Selective Activation of Fas/Fas Ligand-mediated Cytotoxicity by a Self Peptide

By Peter Brossart and Michael J. Bevan

From the Howard Hughes Medical Institute, Department of Immunology, University of Washington, Seattle, Washington 98195

Summary

To study how MHC-associated self antigens may regulate the function of T cells in the periphery, we generated CD8+ T cell lines specific for a single residue variant of a self peptide. The self peptide (GAYEFTTL) was isolated from H-2-Kb class I MHC molecules immunopurified from tumor cells. CD8+ CTL lines from H-2b mice were generated against a variant peptide, pE4R, (arginine for glutamic acid at the TCR contact position 4). In short-term 51Cr-release assays, these CTL lysed H-2Kb targets that were pulsed with picomolar levels of pE4R but did not lyse target cells coated with the self peptide at micromolar levels. However, in overnight assays the CTL lysed Fas-positive target cells in the presence of nanomolar levels of the self peptide. This killing was shown to be entirely Fas/Fas ligand mediated by blocking with anti-Fas antibody and Fas-Fc chimeric molecules. While the self peptide was unable to induce serine esterase release from the CTL, it did induce secretion of IFN-γ. By these criteria then, the unmodified self ligand served as a partial agonist for the CTL raised against a single-residue variant. CD8+ T cell lines raised by in vitro stimulation with the self peptide were likewise unable to kill self peptide-coated targets via the perforin pathway but did lyse targets via Fas. These and similar data from other groups show that self antigens (i.e., MHC/peptide complexes) may be recognized by mature peripheral T cells. The T cell population is tolerant of the self antigen in the sense that they do not respond to physiological levels of the MHC/peptide complex. However, when the level of self antigen is increased (by using synthetic peptide loading) CD8+ T cells may respond by proliferation, IFN-γ secretion, Fas ligand upregulation, and Fas-mediated cytolysis but are still unable to respond by perforin-mediated cytolysis or granzyme release. The physiological significance of such partial activation in regulation of the immune system remains to be demonstrated.

CD4+ and CD8+ peripheral T lymphocytes recognize and respond to foreign peptide antigens presented in the groove of MHC class II and class I molecules, respectively. Interaction of antigen-specific T cells with APC that present the original antigenic MHC/peptide complex usually results in a cascade of T cell responses including proliferation and secretion of a plethora of lymphokines in the case of CD4+ T cells and proliferation, target cell lysis, and IFN-γ secretion by CD8+ T cells (1, 2).

Recent work has shown that T cells can also interact productively with APC expressing subtle variants of the original agonist MHC/peptide complex. Collectively, these less-than-optimal ligands have been referred to as "altered peptide ligands" (APL; 3). In the initial studies, single-residue changes made in an immunogenic peptide of hemoglobin presented by I-Ek molecules, resulted in a partial agonist APL that retained the ability to stimulate IL-4 secretion but not proliferation by a Th2 clone (4). This work was followed by numerous reports of variations in the original MHC/peptide ligand resulting in a partial stimulation of CD4+ T cell responses (5–8). Other studies demonstrated that APL could inhibit the response of CD4+ or CD8+ T cells to their original agonist ligands (9–13). The antagonist MHC/peptide ligand was shown to interact specifically with the TCR in a way that disrupted the signaling provided by the agonist ligand.

Only recently have attempts been made to ask whether APL, serving as non-optimal ligands for the TCR, exist endogenously in a healthy animal and whether they may influence the function of T cells. Using TCR transgenic animals, it has been shown that during CD8+ T lymphocyte positive selection in the thymus, antagonist peptides (14) and other APL (15) serve as highly efficient ligands for signaling this maturation step while agonist ligands usually tip the balance to deletion or inactivation of maturing T cells (16). These studies have fostered the notion that the multitude of self peptides presented by self MHC on thymic epi-

Abbreviations used in this paper: APL, altered peptide ligand; FasL, Fas ligand; MFI, mean fluorescence intensity.
The presence of self peptides on mature, peripheral T cells was systematically investigated. The immune system generally tolerizes to self peptides due to the high affinity of T cells for self antigens. However, the influence of self peptides on mature, peripheral T cells was not well understood.

