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Spontaneous Apoptosis in Human Thymocytes

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Apoptosis seems to be involved in different stages of immune cell development. In particular, experimental evidence suggests that it is a major form of cell death in the thymus. The present analysis of human thymocytes reveals that a fraction of these cells, cultured* in vitro*, undergoes spontaneous apoptosis. This observation is based both on molecular (DNA fragmentation) and morphological (electron microscopic) investigations of the cells. The apoptotic thymocytes are $CD3^-$ or $CD3^{lo}$, $CD4^{lo}$, and $CD8^{lo}$ and do not express Bcl-2 protein. Furthermore, thymocytes die by apoptosis when exposed to pharmacological stimuli, such as tumor necrosis factor-α, dexamethasone, ATP, or Ca^{++} ionophore. Thus the apoptotic machinery in thymocytes can be triggered by an imbalance in growth factors in the* in vitro *culture media and can be modulated by various biochemical signals. The process of spontaneous apoptosis is independent of mRNA or protein synthesis, as actinomycin D and cycloheximide fail to inhibit this phenomenon. Furthermore, apoptosis seems to require active oxidative phosphorylation, as it is prevented by incubation of the cells with inhibitors of the respiratory chain. (Am J Pathol 1995, 147:434–444)

Apoptosis has been proposed as the mechanism responsible for the cell death occurring in murine and human thymus.[1,2] *In vivo* studies examining the kinetics of murine immature $CD4^+CD8^+$ thymocytes indicate that a vast majority of this cell population is doomed to die.[3,4] *In vitro* experiments demonstrate that apoptosis can be triggered in these cells by exposure to low concentrations of various exogenous agents, such as glucocorticoids or γ-irradiation.[5–7] Furthermore, monoclonal antibodies to the T cell receptor complex can induce programmed cell death in immature thymocytes *in vitro*, mimicking the process of autoreactive cell deletion occurring *in vivo*.[8–9] Apoptosis also seems to be involved in antigen-induced tolerance, as thymocytes from mice transgenic for a peptide-specific T cell receptor are eliminated by apoptosis.[10]

Multiple death pathways seem to exist, which can be induced by different stimuli and can be negatively or positively regulated by two oncogene products, Bcl-2 and p53. In murine thymocytes, expression of the p53 gene is required for the programmed death triggered by agents damaging DNA but not by other signals.[11,12] In contrast, Bcl-2 protein, overexpressed in transgenic mice, makes thymocytes resistant to a wide variety of apoptotic stimuli, although it does not interfere with the process of negative selection.[13,14] It has been suggested that Bcl-2 inhibits apoptosis by functioning in an antioxidant pathway.[15] Human thymocytes have also been investigated for their apoptotic potential, although their suicide programs are not yet fully understood. They undergo apoptosis when cultured in the presence of calcium-elevating agents, anti-CD3 monoclonal antibody, or steroid hormones.[16–19] In the above studies the apoptotic process was demonstrated by searching for DNA fragmentation into oligonucleosomal-sized bands, a typical feature of programmed cell death. In some cell types, however, apoptosis is characterized primarily by morphological transformations such as cytoplasmic and nuclear condensation.[20–22] The absence of DNA cleavage is thus not a sufficient criterion to exclude the occurrence of apoptosis.

In view of these observations, we studied apoptosis in human thymocytes by analyzing both the ultrastructural and molecular changes of dying cells. Our data show that a subset of immature, Bcl-2-negative thymocytes are susceptible, when cultured *in vitro*, to

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spontaneous apoptosis, as demonstrated by DNA cleavage and by morphological transformation. This phenomenon can be modulated by different stimuli and does not depend on mRNA or protein synthesis. Apoptosis in human thymocytes requires an active oxidative phosphorylation, as it is prevented by inhibitors of the respiratory chain. It probably reflects a phenomenon occurring in a fraction of thymocytes *in vivo*. Morphological analysis of thymic tissue in fact showed apoptotic cells in the cortical area.

Materials and Methods

Antibodies and Chemicals

The anti-CD69 (MLR3) and the 8B4/20 murine monoclonal antibodies were produced in our laboratory as described.[23,24] The fluorochrome-conjugated monoclonal antibodies and goat fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin (Ig) were from Becton Dickinson (Mountain View, CA) with the exception of anti-Bcl-2 monoclonal antibody provided by Dakopatt (Glostrup, Denmark). All chemicals were from Sigma Chemical Co. (St. Louis, MO), except where otherwise indicated. Human recombinant tumor necrosis factor (rTNF)-α and interleukin-2 were provided by Genzyme (Cambridge, MA). Immobilized CD3 antibody was obtained by binding the antibody purified from ascitic fluid of mice injected with the anti-CD3 hybridoma cell line (OKT3, American Type Culture Collection, Rockville, MD) to goat anti-mouse Ig-coated magnetic beads (Dynabeads from Promega, Madison, WI).

Cell Preparation and Cultures

Normal thymic tissue was obtained from children (aged from a few days to 3 years) undergoing corrective heart surgery. Thymic cell suspensions were obtained as already described.[24] Cells were cultured at 10^6 cells/ml in 25-cm^2 cell culture flasks at 37°C in a CO_2 incubator in RPMI 1640 medium supplemented with 10% fetal calf serum (PAA, Austria), penicillin (50 IU/ml), streptomycin (50 μg/ml), and L-glutamine (2 mmol/L). Metabolic inhibitors, cytokines, and drugs were added at the doses reported in the figure legends at the onset of the cell cultures, which ended after 16 hours.

Immunomagnetic negative selection of $CD69^-$ or of $8B4/20^-$ thymocytes was carried out according to the standard procedure[25] by means of purified anti-CD69 or 8B4/20 monoclonal antibodies, and goat anti-mouse Ig-coated beads. Less than 5% of $CD69^+$ or $8B4/20^+$ cells were found in the negatively selected cell populations.

