A Partially Agonistic Peptide Acts as a Selective Inducer of Apoptosis in CD8 + CTLs

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A Partially Agonistic Peptide Acts as a Selective Inducer of Apoptosis in CD8+ CTLs

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We have analyzed the effect of partially agonistic peptides on the activation and survival of CTL clones specific for a highly immunogenic HLA A11-restricted peptide epitope derived from the EBV nuclear Ag-4. Several analogues with substitutions of TCR contact residues were able to trigger cytotoxic activity without induction of IL-2 mRNA and protein or T cell proliferation. Triggering with these partial agonists in the absence of exogenous IL-2 resulted in down-regulation of the cytotoxic potential of the specific CTLs. One analogue selectively triggered apoptosis as efficiently as the original epitope, subdividing the partial agonists into apoptosis-inducing and noninducing ligands. Analysis of early T cell activation events, induction of Ca2+ influx, and acid release did not reveal significant differences between the two types of analogue peptides. These results demonstrate that some partial agonists can dissociate the induction of CTL death from CTL activation. Peptides with such properties may serve as useful tools to study signal transduction pathways in CD8+ lymphocytes and as therapeutic agents modulating natural immune responses. The Journal of Immunology, 1999, 163: 2601–2609.

The TCR recognizes MHC molecules associated with peptides derived from exogenous or endogenous proteins. MHC/TCR interactions are central for T cell biology since they play a major role in regulating the selection, differentiation, activation, persistence, and death of T lymphocytes. The variety of biological outcomes resulting from the engagement of the same receptor suggests that the TCR may be able to transduce quantitatively and qualitatively different signals. This concept is strongly supported by the existence of antagonists and partial agonists, peptide analogues that can inhibit T cell activation induced by antigenic peptides or selectively stimulate only some T cell effector functions (reviewed in Ref. 1). Partial activation was shown to engage only some of the signal transduction pathways operating in the course of full scale T cell activation (2). Partially agonistic peptides can selectively induce cytolytic function of CTLs (3), Fas-mediated cytotoxicity (4, 5), early activation events without lymphokine production or cell proliferation (6), secretion of some lymphokines without detectable production of others (7, 8), as well as induce anergy in specific T cells (9–12). Recently, peptide analogues that are able to selectively trigger apoptosis in CD4+ lymphocytes have been described (13). These analogues provide a new tool to study the mechanisms regulating T cell function and may also allow the development of therapeutic approaches aiming to selectively down-regulate harmful manifestations of immunity such as autoimmune and allergic reactions or damage accompanying excessive immune responses against viruses and bacteria.

Recent reports demonstrate that the systemic administration of immunogenic peptides can lead to exhaustion and/or tolerization of specific T cells, but is also associated with general immunosuppression and severe damage to the lymphoid organs (14, 15). In contrast, analogue peptides that selectively induce apoptosis were shown to deplete specific CD4+ T cells without associated immunopathology in a TCR transgenic mouse model (16). The identification of functionally similar peptide analogues for CD8+ CTLs is of considerable interest since natural T cell responses include CD4+ and CD8+ effectors, and the therapeutic use of apoptosis-inducing peptide analogues will require the targeting of both T cell populations. Moreover, since different subpopulations of CD4+ T lymphocytes exhibit variable sensitivity to activation-induced cell death (AICD)3 (17) and engagement of CD4 can enhance or inhibit apoptosis induced by specific CD4+ T cells without associated immunopathology (18). The identification of peptide analogues capable of selective induction of apoptosis in CD4+ cells does not necessarily imply that functionally similar peptides exist or operate through similar mechanisms in CTLs. Finally, the induction of cytotoxic activity requires a small amount of antigenic peptide and is usually triggered by partial agonists, potentially limiting their practical application (3, 19). Therefore, it would be important to establish the relationship between the apoptosis- and cytotoxicity-inducing activity of partial agonists.

We have addressed these issues on the model of EBV-specific CTL responses that appear to be critical for the control of primary EBV infection and for limiting the proliferation of EBV-transformed B cells during persistent infection (20). Some of these EBV-specific CTLs were shown to exhibit cross-reactivity with allo-MHC that can mediate graft rejection in transplant recipients (21). Furthermore, EBV-specific CTLs have been suggested to play a role in the pathogenesis of certain autoimmune diseases (22, 23) and in some life-threatening complications of infectious mononucleosis, such as fulminant hepatitis (24, 25). In all these situations, a selective depletion of the CTL repertoire may be of clinical interest. It is noteworthy that EBV-specific CTLs may be a convenient target for peptide-based therapy since EBV-specific
responses are often focused on a limited number of peptide epitopes (26, 27) and the peptide-specific T cell repertoires are often oligo or monoclonal (28, 29). In this study, we have used CTL clones specific for the HLA A11-restricted peptide IIVTDFS-VIK (designated IVT), corresponding to the amino acids 416–424 of the EBV nuclear Ag-4, to characterize the effect of partially agonistic peptides on T cell activation. We demonstrate the existence of peptide analogues that trigger cytotoxicity without induction of IL-2 production and proliferation of the specific CTLs. Culture in the presence of these partial agonists resulted in down-regulation of cytotoxic activity and, in one case, selective triggering of AICD.

