

Pertussis Toxin Inhibits Activation-induced Cell Death of Human Thymocytes, Pre-B Leukemia Cells and Monocytes

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Summary

Activation of human thymocytes and pre-B cells via the CD3/T cell receptor (TCR) complex or the IgM/B cell receptor complex, respectively, results in apoptotic cell death. Similarly, cross-linking of the activation marker CD69, which belongs to the natural killer complex, causes apoptosis of lipopolysaccharide-preactivated monocytes. Here we show that pertussis toxin (PTX) inhibits the activation-induced apoptosis of these three cell types, though it fails to prevent the programmed cell death that follows exposure of cells to the synthetic glucocorticoid dexamethasone (thymocytes, pre-B cells) or to interleukin 4 (monocytes). The capacity of pertussis toxin to suppress activation-induced death is not due to quenching of the activation signal, because thymocytes exposed to PTX are still capable of mobilizing Ca^{2+} after TCR- α/β cross-linking and proliferate in response to costimulation with PTX and CD3/TCR ligation. The apoptosis-inhibitory effect of PTX depends on the presence of an intact adenosine diphosphate (ADP)-ribosylating moiety, since a mutant pertussis toxin molecule that lacks enzymatic activity, but still possesses the membrane translocating activity, fails to interfere with activation-induced cell death. A toxin that induces a different spectrum of ADP ribosylation than PTX, cholera toxin, fails to inhibit apoptosis. To suppress apoptosis, the intact PTX holotoxin must be added to cells before the lethal activation step; its addition 30 min after initial activation remains without effect on apoptosis. These data unravel a PTX sensitive signal transduction event that intervenes during an early step of activation-induced cell death of immune cells.

Apoptosis or programmed cell death concerns immune cells exposed to specific activation signals that are delivered via the antigen receptor, alternative activation pathways, endogenous immunosuppressive molecules (e.g., glucocorticoids, transforming growth factor), cytotoxic effector molecules (e.g., granzymes, TNF), or a deficiency in obligate trophic factors (1). A genetically determined or acquired resistance to apoptosis induction may participate in lymphoproliferative and autoimmune diseases, as well as in the genesis of leukemias or lymphomas. In contrast, an excessive apoptotic decay of lymphoid cells is likely to be involved in the pathogenesis of acute virus-induced immunodepression (2, 3), as well as in chronic AIDS-associated lymphopenia (4, 5). Given the probable role of apoptosis in immunodeficiency, strategies designed to avoid lymphocyte apoptosis could have a prophylactic effect on progressive lymphocyte depletion. With these facts in mind, and to dissect the mechanisms involved in apoptosis regulation, we and others are presently searching substances capable of inhibiting lymphocyte apoptosis (6).

Here, we show that pretreatment with pertussis toxin (PTX) inhibits the subsequent activation-induced apoptosis

of three cell types that are notoriously prone to apoptosis induction: thymocytes, pre-B leukemia cells, and monocytes. However, PTX pretreatment fails to prevent death induced by other stimuli such as glucocorticoids (thymocytes, pre-B cells) or the cytokine IL-4 (monocytes). The inhibitory effect of PTX on activation-induced death depends on the presence of intact ADP-ribosyltransferase activity, thereby implying G protein(s) in the regulation of apoptosis of immunologically relevant cells.

Materials and Methods

Isolation of Cell Populations. Thymocyte suspensions were obtained from thymus fragments removed during corrective surgery from 1–12-mo-old patients. Double negative ($CD4^-CD8^-$), single positive ($CD4^+CD8^-$ or $CD4^-CD8^+$), and double positive ($CD4^+CD8^+$) thymocyte populations were isolated by negative and/or sequential positive selection steps with antibodies (anti-CD4, anti-CD8 α) coupled to magnetic beads according to the manufacturer's instructions (Dynabeads M450; Dynal, Oslo, Norway). To detach beads from positively selected thymocyte populations, cells were incubated during 8–12 h at 37°C in complete culture medium.

