

Reduction in Mitochondrial Potential Constitutes an Early Irreversible Step of Programmed Lymphocyte Death In Vivo

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Summary

In a number of experimental systems in which lymphocyte depletion was induced by apoptosis-inducing manipulations, no apoptotic morphology and ladder-type DNA fragmentation were detected among freshly isolated peripheral lymphocytes *ex vivo*. Here we report that one alteration that can be detected among splenocytes stimulated with lymphocyte-depleting doses of dexamethasone (DEX) *in vivo* is a reduced uptake of 3,3'-dihexyloxycarbocyanine iodide (DiOC₆[3]), a fluorochrome which incorporates into cells dependent upon their mitochondrial transmembrane potential ($\Delta\Psi_m$). In contrast, *ex vivo* isolated splenocytes still lacked established signs of programmed cell death (PCD): DNA degradation into high or low molecular weight fragments, ultrastructural changes of chromatin arrangement and endoplasmic reticulum, loss in viability, or accumulation of intracellular peroxides. Moreover, no changes in cell membrane potential could be detected. A reduced $\Delta\Psi_m$ has been observed in response to different agents inducing lymphoid cell depletion *in vivo* (superantigen and glucocorticoids [GC]), in mature T and B lymphocytes, as well as their precursors. DEX treatment *in vivo*, followed by cytofluorometric purification of viable $\Delta\Psi_m^{\text{low}}$ splenic T cells *ex vivo*, revealed that this fraction of cells is irreversibly committed to undergoing DNA fragmentation. Immediately after purification neither $\Delta\Psi_m^{\text{low}}$, nor $\Delta\Psi_m^{\text{high}}$ cells, exhibit detectable DNA fragmentation. However, after short-term culture (37°C, 1 h) $\Delta\Psi_m^{\text{low}}$ cells show endonucleolysis, followed by cytolysis several hours later. Incubation of $\Delta\Psi_m^{\text{low}}$ cells in the presence of excess amount of the GC receptor antagonist RU-38486 (which displaces DEX from the GC receptor), cytokines that inhibit DEX-induced cell death, or cycloheximide fails to prevent cytolysis. The antioxidant, *N*-acetylcysteine, as well as linomide, an agent that effectively inhibits DEX or superantigen-induced lymphocyte depletion *in vivo*, also stabilize the DiOC₆[3] uptake. In contrast, the endonuclease inhibitor, aurintricarboxylic acid acts at later stages of apoptosis and only retards the transition from the viable $\Delta\Psi_m^{\text{low}}$ to the nonviable fraction. Altogether, these data suggest a sequence of PCD-associated events in which a reduction in $\Delta\Psi_m$ constitutes an obligate irreversible step of ongoing lymphocyte death, preceding other alterations of cellular physiology, and thus allowing for the *ex vivo* assessment of PCD.

It is currently assumed that lymphocyte elimination normally involves programmed cell death (PCD)¹ (1). This notion is supported by two observations. First, a large panel of stimuli (antigen- and coreceptor-mediated activation,

cytokines, hormones, cytotoxic drugs, physical damage, etc.) that deplete lymphocytes *in vivo* also causes lymphocyte apoptosis *in vitro* (1, 2). Second, lymphocyte depletion *in vivo* is accompanied by an enhanced apoptotic decay of lymphocytes kept in culture during several hours *ex vivo*. This latter observation applies to rather disparate systems, such as superantigen-induced depletion of mouse T cells (3), circulating leukemia cells from patients receiving apoptosis-inducing chemotherapy (4, 5), and peripheral blood lymphocytes from HIV carriers (6, 7). However, in several of these experimental systems, no morphological signs of apoptosis

¹ Abbreviations used in this paper: ATA, aurintricarboxylic acid; $\Delta\Psi_m$, mitochondrial transmembrane potential; DEX, dexamethasone; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DiBAC₄(3), bis-(1-3-dibutylbarbituric acid)trimethixine oxonol; DiOC₆(3), 3,3'-dihexyloxycarbocyanine iodide; GC, glucocorticoid; mClCCP, carbamoyl cyanide *m*-chlorophenylhydrazine; NAC, *N*-acetylcysteine; PCD, programmed cell death; PI, propidium iodide; Rh123, rhodamine 123; SEB, *Staphylococcus aureus* enterotoxin B.

and no oligonucleosomal DNA fragmentation were observed among peripheral lymphocytes without previous *in vitro* culture, even when lymphocytes were isolated *ex vivo* during the phase of *in vivo* depletion (3–7). To reveal the “ladder-type” DNA fragmentation, in many systems, cells must be cultured during a short period at 37°C *in vitro* (3, 6, 7). The apparent discrepancy between *in vitro* and *in vivo* data concerning apoptosis in peripheral lymphoid organs may be hypothetically resolved by assuming migration of preapoptotic cells or the efficient engulfment and rapid degradation of dying cells by intact adjacent cells *in situ*, before apoptosis and endonucleolysis become manifest (8).

With these facts in mind, we decided to search for alterations in lymphoid physiology that reveal commitment to PCD *in vivo*. We applied a fluorescence-based method for the determination of mitochondrial transmembrane potential ($\Delta\Psi_m$) (9) to lymphocytes undergoing PCD *in vivo*. Our data indicate a strict correlation between a partial loss in $\Delta\Psi_m$ and later entrance into apoptosis *in vitro* and/or elimination of cells *in vivo*. This allows for the quantitative assessment of ongoing lymphocyte PCD *ex vivo*, i.e., without the necessity of *in vitro* culture. Moreover, a characterization of the temporal and causal relationship between alterations in $\Delta\Psi_m$ and other PCD-associated events is reported in this paper.

Materials and Methods

Animals and *In Vivo* Treatment: 8–10-wk-old female BALB/c mice received injections of the superantigen *Staphylococcus aureus* enterotoxin B (SEB) (50 µg in 200 µl PBS *i.v.*) (Sigma Chemical Co., St. Louis, MO), dexamethasone (DEX) (500 µg in 200 µl PBS *i.p.*) (Sigma Chemical Co.). Linomide (kindly provided by Dr. T. Kalland; Pharmacia LKB, Uppsala, Sweden) was administered together with the drinking water (300 mg/kg per day, 2–3 d before inducing apoptosis by DEX or SEB as described (10)). *N*-acetylcysteine (NAC) was administered orally (1 or 3 g/kg per d, in the drinking water) 24 h before injection of DEX (11). Aurintricarboxylic acid (ATA) was administered 4 and 2 h before DEX at a dose (2 × 1.5 mg *i.p.*) that completely inhibits oligonucleosomal DNA fragmentation *in vivo* (12).

Isolation of Cells and *In Vitro* Culture. Thymuses, spleens, and femurs were immediately transferred to petri dishes containing HBSS kept on ice. Monocellular suspensions were prepared and washed as described (13), while the temperature was kept continuously between 0 and 4°C until staining or culture. Erythrocytes were eliminated from splenic and bone marrow cell suspensions by hypotonic shock at 0°C, and splenic T cells were obtained by panning of B cells, using plastic-immobilized rabbit anti-IgM (Southern Biotechnology Associates, Birmingham, AL) as described (3). Cells (1–2 × 10⁶/ml) were cultured in medium (RPMI 1640, supplemented with 5% FCS, L-glutamine, and antibiotics) at 37°C, during the indicated period in the presence or absence of 100 nM DEX, 10 µM RU-38486 (kindly provided by Dr. Martini, Roussel Uclaf, Romainville, France), 100 U/ml IL-2 (kindly provided by Dr. M. Galiay, Roussel Uclaf), 20 ng/ml IL-4 (Prepro Tech, Inc., Rocky Hill, NJ), and/or 50 µg/ml cycloheximide (Sigma Chemical Co.).

