Soluble Major Histocompatibility Complex-Peptide Octamers with Impaired CD8 Binding Selectively Induce Fas-dependent Apoptosis

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Fluorescence-labeled soluble major histocompatibility complex class I-peptide “tetramers” constitute a powerful tool to detect and isolate antigen-specific CD8⁺ T cells by flow cytometry. Conventional “tetramers” are prepared by refolding of heavy and light chains with a specific peptide, enzymatic biotinylation at an added C-terminal biotinylation sequence, and “tetramerization” by reaction with phycoerythrin- or allophycocyanin-labeled avidin derivatives. We show here that such preparations are heterogeneous and describe a new procedure that allows the preparation of homogeneous tetra- or octameric major histocompatibility complex-p peptide complexes. These compounds were tested on T1 cytotoxic T lymphocytes (CTLs), which recognize the Plasmodium berghei circumsporozoite peptide 252–260 (SYIPSAEKI) containing photoreactive 4-azidobenzoic acid; H9252. We report that mutation of the CD8 binding site of Kd greatly impairs the binding of tetrameric but not octameric or multimeric Kd-PbCS(ABA) complexes to CTLs. This mutation abolishes the ability of the octamer to elicit significant phosphorylation of CD8, intracellular calcium mobilization, and CTL degranulation. Remarkably, however, this octamer efficiently activates CTLs for Fas (CD95)-dependent apoptosis.

CD8⁺ T cells and thymocytes recognize with their T-cell antigen receptor (TCR) cognate MHC-peptide complexes on the surface of antigen-presenting cells (1). CD8, by binding to the constant domain of MHC class I molecules, can increase the avidity of TCR-ligand binding but can also act as an adhesion molecule and strengthen CTL-target cell conjugate formation (1–3). The coordinate binding of CD8 to TCR-associated MHC molecules brings CD8-associated Lck to TCR/CD8, which promotes tyrosine phosphorylation of its immunoreceptor tyrosine-based activation motifs, which is an initial crucial event of TCR-mediated T-cell activation (1–4). The CD8 binding site on MHC class I molecules contains an acidic loop (residues 222–229), and charge inversion in position 227 (e.g. KdD227K) impairs CD8 binding by about 85% (3, 4).

The recognition of sensitized target cells by CD8⁺ CTLs involves rapid and avid conjugate formation, followed by CTL degranulation in the contact zone, resulting in perforin/granzyme-mediated target cell killing (5). With a slower kinetic, CTLs also express surface Fas ligand, which, by interacting with Fas on other cells, induces Fas-mediated cytotoxicity (5, 6). Although perforin-mediated cytotoxicity and Fas-mediated cytotoxicity are both induced by TCR triggering, the activation requirements are very different. For example, certain altered peptide ligands that are unable to elicit perforin-dependent cytotoxicity or cytokine production can efficiently induce Fas-mediated killing (7, 8). We observed previously that blocking of CD8 greatly impairs calcium mobilization, degranulation of CTL, and IFN-γ release but has no effect on Fas-dependent cytotoxicity (8). More recently, it has been shown that antigen-presenting cells or microspheres expressing MHC-peptide complexes with ablated CD8 binding selectively induce Fas-mediated apoptosis of CTLs (9).

The idea of eradicating antigen-specific CD8⁺ T cells or thymocytes via Fas-dependent apoptosis is attractive because it takes place in the absence of full, potentially harmful T-cell activation. Because in vivo application of blocking CD8 by antibodies or the use of MHC-peptide-coated microspheres is risky, we investigated here whether soluble MHC-peptide complexes with impaired CD8 binding can be used to the same end.

