Hypaque gradient separation of mononuclear cells followed by depletion of CD20+ B cells, CD14+ monocytes, and CD56+ natural killer (NK) cells with monoclonal antibody (mAb)-coated immunomagnetic beads (Miltenyl Biotec, Auburn, CA). The aliquots of lymphocyte population were stimulated in vitro by exposure to either irradiated autologous LMP1 or Flu58-66 peptide–loaded, EBV-transformed B cells and cultured in special lymphocyte medium (AIM-V medium; Gibco, Carlsbad, CA) containing 100 IU/mL interleukin 2 (IL-2; BD Biosciences, San Jose, CA). Cells were stimulated weekly and the enriched T-cell cultures were subsequently tested by anti-CD8 mAb and LMP1 tetramer or Flu58-66 tetramer flow cytometry for binding specificity. CD8 T-cell lines positive for tetramer binding were further stimulated and aliquots of the enriched human peptide–specific CD8 cells (> 90% pure) were used in this study. Murine LLO91-99 or p60217-225 specific CD8+ T-cell lines were established from BALB/c splenocytes 1 week after secondary immunization with a sublethal dose of Listeria19-22 The LLO91-99 (GYKDGNEYI) or p60217-225
(KYGVSVQDI) peptide-specific CD8+ T cells were maintained in RPMI medium containing 0.16 µg/mL IL-7 (BD Biosciences) and 0.5 ng/mL IL-2 at 37°C in 5% CO2. Tetramer binding specificity of the CD8+ T cells was reconfirmed by flow cytometry before each experiment. Tetramer binding to CD8+ cell lines was stable over several weeks when cells were maintained in culture with periodic exposure to peptide-pulsed antigen-presenting cells and fresh cytokines. This allowed us to utilize the same mouse cell line repeatedly for study.

**Peptide–MHC class I tetramer constructs**

Tetramers were prepared as previously described18,23 and provided by the Memorial Sloan-Kettering Cancer Center (MSKCC) Tetramer Core Facility. Briefly, recombinant HLA-A2 or H-2Kd and human β2 microglobulin produced in Escherichia coli were solubilized in urea and reacted with synthetic peptide antigens in a guanidine refolding buffer. The peptides used in this study were synthesized by ResGen (Huntsville, AL) and were more than 90% pure. Refolded peptide–MHC I complexes were purified and then biotinylated. Tetrameric peptide–MHC I complexes were subsequently produced by the stepwise addition of streptavidin-conjugated phycoerythrin (PE) to achieve a 1:4 molar ratio.

**Flow cytometry**

The binding specificity of tetramers was analyzed by flow cytometry using the human and mouse antigen-specific CD8 T-cell lines. Human LMP1- or Flt3L-specific CD8 T cells (1 × 105 in 100 µL) were stained with fluorescein isothiocyanate (FITC)-anti-CD8 antibody (BD Biosciences) and PE-labeled cognate peptide/HLA A2 tetramers at different concentrations at 4°C for 60 minutes. In addition, binding of Flt3L-dependent tetramers to LMP1 CD8 T cells and LMP; tetramers to Flt3L-specific CD8 T cells was employed to confirm the specificity of the tetramer staining. Flow cytometry using LLO91-99/H-2Kd or p60217-225/H-2Kd tetramers on mouse CD8 T-cell lines was also conducted using identical conditions. To determine the stability of tetramer binding to TCRs, CD8 T-cell lines were incubated with tetramers at 37°C for 1, 2, 3, 8, and 24 hours in phosphate-buffered saline (PBS) or in 100% serum, and tetramer binding was subsequently quantified by flow cytometry. Fractions of multimeric constructs were collected separately by size exclusion chromatography and binding of dimers, trimers, and tetrameristic MHC constructs to their cognate CD8 T cells was determined by flow cytometry.