Assessment of Cellular and Mitochondrial Potentials, Intracellular Peroxides, Membrane Peroxidation, Cell Viability, and DNA Content. Cell membrane potentials were measured by means of bis-(1-3-

diethylbarbituric acid)trimethixine oxonol (DiBAC₄[3]) (Molecular Probes Inc., Eugene, OR) (14). 5 × 10⁵ cells were incubated for 10 min at 37°C in 1 ml of 150 nM DiBAC₄(3), before cytofluorometric analysis using a cytofluorometer previously equilibrated with the oxonol dye (Epics Profile; Coulter Corp., Hialeah, FL) with excitation and emission settings of 488 and 525 nm, respectively. To evaluate $\Delta\Psi_m$, cells (5 × 10⁵/ml) were incubated with 3,3'-dihexyloxycarbocyanine iodide (DiOC₆[3]) (40 nM in PBS; Molecular Probes, Inc.) for 15 min at 37°C (9), followed by analysis on a cytofluorometer (Epics Profile, excitation: 488 nm; emission: 525 nm). Alternatively, cells were incubated for 20 min at 37°C in 50 ng/ml rhodamine 123 (Rh123) to measure $\Delta\Psi_m$ by cytofluorometry (excitation: 488 nm; emission: 525 nm) (15). Control experiments were performed in the presence of 5 µM carbamoyl cyanide *m*-chlorophenylhydrazone (mClCCP) (15 min, 37°C), an uncoupling agent that abolishes the $\Delta\Psi_m$. Intracellular peroxides were measured by means of 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes Inc.; 5 µM in PBS; 1 h, 37°C; excitation: 488 nm; emission: 525 nm), as described (16). For the determination of lipid peroxidation, 5 × 10⁶ cells were incubated with 5 µM *cis*-parinaric acid (Molecular Probes Inc.) in PBS (1 h, 37°C), washed twice and resuspended in complete medium in the presence or absence of DEX (1 µM) for the indicated period, and analyzed by cytofluorometry (excitation: 334–364 nm; emission: 410 nm) on a cytofluorometer (Elite; Coulter Corp.) (17). To determine the integrity of the cell membrane, cells were incubated for 10 min in propidium iodide (PI) (5 µg/ml in PBS) at 37°C. For cell cycle analysis, 2 × 10⁶ cells were spun down and fixed in 70% ice-cold ethanol/PBS, added dropwise while vortexing, kept overnight at 4°C, and centrifuged. Cells were resuspended in 1 ml PBS containing 50 µg/ml PI and analyzed in a cytofluorometer (Epics Profile).

Cytofluorometric Purification of DiOC₆(3)^{low} and DiOC₆(3)^{high} Cells. Splenic T cells were stained for 15 min with DiOC₆(3) (40 nM, 37°C). During the last 5 min of this incubation period, PI was added (5 µg/ml). Cells were sorted immediately on a cytofluorometer (Elite) (10⁴ cells/s) and recovered in complete medium on ice.

Immunofluorescence Analysis. Cells were stained with mAbs directed against CD3ε (145-C11), CD4 (RM4-5), CD8α (53-6.7), Vβ6 (RR4-7), Vβ8.1, and 8.2 (MR5-2) or anti-IgM^s (DS-1; Pharmingen, San Diego, CA), which were either PE-labeled or biotin-conjugated and developed by means of streptavidin-PE (GIBCO-BRL, Gaithersburg, MD), as described (13). Isotype-matched control antibodies served as negative controls. Mitochondrial F₁ ATPase expression was determined by cytofluorometry on permeabilized cells (70% methanol in PBS, 30 min at –20°C, followed by two washings in PBS), using optimal concentrations of a specific polyclonal rabbit antiserum (kindly provided by Dr. P. V. Vignais, Centre National de la Recherche Scientifique–URA 1130, Grenoble, France) revealed by an FITC-labeled affinity purified F(ab')₂ fragment donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Preliminary experiments on murine fibroblasts revealed specific mitochondrial staining patterns, using this combination of antibodies.

Assessment of DNA Fragmentation and Electron Microscopy. To determine oligonucleosomal DNA fragmentation, nuclear DNA from lysed cells treated with protease K and RNase, according to standard protocols, was subjected to conventional horizontal agarose gel electrophoresis (1%), followed by ethidium bromide staining (3, 13). For PAGE electrophoresis, DNA was prepared from agarose plugs (1 × 10⁶ cells) (18) digested twice with proteinase K (1 mg/ml, 50°C, 12 h) in NDS buffer (0.5 M EDTA, 10 mg/ml lauroyl sarcosine) and washed in Tris-buffered EDTA × 0.5, fol-

lowed by electrophoresis (CHEF-DRII; Bio-Rad Laboratories, Inc., Richmond, CA) (1% agarose, TBE \times 0.5, 200 V, 24 h, pulse wave 60 s, 120° angle). Mol wt standards were purchased from BioRad Laboratories, Inc. (yeast chromosomes) and Appligene (Raoul; Illkirch, France). To assess zinc-resistant changes in ultrastructure and DNA configuration, cells were cultured in the presence of 1 mM ZnCl₂ and/or 1 μ M DEX, followed by PAGE electrophoresis or fixation in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), osmium tetroxide staining, and electron microscopic examination as described (19).

Immunohistochemical Determination of Mitochondrial ATPase Expression. Cells were rinsed twice in cold PBS and lysed by addition of 0.5% NP-40. Crude extracts, normalized for protein content (60 μ g protein/lane), were subjected to SDS-PAGE in 7.5% acrylamide and 0.1% bisacrylamide, followed by Western blot transfer to nitrocellulose membranes (20). Expression of mitochondrial F₁ ATPase was determined by means of a specific polyclonal rabbit antiserum revealed by a second peroxidase-conjugated goat anti-rabbit Ig serum (BioSys, Compiègne, France) for 1 h at room temperature. The immunoreactivity was revealed using the enhanced chemoluminescence kit (Amersham Corp., Arlington Heights, IL) (time of exposure: 2 min).

Results and Discussion

Reduction in $\Delta\Psi_m$ of Splenocytes Can Be Detected Ex Vivo after Injection of DEX. Thymocytes and splenocytes rapidly underwent apoptosis upon exposure to glucocorticoid (GC) in vitro (21, 22). However, splenocytes recovered from animals treated with high doses of DEX in vivo did not exhibit DNA fragmentation or hypoploidy after isolation ex vivo, even during the phase of lymphoid cell depletion, unless they were cultured during a short period (\leq 60 min) at 37°C (Fig. 1). Splenocytes recovered from animals 12 h after the injection of 500 μ g DEX exhibited an intact DNA content, as determined by PI staining of permeabilized cells. Moreover, they possessed a normal electric potential of the cell membrane, as determined with DiBAC₄(3), an agent that incorporates into cells strictly dependent upon their external cell membrane potential (14). Furthermore, at this time point, no increase in the concentration of intracellular peroxides could be detected by means of DCFH-DA.

