Published November 1, 1993

Protease Inhibitors Selectively Block T Cell Receptor-triggered Programmed Cell Death in a Murine T Cell Hybridoma and Activated Peripheral T Cells

By Apurva Sarin, David H. Adams, and Pierre A. Henkart

From the Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Summary

The hypothesis that cytoplasmic proteases play a functional role in programmed cell death was tested by examining the effect of protease inhibitors on the T cell receptor-mediated death of the 2B4 murine T cell hybridoma and activated T cells. The cysteine protease inhibitors *trans*-epoxysuccininyl-L-leucylamido-(4-guanidino) butane (E-64) and leupeptin, the calpain selective inhibitor acetyl-leucyl-leucyl-normethional, and the serine protease inhibitors diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride, all showed dose-dependent blocking of the 2B4 death response triggered by the T cell receptor complex and by anti-Thy-1. These protease inhibitors enhanced rather than inhibited IL-2 secretion triggered by T cell receptor cross-linking, showing that they did not act by preventing signal transduction. Growth inhibition induced by cross-linking the 2B4 T cell receptor, measured by inhibition of thymidine incorporation, was not generally blocked by these protease inhibitors. All five of these protease inhibitors enhanced rather than blocked 2B4 cell death triggered by dexamethasone, an agent previously shown to have a death pathway antagonistic with that of the TCR. 2B4 cytolysis by the cytotoxic agents staphylococcal α-toxin and dodecyl imidazole, and that caused by hypotonic conditions, was not significantly affected by the five protease inhibitors tested. The selected protease inhibitors blocked both the apoptotic nuclear morphology changes and DNA fragmentation induced by T cell receptor cross-linking, and enhanced both these properties induced by dexamethasone in 2B4 cells. The T cell receptor-induced death of activated murine lymph node T cells and human peripheral blood $CD4^+$ T cells was blocked by both cysteine and serine protease inhibitors, showing that the protease-dependent death pathway also operates in these systems.

Cell death has become recognized as an important physiological process in normal development, in hormonal regulation of various tissues, and in regulation of the receptor repertoires of both T and B lymphocytes. A major unresolved problem is the lack of defined molecular pathways for such programmed cell death (PCD).[1] The finding that a pattern of morphological changes characterizes many examples of PCD led to the suggestion of a common death mechanism, termed apoptosis (1). This concept was extended by the finding that nuclear DNA fragmentation correlates well with apoptotic morphology (2), and the current literature contains many examples of PCD accompanied by these features. However, the relationship between DNA fragmentation and the molecular pathway(s) of cell death has not been meaningfully elucidated, and there are examples of PCD in the absence of apoptotic morphology or DNA fragmentation (3, 4). New approaches to defining the molecular pathways of PCD are clearly needed.

Lymphocyte-mediated cytotoxicity has been considered to be an example of apoptotic death since the target cells often (but not always) show DNA fragmentation (5) and apoptotic morphology (6). However, since there is generally no requirement for RNA or protein synthesis, this type of cell death has been considered to be a different category of apoptotic death (7). There is now considerable evidence that the effector cell granule proteases known as granzymes induce an "internal disintegration" pathway (8) in target cells, leading

[1] *Abbreviations used in this paper:* AllnM, acetyl-leucine-leucine-normethional (Calpain inhibitor II); APNE, acetyl-phenylalanine-β-naphthyl ester; DFP, diisopropyl fluorophosphate; E-64, *trans*-Epoxysuccininyl-L-leucylamido-(4-guanidino) butane; E-64d, ethyl-*trans*-Epoxysuccininyl-L-leucylamido-3-methylbutane; PCD, programmed cell death; SEB, staphylococcal enterotoxin B; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

Published November 1, 1993

to DNA breakdown and lysis (9–12; and Nakajima, H., and P. Henkart, manuscript submitted for publication). While the above experiments were directed at clarifying the role of granzymes in lymphocyte-mediated cytotoxicity, they suggested a hypothesis that intracellular protease activation may be part of a molecular pathway of PCD. In support of this, several distinct serine protease inhibitors have been shown to block TNF-induced cytotoxicity (13–15), and in some instances proteolysis has been found to accompany PCD (16, 17).

To assess the role of intracellular proteases in PCD, we have investigated the effects of a variety of protease inhibitors on several PCD systems. We describe here studies principally on the murine T cell hybridoma 2B4, a well-characterized cell that responds to T cell receptor cross-linking with PCD (18–20). Similar TCR-induced death has been reported with other murine T cell hybridomas (21, 22) and with activated peripheral T cells (23). The present results show that inhibitors specific for either cysteine or serine proteases dramatically block this PCD. Remarkably, the inhibitors do not block IL-2 secretion or growth inhibition triggered by the same receptor, indicating that they act on a death-specific molecular pathway. The active protease inhibitors do not block death induced by most other agents, and enhance the death of 2B4 cells triggered by dexamethasone, previously shown to be mediated by an antagonistic PCD pathway (20).

Materials and Methods

Chemicals. Ethyl-*trans*-Epoxysuccininyl-L-leucylamido-3methylbutane (E-64d) was a kind gift of Dr. K. Hanada, Taisho Phamaceuticals, Saitamo, Japan. Dodecyl imidazole was purchased from Toronto Research Chemicals, Toronto, Canada. Staphylococcal α-toxin was a gift of Dr. Sidney Harshman, Vanderbilt University. Hoechst 33342 was obtained from Molecular Probes, Inc., Eugene, OR. Terminal transferase was from Boehringer Mannheim Biochemicals, Indianapolis, IN, and biotinylated dUTP from Clontech, Palo Alto, CA. All other chemicals were from Sigma Chemical Co., St. Louis, MO. Protease inhibitors were made up as stock solutions in dry DMSO at 10 mM (E-64, acetyl-leucine-leucine-normethional (ALLnM), and leupeptin) or 100 mM (diisopropyl fluorophosphate [DFP], PMSF) and stored at −20°C, and then diluted directly into culture medium for use. DMSO controls had no measurable effect.