**Preparation of 225Ac-DOTA-biotin and 111In-DTPA-biotin**

Radionuclide 111In was purchased from PerkinElmer Life Sciences (Billerica, MA) and 225Ac was obtained from the Oak Ridge National Laboratory (Oak Ridge, TN). The 225Ac nitrate residue was dissolved in 0.2 M Optima grade HCl (Fisher Scientific, Pittsburgh, PA) and biotinylated 1,4,7,10-tetraazacyclododecane-1,4,7,19-tetraacetic acid (biotin-DOTA) was prepared by the Organic Core Facility at MSKCC (W.B., June 2003, unpublished). Biotin-DOTA or biotinylated diethylenetriaminepentaacetic acid α, ω-bis (DTPA; Sigma, St Louis, MO) was dissolved in metal-free water to yield a 10 to 20 mg/mL solution. The same procedure was used to label either biotin-DOTA (1 mg) with 1 mCi (37 MBq) of 225Ac or biotin-DTPA (1 mg) with 2 mCi (74 MBq) of 111In. In brief, 5 µL to 20 µL 225Ac dissolved in 0.2 M HCl was added to a NUNC 1.8-mL reaction tube. One milligram of biotin-DOTA solution (100 µL) was dissolved in 0.2 M HCl, 50 µL of 2 M tetramethylethelimonium acetate, and 15 µL of 150 g/L l-ascorbic acid (Aldrich Chemical, Milwaukee, WI). The mixture (pH = 4.5-5.0) was then heated to 60°C for 30 minutes and the reaction was terminated by adding 20 µL of 0.10 M EDTA (ethylenediaminetetraacetic acid; Aldrich Chemical). The 111In-DTPA-biotin mixture was prepared without the heating step before termination.

To quantify incorporation of 225Ac or 111In radionuclide, 1 mL Sepadex C-25 resin (Aldrich Chemical) in 0.9% NaCl was packed into a column. A 2-µL aliquot of the radioactive reaction mixture was applied and the column was eluted with 3 mL of 0.9% NaCl. The column was eluted a second time to determine if all radioactivity had been removed. The column and washes were either counted immediately using a Squibb CRC-17 Radioisotope Calibrator (ER Squibb and Sons, Princeton, NJ) to measure 111In activity or counted 20 hours later to determine 225Ac activity levels. The activity contained in the eluate was considered to be the percent 111In or percent 225Ac that was complexed to the chelant moiety.

Biotin reactivity in the radiolabeled component was assayed after application of the radioactive reaction mixture to an immobilized avidin column (Pierce, Rockford, IL). The column was washed twice with 5 mL of 0.9% NaCl to remove unbound material and the column and washes were counted to determine the 111In or 225Ac activity using the same method previously described in the previous paragraph. The percent activity bound to the column was considered to be the percent 111In or percent 225Ac that contained biotin-avidin binding reactivity.

**Radiolabeled tetramer constructs**

111In has a relatively short half-life (~ 3 days), and it was selected for use in our early experiments to establish the optimal conditions for tetramer labeling. The freshly prepared 111In-DTPA-biotin products were mixed with freshly prepared biotinylated monomers in the presence of streptavidin at a ratio of 1:3:1 in order to construct radiolabeled tetramers. The product was further purified by size exclusion chromatography using a 10-mL Econo-Pac 10DG column (BioRad, Hercules, CA) with a PBS mobile phase. Both radiolabeled-specific and non-specific tetramers were prepared in this fashion for our in vitro studies. In addition, non-radiolabeled tetramers used for controls or blocking experiments were similarly prepared.

**Specific binding and internalization of radiolabeled tetramers**

Different dilutions of radiolabeled tetramers in 5 to 8 µL PBS were added to 1 × 106 CD8+ T cells on ice. To determine the influence of incubation temperature on radiolabeled tetramer binding or tetramer internalization, cells were incubated with either radiolabeled tetramers on ice or at 37°C for 1 hour and 4 hours and overnight. The cells were then centrifuged, washed twice with 1 mL ice-cold PBS, and subjected to counting to determine the amount of specific 111In tetramer binding. To quantify internalization of radiolabeled tetramers, the cell surface–bound radiolabeled tetramers were stripped from the cell pellet by exposure to 1 mL of 50 mM glycine/150 mM NaCl (pH 2.8) for 10 to 15 minutes at room temperature.24 The quantity of surface-bound and internalized radioactivity was determined by counting the samples separately. Both radiolabeled nonspecific tetramers and CD8 T-cell lines bearing TCRs of different peptide specificities served as controls for this assay. All assays were performed in duplicate.