In contrast, splenocytes from DEX-treated mice exhibited a reduction in the incorporation of the fluorochrome DiOC₆(3), as compared to cells from control animals (Fig. 1). DiOC₆(3) incorporated into cells in strict nonlinear correlation with $\Delta\Psi_m$ (9). mClCCCP, an uncoupler of the oxidative phosphorylation that abolishes $\Delta\Psi_m$, abolished staining with DiOC₆(3), demonstrating that the dye uptake was driven by $\Delta\Psi_m$ and did not involve significant binding to other cellular components (Fig. 1, upper left). A fraction of viable (PI⁻) control cells from vehicle-injected animals (15–25%) showed a reduced DiOC₆(3) uptake, as compared to the principal population, correlating with the fact that a significant percentage of splenocytes underwent PCD spontaneously (Fig. 2 D) (22). However, the percentage of DiOC₆(3)^{low} cells was much higher among splenocytes from DEX-treated animals (Fig. 1). Analogous results were obtained with other cationic fluorochromes commonly used for

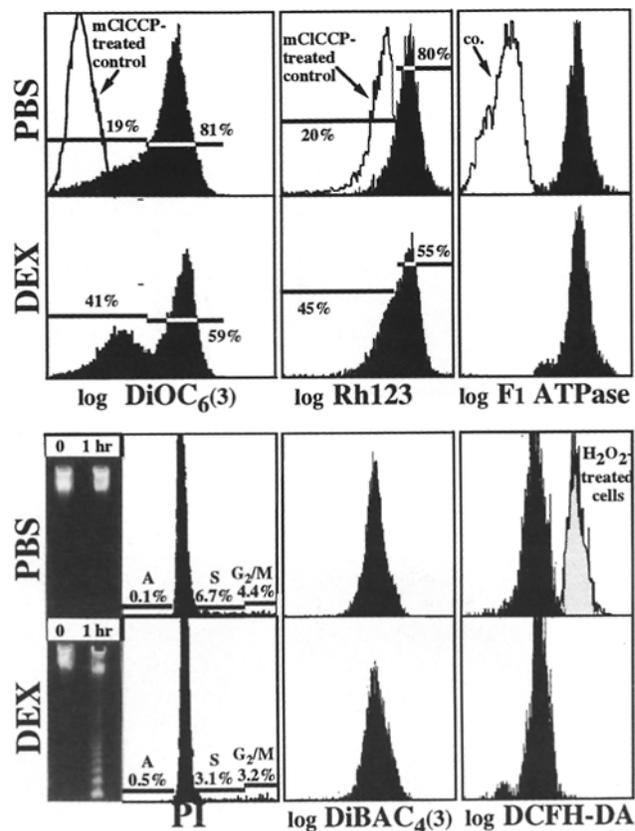


Figure 1. Alterations detectable among splenic lymphocytes primed for cell death in vivo. Splenocytes were recovered from animals treated 12 h before with 500 μ g DEX i.p. or PBS and stained by incubation with DiBAC₄(3), DiOC₆(3), Rh123, DCFH-DA, PI, or an antiserum specific for mitochondrial F₁ ATPase. The distribution of viable (PI⁻) cells is shown for DiBAC₄(3), DiOC₆(3), and DCFH-DA. For DiOC₆(3) and Rh123, negative controls (cells treated with mClCCCP) are shown. For DCFH-DA, a positive control (cells kept 2 min in 15 mM H₂O₂ and washed three times) was inserted. F₁ ATPase was determined by immunofluorescence, using methanol-permeabilized cells. Controls were incubated with a nonimmune rabbit antiserum. DNA content was quantified by means of PI staining of ethanol-permeabilized cells. DNA fragmentation was assessed by horizontal agarose gel electrophoresis, followed by staining with ethidium bromide. DNA was analyzed from either ex vivo isolated cells or from cells kept in culture for 60 min at 37°C. Data are representative for five different experiments.

the quantification of $\Delta\Psi_m$: Rh123 (Fig. 1) (15) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (23) (not shown). Staining of cells with an antibody-recognizing mitochondrial F₁ ATPase revealed no quantitative difference between DEX-treated and control splenocytes, indicating that no loss of mitochondrial mass occurs during PCD (Fig. 1).

Kinetics of Alterations in $\Delta\Psi_m$ and In Vivo Detection of Reduced $\Delta\Psi_m$. To determine the temporal relationship between loss in $\Delta\Psi_m$ and established signs of ongoing PCD, we analyzed the kinetics of these phenomena in vitro and in vivo. In both splenocytes and thymocytes, endonucleolytic DNA fragmentation, associated hypoploidy, loss in cell viability, and membrane lipid peroxidation induced by DEX in vitro