Cells and PCD Induction. The 2B4 hybridoma cells used in this study are a murine T cell recognizing cytochrome C (24), and were a generous gift from Drs. Allan Weissman and Cristina Cenciarelli, National Cancer Institute, Bethesda, MD. Mouse lymph node cells were prepared as cell suspensions from axillary lymph nodes of C57Bl/10 mice. $CD4^+$ human T cells were prepared by exhaustive negative immunomagnetic selection from peripheral blood mononuclear cells of normal donors. Antibody reactive cells were depleted with immunomagnetic beads and a multiantibody cocktail including antibodies against CD20, CD16, CD11b, CD14, glycophorin, and CD8, as described previously (25, 26). The resulting preparations were >98% $CD3^+CD4^+$ by flow cytometry.

Cells were cultured in RPMI 1640 with 10% FCS, plus 20 U/ml IL-2 in the case of activated lymphocytes. Activation of mouse lymph nodes cells was achieved by 2 d of culture with immobilized 2C11 anti-CD3 antibody (27), prepared by precoating flat-bottomed microtiter wells with 20 μg/ml purified mAb in 0.1 M $NaHCO_3$ overnight at 5°C. Human $CD4^+$ T lymphocytes were similarly activated by a 2-d incubation with immobilized OKT3. After activation, the lymphoblasts were cultured in the presence of IL-2 for 5–7 d before induction of PCD, which was induced in mouse cells by 18 h of culture on immobilized 2C11 anti-CD3 antibody (28), or in the case of human cells, a mixture of pokeweed mitogen (PWM) (10 μg/ml) plus staphylococcal enterotoxin B (SEB) (1 μg/ml) both from Sigma Chemical Co., St. Louis, MO. Cell number per well varied with the read-out: 1×10^4 for ^{51}Cr assay, 1×10^5 for trypan, DNA dyes, and IL-2 secretion, and 4×10^5 for growth inhibition and dye reduction. Unless stated otherwise, protease inhibitors were added to cells at the time of their addition to the wells containing immobilized antibody to induce PCD.

^{51}Cr Release Assay. 2B4 cells in 5 ml culture medium were labeled by addition of 300 μCi $Na_2{}^{51}CrO_7$ (Amersham Corp., Arlington Heights, IL) for 2 h in a CO_2 incubator. The cells were then harvested and washed before use in the PCD assay. Spontaneous release for the 12–16-h assays used was 30–45%.

IL-2 Assay. Culture supernatants were harvested and diluted twofold with fresh medium into 96-well plates. CTLL cells (2×10^4/well) were added and cultured for 24 h, after which 0.5 μg [^{3}H]thymidine was added to each well, followed by another 24 h of culture. The plates were harvested on an automated filter harvester and counted. r-Human-IL-2 (Cetus Corp., Berkeley, CA) was similarly diluted and used as a standard, and the results given as Cetus IL-2 units.

Growth Inhibition Assay. The assay for 2B4 growth inhibition was performed as described (19) with 3–6 h of culture followed by 1–2 h of [^{3}H]thymidine pulse.

Apoptotic Morphology and DNA Fragmentation Assays. Apoptotic nuclear morphology was assessed by culturing cells for 15 min with 5 μg/ml of the DNA staining dye Hoechst 33342, transferring them to a slide, and examining them in the fluoroscence microscope with appropriate UV/blue filters. DNA fragmentation in situ was assessed by the TdT-mediated dUPT-biotin nick end labeling (TUNEL) technique as described (29) except that the final staining was with 1 μg/ml FITC-avidin and the slides were read in the fluorescence microscope.

Results

Effect of Selected Protease Inhibitors on TCR-triggered PCD and IL-2 Secretion in 2B4 Cells. A number of protease inhibitors that inhibit candidate activatable intracellular proteases were tested for toxicity on 2B4 cells, and those that were nontoxic at what were judged to be effective concentrations were tested for their ability to reverse PCD induced by overnight culture with immobilized mAb against the TCR complex, or by the soluble anti-Thy-1 mAb G7. Five such inhibitors showed a potent and consistent ability to prevent this PCD. These include three calpain inhibitors: the epoxysuccinyl compound E-64, the peptide aldehyde analogs leupeptin and ALLnM, and the classical serine protease inhibitors DFP and PMSF. Fig. 1 shows an experiment in which trypan blue was used to assess cell death after 16 h of culture; all five protease inhibitors showed a clear dose-related ability to reverse death induced by both immobilized α-CD3 and α-Thy-1, at inhibitor concentrations having minimal effect on the viability of unstimulated control cells. The most potent inhibitor of both anti-Thy-1– and anti-CD3–induced cell death was the calpain selective inhibitor ALLnM, which showed

Published November 1, 1993

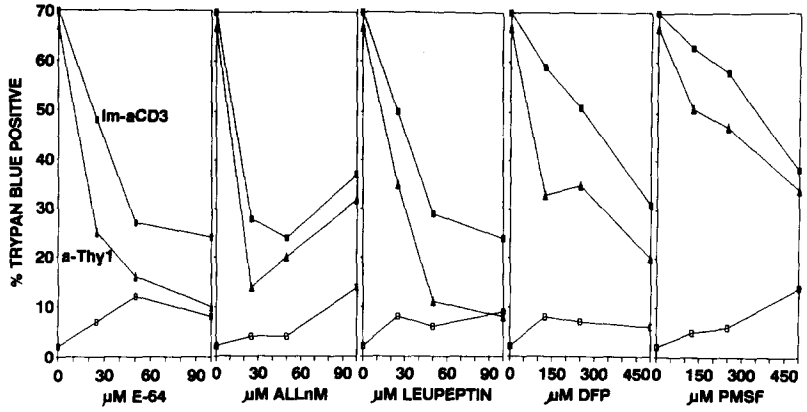

Figure 1. Effect of selected protease inhibitors on α-CD3 and anti-Thy-1-induced programmed cell death in 2B4 cells. Cultures of 10^5 2B4 cells in 200 μl medium were set up in flat-bottomed 96-well plates containing immobilized anti-CD3 (*filled rectangles*), anti-Thy-1 mAb G7 at 5 μg/ml (*triangles*), or control wells (*open rectangles*). Various protease inhibitors were added at the initiation of culture to the final concentration indicated. After 16 h of culture, cell viability was assessed with trypan blue.