**Specific cell killing by 225Ac-labeled tetramers**

The killing efficacy of suicide tetramers was quantified using 1 × 106 LMP1- or LLO91-99-specific CD8+ T cells in 96-well plates. Flt3L-specific or p60217-225-specific CD8+ T cells served as negative controls. Serial dilutions of 225Ac-tetramers were added to the CD8+ T cells and nonspecific cell killing was determined by adding only 225Ac-DOTA or only nondirolabeled tetramers. To confirm specificity of cell killing, some cells were first incubated with a 50-fold excess of nondirolabeled specific tetramers for 30 minutes before addition of suicide tetramers. The cells were incubated at 37°C in 5% CO2 for 72 hours and cell viability was subsequently determined by [3H]thymidine incorporation and trypan blue staining. Each assay was performed in triplicate.

In order to further demonstrate that 225Ac-suicide tetramer killing was T-cell specific, the 225Ac-LLO91-99 tetramers were added to a mixed cell culture of LLO91-99-specific CD8+ T cells and LLO tetramer-negative, p60217-225-specific CD8+ T cells. Serial dilutions of suicide 225Ac-LLO91-99 tetramers were added to the cell mixture (50:50) containing LLO91-99 CD8+ cells and p60217-225-specific CD8+ cells. Cell viability was subsequently determined by both trypan blue staining and [3H]thymidine incorporation after incubation at 37°C in 5% CO2 for 72 hours. The remaining viable CD8+ T cells were washed and then restudied by tetramer flow cytometry to define their specificities. Each assay was performed in triplicate.

**IFN-γ ELISPOT assay**

The interferon γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay was performed in nitrocellulose-lined 96-well microplates (Millipore...
with antibody to murine IFN-γ/H9253 cells bound to both anti-CD8 mAb and LLO 91-99 tetramers. Similarly, tetramers and none of these CD8 cells reacted with LMP 1 /HLA-A2 tetramers. (C) The panel shows that 87% of Flu-specific CD8 T cells bound to Flu58-66/HLA-A2 tetramers. (D) Less than 1% of the human Flu-specific CD8+ T cells tested positive for the LMP 1 /HLA-A2 tetramers. (E) The panel shows that 0% normal mouse spleen CD8 T cells bound the LLO 91-99 /H-2K d tetramers. (F) More than 80% of the mouse splenic CD8 T-cell line bound LLO 91-99 /H-2K d tetramers. (G) The panel shows that 90% of the p60 217-225-specific T-cell line tested positive for p60 217-225 tetramer staining. (H) Only mildly decreased binding to their cognate CD8 T cells by dimeric/trimeric compared with trimeric/tetrameric MHC constructs was observed.

Statistics
Statistical analyses were performed using one-way analysis of variance (ANOVA) on GraphPad Instat 3.0 (GraphPad Software, San Diego, CA).

Results
Peptide-specific tetramers bind CD8+ T-cell lines with high specificity

Human and mouse peptide-specific CD8 T-cell lines were established in order to assess specific cell-killing efficacy of our radiolabeled tetramers. The binding specificity of individual nonradiolabeled tetramers was first analyzed by flow cytometry. Only 0.02% of the EBV-negative healthy human peripheral blood mononuclear cells (PBMCs) tested positive for LMP 1 /HLA-A2 tetramers. (B) The panel shows that 92% of LMP 1-specific CD8 T cells bound to LMP 1 /HLA-A2 tetramers. (C) The panel shows that 87% of Flu-specific CD8 T cells bound to Flu58-66/HLA-A2 tetramers. (D) Less than 1% of the human Flu-specific CD8+ T cells tested positive for the LMP 1 /HLA-A2 tetramers. (E) The panel shows that 0% normal mouse spleen CD8 T cells bound the LLO 91-99 /H-2K d tetramers. (F) More than 80% of the mouse splenic CD8 T-cell line bound LLO 91-99 /H-2K d tetramers. (G) The panel shows that 90% of the p60 217-225-specific T-cell line tested positive for p60 217-225 tetramer staining. (H) Only mildly decreased binding to their cognate CD8+ T cells by dimeric/trimeric compared with trimeric/tetrameric MHC constructs was observed.