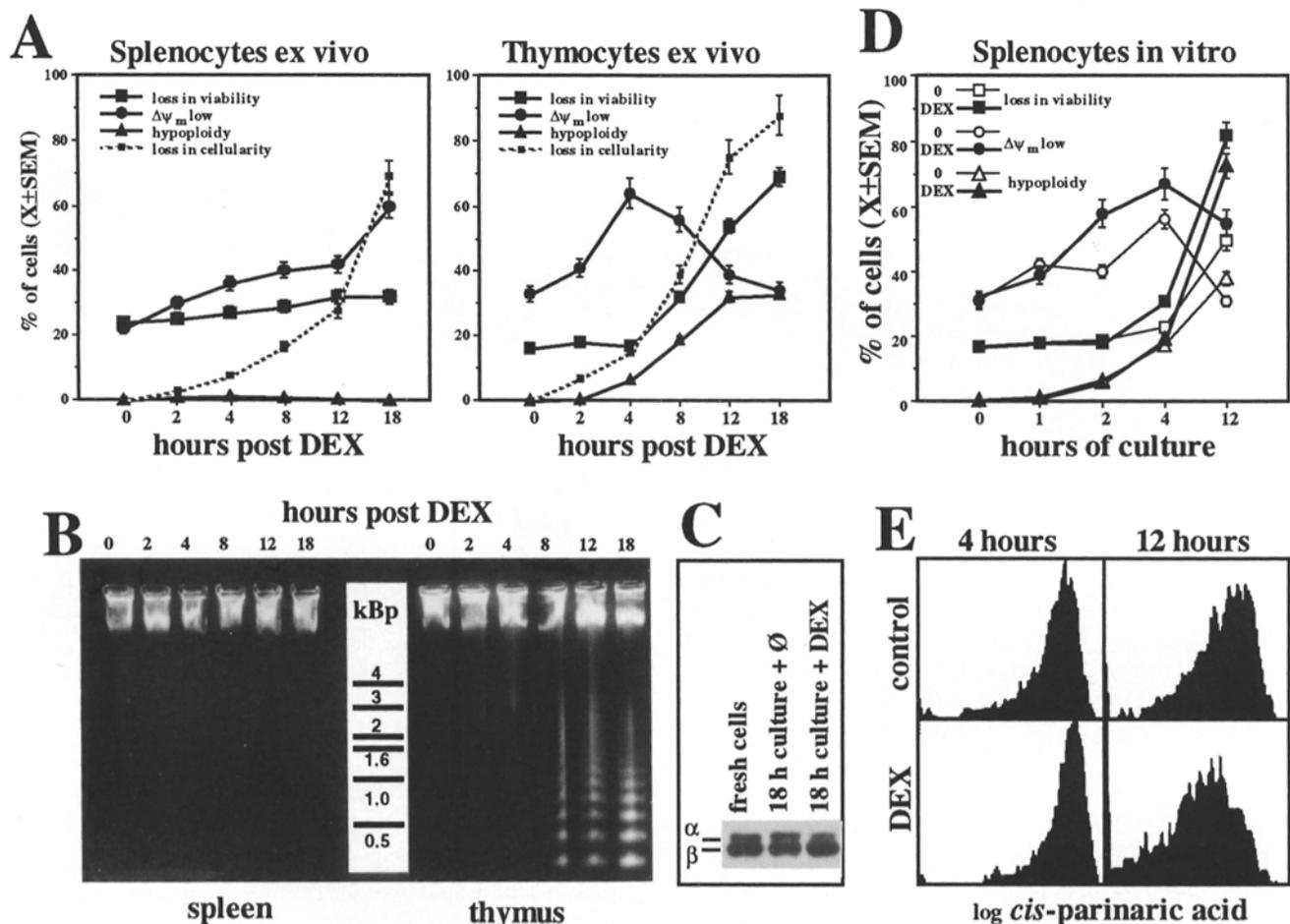


Figure 2. Kinetic analysis of apoptosis-associated changes in vivo and in vitro. Thymocytes or splenic T cells were subjected to the determination of DNA hypoploidy (PI staining of ethanol-fixed cells) or $\Delta\Psi_m$ (DiOC₆(3) incorporation). In addition, lipid peroxidation was determined by means of *cis*-parinaric acid. (A) Kinetics of DEX-induced alterations in thymocytes and splenocytes ex vivo. Mice received 500 μg DEX i.p. and were killed at the indicated time points (two to three mice per point, $X \pm \text{SEM}$). Loss in cell viability was determined by PI staining, loss in $\Delta\Psi_m$ by staining with DiOC₆(3), and hypoploidy by PI staining of ethanol-fixed cells (see Fig. 1). The percentage of $\Delta\Psi_m^{\text{low}}$ cells was calculated among the viable fraction of cells. Loss in cellularity was determined by calculating the total number of viable (trypan blue-negative) thymocytes or splenocytes and comparing them to age- and sex-matched untreated controls. Cell suspensions were prepared while keeping organs on ice and were directly subjected to analysis ex vivo. (B) DNA fragmentation of freshly isolated thymocytes or splenocytes after injection of DEX (same suspensions as in A). After injection of DEX in vivo, cell suspensions were prepared after the indicated interval. DNA fragmentation was assessed by horizontal agarose gel electrophoresis. Data are representative for five different experiments. (C) Stable expression of mitochondrial F₁ ATPase in cells undergoing apoptosis. Freshly isolated splenocytes or cells cultured 18 h in the presence (90% PI⁺ cells) or absence (52% PI⁺ cells) of 100 nM DEX were analyzed by immunoblot using a polyclonal antiserum recognizing both the α and the β subunits of F₁ ATPase (~ 55 kD). (D) Kinetics of DEX-induced alterations in vitro. Splenic lymphocytes from untreated animals were cultured in the presence or absence of DEX (100 nM) during the indicated period, followed by determination of the different parameters. The percentage of $\Delta\Psi_m^{\text{low}}$ cells was calculated among the viable (PI⁻) fraction of cells. (E) Determination of lipid peroxidation induced by treatment with DEX in vitro. Splenocytes were labeled with *cis*-parinaric acid, cultured in the presence or absence of DEX (100 nM) for the indicated period, and analyzed by cytofluorometry.

were preceded by a reduction in $\Delta\Psi_m$ (Fig. 2). This applied to both PCD occurring in vivo (Fig. 2, A and B) and in vitro (Fig. 2 D). Splenocytes isolated ex vivo exhibited a significant reduction in $\Delta\Psi_m$ as little as 4 h after injection of DEX (Fig. 2 A). This applied also to thymocytes 4 h after injection of DEX, i.e., at a time point at which no significant loss in cellularity had occurred and neither DNA fragmentation, nor hypoploidy, could be detected ex vivo, yet $\Delta\Psi_m$ was already perturbed. In contrast to splenocytes, thymocytes did display advanced stages of apoptosis, such as DNA frag-

mentation and hypoploidy ex vivo, 8 h after injection of DEX. However, kinetic analysis revealed that reduced DiOC₆(3) incorporation preceded these alterations (Fig. 2, A and B). Only when the percentage of nonviable (PI⁺) cells increased in culture, did the frequency of DiOC₆(3)^{low} decrease among the viable (PI⁻) population, indicating that DiOC₆(3)^{low} PI⁻ cells were recruited into the pool of PI⁺ cells. In vitro induction of PCD in splenocytes also revealed a loss in $\Delta\Psi_m$ before cells lost their viability and chromosomal DNA was digested. (Fig. 2 D). 4 h after culture with DEX, a significant

reduction in $\Delta\Psi_m$ could be appreciated, yet no significant lipid peroxidation could be determined by means of the fluorescent dye *cis*-parinaric acid at this time point (Fig. 2 E). Western blot analysis revealed that splenocytes incubated 18 h in the presence of DEX (90% PI⁺ cells) did not manifest a consistent reduction in the expression of either subunits of mitochondrial F₁ ATPase, as compared to fresh splenocytes (Fig. 2 C). This correlates with the fact that, even at advanced stages of apoptosis, when nuclear DNA was fragmented, no degradation of mitochondrial DNA was observed (24, 25) and that, by consequence, the number or mass of mitochondria did not diminish during PCD.

To measure $\Delta\Psi_m$ ex vivo, it is necessary to incubate the cells during a short period (10–15 min) at 37°C. To determine whether alterations in $\Delta\Psi_m$ truly occur in vivo, we intravenously injected the fluorochrome DiOC₆(3) animals pretreated with DEX (1 mg i.p., 6 h before) or PBS controls. After 45 min of in vivo incubation, during which animals did not display any signs of toxicity, splenic lymphocytes were prepared on ice and analyzed by cytofluorometry without any prior culture. As shown in Fig. 3, a significant fraction of splenocytes from DEX-treated animals exhibited a lower $\Delta\Psi_m$ -related fluorescence than did cells from a vehicle control.

In synthesis, a reduction in $\Delta\Psi_m$ accompanied DEX-induced PCD in vitro and in vivo. Kinetic data are compatible with the hypothesis that cells lose their $\Delta\Psi_m$ first, and later undergo endonucleolysis, lipid peroxidation, and cytolysis.

Loss in $\Delta\Psi_m$ Occurs in Cells Irreversibly Committed to PCD. To evaluate the relationship of diminished $\Delta\Psi_m$ and apoptotic cell death, splenocytes from DEX-injected animals (500 μ g i.p., 12 h before) were stained simultaneously with DiOC₆(3) plus PI and separated into two fractions, a population of viable (PI⁻) cells with intact $\Delta\Psi_m$ (DiOC₆(3)^{high}) and a yet viable population with low $\Delta\Psi_m$ (DiOC₆(3)^{low}) (Fig. 4 A). None of these populations exhibited oligonucleosomal DNA fragmentation immediately after sorting. After short-term in vitro culture (60 min, 37°C), DiOC₆(3)^{low}, but not DiOC₆(3)^{high}, cells showed typical ladder-type oligo-

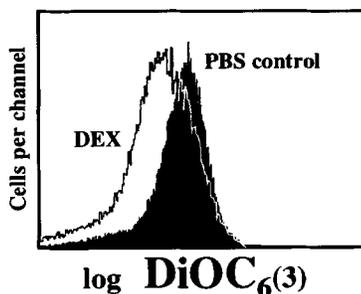


Figure 3. In vivo determination of DEX-induced $\Delta\Psi_m$ reduction. Mice were injected with DEX (1 mg i.p.) or PBS. 6 h later, 10 μ g of the fluorochrome DiOC₆(3)^{low} were injected into the tail vein, and, 45 min later, splenocytes were prepared on ice, as described in Materials and Methods, followed by their immediate cytofluorometric analysis. At the indicated dose, DiOC₆(3) does not exhibit any apparent in vivo toxicity.