maximal inhibition of death in the 25–50 μM range. However, higher concentrations of this compound were clearly toxic, as seen by their increase in trypan blue staining of unstimulated 2B4 cells. The cysteine protease inhibitors E-64 and leupeptin also showed a potent ability to reverse PCD, giving maximal effects at 50–100 μM. The serine protease inhibitors DFP and PMSF also reversed this death, but only concentrations >500 μM achieved a maximal effect. All these protease inhibitors gave greater inhibition of α-Thy-1–induced death than α-CD3–induced death. Similar results were obtained using mitochondrial dye reduction to assess viability after the overnight culture (data not shown).

Protease Inhibitors Generally Do Not Block Other TCR-triggered Functions in 2B4 Cells. One possible explanation of the blocking of cell death by the protease inhibitors is that they interfere with early events in the TCR signal transduction pathway, although this has not been previously reported with the inhibitors used. To test for this, IL-2 secretion was measured by assaying the culture supernatants after an overnight culture. As shown in Fig. 2, IL-2 secretion triggered by immobilized antibody to TCR β was generally increased by the protease inhibitors showing reversal of cell death. In some cases as much as a threefold enhancement was seen in this experiment (with ALLnM), and in other experiments as much as a sevenfold enhancement was seen (also with ALLnM; data not shown). Since the TCR stimulus triggers both death and IL-2 secretion within 16 h, blocking cell death selectively may be expected to enhance IL-2 secretion.

It has been shown previously that 2B4 cells respond to TCR cross-linking by growth inhibition as measured by the early inhibition of [^{3}H]thymidine incorporation. This response is distinct from cell death in that it is not calcium dependent or inhibited by cyclosporin A (19). Fig. 3 shows that the selected protease inhibitors generally have a minimal or negligible effect on this growth inhibition response. The experiment shown was pulsed with thymidine after 6 h of incubation with antibodies. Other experiments in which the cultures were pulsed after 3 h gave similar results. There is some reduction (0–30%) in the level of control thymidine incorporation by several of the protease inhibitors, which complicates the interpretation of these experiments. Leupeptin reproducibly showed a partial restoration (20–35%) of the growth inhibition as seen Fig. 3, and PMSF showed a variable degree of partial restoration of thymidine incorporation. The other inhibitors showed little or no tendency to restore this response to TCR cross-linking, in contrast to the cell death response. These data thus reinforce the view that growth inhibition, measured by thymidine incorporation within 6 h of TCR cross-linking, involves a pathway that diverges from that responsible for cell death.

Protease Inhibitors That Block 2B4 TCR-induced Cell Death Do Not Block Cell Death Induced by Other Agents. In addi-

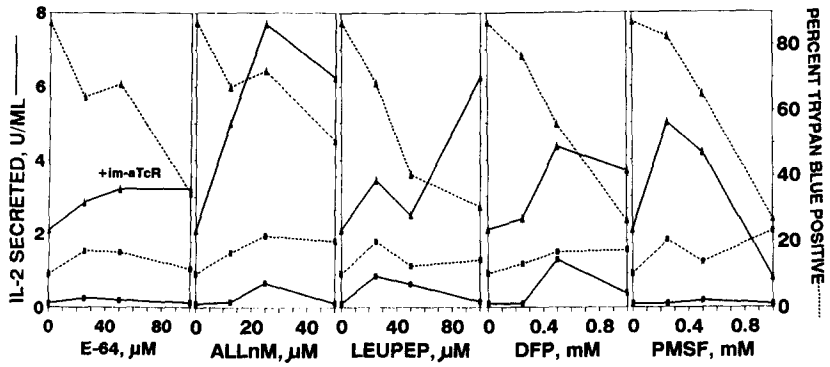

Figure 2. Effect of protease inhibitors on TCR-induced programmed cell death and IL-2 secretion in 2B4 cells. Cultures were established as described in Fig. 1, with immobilized anti-TCRβ (*triangles*) or without stimulation (*rectangles*). At the end of 16 h, supernatants were harvested for assay of IL-2 (*solid lines*) and the cell viability was assessed with trypan blue (*dashed lines*).

Published November 1, 1993

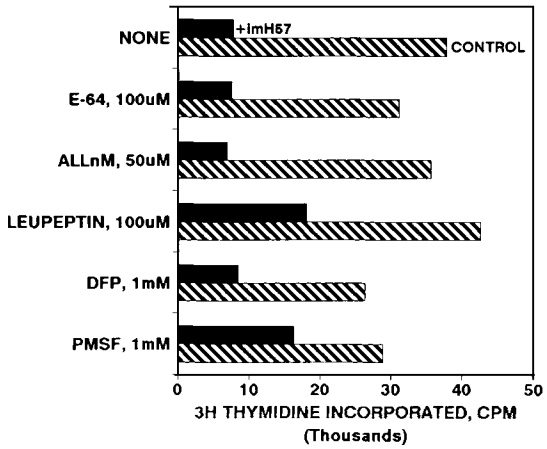

Figure 3. Effect of protease inhibitors on T cell receptor-mediated growth inhibition. 2B4 cells (20,000 per well) were cultured on flat bottom micro wells coated with immobilized H57 (*solid bars*) or control wells (*striped bars*) for 6 h, after which each well was pulsed with 1 μc of [^{3}H]thymidine for 1.5 h before harvest. The partial restoration by PMSF seen in this experiment was not reproduced in other experiments.