To isolate apoptotic cells, thymocytes were layered onto discontinuous Percoll gradients as described by Wyllie et al.[26] Briefly, three layers of Percoll suspensions corresponding to densities of 1.094, 1.077, and 1.063 g/cm^3, respectively, were applied onto 2 ml of Percoll (Pharmacia, Uppsala, Sweden), which had been previously adjusted to a density of 1.124 g/cm^3.[27] After centrifugation (20 minutes at 1200 × *g* at room temperature), four cell fractions were harvested from the interfaces between the layers and designated A, B, C, and D from the lowest to the highest density, respectively.

DNA Extraction, Agarose Gel Electrophoresis, and Quantitation of DNA Fragmentation

DNA cleavage was determined according to Shi et al.[28] Briefly, the cells (3×10^6 for each sample) were lysed with 0.5% Sarkosyl in 50 mmol/L Tris-HCL, 10 mmol/L EDTA, pH 8, with the addition of proteinase K (0.5 mg/ml); after 1 hour of incubation, RNAse A (0.25 mg/ml) was added to each sample. The lysates were analyzed by electrophoresis on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide; λ DNA digested with *Hin*dIII and *Eco*RI (DNA molecular weight marker III, Boehringer, Mannheim, Germany) was used as a marker for molecular size. To quantitate the degree of DNA fragmentation, the negatives of the photographs of the gels were analyzed with the LKB Ultroscan XL densitometer equipped with LKB 2400 Ultroscan software. The areas integrated under the curves were divided into high and low molecular weight DNA at the 4.9-kb marker position. The percentage of DNA fragments was computed as a percentage of low molecular weight areas related to total area under the slope.

Flow Cytometry and Cell Permeabilization

Thymocytes were stained at 4°C with the fluorochrome-labeled antibodies and analyzed with a flow cytometer (FacStar, Becton Dickinson), gating out cell fragments and debris. For detection of Bcl-2, thymocytes were fixed with 1% paraformaldehyde at 4°C for 30 minutes and permeabilized with 70% cold ethanol for 3 minutes. They were then incubated with anti-Bcl-2 monoclonal antibody followed by FITC-conjugated goat anti-mouse Ig as in the standard indirect immunofluorescence procedure.

Ultrastructural Analysis

Cell fractions harvested from Percoll gradients were spun down at 400 × *g* in microcentrifuge tubes. The cell pellets were fixed with 2.5% (v/v) glutaraldehyde in 0.2 mol/L sodium cacodylate buffer (pH 7.4) for 6 hours at 4°C, post-fixed in 2% (w/v) osmium tetroxide in cacodylate buffer for 1 hour, *en bloc* stained in 2% alcoholic uranyl acetate for 15 minutes, and embedded in epoxy resin (SPURR). Ultrathin sections were stained with uranyl acetate and lead citrate before examination with a Zeiss EM 902 electron microscope. The same procedure was used for the ultrastructural analysis of thymic tissue fragments. The apoptotic cells were identified by standard criteria.[20,29]

Measurement of Respiration Rate

O_2 consumption was assessed according to the procedure described by Nobes et al.[30] with slight modifications. Thymocytes (5×10^6 cells/ml) either freshly isolated from thymuses or harvested from the cell cultures were resuspended in respiration buffer (0.25 mol/L sucrose, 5 mmol/L succinate, 5 mmol/L glucose, 1 mmol/L $MgCl_2$, 10 mmol/L Na_2HP0_4, 0.1 mmol/L EGTA, pH 7.4) and analyzed in the Perspex incubation chamber of a Clark-type oxygen electrode (YSI 5300, Yellow Springs Instruments, Inc., Yellow Springs, OH). Respiration rates were measured over a 15-minute period.

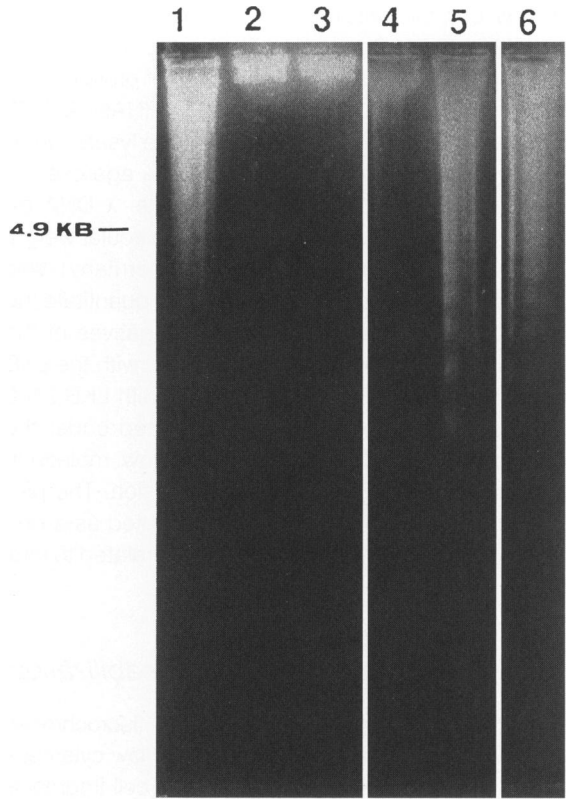

Figure 1. *Spontaneous apoptosis in human thymocytes. Thymocytes were isolated from thymuses and incubated as described in Materials and Methods at 4°C or at 37°C with or without the metabolic inhibitors indicated. DNA was extracted from equivalent numbers of cells (3×10^6 for each sample) and fragmentation was analyzed by electrophoresis on a 1.5% agarose gel. One typical experiment of three is shown: thymocytes incubated at 37°C (lane 1), freshly isolated from thymus (lane 2), incubated at 4°C (lane 3), at 37°C with 10 mmol/L sodium azide (lane 4), with 2 μg/ml actinomycin D (lane 5), and with 50 μg/ml cycloheximide (lane 6).*