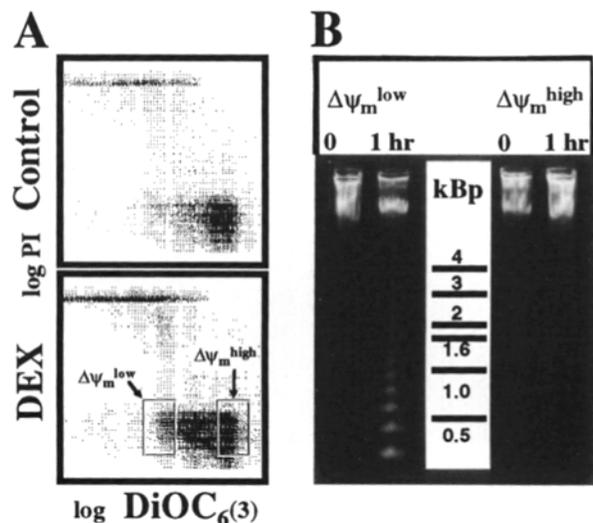


Figure 4. Determination of the status of nuclear DNA in cytofluorometer-purified populations differing in $\Delta\Psi_m$. (A) Purification of viable DiOC₆(3)^{low} and DiOC₆(3)^{high} populations. Splenic T cells from a DEX- (500 μ g) or PBS-injected control animal were subjected to a combined PI/DiOC₆(3) staining, followed by FACS[®] purification of DiOC₆(3)^{low} and DiOC₆(3)^{high} cells, as indicated by the windows. Cells were cultured 60 min at 4 or 37°C, and DNA fragmentation was assessed by horizontal agarose gel electrophoresis. Results are representative for two independent experiments.

nucleosomal DNA degradation (Fig. 4 B). Thus, it appears that only the DiOC₆(3)^{low} population is prone to undergoing apoptosis.

To determine whether the loss in $\Delta\Psi_m$ would be reversible, cytofluorometer-purified PI⁻DiOC₆(3)^{low} splenic T cells derived from a DEX-pretreated animal were incubated in the presence of cycloheximide, the GC receptor antagonist RU-38486, and/or the cytokines IL-2 and/or IL-4, which have been reported to rescue T cells from GC-induced apoptosis (26–29). In spite of this manipulation, PI⁻DiOC₆(3)^{low} cells rapidly lost their viability upon in vitro culture (Fig. 5 A). As a positive control for the effectiveness of one of these manipulations, it is shown in Fig. 5 B that IL-4 and RU-38486 synergize to preserve the viability of splenocytes undergoing PCD upon in vitro culture, either spontaneously, or as a consequence of a short-term in vivo exposure to DEX.

In synthesis, loss in $\Delta\Psi_m$ accompanied DEX-induced PCD at an already irreversible stage of apoptotic cell death.

Ex Vivo Assessment of DEX- and Superantigen-induced Alteration of $\Delta\Psi_m$ in Different Cell Populations. The fluorochrome DiOC₆(3) emits within the spectrum of green light and, thus, is compatible with the use of PE-labeled antibodies. The combined assessment of antigenic phenotype (CD3, CD4, CD8, IgM) and $\Delta\Psi_m$ reveals that treatment with high doses of DEX induces a loss in $\Delta\Psi_m$ in splenic CD4⁺ and CD8⁺ T cells, splenic B cells, as well as thymocytes and bone marrow pre-B cells isolated ex vivo (Fig. 6), correlating with a significant reduction in the cellularity of these populations. Similarly, a reduction in $\Delta\Psi_m$ preceded the PCD-mediated depletion of V β 8⁺ splenic T cells 4 d after intravenous ad-

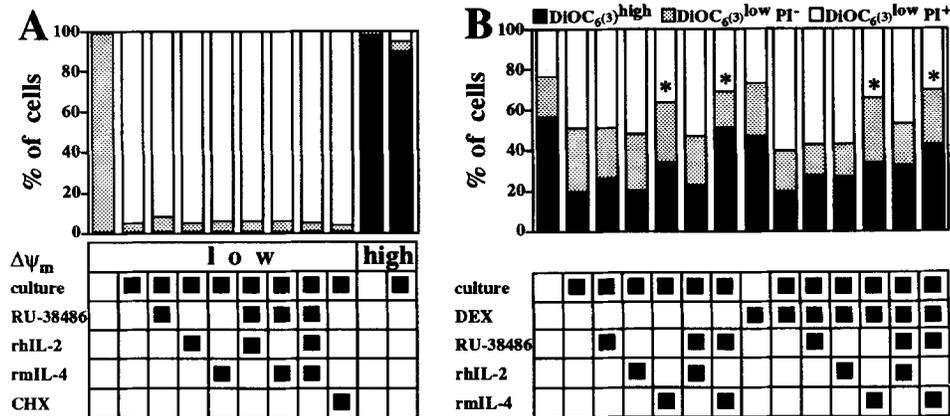


Figure 5. Irreversibility of loss in $\Delta\Psi_m$. (A) DiOC₆(3)^{low} and DiOC₆(3)^{high} splenic T cells from a DEX-injected mouse were obtained by cytofluorometric purification as described in Fig. 4 A, followed by culture of cells at 37°C for 12 h in the presence of the GC receptor antagonist RU-38486 (10 μ M), IL-2 (100 U/ml), IL-4 (20 ng/ml) and/or cycloheximide (50 μ g/ml), and combined PI/DiOC₆(3) staining. The percentage of cells that lost their viability (PI⁺ cells) immediately after sorting (25–40%) was subtracted. (B) Effect of RU-38486 and IL-4 on the maintenance of splenocyte viability in vitro. Splenic lymphocytes from a PBS-injected control mouse or a DEX-primed animal (2 h before necropsy) were cultured 12 h in the presence of RU-38486 (10 μ M), IL-2 (100 U/ml) and/or IL-4 (20 ng/ml). Results are typical for three different experiments. *Significant ($P < 0.01$) reduction of cellular mortality, as compared to controls kept in the absence of the indicated substances.

ministration of the bacterial superantigen SEB (Fig. 7). $V\beta 8^+$ T cells that do not respond to SEB (3) failed to show an alteration of $\Delta\Psi_m$.

Thus, reduced $\Delta\Psi_m$ is not a particularity of GC-induced PCD and also occurs during activation-induced PCD. In addition, nonspecific induction of PCD, e.g., by injection of D-galactosamine (13), is also accompanied by reduced $\Delta\Psi_m$ (data not shown).

Pharmacological Inhibition of $\Delta\Psi_m$ Reduction In Vivo. The immunomodulator linomide inhibited the SEB-driven deletion of $V\beta 8^+$ T cells (10). Accordingly, it fully inhibited the SEB-induced reduction in $\Delta\Psi_m$ (Fig. 7). Moreover, linomide inhibited the DEX-driven depletion of splenic T and B lymphocytes and mature CD3^{high} thymocytes (Reference 10 and Fig. 6), correlating with the maintenance of normal $\Delta\Psi_m$ levels. On the contrary, linomide failed to stabilize $\Delta\Psi_m$ of immature CD3^{low} thymocytes and bone marrow pre-B cells, which both succumbed to DEX, irrespective of whether animals were treated with linomide or not (Fig. 6).

These data suggest that linomide interferes with an early event of T lymphocyte PCD. To confirm this hypothesis, we determined further apoptosis-associated changes in cells from linomide-treated animals. After short-term culture (4 h, 37°C) in the presence of DEX, splenic T cells exhibited early apoptosis-associated morphological changes (dilation of the smooth endoplasmic reticulum, incipient heterochromatin condensation, convolution of the nuclear membrane), even when cells were kept in the presence of zinc ions at doses precluding the action of Ca²⁺- and Mg²⁺-dependent endonucleases (19). This was accompanied by fragmentation of DNA into large (≥ 50 -kb) fragments by zinc-resistant DNAses (18, 19). Cells from linomide-treated animals did not exhibit these PCD-associated changes (Fig. 8), thus

confirming that linomide inhibits a rather early step of the apoptotic cascade.

