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*J Immunol* 2001; 166:989-995; doi: 10.4049/jimmunol.166.2.989

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Different Programs of Activation-Induced Cell Death Are Triggered in Mature Activated CTL by Immunogenic and Partially Agonistic Peptide Ligands

Cheng-Hong Wei,* Hideo Yagita,† Maria G. Masucci,* and Victor Levitsky2*†

Activation-induced cell death (AICD) of mature T cells plays an important role in the control of immune homeostasis and peripheral tolerance. TNFRs and Fas have been implicated in the induction of AICD. However, these molecules were shown to be dispensable, at least in some experimental systems, for downsizing of Ag-induced T cell expansions and development of tolerance in vivo. The conditions of T cell activation leading to T cell deletion in a death receptor-independent manner are not well characterized. Here we show that human CTLs die through a death receptor-independent apoptotic program upon triggering with a partially agonistic peptide ligand. This apoptotic process exhibits some features of T cell death due to lymphokine deprivation and is blocked by exogenous IL-2. Our data demonstrate that engagement of TCR by MHC-peptide complexes can trigger diverse apoptotic programs of AICD and that the choice between these programs is determined by the agonistic potency of MHC-peptide ligand. The Journal of Immunology, 2001, 166: 989–995.

Lymphocytes die en masse during different stages of normal immune response (1, 2). Dysregulation of the death process can lead to lymphopenia and increased incidence of autoimmunity (3–6). Repeated triggering of mature activated T cells results in activation-induced cell death (AICD)7 by apoptosis (reviewed in Ref. 7). Molecules of the TNFR superfamily, Fas and TNFR1 and -2, were implicated by in vitro and in vivo studies in the induction of AICD. Therefore, expression of TNF-a and Fas ligand (FasL) by activated T cells is viewed as an important negative feedback mechanism of immune regulation (8–10). Fas and TNFRs transmit their death signals through activation of caspases, a family of cysteine proteases (11–14). Transduction of apoptotic signals through these receptors requires the activation of caspase-8 induced upon receptor oligomerization. Caspase-8 cleaves and activates caspase-3, which directly initiates the apoptotic changes (15, 16).

Activated T cells also die upon withdrawal of lymphokines (7, 17). This “passive” death is independent of death receptors, requires de novo protein synthesis, and is blocked by the anti-apoptotic proteins Bcl-2 or Bcl-XL (18–20). Anti- and pro-apoptotic Bcl-2-related proteins regulate the sensitivity of cells to death signals at multiple levels (for review, see Refs. 21–23) including the maintenance of mitochondrial transmembrane potential. A drop of mitochondrial potential and increase of membrane permeability result in the release of cytochrome c, its association with the cytosolic protein Apaf-1, and activation of caspase-9 (24, 25). Caspase-9 activates caspase-3 and, as recent evidence suggests, may also act directly as an executioner caspase (26).

IL-2 plays opposing roles in the regulation of the two forms of T cell death. It enhances AICD by up-regulation of FasL and down-regulation of anti-apoptotic Fas-associated death domain-like IL-1-converting enzyme (FLICE)-like inhibitory protein (FLIP) (27, 28) and prevents the development of the passive form of T cell apoptosis through up-regulation of Bcl-2 and Bcl-XL (17, 20).

Recent studies strongly suggest that withdrawal of lymphokines and AICD mediated by death receptors cannot account for all instances of T cell death. Experiments with mice deficient in Fas or FasL (1, 29–32) or lacking both Fas and TNFR1 demonstrate that these molecules are not essential for the termination of specific immune responses (33, 34), suggesting the existence of active but death receptor-independent mechanisms of T cell death. Furthermore, superantigen-induced deletion of mature T lymphocytes in vivo does not require Fas or TNF (35), and the development of transplantation tolerance under conditions of costimulatory blockade involves triggering and proliferation of specific T cells followed by their elimination in a Fas-independent manner (36, 37). However, in these two cases T cell activation does not involve the recognition of specific MHC-peptide ligands and does not lead to productive immune response and establishment of memory.

It is not known whether death receptor-independent apoptotic programs can be directly initiated in mature T lymphocytes by recognition of MHC-peptide complexes on the surface of APCs.

