Extracellular ATP as a Trigger for Apoptosis or Programmed Cell Death

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Abstract. Extracellular ATP is shown here to induce programmed cell death (or apoptosis) in thymocytes and certain tumor cell lines. EM studies indicate that the ATP-induced death of thymocytes and susceptible tumor cells follows morphological changes usually associated with glucocorticoid-induced apoptosis of thymocytes. These changes include condensation of chromatin, blebbing of the cell surface, and breakdown of the nucleus. Cytotoxicity assays using double-labeled cells show that ATP-mediated cell lysis is accompanied by fragmentation of the target cell DNA. DNA fragmentation can be set off by ATP but not the nonhydrolysable analogue ATPγS nor other nucleoside-5'-triphosphates. ATP-induced DNA fragmentation but not ATP-induced ^{51}Cr release can be blocked in cells pretreated with inhibitors of protein or RNA synthesis or the endonuclease inhibitor, zinc; whereas pretreatment with calmidazolium, a potent calmodulin antagonist, blocks both DNA fragmentation and ^{51}Cr release. The biochemical and morphological changes caused by ATP are preceded by a rapid increase in the cytoplasmic calcium of the susceptible cell. Calcium fluxes by themselves, however, are not sufficient to cause apoptosis, as the pore-forming protein, perforin, causes cell lysis without DNA fragmentation or the morphological changes associated with apoptosis. Taken together, these results indicate that ATP can cause cell death through two independent mechanisms, one of which, requiring an active participation on the part of the cell, takes place through apoptosis.

In contrast to accidental cell death (necrosis), which occurs as a result of trauma or stress and can be regarded as a pathological response, apoptosis is a normal and deliberate process that takes place when cell death is part of an organized tissue reaction, such as can be found in embryogenesis, early postnatal life, insect metamorphosis, tissue atrophy, aging, and some cases of tumor regression (12, 19, 46). Apoptosis requires that the dying cell be metabolically active, as protein or RNA synthesis inhibitors can block the process in a variety of tissues (7, 47). In all of the cases thus far investigated, a rise in the cytoplasmic calcium concentration of the susceptible cell serves as a common, early signal for initiation of apoptosis (28).

Presently, apoptosis is known to be induced by glucocorticoids (45), low levels of γ-irradiation (35), lymphotoxin (34), tumor necrosis factor (21) and related cytotoxins (22), and several nonphysiological toxins such as 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (24). Glucocorticoids suffer from the disadvantage of having effects that are restricted to thymocytes, and tumor necrosis factor, while cytotoxic to a number of transformed cells, is not cytotoxic to all tumor cell lines and is innocuous to normal cells (37, 43). During the course of our search for physiological ligands that might induce apoptosis, it became clear that the morphological and biochemical paths taken by cells lysed by ATP closely resemble the classic description of cells undergoing apoptosis. Compared to nonphysiological apoptosis-inducing agents studied in the past (24, 35), ATP is of particular interest as it is an ubiquitous energy source that can be released from the cytoplasm of a number of cell types and can interact specifically with purinoreceptors on the surface of many different cells (18). It was also known that ATP can cause lysis of certain cells (9, 13), although the mechanisms of cell death were not characterized. Here it is shown that ATP, but not other nucleoside-5-triphosphates, causes first an abrupt increase in cytoplasmic calcium levels within susceptible cells, which is followed by cytoplasm condensation, cell blebbing, nuclear segmentation and DNA fragmentation, and ultimately culminates in cell death. This series of events can be blocked by inhibitors of macromolecular assembly, implying that ATP-induced cell death occurs through apoptosis. ATP could thus be used as a convenient trigger for apoptosis in a wide spectrum of responsive cells.

Materials and Methods

Cells and Materials

ATP, ADP, GTP, ITP, UTP, CTP, and adenosine-5'-*O*-(3-thio)triphosphate (ATPγS) were obtained from Boehringer Mannheim, Diagnostics Inc. (Indianapolis, IN), and diadenosine pentaphosphate was from Pharmacia LKB Biotechnology. Dexamethasone, calmidazolium, DRFB, emetine, and actinomycin D were from Sigma Chemical Co. (St. Louis, MO), and valinomycin was purchased from Calbiochem-Behring Corp. (La Jolla, CA).

Murine CTLL-R8 cells, originally obtained from Dr. Michael A. Palladino (Genentech, Inc., So. San Francisco, CA) and fully described elsewhere (48), were maintained in αMEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS and 10% IL 2-containing leukocyte

medium. The murine tumor cell lines, P815 (mastocytoma), Yac-1 (lymphoma), and EL4 (thymoma), were maintained as suspension culture in RPMI-1640 (Gibco Laboratories) supplemented with 5% FBS. The cells were always diluted and cultured in their log phase before experiments.

Thymus glands were aseptically isolated from 3–4-wk-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). The animals were killed, and the thymus was quickly removed and immersed in RPMI. Single cell suspensions of thymocytes were prepared by mincing the thymus with the hub of a plastic syringe and passing the cells through a steel mesh into RPMI. Cells were washed twice with RPMI, then resuspended to 2×10^7 cells/ml in RPMI supplemented with 5% FBS (HyClone Laboratories, Logan, UT) before further use.

Murine perforin was purified from CTLL-R8 cells, after modifications (29) made on a previously published protocol (30, 48). One hemolytic unit (HU) is defined as the amount of perforin required to lyse 10^7 sheep RBC in 140 μl.

Treatment with ATP and Glucocorticoid

Thymocytes were used at either 2×10^7 cells/ml (for short-term experiments) or 1×10^7 cells/ml (for 18–25-h experiments) in RPMI/5% FBS. ATP and other nucleotides were freshly prepared as 100 mM solutions in RPMI or PBS and diluted with the thymocyte suspension to the appropriate final concentrations. Dexamethasone was kept at 4°C as a 5 mM solution in ethanol and used at a 1 μM final concentration in all the experiments performed. Thymocytes treated with either nucleotides, dexamethasone, or medium (control) were incubated at 37°C in a 5% CO_2 humidified chamber. At the indicated times, cells were centrifuged, washed three times with PBS, and subjected to further processing. Murine tumor cells were treated under similar conditions, except that cells were resuspended at 5×10^6 cells/ml.

Transmission and Scanning EM

For transmission EM, cell suspensions were treated with ATP for 15, 30, 45, 90, 120, and 240 min; with glucocorticoid for 2, 4, 8, 18, and 20 h; or with 2 μg/ml cycloheximide and 1 mM ATP for 15, 30, and 90 min. These were then fixed in situ by mixing the same volume of fixative, which contained 4% glutaraldehyde, 1% paraformaldehyde, 0.1 M phosphate, pH 7.2, at 4°C for 2 h. After two washes in 0.2 M phosphate, the cell pellets were postfixed with 2% OsO_4 in the same buffer for 30 min. The pellets were washed and dehydrated in 30, 50, 70, 95, and 100% ethanol, followed by 100% propylene oxide, two times each, for 5 min. The samples were embedded in Epon 812 at 37°C overnight at 60°C for another 3 d. Ultrafine sections were cut on an ultramicrotome (model MT600-XL; Reichert Scientific Instruments, Buffalo, NY). The sections were contrasted with uranyl acetate and lead citrate for examination on a JEOL-100 EX electron microscope.

For scanning EM, cell suspensions were treated with 1 mM ATP, 5 mM ATP, or 2 μg/ml cycloheximide with 5 mM ATP for 15, 30, and 90 min. The samples were then fixed in situ for 4 h with the same fixative as described above. After fixation, 2 ml of the cell suspension was gently dropped on polylysine coated glass coverslips, and was kept in situ overnight for natural sedimentation of cells to occur on the coverslips. The sedimented cells were then washed in 0.2 M phosphate buffer and postfixed in 2% OsO_4 for 2 h. After three buffer washes, the samples were dehydrated in ascending ethanol concentrations, and transferred to isoamyl acetate. The specimens were then critical point dried using liquid CO_2 and coated with 5 nm of vacuum-evaporated gold before examination on a Philips SEM ETEC-AUDOSCAN.