Table 1. Stability of tetramer binding to CD8+ T-cell receptors at 37°C

<table>
<thead>
<tr>
<th>CD8 T cells/tetramers (medium)</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h, %</td>
</tr>
<tr>
<td>LMP1/LMP1, (PBS)</td>
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</tr>
<tr>
<td>Flu58-66/LMP1, (PBS)</td>
<td>0.9</td>
</tr>
<tr>
<td>LLO2/5/LLO2/5 (PBS)</td>
<td>68</td>
</tr>
<tr>
<td>LMP1/LMP1, (serum)</td>
<td>98</td>
</tr>
<tr>
<td>LLO2/5/LLO2/5 (serum)</td>
<td>88</td>
</tr>
</tbody>
</table>

— indicates not determined.
The $^{111}$In-radiolabeled multimers were incubated with cognate and control CD8$^+$ T-cell lines to determine if they displayed specific binding and to determine their binding kinetics and internalization. $^{111}$In-labeled LMP$^1_1$ tetramers exhibited dose-dependent specific binding to the LMP$^1_1$ CD8$^+$ clone (Figure 2A). In contrast, there was little binding of $^{111}$In-LMP$^1_1$ tetramers to the Flu-specific control CD8$^+$ T cells. As another control, $^{111}$In-Flu tetramers showed little binding to LMP$^1_1$ CD8$^+$ T cells even at very high concentrations. These results strongly indicated that the peptide-specific tetramers could be successfully radiolabeled with maintenance of their binding specificity for their cognate CD8$^+$ T cells. These observations were confirmed in the mouse model by a similar experiment testing $^{111}$In-labeled LMP$^1_1$ tetramer binding against murine LLO$^{91-99}$-specific CD8$^+$ T cells (Figure 2B).

While efficacy of killing by the alpha-emitting elements should be increased if the armed tetramers are internalized, it is not a prerequisite for killing. Time-dependent tetramer internalization of the tetramers was seen, but only small amounts (6%-8%) of $^{111}$In-LMP$^1_1$ tetramers were internalized after a 1-hour incubation at 37°C when tested on LMP$^1_1$ CD8$^+$ T cells. Maximum internalization (~22%) was achieved with a 24-hour incubation (Figure 2C).

**Specific killing of CD8$^+$ T cells by armed $^{225}$Ac-labeled tetramers**

$^{225}$Ac, a radioiodine atomic generator that decays to yield 4 net alpha particles, can be stably conjugated to proteins by use of bifunctional DOTA chelates. Therefore, using the MHC multimer radiolabeling method that was developed for $^{111}$In tracing (see the previous section), we next constructed radiolabeled suicide tetramers armed with $^{225}$Ac generators. Biotinylated DOTA was labeled with $^{225}$Ac at high yields (>96%). The armed $^{225}$Ac-LMP$^1_1$ tetramers effectively killed the targeted LMP$^1_1$ CD8$^+$ T-cell clones at small doses (ED$_{50}$ = 5.8 nCi/mL or 1.16 µg/mL; Figure 3A). In contrast, the armed $^{225}$Ac-LMP$^1_1$ tetramers at 5 nCi/mL to 8 nCi/mL exhibited much less toxicity to control Flu-specific CD8$^+$ T cells. Nonselective cytotoxicity was induced at 15- to 40-fold-higher doses (ED$_{50}$ = 110-200 nCi) when using $^{225}$Ac DOTA alone. Much higher levels of unlabeled specific LMP$^1_1$ tetramers (100-140 µg/mL) were required to induce mild cytotoxicity in targeted CD8$^+$ T cells. In the murine system, similar high-potency specific cell killing by suicide LLO$^{91-99}$ tetramers was also demonstrated. LLO$^{91-99}$ peptide-specific CD8$^+$ T cells were effectively killed after incubation with $^{225}$Ac-LLO$^{91-99}$ tetramers (Figure 3B); as another specificity control, the T-cell killing by suicide LLO$^{91-99}$ tetramers was partially blocked by the addition of a 50-fold excess of unlabeled LLO$^{91-99}$-specific tetramers.