Materials and Methods

Cell lines and CTL clones

The HLA A11-transfected subline of the HLA class I-negative mutant cell line C1R (30) was generated by transfection with a pHEBO vector-based HLA A11 expression vector (31) and maintained in medium containing 200 μg/ml hygromycin B. The EBV-transformed lymphoblastoid cell line (LCL) JAC-B2 was produced from lymphocytes of an HLA-typed donor, as previously described (32). All cell lines were maintained in RPMI 1640 medium supplemented with 100 μg/ml streptomycin, 100 IU/ml penicillin, and 10% FCS (standard medium). The generation and analysis of specificity and TCR structure of the EBV-specific HLA A11-restricted CTLs clones BK112 and BK289 were described previously (29).

Synthetic peptides

Peptides synthesized by the Merrifield solid-phase method (33) were purchased from Alta Bioscience (Alta Bioscience, University of Birmingham, School of Biochemistry, Birmingham, U.K.) and purified by HPLC on SuperPac Pep P-5 mm reverse-phase columns (Pharmacia, Uppsala, Sweden). The purified peptides were dried using SpeedVac (Pharmacia) and dissolved in DMSO at a concentration of 1 × 10−2 M, as determined by Biuret assays (34). Peptide dilution in PBS was performed immediately before the assays to obtain indicated concentrations.

Cytotoxicity assays

Cytotoxic activity was measured in standard 4-h 3Cr release assays (35). Peptide-pulsing experiments were performed by adding 13 μl of the indicated peptide preparations diluted in complete medium to triplicate wells of 96 V-shaped well plates containing 4 × 105 labeled targets in 25 μl of complete medium. After brief centrifugation to help conjugate formation, the cells were incubated at 37°C for 3 h and washed twice with ice-cold PBS, and total RNA extraction was performed as previously described (36). The concentration of extracted RNA was measured on a spectrophotometer (Perkin-Elmer, Norwalk, CT), and 0.3 μg of RNA from each sample was separated on ethidium bromide-stained 1% agarose gel. The OD of the RNA bands was analyzed using Kodak DC-40 digital camera and Kodak Digital Science 1D Image Analysis Software (Eastman Kodak, Rochester, NY) to confirm the results of spectrophotometric measurements and to normalize, if necessary, the concentration of each RNA sample. At this step, RNA degradation and contamination of the samples with genomic DNA were also excluded. For first-strand cDNA synthesis, the RNA was denatured for 5 min at 70°C and then chilled on ice. Reverse transcription was performed in a 40-μl reaction containing 2 μg of denatured RNA dissolved in 20 μl RNase-free water, 8 μl 5X buffer (Life Technologies, Rockville, MD), 4 μl dNTP (5 mM each; Pharmacia), 3 μl 100 mM DTT (Life Technologies), 1 μl RNasin (40 U/μl; Promega), 2 μl 0.1 mM random hexamer primers (Pharmacia), and 2 μl M-MLV reverse transcriptase (200 U/μl; Life Technologies). After a 45-min incubation at 40°C and heating at 95°C for 5 min to inactivate the reverse transcriptase, the samples were used directly for PCR reaction. One microtiter of cDNA was diluted with sterile water to 5 μl and mixed with 20 μl PCR mixture containing: 2.5 μl 10X buffer (100 mM Tris-HCl/500 mM KCl/0.1% gelatin (pH 8.3)), 2 μl 25 mM MgCl2, 4 μl dNTP (1.25 mM each; Pharmacia), 2.5 μl each primer (5 μM), 6.375 μl sterile water, and 0.125 μl Taq polymerase (Perkin-Elmer). The reaction mixture was amplified with a PTC-100 thermal cycler (MJ Research, Watertown, MA). The cycling conditions were 1 min at 95°C for denaturation, 1 min at 58°C for annealing, and 1 min at 72°C for elongation. The PCR reaction was performed by 7-min incubation at 72°C for final elongation. Serial dilutions of the template were performed with each primer set to establish the number of cycles required to reach the exponential phase of the amplification reaction (20 cycles for amplification of β2m and 32 cycles for amplification of cDNA primers: IL-2, 5’-TGTCAGGATGCACTCTGCTC-3’ and 5’-CAATGTTGCTGTCTCATCAG-3’; βm, 5’-GAATTGCTATGT-GTCTGGGT-3’ and 5’-CATCTCCAACCTCCATGTG-3’ were purchased from Biosource Europe (Fleurus, Belgium). The PCR products were separated on 1.6% agarose gel (Eastman Kodak) and visualized by ethidium bromide staining.