Cytotoxicity and DNA Fragmentation Assays

For the cytotoxicity assay, 10^7 cells were washed in RPMI 1640 containing 5% BCS and resuspended in 400 μl of serum containing 3 mCi of ^{125}I-deoxyuridine (New England Nuclear), and incubated at 37°C for 30 min, at which point 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) were added. After another 30-min incubation, the cells were washed three times and resuspended in serum-containing medium. 5×10^4 labeled cells were added per well in triplicate to round bottom microtiter wells and mixed with the appropriate concentration of nucleotide in a final volume of 100 μl. When inhibitors were used, the cells were incubated for 30 min in the presence of the inhibitors before adding ATP. After incubation at 37°C for 3 h, 100 μl of medium were added to each well and centrifuged at 200 *g* for 5 min, and 70 μl of supernatant was collected from each well for determination of radioactivity in a gamma counter. For quantification of DNA fragmentation, the plates were incubated for 6 h at 37°C and 100 μl of 2× lysis buffer (20 mM Tris, pH 7.4, 4 mM EDTA, 0.4% Triton X-100) were added. The cells were incubated for 15 min at room temperature, then shaken and centrifuged at 800 *g* for 5 min. The radioactivity in 70 μl of the supernatant was determined. Spontaneous release was considered as the release in cells incubated with medium or, in the case of inhibitors, of cells incubated in medium with the inhibitor but without ATP. In all cases, spontaneous release was <15% for ^{51}Cr release and 10% for ^{125}I release. The counts of cells lysed with 1% SDS were used as total release. The percent of specific ^{51}Cr or ^{125}I release was determined as % specific release = (experimental release − spontaneous release)/(total release − spontaneous release) × 100 (3). All experiments were repeated at least three separate times.

For DNA gels, 10^5 cells were incubated in the indicated conditions in 400 μl of medium and incubated for 5 h. After adding lysis buffer, the cells were centrifuged at 13,000 *g* for 5 min. The supernatant was precipitated first with 0.5 M NaCl and isopropanol and then washed with 70% ethanol. The DNA was resuspended in TE (10 mM Tris, 1 mM EDTA), treated with RNAse, and run on 0.8% agarose gels containing ethidium bromide and visualized under ultraviolet light. For ^{125}I-deoxyuridine-labeled cells, the DNA was transferred by capillary action onto a nylon membrane (Micro Separation Inc., Westboro, MA) overnight. The membrane was air dried and exposed to an x-ray film (X-OMAT, Kodak). 1-kb ladder (Bethesda Research Laboratory, Gaithersburg, MD) was used as relative molecular mass standard.

Calcium Flux Measurements

Thymocyte, EL4, Yac-1, P815, or CTLL-R8 were incubated at $2\text{–}5 \times 10^6$ cells/ml at 37°C for 30 min with fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes Inc., Eugene, OR). The fura-2 AM solution had originally been prepared by premixing 5 μl of a 1 mM fura-2 AM stock solution with 2.5 μl of 20% Pluronic F-127 (Molecular Probes Inc.) in DMSO, to which 75 μl FCS was added. This mixture was diluted with the final concentration of cells in PBS, pH 7.6, containing 1 mM Mg^{2+} and 1 mM Ca^{2+}, to give 1 μM fura-2 AM. The cells were washed three times in the same buffer at room temperature to remove extracellular fura-2 AM, and then resuspended at $\sim 5 \times 10^7$ cells/ml. These cells were then diluted 100-fold directly into quartz cuvettes in a spectrofluorometer (model SLM 500C; Aminco) equipped with magnetic stirrer for calcium concentration measurements. The excitation/emission wavelength pair used for continuous monitor of the dye fluorescence was 335/510 nm. The internal calcium concentration was calculated as previously described (20).

ATP, ATP analogues, or perforin were diluted into the cuvette from stock suspensions. 10 min after the addition of ATP, 20 mM EGTA was added to the cuvette to verify that the increases in fura-2 fluorescence were not due to dye leakage. For the experiments with $ZnSO_4$, a 100 mM $ZnSO_4$ solution was diluted a 100-fold into the cuvette before addition of cells. Treatment of cells with calmidazolium was done during the 30 min fura-2 incubation step at 37°C.

Results

Morphological Changes Due to ATP and Glucocorticoids

Addition of 0.5–5 mM ATP to thymocytes induces the morphological changes characteristic of apoptosis (46), as gauged by transmission EM. Untreated thymocytes have relatively thin cytoplasms, with most of the cell volume being occupied by the nucleus. The chromatin in the nucleus is heterogenously distributed, and the Golgi complex and the mitochondria tend to be localized in one pole of the cell. Within 30 min of ATP treatment, ~10% of the cells already have distinctly shrunken volumes. Surface microvilli disappear; with the exception of the mitochondria, which still appear normal, the cytoplasmic organelles become compacted together; protrusions known as blebs have already formed on the plasma membrane, and the nucleus is often displaced to one edge of the cell; and the chromatin aggregates into a dense mass with no discernible fine structure. Progression

of this process leads to crowding of the organelles, more widespread blebbing of the plasma membrane, and complete condensation of the chromatin, which at this stage is also beginning to fragment. The surface blebs eventually separate, forming apoptotic bodies. Within 4 h after steroid treatment, 70% of the thymocytes display fully fragmented nuclei. Nucleoside-5′-triphosphates other than ATP were unable to bring about these morphological changes.

Fig. 1 *a* shows thymocytes in several stages of apoptosis. The thymocyte in the center of the figure shows extensive blebbing and condensation of the nucleus. Fragmentation of the nucleus and an apoptotic body are observed at the bottom of the figure. In Fig. 1, *b* and *c*, a normal cell (Fig. 1 *b*) is contrasted with a cell already undergoing blebbing but no noticeable nuclear condensation (Fig. 1 *c*). Progression of apoptosis leads to a condensed nucleus (Fig. 1 *d*) and culminates in nuclear fragmentation (Fig. 1 *e*) and cell death (Fig. 1 *f*). "Boiling" of the cytoplasm always starts on one side of the cell. Similar ATP-induced morphological changes were observed with the murine thymoma cell line, EL4 (data not shown).

Incubation of thymocytes with 1 μM glucocorticoid leads to ultrastructural changes similar to those seen with ATP, except that surface blebbing is not as prominent (Fig. 1, *g–i*). The kinetics of the changes are the same as for ATP, and, likewise, there is marked heterogeneity in the cells undergoing the various stages of apoptosis at any given time (Fig. 1 *g*).

Scanning EM was used to observe the thymocyte surface changes due to ATP treatment. As seen in Fig. 2, microvilli disappear from the cell surface 1 h after addition of 1 mM ATP, and there is extensive surface blebbing.

At lower ATP concentrations, a smaller fraction of cells undergoes apoptosis, although, for the dying cells, the morphological changes are always those described in Fig. 1, *c–f* for thymocytes. Apoptosis could be elicited with 1 mM ATP, but not with the same concentration of either ADP, GTP, CTP, or UTP (data not shown). In addition, the morphological changes associated with ATP-induced apoptosis could be effectively blocked by the protein synthesis inhibitor, cycloheximide (Fig. 1 *j*).