Small structural modifications in the antigenic peptides recognized by specific T cells often result in significant changes in both the amplitude and profile of T cell activation events (38). Variants of immunogenic T cell epitopes selectively inducing apoptosis in CD4+ or CD8+ T cells have been described (39–41). In the case of mouse CD4+ T cells, the activity of such partial agonists has been attributed to the selective induction of FasL and TNF-α. In this study we demonstrate that upon triggering with a partially agonistic peptide mature specific T cells die through an apoptotic program that does not require Fas and TNF-α and is similar in several characteristics but yet distinct from T cell death due to lymphokine deprivation.

Materials and Methods

Cell lines and CTL clones

The generation, fine peptide specificity, and TCR structure of the EBV-specific HLA-A11-restricted CTL clones BK289 and CAR13 that recognize the EBV nuclear Ag (EBNA)-4-derived epitope IVTDFSVK (IVT) were described previously (42, 43). An HLA-A11-transfected subline of the MHC class I-deficient mutant cell line C1R (44) was maintained in RPMI 1640 medium supplemented with 100 μg/ml streptomycin, 100

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Received for publication July 27, 2000. Accepted for publication October 27, 2000.

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This work was supported by grants from the Swedish Cancer Society, the Children’s Cancer Foundation, the Foundation for Strategic Research, and the Karolinska Institution, Stockholm, Sweden.

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Abbreviations used in this paper: AICD, activation-induced cell death; CMFDA, carboxyfluorescein diacetate acetyl ester; CsA, cyclosporine A; EBNA, EBV nuclear Ag; FasL, Fas ligand; FLIP, FLICE-like inhibitory protein; IVT, IVTDFSVK; IFN-γ, interferon-γ; TRAIL, TNF-related apoptosis-inducing ligand; Y5, IVTDFSVK.
IU/ml penicillin, 10% FCS (complete medium), and 200 μg/ml hygromycin B. The cell line is transformed by the B-type EBV strain, which carries mutations in the IVT epitope abolishing its recognition by IVT-specific CTLs (45). The TNP-sensitive mouse fibrosarcoma cell line WEHI 164 (no. CRL-1751; American Type Culture Collection, Manassas, VA) was maintained in complete medium.

Abs and reagents

The following Abs were used in this study: FITC-conjugated anti-human Fas mAb (Dako, Copenhagen, Denmark), FITC-conjugated anti-human and anti-mouse FasL mAb (Alexis, San Diego, CA), anti-human TNFR1, TNFR2 mAbs (R&D Systems, Abingdon, U.K.), FITC or PE-conjugated anti-human CD3 mAbs and isotype control Abs (PharMingen, San Diego, CA), and Bcl-2-specific mAb (clone Bcl-2-100; Zymed Laboratories, Carlsbad, CA). Neutralizing polyclonal rabbit anti-human TNF-α antiserum was purchased from Pharmingen (San Diego, CA). Recombinant human TNF-α was purchased from Boehringer Mannheim Scandinavia AB (Bromma, Sweden). A neutralizing anti-human FasL mAb (NOK-2) and a neutralizing anti-human TRAIL mAb (RK-2) were prepared as described previously (9, 46). The cell permeable, irreversible inhibitor of caspase-3, Z-DEVD-FMK, caspase-8, Z-LETD-FMK, caspase-9, Z-LEHD-FMK, and general caspase inhibitor, BOC-D-FMK, (Enzyme System Products, Livermore, CA) were dissolved in DMSO and stored at −20°C as a 20 mM stock solutions. Cyclosporine A (CsA) was purchased from Sigma (St. Louis, MO).

Synthetic peptides

Peptides were synthesized by the Merrifield solid-phase method at Alta Bioscience (University of Birmingham, School of Biochemistry, Birmingham, U.K.) and purified by HPLC on SuperPac Pep-S 5-mm reverse-phase columns (Pharmacia, Uppsala, Sweden). The purified peptides were dried using SpeedVac (Pharma) and dissolved in DMSO at a concentration of 1 × 10^6 M, as determined by Biuret assays. Peptide dilution in PBS was performed immediately before the assays to obtain the indicated concentrations.