Results

Stimuli Inducing Apoptosis in Human Thymocytes

When human thymocytes were cultured overnight in medium either enriched with or deficient in fetal calf serum, a percentage of cells appeared to undergo apoptosis as demonstrated by DNA fragmentation (Figure 1, lane 1, and Table 1). Different batches of fetal calf serum, or the switch from fetal calf serum to 5% bovine serum albumin, did not change the results. The phenomenon was neither evident in thymocytes freshly isolated from thymuses nor in cells incubated overnight at 4°C or at 37°C in the presence of inhibitors of oxidative phosphorylation, such as potassium cyanide or sodium azide (Figure 1, lanes 2 to 4). In contrast, actinomycin D and cycloheximide, which inhibit, respectively, RNA and protein synthesis, not only failed to prevent apoptosis but also increased DNA fragmentation (Figure 1, lanes 5 and 6, and Table 1). To extend our analysis we challenged thymocytes with a number of stimuli reported to trigger programmed cell death in murine T cell precursors, namely reagents that modify the cell membrane

Table 1. *Quantitative Analysis of DNA Fragmentation*

	Additions*	Fragmented DNA (%)†
Time 0		5.8 ± 3
4°C		3.5 ± 1
37°C	None	26.2 ± 4
	NaN_3 (10 mmol/L)	5.7 ± 4
	Cycloheximide (50 μg/ml)	37.7 ± 3
	Actinomycin D (2 μg/ml)	33.1 ± 2
	ATP (2 mmol/L)	30.4 ± 2
	A23187 (2 μmol/L)	34.2 ± 2
	TNF-α (1000 U/ml)	43.5 ± 2
	Dexamethasone (5 μmol/L)	39.3 ± 5
	Human recombinant interleukin-2 (100 U/ml)	31.2 ± 4
	Anti-CD3 (insolubilized)	27.3 ± 2

*Thymus cells were prepared and cultured alone or with metabolic inhibitors or exogenous stimuli as described in Figures 1 and 2.

†The quantitation of DNA cleavage was determined by densitometric analysis as reported in Materials and Methods. Results are expressed as the mean ± SD of three separate experiments.

permeability (ATP or calcium ionophore; see Refs. 31,32), or molecules that transduce biochemical signals to the cells (dexamethasone, TNF-α, anti-T cell receptor monoclonal antibody; see Refs. 1,2).

Both ATP and A23187 induced DNA fragmentation, as did TNF-α and dexamethasone. The magnitude of the phenomenon was increased as compared with that occurring under normal culture conditions. Human recombinant interleukin-2 also augmented the level of DNA fragmentation. In contrast, thymocytes cultured in the presence of anti-CD3 monoclonal antibody either in solution or insolubilized did not show any appreciable difference in the amount of fragmented DNA with respect to the untreated cells (Figure 2 and Table 1).

Apoptosis in Thymocyte Subsets

As the percentage of DNA fragments observed in our experiments represented a fraction of total DNA, it was conceivable that only a subset of thymocytes underwent apoptosis. In an attempt to identify this cell population, we depleted the thymocytes from mature $CD3^{hi}$, $CD4^+$, or $CD8^+$ cells by means of anti-CD69 antibody-coated magnetic beads. In fact, the CD69 molecule is constitutively expressed by mature thymocytes.[33] The remaining negatively selected population on cytofluorimetric analysis displayed a $CD3^+$ medium and low, $CD4^+CD8^+$ double-positive or $CD3^-CD4^-CD8^-$ phenotype (data not shown). DNA fragmentation was detectable in this cell subset cultured with medium alone and was increased by incubation with 5 µmol/L dexamethasone (Figure 3, lanes 4 to 6). We then enriched the thymocyte population in mature $CD3^{hi}$ cells by using the 8B4/20 monoclonal antibody. The $8B4/20^+$ population consists of immature thymocytes. In contrast, the cell fraction negative for 8B4/20 molecule is composed of $CD3^{hi}$ $CD4^+$ or $CD8^+$ thymocytes.[24] In this latter subset, no substantial DNA fragmentation was observed, even after addition of dexamethasone (Figure 3, lanes 1 to 3).

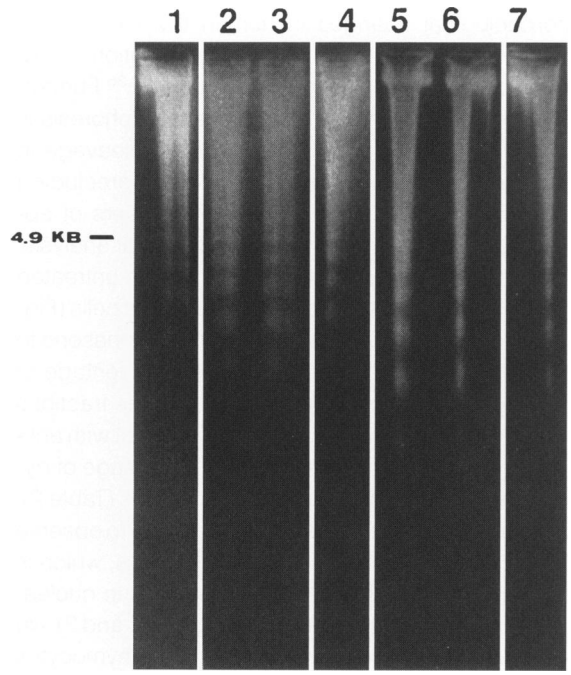

Figure 2. *Modulation of thymocyte apoptosis by various stimuli. Thymocytes were prepared and cultured as in Figure 1. DNA was extracted and analyzed by electrophoresis on a 1.5% agarose gel. One typical experiment of three is shown: thymocytes incubated at 37°C (lane 1), with 2 mmol/L ATP (lane 2), with 5 µmol/L dexamethasone (lane 3), with 2 µmol/L A23187 (Calbiochem Co., San Diego, CA) (lane 4), with 1000 U/ml human rTNF-α (lane 5), with 100 U/ml human recombinant interleukin-2 (lane 6), and with anti-CD3 monoclonal antibody insolubilized by binding to anti-mouse Ig magnetic beads (lane 7).*

