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Caspase-Independent Cell Death Induced by Anti-CD2 or Staurosporine in Activated Human Peripheral T Lymphocytes

Olivier Déas,* Céline Dumont,† Marion MacFarlane,‡ Matthieu Rouleau,* Chafika Hebib,* François Hirsch,* Bernard Charpentier,* Gerald M. Cohen,‡ and Anna Senik2,3*†

We examined the effects of the cell-permeable, broad spectrum peptide caspase inhibitors, benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD.fmk), and BOC-Asp(OMe)-fluoromethyl ketone (BOC-D.fmk), on apoptosis induced by anti-CD2, anti-Fas, and the protein kinase inhibitor staurosporine in activated human peripheral T lymphocytes. We monitored ultrastructural, flow cytometric, and biochemical apoptotic changes, including externalization of phosphatidylserine, cleavage of poly(ADP-ribose) polymerase (PARP) and lamins, activation of caspase-3 and caspase-7, decrease in mitochondrial membrane potential, and DNA fragmentation. Z-VAD.fmk and BOC-D.fmk completely inhibited all the biochemical and ultrastructural changes of apoptosis in anti-Fas-treated cells. In marked contrast, neither Z-VAD.fmk nor BOC-D.fmk inhibited CD2- or staurosporine-mediated cell shrinkage, dilatation of the endoplasmic reticulum (seen in anti-CD2-treated cells), externalization of phosphatidylserine, and loss of mitochondrial membrane potential that accompanied cell death. However, these inhibitors did inhibit the cleavage of PARP and lamins and the formation of hypodiploid cells, and partially inhibited chromatin condensation. These results demonstrate that in activated T cells, anti-CD2 and staurosporine induce a caspase-independent cell death pathway that exhibits prominent cytoplasmic features of apoptosis. However, caspase activation is required for the proteolytic degradation of nuclear substrates such as PARP and lamins together with the DNA fragmentation and extreme chromatin condensation that occur in apoptotic cells. The Journal of Immunology, 1998, 161: 3375–3383.

Apoptosis is a form of cellular suicide characterized by well-defined ultrastructural changes, including membrane blebbing, condensation of the chromatin and the cytoplasm, and ultimately internucleosomal cleavage of DNA (1). Apoptotic cell death occurs in two phases, an initial commitment phase followed by an execution phase characterized by dramatic stereotypic changes in cell morphology (2). In the nematode Caenorhabditis elegans, the ced-3 gene product is essential for apoptotic cell death (3). This gene encodes a protein that is homologous to the IL-1β-converting enzyme (4), a cysteine protease whose specific substrate is pro-IL-1β (5) and whose active form, derived from an inactive precursor, is composed of large and small subunits associated in a (p20)7/(p10)2 tetramer (6). A family of at least 10 related cysteine proteases has been identified and are now known as caspases (7). Caspases pre-exist in the cytoplasm as single-chain inactive proenzymes that are proteolytically processed during apoptosis to form active heterodimeric enzymes with a specific substrate cleavage activity after an aspartate residue at the P1 subsite (8). Activation of caspasas during apoptosis results in the cleavage of critical cellular substrates, including poly-(ADP-ribose) polymerase (PARP)4 and lamins (for review, see Refs. 9–11), thus precipitating the dramatic morphologic changes of apoptosis.

In various tumor cells undergoing apoptosis, multiple species of caspase-3 and caspase-6 constitute the major pool of activated caspases regardless of the initial apoptotic stimulus (12). In addition to caspase-3 and caspase-6, caspase-2 and caspase-7 are activated during the execution phase of apoptosis in human monocytic tumor cells (13). In the cells (except those in the brain) of caspase-3-deficient mice, both PARP cleavage and apoptosis occur normally, demonstrating that other caspases with overlapping substrate specificity (probably caspase-7) can replace caspase-3 (14). Thus, it appears that caspases-3, -6, and -7 are the major effector caspases, with some possible contribution of caspase-2. It has been proposed that activator caspases, such as caspase-8, acting near or at the apex of the hypothetical caspase cascade, activate the effector caspases. The identity of the activator caspases is less clear, although there is evidence that caspase-8 (MACH/FLICE/Mch5) is recruited to the Fas signaling complex and initiates the caspase cascade involved in Fas-mediated apoptosis (15–18). Also, activation of caspase-10 (Mch4) has been proposed to be the primary mechanism by which granzyme B initiates cell death (19). Caspases-8 and -10 can, in fact, process/activate all known pro-caspases as well as each other (17, 20).

