Biochemical Pharmacology, Vol. 51, pp. 1331–1340, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00041-X

Apoptosis as a Mechanism of Cell Death Induced by Different Chemotherapeutic Drugs in Human Leukemic T-Lymphocytes

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ABSTRACT. The involvement of apoptosis in the mechanism of cell death induced by six clinically relevant anticancer drugs [methotrexate (MTX), doxorubicin (ADR), daunorubicin (DNR), vincristine (VCR), 6-mercaptopurine (6MP), and prednisolone (PRD)] in human leukemic T-lymphocytes (CCRF-CEM and Jurkat) was investigated by analysing changes in cell size and morphology, changes in membrane integrity, alterations in $[Ca^{2+}]_i$ and induction of DNA fragmentation. MTX, ADR, and DNR showed pronounced dose- and time-dependent cytotoxic effects on both cell lines, whereas cell viability was not considerably reduced by 6MP or PRD. On the other hand, the cytotoxic activity of VCR was much higher on Jurkat cells than on CEM cells. With the exception of 6MP and PRD, all the other compounds induced extensive chromatin condensation, nuclear fragmentation, plasma membrane blebbing, and formation of apoptotic bodies and fragmentation of DNA in both cell lines. Occurrence of DNA fragmentation always preceded loss of membrane integrity. These observations are consistent with cell death being mediated by apoptosis. Significant increases in $[Ca^{2+}]_i$ were only observed in CEM cells preincubated with MTX or DNR (10 μM). In contrast, MTX as well as VCR induced a reduction in the basal intracellular Ca^{2+} concentration in Jurkat T-cells. Although the ability to induce changes in $[Ca^{2+}]_i$ correlated with higher cytotoxic potency of the anticancer drugs, a causal relationship between increased $[Ca^{2+}]_i$ and induction of apoptosis could not be clearly established. These results, therefore, suggest no determinant role for Ca^{2+} in triggering the process of endonucleolytic cleavage of genomic DNA in these leukemic T-lymphocytes. BIOCHEM PHARMACOL 51;10:1331–1340, 1996.

KEY WORDS. apoptosis; anticancer drugs; DNA fragmentation; human T-lymphocytes; intracellular calcium; leukemia

Treatment of acute lymphoblastic leukemia can often be achieved with the use of a wide variety of chemotherapeutic drugs [1, 2]. Among such drugs, the Vinca alkaloids, such as VCR,[‖] have been thought to exert their antileukemic effects through inhibition of mitosis [2, 3], whereas the cytotoxic activity of several antimetabolites, including MTX, 6MP, ADR, and DNR, has been mainly attributed to their inhibition of DNA synthesis [2, 4]. However, other important biological effects have also been reported for these compounds [5, 6] and the precise mechanisms by which many of these anticancer drugs induce cell death are still not fully understood.

Apoptosis has recently been recognized as a mode of cell death that can be activated in many systems, including T-lymphocytes and leukemic cells, by a wide variety of chemical and physical stimuli [7–9]. Observations from several laboratories indicate that many anticancer agents can induce apoptosis in different types of cells [9, 10]. We, therefore, addressed the question as to whether or not apoptosis could be one mode of cell death induced by some chemically distinct anticancer drugs in human leukemic T-cells.

Cells undergoing apoptosis show characteristic morphological and biochemical changes, which include chromatin condensation, fragmentation of DNA into oligonucleosome-sized fragments, cell shrinkage, loss of membrane architecture, and appearance of blebbing [7, 11]. An important biochemical event in apoptosis is the activation of an endonuclease that cleaves the DNA at internucleosomal linker sites [12]. Another common, although not universal, observation in apoptosis is a sustained increase in $[Ca^{2+}]_i$, which is thought to trigger activation of the endonuclease responsible for fragmentation of DNA [7, 13]. However, a definitive role for Ca^{2+} in the induction of apoptosis is still not clear [7, 14].

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‖ *Abbreviations*: ADR, Adriamycin®, doxorubicin; $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; DNR, daunorubicin; 6MP, 6-mercaptopurine; MTX, methotrexate; PRD, prednisolone; VCR, vincristine.

Received 30 June 1995; accepted 18 December 1995.