Intracellular IL-2 staining

A mixture of 1 × 105 BK289 cells and 1 × 105 C1R/A11 cells, untreated or preincubated with the indicated final concentration of synthetic peptide, was cultured at 37°C overnight in 2 ml of complete medium, containing 1 μM Brefeldin A (Golfgang; PharMingen, San Diego, CA) to inhibit IL-2 secretion. To distinguish the HLA A2-positive CTLs and HLA A2-negative C1R/A11 cells, the cells were stained with mouse anti-human HLA A2-specific Ab (American Type Culture Collection, Manassas, VA; HB-54) and PE-conjugated rat anti-mouse Ig (Dako, Glostrup, Denmark). After removing the unbound Ab by washing in PBS, cells were fixed and permeabilized using the Cytofix/Cytoperm kit in accordance with the manufacturer’s instruction (PharMingen). A FITC-conjugated rat anti-human IL-2 mAb and its isotype control (PharMingen) were used for intracellular IL-2 staining. The data were acquired and analyzed on a FACS analyser using the CELLQuest software (Becton Dickinson, San Jose, CA).

Proliferation assays

Peptide-pulsed C1R/A11 cells were irradiated (5000 rad), washed extensively in PBS to remove the unbound peptide, and mixed with T cells in complete medium alone or supplemented with 10 U/ml of IL-2 at an E:T ratio of 3:1. The cell suspension was adjusted to a density of 1.5 × 105 cells/ml and distributed in triplicates to 96-well U-bottom microtiter plates (200 μl/3 × 104 T cells/well). The cells were cultured in a CO2 incubator for 72 h and pulsed with [3H]thymidine (0.5 μCi/well) during the last 8 h of incubation. The plates were then harvested to glass filters on a Tomtec harvester 96 (Orange, CT), and filters were counted on a Wallac 1450 Microbeta liquid scintillation counter (Wallac, Finland).

Monitoring of cell death and apoptosis

PepTide-pulsed and irradiated C1R/A11 cells were mixed at an E:T ratio of 3:1 with 2–3 × 105 BK289 cells in complete medium with or without IL-2. After 3 days, viable CTLs were counted by the trypan blue exclusion method. Mean value of four separate fields was determined for each individual sample. At this time, almost all the live cells were CD3+, as determined by FACS analysis with FITC-conjugated CD3-specific Ab (Becton Dickinson). Induction of apoptosis in BK289 cells was monitored by Hoechst staining, as previously described (37).

Acid release

Acid release was measured as described previously (38). Briefly, T cells rested from stimulation (14 days) were mixed with C1R/A11 cells at a ratio of 3:1 and collected by centrifugation. The cell pellet (3–6 × 106 cells) was resuspended in 105 μl complete medium mixed with 35 μl modified low temperature-melting agarose (Molecular Devices, Sunnyvale, CA) at 37°C. The agarose cell mixture (10 μl) was immediately spotted onto the membrane of a Cytosensor cell capsule (Molecular Devices). After 10 min, the cell capsule was assembled and loaded in the microphysiometer chamber maintained at 37°C. The chamber was perfused (50 μl/min) with low buffer RPMI 1640 medium (Molecular Devices) containing 1 μM sodium phosphate, 1 mg/ml endotoxin-free BSA (Calbiochem, San Diego, CA), and no bicarbonate (pH 7.4). The rate of acid release was determined with 20-s potentiometric rate measurements after a 58-s pump cycle and 10-s delay (total cycle time, 90 s).

Calcium influx

Changes in the concentration of intracellular calcium were analyzed according to standard methods (39). Briefly, C1R were loaded with Flock by
incubation in loading medium (HBSS, Life Technologies; 1% FCS) containing 4 mM probenecid (Sigma, St. Louis, MO) and 4 μg/ml Fluo3 (Calbiochem) for 30 min at 37°C. The cells were then mixed with peptide-pulsed C1R/A11 cells at an E:T ratio of 3:1, and the cell suspension was pelleted down and incubated for 2 min in a 37°C water bath. The pellet was resuspended by vortexing, and cells were analyzed on a FACS analyzer (Becton Dickinson). The sample tube was kept at 37°C throughout the analysis by means of tubing connected to a 37°C water bath through a peristaltic pump.

Results

Several IVT analogues can selectively trigger cytotoxic activity without induction of IL-2 mRNA

Two sets of IVT analogues were compared for their ability to trigger cytotoxic activity and IL-2 mRNA expression in the CD8+ CTL clones BK112 and BK289, which express structurally different TCRs (29). In the first set of analogues, each residue of the IVT peptide was consecutively substituted with alanine. In the second set, the common TCR contact residue, phenylalanine in position 5 (F5) (29), was substituted to hydrophobic (G, L, I), polar (S, Y, W), or charged (K) residues. C1R/A11 cells prepulsed with 1×10^{-8} M of the various peptides were used as APC. Cytotoxic activity was measured in standard 51Cr release assays, while IL-2 mRNA expression was monitored by semiquantitative RT-PCR.