Biological assay for TNF-α

WEHI 164 cells were seeded on flat-bottom 96-well plates (20,000 cells/well) in 100 μl of complete medium. After incubation at 37°C for 2 h, medium was removed, and 100 μl of culture supernatant of stimulated or unstimulated T cells or complete medium with or without serial dilutions of recombinant TNF-α (from 10 ng/ml to 2 pg/ml) were added to wells in triplicates. Actinomycin-D was added to each well to a final concentration of 2 μg/ml. The plates were incubated for 20 h at 37°C, and 50 μl of 2 mg/ml MTT solution (Sigma) in PBS were added to each well. After an additional 3–4 h of incubation, 100 μl of 10% SDS, 0.01 N HCl solution were added to each well and crystals of enzymatically modified MTT were solubilized during 4 h incubation at 37°C. The OD of the resulting solution was measured at 540 nm. The concentration of TNF-α in culture supernatants was determined by a standard curve generated in each experiment using a serial dilution of recombinant TNF-α.

T cell stimulation and monitoring of cell death

C1R/A11 cells, untreated or pulsed with the indicated concentrations of synthetic peptides, were stained during a 10- to 20-min incubation at room temperature with 2 μM carboxyfluorescein diacetate aceetylperoxy (CMFDA; Molecular Probes, Eugene, OR), irradiated (8000 rad), extensively washed, and mixed with 1–2 × 10^6 BK289 CTLs in round-bottom tubes at an E:T ratio of 3:1. After a brief centrifugation to help conjugate formation, cells were incubated at 37°C for 1 h and then transferred to a 24-well plate. Each sample was incubated in duplicate wells for 24 or 72 h. In the 24-h assay, cells were stained with propidium iodide (PI), and flow cytometry analysis was performed by acquiring all events at a constant flow rate during a fixed percent of the total time using FACSScan with CellQuest software (Becton Dickinson, Mountain View, CA). Cell recovery was estimated as the number of PI and fluorescein double-negative cells. Acquisition was performed immediately before the assays to obtain the indicated concentrations.

Analysis of surface marker expression

To analyze TNFR expression, 1–2 × 10^6 BK289 CTLs were mixed with untreated or peptide-pulsed C1R/A11 cells in round-bottom tubes at an E:T ratio of 3:1. After 24 h of incubation at 37°C, the cells were incubated with TNFRI- or -2-specific mAb for 30 min on ice, washed, and then stained with PE-conjugated rabbit anti-mouse Ig (Dako). For measurement of Fas or FasL expression, the mixtures of CTLs (HLA-A2^+^) and APCs (HLA-A2^+^) were cultured with or without the metallocorticosteroidase inhibitor KB8301 (PharMingen), incubated with HLA-A2-specific mAb HB54, washed, and stained with PE-conjugated anti-mouse Ig. After washing in PBS, the cells were stained with FITC-conjugated Fas-L or Fas-specific mAbs. FACs analysis was performed using a FACScan with CellQuest software (Becton Dickinson). Background staining was assessed using isotype-matched Abs conjugated with the relevant fluorescent dye (PharMingen).

Analysis of Bcl-2 and Bcl-XL expression

BK289 CTLs untreated or incubated for 12 or 24 h with irradiated C1R/ A11 cells pulsed with the synthetic peptides were lysed in electrophoresis sample buffer, and the amount of lysate corresponding to 1 × 10^6 CTLs was separated by discontinuous SDS-PAGE. The gels were blotted onto nitrocellulose filters, which were then incubated with 1:1000 diluted Bcl-2-specific mAb or with 1:1000 diluted Bcl-XL-specific rabbit antiserum. After incubation with anti-mouse or anti-rabbit HRP-conjugated Abs, the blots were visualized by ECL, and the intensities of specific bands were quantified by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Results

Fas-FasL interactions are not required for AICD induced upon triggering with the partially agonistic peptide ligand

The CTL clone BK289 recognizes the HLA-A11-restricted peptide IVTDFSVIK (designated IVT) corresponding to the amino acid residues 416–424 of EBNA4. An analogue of IVT containing an F to Y substitution in position 5 of the peptide (Y5) acts as a partial agonist for BK289 CTLs triggering cytotoxic activity without induction of IL-2 production or thymidine incorporation. We have previously shown that a comparable proportion of BK289 cells die by apoptosis upon IVT- or Y5-mediated triggering (41); however, the molecular mechanisms of apoptosis induced by the two peptides have not been characterized.