tion to antibodies against the TCR complex and Thy-1, corticosteroids have been shown to induce programmed cell death in 2B4 cells (20). Fig. 4 shows ^{51}Cr release experiments to test whether the selected protease inhibitors blocked PCD induced by dexamethasone, a synthetic corticosteroid. While the protease inhibitors showed the expected blocking of PCD triggered by the TCR or Thy-1, death induced by dexamethasone was modestly but clearly enhanced by all five of the protease inhibitors in a dose-dependent response (Fig. 4). ^{51}Cr release was not induced by the protease inhibitors alone at the concentrations used. The enhancement of corticosteroid-induced death occurred with both low and high levels of steroid-induced PCD as shown by the two experiments depicted in Fig. 4. Thus, the two antagonistic pathways giving

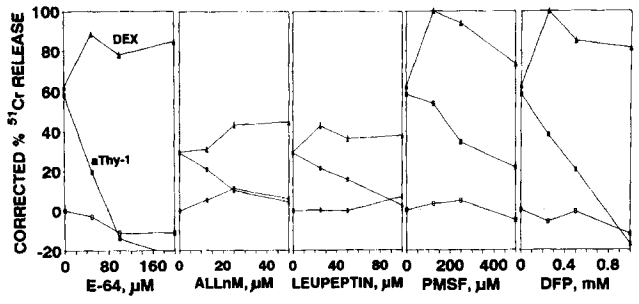

Figure 4. Effect of protease inhibitors on 2B4 programmed cell death induced by the TCR, Thy-1, and corticosteroid. ^{51}Cr-labeled 2B4 cells were cultured overnight with the indicated stimuli, and the ^{51}Cr label released into the supernatant measured. Triangles represent cultures containing dexamethasone (2.5×10^{-6} M), and open rectangles control cultures with medium only. Representative panels from two separate experiments are shown here. Filled rectangles show stimulation by αThy-1 (5 μg/ml) in the E-64, PMSF, and DFP panels, and by immobilized αCD3 (2C11) in the ALLnM and leupeptin panels. The spontaneous releases were 54% in the former experiment and 35% in the latter.

PCD in 2B4 are influenced in opposite directions by the protease inhibitors.

The effect of protease inhibitors on a number of other cytotoxic agents and conditions applied to 2B4 cells during an overnight culture was examined. In these experiments, the 2B4 cells were pretreated with protease inhibitors for half an hour before culture, since the toxic effects might be more rapid than the PCD studied previously. A negligible effect was observed with all the inhibitors tested in some cases, such as with the membrane pore-forming agent staphylococcal α-toxin (30) (shown in Fig. 5), the lysosomotropic detergent dodecyl imidazole (31), hypotonic conditions (50% water/50% medium) (data not shown for the last two).

Fig. 5 also shows that death induced by some other toxic agents can be either enhanced or suppressed by protease inhibitors. Death by the topoisomerase II inhibitor and chemotherapeutic agent etoposide (32) was generally blocked by the protease inhibitors, albeit not as completely as that by TCR cross-linking. Surprisingly, cell death elicited by sodium azide was blocked by cysteine but not serine protease inhibitors (Fig. 5).

Protease Inhibitors Block TCR-mediated and Enhance Steroid-mediated Apoptotic Nuclear Morphology Changes and DNA Fragmentation in 2B4 Cells. Fig. 6 shows an experiment that tested the effect of protease inhibitors on the apopotic features of 2B4 PCD. DNA fragmentation was assessed by the recently described sensitive TUNEL technique, which measures DNA fragmentation in situ by using terminal transferase and biotinylated dUTP (29), and nuclear morphology was assessed by fluorescence microscopy using the DNA staining dye Hoechst 33342. The results show that both the TCR-induced DNA fragmentation and nuclear morphology changes are reversed by the protease inhibitors in parallel with their inhibition of cell death measured by membrane permeability. Furthermore, the increased death seen with dexamethasone in

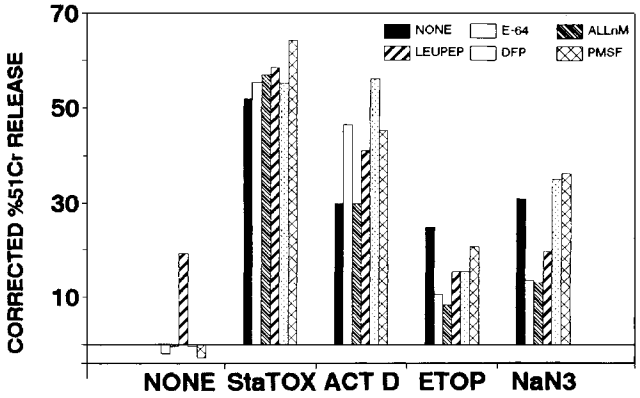

Figure 5. Effect of protease inhibitors on 2B4 cell death induced by various agents. ^{51}Cr-labeled 2B4 cells were precultured with protease inhibitors for 30 min before addition of the toxic agent. Label release was measured after a 13-h incubation. The spontaneous release in this experiment was 26%. The inhibitor final concentrations used were: E-64, 100 μM; ALLnM, 50 μM; DFP, 1 mM; PMSF, 1 mM. Final concentrations of cytotoxic agents were: staphylococcal α-toxin, 5 μg/ml; etoposide, 5 μg/ml; NaN_3, 1 mg/ml.