4 Abbreviations used in this paper: PARP, poly(adenosine diphosphate-ribose) polymerase; Z-VAD.fmk, benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone; BOC-D.fmk, BOC-Asp(OMe)-fluoromethyl ketone; ΔΨm, mitochondrial membrane potential; AICD, activation-induced cell death; ZFA.fmk, benzoyloxycarbonyl-Phe-Ala-fluoromethylketone; Ac-IETD-CHO, Ac-Ile-Glu-Thr-aspaptic acid aldehyde; Z-AAD-mmk, Z-Ala-Ala-Asp-CH2Cl; DiOC4, 3,3’-diethylxothiocarbocyanine; PI, propidium iodide.
Caspase inhibitors of differing specificities have also been used to implicate a critical role for caspases in the execution phase of apoptosis (10, 11). Both Z-VAD.fmk and BOC-D.fmk, two cell-permeable, broad spectrum caspase inhibitors, suppress apoptosis induced by a wide variety of stimuli (21–25). Additionally, BOC-D.fmk suppresses apoptosis in some Z-VAD.fmk-resistant systems, such as apoptosis triggered in activated mouse lymphoblasts by several independent stimuli (22), suggesting that each inhibitor can target distinct members of the caspase family. In intact cells, Z-VAD.fmk inhibits the processing of caspases-2, -3, -6, and -7 (13, 23) and hence may prevent the activity of an upstream caspase, possibly caspase-8 and/or caspase-10. This assumption is supported by the fact that Z-VAD.fmk prevents the activity of caspase-8 (18). Bcl-2, but not Z-VAD.fmk, blocks the release of cytochrome c from mitochondria during apoptosis (26, 27). Cytochrome c binds to apoptosis protease-activating factor-1, a human protein homologous to C. elegans CED-4 (28), and in the presence of dATP results in the activation of caspase-9 and the initiation of the caspase cascade (29).

In this study, we compared the effects of Z-VAD.fmk and BOC-D.fmk on apoptosis induced in activated human T lymphocytes via CD2 and via Fas, two receptors that initiate independent death pathways (30, 31). We extended this approach to the apoptotic pathway elicited in these cells by staurosporine, a broad spectrum kinase inhibitor and a ubiquitous inducer of apoptosis (32) whose lethal effect is generally blocked by Z-VAD.fmk (23).

We demonstrate that in activated T cells, CD2 and staurosporine can induce a caspase-independent death pathway, contrasting with the caspase-dependent pathway induced by Fas. It appears that in the absence of caspase activation, cytoplasmic events related to apoptosis, such as cell shrinkage, cytoplasmic condensation, loss of mitochondrial membrane potential (ΔΨm), and exposure of phosphatidylserine, are readily triggered. However, caspase activation is required for the triggering of characteristic biochemical and morphologic nuclear changes associated with apoptosis, including extreme chromatin condensation, internucleosomal cleavage of DNA, and proteolysis of nuclear substrates.

Materials and Methods

Cell separation

PBL were isolated from blood bank leukopheresis packs obtained from healthy volunteers of both sexes (through the courtesy of the Blood Transfusion Center from Hôpital Saint Louis, Paris, France). After Ficoll-Isoopaque density (density = 1.078) gradient centrifugation, adherent cells were removed by incubation on plastic dishes for 30 min at 37°C, and the rest of the cells was fractionated by filtration over nylon wool columns.

mAbs and other reagents

Purified CD2 mAb were GT2 (IgG1), T11 (IgG1), and D66 (IgM) from Dr. D. Lynch from Immunex Research and Development, Seattle, WA. Purified CD3 mAb were GT2 (IgG1), T11 1 (IgG1), and D66 (IgM) from American Type Culture Collection (IgM) was purchased from DIACLONE (Besancón, France), and anti-CD3 mAb OKT3 (IgG2a) was obtained from American Type Culture Collection (Manassas, VA). In some experiments, an antagonistic anti-CD3 mAb (provided by Dr. D. Lynch from Immunex Research and Development, Seattle, WA) and a neutralizing rabbit anti-CD3 mAb (provided by Dr. J. Wietzerbin, INSERM, Unit 365) were used to block activation-induced cell death (AICD). Z-VAD.fmk, BOC-D.fmk, and ZFA.fmk (benzylxycarbonyl-Phe-Ala-fluoromethylketone) were purchased from Enzyme Systems Products (Dublin, CA), dissolved at stock solutions of 100 mM in DMSO, and stored at −20°C. Ac-Ile-Glu-Thr-t-arapastic acid aldehyde (Ac-IETD-CHO) was purchased from Bachem (Voisins-le-Bretonneux, France), and Z-Ala-Ala-Asp-Ch2Cl (Z-AAD-cmk) was obtained from Calbiochem (France Biochem, Meudon, France). Etoposide (VP16) was purchased from Sigma (St. Quentin Fallavier, France).