In the present work, we investigated the possible involvement of apoptosis in the mechanism of cell death induced by six clinically relevant anticancer drugs (MTX, ADR, DNR, VCR, 6MP, and PRD) in human leukemic T-lymphocytes (CCRF-CEM and Jurkat) by analysing changes in cell size and morphology, changes in membrane integrity, alterations in $[Ca^{2+}]_i$, and induction of DNA fragmentation. Potential correlations between increases in $[Ca^{2+}]_i$ and induction of apoptosis were also evaluated.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, MTX (sodium salt), ADR (hydrochloride), DNR (hydrochloride), VCR (sulphate salt), 6MP, PRD, proteinase K, ribonuclease T1, and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal calf serum was obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Fura-2/AM and ionomycin were obtained from Calbiochem (Frankfurt, Germany). Agarose was from Pharmacia/LKB (Uppsala, Sweden). All other chemicals were of the highest purity available.

Cells and Cell Culture

The human T-lymphoblastoid cell lines CCRF-CEM and Jurkat were obtained from the cell culture facilities at the University of California (San Francisco, CA, U.S.A.) and the Max-Planck Institut of Erlangen-Nuremberg, respectively. Both cell lines were maintained in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 20 mM HEPES (pH 7.4), 100 units/mL penicillin and 100 μg/mL streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell Proliferation Studies

To study the growth-inhibitory effects of the anticancer drugs, cells were incubated with the indicated concentrations of the compounds for 24 to 72 hr, at an initial cell density of 3–4 × 10^5 cells/mL. An appropriate volume of drug vehicle was added to untreated cells. After each period of incubation, aliquots of the cell suspensions were removed and cellular growth was evaluated by cell counting in a Coulter counter or in a hemocytometer. Cell viability was assayed by Trypan blue exclusion.

Morphological Studies

Following incubation for 8 or 24 hr with the anticancer drugs (50 nM or 1 μM), cells were collected by centrifugation and used for preparation of cell smears. The cells were stained with May-Grünwald-Giemsa for 20 min and cell morphology was examined by light microscopy, using a Leitz Dialux 20 microscope. In addition, ultrastructural changes were evaluated by electron microscopy. After the indicated drug treatment, approximately 4 × 10^6 cells were collected by centrifugation and fixed with 1 mL of 3% glutaraldehyde, 1% paraformaldehyde in 0.2 M cacodylate buffer, pH 7.4, for 2 hr at 4°C. The cells were subsequently washed twice with 0.2 M cacodylate buffer, pH 7.4, at 4°C and postfixed with 1% osmium tetroxide in the same buffer (2 hr, 4°C). The cells were dehydrated through an ascending ethanol series, impregnated with epoxipropane, and embedded in a mixture of Epon-Araldite (1:1). Ultrafine sections were made, stained with uranyl acetate for 30 min at 40°C and, subsequently, with lead acetate for 3 min. Specimens were examined at 80 KV in a Jeol 100C transmission electron microscope.

Measurement of Cellular DNA Content by Flow Cytometry

Cell cultures maintained in a density of 4 × 10^5 cells/mL were treated with the drugs (50 nM or 1 μM) for 24, 48, and 72 hr. Cells (approximately 4 × 10^6 cells) were collected by centrifugation (200 × g, 6 min), washed in PBS, and resuspended in the same buffer supplemented with 0.1% SDS and 3% trypsin. Samples were incubated for 10 min at room temperature, under continuous shaking. Then, 1.5 mL of a solution containing trypsin inhibitor (1%) and RNAase A (50 μg) were added to each sample and incubations continued for a further 10 min. DNA was stained with propidium iodide (100 μg/mL) for 10 min and cellular DNA content was measured in a Becton-Dickinson flow cytometer (FACStar Plus) with excitation at 488 nm and emission above 585 nm. Approximately 20,000 cells were measured for each DNA content histogram. Chicken erythrocyte nuclei (DNA-QC particles; Becton Dickinson) stained with propidium iodide were used as a standard to optimize the instrument settings, check the CV and linearity, and set proper electronic compensation. The CellFIT software program (Becton-Dickinson) was used for the analysis of cell cycle phase distributions.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis

CEM and Jurkat cells (approximately 2 × 10^6 cells/sample) were treated with the anticancer drugs (50 nM or 1 μM) for 8 or 24 hr. At the end of the incubation period, cells were harvested by centrifugation (200 × g, 6 min), washed twice in phosphate-buffered saline, pH 7.4, and resuspended in lysis buffer containing 0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0, in a concentration of 2 × 10^6 cells/200 μL. To each 200 μL of cell suspension, 25 μL of proteinase K (10 μg/μL) and 25 μL of 10% SDS were added. Samples were incubated at 70°C for 90 min. Ribonuclease T1 was added (100 units/200 μL) and the incubation continued for a further 60 min. Quantification of DNA was performed spectrophotometrically by measuring absorbance at 260 nm [15]. Samples containing approximately 10–20 μg of DNA were supplemented with loading buffer (10 mM EDTA, pH

8.0, containing 0.04% bromophenol blue, 0.04% xylene cyanol, and 2.5% Ficoll 400), at a 1:5 volume ratio, before application onto 1.5% agarose gels. Electrophoresis was carried out in a buffer containing 90 mM Tris, 90 mM sodium borate, 2 mM EDTA, pH 8.0 (TBE buffer), and the gels were run at 20 V for 20 hr. The pattern of DNA fragmentation was visualized under UV light, after staining the gel with ethidium bromide (0.5 μg/mL in TBE buffer).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in cells loaded with the fluorescent indicator Fura-2. In brief, CEM or Jurkat T-cells (10–20 × 10^6 cells) were incubated with 4 μM Fura-2/AM in RPMI 1640 medium for 30 min at 37°C, washed twice with assay buffer, and resuspended in the same buffer. Fluorescence was measured in batches of 2–4 × 10^6 cells/2 mL, maintained under continuous stirring in a Perkin-Elmer LS-5B fluorimeter, at excitation and emission wavelengths of 340 ± 5 nm and 505 ± 10 nm, respectively. Measurements were carried out at 30°C in a medium containing (in mM): 140 NaCl, 5 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 1 NaH_2PO_4, 5.5 glucose, and 20 HEPES, pH 7.4. In some experiments, EGTA/Tris (3 mM/30 mM, pH 7.4) was added to chelate extracellular Ca^{2+}. At the end of each single measurement, maximal fluorescence (F_{max}) was determined by addition of ionomycin (2 μM) and Ca^{2+} (1 mM) and minimal fluorescence (F_{min}) was determined by addition of EGTA/Tris (4 mM/40 mM). $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz *et al.* [16]. The effects of the anticancer drugs on $[Ca^{2+}]_i$ were analysed in cells treated for 2 hr (or 24 hr in some experiments) with concentrations of 1–10 μM of the drugs, prior to loading with Fura-2. The fluorescent properties of Fura-2 were not affected by the anticancer agents used in this work.

RESULTS

Antiproliferative and Cytotoxic Effects of the Anticancer Drugs

Exposure of CEM and Jurkat T-lymphocytes to each of the anticancer drugs induced a dose- and time-dependent decrease in cell growth (Figs. 1 and 2) and in cell viability (Figs. 3 and 4). With the exception of VCR and, to a lesser extent, ADR and 6MP, the antiproliferative and cytotoxic effects of the anticancer drugs tested were similar for both cell lines. MTX, ADR and DNR (in concentrations between 50 nM and 10 μM) induced marked decreases in cell number and in cell viability, especially after 48 hr of treatment (less than 50% of cells survived at the highest drug concentrations). The number of nonviable cells measured by the uptake of Trypan blue always reached 100% after 72 hr of incubation with the highest concentrations of these drugs. The antiproliferative and cytotoxic effects of 6MP and PRD were much smaller and, in the range of concentrations analysed (50 nM–50 μM), neither cell growth nor cell viability were significantly changed following treatment with these compounds (70–100% of viable cells, after 72 hr) (Figs. 1–4, D–E). Only in the case of CEM cells was a pronounced decrease in cell viability observed after 72 hr of incubation with 10 μM of 6MP. Major differences were observed between the responses of each cell line to VCR (Figs. 1–4, F), with Jurkat cells showing a much higher sensitivity to this drug than CEM cells.

Drug Effects on Cellular Morphology and DNA Content

The morphological changes produced in CEM and Jurkat T-cells following a 24-hr exposure to the anticancer drugs MTX, ADR, DNR, and VCR included considerable chromatin condensation and nuclear fragmentation, as well as decreases in cell size, blebbing of the plasma membrane, and formation of apoptotic bodies (data not shown). Although less pronounced, these drug effects were already detectable after 8 hr of incubation with MTX or DNR, in the case of CEM cells, and with DNR, ADR, and VCR in the case of Jurkat cells (data not shown). On the other hand, 6MP and PRD did not considerably change CEM or Jurkat T-cell morphology under the experimental conditions used (not shown).