Before the *in vitro* induction of apoptosis, nonviable cells were removed by Ficoll Hypaque gradient centrifugation. The purity of the different subsets assessed by immunofluorescence analysis was >90%, and viability was >98%. Monocytes were isolated from heparinized peripheral blood of healthy adult donors. Buffy coat cells were subjected to differential gradient centrifugation (Ficoll/Hypaque; Pharmacia LKB, Uppsala, Sweden) to enrich monocytes and enriched for CD14⁺ expression (Dynabeads). Pre-B leukemia cells (IgM⁺IgD⁺) were obtained from peripheral blood of a 69-yr-old male patient. Blood leukocytes were depleted from CD3⁺, CD5⁺, and CD56⁺ cells (Dynabeads), as well as plastic-adherent cells (12 h, 37°C). The phenotype of these cells was determined to be CD5⁺ (41%) CD19⁺ (71%) CD20⁺ (30%) CD21⁻CD22⁺ (27%) CD24⁻ and HLA-DR⁺ (89%).

Culture Conditions. Cells were cultured at the indicated concentration in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM Hepes, 10% FCS, and antibiotics (200 μ l/well in flat-bottomed 96-well microtiter plates; Nunc, Roskilde, Denmark). To induce apoptosis in thymocytes, cells were exposed to plastic-bound anti-CD3 (Leu 4b; Serotec Ltd., Oxford, UK; 20 μ g/ml in 50 μ l/well PBS containing 0.1% BSA incubated for 4 h at 37°C, followed by three washes with PBS). 4 h after starting cell cultures, anti-TCR- α/β mAb (TA 1043, 2 μ g/ml; T Cell Diagnostics, Cambridge, UK) was added. Alternatively, thymocytes were exposed for 24 h to 0.1 μ M dexamethasone (DEX). Pre-B leukemia cells were exposed during 18–24 h to plastic-immobilized anti-IgM mAb (SERT 116; Serotec Ltd.). 1 h after addition to plastic-bound anti-IgM, an anti-CD19 mAb (J4.119, 2 μ g/ml; Serotec) (7) was added to wells. Alternatively, cell death was induced by culture with DEX (1 μ M). Monocytes were preactivated in the presence of LPS (from *Escherichia coli*, 25 ng/ml, 12 h at 37°C; Sigma Chemical Co., Poole, UK). After three washes (600 g, 10 min, at room temperature) cells were exposed to anti-CD69 (MCA736, 2 μ g/ml; Serotec Ltd.) or to recombinant human IL-4 (500 U/ml; British Biotechnology, Oxford, UK) (8). In most experiments, cells were cultured for 30 min in the presence of PTX (100 ng/ml; Sigma Chemical Co.), followed by three washes (600 g, 10 min at room temperature), to prevent the induction of apoptosis. A mutant PTX molecule (mPTX; PT9K 129G, 100 ng/ml, reference 9), cholera toxin (1 μ g/ml; Sigma Chemical Co.), or incubation in medium only served as controls.

Proliferation Assays, Assessment of Apoptotic Cell Death, Determination of Intracellular Calcium. Proliferation was measured after addition of 1 μ Ci/well of [³H]thymidine (Amersham International, Little Chalfort, UK) during the last 18 h of culture. Apoptosis was quantitated by three different methods: (a) Mayer (Azur II/Eosin) staining of cytospin preparations allowing for the detection of apoptotic morphology; (b) assessment of the apoptosis-associated increase in membrane permeability for propidium iodide; or (c) determination of oligonucleosomal DNA fragmentation on horizontal agarose gels (3). To measure the mobilization of intracellular calcium, PTX, and/or anti-CD3-treated CD4⁺CD8⁺ thymocytes were loaded with quin-2-tetrakis(acetomethyl)ester (2.5 μ g/ml; Molecular Probes Inc., Eugene, OR) and stimulated with soluble anti-TCR- α/β (2 μ g/ml). Fluorescence of individual cells was measured using a microscope (Axiovert 35; Zeiss Ltd., Welwyn Garden City, UK).

Results and Discussion

PTX Selectively Inhibits the TCR/CD3-induced Apoptosis of Human Thymocytes. A significant percentage of human thymocytes undergoes apoptosis after sequential activation

of the CD3 ϵ and the TCR complexes. Using a system in which purified thymocyte subpopulations were exposed to immobilized anti-CD3 α during a 4-h culture period, followed by addition of a mAb specific for the TCR- α/β (10), we observed that CD4⁺CD8⁺ and CD4⁺CD8⁻ thymocytes are more susceptible to apoptosis induction than CD4⁻CD8⁺ or CD4⁻CD8⁻ cells (Fig. 1 A). Preincubation of these thymocyte subpopulations with PTX for a minimum of 30 min completely prevents the CD3/TCR-mediated induction of apoptosis (Fig. 1, A and B) and apoptosis-associated DNA fragmentation (Fig. 1 C). This apoptosis-inhibitory effect is specific for activation-induced death, because PTX does not block the glucocorticoid (DEX)-induced death of thymocytes (Fig. 1 A). PTX inhibits all signs of apoptosis induced via the anti-CD3/TCR complex: acquisition of apoptotic morphology (Fig. 1 A); loss of cell viability (Fig. 1 B); and DNA fragmentation (Fig. 1 C).