Culture conditions and induction of cell death

T lymphocytes (6 × 10⁶) were cultured in the wells of six-well flat-bottom plates (Nunc, Roskilde, Denmark) in 6 ml of RPMI 1640 medium supplemented with 10% FCS and antibiotics. Stimulation of the cells was performed by using the mitogenic GT2+T11, CD2 mAb pair (2 μg/ml) plus 100 U/ml IL-2 (Rousset-UCLA, Romainville, France) or by using 0.25 μg/ml OKT3 plus IL-2. After a 4- or 5-day culture period, the cells were washed, replaced in IL-2-containing medium, then exposed to the death signals delivered by the T11+, D66 CD2 mAb pair (2 μg/ml), by anti-Fas mAb (2 μg/ml), or by staurosporine (0.1–0.5 μM).

Flow cytometric analysis of cell death

Cells were stained with 5 μg/ml propidium iodide (PI) 10 min before examination to detect permeable dead cells (FL3 positive) using a FACScan (Becton Dickinson, Paris, France) or a Coulter EPICS Profile II cytofluorometer (Margency, France). To evaluate mitochondrial ΔΨm, cells were stained for 15 min at 37°C with 40 nM of the potential sensitive fluorescent dye DiOC6 (3,3′-diethyloxacarbocyanine) from Molecular Probes (Interchim, Montluçon, France). Measurement of phosphatidylserine externalization was performed by staining the cells with annexin VFITC from Boehringer Mannheim (Meylan, France), according to the manufacturers instructions.

Hypodiploid cell assessment and microscopic detection of chromatin condensation

Cells (5 × 10⁶) were washed twice in PBS with 5.5 mM glucose and fixed overnight in ethanol (70% in water, at 4°C). Cells were then resuspended in 0.5 ml of PBS containing 50 μg/ml PI and 100 U/ml RNase A (Sigma) and incubated for 30 min at room temperature under agitation. The DNA content of 10⁶ cells was monitored by cytofluorometry using a Coulter EPICS profile II analyzer.

Immunoblot analysis

Cells (5 × 10⁶) were washed and solubilized in 20 μl of Laemmli buffer. Cell lysates were then subjected to SDS-PAGE and electrophoresed onto a nitrocellulose membrane. Cleavage of PARP was determined using the C2-10 mAb (purchased from G. Poitier, Montreal University, Montreal, Canada) (33); that of lamin B was determined using an anti-lamin B polyclonal rabbit antisera provided by J. C. Courvalin (34). For detection of the cleavage products of caspase-3, we used an anti-caspase-3 rabbit antisera (PharMingen, represented by Becton Dickinson, Le Pont de Claira, France) or an anti-caspase-3 mAb from Transduction Laboratories (Interchim, Montluçon, France) raised against the p17 subunit; both detected procaspase-3 and its large subunits. The rabbit polyclonal Abs to caspase-2, which detected procaspase-2 and its p12 small subunit, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal Abs to caspase-7, which detected procaspase-7 and the p20 large subunit, were raised as previously described (13). Blots were stained with either anti-mouse or anti-rabbit horseradish peroxidase-labeled secondary Ab (Amersham, Les Ulis, France). They were then developed using an enhanced chemiluminescence detection system (ECL kit, Amersham). Films were exposed for 1 to 15 min.

Results

Z-VAD.fmk and BOC-D.fmk inhibit Fas-mediated, but not CD2- or staurosporine-mediated, cell death in activated peripheral T lymphocytes