The results of one representative experiment illustrating the differential induction of IL-2 mRNA by stimulation of BK289 CTLs with C1R/A11 cells pulsed with the set of alanine-containing analogues are shown in Fig. 1. A compilation of the screening performed with the entire set of analogue peptides is shown in Fig. 2. Based on their functional properties, the IVT analogues could be divided into three groups: 1) inert peptides that did not trigger any of these effects; 2) full agonists that triggered both functions; and 3) partial agonists that selectively activated the cytotoxic activity of the CTLs. In the last group, the A7 and A8 peptides appeared to act as partial agonists for BK112 and BK289, respectively. The W5 analogue acted as partial agonist for BK112, but exhibited full agonistic activity for BK289. The Y5 analogue selectively activated the cytotoxicity of both clones, but with different efficiency. Our subsequent analysis was focused on the activation of BK289 by the A8 and Y5 analogues.
The Y5 and A8 analogues act as partial agonists for BK289 CTLs

Partially agonistic T cell ligands are characterized by their ability to selectively trigger certain T cell functions over a wide range of peptide concentrations (19). We therefore compared the ability of the IVT, Y5, and A8 peptides to induce cytotoxic activity, IL-2 production, and proliferation of BK289 cells in peptide titration experiments. A representative experiment illustrating the cytotoxic activity of BK289 against C1R/A11 cells prepulsed with the three peptides is shown in Fig. 3. The Y5 and A8 analogues triggered comparable cytotoxic responses reaching half-maximal lysis at concentrations between $5 \times 10^{-9}$ and $1 \times 10^{-8}$ M, while the IVT peptide induced similar responses at 50–100-fold lower concentrations. Maximal levels of killing were achieved with the three peptides at concentrations between $1 \times 10^{-6}$ and $1 \times 10^{-7}$ M. 

Induction of IL-2 expression was analyzed at the protein level by intracellular staining and FACS analysis. BK289 cells were double stained with mAbs specific for IL-2 and HLA A2 to distinguish them from the A2-negative C1R/A11 cells. Representative FACS plots of BK289 cells triggered with C1R/A11 cells pulsed with $1 \times 10^{-6}$ M and $1 \times 10^{-7}$ M of the three peptides are shown in Fig. 4A, and the net increase of percentage of IL-2-positive cells recorded in three experiments in which each peptide was tested over a 5 log dilution range is shown in Fig. 4B. While a significant increase in the percentage of IL-2-positive cells was detected upon triggering with the IVT peptide, the Y5 and A8 analogues failed to induce any measurable up-regulation of IL-2 expression. Similar results were obtained when the IVT peptide and the two analogues were compared for their capacity to induce DNA synthesis of BK289 cells, as determined by [3 H]TdR incorporation following stimulation with peptide-pulsed C1R/A11 in the absence (Fig. 4C) or presence of exogenous IL-2 (not shown). The Y5 and A8 analogues bind to HLA A11 as efficiently as IVT and compete as efficiently with irrelevant peptides in cytotoxicity sensitization assays (40, and data not shown). Therefore, the differences in T cell activation induced by the three peptides are likely to be due to alterations in TCR/MHC:peptide interactions.

Induction of apoptosis in BK289 cells by the immunogenic and partially agonistic peptides

T cell triggering in vivo and in vitro may result in AICD (41, 42). Several mechanisms were shown to operate upon induction of

![FIGURE 3. Dose-response curves of peptide-triggered cytotoxicity. C1R/A11 cells prepulsed with the indicated concentrations of peptide were used as targets for BK289 CTLs in 4-h $^{51}$Cr release assays. The percent specific lysis was normalized to the maximal response induced by C1R/A11 cells prepulsed with $1 \times 10^{-7}$ M of the IVT peptide (range 60–82%). The mean values of four independent experiments are shown.](http://www.jimmunol.org/)