Cell cycle analysis of drug-treated cells, using flow cytometry, revealed the presence of a distinct cell cycle region below the G0/G1 region. This "sub-G1" peak, displaying particles with lower DNA content (<2N), represents apoptotic bodies with their characteristic reduced volume and nuclear condensation [17]. As shown in Table 1, the percentage of apoptotic bodies in both cell lines increases with the time of incubation with the anticancer drugs MTX, ADR, DNR, and VCR. For CEM cells, the highest increase in the number of apoptotic bodies was observed following exposure to VCR (20–25%, after 24 and 48 hr, respectively), whereas between 5 and 10% of apoptotic bodies were found in MTX-, ADR-, and DNR-treated samples. In contrast, the number of apoptotic bodies measured in Jurkat T-cells exposed to these drugs was consistently higher than in CEM cells. At 48 hr, the percentage of apoptotic bodies was 47.3 and 47.5 for DNR and VCR, respectively, whereas the corresponding values for MTX and ADR were 28.5 and 34.5. It should be noted, however, that higher levels of apoptotic and dead cells were also found in control samples of Jurkat lymphocytes, in comparison to untreated CEM cells.

Drug-Induced DNA Fragmentation

Fragmentation of the genomic DNA was analysed in CEM and Jurkat cells following incubation with the anticancer agents, in concentrations of 50 nM or 1 μM for different periods of time. Under the experimental conditions used, 6MP and PRD had no effect on DNA fragmentation in any of the cell lines tested. In contrast, a characteristic pattern of DNA fragments, corresponding to the nucleosome ladder typical of apoptosis, was observed in cells previously ex-