We have previously shown that the induction of apoptosis in activated human T lymphocytes via Fas and CD2 receptors occurs by two independent pathways (30, 31). To determine whether caspase activation was equally involved in these pathways, we examined their sensitivities to the caspase inhibitors, Z-VAD.fmk and BOC-D.fmk. The irreversible caspase B inhibitor, Z-FA.fmk, was used as a control, as it lacks an Asp in the P1 position and therefore does not inhibit caspase activity (22). We also studied the effects of these inhibitors on apoptosis induced by staurosporine, a universal inducer of apoptosis (32). The cells were preincubated for 4 h with a range of concentrations of the peptide inhibitors (0.01–100 μM) to allow them to enter the cells. Z-VAD.fmk and BOC-D.fmk, but not Z-FA.fmk, protected activated T lymphocytes from anti-Fas-mediated apoptosis (routinely visualized by PI uptake) in a concentration-dependent manner (Fig. 1). Z-VAD.fmk was somewhat more effective than BOC-D.fmk, with significant
inhibition observed at 1 μM and almost complete protection afforded at 10 μM. In marked contrast, these inhibitors, even at 100 μM, did not afford any protection against CD2- and staurosporine-induced cell death, at odds with the blocking effect exerted by Z-VAD.fmk in numerous cellular models (23). Dose-response experiments further indicated that Z-VAD.fmk and BOC-D.fmk (at 50 μM) were unable to prevent cell death regardless of the strength of the CD2 or staurosporine apoptotic stimulus (Fig. 2), suggesting that the caspase inhibitors were present at concentrations adequate to fully control their target caspases.

Externalization of phosphatidylserine at the external leaflet of the plasma membrane is a feature of apoptotic cells and constitutes an important signal for cellular recognition and ingestion by phagocytes (35). Flow cytometric analyses, monitoring the appearance of annexin V+/PI- apoptotic T lymphocytes, were therefore undertaken to determine whether CD2- and staurosporine-induced cytolysis, occurring in the presence of Z-VAD.fmk and BOC-D.fmk, displayed this apoptotic feature. Preliminary experiments indicated that 2 h after induction of cell death, annexin-V+/PI- cells appeared and rapidly evolved to annexin-V+/PI+ dead cells. Z-VAD.fmk and BOC-D.fmk (both at 50 μM) prevented phosphatidylserine externalization in anti-Fas-treated cells, but not in anti-CD2 and staurosporine-treated cells (Fig. 3).
lamin B1 (the most abundant lamin in T lymphocytes (34)) were cleaved to their characteristic 85- and 46-kDa signature fragments, respectively. Z-VAD.fmk and BOC-D.fmk, but not Z-FA.fmk, prevented PARP and lamin B1 cleavage irrespective of the apoptotic stimulus (Fig. 5, lanes 3–5). Inhibition of PARP and lamin B1 proteolysis by the caspase inhibitors was long lasting; it was still effective 24 h after apoptotic signaling (not shown). Thus, the activities of at least caspase-3, -6, and -7 were apparently not required for the execution of anti-CD2- and staurosporine-induced cell death in activated human T lymphocytes.

Ultrastructural features of T cells undergoing anti-CD2- and staurosporine-induced cell death in the presence of Z-VAD.fmk and BOC-D.fmk

To determine whether inhibition of caspases with PARP- and lamin-cleaving activities altered the morphology of T lymphocytes undergoing anti-CD2- and staurosporine-mediated cell death, we examined the ultrastructure of these cells. In the absence of Z-VAD.fmk and BOC-D.fmk, treatment of activated T cells with anti-Fas, anti-CD2, or staurosporine induced varying degrees of ultrastructural changes characteristic of apoptosis, including condensation of the cytoplasm and the chromatin, disintegration of the nuclear envelope, and, in the case of anti-CD2- and anti-Fas-mediated apoptosis, dilatation of the endoplasmic reticulum and subsequent formation of vesicles, some of which were fused with the cell membrane to give a bubbling appearance (Fig. 6). Z-VAD.fmk blocked all of these ultrastructural changes in anti-Fas-treated cells. In marked contrast, it blocked only some, not all, of the nuclear changes induced by anti-CD2 and staurosporine. In these cases, the cells exhibited clumps of partially condensed chromatin but did not possess the crescents of completely condensed chromatin seen in cells exposed to these apoptotic stimuli in the absence of Z-VAD.fmk (Fig. 6). It has been proposed that such changes are consistent with the inactivation of caspase-6 and the subsequent lack of lamin cleavage (43). Similar results were obtained with BOC-D.fmk (data not shown). In anti-CD2- and staurosporine-treated cells, many of the prominent features of apoptosis, such as condensation of the cytoplasm and dilatation of the endoplasmic reticulum (mainly seen in anti-CD2-treated cells), were not blocked by Z-VAD.fmk, although in the latter cells the dilated endoplasmic reticulum was not fused with the plasma membrane. Cell shrinkage was also confirmed by forward scatter analysis of the cells (not shown). Thus, many, but not all, of the ultrastructural changes of apoptosis were taking in place in cells whose PARP- and lamin-cleaving activities were inhibited by Z-VAD.fmk and BOC-D.fmk.