BK289 CTLs maintained in vitro in IL-2 medium constitutively expressed low levels of both Fas and FasL. Stimulation with IVT-pulsed C1R/A11 cells in the presence of metallocorticosteroidase inhibitor resulted in a 2- to 3-fold up-regulation of FasL, whereas Y5-mediated triggering induced only 40–50% increase of FasL expression (Fig. 1A). The surface expression of Fas was not affected by stimulation with either of the peptides (data not shown). To assess the role of Fas-FasL interactions in the IVT- and Y5-mediated apoptotic process, the number of viable BK289 cells was determined after coculture for 72 h with peptide-pulsed APCs in the presence or absence of the FasL-blocking Ab NOK-2. In agreement with our previous report, both IVT- and Y5-mediated triggering decreased the recovery of T cells by 60–70% (Fig. 1B). In the presence of NOK-2 Ab, the recovery of IVT-stimulated CTLs was completely rescued and in some experiments exceeded that of control samples, which was most likely due to IVT-induced cell proliferation. In contrast, addition of the same mAb did not improve the recovery of CTLs stimulated with Y5-pulsed APCs, suggesting that Fas-FasL interaction is not involved in Y5-mediated apoptosis.

The generality of this phenomenon was assessed using the IVT-specific CTL clone, CAR13. Although CAR13 and BK289 CTLs are derived from different individuals and express structurally different TCRs (42), the Y5 peptide induces comparable patterns of partial activation in both clones (V. Levitsky and C.-H. Wei, unpublished data). As observed with BK289 cells, the NOK-2 mAb rescued the recovery of IVT-stimulated but not Y5-stimulated CAR13 CTLs (Fig. 1B). These results demonstrated that the ability of the partially agonistic peptide to trigger Fas-independent apoptosis in specific CTLs is not clone or donor dependent.

Addition of NOK-2 Abs rescued BK289 cells from apoptosis induced by the IVT peptide over a wide range of peptide concentrations,
while the recovery of Y5-stimulated cells was not affected (Fig. 1, C and D). Therefore, the Y5-induced Fas-independent AICD does not mimic the process occurring upon weak stimulation induced by small amounts of the immunogenic epitope.

Triggering with either the IVT or Y5 peptide resulted in up-regulation of TNFR2 expression on the surface of BK289 cells but did not affect the levels of TNFR1 (data not shown). In several independent experiments, 3- to 4-fold higher amounts of soluble TNF-α were detected in supernatants of IVT-triggered CTLs (300–2500 pg/ml) as compared with supernatants of Y5-activated cells (100–700 pg/ml). However, the recovery of BK289 cells stimulated with IVT- or Y5-pulsed APCs was not affected by the addition of neutralizing Abs to TNF-α or blocking mAbs against TNFR1 and -2. Blocking of the TNF-related apoptosis-inducing ligand (TRAIL), which has been implicated in the development of T cell apoptosis in some models (47, 48), also had no effect (data not shown). We concluded that TNF-α and TRAIL do not play any significant role in the induction of apoptosis in BK289.

Collectively, these results demonstrate that Fas-FasL interactions account for the bulk of apoptosis in BK289 CTLs upon IVT-mediated activation but are not essential for the induction of cell death in Y5-activated CTLs.

The Fas-independent Y5-induced apoptosis of BK289 CTLs develops with delayed kinetics

The Fas-mediated apoptotic program can be completed within hours while other forms of apoptosis progress relatively slowly (49, 50). To investigate the kinetics of CTL death induced by IVT- or Y5-triggering, BK289 cells were cocultured with untreated or peptide-pulsed APCs and the recovery of viable CTLs was evaluated at the indicated time points. The recovery of IVT-stimulated CTLs dropped by 50% during the first 24 h of stimulation but remained stable during the following 48 h of culture (Fig. 2A). Death of the IVT-stimulated BK289 cells was inhibited by the NOK-2 Ab and the caspase-3 inhibitor, Z-DEVD-FMK, both at 24 and 72 h after triggering. Following Y5-mediated activation, the number of viable cells decreased only by 25% after 24 h, and the loss of cells was at this time point inhibited by reagents that block Fas-mediated apoptosis (Fig. 2B). However, the number of viable cells declined further, amounting only to about 25% of that in the controls after 72 h, and this slower apoptotic process was not prevented by NOK-2 or Z-DEVD-FMK (Fig. 2C). These data suggest that upon Y5-mediated triggering at least two independent apoptotic pathways are activated in BK289 CTLs. One pathway proceeds with a relatively rapid kinetics and requires the engagement of Fas, while another pathway proceeds relatively slowly and is independent of Fas-FasL interactions.