Soluble fluorescence-labeled MHC class I-peptide multimers, so-called “tetramers,” are widely used for the detection and isolation of antigen-specific CD8⁺ T cells (10, 11). The conventional way to prepare such molecules involves enzymatic biotinylation of an added C-terminal biotinylation sequence (BSP) with the biotin ligase BirA (12). The biotinylated MHC-peptide monomers are then reacted with phycoerythrin (PE)- or allophycocyanin-labeled avidin derivatives. Although PE and allophycocyanin are extremely high fluorescence intensities, their conjugates with avidin are heterogeneous. Because of their large size, their conjugation with streptavidin yields ill-defined mixtures of conjugates with different stoichiometries and configurations (the molecular size of unconjugated PE is about 240,000 Da, the molecular mass of allophycocyanin is about 104,000 Da, and the molecular mass of streptavidin is about 60,000 Da). In consequence, saturation of such conjugates with biotinylated...
MHC-peptide complexes result in heterogeneous MHC-peptide complexes, referred to as multimers. To prepare well-defined soluble MHC class I-peptide complexes of different sizes, we introduced by point mutation a free cysteine at the C terminus of the MHC heavy chain, which can be alkylated with biotin containing iodoacetamide or maleimide derivatives. For oligomerization, we used homogeneous streptavidin conjugates containing the low molecular weight fluorochrome Cychrome Cy5. By using a branched peptide containing one biotin and two maleimide moieties (DMGS), this peptide allowed (1) the preparation of oligomeric MHC class I-peptide complexes that co-engage or do not co-engage CD8 and their ability to activate perforin- and Fas-dependent cytotoxicity on cloned T1 CTLs. T1 CTLs recognize the Phbs peptide 252–260 (SYTIPSAEK) containing photoreactive 4-azidobenzoic acid on Lys\textsuperscript{359} (Phbs(ABA)) in the context of K\textsuperscript{d} (4, 13). We find that the binding of tetrameric but not octameric or multimeric K\textsuperscript{d} Phbs(ABA) complexes to T1 CTLs is greatly reduced when CD8 co-engagement is ablated by the charge inversion K\textsuperscript{d}-DMGS. Although K\textsuperscript{d}-DMGS-biotin (Phbs(ABA)) complexes fail to elicit significant tissue phosphorylation, calcium mobilization, and degranulation, they efficiently induced Fas-dependent cytotoxicity.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—The branched DMGS-biotin peptide was synthesized using conventional solid phase Fmoc strategy. Biotin was introduced by using Fmoc-Res-Ahx-biotin (Bachem AG, Bubendorf, Switzerland). The protected G8S-biotin peptide was purified by GFC on a Superdex S75 column (150 cm × 30 cm; Pharmacia) and reacted with N\textgamma-\textgamma-maleimidobutyryllysycussinicinamide ester (GMBS; Pierce) in DMSO containing 1% di-isopropyl-ethylamine. The resulting di-maleimide conjugate was purified by high pressure liquid chromatography on a C18 reverse phase column (2 × 30 cm; Machery & Nagel, Oensingen, Switzerland). The column was eluted with 0.1 trifluoroacetic acid and a linear gradient of acetonitril in 1 h from 0 to 75%. The purified DMGS-biotin peptide eluted at 26.4 min, and the mono-maleimide derivative eluted at 25.3 min. All products had the correct molecular weight as assessed by mass spectrometry. Iodoacetyl-PEO-biotin was from Pierce, and SYTIPSAEK(ABA)I (Phbs(ABA)) was prepared by the conventional Fmoc-Lys(ABA) (Bachem) following the solid phase Fmoc strategy using Fmoc-Lys(ABA) (Bachem) following a 24h deprotection GS-biotin peptide was purified by GFC on a Superdex 200 column (150 cm × 30 cm; Pharmacia), which was eluted in Tris buffer containing 5 mM glutathione to reduce the C-terminal free cysteine. After GFC over a Superdex S75 column (150 cm × 30 cm; Pharmacia), the reduced monomers were reacted in Tris buffer containing 15 mM glutathione to reduce the C-terminal free cysteine. The Coomassie Blue-stained gels were evaluated by densitometry, and the alkylation efficiency, calculated as a percentage, was as follows: 90%.

**Preparation of Fluorescence-labeled K\textsuperscript{d}-Peptide Oligomers—**K\textsuperscript{d}-peptide tetramers and octamers were obtained by reacting the biotinylated K\textsuperscript{d}-peptide complexes with 20% ethyl-2imidazolidinone. The resulting di-maleimide conjugate was purified by gel filtration on a Superdex S75 column. Dimeric K\textsuperscript{d}-DMGS-biotin complexes were obtained by reacting monomeric K\textsuperscript{d}-DMGS-biotin with a 2-fold excess of K\textsuperscript{d}-cysteine monomers. Dimeric K\textsuperscript{d}-peptide complexes were separated from monomers by anion exchange chromatography on a Resource Q fast flow anion exchange column (Amersham Biosciences). The biotinylation efficiency was determined by SDS-PAGE (15%, non-reducing) and stained with Coomassie Blue. The Coomassie Blue-stained gels were evaluated by densitometry, and the alkylation efficiency, calculated as a percentage, was as follows: (amount of avidin-bound K\textsuperscript{d}-peptide/amount of K\textsuperscript{d}-peptide) × 100.

**Preparation of Fluorescence-labeled K\textsuperscript{d}-Peptide Oligomers—**K\textsuperscript{d}-peptide tetramers and octamers were obtained by reacting the biotinylated K\textsuperscript{d}-Phbs(ABA) complexes with Cy5-labeled streptavidin (Amersham Biosciences), and the multimers were obtained by reacting biotinylated K\textsuperscript{d}-Phbs(ABA) monomers with Extravidin-PE (Sigma) at a molar ratio of 4:1. Labeled oligomers were purified by GFC on a Superdex 200 column (150 cm × 30 cm; Pharmacia), which was eluted in Tris buffer (20 mM, pH 8.0, 150 mM NaCl) at a flow rate of 0.7 ml/min.