Z-VAD.fmk and BOC-D.fmk inhibit the appearance of hypodiploid cells

The partial condensation displayed by apoptotic T lymphocytes resisting the protection afforded by Z-VAD.fmk and BOC-D.fmk suggested that the caspase inhibitors may have altered DNA fragmentation. We therefore measured the effects of these inhibitors on the appearance of hypodiploid cells whose decreased DNA content has been shown to result from internucleosomal DNA cleavage (44). The generation of hypodiploid cells induced by anti-Fas, anti-CD2, and staurosporine was, in fact, inhibited by Z-VAD.fmk and BOC-D.fmk in all cases (Fig. 7). These results, together with the lack of highly condensed chromatin observed in the ultrastructural studies, confirm that the caspase inhibitors preferentially affected some, but not all, nuclear manifestations of CD2- and staurosporine-induced apoptosis.
Activation status of caspases-2, -3, and -7 in activated T lymphocytes undergoing apoptosis in the presence of Z-VAD.fmK and BOC-D.fmK

Given that Z-VAD.fmK and BOC-D.fmK were unable to inhibit all the features of a classical apoptotic phenotype in anti-CD2- and staurosporine-treated cells, we wished to examine the processing of some of the caspases classically involved in the execution phase of apoptosis. The cells were lysed with Laemmli buffer containing 3% SDS, which should avoid the postlysis proteolytic processing of caspases-3 and -7 that can occur when activated T cells are lysed with RIPA buffer (45). Granzyme B is, in fact, released from cytotoxic granules during the lysis procedure of activated T cells, and if not neutralized, it will activate several caspases, including caspase-3 (46) and caspase-7 (47). In some experiments (not shown), the cells were incubated, before lysis, with PBS containing 300 μM Ac-IETD-CHO or Z-AAD-cmk, two peptides that serve as granzyme B substrates and should inhibit its proteolytic action. In these conditions, immunoblot analysis revealed that control activated T cells possessed the 32-kDa proform of caspase-3 together with the p20 subunit and small amounts of the p19 subunit (Fig. 8A, lane 2). Both the p20 subunit resulting from cleavage of full-length procaspase-3 at Asp175 and the p19 subunit formed following subsequent autocatalytic cleavage at Asp9 (20) are potentially catalytically active, as evidenced from their capacity to interact with biotinylated DEVD-CHO (48, 49). However, despite the presence of both the p20 and p19 subunits in control activated cells, PARP remained intact, as was the case for resting T cells, which only exhibited the latent 32-kDa proform of caspase-3 (Fig. 8A, lane 1). In contrast, in activated T cells induced to undergo apoptosis with anti-Fas, anti-CD2, or staurosporine, an additional p17 protein was observed (Fig. 8A, lanes 3, 8, and 12). The formation of the p17 subunit, which is due to further processing of the p19 subunit at Asp28 (20), coincided with the cleavage of PARP (Fig. 5). With all three apoptotic stimuli, processing of caspase-3 to the p17 subunit was completely blocked by Z-VAD.fmK (Fig. 8A, lanes 5, 10, and 14) and BOC-D.fmK (Fig. 8A, lanes 6, 11, and 15), whereas Z-FA.fmK had little effect (Fig. 8A, lanes 4, 9, and 13).

In additional experiments we examined whether the cells with low ΔΨm that were present (at ~15–20%) in control activated T cell preparations (see Fig. 4), would account for the partial processing of caspases-3 and -7 observed in such cell preparations. DiOC6high and DiOC6low cells present in control activated T cells were separated by cell sorting. For comparison, T cells exposed to anti-CD2 were fractionated in a similar manner. It can be seen that >98% pure DiOC6high T cells isolated from control populations (Fig. 8B, lane 4) and from populations exposed to anti-CD2 (Fig. 8B, lane 5) still exhibited partial processing of caspase-3 to its p20 subunit. In marked contrast, in DiOC6low/PI-negative cells (Fig. 8B, lane 6), the p32 proform was quite reduced, and the large subunit was entirely processed to both the p19 and p17 subunits. In DiOC6low/PI-positive cells (Fig. 8B, lane 3), the subunits of caspase-3 were hardly detected (they were probably degraded). These data did not support the suggestion that the p20 observed in control activated T cells was due to the low percentage of cells with low ΔΨm. Rather, they suggested that in live DiOC6high T cells, the p20/p12 tetramer of caspase-3 was present, but was catalytically less active than the p17/p12 tetramer; its activity was possibly blocked by endogenous inhibitors.