Published November 1, 1993

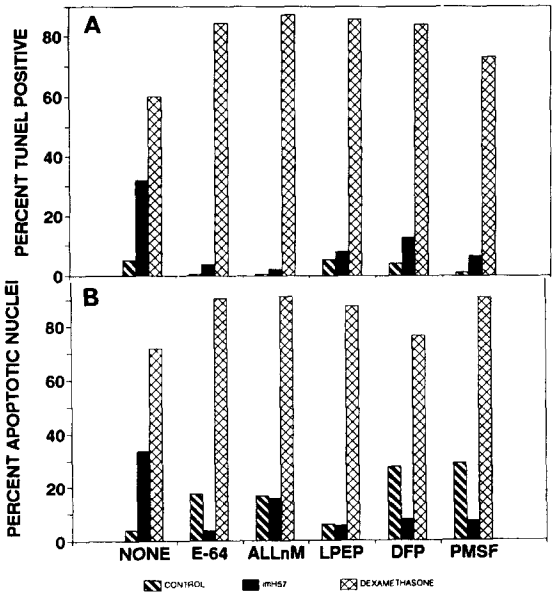

Figure 6. TCR-induced apoptotic nuclear morphology changes and DNA fragmentation are blocked by protease inhibitors. 2B4 cells were cultured for 17 h alone (*diagonal striped bars*), on immobilized α-TCRαβ (*filled bars*) or with 1×10^{-8} M dexamethasone (*hatched bars*), in the presence of the protease inhibitors indicated. Cells were analyzed for DNA fragmentation by the TUNEL technique, or for nuclear morphology by examination of Hoechst 33342 stained cells.

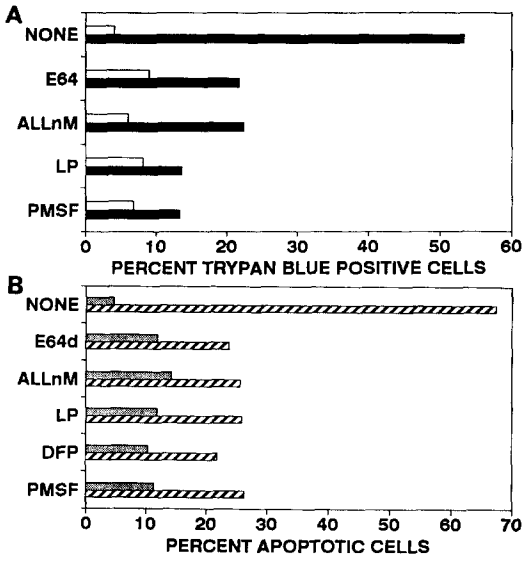

Figure 7. TCR-induced death of activated peripheral T cells is blocked by protease inhibitors. *A* shows the effects of protease inhibitors on the death of such activated murine lymph node cells after a subsequent culture for 18 h on immobilized anti-CD3 (*solid bars*) or normal wells (*open bars*). Results are the means of two experiments, with death assessed by trypan blue exclusion. The final inhibitor concentrations 1 μM were: E-64, 50; ALLnM, 12; leupeptin, 50; PMSF, 500. *B* shows an experiment using purified human CD4+ peripheral blood T cells, in which the activated cells were cultured for 20 h with a mixture of PWM and SEB (*striped bars*) or without additional activation (*stippled bars*). The inhibitor concentrations were the same as in *A*, except ALLnM was 25 μM and DFP was 500 μM. In this case, nuclear morphology was assessed by the dye Hoechst 33342. In the absence of protease inhibitors, propidium iodide stained 1.8% of these cells in the control cultures without stimulation and 19.4% in the cultures with PWM/SEB.

the presence of protease inhibitors is also clearly apoptotic by both criteria. Both these read-outs of apoptotic changes correlate with each other and with death measured by membrane integrity.

The nuclear morphology of 2B4 cells treated with both etoposide and sodium azide was clearly apoptotic, while that of 2B4 cells treated with hypotonic shock, staphylococcal α-toxin and dodecyl imidazole was predominantly nonapoptotic. Thus the protease inhibitors tended to have either positive or negative influences on apoptotic 2B4 deaths, and little or no effect on nonapoptotic deaths.

Protease Inhibitors Block TCR-mediated Cell Death of Activated Murine Lymph Node T Cells and Human CD4+ Peripheral Blood T Cells. To test whether the protease-dependent TCR-mediated death pathway operates in normal T cells, we tested the ability of the selected protease inhibitors to block the death of activated peripheral T cells induced by this receptor (23) in both mouse and man. After in vitro activation, lymphocytes were cultured in the presence of IL-2 for several days without other stimulation. With murine lymph node cells, subsequent exposure to immobilized anti-CD3 induced substantial cell death within 1 d, as was the case with 2B4. Fig. 7 *A* shows that this death was blocked by four of the protease inhibitors found to block 2B4 death, and other experiments indicated that DFP also blocks death in this system (data not shown). Activated human CD4+ peripheral blood T cells die considerably more slowly when cultured on immobilized anti-CD3, requiring 3–5 d for death as seen by membrane permeability breakdown (33). Following the suggestions of Groux et al. (34), we found that a mixture of pokeweed mitogen and the superantigen staphylococcal enterotoxin B induced death in 2 d, although apoptotic nuclear morphology changes were clearly evident after overnight culture. Fig. 7 *B* shows that the protease inhibitors active in the 2B4 system also gave substantial blocking of these apoptotic changes.

Discussion

Previous studies of TCR-triggered PCD in 2B4 and other murine T cell hybridomas have shown that it shares many properties with other PCD systems. Several laboratories have studied the mechanism of this PCD, but no molecular death pathway has been elucidated. Intracellular signaling associated with this response appears generally similar to that seen with activation responses in T cells (35). The death response is blocked by inhibitors of protein and RNA synthesis (18), implying active participation of the cells in their death. It requires calcium in the external medium (19) and is accompanied by nuclear DNA fragmentation (21) and apoptotic morphology (22). Interestingly, recent studies show that the TCR-triggered and corticosteroid-triggered PCD pathways are antagonistic, since addition of the two lethal stimuli to-

Published November 1, 1993

gether result in survival (20, 36). The present results provide the first evidence that a protease is one step of the TCR-triggered cell death pathway. The clear finding that cysteine protease inhibitors do not inhibit IL-2 secretion in these cells argues that this protease step is part of a unique death pathway that diverges from other signaling pathways associated with activation.