The Apoptosis Inhibitory Effect of PTX Requires Pretreatment and Depends on ADP Ribosyltransferase Activity. As shown in Fig. 1 D, to obtain an apoptosis-inhibitory effect, PTX must be added to thymocytes shortly before the lethal activation signal is provided. Addition of PTX together with the CD3-cross-linking antibody is far less effective than PTX pretreatment, and addition of PTX as little as 30 min after CD3 cross-linking is completely inefficient in preventing apoptosis. Resting of cells at 37°C for more than 150 min after incubation of cells with the PTX standard dose (100 ng/ml, 30 min), before CD3/TCR cross-linking, abolishes the apoptosis-inhibitory effect of PTX, indicating that the changes induced by PTX are reversible. The antiapoptotic effect of PTX critically depends on its ADP ribosyltransferase activity, as revealed by analysis of a mutant PTX (mPTX) molecule. The mutant PTX holotoxin PT9K 129G (mPTX) lacks this enzymatic activity due to two amino acid substitutions in the S1 subunit (residues 9 and 129), though conserves its overall tertiary and quaternary structure and retains its cell-binding and membrane translocation potentials, as well as T cell-mitogenic and hemagglutinating activities (9). mPTX fails to inhibit activation-induced thymocyte death (Fig. 1, A and D), indicating that the ADP-ribosylating activity of PTX is essential for its apoptosis-inhibitory effect. Pretreatment of thymocytes with another ADP-ribosylating toxin, the exotoxin elaborated by cholera toxin (1 μ g/ml, 30 min) that covalently modifies a different set of G proteins than PTX (11), has no effect on the induction of apoptosis (Fig. 1 D).

Failure of PTX to Inhibit TCR-mediated Signal Transduction. To exclude the possibility that PTX might inhibit activation-induced cell death by grossly perturbing cellular metabolism and/or signal transduction, we studied the effect of preincubation with PTX on TCR-triggered intracellular signals. TCR-induced calcium mobilization was not affected by prior exposure to PTX (Fig. 2 A). Another indication that PTX does not perturb TCR-mediated signal transduction comes from the observation that CD4⁺CD8⁺ thymocytes stimulated with PTX plus anti-CD3/TCR exhibit a higher incorporation of [³H]thymidine than controls exposed to PTX or anti-CD3/TCR only. In contrast to intact PTX, mPTX is not comitogenic in this system (Fig. 2 B).

Thus, PTX does not interfere with TCR-triggered signal transduction and cell cycle progression.

PTX Inhibits Anti-IgM- but not IL-4-induced Apoptosis of Human Pre-B Leukemia Cells. Leukemia cells bearing a pre-B cell phenotype were pretreated in the absence or presence of PTX (or mPTX as a control), followed by cross-linking of the IgM antigen receptor, as well as CD19. In this type

of pre-B leukemias, only a combination of anti-IgM or CD19 cross-linking was found to induce a significant (>10%) apoptotic index. Whereas a significant percentage of cells pretreated with medium only (or mPTX as a control) undergoes apoptosis after exposure to anti-IgM/CD19, pre-B leukemia cells pretreated with intact PTX fail to do so (Fig. 3). PTX-mediated resistance to apoptosis induction is restricted to anti-IgM/CD19 triggered death and does not extend to death induced by DEX (Fig. 3).

PTX Inhibits the Activation-induced Death of Monocytes. As shown in Fig. 4 A, LPS-pretreated monocytes undergo apoptosis if they are stimulated with an antibody specific for the activation antigen CD69 that belongs to the NK complex (12). Only a combination of LPS pretreatment and CD69 cross-linking but not either of these manipulations alone induce apoptosis of a significant percentage of cells. Again, a short (30 min) preincubation step with PTX (but not mPTX) has a selective apoptosis inhibitory effect, rescuing monocytes from anti-CD69-induced cell death. In contrast, PTX does not inhibit the death of LPS-pretreated monocytes incubated in the presence of IL-4 (Fig. 4 A) (8), thus unraveling a dichotomy between two death pathways operating in monocytes, one that is PTX-sensitive and mediated via CD69 and another that is PTX-resistant and triggered via IL-4 receptors.