ATP-mediated Cytolysis and DNA Fragmentation

Treatment of cells with ATP leads to cell death as determined by at least two criteria, release of ^{51}Cr-labeled proteins from the cells and fragmentation of the cell's DNA. Fig. 3 *A* shows the effect of ATP, ADP, and five other nucleoside-5′-triphosphates on lysis of the tumor cell line, EL4. By far the most effective compound was ATP, with about half of the cells being lysed after incubation of EL4 with 1 mM ATP for 6 h at 37°C. Next in potency, 1 mM GTP lysed 22% of the cells, followed by 1 mM UTP, which lysed 15%, and 1 mM CTP, which lysed 8%. Both ADP and the nonhydrolysable ATPγS were about equally effective, with each lysing ~15% of the cells. Incubation with 2 mM ATPγS resulted in 32% lysis (data not shown). Although the magnitude and time course of cell lysis depended very much on the cell type, the hierarchy of effects observed with the different nucleotides was qualitatively similar for all susceptible cells tested (data not shown).

The difference between ATP and the ATP analogues and other nucleotides was much more pronounced when DNA fragmentation was measured (Fig. 3 *B*). More than one-third of the DNA was cleaved after a 6-h incubation of EL4 cells with 1 mM ATP. In contrast, the same concentration of ADP, ATPγS, CTP, GTP, ITP, and UTP was totally ineffective. Increasing the ATPγS to 2 mM was not sufficient to cause DNA fragmentation.

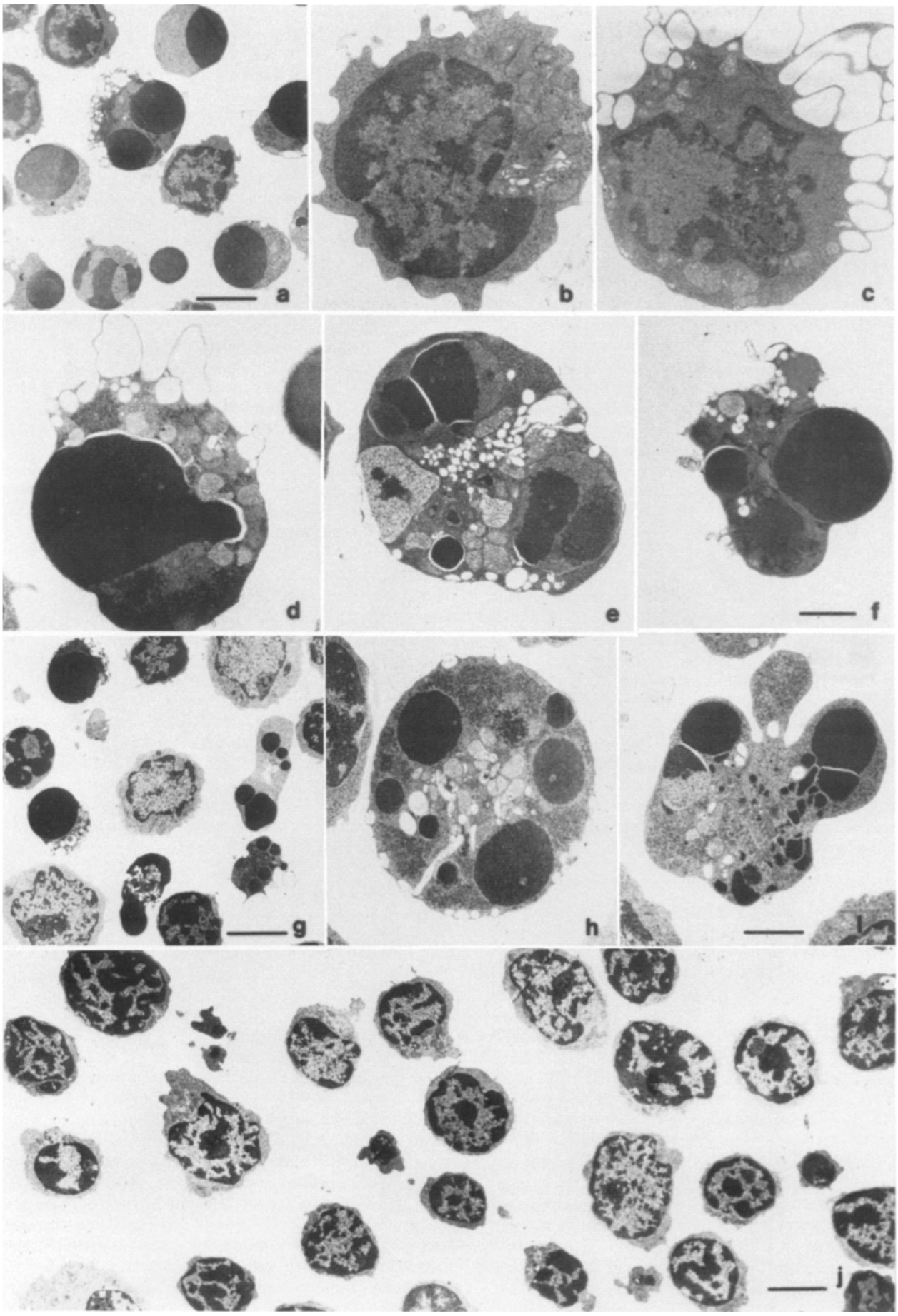
The concentration dependence for ATP-induced cytotoxicity and DNA fragmentation in EL4 cells is shown in Fig. 3, *C* and *D*. There was a gradual increase in ^{51}Cr release which reached a plateau at ~2 mM ATP, with the half-maximal effect being at slightly over 1 mM ATP (Fig. 3 *C*). The DNA fragmentation was more sensitive to ATP concentration, being greatest at 0.5 mM ATP but then decreasing towards higher ATP concentrations (Fig. 3 *D*). It should be noted that these results were also a function of the cell type; for example, the ATP concentration required for maximal DNA fragmentation in thymocytes was >1 mM (data not shown).

It has previously been reported that ATP^{4-} is the active agent responsible for cation fluxes triggered by ATP in certain cell lines (39, 40). Accordingly, lysis of EL4 due to ATP was measured in the presence and absence of magnesium (Table I). ATP-induced cytolysis was significantly enhanced in the absence of magnesium, implying that cytolysis is due to the tetrabasic species of ATP.

As actinomycin D can block the morphological changes of apoptosis in thymocytes, EL4 cells were preincubated with the RNA synthesis inhibitor before treatment with ATP. Although only marginal effects were observed in the ability of actinomycin D to inhibit ^{51}Cr release (Fig. 3 *E*), actinomycin D completely blocked DNA fragmentation due to ATP (Fig. 3 *E*), as measured by the ^{125}I-labeling method. Likewise, 125 μM of the RNA synthesis inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRFB) (35), or 1 mM of the endonuclease inhibitor, Zn^{2+}, blocked DNA fragmentation but not ^{51}Cr release. On the other hand, pretreatment with the calmodulin antagonist, calmidazolium (also known as R24571) (16), resulted in complete suppression of both ^{51}Cr release and DNA fragmentation.

The effect on DNA fragmentation was visualized qualitatively by migrating the DNA from EL4 cells and thymocytes on agarose gels. After incubation with 1 mM ATP, the DNA of both thymocytes (Fig. 4 *A*, lane *2*) and EL4 cells (Fig. 4 *B*, lane *2*) was degraded into discrete multiples of ~200 bp. This pattern was not seen when the cells were incubated with 1 mM ATPγS (Fig. 4 *B*, lane *3*), and both actinomycin D (Fig. 4 *B*, lane *4*) and Zn^{2+} (lane *5*) blocked the fragmentation due to ATP. The potassium ionophore, valinomycin, used as a positive control (1, 11), causes DNA fragmentation in a stepladder fashion (Fig. 4 *B*, lane *6*). Since ATP-induced DNA fragmentation, as assayed by the ^{125}I-labeling method, decreases at higher ATP concentrations (Fig. 3 *D*), a DNA sample from EL4 cells treated with 2 mM ATP was also migrated on a gel (Fig. 4 *B*, lane *7*). Consistent with the results shown in Fig. 3 *D*, there is a marked decrease in the amount of DNA fragmentation due to 2 mM ATP.