**Preparation of Fluorescence-labeled K\textsuperscript{d}-Peptide Oligomers—**Bystander Cytolytic Assay—T1 CTLs (5 × 10\textsuperscript{6} cells/ml) were incubated in Dulbecco’s modified Eagle’s medium (Invitrogen) at 37 °C with 10 μg/ml human TNFα, 10 μM Human α2M, and 10 μg/ml human IFNγ. After 3 h, the CTLs were incubated in 96-well plates (15,000 cells/well) with [Cr] labeled P815 cells (5,000 cells/well) at 37 °C for 4 h. The specific chromium release, calculated as a percentage, was as follows: (experimental release – spontaneous release)/total release × 100.
100. The total release was the esterase content after lysis of the cells with 1% Triton X-100.

**Esterase Release**—T1 CTLs were adhered in 96-well plates previously coated with super fibronectin (Sigma) and incubated in OptiMEM medium (Invitrogen) supplemented with 2 µg/ml human β2m and 10 µM PbCS 252–262 peptide for 90 min with 25 nM K<sup>D</sup>-PbCS(ABA) octamer in the absence or presence of 100 nM concanamycin A (Sigma), anti-CD8<sup>α</sup> mAb H55 (10 µg/ml), or PbCS(ABA) peptide (1 µM). Released esterases were measured in the supernatants as described previously (16). All incubations were performed in triplicates.

**Immunoprecipitation and Western Blotting**—T1 CTLs (1 x 10<sup>5</sup> cells/ml) were incubated or not incubated with K<sup>D</sup>-PbCS(ABA) complexes (25 nM) for 5 min at 37 °C. After washing with chilled phosphate-buffered saline, the cells were lysed on ice for 1 h in phosphate-buffered saline containing Brj78 (1%) and protease inhibitor mixture (Roche Molecular Biochemicals), and from the detergent-soluble fraction, TCR/CD3 was immunoprecipitated with anti-TCR mAb H57. The immunoprecipitates were resolved on SDS-PAGE (15%, reducing) and Western blotted using anti-phosphotyrosine mAb 4G10 or anti-CD3ε antibody. For detection, the enhanced chemiluminescence Western blotting detection kit (ECL, Amersham Biosciences) was used as recommended by the supplier.

**Confocal Microscopy**—T1 CTLs were incubated with Cy5-labeled K<sup>D</sup>-PbCS(ABA), K<sup>D227K</sup>-PbCS(ABA), or K<sup>Cw3</sup> 170–179 octamer for 30 min at 37 °C or 18 °C. After washing, cells were fixed with 3% paraformaldehyde for 10 min at room temperature and laid onto poly-L-lysine-coated slides for 10 min and mounted. Internalization of Cy5-conjugated octamer was analyzed by confocal microscopy on an Axiovert 100 microscope (LSM510; Carl Zeiss, Jena, Germany) with a ×63 oil objective. Cy5 fluorescence was measured upon excitation with neon/helium laser at 633 nm. Each image was the average of four scans.

Digital images were prepared using Adobe Photoshop.

**Apoptosis Assay**—T1 CTLs (0.5 x 10<sup>5</sup> cells/ml) were resuspended in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and 20 nM HEPES and incubated in 50-µl aliquots at 37 °C for 30 min with 25 nM K<sup>D</sup>-PbCS(ABA), K<sup>D227K</sup>-PbCS(ABA), or K<sup>Cw3</sup> 170–179 octamers or left untreated. After one wash, the cells were incubated for 4.5 h in the same medium, stained with Cy5-labeled annexin V, and analyzed by FACS.

**Intracellular Calcium Mobilization**—T1 CTLs (1 x 10<sup>5</sup> cells/ml) were incubated with 5 µM Indo-1 (Sigma) at 37 °C for 45 min, washed, and incubated at 37 °C with 25 nM K<sup>D</sup>-peptide octamers or medium, and calcium-dependent Indo-1 fluorescence was measured by FACS on a FACStar as described previously (14).

**RESULTS**

**Preparation of Fluorescence-labeled Soluble K<sup>D</sup>-Peptide Complexes**—We prepared and examined three different types of soluble K<sup>D</sup>-peptide complexes: multimers, tetramers, and octamers. All complexes were produced with either wild type K<sup>D</sup> or K<sup>D227K</sup>, which has greatly impaired CD8 binding (4).

Monomeric K<sup>D</sup>-PbCS(ABA) complexes were obtained by refolding of different K<sup>D</sup> heavy chains and human β2m in the presence of PbCS(ABA) peptide. The refolding efficiency of K<sup>D</sup>-PbCS(ABA) complexes containing the heavy chain comprising residues 1–277 or the heavy chain containing an added BSP–residues 1–270 or the heavy chain containing an added BSP–residues 1–277 (Fig. 1A) of the K<sup>D</sup> heavy chain, in which residue 273, 275, or 277 or the heavy chain containing an added BSP–residues 1–270 or the heavy chain containing an added BSP–residues 1–277 (Fig. 1B) of the K<sup>D</sup> heavy chain, in which residue 273, 275, or 277 (triangles) was mutated to cysteine. Structures of iodooctyl-PEO-biotin (C) and DMGS-biotin (D).