Concluding Remarks. As to the mechanism by which PTX selectively impedes activation-induced death, two observations may be relevant. First, only pretreatment of cells with

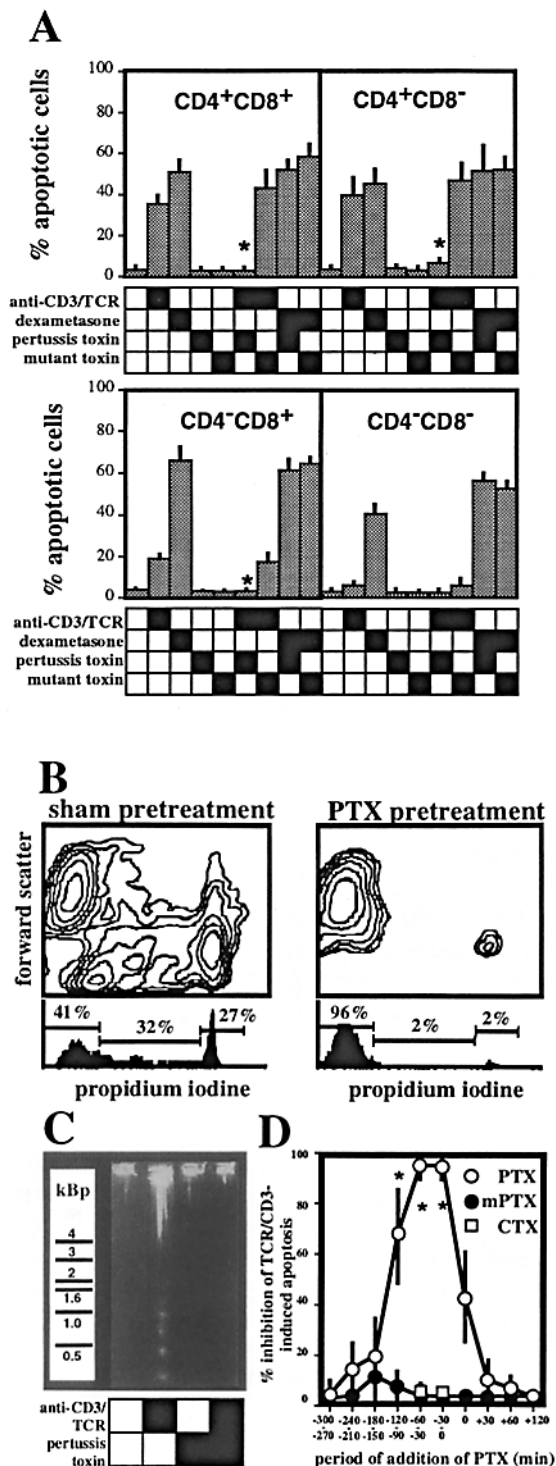


Figure 1. PTX inhibits activation-induced thymocyte apoptosis. (A) Effect on apoptotic cell death induced in purified thymocyte populations. Purified human thymocyte subpopulations were incubated for 30 min in PTX (100 ng/ml), a mutant PTX derivate lacking ADP-ribosylating activity (mPTX, 100 ng/ml) or medium only (0), washed three times and exposed to immobilized anti-CD3 ϵ (0–24 h) and soluble anti-TCR- α/β mAbs (12–24 h) to induce cell death. Alternatively, apoptotic death was induced by DEX. Black squares symbolize the execution of the indicated treatment. Apoptosis was quantitated on Mayer-stained cytospin preparations by morphological criteria after 24 h of culture. Mean results (\pm SEM) out of five different experiments are shown. Asterisks indicate significant effects ($p < 0.01$, unpaired Student's t test) of PTX as compared with controls cultured in the absence of PTX or in the presence of mPTX. (B) Effect of PTX pretreatment on anti-CD3/TCR-induced death of CD4⁺CD8⁺ cells. Cells pretreated in the presence or absence of PTX were stimulated by sequential treatment with anti-CD3 ϵ and anti-TCR- α/β (same treatment as in A). After 24 h cell viability was determined by staining with propidium iodine. (C) Effect of PTX on apoptosis-associated DNA fragmentation. CD4⁺CD8⁺ thymocytes were preincubated with PTX and/or were stimulated sequentially via the CD3 and the TCR- α/β . DNA from 10⁶ cells was analyzed by agarose gel electrophoresis and staining with ethidium bromide. (D) Time-dependence of the PTX effect. Apoptosis was induced in CD4⁺CD8⁺ thymocytes as in A, i.e., by exposure of cells to plastic-bound anti-CD3 (0–24 h) and soluble anti-TCR- α/β (4–24 h). To prevent the induction or execution of cell death, cells were preincubated during the indicated intervals before stimulation with anti-CD3 mAb (negative symbols) in the presence of PTX (100 