**Materials and Methods**

**Animals.** Adult female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used at 6-8 wk of age.

**Cell Lines.** RMA-S (H-2b), EL-4 (H-2k), MC57G (H-2b), P815 (H-2k), and Jurkat (human T cell line) cells were maintained in RPMI 1640 containing G418 at 3,000 µg/ml, supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, and antibiotics (RP10). The cell line RMA-S/B7, a kind gift of Dr. Matteo Bellone (Laboratorio I. A., Instituto Scientifico H. San Raffaele, Milan, Italy), is stably transfected with a cDNA encoding the human B7.1 molecule (25). The human cell line, Cao et al. showed recently that a H-2Kd-restricted CTL clone specific for an influenza hemagglutinin peptide was antagonized in vitro with the self ligand induced Fas-mediated but not perforin-mediated cytolytic activity.

**Peptides.** The 40-kD peptide (GAYEFTTL) and its single-amino acid variants pE4R (GAYRFTTL), pE4K (GAYKFTTL), pE4Q (GAYQFTTL) and pE4D (GAYDFTTL) were synthesized using a Synergy peptide synthesizer (Applied Biosystems; Foster City, California) and analyzed by HPLC. Peptide concentrations were determined using the BCA assay (Pierce Chemical Co., Rockford, IL). The K⁺-binding OVA (SIINFEKL) and the D⁺-binding influenza (ASSENMDAM) peptides were described previously (16).

**RMA-S Stabilization Assay.** RMA-S cells were incubated at 31°C overnight to provide maximum MHC expression at the cell surface (29). Various concentrations of peptide were added for 30 min at 31°C and cultures were washed and shifted to 37°C for another 4 h. Cells were washed and stained for K⁺ expression with anti-K⁺ antibody Y3 followed by FITC-conjugated goat anti-rabbit Ig (Accurate Chemical and Scientific Corp., Westbury, NY).

**In Vitro Priming of Cytotoxic Lymphocytes.** RMA-S/B7 cells were cultured at 31°C overnight in RP10 medium and pulsed with 100 µM peptide for 1 h at 31°C. Cells were then washed, irradiated (20,000 rad), and 5 x 10⁵ peptide-pulsed cells were cultured with 50 x 10⁴ spleen cells from naive mice in 10 ml medium. Medium used was RP10. One-half of the culture medium was changed daily. At day 5 cells were tested for cytolytic activity.

**CTL Assays.** Target cells were labeled with ¹⁰⁴ C⁶-sodium chromate in RP10 for 1 h at 37°C. After washing, cells were incubated with peptide at the indicated concentration for another hour, washed three times, and 10⁴ cells were transferred to a well of a round-bottomed 96-well plate. Varying numbers of CTL were added to give a final volume of 200 µl. The plates were incubated for 4 or 18 h at 37°C, as indicated. At the end of the assay, the plates were centrifuged and supernatants (100 µl/well) were harvested and counted in a gamma counter. The percent specific lysis was calculated as: 100 X (experimental release-spontaneous release)/maximal release-spontaneous release. Spontaneous and maximal release were determined in the presence of either medium or 1% Triton X-100, respectively. Spontaneous release after 4 h was 10-15%, after 18 h was 25-38%. For titration experiments, peptide was titrated before the addition of target and effector cells. In some cultures emetine (0.6 µg/ml), anti-huFas M3 (10 µg/ml), M33 (10 µg/ml), or human Fas.Fc (15 µg/ml) were added, as noted. The presence of the anti-Fas (M3, M33) or Fas.Fc had no effect on the spontaneous release of any of the target cells.