To validate that the cell selectivity of killing by $^{225}$Ac-LLO$^{91-99}$ tetramers of LLO$^{91-99}$-peptide–specific CD8$^+$ T cells within a mixture of possible target cells (as might occur in most applications), $^{225}$Ac-LLO$^{91-99}$ tetramers at concentrations of 1 nCi/mL to 30 nCi/mL (0.26-6 µg/mL) were added to mixed cultures of LLO$^{91-99}$-specific CD8$^+$ and p60$^{217-225}$-specific CD8$^+$ T cells. After a 72-hour incubation with $^{225}$Ac-LLO$^{91-99}$ tetramers, significant cell killing was demonstrated in the whole population as judged by [H]$^3$Hthymidine incorporation and confirmed by reductions in viable cells determined with trypan blue staining. When the remaining viable cells in the population were analyzed by tetramer flow cytometry to define their specificities, there was a significant reduction in LLO$^{91-99}$-specific CD8$^+$ T cells ($P < .001$; Figure 3C). In contrast, the nontargeted p60$^{217-225}$-specific CD8$^+$ T-cell population in the mixed cell culture showed only modest reductions even when exposed to higher quantities of $^{225}$Ac-LLO$^{91-99}$ tetramer (10 nCi/mL or 2 µg/mL).

Functional assays confirmed a significant reduction in the level of IFN-γ secretion in suicide tetramer–treated LLO$^{91-99}$ CD8$^+$ T cells when compared with the nontreated control T cells (Figure 3D). In the control p60$^{217-225}$-specific CD8 T-cell population, modest nonspecific reduction in IFN-γ secretion was observed. These results demonstrate that armed tetramers can selectively delete both numbers and function of specific cytotoxic T lymphocytes (CTLs) with high specificity and induce little cytotoxicity within the other CD8$^+$ T-cell populations, mimicking most applications envisioned.

**Discussion**

The choice of agents to arm MHC tetramers for specific cytotoxicity is limited. The selected agent must be capable of extraordinary potency, killing with a limited number of bound tetramers, most or all of which remain outside the targeted T cell. In addition, the cytotoxic effect must be of short range to allow selective killing of only the individual targeted...
cognate T cell while sparing bystander cells. As a consequence, nearly all drugs and toxins will be weakly effective. Among the radioisotopes, only alpha-emitting nuclides offer a solution. The $^{225}$Ac isotope (an atomic monolabeled tetramer) has a 10-day half-life, emits one alpha, and is the parent molecule in a decay cascade that produces a net of 6 new elements and 4 alpha particles. $^{225}$Ac-radiolabeled monoclonal antibodies have been proposed for use in cancer therapy, as these targeted nanogenators are capable of specifically killing individual cancer cells at extremely low doses without significant toxicity. Because it takes several hours to prepare radiolabeled tetramers, the much shorter half-life of other alpha particle emitters of possible clinical utility (At-211, Bi-213) may limit their potential application for these purposes. These initial experiments now can be extended to study clonal deletion of antigen-specific CD8 $^+T$ cells in vivo. Alternatively, this strategy may hold potential for ex vivo purging of specific CTLs prior to bone marrow and stem cell transplantation to prevent graft-versus-host disease. Such an approach would require identification of key immunodominant targets in the human system, which are currently unknown. Previous studies showed that soluble MHC tetramers injected into mice could modulate antigen-specific T-cell response in vivo, suggesting stability in vivo. Moreover, our previous work with the alpha-emitting Bi-213–labeled antibodies has demonstrated the ability to target hematopoietic cells in the bone marrow, liver, spleen, and blood within 10 minutes, suggesting that significant depletions within these compartments in a short time frame is possible. However, the induction of clonal deletion of T cells distant from the well-vascularized organs in vivo by suicide tetramers may be limited by structural instability of the molecules; techniques to stabilize the constructs may be necessary. Predictions as to the consequences of the rapid expected clearance of these molecules from plasma are difficult without in vivo study. These methods for selective T-cell clonal deletion may allow advances in our understanding of the role of antigen-specific CD8 $^+T$ cells in diverse disease processes including infection, autoimmune disease, and other CD8 $^+T$-mediated disorders and may provide a pathway to possible T-cell–selective therapies of autoimmune or neoplastic origin.

Acknowledgments
We thank Patrick Guirnalda and Eva Menet for technical assistance.
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