**RESULTS**

**Preparation of Fluorescence-labeled Soluble K<sup>D</sup>-Peptide Complexes**—We prepared and examined three different types of soluble K<sup>D</sup>-peptide complexes: multimers, tetramers, and octamers. All complexes were produced with either wild type K<sup>D</sup> or K<sup>D227K</sup>, which has greatly impaired CD8 binding (4).

Monomeric K<sup>D</sup>-PbCS(ABA) complexes were obtained by refolding of different K<sup>D</sup> heavy chains and human β2m in the presence of PbCS(ABA) peptide. The refolding efficiency of K<sup>D</sup>-PbCS(ABA) complexes containing the heavy chain comprising residues 1–277 or the heavy chain containing an added BSP sequence was, on average, 20% (Fig. 1A). The BSP-containing K<sup>D</sup>-PbCS(ABA) complexes were biotinylated by using the biotin ligase BirA (12). The efficiency of the biotinylation was 70–80%, and the efficiency of the refolding of K<sup>D</sup>-PbCS(ABA) or K<sup>D</sup>-PbCS(ABA)-BSP complexes, on average, was 20% (Fig. 1S).

Alternatively, biotinylation was accomplished by site-specific alkylation of a free cysteine introduced by point mutation at the C-terminal portion of the K<sup>D</sup> heavy chain. To find out what position is most suitable, a free cysteine was introduced in position 273, 275, or 277 (Fig. 1B). The former two flank the conserved Trpr<sup>274</sup>, which is the last residue of the folded α3 domain of MHC class I molecules (17). Refolding under the same conditions gave yields of 14% for K<sup>D</sup>K275C, 12% for K<sup>D</sup>R273C, and 6% for K<sup>D</sup>A277C (Fig. 1S, A). The refolding efficiency of K<sup>D</sup>K275C was increased to 20% when 0.3 mM dithiothreitol was added to the urea buffer.

The alkylation efficiency of the different K<sup>D</sup>-PbCS(ABA) cysteine mutants was assessed after incubation at 4 °C overnight with a 5-fold molar excess of iodoacetyl-PEO-biotin (Fig. 1C).

The alkylation efficiency was 16% for K<sup>D</sup>R273C, 24% for K<sup>D</sup>K275C, and 12% for K<sup>D</sup>A277C (Fig. 1S, B). Based on these results, K<sup>D</sup>K275C was selected. Its alkylation efficiency was increased to about 80% upon reduction of K<sup>D</sup>K275C-PbCS(ABA) complexes with 15 mM glutathione before the alkylation (Fig 1S, C). The alkylation was selective for the free cysteine because K<sup>D</sup>-PbCS(ABA) wild type complexes were not significantly alkylated (Fig. 1S, B and C). A slightly higher alkylation efficiency (about 85%) was obtained for the monoalkylation of K<sup>D</sup>K275C-PbCS(ABA) complexes with bi-maleimide-biotin reagent DMGS-biotin (Fig. 1D). The purified monoalkylated K<sup>D</sup>-DMGS-biotin complexes were then reacted with a 2-fold molar excess of reduced K<sup>D</sup>K275C-PbCS(ABA). The efficiency for this reaction was 60–70%. Together, these results show that K<sup>D</sup>K275C can be refolded and biotinylated by alkylation with the same efficiencies as by the BSP/BirA strategy. The same findings were obtained for HLA-A2 (data not shown).

Defined K<sup>D</sup>-peptide tetramers and octamers were obtained by reacting K<sup>D</sup>-peptide-PEO-biotin and dimeric K<sup>D</sup>-peptide-DMGS-biotin complexes with homogeneous Cy5-labeled streptavidin. For multimeric K<sup>D</sup>-peptide complexes, K<sup>D</sup>-BSP-biotin-peptide monomers were reacted with heterogeneous PE-extravidin. The different compounds were analyzed by gel filtration on a Superdex S200 column and anion exchange chromatography on a Source 15Q column, respectively. As shown in Fig. 2, the K<sup>D</sup>-PbCS(ABA) monomers, dimers, tetramers, and octamers were homogenous, except for some minor contaminants, and eluted in both types of chromatography as expected. By contrast, the PE-labeled K<sup>D</sup>-PbCS(ABA) multimers eluted in a heterogeneous manner from the anion exchange column. The majority of the PE-labeled K<sup>D</sup>-PbCS(ABA) multimers eluted from the Superdex S200 column at around 10 min, i.e. in the void volume. Because the size exclusion of this column is about M<sub>e</sub> 600,000, and PE-extravidin K<sup>D</sup>-peptide tetramers have a M<sub>e</sub> of about 470,000, this preparation contained mainly conjugates that were larger than tetramers containing one PE. In agreement with this is the late elution of these complexes from the anion exchange column. The same results were obtained for the K<sup>D</sup>-peptide complexes containing the D227K mutation (data not shown).
Binding of Soluble K\textsuperscript{d}-Peptide Complexes to T1 CTLs—To study the binding of Cy5-labeled K\textsuperscript{d}-peptide complexes, we first measured the binding kinetics of the K\textsuperscript{d}-PbCS(ABA) octamers and multimers. As shown in Fig. 3, A–D, the binding of both complexes was rapid at 37 °C, 18 °C, and 4 °C, with ≥90% of maximal binding reached within the first few minutes of incubation. For octamer at 37 °C (Fig. 3A), but not at 18 °C or 4 °C (Fig. 3, C and D), a transient binding maximum was observed at about 10 min, followed by a modest decrease, to reach a stable plateau after 1 h. A similar biphasic binding kinetics was recorded at 37 °C for K\textsuperscript{d}-PbCS(ABA) monomer and tetramer (data not shown) (4). In all cases, the binding of noncognate K\textsuperscript{d}-Cw3 170–179 octamer or multimer was no more than a few percent of the binding of the corresponding cognate complexes, indicating that nonspecific binding, namely, binding to CD8 under these conditions, is insignificant.