ng/ml, 37°C, 30 min), mPTX (same concentration and treatment) or cholera toxin (CTX; 1 μ g/ml) followed by washing and incubation at 37°C until the addition of cells to anti-CD3-coated microtiter plates. Alternatively, PTX was added together (0) or after (positive symbols) incubation of cells with an immobilized anti-CD3 antibody. Mutant PTX or incubation in medium served as controls. Values of apoptosis obtained for cells with medium only were constant (range: 41–50%).

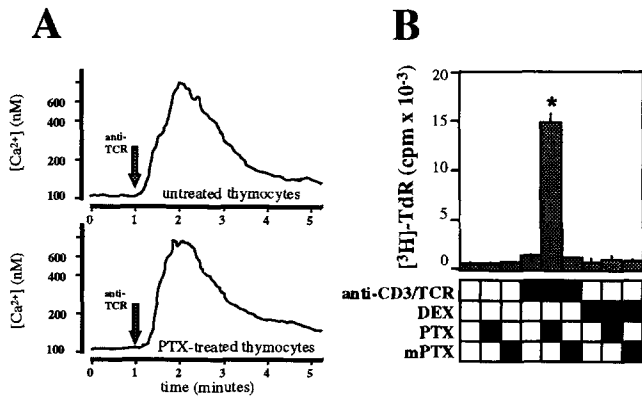


Figure 2. PTX does not inhibit TCR-triggered T cell activation. (A) Effect of PTX on anti-TCR- α/β induced calcium mobilization. CD4⁺CD8⁺ thymocytes having been exposed to PTX or control cells were stimulated during a 4-h period with plastic-bound anti-CD3. After loading with quin-2, cells were stimulated with anti-TCR- α/β to mobilize intracellular calcium. Results are typical for 50 individual measurements. (B) Effect of PTX on thymocyte proliferation. Cells were prestimulated with PTX and/or stimulated with anti-CD3 plus anti-TCR- α/β during 72 h. [³H]thymidine incorporation was assessed during the last 18 h of culture.

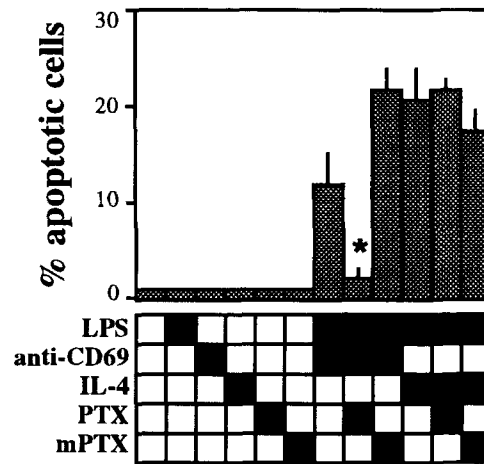


Figure 4. PTX inhibits activation-induced cell death of monocytes. LPS-primed or control monocytes were preincubated for 30 min in PTX (100 ng/ml), mPTX, or medium only, followed by apoptosis induction with IL-4 or anti-CD69. DNA hypodiploidy was assessed 18 h later. Mean values of four different experiments are shown. Asterisks mark significant PTX effects.

PTX inhibits activation-induced cell death, indicating that it has to interfere with an early step in the apoptosis inducing cascade and/or reprogram per se apoptosis-susceptible cells towards a refractory state. Second, to exert its activation death-inhibitory effect, the PTX molecule has to contain an intact ADP-ribosylating moiety. The PTX holotoxin is composed of five different subunits (S1–S5). The S1 subunit ADP-ribosylates PTX-sensitive G α subunits so that ligand-induced exchange of GDP for GTP on the G α subunit is blocked.