In resting T cells, caspase-7 was present primarily as its 35-kDa proform (Fig. 8C, lane 1), whereas in activated T cells, it was already processed to a p23 fragment (Fig. 8C, lane 2), probably following cleavage between the large and small subunits. Induction of apoptosis by all three apoptotic stimuli resulted in the appearance of a p20 fragment (Fig. 8C, lanes 3, 6, and 9) following further cleavage at Asp23 (20). Formation of the p20 subunit was inhibited by Z-VAD.fmK (Fig. 8C, lane 5), but not by Z-FA.fmK (Fig. 8C, lanes 4, 7, and 10).

Recently, we and others have shown that procaspase-2 is activated at an early stage of the apoptotic process in some cells (13, 50). Using an Ab that recognizes both the proform and the small p12 subunit of caspase-2, it appeared that procaspase-2 was not processed during anti-Fas and anti-CD2-induced apoptosis of activated human T cells (Fig. 8D). Under similar conditions, this Ab detected the p12 subunit of caspase-2 in human monocytic THP.1...
cells induced to undergo apoptosis by etoposide, a DNA topoisomerase II inhibitor (Fig. 8D, lane 4). Thus, apoptosis induced by several stimuli in activated T cells leads to the selective activation of some, but not all, caspases.

Discussion
This study shows that broad spectrum caspase inhibitors, Z-VAD.fmk and BOC-D.fmk, were unable to inhibit the anti-CD2- and staurosporine-induced cell death of activated T lymphocytes, with the dying cells exhibiting several typical features of apoptosis, including cell shrinkage, dilatation of the endoplasmic reticulum (in the case of CD2-induced cell death), partial chromatin condensation, externalization of phosphatidylserine, and decrease in mitochondrial membrane potential, under conditions where they inhibited the activation of caspases-3 and -7 and the proteolytic cleavage of PARP and lamins. This strongly suggests that the substrates degraded in this situation were different from those usually associated with apoptotic cell death mediated by caspases.

In numerous cell models, Z-VAD.fmk or BOC-D.fmk block apoptosis (21–23, 25) by irreversibly inhibiting the activity/process of a number of caspases (13, 18, 22). However, in a small number of recent studies that analyzed the induction of apoptosis by Bax, Bak, GD3 ganglioside, or the CTL granule exocytosis pathway, Z-VAD.fmk and BOC-D.fmk were ineffective in blocking cell death (19, 51–54). In most of these studies, the mode of cell death, apoptosis, necrosis, or a combination of both was not clearly assessed. In addition, in many of these studies, cell death was induced over a period of days, raising the possibility that the caspase inhibitors were ineffective because they were present intracellularly at inadequate concentrations and for insufficient periods of time. In the present study, although this problem was circumvented by inducing apoptosis over a short period of time (up to 4 h), the caspase inhibitors failed to block anti-CD2- and staurosporine-mediated cell death in conditions where they inhibited Fas-induced apoptosis. In addition, both inhibitors were present in the cells at concentrations that blocked the activities of caspase-3 and caspase-7 (the caspases primarily responsible for PARP cleavage), caspase-6 (the caspase primarily responsible for lamin...
cleavage), and caspase-8 (probably by inhibiting the activity of caspase-8 recruited to the death-inducing signaling complex after Fas ligation) (15, 16). While this manuscript was in preparation, we became aware of the work of Toyoshima et al. (55), which showed that caspase-3-like peptide inhibitors (such as Z-DEVD.fmk) were unable to block Fas-mediated cell death of Jurkat cells. Interestingly, cell death occurring in these conditions was characterized by cytoplasmic apoptotic responses such as cell shrinkage and surface blebbing, but lacked the prominent features of apoptotic nuclear responses (chromatin condensation and DNA fragmentation), reminiscent of the changes elicited by anti-CD2 and staurosporine in the presence of Z-VAD.fmk and BOC-D.fmk. Sarin et al. have reported that primary murine lymphoblasts are largely protected by BOC-D.fmk (and much less by Z-VAD.fmk) from apoptosis induced by dexamethasone, etoposide, and irradiation (22). However, if the nuclear events of apoptosis were almost completely prevented by this caspase inhibitor, a substantial proportion of the murine lymphoblasts of that study still died, in line with our own results. All these data strongly support the presence of a caspase-independent pathway of cell death operating in concert with a caspase-dependent one, both leading to the complete set of changes characterizing the apoptotic phenotype. Interestingly, it was recently shown that lymphocytes undergoing in vivo cell death in HIV-infected lymph nodes are characterized by reduced size, different degrees of chromatin condensation, and absence of fragmented DNA (56). With the exception of the mitochondrial swelling noticed in these lymphocytes, these features are similar to those observed in the lymphocytes undergoing the caspase-independent cell death pathway in the present study. Examination of the activation status of caspases within the dying lymphocytes of HIV-infected individuals would establish whether the caspase-independent pathway described here has an in vivo relevance. It is not possible to completely exclude a possible role of the activation of an as yet uncharacterized caspase in anti-CD2- and staurosporine-induced apoptosis in the presence of caspase inhibitors. However, this seems unlikely, as Z-VAD.fmk and BOC-D.fmk are capable of inhibiting the caspase cascades initiated by a large number of apoptotic stimuli, including the cascade initiated by caspase-10 in the course of granzyme B-mediated apoptosis (19). It is possible that the strength of the apoptotic stimuli might have overcome the inhibitory effect of Z-VAD.fmk and BOC-D.fmk against these putative uncharacterized caspases. However, regardless of the strength of the apoptotic signal delivered by anti-CD2 or staurosporine in our cells, the caspase inhibitors failed to prevent cell death, arguing against this possibility (Fig. 2).