**Proliferative Response.** To measure the proliferative response, 10⁴ to 10⁵ CTL, 10 d after their last restimulation, were cultured in flat-bottom 96-well plates with 7 x 10⁶ irradiated spleen cells
coated with peptide. 4 h later cultures were pulsed with 1 μCi [3H]-Tdr and harvested after 16 h. Data represent the mean of triplicate cultures. SD were generally within 5–15% of the mean.

**Serine Esterase Assay and IFN-γ ELISA.** EL-4 cells were irradiated (20,000 rad) and then coated with 1 μM peptide for 1 h at 37°C. 10^4 washed cells were added to a well of a 96-well plate containing 2 × 10^5 CTL. After 16 h 80 μl of the supernatant was assayed for IFN-γ content and 20 μl supernatant was analyzed for containing 2 × 10^4 M N-benzyloxycarbonyl-r-lysine thiobenzyl ester and 2.2 × 10^4 M 5,5'-dithio-bis-2-nitrobenzoic acid in PBS, pH 7.4. After incubation for 2 h, the absorbance was measured at 405 nm using an ELISA plate reader. The percentage serine esterase release was determined following the formula used for the CTL lysis assay.

The peptide-induced IFN-γ production by the CTL was determined using a commercially available IFN-γ ELISA (Genzyme, Cambridge, MA; sensitivity 125 pg/ml). All experiments were done in quadruplicate.

**RT-PCR.** For assessment of FasL expression by PCR, 5 × 10^6 CTL were mixed with 1 × 10^5 irradiated MC57G cells coated with 1 μM peptide in 24-well plates. After 6 h incubation, total RNA was isolated from the T cell lines using RNA STAT-60 reagent (Tel-Test "B", Inc., Friendswood, TX). For reverse transcription, 1 μg of total cellular RNA was added to 25 ml of reverse transcription buffer containing 0.4 μM oligo-dT (12–18), 1 mmol/l dNTP (dATP, dCTP, dTTP, dGTP), 1 μl of Super-script II RNaseH⁻ reverse transcriptase (GIBCO BRL, Gaithersburg, MD), and 40 units RNasin (Promega Corp., Madison, WI). After incubation at 42°C for 1 h, 2 μl of the sample was suspended in 50 ml PCR-buffer containing 0.3 μg of each primer and 2 units Taq DNA polymerase (Promega). PCR was carried out with a Thermal cycler (Perkin-Elmer Corp., Norwalk, CT) programmed for denaturation at 94°C for 90 s, annealing at 50°C for 90 s and extension at 72°C for 90 s. Primers were devised from published sequences. Fas ligand: 5'-CGTGAAGTCTCACACCAAGC (FasLs, sense), 5'-GAGTTCTCATATAGCCTTG (FasLs, antisense) (30); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CCATCACCACATTTCCAGAG (GAPDHs, sense), 5'-CCTGCTTACACACCTCTTT (GAPDHs, antisense) (31).

8 μg of reaction product was run on a 2% agarose gel and stained with ethidium bromide. RNA integrity was checked by reverse transcription and PCR with primers for GAPDH. The origin of the amplified product was confirmed by direct sequencing of the PCR product using Sequenase Version 2.0 DNA sequencing kit (USB, Amershams Corp., Arlington Heights, IL).

To permit quantitative analysis of PCR signal strength, primer and RNA concentrations, as well as PCR cycles, were titrated as described (32). PCR comprised 24 cycles for GAPDH and 27 cycles for FasL.

**Results**

**Induction of CTL Against a Self Peptide Variant.** The sequence GAYFETTL was identified as a naturally processed peptide isolated from H-2K^b^ molecules purified from LB 27.4 cells (33). It has homology to a bacterial 40-kD ribosomal protein and is referred to as the 40-kD peptide (Imaeda, S., and C.A. Janeway, personal communication). As would be expected of a naturally presented peptide, it efficiently binds and stabilizes H-2K^b^ (14). Structural analyses of K^b^ complexes with single peptides indicate that the side chain of position 4 of an octameric peptide points up, out of the K^b^ groove (34, 35). To study the H-2K^b^-restricted CD8⁺ T cell response to a self peptide and its single-residue variants, we therefore made a number of analogues of the 40-kD peptide with substitutions at position 4. The RMA-S stabilization data presented in Fig. 1 show that the 40-kD peptide and all position four variants bind K^b^ at least as well as another K^b^-presented epitope from Ova (SIINFEKL).