A number of cell lines was next screened for their ability to partially or fully resist ATP-mediated lysis. Variable results were obtained. Whereas many cell lines respond markedly to ATP, it was found, for example, that ATP concentrations as high as 5 mM ATP result in only 15% specific ^{51}Cr release from the mastocytoma cell line, P815, and that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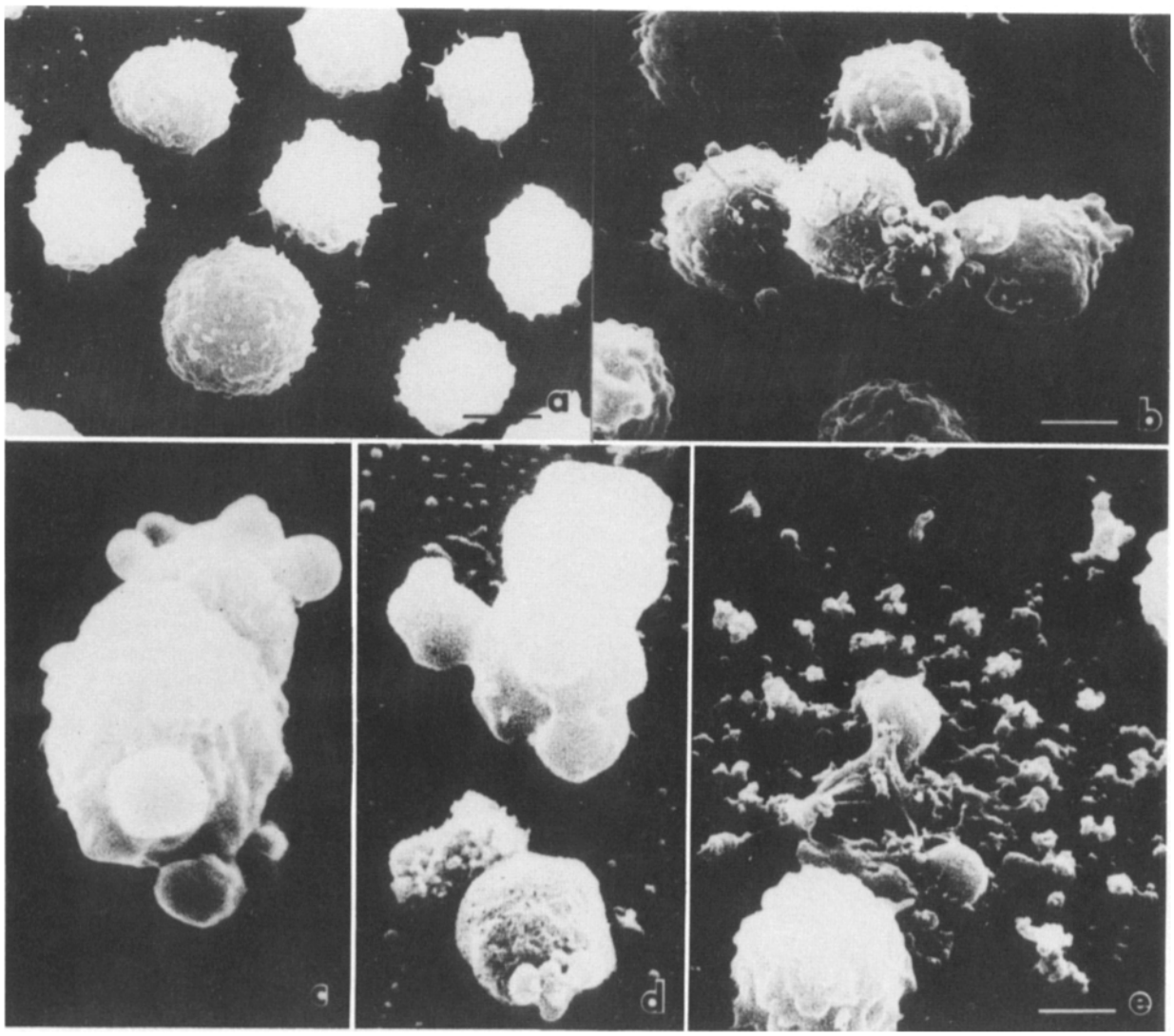

Figure 2. Scanning EM of thymocytes undergoing apoptosis due to treatment with ATP. (*a*) A representative view of normal thymocytes. (*b*) A high proportion of the cells show signs of apoptosis after a 60-min treatment with 1 mM ATP. (*c*) High magnification of a thymocyte undergoing apoptosis, showing extensive surface blebbing. (*d*) Dead cell next to an apoptotic body. (*e*) Final cell lysis. After a 90-min treatment with 1 mM ATP, cells are variously found at stages (*c*, *d*, and *e*). Bars: 5 μm (*a*); 4 μm (*b*); 3 μm (*c–e*).

the lymphoma cells, Yac-1, and the cytotoxic T lymphocytes, CTLL-R8, are refractory to lysis due to ATP (data not shown).

Calcium Fluxes Caused by ATP

In all the cases studied, agents that cause apoptosis also trigger a rapid, sustained increase in the internal calcium concentration, $[Ca^{2+}]_i$, of the cell. To determine whether there is any correlation between the effects of ATP on the morphological changes of cells and on an initial calcium flux, thymocytes were loaded with the calcium-specific fluorescent dye fura-2 (20). In a buffer containing 1 mM Mg^{2+} and 1 mM Ca^{2+}, addition of 1 mM ATP results in an almost immediate increase of $[Ca^{2+}]_i$. Neither ITP, ADP, CTP, GTP, diadenosine pentaphosphate, nor UTP at 1 mM were able to elicit the same response. The nonhydrolysable analogue ATPγS

Figure 1. Comparison of ultrastructural characteristics of thymocyte apoptosis induced by ATP and glucocorticoids. (*a*) A representative view of thymocytes treated with 1 mM ATP for 90 min, showing most of the cells undergoing apoptosis. (*b*) A normal thymocyte. (*c*) Beginning stages of apoptosis, showing surface blebbing; thymocytes at this stage are first seen after 30-min treatment with 1 mM ATP. Dying thymocytes showing (*d*) nuclear condensation and (*e*) nuclear fragmentation due to 1 mM ATP; cells at these stages are first observed after 45- and 90-min treatment, respectively. (*f*) Final stage of apoptosis, usually first seen after a 120-min treatment with 1 mM ATP. (*g*) A representative view of thymocytes treated with 1 μM glucocorticoid for 18 h. (*h* and *i*) Dying thymocytes showing nuclear condensation at different stages; cells incubated with 1 μM glucocorticoid are usually first seen at these two stages after 8- and 18-h treatment, respectively. (*j*) Lack of effect of 1 mM ATP on thymocytes that had been pretreated for 90 min with 2 μg/ml cycloheximide. Bars: 5 μm (*a*, *g*, and *j*); 2 μm (*b–f* and *h* and *i*).