Another substance that partially inhibits the DEX-mediated depletion of lymphocytes is NAC. In contrast to linomide (10), it acts on both immature (CD4⁺CD8⁺) thymocytes and splenocytes (Fig. 9, A and B). However, to obtain an apoptosis-inhibitory effect, NAC has to be administered at doses that, per se, are toxic and provoke a significant lymphopenia (Fig. 9 A). NAC treatment in vitro reduced the DEX-induced loss in DiOC₆(3) incorporation (Fig. 9 C). ATA, an inhibitor of endonuclease activation, only partially reduces the DEX-induced lymphocyte depletion (Fig. 10 A), even if it is administered at sublethal doses that inhibit the SEB-driven deletion of $V\beta 8^+$ thymocytes in vivo (12) and fully suppress DEX-induced DNA fragmentation (Fig. 10 B). ATA by itself reduces lymphocyte $\Delta\Psi_m$, a finding that may explain its apparent toxicity. It fails to maintain the pool of cells with an intact $\Delta\Psi_m$. Rather, it inhibits the cytolysis of DiOC₆(3)^{low} cells (Fig. 10 C). ATA did not confer long-term protection (>24 h) to lymphocytes cultured in the presence of DEX (data not shown).

In conclusion, efficient pharmacological inhibition of apoptosis is accompanied by maintenance of stable $\Delta\Psi_m$ values. Inhibition of DNA endonucleases does not stabilize $\Delta\Psi_m$. Rather, it retards the cytolysis of cells that are irreversibly committed to PCD.

Concluding Remarks. In the present paper, we demonstrate that peripheral lymphocytes exhibit a reduction in $\Delta\Psi_m$ before exhibiting other PCD-associated changes: DNA fragmentation and concomitant loss in nuclear DNA content, reduction of cell membrane potential, and membrane disruption. Reduced $\Delta\Psi_m$ is the only alteration in cellular physiology accompanying GC- or activation-induced death of pe-

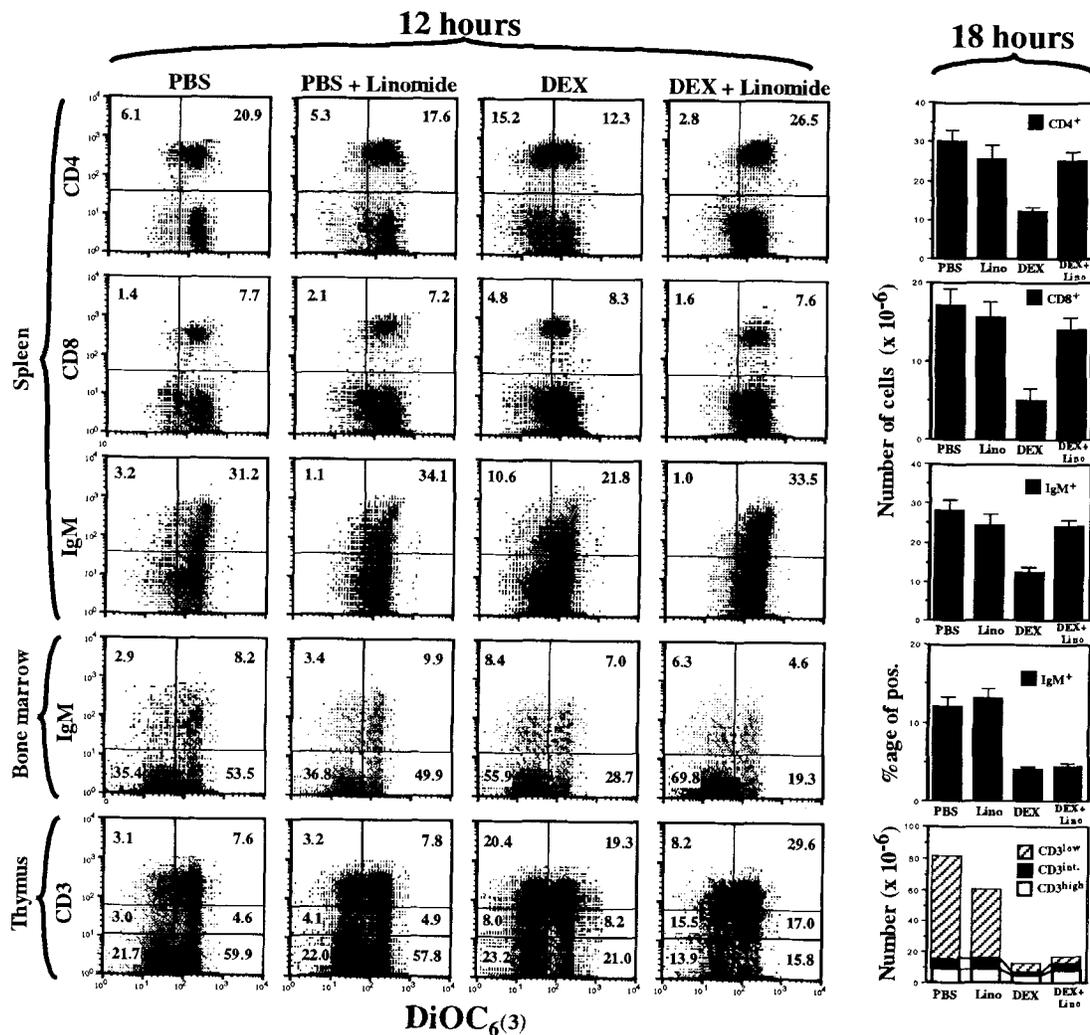


Figure 6. Effect of DEX and linomide on the $\Delta\Psi_m$ of T and B lymphocytes and their precursors. DEX (500 μg i.p.) or PBS were injected into mice pretreated with linomide or controls. 12 h later, splenocytes, thymocytes, or bone marrow leukocytes were recovered, stained with the indicated PE-labeled mAbs, followed by a short-term incubation (15 min, 37°C) with DiOC₆(3). Viable cells were gated on the basis of the light and forward scatters. Numbers indicate the percentage of cells found in the respective window. On the right, the cellularity of the respective populations recovered 18 h after treatment with DEX and/or linomide is indicated.

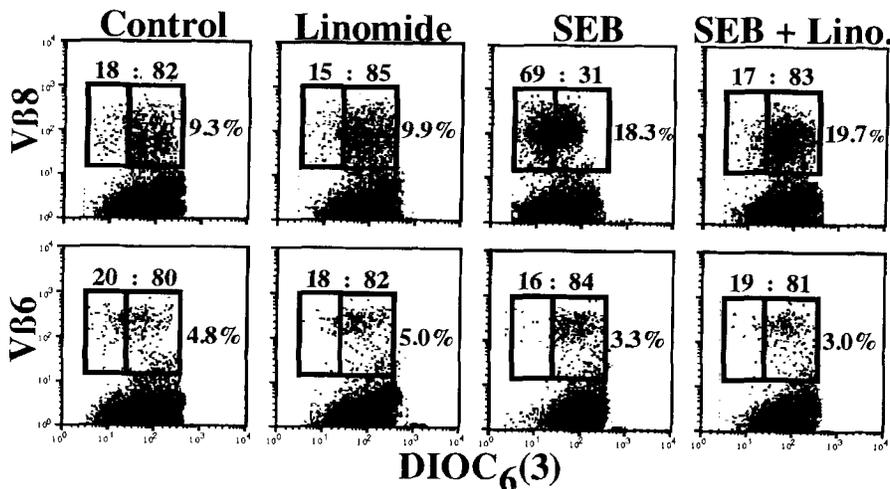


Figure 7. Effect of SEB and linomide on the $\Delta\Psi_m$ of splenic T cells. SEB (50 μg i.v.) or PBS was injected into mice pretreated with linomide or controls. 4 d later, splenocytes were stained with biotin-labeled anti-V β 8.1/2 or anti-V β 6, revealed with a PE-streptavidin conjugate and labeled with DiOC₆(3). Light and forward scatters were gated on viable cells. Numbers indicate the proportion of DiOC₆(3)^{low} and DiOC₆(3)^{high} cells. The percentages refer to the entire V β 8⁺ or V β 6⁺ population.