Exogenous IL-2 renders Y5-stimulated CTLs responsive to FasL blockade

We have previously shown that triggering of BK289 cells with the IVT but not with the Y5 peptide induces a substantial increase in IL-2 gene transcription and IL-2 protein secretion (41). Because IL-2 has opposing effects on different apoptotic programs in T cells, we tested the ability of this lymphokine to affect the survival of BK289 cells upon
IVT- or Y5-mediated triggering in the absence or presence of different apoptosis blocking reagents. The number of viable cells was determined in CTL cultures with or without IL-2 72 h after triggering. The recovery of Y5-stimulated CTLs was increased 2-fold by addition of exogenous IL-2, while a slight decrease of recovery was observed in IVT-stimulated cultures (Fig. 3A). FasL blockade resulted in a strong increase of CTL recovery after IVT-triggering regardless of the presence of exogenous IL-2. In contrast, the NOK-2 Ab increased the recovery of Y5-stimulated CTLs only in the presence of exogenous IL-2 (Fig. 3A). Similarly, specific inhibitors of caspase-3 (Z-DEVD-FMK) and caspase-8 (Z-LETD-FMK) blocked IVT-induced apoptosis both in the absence and in the presence of exogenous IL-2 but inhibited Y5-induced CTL death only when IL-2 was added (Fig. 3, B and C). The general inhibitor of caspase activity, BOC-D-FMK, and the specific caspase-9 inhibitor, Z-LEHD-FMK (51, 52), had a strong protective effect against both IVT- and Y5-induced cell death independently of exogenous IL-2. These results indicate that IL-2 enhances apoptotic signals transmitted through Fas but blocks the alternative apoptotic pathway activated in Y5-stimulated BK289 cells.

The Y5 analogue fails to induce up-regulation of Bcl-2 and Bcl-X \textsubscript{L} in BK289 cells

Anti-apoptotic proteins of the Bcl-2 family are up-regulated by IL-2 and protect a variety of cells from apoptosis involving mitochondrial damage but they have little effect on Fas-mediated death of T cells (7, 53). Expression of Bcl-2 and Bcl-X \textsubscript{L} in BK289 cells was analyzed by immunoblotting before and after IVT or Y5 triggering. BK289 cells expressed a relatively high level of Bcl-2, which was slightly increased by IVT triggering or addition of IL-2 but was not affected by Y5 stimulation. The IVT peptide induced ~10-fold up-regulation of Bcl-X \textsubscript{L} expression (Fig. 4). In contrast, only a relatively small increase (~2-fold) of Bcl-X \textsubscript{L} expression was induced by stimulation with the Y5 peptide. Addition of IL-2 alone (data not shown) or in combination with Y5-pulsed APC resulted in a significantly stronger Bcl-X \textsubscript{L} up-regulation although in this case the expression of Bcl-X \textsubscript{L} was only half.

FIGURE 2. Kinetics of apoptotic death and time-dependent changes in the sensitivity to FasL blocking or caspase inhibition in BK289 CTLs after IVT- or Y5-mediated triggering. A, Recovery of viable cells was evaluated after 24, 48, and 72 h using trypan blue staining, and T cell recovery was calculated after measuring the percentage of viable APCs in the same samples by FACS analysis. One representative of three experiments is shown. In separate experiments, BK289 CTLs were cocultured with CMFDA-labeled APCs pulsed with either the IVT or Y5 peptide in the presence or absence of FasL blocking Ab (NOK-2; 10 µg/ml) or Z-DEVD-FMK (50 µM). The recovery of viable T cells was determined after 24 (B) and 72 h (C) by PI staining and FACS analysis and expressed as percentage relative to control samples stimulated by APCs without peptide. Mean and SDs of three experiments.

FIGURE 3. Effect of exogenous IL-2 on the recovery of IVT- or Y5-stimulated BK289 cells and their sensitivity to FasL blocking and caspase inhibition. BK289 CTLs were cocultured with IVT- or Y5-pulsed irradiated APCs in the presence or absence of exogenous IL-2 (10 U/ml) with or without addition of FasL blocking Ab (A). The mean and SDs of three experiments are shown. Alternatively, cells were cultured in the absence (B) or presence (C) of rIL-2 with or without addition of either Z-DEVD-FMK (50 µM), Z-LETD-FMK (50 µM), Z-LEHD-FMK (100 µM), or BOC-D-FMK (100 µM). After 72 h of culture, the recovery of viable T cells was determined by PI staining and FACS analysis and expressed as percentage relative to control samples stimulated by APCs without peptide in the presence or absence of rIL-2, respectively. One representative of two experiments is shown. Individual caspase inhibitors were tested in four to eight separate experiments with similar results.
of that detected in IVT-stimulated BK289 cells. Therefore, resistance of BK289 cells to Y5-mediated apoptosis correlates with increased levels of Bcl-XL expression.