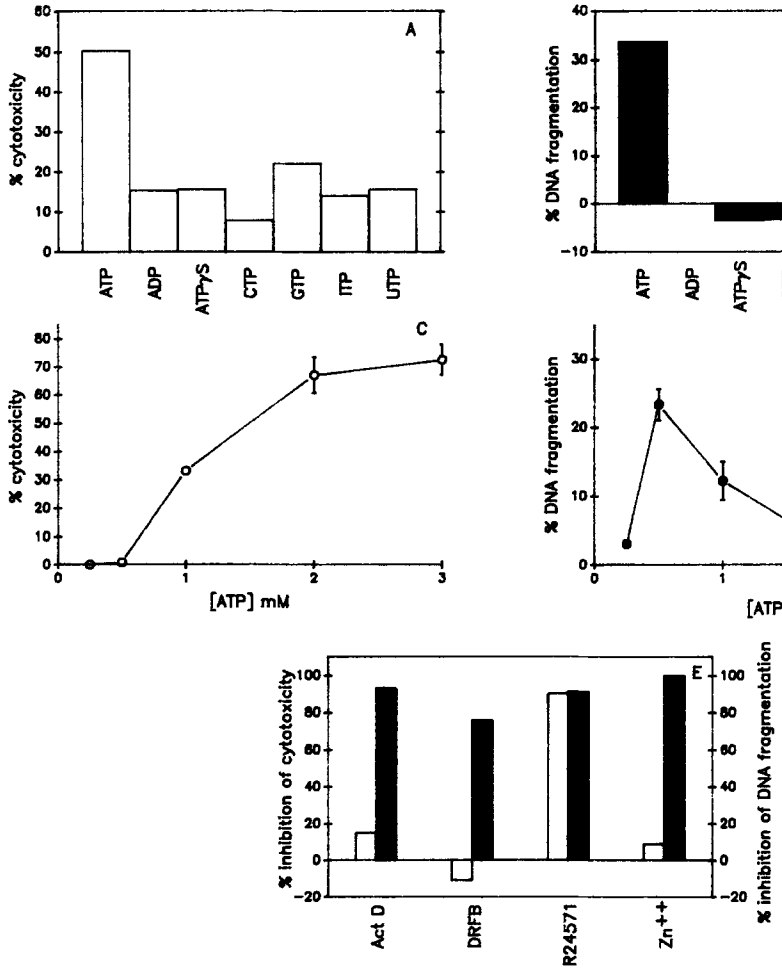

Figure 3. Effect of ATP, ATP analogues, other nucleotides, or ATP with inhibitors on double-labeled EL4 cells. Percent specific cytotoxicity and DNA fragmentation of ^{51}Cr- and [^{125}I]dU-labeled EL4 cells was determined as described in Materials and Methods. (*A*) ^{51}Cr release from EL4 cells incubated with ATP or the indicated analogue at a final concentration of 0.5 mM. (*B*) DNA fragmentation in an experiment parallel to that in *A*. (*C*) Dose dependence of the effect of ATP on specific cytotoxicity. (*D*) Dose dependence of the effect of ATP on DNA fragmentation in the same EL4 cells as used in *C*. (*E*) Effect of pretreatment with the indicated inhibitors on cytotoxicity (*open bars*) and DNA fragmentation (*solid bars*) due to 1 mM ATP. The results are plotted as percent inhibition of the cytotoxicity or DNA fragmentation that would have taken place in the absence of inhibitors. The final concentrations of the inhibitors were: actinomycin D, 5 μg/ml; DRFB, 125 μM; R24571 (calmidazolium), 6.25 mM; and $ZnSO_4$, 500 μM.

was also ineffective at this concentration, but at 2 mM gave a response similar to that of 1 mM ATP. Dose–response measurements indicate that most of the calcium flux can be evoked with ATP at a concentration of 0.5 mM (Fig. 5 *a*). Although a sustained response was also achieved with EL4 and Yac-1 cells, with $[Ca^{2+}]_i$ increasing from near 100 nM to values between 200 nM and 1 μM (data not shown), 1 mM ATP triggered a transient response in P815 cells. In the latter cells, $[Ca^{2+}]_i$ increased from ~100 to 500 nM within 15 s, but returned to above the basal level (300 nM) after ~2 min (Fig. 5 *b*). Unlike the above mentioned cells, however, 1 mM ATP was unable to elicit any $[Ca^{2+}]_i$ changes in CTLL-R8 cells (data not shown). Neither $ZnSO_4$ nor calmidazolium were able to suppress the ATP-induced $[Ca^{2+}]_i$ changes in responsive cells.

*Table I. Effect of Magnesium on ATP-induced Cytolysis**

ATP conc.	Percent specific ^{51}Cr release	
	+ 1 mM Mg^{++}	No Mg^{++}
mM		
0	0	0
0.25	7.4 ± 2.9	66.2 ± 7.6
0.5	43.3 ± 8.5	78.4 ± 10.5
1.0	72.8 ± 7.5	88.3 ± 9.5
1.5	82.3 ± 12.9	90.2 ± 13.6

* EL4 cells were treated with ATP with or without 1 mM $MgCl_2$. Results represent the average from two separate experiments performed in triplicate.

Cytolysis Due to Pore-forming Proteins

The morphological changes taking place during cell lysis were studied for thymocytes treated with perforin, the pore-forming protein from cytolytic lymphocytes (48). When thymocytes are treated with 33 HU/ml perforin, there is soon blebbing of the cell surface, dilation of the ER, expansion of the mitochondrial volume accompanied by disruption of its structure, and flocculation of the nuclear chromatin (Fig. 6). The chromatin eventually disappears, and the cells swell and many are observed to burst. In contrast to thymocytes undergoing apoptosis, there is no condensation of the nuclear chromatin nor later breakdown of the nucleus. These features of perforin-induced lysis are characteristic of those produced by necrosis.

In agreement with a previous report (11), DNA from cells treated with perforin (Fig. 4 *a*, lane *4*) is indistinguishable from the control (lane *1*).

Perforin, at the same concentration as used to induce necrosis in thymocytes, gives rise to an almost instantaneous calcium flux, which saturated the fura-2 signal (data not shown). Perforin produces a passageway for ions through the cell plasma membrane, causing salts and, soon afterwards,