The analogue peptide pE4R was used to induce a C57BL/6 CTL response in vitro using RMA-S cells expressing the B7 costimulator molecule as APC. After a number of weekly stimulations the line demonstrated peptide-specific killing of target cells that was entirely H-2K^b^ restricted (Fig. 2A). This uncloned CD8⁺ T cell line is referred to as CTL.R. In a 4-h. ^51^Cr-release assay using EL4 cells as targets and high doses of peptide, the CTL.R line showed some cross-reactive lysis of targets coated with the pE4Q variant, but no lysis of targets coated with pE4K, pE4D, or wild-type, 40-kD peptide (Fig. 2B). Consistent with this result, only pE4Q and the immunizing peptide, pE4R induced serine esterase release by the CTL.R line when EL4 cells coated with 1 μM levels of peptide were used as stimulators (Fig. 3).

**Lysis of Fas-positive Targets.** The CTL.R line exhibited a different pattern of cytolytic specificity when target cells expressing Fas were used in an 18-h ^51^Cr-release assay. With Jurkat-K^b^ cells as targets it was evident that coating with the 40-kD peptide at 10⁻⁹ M or higher concentrations sensitized the cells for lysis (Fig. 4). Lysis mediated by the inducing, pE4R peptide occurred at 10⁻¹¹ M concentrations in this assay. This cross-reaction on 40-kD was specific since the pE4K and pE4D peptides caused no lysis of the Jurkat-

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Position 4 variants of a self peptide bind and stabilize K^b^ on RMA-S cells. The RMA-S stabilization assay was performed to analyze binding of the peptides to the K^b^ molecule. RMA-S cells were incubated with various peptide concentrations at 31°C and the level of K^b^ on the cell surface was determined by FACS® analysis. The mean fluorescence intensity (MFI) is shown. Flu refers to a control D^b^ binding peptide from influenza nucleoprotein.
Figure 2. MHC and peptide specificity of the in vitro-induced CTL.R line. Mouse and human tumor cell lines were coated with 1 µM of the pE4R peptide and used as targets (A). EL-4 cells were coated with 1 µM of the 40-kD peptide or its variants and used as targets (B).

Figure 3. Serine esterase release by CTL.R is induced after stimulation with pE4R and pE4Q peptides. CTL.R was cultured with irradiated EL-4 cells coated with 1 µM of the self-peptide or its variants and serine esterase release was determined after 16 h. The assay was conducted in quadruplicate and error bars show the means and standard deviations.

Figure 4. Fas* target cells coated with the self-peptide are lysed in a 18 h 51Cr-release assay by CTL.R. 51Cr-labeled Jurkat-Kb cells were incubated with the indicated concentrations of the 40-kD self peptide or its analogs and used in a 18 h Cr-release assay as described in Materials and Methods. CTL were added at an E:T ratio of 10:1.

Kb targets. Jurkat cells express high levels of the Fas molecule (CD95) on their surface as detected by staining with the M33 antibody (25, and data not shown). MC57G fibroblast cells from C57BL/6 mice can also retain 51Cr well enough to be used in an 18-h 51Cr-release assay, and are Fas-negative. The data in Table 1 show that MC57G cells are not damaged by the CTL.R line when coated with the 40-kD peptide, but they are lysed efficiently in the presence of the pE4R peptide.