CsA rescues survival of Y5-activated BK289 cells but does not affect IVT-mediated apoptosis or CTL death upon IL-2 withdrawal

Dysregulation of Ca\(^{2+}\) homeostasis can result in apoptotic cell death that is inhibited by Bcl-2 (21, 54). Because both IVT and Y5 induce Ca\(^{2+}\) influx in BK289 cells (41), we tested the effect of the calcineurin inhibitor, CsA, on the CTL apoptosis induced by the two peptides. The recovery of IVT-stimulated CTLs estimated 72 h after activation was not significantly affected by the presence of CsA, while the survival of Y5-stimulated CTLs increased by ~2-fold (Fig. 5A). Although CsA is a strong inhibitor of IL-2 production and blocks IL-2-dependent up-regulation of Fas, the death of IVT-activated cells occurred in a Fas-dependent manner also in the presence of the inhibitor (Fig. 5B). The rate of passive death induced by culture of BK289 cells in the absence of exogenous IL-2 was also not affected by CsA (Fig. 5C). These results indicate that the effect of CsA on Y5-induced apoptosis is due to its capacity to interfere with an apoptotic program that differs from both Fas-mediated and lymphokine deprivation-induced programs of T cell death.

Discussion

In this study, we demonstrate that small alterations in the structure of an immunogenic peptide can modify its capacity to trigger different apoptotic pathways in mature CTLs. While triggering of CTL death by the immunogenic peptide is mediated by Fas-Fasl interactions, some partially agonistic peptide ligands, represented by the Y5 peptide in our experimental system, are capable of triggering both Fas-mediated and death receptor-independent programs of apoptosis.

Stimulation with Y5-pulsed APCs weakly induces surface expression of FasL on BK289 cells (Fig. 1), and a FasL-blocking Ab improves the recovery of Y5-activated CTLs 24 h after triggering (Fig. 2). However, at later time points the CTLs die through a slowly progressing Fas-independent apoptotic program as revealed by a significant decrease of cell survival that cannot be rescued by FasL blocking or inhibition of caspase-3 or caspase-8 activity. While exogenously added IL-2 promotes IVT-induced apoptosis, CTLs exposed to Y5-pulsed APCs are rescued. Exogenous IL-2 and FasL-blocking have additive effect because IL-2 alone protects only a proportion of Y5-stimulated cells but its combination with FasL-blocking Ab completely prevents the development of Y5-induced death. The cell population rescued by IL-2 alone appears to die exclusively through a Fas-independent mechanism because, upon prolonged incubations, Fas-blockade does not rescue any cells, unless exogenous IL-2 is also provided. This demonstrates the predominant role of Fas-independent apoptosis in Y5-stimulated CTLs.

The differential effect of IL-2 on the two apoptotic programs is consistent with its dualistic effect in vivo. Although IL-2 deficiency leads to abnormal lymphoproliferation probably due to inability of T cells to up-regulate FasL at physiological levels (55–57), the lymphokine also promotes survival of activated T cells (58). This latter effect may be explained, at least in part, by interference with the apoptotic pathway described in this study. In vivo this type of apoptosis might be induced in CTLs by a subset of endogenous peptide ligands or by other types of triggering leading to partial activation (e.g., in the absence of costimulation).

Several lines of evidence suggest that the Y5-induced apoptosis represents an entity distinct from both Fas-mediated and passive cell death due to lymphokine withdrawal. First, it is initiated by TCR engagement because the control unstimulated cells do not die within the time frame of our experiments without exogenous IL-2 (Fig. 2A). This might still be viewed as sensitization of cells to IL-2 deprivation. However, this seems unlikely because death due to lymphokine withdrawal is known to require caspase-3 activity (59), while inhibition of the enzyme did not rescue Y5-stimulated cells. Moreover, CsA, which protects a significant proportion of Y5-stimulated cells, had no effect on Fas-mediated or passive T cell death in our system (Fig. 5).