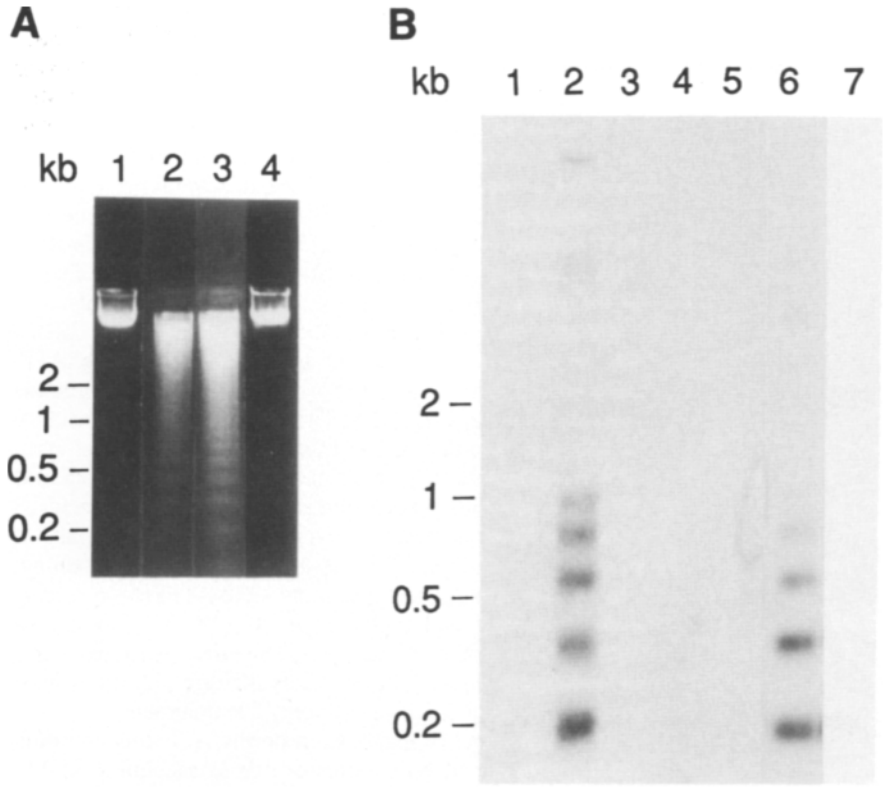

Figure 4. DNA fragmentation due to ATP treatment. (*A*) Thymocytes were incubated with the different agents for 6 h. Fragmented DNA and intact chromatin were separated and precipitated overnight as described in Materials and Methods. The DNA was resolved on a 0.8% agarose gel and stained with ethidium bromide. Lane *1*, untreated cells; lane *2*, ATP-treated cells; lane *3*; glucocorticoid-treated cells; and lane *4*, perforin-treated cells. (*B*) [^{125}I]dU-labeled EL4 cells were pretreated with the indicated inhibitors and incubated with ATP for 6 h. The fragmented DNA was separated from intact chromatin and precipitated overnight as described in Materials and Methods. The DNA was run on a 0.8% agarose gel and transferred to a nylon membrane. The autoradiogram of this membrane is shown. The cells were treated as follows: lane *1*, control; lane *2*, 1 mM ATP; lane *3*, 2 mM ATPγS; lane *4*, 1 mM ATP after pretreatment with 5 μg/ml actinomycin D; lane *5*, 1 mM ATP after pretreatment with 500 μM Zn^{++}; lane *6*, 100 μM valinomycin; and lane *7*, 2 mM ATP. Molecular weights are shown.

water to move into the cell. This leads ultimately to the swelling observed under EM.

Discussion

Apoptosis is a widespread phenomenon that plays a crucial role in embryogenesis and other developmental processes where a concerted removal of cells must take place. While apoptosis can be induced by glucocorticoids and a number of different nonphysiological toxins, the metabolic pathways common to the cell death due to these various agents are not fully understood. With the view of providing a physiological ligand that might be conveniently exploited as a trigger of apoptosis, this work presents a morphological and biochemical characterization of cell death caused by extracellular ATP.

The most characteristic traits of apoptosis are condensation of the cytoplasm and nuclear chromatin, segmentation of the nucleus, and extensive membrane blebbing (46). All of these morphological features are observed in thymocytes and EL4 cells during ATP-induced cell lysis. Moreover, apoptosis is caused specifically by ATP, as neither the nonhydrolysable analogue ATPγS nor other nucleoside-5′-triphosphates are able to produce this series of events. The contrasting mode of cell death is exemplified in the morphological changes brought about by complement or perforin, which are caused initially by major structural damage to the plasma membrane and thus result primarily from a loss of the cell's capacity for homeostasis.

Although a rigorous set of biochemical criteria for apoptosis has yet to be established, a requirement for an active metabolic participation on the part of the dying cell has usually been observed (6, 31, 41). During the development of the nervous system, for instance, large numbers of cells die before the completion of differentiation (27), and it has been shown with neurons grown in vitro that death of cells due to nerve growth factor deprivation is prevented by inhibitors of protein and RNA/DNA synthesis (23). However, the most reliable marker for apoptosis is endonuclease activation, which causes DNA fragmentation into integer multiples of ~200 bp. The classic example for this type of degradation is found in the apoptotic thymocyte after glucocorticoid treatment (45).

The morphological changes associated with ATP-induced apoptosis are blocked in thymocytes by the inhibitors of macromolecular assembly, cycloheximide and actinomycin D. Likewise, ATP-mediated DNA fragmentation in EL4 cells is suppressed by inhibitors of transcription as well as by zinc, an endonuclease inhibitor (6, 8, 10). The extent of fragmentation is not directly proportional to the ATP concentration, however, as the fragmentation decreases when the ATP concentration is increased beyond 0.5 mM. On the other hand, specific release of ^{51}Cr-labeled protein, a nonspecific indicator for cell lysis, increases monotonously with ATP concentration. ATP thus appears to cause both apoptotic and necrotic death in EL4 cells, and this interpretation is supported by the observation that DRFB and actinomycin D have only a minor effect on ^{51}Cr release. In contrast to the synthesis inhibitors, the potent calmodulin antagonist, calmidazolium (16), inhibits both DNA fragmentation and ^{51}Cr release. Since higher ATP concentrations lead to lower levels of apoptosis but higher amounts of nonspecific ^{51}Cr release, it suggests that the extent of apoptosis or necrosis induced may depend on the severity of the assault with ATP.

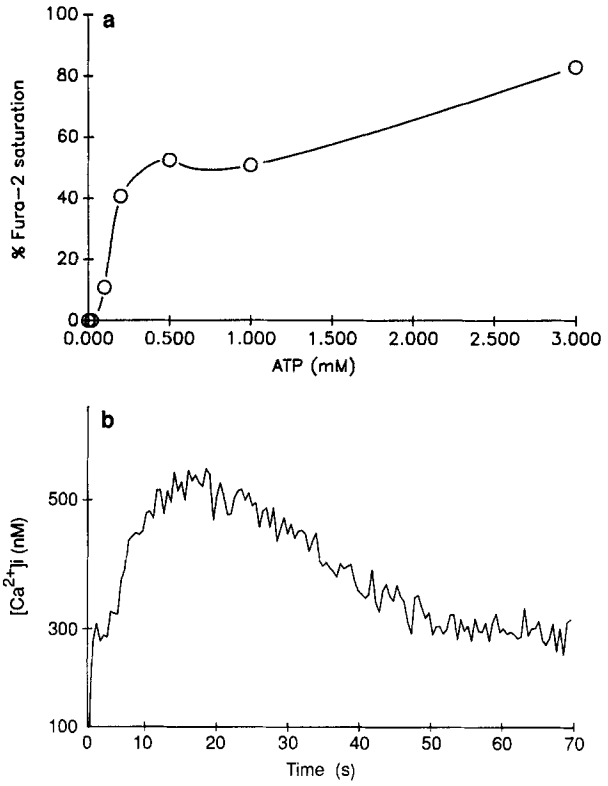

Figure 5. ATP-induced calcium fluxes in thymocytes and P815 cells. The internal calcium concentration was measured with fura-2 as described in Materials and Methods. (*a*) Concentration dependence of ATP-mediated calcium fluxes in thymocytes. Data points were taken at 10 s after addition of the indicated concentrations of ATP. (*b*) Transient calcium concentration change in P815 cells due to 1 mM ATP.

To address the question of what role the cytoplasmic concentration, $[Ca^{2+}]_i$, may play in triggering ATP-mediated DNA fragmentation and ^{51}Cr release, cells were loaded with the calcium-sensitive dye, fura-2 (20). ATP, but not other nucleoside-5-triphosphates, was able to stimulate a sustained $[Ca^{2+}]_i$ increase in thymocytes, EL4 cells and Yac-1 cells, and a transient increase in P815 cells. Although it has been proposed that calcium fluxes represent an obligatory prerequisite for glucocorticoid-induced DNA fragmentation in thymocytes (6, 25) and toxin-induced cell death in a number of cell types (24, 28, 33, 36), our results suggest that a rise in $[Ca^{2+}]_i$ is by itself an insufficient condition for DNA fragmentation, since Yac-1 cells, unlike the highly susceptible thymocytes and EL4 cells and the mildly susceptible P815 cells, are fully resistant to the cytotoxic effects of ATP. This conclusion is reinforced by the observation that perforin, which causes larger calcium fluxes than ATP, is unable to bring about either the morphological changes related to apoptosis or DNA fragmentation in any of the cell types tested.

In normally proliferating cells, $[Ca^{2+}]_i$ is rigorously maintained at ~100 nM against an extracellular calcium concentration of >1 mM by calcium-transporting ATPases on the plasma membrane (5). It is thought that the immediate effect of many cytotoxins is to collapse the calcium gradient across the plasma membrane, and that the internucleosomal DNA cleavage typical of apoptosis depends on the synthesis or activation of a calcium-activated endonuclease. Inasmuch as initial $[Ca^{2+}]_i$ increases have been observed in every case where apoptosis subsequently occurred, our results with ATP-mediated calcium fluxes in Yac-1 cells and perforin-mediated calcium fluxes in thymocytes and EL4 cells indicate that a rise in $[Ca^{2+}]_i$ is only one of several prerequisites for apoptosis to take place. The varying responsiveness to ATP-induced apoptosis of the cell lines tested in this work should provide experimental clues as to the identity of some of the additional endonuclease-activating components.