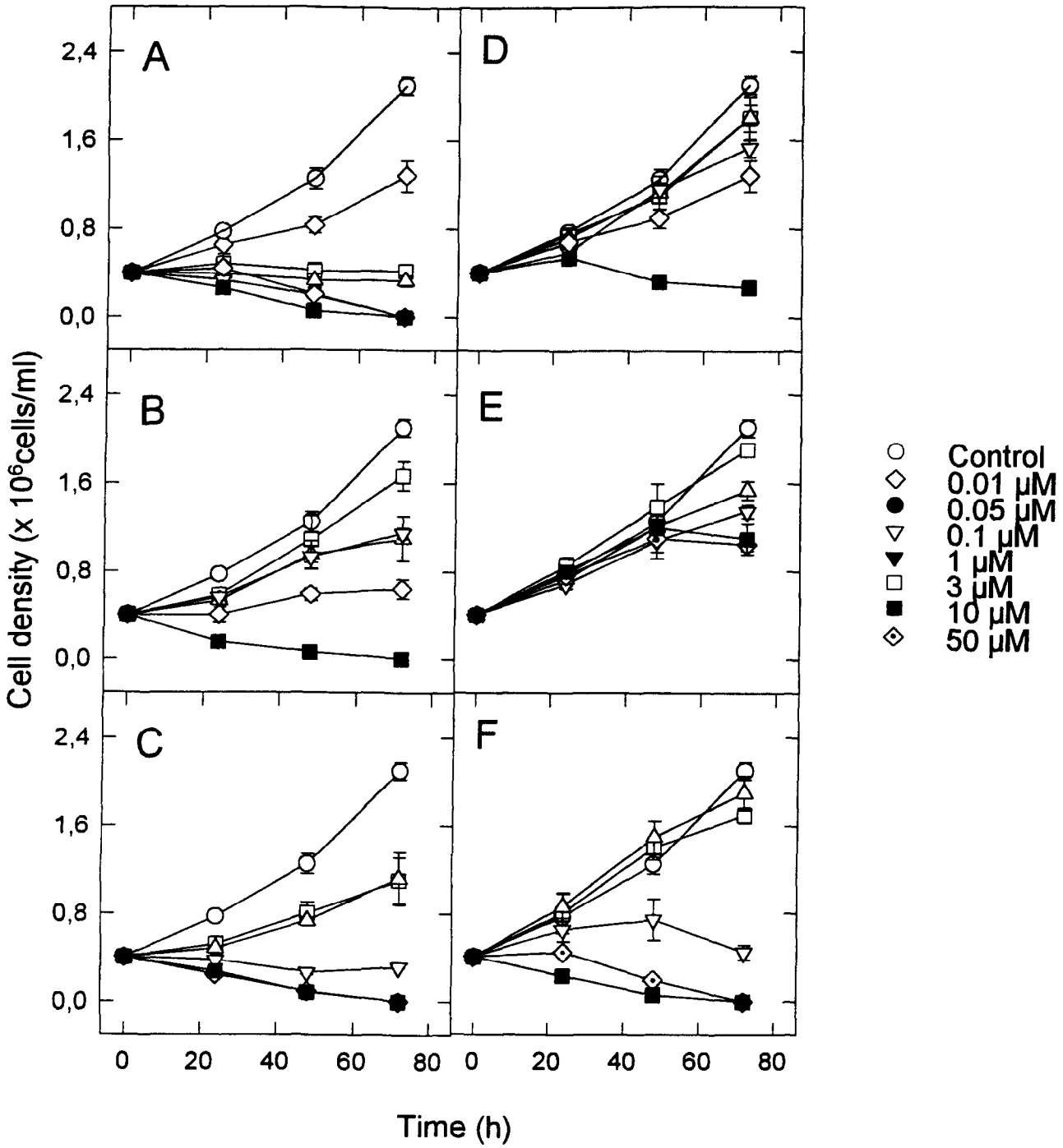

FIG. 1. Time- and dose-dependent antiproliferative effects of anticancer drugs on CEM T-lymphoblasts. Cells (4 × 10^5 cells/mL) were incubated for 24–72 hr with the anticancer drugs, in the concentrations indicated. Every 24 hr, aliquots of the cell suspensions were removed and cells counted in a Coulter counter or in a hemocytometer. Cell numbers represent only viable cells, as measured by the exclusion of Trypan blue. Results are given as mean ± SE of 4–5 independent experiments performed in duplicate. (A) MTX; (B) ADR; (C) DNR; (D) 6MP; (E) PRD; (F) VCR.

posed to concentrations of 1 µM of the other compounds (Fig. 5). At this drug concentration, DNA fragmentation was already visible in CEM cells after 8 hr of treatment (not shown). However, with increasing time of exposure (24 hr) to the anticancer drugs, a more intense pattern of nucleosome fragments was detected (Fig. 5A, B). In the case of Jurkat cells, degradation of DNA could only be detected at 24 hr, although MTX- and DNR-treated cell samples already showed small amounts of fragmented DNA after 8 hr (not shown). No DNA fragmentation was detected in cell samples treated with lower concentrations (50 nM) of the drugs, for up to 24 hr (data not shown).