Alterations in mitochondrial function, as evidenced by an early fall in mitochondrial membrane potential, were proposed to be responsible for the Bax-induced caspase-independent cell death pathway (51). Our data also show a decrease in mitochondrial membrane potential, which, in the case of anti-CD2- and staurosporine-treated cells, was not blocked by the caspase inhibitors. One currently favored hypothesis for the induction of apoptosis is that perturbations of mitochondria allow the release of cytochrome c (26, 27), which upon binding to apoptosis protease-activating factor-1 and in the presence of dATP leads to the activation of a caspase cascade (28, 29). These events are responsible for many of the biochemical changes characteristic of apoptosis. For example,

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**FIGURE 8.** Immunoblot analysis of the activation status of caspases-2, -3, and -7. Total cell lysates were prepared as described in Figure 5. A, Immunoblot analysis of caspase 3; the positions of the 32-kDa proenzyme of caspase-3 and of its 20-, 19-, and 17-kDa cleavage products are indicated. Lane 1 contains a lysate from 2 × 10^6 resting T cells, whereas all other lanes (lanes 2–15) contain lysates from 5 × 10^6 activated T cells. Lysates were prepared from control activated T cells (lanes 2 and 7), from activated T cells subjected to the apoptotic stimuli after preincubation with medium (lanes 3, 8, and 12), with Z-FA.fmk (lanes 4, 9, and 13), with Z-VAD.fmk (lanes 5, 10, and 14), or with BOC-D.fmk (lanes 6, 11, and 15). B, Immunoblot analysis of caspase-3. Lanes 1 through 3, Lysates from 5 × 10^5 control activated T cells; lanes 4 through 6, lysates from 5 × 10^6 resting T cells exposed for 3 h to the CD2 apoptotic stimulus; lanes 2 and 5, sorted DiOC6^low cells; lanes 3 and 6, sorted DiOC6^high cells. C, Lysates examined for caspase-7. The positions of the 35-kDa proenzyme of caspase-7 and of its 23- and 20-kDa cleavage products are indicated. Lane 1 contains a lysate from 2 × 10^6 resting T cells, whereas all other lanes (lanes 2–11) contain lysates from 5 × 10^6 activated T cells; lysates were prepared from activated T cells preincubated with medium (lanes 3, 6, and 9), Z-FA.fmk (lanes 4, 7, and 10), or with Z-VAD.fmk (lanes 5, 8, and 11). D, Lysates examined for caspase-2. The lysates were prepared from 5 × 10^5 control activated T cells either alone (lane 1) or following treatment with either anti-CD2 or anti-Fas (lanes 2 and 3, respectively). No processing of caspase-2 was observed, in contrast to a positive control of THP.1 cells exposed to etoposide (lane 4) (13). The experiment shown is representative of five others performed.
activation of caspase-3 results in the cleavage of DNA fragmentation factor, leading to internucleosomal cleavage of DNA (57), as well as to the cleavage of numerous other substrates, including PARP and retinoblastoma protein (reviewed in Ref. 11). In the present study both Z-VAD.fmk and BOC-D.fmk inhibited internucleosomal cleavage of DNA as well as PARP cleavage, demonstrating that events downstream of caspase-3 and caspase-7 were effectively inhibited. Beside the loss of mitochondrial cytochrome c, other mitochondrial components might participate in the induction of the characteristic biochemical and morphologic changes associated with apoptosis. One partially characterized component is apoptosis-inducing factor, which may also be responsible for DNA fragmentation and for proteolytic degradation of caspase-3 (58). However, as apoptosis-inducing factor is potentially inhibited by Z-VAD.fmk (59), it is highly unlikely that it is responsible for the caspase-independent cell death in our study. Our data, demonstrating a decrease in \( \Delta \psi \)m in CD2- and staurosporine-treated activated T cells, even in the presence of the caspase inhibitors, are compatible with the possibility that the caspase-independent cell death pathway may be mediated in these cells by alterations in mitochondrial function. Whether such alterations constitute commitment for the demise of the cells or whether they function as a potent amplificatory loop remains to be established in anti-CD2- and staurosporine-treated cells.