Apoptotic Cells: Phenotype and Bcl-2 Expression

To characterize the phenotype of apoptotic cells, we analyzed the expression of CD3, CD4, CD8, and Bcl-2 molecules in the dense, apoptotic cells isolated from the viable ones by isopycnic centrifugation on a discontinuous Percoll gradient. Of the four fractions obtained by centrifugation of cells cultured with no

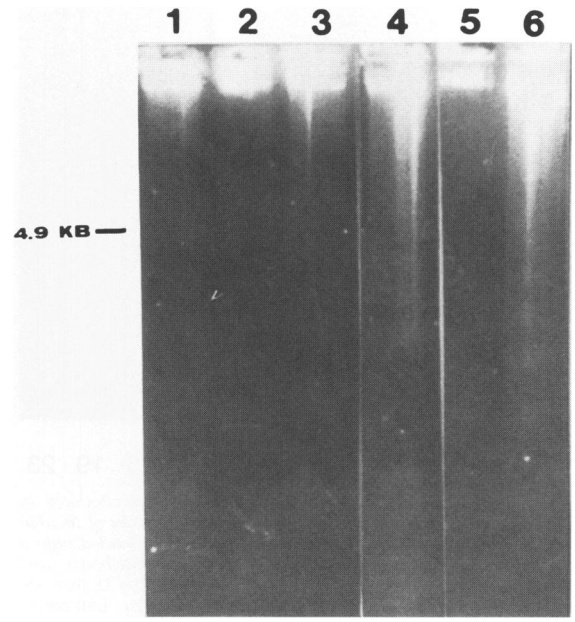

Figure 3. *Analysis of DNA fragmentation in $8B4/20^-$ or in $CD69^-$ thymocytes. Thymocytes were negatively selected with 8B4/20 (lanes 1 to 3) or anti-CD69 (lanes 4 to 6) monoclonal antibodies and goat anti-mouse Ig magnetic beads. The DNA was then extracted and its fragmentation was analyzed after 16 hours of incubation of the cells at 37°C (lanes 1 and 4), at 4°C (lanes 2 and 5), and at 37°C with 5 µmol/L dexamethasone (lanes 3 and 6).*

additional reagent, the high density ones (D and C) contained a significant percentage of cells undergoing apoptosis, as shown by DNA analysis (Figure 4, left panel). In these fractions we indeed found only 5 to 10% of the total number of cells recovered, probably because the high density condition is transient and cells do not accumulate over the apoptotic process occurring in the *in vitro* culture. An immature phenotype characterized thymocytes from C and D fractions, as they were $CD3^-$ or $CD3^{lo}$ and the double-positive cells ($CD4^+CD8^+$) showed a dim fluorescence. $CD4^+$ or $CD8^+$ single-positive cells were present in a very low percentage. Both low density fractions contained a few $CD3^-$ thymocytes, but many $CD3^{hi}$ and $CD3^+$ medium cells. The lowest density fraction (A) was enriched in single $CD4^+$ or $CD8^+$ thymocytes, whereas the majority of cells in fraction B were $CD4^+CD8^+$ double positive (Figure 5a, lower panel). Bcl-2 protein was present in a percentage of low density cells, although its expression was markedly reduced in the other cell fractions (Figure 5b, left panel).

The dexamethasone-treated cultures showed in fraction A an increase in CD4 or CD8 single-positive cells and a corresponding decrease in the double-positive subset. Bcl-2 was expressed in a higher percentage of cells with respect to fraction A from untreated cultures, probably reflecting the enrichment in single-positive thymocytes observed in this cell population after dexamethasone addition. In fact, nearly all mature single-positive thymocytes express Bcl-2.[34] The phenotype of cells from fractions B, C, and D was similar to the one found in untreated samples with regard to the CD3, CD4, and CD8 markers and Bcl-2 expression (Figure 5a, upper panel, and 5b, right panel). DNA degradation was evident in each cell population (Figure 4, right panel). Thus, dexamethasone induces cell death in thymocytes at several developmental stages. Furthermore, it likely amplifies the apoptosis phenomenon in immature thymocytes, as cells recovered from fractions C and D represented 30% of total cells harvested.

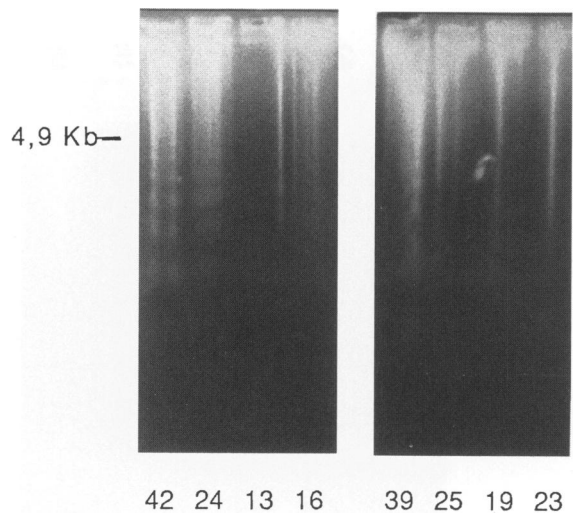

Figure 4. *Analysis of DNA cleavage in thymocytes fractionated in high and low density subsets. Thymocytes, after 16 hours of incubation with or without 5 μmol/L dexamethasone, were loaded onto a discontinuous Percoll gradient (see Materials and Methods), and identical aliquots of the different fractions (A, B, C, and D, from the lowest to the highest density) were used for DNA analysis.* Left panel: *From left to right, D, C, B, and A cell fractions from thymocytes incubated in medium alone.* Right panel: *D, C, B, and A cell fractions from thymocytes incubated with 5 μmol/L dexamethasone. The numbers reported under the lanes indicate the percentage of DNA cleavage determined as described in Materials and Methods. The results of one typical experiment of three are shown.*