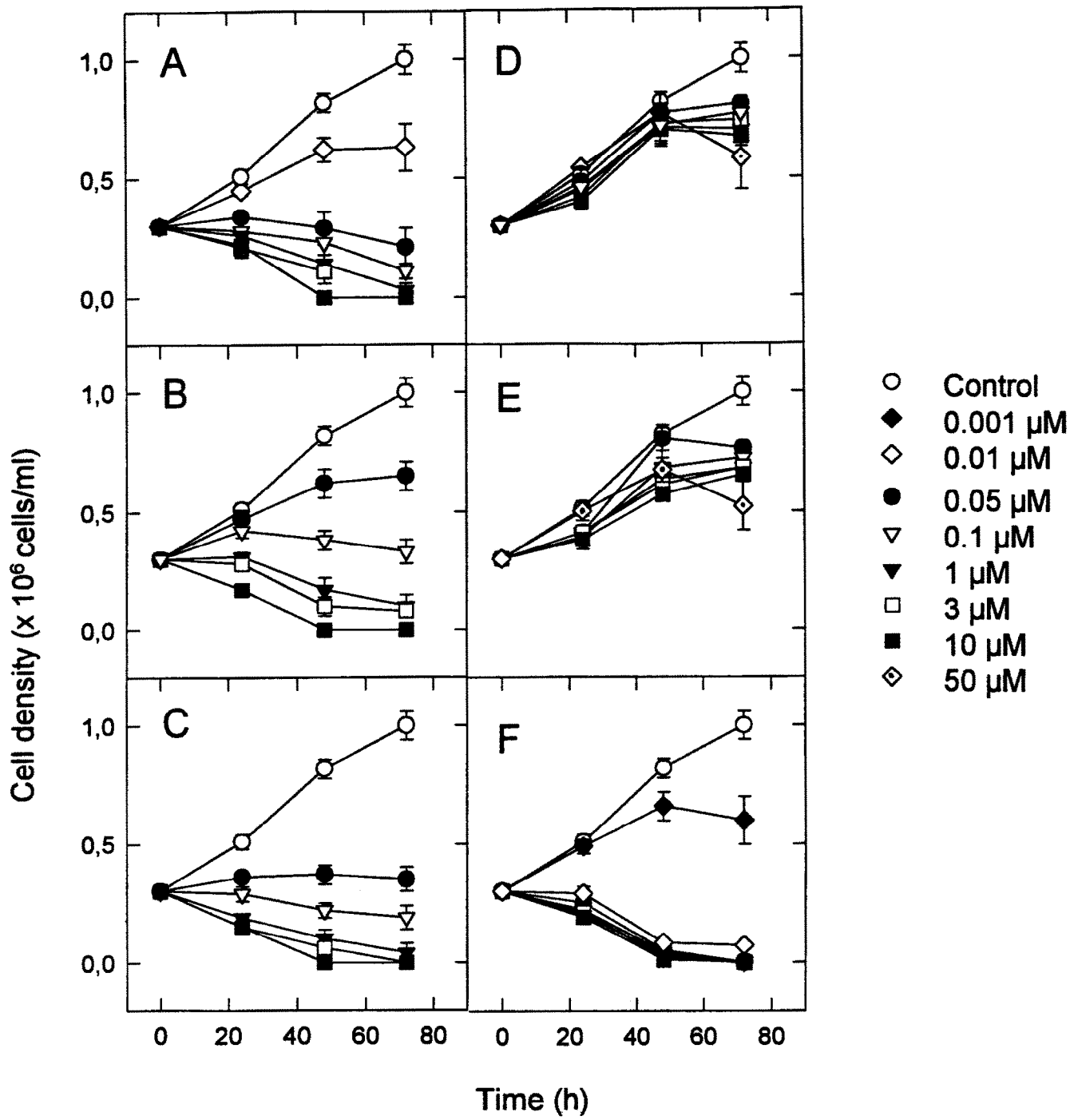

FIG. 2. Time- and dose-dependent antiproliferative effects of anticancer drugs on Jurkat T-lymphoblasts. Cells (3 x 10^5 cells/mL) were incubated for 24–72 hr with the anticancer drugs, in the concentrations indicated. Cell number and cell viability were measured every 24 hr as described in Materials and Methods and in the legend to Fig. 1. Results are given as mean ± SE of 4–5 independent experiments performed in duplicate. (A) MTX; (B) ADR; (C) DNR; (D) 6MP; (E) PRD; (F) VCR.

Effects of Drugs on $[Ca^{2+}]_i$

The results shown in Table 2 illustrate the effects of the anticancer drugs on basal $[Ca^{2+}]_i$ in CEM and Jurkat T-cells. In the case of CEM cells, only MTX and DNR induced significant increases in the resting $[Ca^{2+}]_i$. Following incubation with these compounds, in concentrations up to 10 µM, the free cytosolic Ca^{2+} concentration changed from a basal level of 119.0 ± 8.6 (mean ± SE, n = 20) to 160.5 ± 11.1 (n = 15) with MTX, and to 148.1 ± 8.8 (n = 12) with DNR. In comparison to control cells, increased levels of $[Ca^{2+}]_i$ in MTX- and DNR-treated CEM cells were also observed in a nominally Ca^{2+}-free medium (data not