We next assessed the binding isotherms at 37 °C and 18 °C for the compounds under study (Fig. 3, E–H). At 37 °C, the binding of Cy5-labeled K\textsuperscript{d}-PbCS(ABA) octamers increased steeply in the concentration range of up to 30 nM and then gradually up to 100 nM, the highest concentration tested (Fig. 3E). By contrast, for the tetramer, much lower levels of binding were observed, and the increase in binding required higher concentrations. For the corresponding K\textsuperscript{d}-D227K-PbCS(ABA) complexes, the binding of the octamer was reduced by about 10%, but binding of the tetramer was close to the background binding observed for the noncognate K\textsuperscript{d}-Cw3 171–179 peptide complexes. A similar binding pattern was observed at 18 °C, except that nearly maximal binding was reached already at 12.5 nM for the octamer and at 30 nM for the tetramer (Fig. 3G). This difference seems mainly accounted for by endocytosis, which is taking place at 37 °C, but not at 18 °C (see below).

The binding of PE-labeled multimers increased continuously over the range of concentrations tested at 37 °C (Fig. 3F) and at 18 °C (Fig. 3H). Strikingly, at both temperatures, K\textsuperscript{d} wild type and K\textsuperscript{d}-D227K complexes exhibited about the same binding...
was a time-dependent down-modulation of TCR and CD8 of about 40% after 1 h of incubation with Kd-PbCS(ABA) octamer (Fig. 4E). The Kd-D227K octamer induced the same TCR down-modulation, but the down-modulation of CD8 was less than half (18% after 1 h; Fig. 4F).

To visualize endocytosis of Cy5-labeled Kd-PbCS(ABA) and Kd-D227K-PbCS(ABA) octamers, they were incubated with T1 CTLs for 30 min at 18 °C or 37 °C and analyzed by confocal microscopy. As shown in Fig. 4G, at 18 °C, Kd-PbCS(ABA) complexes were distributed on the cell surface in a wide cap. This was also true for the Kd-D227K-PbCS(ABA) octamer, but the cap formation was less pronounced. By contrast, at 37 °C, the majority of Kd-PbCS(ABA) complexes were internalized in the form of a bright patch. For Kd-D227K-PbCS(ABA), about half of the complexes were localized in a wide cap on the surface, and half were internalized in patch, distal to the cap. In the presence of Fab' fragments of the anti-Kd mAb SF1–1.1.1, which block residual CD8 co-engagement (4), internalization and patch formation of both complexes were strongly inhibited; in particular, the Kd-D227K-PbCS(ABA) complexes were localized predominately at the cell surface.

Kd-PbCS(ABA) Octamers Elicit Fas-mediated Cytotoxicity—To assess the ability of Kd-PbCS(ABA) octamers to elicit Fas-dependent cytotoxicity, T1 CTLs were pulsed with different Kd-peptide complexes, washed, and then incubated for 4 h with 51Cr-labeled P815 cells transfected with Fas. As shown in Fig. 5A, tetrameric, octameric, and multimeric Kd-PbCS(ABA) and Kd-D227K-PbCS(ABA) complexes induced bystander cell killing. The most efficient killing was observed for Kd-D227K-PbCS(ABA) octamer (65%). The Kd-PbCS(ABA) octamer induced slightly less efficient killing, but for both tetramers and multimers, target cell killing was about 20% lower. T1 CTLs pulsed or not pulsed with the corresponding Kd-Cw3 170–179 complexes exhibited only faint background lysis. By contrast, very strong lysis was observed in the presence of anti-Fas antibody, which induces apoptosis by cross-linking of Fas on the target cells.

A concern of the present experiments was that during the assay, Kd-PbCS(ABA) complexes decay, and liberated PbCS(ABA) peptide binds to cell-associated Kd and induces target cell killing. To consolidate this, we assessed the stability of Kd-PbCS(ABA) monomers at 37 °C. As shown in Fig. 5B, ~50% of the Kd-PbCS(ABA) complexes were decayed after 2.5 h of incubation, and after 3.5 h, nearly 80% were decayed. However, in the presence of β2m, the dissociation was greatly reduced; after 3.5 h, only 10% of dissociation took place. The same results were obtained for Kd-D227K-PbCS(ABA) complexes (data not shown). Thus, to reduce the liberation of PbCS(ABA) peptide, β2m was added to all incubations. In addition, in all assays, 10 μM PbCS 252–260 peptide was added to prevent free PbCS(ABA) peptide from binding to cell-associated Kd. Under these conditions, 10 μM PbCS(ABA) peptide caused no significant lysis. However, at a very high concentration (10 μM), free PbCS(ABA) caused strong lysis (Fig. 5A).