Definitive evidence that the CTL.R-mediated lysis of Jurkat-Kb cells coated with the self peptide was Fas-mediated was obtained in blocking studies. The data presented in Fig. 5 A show that the 40-kD peptide-induced lysis of Jurkat-Kb targets can be completely blocked by the M3 mAb specific for human Fas whereas the control M33 mAb had no effect. Furthermore, addition of a Fas.Fc chimeric molecule to the assay completely blocked the self peptide-mediated lysis of Jurkat-Kb (Fig. 5 A). In contrast to this, the blocking mAb M3 and the Fas.Fc showed no significant inhibition of the lysis of pE4R-coated target cells (Fig. 5 B).

Fas/FasL-mediated killing has been shown to require macromolecular synthesis for effector cell activation and to be sensitive to inhibition of protein synthesis, for example by emetine (36, 37). In line with this, we show that the presence of emetine inhibited the lysis of Jurkat-Kb cells coated with the 40-kD peptide by CTL.R (Fig 5 A), whereas it had only a modest effect on the CTL.R mediated cytotoxicity of targets coated with pE4R (Fig. 5 B).

Self Peptide Induction of Proliferation and IFN-γ Secretion by CTL.R. The 40-kD self peptide was able to stimulate the
Table 1. Only Fas+ Target Cells are Lysed by CTL.R when Coated with the Self Peptide

| Peptide  | MC57G targets | Jurkat-Kb targets |
|----------|---------------|-------------------|
|          | %51Cr-release at E:T |          |
| None     | 16 10 8 7 21 18 13 9 |          |
| pE4R     | 71 53 34 17 88 40 38 29 |          |
| 40 kD    | 14 11 8 7 57 34 27 14 |          |

51Cr-Labeled Jurkat-Kb cells (Fas-positive) and fibrosarcoma cell line MC57G (Fas-negative) were coated with 1 μM of the 40-kD self-peptide or the cognate pE4R peptide and used as targets in an 18-h 51Cr-release assay.

despite the fact that the 40-kD self peptide was unable to stimulate the release of serine esterase from CTL.R (Fig. 3), these same supernatants did show the presence of secreted IFN-γ (Fig. 6 B). Again, the 40-kD and pE4Q were about equally effective in stimulating IFN-γ secretion whereas pE4K and pE4D were ineffective.

Induction of CTL against the 40-kD Self Peptide. Repeated in vitro stimulation of C57Bl/6 splenocytes with RMA-S/B7 cells coated with the 40-kD self peptide resulted in the production of a CD8+ line referred to as CTL.40kd. FACS® analysis of this line and the CTL.R line showed that both expressed equivalent levels of CD8, TCR, and CD44, whereas the CTL.40kd had slightly higher levels of CD25 (IL2Ra) expression (data not shown).

The CTL.40kd line mediated no peptide-specific lysis of any target cell in a 4-h 51Cr-release assay (data not shown). However, in an 18-h assay, with Jurkat-Kb targets, the 40-kD peptide induced specific 51Cr-release at nanomolar and higher concentrations (Fig. 7). None of the four analogue peptides, including pE4R, was able to stimulate cytotoxicity (Fig. 7). The 40-kD directed lysis of Jurkat-Kb was entirely Fas-dependent since no lysis of Fas-negative MC57G targets could be detected (Table 2) and the lysis of Jurkat-Kb was blocked completely by the M3 antibody, by the Fas.Fc chimera, and by emetine (Fig. 8).

Figure 5. The cytotoxic activity of CTL.R targeted by the 40-kD peptide is entirely Fas/FasL mediated. 51Cr-labeled Jurkat-Kb cells were coated with 1 μM of the 40-kD self-peptide (A) or the cognate pE4R peptide (B) and used as targets in an 18 h 51Cr-release assay. Emetine (0.6 μg/ml), anti-human M3 (10 μg/ml), M33 (10 μg/ml), or human Fas.Fc (15 μg/ml) were added, as noted.