![FIGURE 4. Induction of IL-2 production and DNA synthesis. Induction of IL-2 production and DNA synthesis were measured in BK289 CTLs stimulated with irradiated peptide-pulsed C1R/A11 cells. A. Induction of IL-2 was measured by intracellular staining after overnight culture, as described in Materials and Methods. Double staining with an HLA A2-specific mAb was used to distinguish between the A2-positive CTLs and A2-negative stimulator cells. The results of one representative experiment demonstrating the effect of peptide stimulation on the percentage of IL-2-positive cells and mean green fluorescence intensity (IL-2 staining) of A2-positive cells. B. The increase in the percentage of IL-2-positive cells induced at a range of peptide concentrations was calculated as the difference between samples of CTLs stimulated with untreated and peptide-pulsed C1R/A11 cells. The numbers in the dot plots indicate the percentage of IL-2-positive cells and mean green fluorescence intensity (IL-2 staining) of A2-positive cells. C. Induction of DNA synthesis in BK289 CTLs was measured by [3 H]TdR incorporation after triggering for 3 days with C1R/A11 cells prepulsed with indicated concentrations of the IVT, Y5, or A8 peptide. One representative experiment of two performed in the absence of exogenously added IL-2 is shown in the figure. The [3 H]TdR incorporation of control cells incubated with irradiated C1R/A11 cells alone is indicated by diamonds.](http://www.jimmunol.org/)
apoptosis in T cells, each acting with a different kinetics (43). Therefore, we decided to monitor T cell death 3 days after specific stimulation when the majority of cells triggered to die should have completed their apoptotic program. Cultures of BK289 CTLs stimulated for 3 days with control-irradiated C1R/A11 cells yielded significantly higher cell numbers compared with cultures stimulated with APC pulsed with $1 \times 10^{-7}$ M of the IVT or Y5 peptides. In contrast, the recovery in cultures stimulated with C1R/A11 pulsed with the A8 peptides was only marginally lower than the control (Fig. 5A). This was not due to differential survival of the peptide-pulsed APCs since 93–98% of the cells recovered were T lymphocytes, as determined by staining with CD3-specific mAbs. Furthermore, similar data were obtained after depletion of the APCs with immunomagnetic beads coupled with CD19-specific Abs (data not shown).

To further compare the apoptosis-inducing activity of the IVT and Y5 peptides, cell recovery was monitored after stimulation for 3 days with C1R/A11 cells pulsed with different peptide concentrations. In contrast to its poor ability to induce IL-2 production and cell proliferation, the Y5 peptide was as efficient as IVT in inducing death of the BK289 cells (Fig. 5B).

To confirm that apoptosis represents the mechanism of AICD in our system, the percentage of apoptotic nuclei in BK289 CTLs

FIGURE 5. Triggering of apoptosis and AICD. A, Cell recovery in cultures of BK289 CTLs cultured for 3 days with C1R/A11 cells pulsed with $1 \times 10^{-7}$ M of the indicated peptide in the absence or presence of exogenously added IL-2. The number of viable cells was evaluated by trypan blue exclusion. The mean ± SD of five independent experiments is shown in the figure. B, Activity of the IVT and Y5 peptides over a range of peptide concentrations. The results of one representative experiment are shown. C, CTLs cultured for 24 h with irradiated C1R/A11 cells untreated (control) or pulsed with the indicated concentrations of peptides were double stained with Hoechst reagent and CD3-specific FITC-conjugated mAb. The percentage of T cells with apoptotic nuclei was evaluated by fluorescence microscopy.

FIGURE 6. Induction of Ca$^{2+}$ influx. CTLs were loaded with Fluo3 and then mixed with peptide-pulsed C1R/A11 cells at an E:T ratio of 3:1. The cells were then pelleted down, incubated for 2 min at 37°C, resuspended, and analyzed by flow cytometry. Each experimental point represents the mean fluorescence intensity of cells analyzed during a 40-s interval. Results obtained with C1R/A11 cells preincubated with $1 \times 10^{-6}$ M or $1 \times 10^{-8}$ M of the indicated peptides are shown by filled and open symbols, respectively. Diamonds represent the baseline obtained with C1R/A11 cells prepulsed with $1 \times 10^{-6}$ M of an irrelevant HLA A11-binding peptide.

FIGURE 7. Induction of acid release. BK289 cells ($1 \times 10^5$ cells/channel) were perfused with media in a microphysiometer, and the basal rate of acid release was potentiometrically monitored every 90 s. The basal rates ranged from 20–30 $\mu$V/s. Upon exposure to the peptide, the rate of acid release increased, reaching a maximum at either 90 or 180 s. The acid release was normalized to the maximum response observed upon exposure of T cells to 10 $\mu$M of the IVT peptide and ranged from 70–90 $\mu$V/s. An irrelevant HLA A11-binding peptide derived from p53 protein did not induce increase of acid release at any peptide concentration tested (not shown).
cultured for 24 h with untreated or peptide-pulsed C1R/A11 cells was monitored by Hoechst staining. The results presented in Fig. 5C demonstrate that the IVT and Y5 were equally potent in inducing apoptosis of the specific CTLs over a broad range of peptide concentrations, while the A8 analogue did not cause nuclear condensation above background levels.

Induction of early T cell activation events
The increase of intracellular Ca^{2+} and the release of acid into the extracellular space are early T cell activation events that can be differentially triggered by partially agonistic peptides (6, 38, 44). The IVT peptide and its analogues included in our analysis were capable of inducing Ca^{2+} influx, as determined by the increase of fluorescence of Fluo3-loaded T cells upon stimulation with peptide-pulsed C1R/A11 cells (Fig. 6). The response induced by C1R/A11 cells pulsed with 1 \times 10^{-8} M of the analogues was weaker and not as sustained as that induced by the IVT peptide, whereas, at 1 \times 10^{-6} M, the Ca^{2+} influx induced by Y5 was similar, in magnitude and kinetics, to that induced by the IVT peptide. A sustained but lower level response was induced by the A8 analogue at this concentration. Only a marginal increase in acid release, as analyzed by potentiometric measurements using a microphysiometer, was induced by the partial agonists at peptide concentrations sufficient to activate full-scale Ca^{2+} influx (Fig. 7).