Partial processing of procaspase-3 and procaspase-7, yielding their respective prodomains with their large subunits, was observed in our control activated peripheral T cells (displaying otherwise excellent cell viability), whereas in unstimulated cells only the proforms of these caspases were found. Postlysis activation of these caspasas, performed by granzyme B (released from its intracellular stores during the lysis procedure), was unlikely to occur in our experimental conditions. The cells were indeed lysed in Laemmli buffer containing 3% SDS and thereafter immediately boiled, which should avoid artifactual caspase processing (45). Moreover, adding granzyme B inhibitors such as Ac-IETD-CHO and Z-AAD-cmk at 300 \( \mu \)M in the last washing buffer just before cell lysis did not impair the formation of the large p20 subunit of caspase-3 (not shown). AICD, occurring at background levels during the primary stimulation period, may be responsible for the partial processing of caspase-3 and -7 in control activated T cells. However, adding antagonistic anti-Fas mAb, neutralizing anti-TNF-\( \alpha \), or BOC-D.fmk during the primary stimulation period of the cells did not attenuate the occurrence of the p20 subunit of caspase-3 in those cells (our unpublished data). Interestingly, using T cells from perforin-deficient mice, Spaner et al. have recently demonstrated that perforin may act as an internal trigger of cell death in short term activated peripheral CD8\(^+\) T cells upon re-cross-linking the CD3/TCR, and that this novel form of AICD preceded the two other forms of AICD, respectively represented by the CD95 and the TNF receptor pathways (60). The mechanism of perforin action is currently unknown. In our experiments, stimulation of primary T cells with OKT3 or the GT2+T11 mAb pair in the presence of IL-2 probably resulted in perforin synthesis. In view of the results reported by Spaner et al. (60), it is conceivable that in control activated live T cells, perforin was activated to a level sufficient to trigger partial processing of caspases-3 and -7 (such a possibility will be investigated). However, the partially processed caspases did not appear to be catalytically active in our cells, as no cleavage of PARP or lamins was observed. It is possible that in control activated T cells (and in live T cells surviving anti-CD2 and staurosporine treatments), putative endogenous inhibitors inhibited the proteolytic activity of the processed caspases. Some support for this suggestion is provided by a recent report showing that recombinant X-linked inhibitor of apoptosis protein is a direct and specific inhibitor of activated caspases-3 and 7, capable of binding to the partially processed large subunits and of inhibiting their catalytic activity (61). On induction of apoptosis in our model, the large subunits of both caspase-3 and caspase-7 were further processed to their p17 and p19 subunits, respectively. These subunits appeared to be catalytically active, as their appearance was accompanied by PARP and lamin proteolysis. Further support for this was provided by the studies with Z-VAD.fmk and BOC-D.fmk, which prevented the further processing of caspase-3 and caspase-7 and inhibited cleavage of PARP and lamins.

In summary, we have demonstrated that in activated human peripheral T lymphocytes, anti-CD2 and staurosporine induce a caspase-independent cell death that may act either independently or in concert with a caspase-dependent pathway. Alone, the caspase-independent pathway triggers major cytoplasmic events related to apoptosis, but it does not trigger the characteristic morphologic and biochemical nuclear changes associated with apoptosis, including proteolysis of lamins and PARP, internucleosomal cleavage of DNA, and intensive chromatin condensation, although partial chromatin condensation still occurs. These nuclear morphologic and biochemical changes of apoptosis require the activation of caspases.

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References