Electron Microscopy Analysis of Thymocytes

The ultrastructural analysis of Percoll gradient-derived cell populations revealed high percentages of dying cells in all cell fractions. Overall, the results were consistent with the data of DNA fragmentation assays, although the numbers of apoptotic cells were higher when compared with the percentages of chromatin degradation observed in each fraction. The morphological changes in human thymocyte apoptosis likely occur before DNA fragmentation, as already shown in rat and mouse systems.[22,35] Furthermore, it is known that agarose gel electrophoresis is not sufficiently sensitive to detect DNA cleavage in relatively low numbers of cells,[36] thereby precluding a quantitative comparison with the numbers of apoptotic cells detected by the ultrastructural analysis. The high density fractions (C and D) from untreated cultures contained the majority of apoptotic cells (Figure 6 and Table 2). The addition of dexamethasone to the thymocyte cultures increased the percentage of cells with apoptotic morphology in all of the fractions from the Percoll gradient, whereas treatment with anti-CD3 antibody failed to change the percentage of dying cells observed in the untreated cultures (Table 2). In the electron micrographs it was possible to observe the different steps of the apoptotic process, which is characterized by dramatic changes both in nuclear and cytosolic structures (Figure 7, panels 1 and 2). No apoptotic morphology was evident in thymocytes treated with sodium azide (Figure 7, panel 3). To support these observations on thymocytes cultured *in vitro*, we looked for a comparable apoptotic phenomenon *in vivo*. Indeed, in thymic tissue fragments processed immediately after surgical removal, some apoptotic cells were scattered in the cortical area;

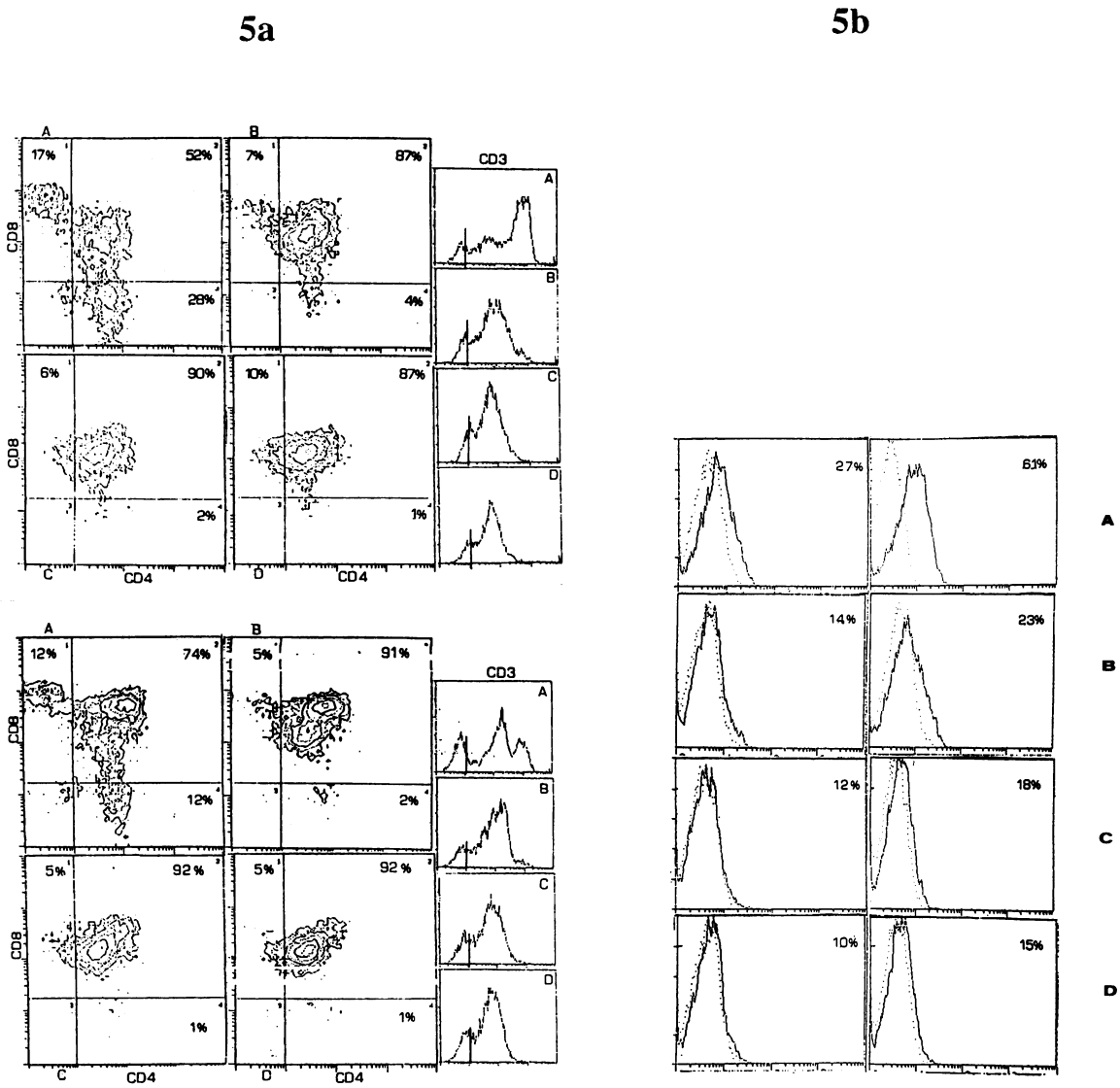

Figure 5. *Phenotype of high density and low density cell fractions. The same cell fractions analyzed for DNA cleavage (see Figure 4) were analyzed by flow cytometry for the expression of CD3, CD4, and CD8* (a) *and Bcl-2 proteins* (b). a: *Green (CD4) and red (CD8) fluorescence is displayed on the abscissa and the ordinate, respectively (4 log decade scales). Quadrant settings were determined by staining with FITC- and phycoerythrin-conjugated mouse Igs. The fraction of double- and single-positive cells is indicated in each quadrant as the percentage of total cells analyzed. CD3 expression was determined by direct immunofluorescence. The fluorescence intensity expressed in arbitrary units is shown on the abscissa of the histograms (4 log decade scales). The cursor was set on the background determined by staining the cells with FITC-conjugated mouse Igs isotype matched with anti-CD3 antibody.* Lower panel: *A, B, C, and D fractions from thymocytes incubated in medium.* Upper panel: *Fractions from thymocytes treated with dexamethasone.* b: *Expression of Bcl-2 protein in the A, B, C, and D subsets from thymocytes cultured alone* (left panels) *or in the presence of dexamethasone* (right panels). *The percentage of Bcl-2-positive cells is indicated in the upper right corner. The solid line indicates anti-Bcl-2 staining determined by indirect immunofluorescence and the dashed line indicates fluorescence in parallel samples stained with FITC-conjugated mouse Igs. The results of one typical experiment of three are shown.*

engulfment of apoptotic bodies by macrophages was also detectable (Figure 8 and Table 2).