Besides Yac-1 cells, the cytotoxic T lymphocytes, CRLL-R8, were resistant to either apoptotic or necrotic ATP-induced cell death. Moreover, ATP was unable to elicit any calcium fluxes in CTLL-R8. Thus, their mode of resistance is different from that of Yac-1 cells. The resistance of cytolytic lymphocytes to the cytotoxic effects of 20 μM to 5 mM ATP had previously been observed (9, 13), and this resistance has been ascribed to ecto-ATPases on the surface of cytolytic lymphocytes (13). Following the same reasoning, one would also expect that any ecto-ATPase-expressing cells, including endothelial cells (17, 18, 32), should be refractory to ATP-mediated lysis. The most instructive cases to investigate would be those cells that are able to at least give rise to $[Ca^{2+}]_i$ changes upon ATP treatment.

The large effect of magnesium on the ATP concentration dependence of cell lysis indicates that extracellular ATP in its tetra-anionic form (ATP^{4-}) causes cell death. In this respect, our results appear related to a previous report (4) that extracellular ATP^{4-}, through ligation of a plasma membrane receptor for ATP^{4-}, induces a large nonselective conductance in a macrophage-like cell line and in resident macrophages. Yet it is safe to assume that ATP does not cause apoptosis through pore-formation alone, since treatment with perforin, which creates nonselective pores with diameters of up to 20 nm (30, 48), results in cell death but not apoptosis. It will be interesting to determine what additional effects of ATP give rise to the cellular phenomena observed in this work.

In the body, ATP is known to be released during platelet thrombus formation (15), catecholamine release from the adrenal medulla (44), shock (42), and strenuous exercise (14). In addition, extracellular ATP plays an important role in many physiological processes. In the nervous system, for instance, ATP appears to behave as a neurotransmitter and neuromodulator (18), and it has been reported that extracellular ATP can also depolarize and cause an increased membrane permeability in both excitable and nonexcitable cells (2, 38–40) and is cytotoxic to isolated hepatocytes (26). Nonetheless, the in vitro ATP effects observed by us took place only at relatively high concentrations of ATP. While these concentrations may be produced physiologically in limited intracellular microenvironments, such as may be found near sensory neurons, platelets and injured cells, in the thymus, or during tumor regression, one cannot at this time exclude the possibility that the apoptosis observed in this work may be produced in vivo by an ATP-like molecule with higher affinity than ATP for the purinoreceptor on the surface of responsive cells. ATP at concentrations between 10 μM and 1 mM would thus mimic the behavior of the actual physiological ligand.

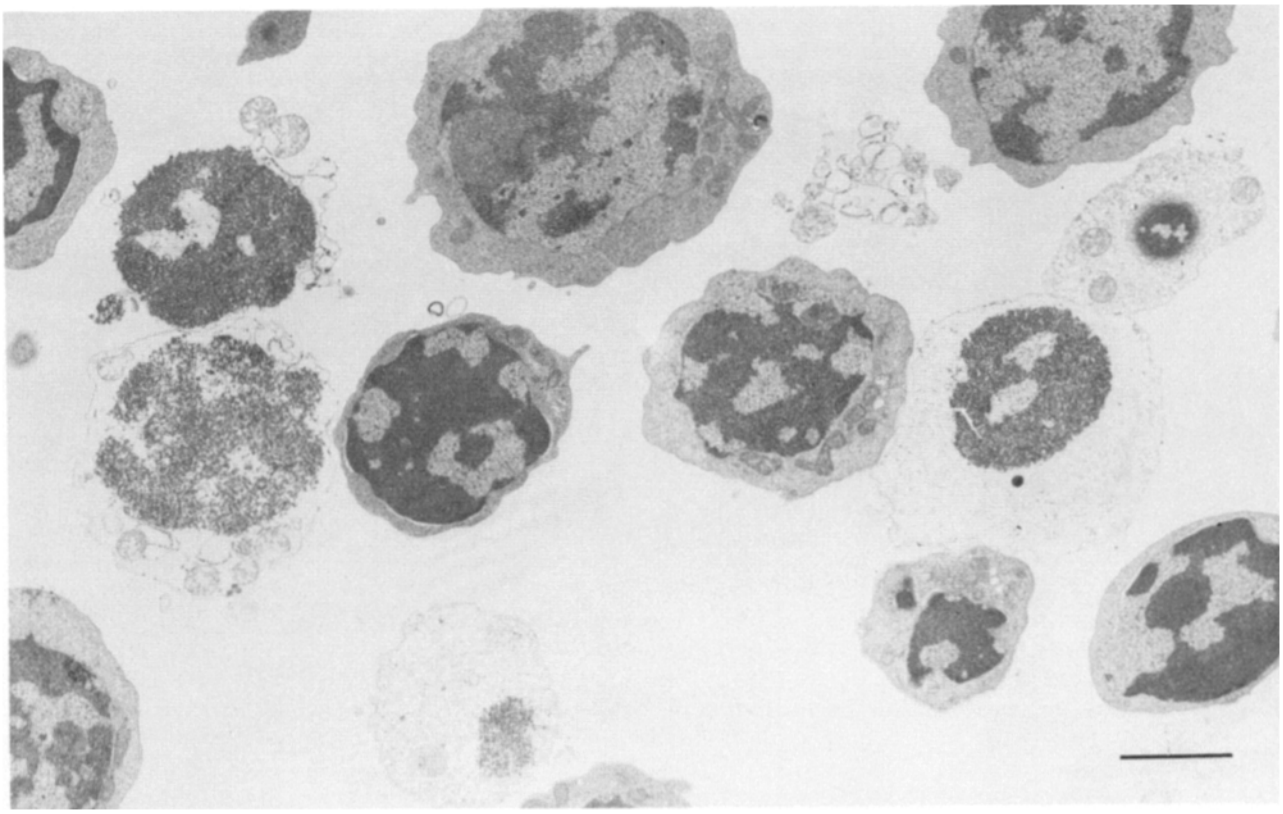

Figure 6. A representative view of thymocytes treated with perforin (33 HU/ml) for 1 h, showing many of the cells undergoing necrosis. Bar, 3 μm.