The experiments described were suggested by two sets of recent investigations: (*a*) multiple lines of evidence that granule proteases are responsible for target DNA breakdown in lymphocyte-mediated cytotoxicity (9–12); and (*b*) injection of several different proteases into living cells results in cell death generally accompanied by apoptotic morphology and DNA breakdown (Williams, M., and P. Henkart, manuscripts submitted for publication). Thus, in these systems proteases seem to have a potent ability to trigger an as yet uncharacterized internal disintegration process leading to cell death. It appears likely that a similar process is triggered by calpain and/or a serine protease in the T lymphocyte systems studied here.

In considering candidate intracellular proteases that might be activated in response to TCR cross-linking, calpain was the most obvious choice among known proteases because of its activation by calcium, whose cytoplasmic concentration is known to be rapidly increased in T lymphocytes by activating stimuli (37). The two purified calpain isozymes, calpain I and calpain II, become proteolytically active at micromolar and millimolar Ca concentrations, respectively. The regulation of their in situ activity is complex, involving a calcium-dependent interaction with the endogenous inhibitory protein calpastatin, proteolytic processing, and interaction with membranes (38). Lymphocytes have the highest levels of both calpains I and II of any cells measured (39).

The conclusion that calpain or another cysteine protease is part of the PCD pathway in the cells studied here rests on the specificity of the inhibitors used. Leupeptin, ALLnM, and E-64 do not react generally with protein -SH groups, but are peptide analogs that are bound at the active site of the proteases before formation of an adduct with the active site cysteine. Calpains are efficiently inhibited by all three of these compounds (40). The possibility of reaction with non-cysteine protease molecules is unlikely because similar results were obtained using different reagents with two different types of reactive groups. E-64 (an epoxysuccinyl peptide analogue) and leupeptin (a peptide aldehyde) have been characterized as reagents specific for cysteine proteases generally including lysosomal cathepsins. While the peptide aldehyde ALLnM is highly selective for calpain, it does react with other cysteine proteases such as papain at a >10× lower rate, and the possibility that it reacts with another cysteine protease in the death pathway cannot be ruled out. Thus the data presented here strongly implicate a cysteine protease in the TCR-mediated cell death of 2B4 and activated T cells, and specifically suggest a role for calpain. Further work is in progress to confirm intracellular calpain activation subsequent to TCR cross-linking.

It is interesting to note that calpains have been shown to digest cytoskeletal proteins such as spectrin and actin-binding protein involved with membrane interactions (41, 42). Thus calpain activation has been implicated in the formation of membrane blebs (43, 44), which have been regarded as a characteristic morphological property of apoptosis (45).

Since neither calpain nor other cysteine proteases are likely to be significantly inhibited by DFP and PMSF, it is possible that the TCR-triggered death pathway consists of at least two proteolytic steps, involving a serine protease in addition to the E-64-sensitive step. Such a cytoplasmic protease cascade has not been previously described, but an obvious speculation would be that TCR cross-linking elevates Ca_i which activates calpain, which in turn cleaves a serine protease proenzyme. Nevertheless, the involvement of a serine protease is less strongly implicated by the evidence presented here than that of a cysteine protease. First, the inhibitors used, PMSF and DFP, are known to react with other enzymes in some instances. Second, Fig. 3 shows that PMSF inhibits IL-2 secretion at high concentrations, in accord with previous results showing that it inhibits the TCR-induced Ca_i increase in Jurkat (46). *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) also appears to react with the functional molecule in this system, suggested to be a 42-kD serine protease (47). (TPCK is, however, very toxic to 2B4 cells.) Further experiments are in progress with other serine protease inhibitors to confirm the tentative interpretation that such a protease is involved.

The cell death induced by TNF, which is apoptotic with some targets but not others (48), also appears to require proteolysis. In this case, the functional proteases may be different from those in 2B4, as the most effective small molecule inhibitors of TNF cytotoxicity, TPCK, *N*-tosyl-L-lysine chloromethyl ketone (TLCK), and acetyl-phenylalanine-β-naphthyl ester (APNE) (13, 14), failed to inhibit TCR mediated cell death of 2B4 at nontoxic concentrations (data not shown). Expression of endogenous plasminogen activator type 2 (PAI-2, a serpin family protease inhibitor) is induced by TNF in several targets, and it has been proposed that its expression explains why TNF cytotoxicity often requires RNA or protein synthesis inhibitors (15). Cells expressing high levels of PAI-2 after transfection with its cDNA are resistant to TNF, and selection of TNF-resistant variants results in increased expression of PAI-2 (15). Curiously, the PAI-2 gene is the closest mapped locus to the gene for bcl-2, a potent antagonist of many examples of cell death (but the two genes are 600 kb apart) (49).

A role for proteases in other systems of PCD has been considered by several laboratories. Since leupeptin failed to block corticosteroid-induced death of thymocytes, it was concluded that calpain was not involved in this mechanism (50), a finding compatible with our results with corticosteroid-induced death of 2B4 cells (Fig. 4). More recently it has been shown that the serine protease inhibitors TPCK and TLCK block thymocyte or HL60 cell DNA fragmentation triggered by steroid or topoisomerase inhibitors (51, 52). In the HL60 cells, proteolysis of nuclear proteins accompanies PCD induced by etoposide (17). However, in none of these cases was it clear that cell death as assessed by nonnuclear criteria was reversed by protease inhibitors, and the role of proteases in these PCD

systems is still obscure. Studies are currently underway in our laboratory to evaluate the role of proteases in other PCD systems. Although these results will be described fully elsewhere, they show that most PCD systems are not blocked by cysteine protease inhibitors (Sarin, A., and P. Henkart, manuscript in preparation). Thus it appears that PCD mechanistic pathways are varied, as implied by the differences between steroid- and TCR-triggered PCD pathways in 2B4.