The general caspase inhibitor BOC-D-FMK protected IVT- and Y5-stimulated cells from apoptosis, indicating that caspases are essential for the induction of CTL death by both peptides. However, the mechanisms and the patterns of caspase activation seem to be different in the two cases. IVT-induced apoptosis required the activity of caspase-3...
and -8 while apoptosis of Y5-stimulated CTLs was only marginally affected by the specific inhibitors (Figs. 2 and 3). Oligomerization of death receptors and mitochondrial damage are two major causes of caspase activation (54, 60). Because the IL-2-sensitive apoptotic pathway activated by Y5 does not appear to involve known death receptors, it is very likely that a dysregulation of mitochondrial function plays a major role in the activation of caspases under these conditions. In agreement with this possibility, Y5-stimulated cells were rescued from apoptosis by inhibition of caspase-9, which is activated by the release of cytochrome c from mitochondria (24, 61). Surprisingly, the specific inhibitor of caspase-9, Z-LEHD-FMK, also prevented IVT-induced apoptosis (Fig. 3). This suggests that activation of caspase-9 in IVT-stimulated BK289 cells may involve caspase-8-mediated cleavage of the pro-apoptotic protein Bid, subsequent cytochrome c release, and caspase-9 activation (62, 63). In agreement, we observed a drop of mitochondrial membrane potential following CTL stimulation with both IVT and Y5 peptides (our unpublished data). It remains unclear why caspase-3 activity is not essential for Y5-mediated apoptosis while caspase-9 appears to be active also in this case. Conceivably, in the absence of IL-2, caspase-9-dependent activation of caspase-3 is blocked by an unknown cellular factor. Additional experiments are needed to investigate the role and mechanisms of caspase activation in this experimental system.

The anti-apoptotic effect of IL-2 is also consistent with a major role of mitochondria in Y5-induced CTL death. In IVT-stimulated BK289 cells the Fas-independent apoptotic pathway is likely to be blocked by high levels of Bcl-2 and Bcl-XL expression induced by IVT-mediated triggering and endogenous IL-2 secretion. However, this is obviously not sufficient to protect the cells from Fas-mediated apoptosis, even if it involves mitochondrial damage. This can be explained by massive caspase activation induced by Fas engagement. Y5 triggering fails to increase significantly the expression of the anti-apoptotic proteins and their up-regulation by exogenous IL-2 is associated with protection of Y5-stimulated cells from apoptosis. It cannot be excluded that other anti-apoptotic effects of IL-2, such as induction of NF-kB activity, contribute to the protective effect of the lymphokine.

Although the Y5 peptide does not induce IL-2 production by BK289 CTLs, it stimulates a considerable and sustained increase in the concentration of intracellular Ca\(^{2+}\) (41). This activity of the partial agonist seems to be critical for the initiation of Fas-independent apoptosis in BK289 cells, because the inhibition of calcineurin by CsA rescues the survival of Y5- but not IVT-stimulated cells. Ca\(^{2+}\) influx is known to be required for the induction of apoptosis in several cell types, involving, at least in some cases, the calcineurin-mediated dephosphorylation of Bad that results in its release from the complex with the 14-3-3 protein (64, 65). The pro-apoptotic activity of liberated Bad can be counterbalanced by up-regulation of Bcl-2 and Bcl-XL. Whether the death of CTLs induced by partial agonists and the anti-apoptotic effect of exogenous IL-2 can be explained by interference with this pathway is currently under investigation.

Although AICD is traditionally viewed as a process mediated by Fas and TNFR, a number of fundamental immunological phenomena involve the activation of T cells followed by their elimination independently of these molecules. These include central- and superantigen-induced peripheral T cell deletion, down-regulation of specific T cell responses, and induction of peripheral tolerance under conditions of costimulatory blockade (see Ref. 66 and Introduction). The apoptotic programs involved in these processes are poorly characterized.

In this study, we demonstrate that partially agonistic peptides can switch the mode of AICD from Fas-mediated apoptosis to a form of death that is apparently independent of molecules of the TNFR superfamily. Therefore, altered peptide ligands should be viewed as agents capable of modulating not only the process of T cell activation but also the mechanisms, kinetics, and regulation of T cell death. This may have important implications for our understanding of CTL interaction with the pool of endogenous peptides and variant forms of immunogenic epitopes generated by mutations in tumor cells and microorganisms.

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