Figure 6. Proliferative response and IFN-γ production by CTL.R after stimulation with the self peptide or its variants. 10 d after their last restimulation, CTL.R cells were cultured in 96-well plates with irradiated spleen cells plus 1 μM peptide (A). The induced IFN-γ release was assayed in supernatants from cultures following 16 h stimulation with irradiated EL-4 cells coated with 1 μM peptide (B).
Discussion

We generated a polyclonal CD8+ T cell line by in vitro stimulation with a single residue variant of a self peptide presented by the H-2Kb molecule. This line, CTL.R, was able to lyse Fas+ and Fas− target cells that were coated with the immunizing peptide, pE4R. A full spectrum of CD8+ T cell responses was elicited by pE4R, including: perforin-mediated cytolysis, proliferation, serine esterase release, IFN-γ secretion, FasL upregulation and Fas-mediated cytolysis of...
targets. When APC were coated with the 40-kD self peptide, no lysis of Fas-negative targets was obtained with CTL.R even at micromolar levels of peptide addition. However, with Fas+ Jurkat-Kb cells as targets, the 40-kD peptide induced cytolysis. Whereas approximately picomolar levels of the original antigenic pE4R targeted Jurkat-Kb for lysis, nanomolar levels of the self peptide were required to observe lysis. Although the self peptide, acting as an APL for CTL.R, was not able to stimulate perforin-mediated cytolysis or serine esterase release, it was able to stimulate the secretion of IFN-γ and proliferation in addition to FasL surface expression and Fas-mediated cytolysis.

The 40-kD peptide was isolated from the groove of H-2Kb molecules prepared from LB 27.4 cells. However, LB 27.4 cells, which do express Fas, were not targets for the CTL line (data not shown). Thus, whereas at the higher levels of ligand density achieved by synthetic peptide loading, the self peptide acts as a partial agonist for the CTL, at the endogenous level of presentation this ligand is not stimulatory. We do not have biochemical proof for the in vivo presentation of the 40-kD peptide by B cell, or any other cell type in vivo. Since the sequence was isolated from a tissue culture-maintained B cell line, it could represent a mutation. We consider this to be quite unlikely, especially since the 40-kD sequence has unique properties in the way it interacts with CD8+ T cells from B6 mice. Thus, whereas a number of single-residue variants (pE4R and pE4Q) readily induce perforin-mediated cytolysis, the 40-kD peptide does not.

These results complement those recently presented by Cao et al., who reported that a germline VH peptide acted as a partial agonist for a hemagglutinin-specific CTL clone (24). In that case Fas-mediated cytolysis of peptide-coated targets, could be selectively induced by a germline VH peptide. It is apparent from these two reports that CD8+ lymphocytes, like CD4+ lymphocytes, can give partial responses to APL.

To follow up on our finding that the CTL.R line treated the 40-kD as a partial agonist, we used the same in vitro stimulation protocol to generate a CTL line using the 40-kD self peptide as the immunogen. A CD8+ line, CTL.40kd, grew out slowly and responded to the immunizing peptide as if it were a partial agonist. Thus, the 40-kD peptide at high concentration stimulated FasL upregulation, Fas-mediated killing and IFN-γ secretion but not perforin-mediated killing or serine esterase release. Despite their similar response to the 40-kD peptide, the T cells in the CTL.40kd line had a
Fluorescence intensity (log)

Figure 11. Induction of FasL expression on the cell surface. FasL expression on stimulated and unstimulated CTL was analysed using a polyclonal antibody PE62. CTL-R (A–D) and CTL.40kd (E–G) were stimulated with 1 μM peptide coated, irradiated MC57G cells or PMA/ionomycin for 12 h. Cells were stained with PE62 followed by sheep anti-rabbit IgG coupled to FITC. (A) Unstimulated CTL-R, (B) CTL-R stimulated with pE4R peptide, (C) CTL-R stimulated with 40-kD peptide, (D) CTL-R stimulated with PMA and ionomycin, (E) unstimulated CTL.40kd, (F) CTL 40-kD stimulated with 40-kD peptide, (G) CTL.40kd stimulated with PMA and ionomycin.

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Address correspondence to Michael J. Bevan, University of Washington, Department of Immunology, Howard Hughes Medical Institute, Box 357370, Seattle, WA 98195.

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