The protease-dependent death pathway described here operates in activated T cells as well as the T cell hybridoma line 2B4. Control of this death pathway appears critical for regulation of immune responses after the initial encounter with antigen (23). In addition the systems studied here can be considered to be a model for two different immunological phenomena important for different reasons: (*a*) the TCR-triggered PCD that deletes both immature autoreactive thymocytes (53); and (*b*) the activation-induced PCD of T cells in HIV-infected asymptomatic individuals (34). We have found that protease inhibitors inhibit the latter system (Sarin, A., M. Clerici, S. Blatt, C. Hendrix, G. Sharer, and P. Henkart, manuscript submitted for publication). If this PCD plays a significant role in HIV pathogenesis, calpain inhibitors may provide a novel therapeutic approach to HIV infection.

We thank Drs. Allan Weissman, Jon Ashwell, and Mark Williams for helpful discussions and comments on the manuscript. We are grateful to Dr. Bonnie Bermas for help with IL-2 assays, and Heidi Park for cell culture.

Address correspondence to Dr. Pierre A. Henkart, Experimental Immunology Branch, NCI, Building 10, Room 4B17, National Institutes of Health, Bethesda, MD 20892.

Received for publication 21 June 1993 and in revised form 16 August 1993.

References

1. Kerr, J.F.R., A.H. Wyllie, and A.R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer.* 26:239.
2. Arends, M.J., R.G. Morris, and A.H. Wyllie. 1990. Apoptosis. The role of the endonuclease. *Am. J. Pathol.* 136:593.
3. Mesner, P.W., T.R. Winters, and S.H. Green. 1992. Nerve growth factor withdrawal-induced cell death in neuronal PC12 cells resembles that in sympathetic neurons. *J. Cell Biol.* 119:1669.
4. Ishigami, T., K.-M. Kim, Y. Horiguchi, Y. Higaki, D. Hata, T. Heike, K. Katamura, M. Mayumi, and H. Mikawa. 1992. Anti-IgM antibody-induced cell death in a human B lymphoma cell line, B104, represents a novel programmed cell death. *J. Immunol.* 148:360.
5. Sellins, K.S., and J.J. Cohen. 1991. Cytotoxic T lymphocytes induce different types of DNA damage in target cells of different origins. *J. Immunol.* 147:795.
6. Webb, S., C. Morris, and J. Sprent. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell.* 63:1249.
7. Golstein, P., D.M. Ojcius, and J.D. Young. 1991. Cell death mechanisms and the immune system. *Immunol. Rev.* 121:29.
8. Russell, J.H. 1983. Internal disintegration model of cytotoxic lymphocyte-induced target damage. *Immunol. Rev.* 72:97.
9. Hayes, M.P., G.A. Berrebi, and P.A. Henkart. 1989. Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. *J. Exp. Med.* 170:933.
10. Shiver, J.W., L. Su, and P.A. Henkart. 1992. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. *Cell.* 71:315.
11. Shi, L., R.P. Kraut, R. Aebersold, and A.H. Greenberg. 1992. A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *J. Exp. Med.* 175:553.
12. Shi, L., C.-M. Kam, J.C. Powers, R. Aebersold, and A.H. Greenberg. 1992. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. *J. Exp. Med.* 176:1521.
13. Ruggiero, V., S.E. Johnson, and C. Baglioni. 1987. Protection from tumor necrosis factor cytotoxicity by protease inhibitors. *Cell. Immunol.* 107:317.
14. Suffys, P., R. Beyaert, P. Van Roy, and W. Fiers. 1988. Involvement of a serine protease in tumor-necrosis-factor-mediated cytotoxicity. *Eur. J. Biochem.* 178:257.
15. Kumar, S., and C. Baglioni. 1991. Protection from tumor necrosis factor-mediated cytolysis by overexpression of plasminogen activator inhibitor type-2. *J. Biol. Chem.* 266:20960.
16. Hogquist, K.A., M.A. Nett, E.R. Unanue, and D.D. Chaplin. 1991. Interleukin 1 is processed and released during apoptosis. *Proc. Natl. Acad. Sci. USA.* 88:8485.
17. Kaufmann, S.H. 1989. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res.* 49:5870.
18. Ucker, D.S., J.D. Ashwell, and G. Nickas. 1989. Activation-driven T cell death. I. Requirements for de novo transcription and translation and association with genome fragmentation. *J. Immunol.* 143:3461.
19. Merćep, M., P.D. Noguchi, and J.D. Ashwell. 1989. The cell cycle block and lysis of an activated T cell hybridoma are distinct processes with different $Ca2^+$ requirements and sensitivity to cyclosporine A. *J. Immunol.* 142:4085.
20. Zacharchuk, C.M., M. Mercep, P.K. Chakraborti, S.S. Simons, Jr., and J.D. Ashwell. 1990. Programmed T lymphocyte death: cell activation- and steroid-induced pathways are mutually antagonistic. *J. Immunol.* 145:4037.
21. Okada, C., H. Kizaki, and T. Tadakuma. 1990. T cell receptor-mediated DNA fragmentation and cell death in T cell hybridomas. *J. Immunol.* 144:2096.