Triggering with partial agonists inhibits the cytotoxic activity of BK289 CTLs
In the course of these studies, we repeatedly observed that CTLs cultured for 3 days with C1R/A11 cells prepulsed with the IVT peptide in the absence of exogenous IL-2 retained their cytotoxic activity, while the cytotoxic activity of CTLs triggered with the A8 and Y5 analogues was significantly decreased. To investigate this phenomenon in more details, BK289 CTLs were cultured with irradiated C1R/A11 cells pulsed with different concentrations of the IVT, Y5, or A8 peptides with or without addition of exogenous IL-2. Viable CTLs recovered after 3 days were then tested for their ability to lyse the A11-positive LCL JAC-B2 and to express IL-2.

FIGURE 8. Inhibition of cytotoxic activity by triggering with partial agonists. C1R/A11 cells were preincubated with the indicated concentrations of the IVT, Y5, or A8 peptide, irradiated, and extensively washed to remove the unbound peptide. BK289 CTLs were mixed with peptide-pulsed C1R/A11 cells at E:T ratio of 3:1, and were incubated in medium alone (closed symbols and bars) or medium containing 10 U/ml of IL-2 (open symbols and bars). After 3 days of incubation, the number of viable CTLs was detected and their cytotoxic activity was tested against JAC-B2 LCL, and the production of IL-2 mRNA was monitored by semiquantitative RT-PCR after triggering with C1R/A11 cells preincubated with 1 \times 10^{-8} M of the IVT peptide. The normalized OD of the specific RT-PCR products are presented as percentage relative to the OD of the specific IL-2 band induced in control BK289 cells.

FIGURE 9. TCR-independent cytotoxic activity of peptide-triggered CTLs. Viable BK289 cells were harvested after 3 days stimulation with untreated C1R/A11 cells or C1R/A11 pulsed with 1 \times 10^{-7} M of indicated peptides in the absence of exogenous IL-2 and then used as effectors in standard \textsuperscript{51}Cr release assay. Untreated and IVT-pulsed (1 \times 10^{-7} M) autologous LCL, BK-B5, and BK-B5 + IVT, and the A11-negative LCL MTB-B1 were used as targets. Killing of MTB-B1 cells was also tested in the presence of 5 \mu g/ml of Con A (MTB-B1 + Con A). The percent specific lysis at an E:T ratio of 3:1 is shown in the figure.
triggered CD19 TCR expression was calculated as the mean green fluorescence of peptide-fluorescence intensity was analyzed by flow cytometry. The percentage of cells relative to control cells incubated with untreated C1R/A11.

with the controls, but significantly higher than in IVT-stimulated CTLs (Fig. 8). It was previously shown that T cell unresponsiveness correlates with TCR down-regulation (19, 48). We have therefore analyzed the kinetics of this process in CTLs stimulated with the IVT, Y5, or A8 peptides in the absence of exogenous IL-2. TCR expression was assessed by staining with anti-CD3 mAb, and double staining with anti-CD19 was performed to distinguish between T cells and APCs. A rapid decrease of TCR expression was induced by the immunogenic epitope and, to a lower extent, by the Y5 and A8 analogues. In all cases, TCR expression was completely reconstituted after 3 days of culture (Fig. 10).

Discussion

TCR engagement by specific and nonspecific ligands results in T cell activation and proliferation, but can at the same time lead to AICD in a population of activated cells. The balance between activation and death appears to be determined by the strength of the activating signal, the proliferative stage of the responder cells (49), and the type of cells involved (17). However, the interplay between activating and apoptosis-inducing signals in T cells remains still elusive. We have compared the induction of CTL activation and death by the immunogenic peptide IVT and its partially agonistic analogues. The major finding of our study is the identification of a partially agonistic peptide that dissociates death-inducing signals from the triggering of IL-2 production and cell proliferation.

Analysis of a relatively small panel of synthetic analogues of the IVT peptide identified a number of ligands that efficiently triggered cytotoxic activity of the CTL clones BK289 and BK112 without induction of IL-2 mRNA. Similar observations were reported in other models (3, 19). This suggests that selective induction of cytotoxic activity is a common feature of peptide analogues carrying substitutions in TCR contact residues of CTL epitopes. A detailed characterization of the Y5 and A8 peptides confirmed that they both fulfill the criteria for partial agonism since the dissociation of cytotoxicity and IL-2-inducing functions occurred over a wide range of peptide concentrations (Figs. 3 and 4). However, only the Y5 peptide was as active as the original epitope in inducing apoptosis of BK289 cells, while triggering with the A8 analogue did not result in any measurable AICD (Fig. 5).