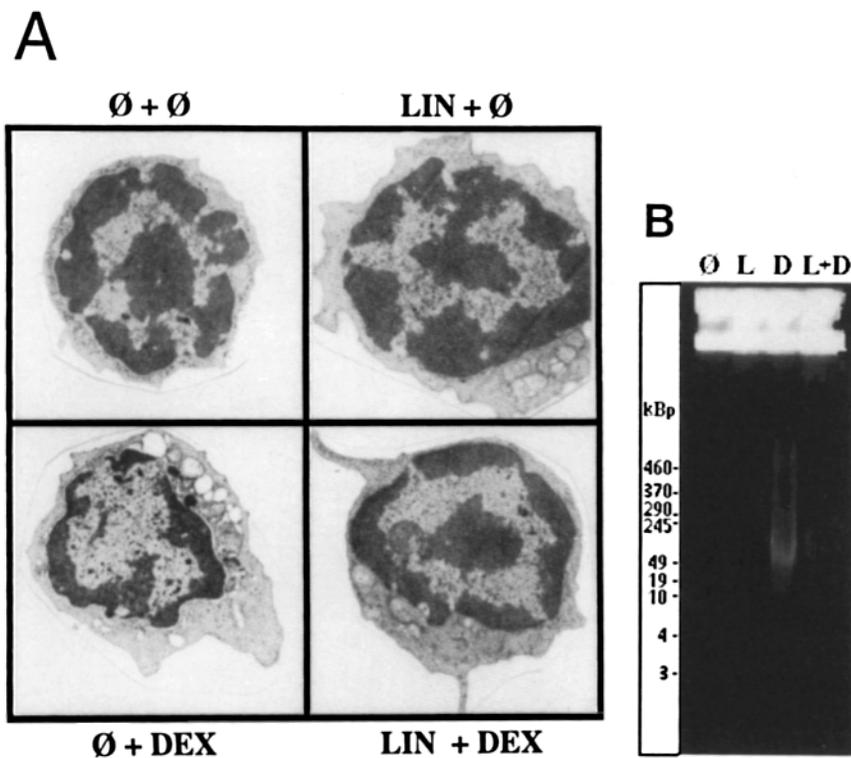


Figure 8. Effect of linomide on early apoptotic changes. Splenocytes were recovered from linomide (*LIN, L*)-treated or control animals and incubated for 4 h in medium supplemented with 1 mM ZnCl₂ in the presence or absence of 1 μ M DEX (*D*). Aliquots of cells were subjected to transmission electron microscopic analysis (*A*) or analyzed for high molecular weight DNA fragmentation by means of PAGE (*B*). Representative cells out of 50 are shown.

ripheral lymphocytes that can be detected directly *ex vivo*. Functional analysis of preapoptotic cells exhibiting reduced $\Delta\Psi_m$ suggests that such cells are committed to PCD, given that they undergo DNA fragmentation within as little as 1 h

of culture and lyse, even when they are cultured in the presence of an excess of RU-38486 (which should displace the apoptosis-inducing agent DEX from the GC receptor) (26) and cytokines that have been reported to block GC-induced

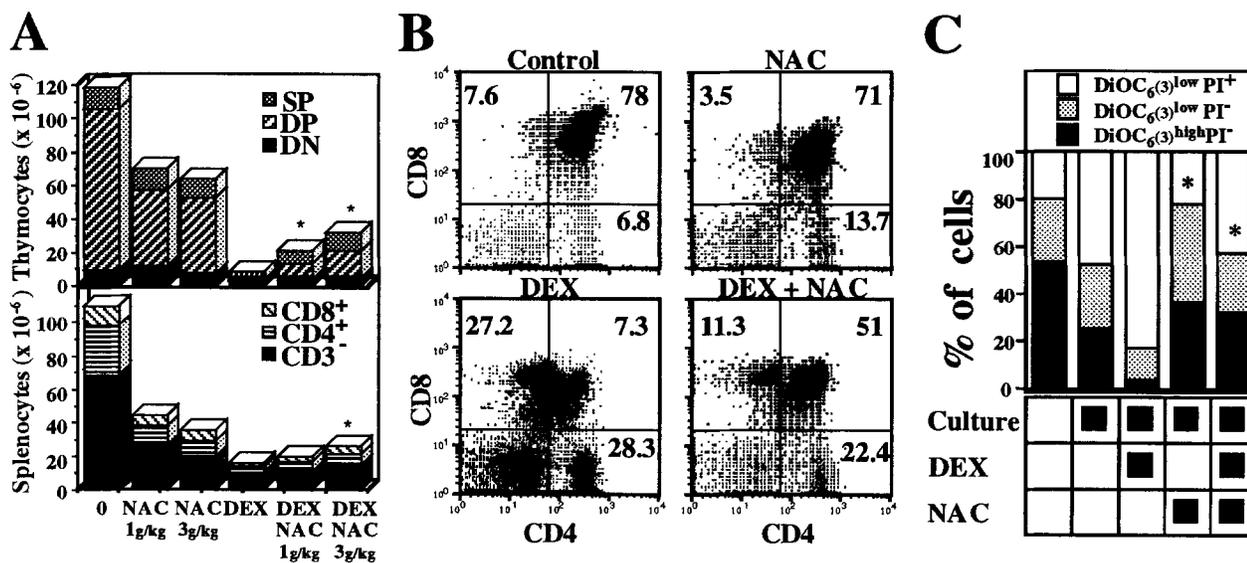


Figure 9. Effect of NAC on DEX-induced lymphocyte depletion and reduction of $\Delta\Psi_m$. (*A*) Effect of NAC on the DEX-triggered depletion of thymocytes and splenocytes *in vivo*. Animals were pretreated with NAC at the indicated dose for 24 h, before injection of DEX (500 μ g *i.p.*). NAC application was continued for another 18 h, followed by immunofluorescence analysis of splenic and thymic cell suspensions. *Significant increase in NAC+DEX-treated animals over controls receiving DEX only. Data are representative for three independent experiments. (*B*) NAC partially rescues the DEX-induced deletion of CD4⁺CD8⁺ thymocytes. Thymic cell suspensions from mice treated with DEX (500 μ g *i.p.*) and/or NAC (3 μ g/kg per d) were stained for CD4 and CD8 expression. (*C*) Effect of NAC on the DEX-driven reduction of $\Delta\Psi_m$. Thymocytes were cultured with DEX (100 nM, from 2 to 12 h) and/or NAC (50 mM, from 0 to 12 h), followed by staining with PI and DiOC₆(3). *Significant effects of NAC on the maintenance of $\Delta\Psi_m$, as compared to cells cultured in the absence of NAC.

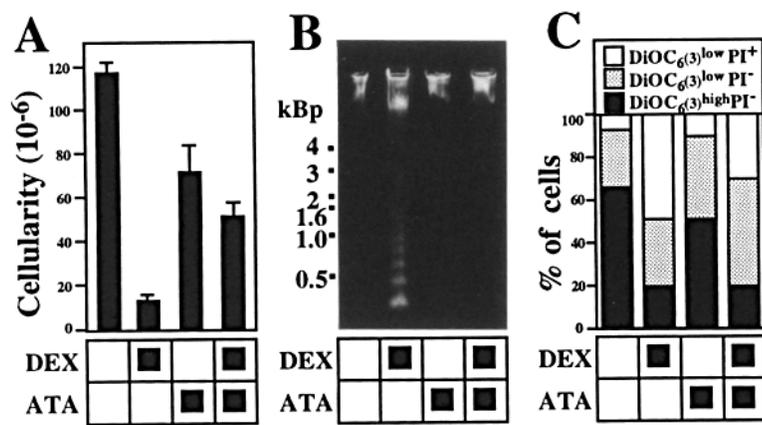


Figure 10. Effect of ATA on DEX-induced lymphocyte depletion and reduction of $\Delta\Psi_m$. (A) Effect of ATA and/or DEX on the cellularity of lymphoid organs. Animals received 1.5 mg ATA twice (4 and 2 h before DEX). 12 h after DEX (500 μ g i.p.) injection, the cellularity of the thymus was determined. (B) Assessment of DNA fragmentation on freshly isolated thymocytes. (C) Effect of ATA on $\Delta\Psi_m$. Thymocytes recovered from the animals treated with ATA and/or DEX were stained with PI and DiOC₆(3), as shown in Fig. 9.