The same experiment performed on normal P815 cells showed no significant lysis, except in the incubation where a high concentration of free PbCS(ABA) peptide was used (Fig. 5B). This is consistent with the finding that normal P815 cells, which express low amounts of Fas, in 4-h cytolytic assays are sensitive to perforin/granzyme-mediated but not Fas-dependent killing (8). Taken together, these results indicate that CTLs pulsed with soluble Kd-D227K-PbCS(ABA) octamer induce strong Fas-dependent but no perforin/granzyme-dependent bystander cell killing.

Kd-PbCS(ABA) but not Kd-D227K-PbCS(ABA) Complexes Induce Degranulation of Adherent CTLs—We next examined the...
ability of the different soluble K\textsuperscript{d}-peptide complexes to elicit degranulation of T1 CTLs, which reflects perforin/granzyme-dependent lysis. Because degranulation requires adhesion and polarization of CTLs (18–20), we adhered T1 CTLs to immobilized fibronectin and incubated them with the soluble K\textsuperscript{d}-peptide complexes. As shown in Fig. 6, all K\textsuperscript{d}-PbCS(ABA) complexes elicited CTL degranulation. The strongest degranulation was observed for PE-labeled K\textsuperscript{d}-PbCS(ABA) multimers (75%) and K\textsuperscript{d}-PbCS(ABA) octamer (68%); K\textsuperscript{d}-PbCS(ABA) tetramers induced about 48% esterase release. In the presence of concanamycin A, which blocks CTL degranulation (21), the strong degranulation induced by K\textsuperscript{d}-PbCS(ABA) tetramers was reduced to background levels, as observed for the K\textsuperscript{d}-Cw3 170–179 complexes.

Remarkably, the corresponding K\textsuperscript{dD227K}-PbCS(ABA) complexes elicited no or scant esterase release, which was ablated.
upon blocking of the residual CD8 co-engagement by anti-CD8 mAb H35 (Fig. 6; Ref. 4). Together, these results indicate that soluble Kd-PbCS(ABA) complexes, namely, octamer and multimers, efficiently elicit CTL degranulation by soluble MHC-peptide complexes under the condition that they co-engage CD8. The same observations were made on S14 CTL clones (data not shown).

Kd-PbCS(ABA) but not KdD227K-PbCS(ABA) Octamers Induce Calcium Mobilization and Strong Tyrosine Phosphorylation—A hallmark of TCR/CD8-mediated T-cell activation is a rapid increase in intracellular calcium and tyrosine phosphorylation of CD3. To assess the ability of Kd-PbCS(ABA) octamers to elicit intracellular calcium mobilization, Indo-1-labeled T1 CTLs were incubated with Kd-PbCS(ABA) and KdD227K-PbCS(ABA) complexes, and calcium flux was measured by FACS. As shown in Fig. 7A, wild type octamer induced strong calcium mobilization that was maximal about 2 min after the addition of octamer. By contrast, KdD227K-PbCS(ABA) octamer elicited a calcium flux close to background levels, i.e. unpulsed CTLs. The same findings were obtained on S14 CTLs (data not shown). This is consistent with our previous finding that soluble MHC-peptide complexes elicit intracellular calcium mobilization only when they co-engage CD8 (14, 22).

Moreover, upon brief incubation with Kd-PbCS(ABA) octamers
ers, T1 CTLs exhibited strong tyrosine phosphorylation of the CD3ε and ζ chain, including its pp23 phospho form, as is typically observed upon TCR triggering with agonists (Fig. 7B) (14, 23, 24). By contrast, KdD227K-PbCS(ABA) octamers induced only faint phosphorylation of the pp21 phospho form of ζ chain, as typically occurs upon T-cell triggering by weak agonists or antagonists (Fig. 7B) (14, 23, 24). Blotting with anti-CD3ε antibody showed that equal amounts were loaded in all lanes. The soluble Kd-PbCS(ABA) complexes used were homogeneous according to SDS-PAGE (Fig. 7C). The same results were obtained on S14 CTLs (data not shown). Taken together, these results show that soluble Kd-PbCS(ABA) octamers efficiently induce intracellular calcium mobilization and CD3 phosphorylation, given that they co-engage CD8.