Cell Respiration during Apoptosis

To assess the role of the respiratory chain in the apoptosis phenomenon, O_2 consumption was measured in thymocytes either freshly isolated or after 16 hours of culture in the presence or in the absence of 5 μmol/L dexamethasone. No striking difference was observed between viable cells and thymocytes undergoing apoptosis. The O_2 consumption was a result of oxidative phosphorylation, as it was prevented by 5 mmol/L KCN (Table 3). The determination of the respiratory control ratio confirmed the data described above (data not shown).

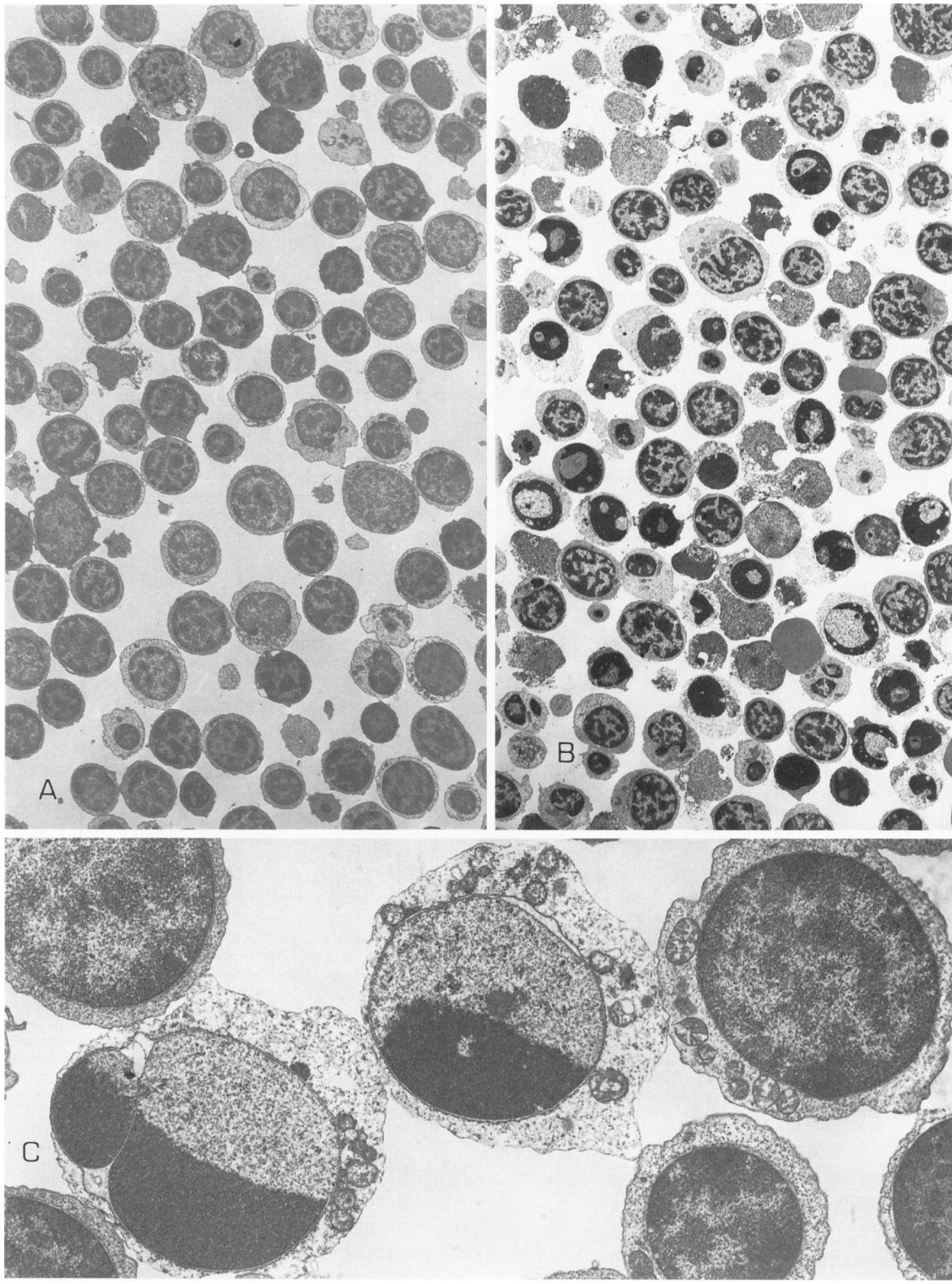

Figure 6. *Ultrastructure of thymocytes undergoing spontaneous apoptosis. Cells were incubated at 37°C in culture medium for 16 hours. Then, after fractionation onto a discontinuous Percoll gradient, they were processed for electron microscopic investigation as described in Materials and Methods.* **A:** *Cells recovered from low density* (**A**) *fraction.* **B:** *Cells recovered from pooled high density fractions* (*C and D*) (*uranyl acetate and lead citrate, ×1200*). **C:** *Morphology of two apoptotic cells from the high density fractions shown at higher magnification* (*uranyl acetate and lead citrate, ×6500*).