PCD (27–29). Although mitochondrial DNA is not fragmented in apoptotic cells whose nuclear DNA has been digested by endonucleases into oligonucleosomal fragments (24, 25), ultrastructural changes in mitochondria occurring during apoptosis have been detected (30). Thymocytes undergoing DEX-induced PCD *in vitro* exhibit uncoupling of electron transport from ATP production, correlating with decreased $\Delta\Psi_m$, and similar results have been obtained in other systems of apoptosis induction (31, 32). Mitochondrial respiratory chain inhibitors such as rotenone and antimycin A, as well as the highly specific mitochondrial ATP-synthetase inhibitor oligomycin, induce PCD of many different mammalian cell types (33); inhibition of mitochondrial protein synthesis with doxycycline, as well as rotenone, render GC resistant cell lines sensitive to PCD induction by DEX (34, 35). These data argue in favor of the possibility that loss in $\Delta\Psi_m$ and concomitant breakdown of mitochondrial respiration actively participate in the apoptotic cascade. The deficit in the generation of energy-rich phosphates may be expected to ultimately cause cytolysis. However, at least at one stage of early PCD, that is represented by DEX-treated peripheral lymphocytes isolated *ex vivo* (Fig. 1), loss in $\Delta\Psi_m$ precedes loss in cell membrane potential and cytolysis.

Cell lines that lack mitochondrial DNA and, therefore, lack a functional respiratory chain are resistant to the induction of death by drugs such as rotenone, antimycin A, and oligomycin (33), yet are susceptible to the induction of apoptosis by growth factor withdrawal (36). Although these data suggest that ethidium bromide-selected cell lines relying on anaerobic glycolysis may undergo PCD in the absence of a modulation of their respiratory function, they do not exclude the possibility that, under physiological conditions, mitochondria are involved in PCD execution. In this context, it is intriguing that cells lacking mitochondrial DNA do possess mitochondria that retain important functions, including succinate dehydrogenase activity (37) and generation of a near-normal electrochemical gradient (including $\Delta\Psi_m$), that, in contrast to normal cells, depends on glycolytic ATP generation (38).

The capacity of drugs (linomide, NAC, ATA) to inhibit

lymphocyte depletion *in vivo* correlates well with their potential to stabilize $\Delta\Psi_m$. NAC is a well-established thiol antioxidant, which, after uptake, deacylation, and conversion to glutathione, functions as both a redox buffer and a reactive oxygen intermediate scavenger. NAC has been reported to prevent or postpone the death of cell lines deprived from essential growth factors (16), as well as the antigen-driven apoptosis of hybridomas (39). In accord with its limited effect on DEX-driven lymphocyte depletion, NAC partially inhibits the DEX-driven loss in $\Delta\Psi_m$. Similarly to NAC, overexpression of the protooncogene product Bcl-2, a protein that is thought to protect against apoptosis via an antioxidant pathway (16), has been shown to actively enhance $\Delta\Psi_m$ in transfected cells and prevents $\Delta\Psi_m$ loss induced by TNF- α *in vitro* (40). High expression of Bcl-2 also correlates with enhanced ATP generation by mitochondria (35). In addition, short-term exposure of cells to H₂O₂ for several minutes, causes an immediate reduction in $\Delta\Psi_m$ (data not shown). These data suggest that reactive oxygen species may be involved in the disruption of $\Delta\Psi_m$.

Although antioxidants contribute to maintain $\Delta\Psi_m$, several observations reported in this paper suggest that the accumulation and/or action of reactive oxygen species are secondary to the breakdown of $\Delta\Psi_m$. First, when $\Delta\Psi_m$ is decreased in cells recovered from DEX-treated animals, the concentration of intracellular hydroperoxide is normal (Fig. 1). Second, lipid peroxidation induced by DEX *in vitro* can be detected only after $\Delta\Psi_m$ is lost (Fig. 2E). To integrate these data, it is tempting to speculate that both events, loss in $\Delta\Psi_m$ and accumulation/action of reactive oxygen species, could result in a self-amplification loop in which disruption of the mitochondrial compartments favors the accumulation of radicals and radical-mediated damage, as well as the breakdown of respiratory metabolism, and that, together, they participate in the execution of PCD.

ATA, an agent that, among other effects, inhibits the activation of DNA endonucleases, also partially protects against DEX-induced lymphocyte depletion *in vivo*. ATA, by itself, reduces the lymphocyte $\Delta\Psi_m$, both *in vivo* (Fig. 10) and *in vitro* (not shown), and fails to inhibit the DEX-induced reduc-

tion in $\Delta\Psi_m$, but retards the cytolysis of $\Delta\Psi_m^{\text{low}}$ cells. These data are compatible with the finding that ATA maintains lymphocyte viability during in vitro short-term cultures (12), although it fails to preserve the mitochondrial function of anti-CD3-stimulated T cell hybridomas (41). The fact that ATA can inhibit DNA fragmentation without preserving lymphoid organ cellularity and $\Delta\Psi_m$ (Fig. 10), as well as the kinetic data (Fig. 2), clearly indicates that DNA fragmentation is not a primary event of PCD. In accordance with this notion, substances other than ATA that block DNA fragmentation are also incapable of providing long-term protection against cytolysis: zinc (18, 42), calcium chelators (19, 43), and enediynes (44). In contrast to ATA, an apoptosis-inhibitory agent that operates via yet unknown mechanisms, linomide (10), stabilizes $\Delta\Psi_m$ in those cell populations that are protected against DEX or superantigen-driven depletion: peripheral lymphocytes and mature (CD3^{high}) thymocytes, but not immature (CD3^{low}) T and B cell precursors from the thymus and the bone marrow. Moreover, it interferes with relatively early (zinc inhibitable) apoptosis-associated changes in the morphological appearance of splenic T cells and with the degradation of DNA into large fragments of ≥ 50 kb.

In synthesis, our data are compatible with the following sequence of catabolic PCD-associated events. After an initial

phase of commitment to PCD, which can be inhibited by linomide, growth factors, interventions on signal transduction (45), exogenous antioxidants, and Bcl-2 (40), mitochondrial function is perturbed, perhaps via a regulated process (46), or perhaps as a consequence of the opening of mitochondrial permeability transition pores or mitochondrial megachannels, accompanied by a partial, yet irreversible reduction in $\Delta\Psi_m$. Opening of such megachannels situated in the inner mitochondrial membrane has indeed been reported to contribute to the death of anoxia-damaged myocardial cells (47). As an alternative, reduced $\Delta\Psi_m$ might be secondary to alterations in glucose alterations, as they have been reported for growth factor-deprived cells (48). Reduced $\Delta\Psi_m$ marks an irreversible early event in the cascade of metabolic events accompanying PCD. In cells that already are irreversibly committed to PCD and whose $\Delta\Psi_m$ is reduced, inhibitors of endonucleolysis may still retard cytolysis. Recognition and removal of peripheral lymphocytes undergoing PCD by adjacent cells would intervene after the loss of $\Delta\Psi_m$, but before later PCD-associated changes occur. Thus, cytofluorometric measurement of $\Delta\Psi_m$ provides a tool for the ex vivo assessment of PCD. These findings have major implications for the detection, as well as the modulation, of PCD in vivo.

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