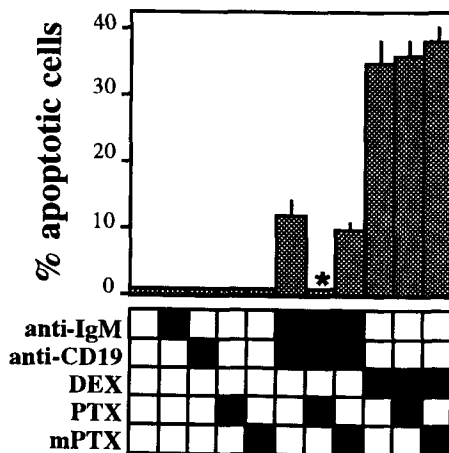


Figure 3. PTX inhibits the antigen receptor-triggered death of pre-B leukemia cells. Pre-B leukemia cells were incubated during 24 h in the presence of the indicated stimulus, followed by determination of the frequency of apoptotic cells. Mean values (\pm SEM) of four independent experiments from cells of the same patient are shown. Results are representative for three different patients. Asterisks mark significant PTX effects.

It thus interferes with G_i-protein-dependent signal transduction from cell surface receptors. The B oligomer of PTX (made up of the S2–S5 subunits) alone has several biological effects, namely T cell mitogenicity, IL-2 induction (13, 14), and downmodulation of the TCR/CD3 complex (15, 16). Given that these effects do not require ADP ribosyl transferase activity, it is improbable that they determine the antiapoptotic potential of PTX. Moreover, the incapacity of PTX to inhibit TCR-mediated calcium mobilization (Fig. 2 A) argues against the possibility that PTX inhibits activation-induced cell death by downmodulating functional TCD/CD3 signal transduction modules (15, 16) or by quenching activation signals. It is improbable that PTX exerts its antiapoptotic effect by up-regulating cAMP levels (17), because elevation of cAMP induces DNA fragmentation of murine thymocytes (18). In addition, CTX, another ADP ribosylating toxin that also causes cAMP elevations, fails to inhibit thymocyte apoptosis (Fig. 1 D).

PTX has a strong proautoimmune effect. It accelerates the development of spontaneous autoimmunity (19) and elicits organ-specific autoimmune diseases when coadministered together with the relevant autoantigen (19, 20). This proautoimmune effect may be hypothetically ascribed to the capacity of PTX to interfere with clonal deletion. PTX inhibits activation-induced cell death of human thymocytes and pre-B cells, thus acting in an experimental setting that is thought to mimic negative selection in the thymus or in the bone marrow (this paper). Similarly, it inhibits the superantigen-mediated deletion of peripheral T cells in an in vivo mouse model without interfering with other biological effects of superantigens such as lymphokine secretion and T cell proliferation (21). In this context it may be intriguing that, in the mouse, a locus controlling the in vivo responsiveness to PTX

is strongly associated with a gene that determines susceptibility to certain organ-specific autoimmune diseases including experimental autoimmune orchitis and encephalomyelitis, a model of multiple sclerosis (22). In addition, it has recently been shown that peripheral T lymphocytes from patients with sarcoidosis, i.e., a disease with a putative autoimmune component, exhibit a selective defect in the major substrate of

PTX-mediated ADP ribosylation (23). These observations strongly suggest the involvement of PTX-sensitive G proteins in the control of the function of the normal immune system. By preventing activation-induced cell death, genetically determined or acquired defects in PTX-sensitive G proteins could lead to the uncontrolled proliferation/accumulation of self-reactive lymphocytes or tissue macrophages.

We thank Ana Senik for constant support and critical comments. We are indebted to Dr. Rino Rappuoli (Istituto di Scienze Immunologiche di Siena, ISIS, Siena, Italy) for providing us with mPTX.

The work was supported in part by grants from the Dirección General de Ciencia y Tecnología (PB92-0910), Fundación Hospital Reina Sofía-Casasur (to R. Ramirez), Centre National de la Recherche Scientifique, Association pour la Recherche sur la Cancer, Fondation Nationale pour la Recherche Médicale, and Institut National de la Santé et de la Recherche Médicale (to G. Kroemer). N. Zamzami receives a fellowship from Roussel-Uclaf.

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Received for publication 22 April 1994 and in revised form 24 May 1994.

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