Table 2. *Percentage of Thymocytes with Apoptotic Morphology in* in vitro *Culture and in Thymic Tissue*

	No addition	5 μmol/L dexamethasone	Anti-CD3
Low density fraction (A)*	17 ± 5	88 ± 4	17 ± 3
High density fractions (C and D)*	67 ± 5	84 ± 9	64 ± 5
Thymic cortex†	5 ± 2		

*Cells were incubated at 37°C alone, with 5 μM dexamethasone or with anti-CD3 antibody as described in Materials and Methods, then fractionated onto Percoll gradient and processed for electron microscopy investigation as described in Figure 6. Numbers represent the percentage of apoptotic cells derived from analyzing 300 cells in the electron micrographs of each sample. The data are the mean ± SD of three separate experiments.

†Fragments of thymic tissue were processed for electron microscopy investigation as described above. The number represents the percentage ± SD of apoptotic cells observed in electron micrographs of thymic cortex examined in three separate experiments.

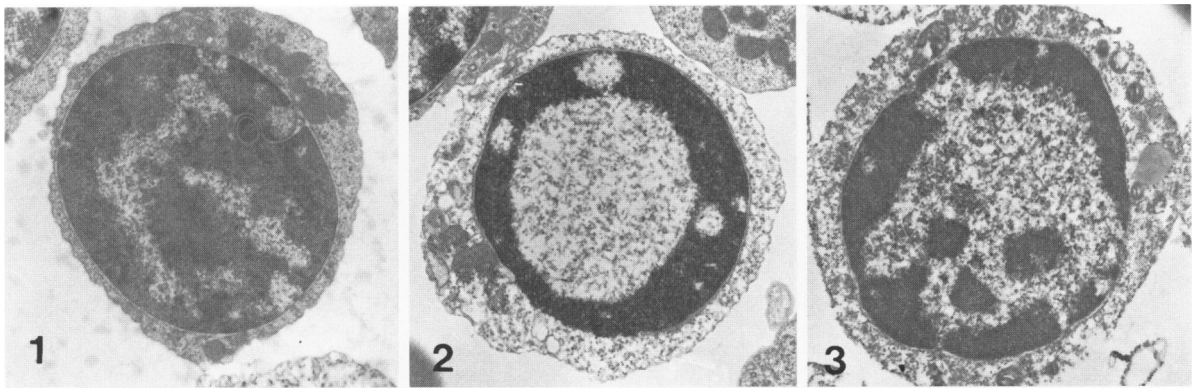

Figure 7. *Ultrastructure of dying cells.* Panel 1: *Early phase of apoptosis with invagination of the nuclear membrane.* Panel 2: *Late phase of apoptosis with condensed chromatin segregated along the margin of the nuclear membrane, vacuoles in the cytoplasm, and morphologically normal mytochondria (uranyl acetate and lead citrate, ×10,000).* Panel 3: *Morphology of a necrotic thymocyte (from thymocyte population incubated for 16 hours with 10 mmol/L sodium azide) with changes in mitochondrial shape and plasma membrane integrity (uranyl acetate and lead citrate, ×11,000).*

Discussion

Our data show that a fraction of human thymocytes undergoes spontaneous apoptosis when cultured *in vitro* for 16 hours. The capability of thymocytes to undergo apoptosis is supported by the analysis of thymic tissue, indicating that programmed death also occurs *in vivo* in cortical thymocytes. The dying cells are $CD3^-$ or $CD3^{lo}CD4^{lo}CD8^{lo}$ and express Bcl-2 protein at a very low level. They likely represent a subset of immature thymocytes. It is conceivable that the low expression of surface antigens, far from being an intrinsic property of the apoptotic cells, is a result of a loss of membrane molecules, occurring while apoptotis is under way. However, this seems unlikely as the expression of the CD1 molecule by dying cells present in high density fractions did not significantly change as compared with the cells from the same donors analyzed at the onset of *in vitro* culture (data not shown). Furthermore, the data on the $CD69^-$ and $8B4/20^-$ cell subsets support the view that a small fraction of immature T cell precursors included in the $8B4/20^+$ subpopulation is particularly susceptible to apoptosis. In agreement with this notion are the data on Bcl-2 expression by dying thymocytes (see Figure 5b).

Spontaneous apoptosis seems independent of mRNA or protein synthesis, as drugs inhibiting macromolecule synthesis not only failed to abolish the apoptotic process, but also enhanced it. It is thus conceivable that an apoptotic machinery that is already present in the cells can be triggered by external conditions such as the absence of nutrients and/or signals promoting the survival of immature thymocytes. It has been reported that in animal tissues failure of specific survival factors can lead to programmed cell death,[37,38] and apoptosis caused by a lack of growth factors has been demonstrated both in developing tissues and in growth factor-dependent cell lines.[20,38–39] Spontaneous apoptosis seems to require an active oxidative phoshorylation, as addition to the cell cultures of inhibitors of the respiratory chain apparently prevented the apoptotic phenomenon (see Figure 1). These results are at variance with the data of Jacobson et al[40,41] on the susceptibility to apoptosis of a human fibroblast cell line lacking mitochondrial DNA and on the loss of mitochondrial function in cell lines undergoing apoptosis. However, it cannot be ruled out that the metabolic pathway leading to suicide in human thymocytes differs somehow from the one operating in cell lines. The presence