Kd-PbCS(ABA) Octamers Induce Apoptosis of T1 CTLs—Based on the observation that Kd-PbCS(ABA) octamers induce Fas-dependent cytotoxicity of bystander cells (Fig. 5), we investigated whether they also induce apoptosis of the CTLs. As shown in Fig. 8, T1 CTLs exhibited a marked increase in annexin V, which is a marker for apoptotic cells (9), upon incubation with Kd-PbCS(ABA) and a slightly smaller increase in annexin V upon incubation with KdD227K-PbCS(ABA) octamers. For CTLs incubated with Kd-Cw3 170–179 octamer, the annexin V expression was at the background level.

DISCUSSION

The present study shows that conventional “tetramers” are ill-defined mixtures of MHC-peptide conjugates (Fig. 2) and that this precludes precise binding studies (Fig. 3). The cause for this heterogeneity is the high molecular weight of PE (or allophycocyanine), which renders defined conjugation with the smaller avidin or avidin derivatives difficult, if not impossible. We find that defined MHC-peptide complexes can be obtained by using Cy5-labeled streptavidin. Although the fluorescence intensity of Cy5-labeled streptavidin is 4–5-fold lower as compared with PE-streptavidin, it is, unlike PE, remarkably resistant to photobleaching, which allows analysis other than FACS. Other low molecular weight fluorochromes can be used instead of Cy5, such as Cy3 or various Alexa dyes.

Moreover, the conventional strategy to derivatize monomeric MHC-peptide complexes by enzymatic biotinylation of an added BSP sequence permits only the preparation of avidin-based MHC-peptide “tetramers.” To produce different soluble MHC-peptide complexes, we investigated the derivatization of MHC-peptide monomers by site-specific alkylation. It has been reported that the heavy (25) or light chain (26) of MHC class I molecules can be biotinylated by alkylation of a free cysteine with maleimide containing biotin derivatives. Because on living cells under physiological conditions β2m is rapidly exchanged (27), we examined how site-specific alkylation of the heavy chain is best accomplished. Our results show that position 275 of the heavy chain is most suitable. For Kd the refolding and the alkylation efficiency of the K275C heavy chain were higher as compared with the R273C and A277C mutants (Fig. 1S). This is consistent with the fact that the conserved Trp274 marks the end of the folded α3 domain (17). Our results further indicate that the efficiency of refolding and alkylation critically depend on appropriate reduction of the introduced free cysteine (Fig. 1S). Because the same results were obtained for HLA-A2, 2 this may be generally applicable.

The biotinylation of MHC peptide complexes has several important advantages compared with the conventional enzymatic biotinylation. 1) The biotinylation can be performed in the cold, which is advantageous in particular in case of thermolabile MHC-peptide complexes. 2) It is significantly lower in terms of cost because alkylation reagents are much cheaper than BirA. 3) The thioether bond formed by alkylation of a free cysteine is very stable and resists proteolytic and chemical degradation. 4) The alkylation method is remarkably versatile. In addition to biotinylation of MHC class I-peptide complexes (Figs. 1 and 2) (25, 26), site-specific alkylation allows the preparation of MHC-peptide complexes of diverse valence and configuration by using branched, maleimide containing linkers for alkylation. Also, fluorescence-labeled MHC-peptide complexes can be prepared by alkylation with fluorescence-labeled maleimides or maleimide containing linkers.

Our MHC-peptide binding studies allow three conclusions. First, the increase of Kd-PbCS(ABA) binding to T1 CTLs is dependent on the valence of the complexes. CD8 increased the binding of monomeric complexes about 10-fold at 37 °C (4, 14), about 5-fold for tetrameric complexes, and <2-fold and hardly at all for multimeric complexes (Fig. 4). With regard to the CD8 dependence of multimer binding to CD8 T cells, there exists a controversy in the literature. Whereas according to some studies multimer binding is markedly CD8-dependent (28, 29), it is not so according to others (23, 30). This discrepancy may be explained in part by differences in the MHC-peptide multimer composition used in the different studies. However, we observed that under the same conditions as described here, the multimer binding to HLA-Cw3-specific CTLs, which express low affinity TCR, is substantially strengthened by CD8. Similarly, Daniels and Jameson (28) found that the CD8 dependence of multimer binding depends on the affinity of the TCR of the cells under study. It thus appears that the binding of MHC-peptide complexes to CD8 T cells is essentially determined by the overall binding avidity. Thus, the higher the affinity of the TCR and the higher the valence of the complexes, the less important the contribution of CD8 is to the binding. In
our hands, the binding of MHC-peptide octamers, but not of smaller complexes, is not CD8-dependent, except on CTLs that express exceptionally low affinity TCR.

Second, the kinetics of K<sup>+</sup>-PbCS(ABA) octamer binding to T1 CTLs is remarkably rapid, taking place within few minutes at all temperatures tested (Fig. 3, A, C, and D). This was also true for tetrameric complexes and on S14 CTLs. By contrast, the binding of monomeric K<sup>+</sup>-PbCS(ABA) complexes was considerably slower, especially in the cold (4). This argues that the kinetics of MHC-peptide complex binding increases with their valence.