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References

1. Allbritton, N. L., C. R. Verret, R. C. Wooley, and H. N. Eisen. 1988. Calcium ion concentrations in target cell destruction by murine cloned cytotoxic T lymphocytes. *J. Exp. Med.* 167:514–527.
2. Benham, C. D., and R. W. Tsien. 1987. A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature (Lond.).* 328:275–278.
3. Blakely, A., K. Gorman, H. Ostergaard, K. Svoboda, C.-C. Liu, J. D.-E Young, and W. R. Clark. 1987. Resistance of cloned cytotoxic T lymphocytes to cell-mediated cytotoxicity. *J. Exp. Med.* 166:1070–1083.
4. Buisman, H. P., T. H. Steinberg, J. Fischbarg, S. C. Silverstein, S. A. Vogelzang, C. Ince, D. L. Ypey, and P. C. J. Leijh. 1988. Extracellular ATP induces a large nonselective conductance in macrophage plasma membranes. *Proc. Natl. Acad. Sci. USA.* 85:7988–7992.
5. Carafoli, E. 1987. Intracellular calcium homeostasis. *Annu. Rev. Biochem.* 56:395–433.
6. Cohen, J. J., and R. C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* 132:38–42.
7. Connor, J., I. S. Sawczuk, M. C. Benson, P. Tomashefsky, K. M. O'Toole, C. A. Olsson, and R. Buttyan. 1988. Calcium channel antagonists delay regression of androgen-dependent tissues and suppress gene activity associated with cell death. *Prostate.* 13:119–130.
8. Dealtry, G. B., M. S. Naylor, W. Fliers, and F. R. Balkwill. 1987. DNA fragmentation and cytotoxicity caused by tumor necrosis factor is enhanced by interferon-γ. *Eur. J. Immunol.* 17:689–693.
9. Di Virgilio, F., V. Bronte, D. Collavo, and P. Zanovello. 1989. Responses of mouse lymphocytes to extracellular adenosine 5′-triphosphate (ATP). *J. Immunol.* 143:1955–1960.
10. Duke, R. C., R. Chervenak, and J. J. Cohen. 1983. Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. *Proc. Natl. Acad. Sci. USA.* 80:6361–6365.
11. Duke, R. C., P. M. Persechini, S. Chang, C.-C. Liu, J. J. Cohen, and J. D.-E Young. 1989. Purified perforin induces target cell lysis but not DNA fragmentation. *J. Exp. Med.* 170:1451–1456.
12. Duvall, E., and A. H. Wyllie. 1986. Death and the cell. *Immunol. Today.* 7:115–119.
13. Filippini, A., R. E. Taffs, T. Agui, and M. V. Sitkovsky. 1990. Ecto-ATPase activity in cytolytic T-lymphocytes. *J. Biol. Chem.* 265:334–340.
14. Forrester, T., and A. Lind. 1969. Adenosine triphosphate in the venous effluent and its relationship to exercise. *Fed. Proc.* 28:1280–1283.
15. Gaarder, A., J. Jonse, S. Laland, A. Hellem, and P. A. Owren. 1961. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature (Lond.).* 192:531–532.
16. Gietzen, K., A. Wuthrich, and H. Bader. 1981. R24571: a new powerful inhibitor of red blood cell Ca^{++}-transport ATPase and of calmodulin-regulated functions. *Biochem. Biophys. Res. Commun.* 101:418–425.
17. Gordon, E. L., J. D. Pearson, and L. L. Slakey. 1986. The hydrolysis of extracellular adenine nucleotides by cultured endothelial cells from pig aorta. *J. Biol. Chem.* 261:15496–15504.
18. Gordon, J. L. 1986. Extracellular ATP: effects, sources and fate. *Biochem. J.* 233:309–319.
19. Goya, R. G. 1986. Role of programmed cell death in the aging process: an unexplored possibility. *Gerontology.* 32:37–42.
20. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.
21. Laster, S. M., J. G. Wood, and L. R. Gooding. 1988. Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. *J. Immunol.* 141:2629–2634.
22. Liu, C.-C., M. Steffen, F. King, and J. D.-E Young. 1987. Identification, isolation, and characterization of a novel cytotoxin in murine cytolytic

lymphocytes. *Cell.* 51:393–403.
23. Martin, D. P., R. E. Schmidt, P. S. DiStefano, O. H. Lowry, J. G. Carter, and E. M. Johnson. 1988. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* 106:829–844.
24. McConkey, D. J., P. Hartzell, S. K. Duddy, H. Håkansson, and S. Orrenius. 1988. 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin kills immature thymocytes by Ca^{2+}-mediated endonuclease activation. *Science (Wash. DC).* 242:256–259.
25. McConkey, D. J., P. Nicotera, P. Hartzell, G. Bellomo, A. H. Wyllie, and S. Orrenius. 1989. Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca^{2+} concentration. *Arch. Biochem. Biophys.* 269:365–370.
26. Nicotera, P., P. Hartzell, G. David, and S. Orrenius. 1986. The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic Ca^{2+} is mediated by the activation of a non-lysosomal proteolytic system. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 209:139–144.
27. Oppenheim, R. W. 1985. Naturally occurring cell death during neural development. *Trends Neurosci.* 8:487–493.
28. Orrenius, S., D. J. McConkey, G. Bellomo, and P. Nicotera. 1989. Role of Ca^{2+} in toxic cell killing. *Trends Pharmacol. Sci.* 10:281–285.
29. Persechini, P. M., and J. D.-E Young. 1988. The primary structure of the lymphocyte pore-forming protein perforin: partial amino acid sequencing and determination of isoelectric point. *Biochem. Biophys. Res. Commun.* 156:740–745.
30. Podack, E. R., J. D.-E Young, and Z. A. Cohn. 1985. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T cell granules. *Proc. Natl. Acad. Sci. USA.* 82:8629–8633.
31. Pratt, R. M., and R. M. Greene. 1976. Inhibition of palatal epithelial cell death by altered protein synthesis. *Dev. Biol.* 54:135–145.
32. Ryan, U. S. 1982. Structural bases for metabolic activity. *Annu. Rev. Physiol.* 44:223–239.
33. Schanne, R. A., A. B. Kane, and E. R. Young. 1979. Calcium dependence of toxic cell death: a final common pathway. *Science (Wash. DC).* 206:701–702.
34. Schmid, D. S., J. P. Tite, and N. H. Ruddle. 1986. DNA fragmentation: manifestation of target cell destruction mediated by cytotoxic T-cell lines, lymphotoxin-secreting helper T-cell clones, and cell-free lymphotoxin-containing supernatant. *Proc. Natl. Acad. Sci. USA.* 83:1881–1885.
35. Sellins, K. S., and J. J. Cohen. 1987. Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol.* 139:3199–3206.
36. Stevenson, M. A., S. K. Calderwood, and G. M. Hahn 1986. Rapid increases in inositol triphosphate and intracellular calcium after heat shock. *Biochem. Biophys. Res. Commun.* 137:826–833.
37. Sugarman, B. J., B. B. Aggarwal, P. E. Hass, I. S. Figari, M. A. Palladino, Jr., and H. M. Shepard. 1985. Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells *in vitro*. *Science (Wash. DC).* 230:943–945.
38. Sung, S.-S. J., J. D.-E Young, A. M. Origlio, J. M. Heiple, H. R. Kaback, and S. C. Silverstein. 1985. Extracellular ATP perturbs transmembrane ion fluxes, elevates cytosolic $[Ca^{2+}]$, and inhibits phagocytosis in mouse macrophages. *J. Biol. Chem.* 260:13442–13449.
39. Steinberg, T. H., and S. C. Silverstein. 1987. Extracellular ATP^{4-} promotes cation fluxes in the J774 mouse macrophage cell line. *J. Biol. Chem.* 262:3118–3122.
40. Steinberg, T. H., A. S. Newman, J. A. Swanson, and S. C. Silverstein. 1987. ATP^{4-} permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J. Biol. Chem.* 262:8884–8888.
41. Tata, J. R. 1966. Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. *Dev. Biol.* 13:77–94.
42. Trams, E. G., H. Kaufmann, and G. Burnstock. 1980. A proposal for the role of ecto-enzymes and adenylates in traumatic shock. *J. Theor. Biol.* 87:609–621.
43. Tsujimoto, M., Y. K. Yip, and J. Vilcek. 1985. Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. *Proc. Natl. Acad. Sci. USA.* 82:7626–7630.
44. Winkler, H. 1976. The composition of adrenal chromaffin granules: an assessment of controversial results. *Neuroscience.* 1:65–80.
45. Wyllie, A. H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature (Lond.).* 284:555–556.
46. Wyllie, A. H., J. F. R. Kerr, and A. R. Currie. 1980. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68:251–306.
47. Wyllie, A. H., R. G. Morris, A. L. Smith, and D. Dunlop. 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.* 142:67–77.
48. Young, J. D.-E, H. Hengartner, E. R. Podack, and Z. A. Cohn. 1986. Purification and characterization of a cytolytic pore-forming protein from granules of cloned lymphocytes with natural killer activity. *Cell.* 44: 849–859.