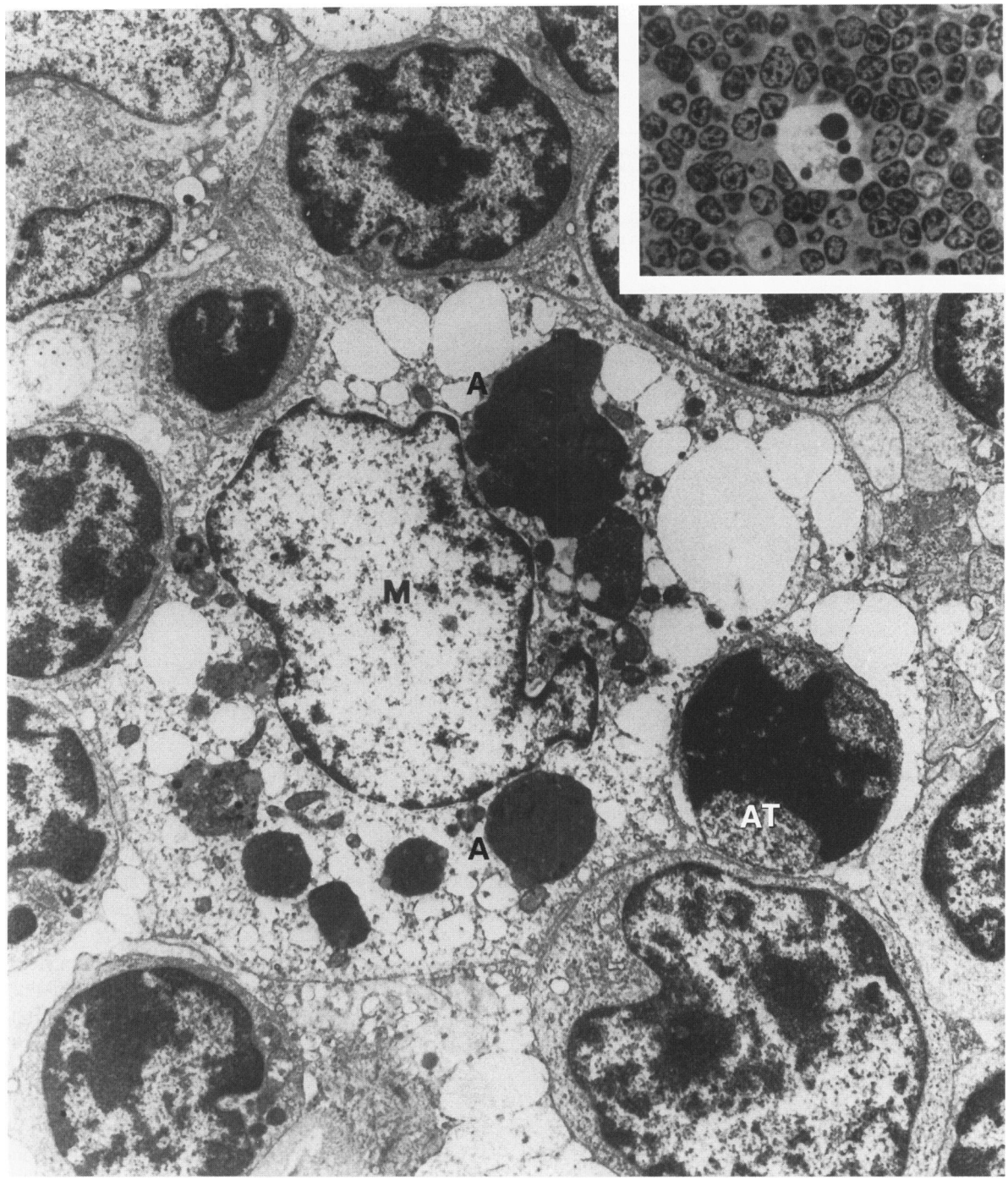

Figure 8. *Electron micrograph of a thymic macrophage (M) with engulfed apoptotic bodies (A) and in close contact with an apoptotic thymocyte (AT), distinguishable by the condensed chromatin and vacuolized cytoplasm from normal thymocytes surrounding the macrophage (uranyl acetate and lead citrate, ×8000). The inset shows a region of the cortex thymic tissue with a macrophage engulfing apoptotic bodies (0.9-μm semithin section; toluidine blue/azur II, ×1000).*

of mitochondria with apparently normal morphology and functions in apoptotic cells (see Figure 7 and the data on cell respiration) further suggests their relevance in human thymocyte apoptosis. The report by Newmeyer et al[42] on the requirement for mitochondria in a cell-free apoptotic system seems in keeping with this view, although in this latter system mitochondrial functions other than oxidative phosphorylation are probably involved in apoptosis. It is also likely that multiple death pathways exist in human T cell pre-

Table 3. *Respiration Rate of Thymocytes*

	nmol/L O_2/minute × 10^6 cells^{-1}		
	Time 0	Medium	5 μmol/L dexamethasone
Endogenous	0.38	0.35	0.42
With 5 mmol/L KCN	0.02	0.01	0.04

The O_2 consumption of thymocytes after thymus dissection or incubation at 37°C with or without 5 μmol/L dexamethasone was determined as described in Materials and Methods. The data of an experiment representative of three are shown.

cursors and that only some of them require oxidative phosphorylation. In fact, a variety of apparently unrelated signals are able to modulate thymocyte apoptosis. Similarly to murine thymocytes,[1] human thymus cells are sensitive to ATP, calcium ionophore, TNF-α, and dexamethasone. However, in contrast to the murine system, in which thymocyte apoptosis mediated by antibodies to the T cell receptor complex has been reported,[1,9] in our analysis of DNA fragmentation and ultrastructural morphology we could not see any significant enhancement of apoptosis mediated by anti-CD3 antibody, as compared with the cells cultured in medium alone. Indeed, a suicide pathway, mediated by perturbation of the CD3 T cell receptor complex, is triggered in medullary $CD1^-CD4^+/CD8^+$ single-positive human thymocytes.[18] As these subsets represent only a small fraction of thymus cells, we could not observe in the total thymocyte population a significant apoptotic effect mediated by CD3-T cell receptor cross-linking that could enhance the one caused by the culture conditions. It is also possible that in our experimental system the anti-CD3 antibody was unable to mediate a significant death signal.

In conclusion, our data indicate that a subset of immature T cell precursors when cultured *in vitro* spontaneously die by a mechanism that, according to morphological and biochemical criteria, may be defined as apoptosis. This phenomenon probably reflects the apoptosis occurring *in vivo*, as indicated by the analysis of thymic tissue; however, we failed to observe it in thymocytes freshly isolated from thymuses (see Figure 1, lane 2), probably because dying cells *in vivo* are cleared off rapidly by phagocytes (see Figure 8). Furthermore, it is likely that the procedures of organ dissection and cell separation caused an enrichment in viable cells, thereby precluding the detection of dying cells.

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