Third, the heterogeneity of MHC-peptide multimers (Fig. 2) precludes precise binding studies. For example, the binding of K<sup>+</sup>-PbCS(ABA) multimers increased continuously with concentration and also at 18 °C, where internalization is scant, whereas octamer binding at 18 °C reached saturation at low concentrations (Figs. 3 and 4). It thus appears that low valence complexes in multimer preparations bind significantly only at higher concentrations, whereas high valence ones bind already at low concentrations. However, it is also conceivable that MHC-peptide complexes that have an appropriate configuration can elicit TCR (and CD8) aggregation. Such aggregation effects may explain why tetrameric K<sup>+</sup>-PbCS(ABA) complexes fail to reach the high levels of binding observed for octameric complexes (Fig. 4G) (tested up to 500 nM).

A key finding of the present study is that soluble MHC class I-peptide complexes that are unable to co-engage CD8 induce strong Fas-mediated cytotoxicity but no perforin-mediated cytotoxicity (Figs. 5, 6, and 8). This is in accordance with the previous observations that blocking of CD8 by antibody blocks perforin- but not Fas-dependent killing of target cells (8) and that target cells or microspheres expressing MHC class I-peptide with ablated CD8 binding induce Fas-dependent apoptosis of human CTLs (9). Common to these strategies is that CD8 co-receptor function is blocked. In the present study, where soluble MHC-peptide complexes are used, CD8-mediated adhesion and MHC-peptide binding or involvement of other auxiliary molecules are excluded (Fig. 3). It thus appears that engagement and cross-linking of TCR in the absence of CD8 co-engagement induce Fas-dependent cytotoxicity, including Fas-mediated apoptosis of the CTLs, in the absence of other cell activation (Figs. 5–8).

What implications has blocking of CD8 co-receptor function on CTL activation? On one hand, the lack of CD8 co-engagement by MHC-peptide complexes impairs the avidity of TCR-ligand binding (4, 14, 28, 29). We show here that for soluble MHC-peptide complexes, this can be compensated for by increasing their valence (Fig. 3). On the other hand, the lack of CD8 co-engagement impairs Lck-mediated phosphorylation of CD3. This is so because normally the coordinate binding of MHC-peptide to CD8 and TCR brings CD8-associated Lck to CD3, which upon cross-linking-mediated activation of Lck results in their phosphorylation (14, 22–24, 31). Once phosphorylated by Lck, CD3 and ζ chain immunoreceptor tyrosine-based activation motifs recruit ZAP-70 (and Syk), which upon phosphorylation by Lck phosphorylates LAT and other substrates, thus initiating various downstream signaling cascades (32–34).

The lack of CD8 co-engagement hence results in impaired Lck activation and by consequence reduced tyrosine phosphorylation of CD3 and recruitment and activation of Zap-70 (Fig. 7B) (8, 9, 23, 32). As a result of this, two downstream signaling pathways are compromised. The first is the recruitment and activation of phospholipase Cγ, which is involved in the observation that in the absence of CD8 co-engagement, there is no significant generation of inositol 1,4,5-triphosphate, which in turn mediates the release of intracellular calcium from stores (34, 35). In agreement with this is our intracellular calcium mobilization (Fig. 7A) (8). It is well established that TCR degranulation requires a rapid intracellular calcium mobilization, followed by a sustained influx of extracellular calcium, whereas for Fas-dependent killing, the latter is sufficient (36, 37).

The second signaling pathway that is compromised involves the recruitment of phosphatidylinositol 3-kinase to phosphorylated CD3 and ζ chain. This kinase phosphorylates phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate and is critically involved in early TCR signaling, including calcium mobilization, activation of Rho family GTP-binding proteins, and cytoskeleton function (38). Inhibition of phosphatidylinositol 3-kinase by wortmannin blocks CTL degranulation but has no effect on Fas-dependent cytotoxicity (39). It thus appears that MHC-peptide complexes that do not co-engage CD8 are unable to significantly activate Lck and hence most CTL effector function, including degranulation, cytokine release, and proliferation. The remarkable exception is Fas-dependent killing, which is Lck-independent, which makes it possible to selectively induce this cytotoxicity in the absence of any other cellular response (8, 40).

The physiological significance of this is not clear. Because CTLs prone to apoptosis once they express Fas ligand (Fig. 8) (9), it is conceivable that this way, CD8<sup>+</sup> T cells with defective CD8 co-receptor function are eliminated. Indeed, it has been shown that misselected CD8<sup>+</sup> T cells, which express TCRs that are not MHC class I-restricted, are eliminated this way (41). The observation that large soluble MHC-peptide complexes with ablated CD8 binding permit eradication of antigen-specific CTLs (Fig. 8) gives them a therapeutic potential. They are more attractive to this end than the previously described use of anti-CD8 antibodies (8) or MHC-peptide-coated microspheres (9) because such molecules can be produced in a well-defined form in adequate quantities and purity. Also, because soluble MHC-peptide complexes with ablated CD8 binding are unable to elicit any cell activation other than Fas-dependent cytotoxicity, they harbor a minimal risk to induce unwanted, potentially harmful immunological reactions (Figs. 5, 6, and 8) (22).

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