The in vitro expansion of specific CTLs requires periodic re-stimulation with the specific Ag. This is usually done at responder:stimulator ratios higher than 20:1 and in the presence of feeder cells that probably provide additional costimulatory signals to T cells. In contrast, we have observed induction of apoptosis at responder:stimulator ratios of 3:1 or lower and in the absence of feeders. Interestingly, under these conditions, DNA synthesis was also triggered, suggesting that apoptosis affects cells in different phases of the cell cycle. Alternatively, apoptosis and DNA synthesis may be triggered in different subpopulations of CTLs, perhaps depending on their intrinsic state of activation. This possibility appears less likely since the recovery of cells cultured for 3 days with IVT- or Y5-pulsed APCs was similar, but only IVT induced DNA synthesis (Figs. 4 and 5).

It is noteworthy that most of the published work on AICD in human T cells was performed using TCR cross-linking with anti-CD3 or TCR chain-specific Abs. In one study investigating the effect of a natural peptide ligand on CTLs, AICD was seen only at very high, supraoptimal peptide concentrations (37, 50). We have observed death of BK289 CTLs at relatively low peptide concentrations, suggesting that AICD could play a role in physiological regulation of EBV-specific CTL responses. Although most of our experiments were performed on CTLs that had reached a resting state about 2 wk after stimulation with PHA-pulsed feeders, similar results were obtained with actively proliferating CTLs (data not shown). This is in agreement with previous studies that did not reveal any difference in the levels of AICD in proliferating or resting CTLs (50). Therefore, it is unlikely that the differences in peptide concentrations required for the induction of AICD are due to differences in the proliferative state of CTLs utilized in different laboratories, but it may be accounted for by the use of different APCs for peptide presentation (EBV-transformed B cells vs splenocytes). We cannot exclude the possibility that CTLs may belong to functionally different subpopulations with variable sensitivity to AICD, as observed in mouse CD4+ Th1 and Th2 lymphocyte subsets (17). Recently, different patterns of lymphokine production have been described for EBV-specific CTL clones (51). It would be interesting to investigate the correlation between the sensitivity to AICD and the pattern of lymphokine production of these cells.

Several lines of evidence suggest that IL-2 plays a critical role in the induction of apoptosis in certain T cell types by up-regulating apoptosis-inducing molecules and increasing the sensitivity of cells to apoptotic signals (52, 53). Our results suggest that IL-2 signals are not required for the induction of AICD in CTLs. The addition of exogenous IL-2 did not affect the induction of apoptosis by the immunogenic peptide and, more importantly, apoptosis was efficiently induced in BK289 by the Y5 analogue at peptide concentrations, which did not trigger detectable levels of IL-2 protein or mRNA (Figs. 4 and 5). We cannot exclude, however, that the exposure of CTLs to rIL-2 before specific stimulation is necessary and sufficient to render the cells sensitive to apoptotic signals. In conclusion, the death of BK289 CTLs induced by the immunogenic peptide and Y5 analogue was seen at relatively low peptide concentrations, was not affected by the presence of exogenous or induction of endogenous IL-2, and was not dependent on the proliferative state of CTLs.

In an effort to explore the mechanisms underlying the difference between the IVT, Y5, and A8 peptides, we have compared their ability to induce the activation of acid release and Ca2+ influx in BK289 cells. The induction of acid release was not previously studied in CD8+ cells. The dissociation of cytotoxic activity from acidification observed upon triggering with partial agonists or with low concentrations of the IVT peptide (Figs. 3 and 7) demonstrates...
that acidification of the extracellular space is not accounted for by the release of cytotoxic granules from the CTLs or leakage of intracellular products from the lysed targets. The fast kinetics of acidification suggests that this process may be dependent on the activation of proton transport. Interestingly, this activation parameter showed a very good correlation with the induction of IL-2 mRNA and cell proliferation. In contrast, both analogues induce Ca\(^{2+}\) influx at the level comparable with that induced by the IVT peptide at high peptide concentrations (\(1 \times 10^{-6}\) M). This correlates with their ability to induce cytotoxicity, which is known to require Ca\(^{2+}\) influx. In summary, we failed to pinpoint any clear difference in the early activation events triggered by apoptosis-inducing and noninducing ligands. Recently, it was shown that these two types of ligands cannot be distinguished on the basis of the pattern of tyrosine phosphorylation induced in CD4\(^+\) lymphocytes (13). Therefore, it remains to be determined which signaling pathways are selectively or predominantly activated by apoptotic peptide analogues. In this context, it should be noticed that the Y5 peptide was slightly more efficient than A8, in most of the assays. According to the kinetics discrimination model of T-cell activation, the TCR must interact with its peptide:MHC ligand for a time sufficient to form a complete signaling complex (54, 55). It is possible that A11 molecules containing the Y5 peptide form more stable complexes with the TCR expressed by BK289 cells compared with complex containing A8. This stability may be sufficient for the induction of apoptosis, but not for the induction of full scale T-cell activation. The effector mechanisms of apoptotic death as well as signaling pathways induced by the Y5 peptide in BK289 CTLs are currently under investigation.