22. Shi, Y.F., M.G. Szalay, L. Paskar, B.M. Sahai, M. Boyer, B. Singh, and D.R. Green. 1990. Activation-induced cell death in T cell hybridomas is due to apoptosis. Morphologic aspects and DNA fragmentation [published erratum appears in *J. Immunol.* 1990 Dec 1;145(11):3945]. *J. Immunol.* 144:3326.
23. Kabelitz, D., T. Pohl, and K. Pechhold. 1993. Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. *Immunol. Today.* 14:338.
24. Ashwell, J.D., R.E. Cunningham, P.D. Noguchi, and D. Hernandez. 1987. Cell growth cycle block of T cell hybridomas upon activation with antigen. *J. Exp. Med.* 165:173.
25. Horgan, K.J., and S. Shaw. 1991. Immunomagnetic purification of T cell subpopulations. *In* Current Protocols in Immunology. J.E. Coligan, A.M. Kruisbeek, D.M. Margulies, E.M. Shevach, and W. Strober, editors. Wiley Interscience, New York. Chapter 7.4.
26. Horgan, K.J., G.A. Van Seventer, Y. Shimizu, and S. Shaw. 1990. Hyporesponsiveness of naive (CD45RA+) human T cells to multiple receptor-mediated stimuli but augmentation of responses by costimuli. *Eur. J. Immunol.* 20:1111.
27. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84: 1374.
28. Kubo, R.T., W. Born, J.W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine alpha-beta T cell receptors. *J. Immunol.* 142:2736.
29. Gavrieli, Y., Y. Sherman, and S.A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* 119:493.
30. Bhakdi, S., and J. Tranum-Jensen. 1991. Alpha toxin of Staphylococcus aureus. *Microbiol. Rev.* 55:733.
31. Wilson, P.D., R.A. Firestone, and J. Lenard. 1987. The role of lysosomal enzymes in killing of mammalian cells by the lysosomotropic detergent *N*-dodecylimidazole. *J. Cell Biol.* 104:1223.
32. Liu, L.F. 1989. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* 58:351.
33. Banda, N.K., J. Bernier, D.K. Kurahara, R. Kurrle, N. Haigwood, R.-P. Sekaly, and T.H. Finkel. 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. *J. Exp. Med.* 176:1099.
34. Groux, H., G. Torpier, D. Monté, Y. Mouton, A. Capron, and J.C. Ameison. 1992. Activation-induced death by apoptosis in $CD4^+$ T cells from human immunodeficiency virus-induced asymptomatic individuals. *J. Exp. Med.* 175:331.
35. King, L.B., and J.D. Ashwell. 1993. Signaling for death of lymphoid cells. *Curr. Opin. Immunol.* 5:368.
36. Iwata, M., S. Hanaoka, and K. Sato. 1991. Rescue of thymocytes and T cell hybridomas from glucocorticoid-induced apoptosis by stimulation via the T cell receptor/CD3 complex: a possible *in vitro* model for positive selection of the T cell repertoire. *Eur. J. Immunol.* 21:643.
37. Gartner, P. 1989. Calcium and T lymphocyte activation. *Cell.* 59:15.
38. Croall, D.E., and G.N. Demartino. 1991. Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol. Rev.* 71:813.
39. Murachi, T. 1989. Intracellular regulatory system involving calpain and calpastatin. *Biochem. Int.* 18:263.
40. Wang, K.K.W. 1990. Developing selective inhibitors of calpain. *Trends Pharmacol. Sci.* 11:139.
41. Arai, A., P. Vanderklish, M. Kessler, K. Lee, and G. Lynch. 1991. A brief period of hypoxia causes proteolysis of cytoskeletal proteins in hippocampal slices. *Brain Res.* 555:276.
42. Fox, J.E.B., C.D. Austin, J.K. Boyles, and P.K. Steffen. 1990. Role of the membrane skeleton in preventing the shedding of procoagulant-rich microvesicles from the platelet plasma membrane. *J. Cell Biol.* 111:483.
43. Nicotera, P., P. Hartzell, G. Davis, and S. Orrenius. 1986. The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic Ca+2 is mediated by the activation of a non-lysosomal proteolytic system. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 209:139.
44. Fox, J.E.B., C.D. Austin, C.C. Reynolds, and P.K. Steffen. 1991. Evidence that agonist-induced activation of calpain causes the shedding of procoagulant-containing microvesicles from the membrane of aggregating platelets. *J. Biol. Chem.* 266: 13289.
45. Wyllie, A.H., J.F.R. Kerr, and A.R. Currie. 1980. Cell death: the significance of Apoptosis. *Int. Rev. Cytol.* 68:251.
46. Auberger, P., D. Mary, J.P. Brettmayer, C. Aussel, and M. Fehlmann. 1989. Chymotryptic-type protease inhibitors block the increase in Ca+2 and IL-2 production in activated Jurkat T cells. *J. Immunol.* 142:1253.
47. Auberger, P., S. Sonthonnax, J.-F. Peyron, B. Mari, and M. Fehlmann. 1990. A chymotryptic-type serine protease is required for IL-2 production by Jurkat T cells. *Immunology.* 70:547.
48. Laster, S.M., J.G. Wood, and L.R. Gooding. 1988. Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. *J. Immunol.* 141:2629.
49. Silverman, G.A., J.I. Jockel, P.H. Domer, R.M. Mohr, P. Taillon-Miller, and S.J. Korsmeyer. 1991. Yeast artificial chromosome cloning of a two-megabase-size contig within chromosmal band 18q21 establishes physical linkage between BCL2 and plasminogen activator inhibitor type-2. *Genomics.* 9:219.
50. McConkey, D.J., P. Hartzell, P. Nicotera, and S. Orrenius. 1989. Calcium-activated DNA fragmentation kills immature thymocytes. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:1843.
51. Bruno, S., P. Lassota, W. Giaretti, and Z. Darzynkiewicz. 1992. Apoptosis of rat thymocytes triggered by prednisolone, camptothecin, or teniposide is selective to G0 cells and is prevented by inhibitors of proteases. *Oncol. Res.* 4:29.
52. Bruno, S., G. Del Bino, P. Lassota, W. Giaretti, and Z. Darzynkiewicz. 1992. Inhibitors of proteases prevent endonucleolysis accompanying apoptotic death of HL-60 leukemic cells and normal thymocytes. *Leukemia.* 6:1113.
53. Murphy, K.M., A.B. Heimberger, and D.Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+ TcRlo thymocytes in vivo. *Science (Wash. DC).* 250:1720.