The ability of the Y5 peptide to selectively trigger apoptosis may prove useful for the down-regulation of pathological or excessive immune responses. The validity of this concept was experimentally proven in a TCR transgenic mouse model in which CD4\(^+\) T lymphocytes have been depleted by systemic administration of partial agonists with apoptosis-inducing activity (16). The effect was not associated with the immunopatohology and general immunosuppression observed upon systemic administration of fully agonistic peptides (14, 16). The use of partially agonistic peptides for the modulation of CTL responses may be limited by their capacity to trigger cytotoxicity. We have tested the cytotoxic activity of BK289 cells against the HLA A11-positive LCL JAC-B2 3 days after challenge with APCs pulsed with the IVT, Y5, or A8 peptides. In agreement with previous studies (56–58), the cytotoxic activity of CTLs triggered with the immunogenic peptide was not affected, while pretriggering with the Y5 and A8 peptides in the absence of exogenous IL-2 resulted in significant inhibition of LCL lysis (Fig. 8). The effect was not attributed to sustained TCR down-regulation since the expression was completely reconstituted after 3 days of culture irrespective of the addition of exogenous IL-2 (Fig. 10). This phenomenon appears to be different from other forms of CTL anergy or CTL paralysis that either do not affect cytotoxicity (56–58) or require CTLs as APC (paralysis) (58, 59). Somewhat surprisingly, the IVT-triggered cells produced low amounts of IL-2 mRNA in response to specific rechallenge in spite of their full cytotoxic potential. This is likely to reflect the induction of a refractory state, as described in other systems (45–47). The Y5- and A8-triggered cells produced higher levels of IL-2 upon rechallenge (Fig. 8), indicating that they were not generally less functional or more prone to apoptosis. The possibility that activation with partial agonists consumes the cytolytic granules that are not reconstituted in the absence of endogenous or exogenously added IL-2 is supported by the inability of Y5- and A8-triggered cells to execute MHC-independent Con A-induced lysis of A11-negative target cells (Fig. 9).

In conclusion, the characterization of partially agonistic peptides performed in this study demonstrates that death and activation signals can be dissociated in CTLs. Peptide analogues that selectively induce apoptosis may provide a valuable tool for dissecting the relevant signaling pathways and may have a potential application as therapeutic agents for specific inhibition of CTL responses.

References


17. Tsitoura, D. C., W. Holter, A. Cerwenka, C. M. Gelder, and J. R. Lamb. 1996. Induction of partial agonists with apoptosis-inducing activity (16). The ability of the Y5 peptide to selectively trigger apoptosis may prove useful for the down-regulation of pathological or excessive immune responses. The validity of this concept was experimentally proven in a TCR transgenic mouse model in which CD4\(^{+}\) T lymphocytes have been depleted by systemic administration of partial agonists with apoptosis-inducing activity (16). The effect was not associated with the immunopathology and general immunosuppression observed upon systemic administration of fully agonistic peptides (14, 16). The use of partially agonistic peptides for the modulation of CTL responses may be limited by their capacity to trigger cytotoxicity. We have tested the cytotoxic activity of BK289 cells against the HLA A11-positive LCL JAC-B2 3 days after challenge with APCs pulsed with the IVT, Y5, or A8 peptides. In agreement with previous studies (56–58), the cytotoxic activity of CTLs triggered with the immunogenic peptide was not affected, while pretriggering with the Y5 and A8 peptides in the absence of exogenous IL-2 resulted in significant inhibition of LCL lysis (Fig. 8). The effect was not attributed to sustained TCR down-regulation since the expression was completely reconstituted after 3 days of culture irrespective of the addition of exogenous IL-2 (Fig. 10). This phenomenon appears to be different from other forms of CTL anergy or CTL paralysis that either do not affect cytotoxicity (56–58) or require CTLs as APC (paralysis) (58, 59). Somewhat surprisingly, the IVT-triggered cells produced low amounts of IL-2 mRNA in response to specific rechallenge in spite of their full cytotoxic potential. This is likely to reflect the induction of a refractory state, as described in other systems (45–47). The Y5- and A8-triggered cells produced higher levels of IL-2 upon rechallenge (Fig. 8), indicating that they were not generally less functional or more prone to apoptosis. The possibility that activation with partial agonists consumes the cytolytic granules that are not reconstituted in the absence of endogenous or exogenously added IL-2 is supported by the inability of Y5- and A8-triggered cells to execute MHC-independent Con A-induced lysis of A11-negative target cells (Fig. 9).