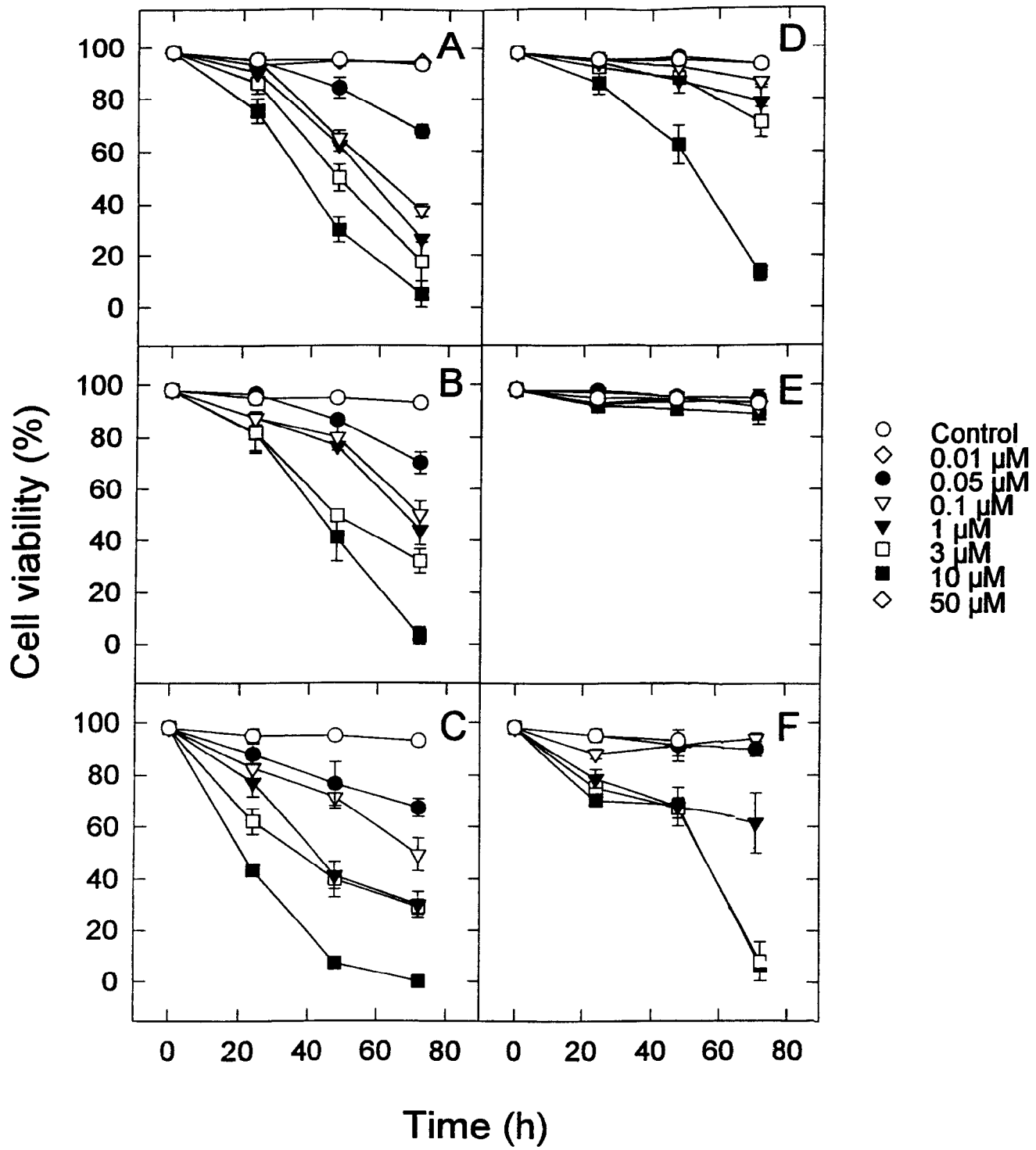

FIG. 3. Time- and dose-dependent cytotoxic effects of anticancer drugs on CEM T-lymphoblasts. Cells (4×10^5 cells/mL) were incubated for 24–72 hr with the anticancer drugs, in the concentrations indicated. Every 24 hr, aliquots of the cell suspensions were removed and cells counted in a Coulter counter or in a hemocytometer. Cell viability was measured by the exclusion of Trypan blue. Results are given as mean ± SE of 4–5 independent experiments performed in duplicate. (A) MTX; (B) ADR; (C) DNR; (D) 6MP; (E) PRD; (F) VCR.

shown). Measurements of $[Ca^{2+}]_i$ in drug-treated Jurkat T-lymphocytes indicated that, for this cell line, MTX and VCR were the only drugs that induced significant changes in the intracellular Ca^{2+} concentration. However, instead of an increase in $[Ca^{2+}]_i$, treatment of Jurkat cells with these compounds induced a 1.6–2.5-fold decrease in the basal levels of Ca^{2+} over controls. $[Ca^{2+}]_i$ changes similar to the ones described above were also obtained when CEM and Jurkat cells were preincubated with some of the compounds for longer periods of time (24 hr).

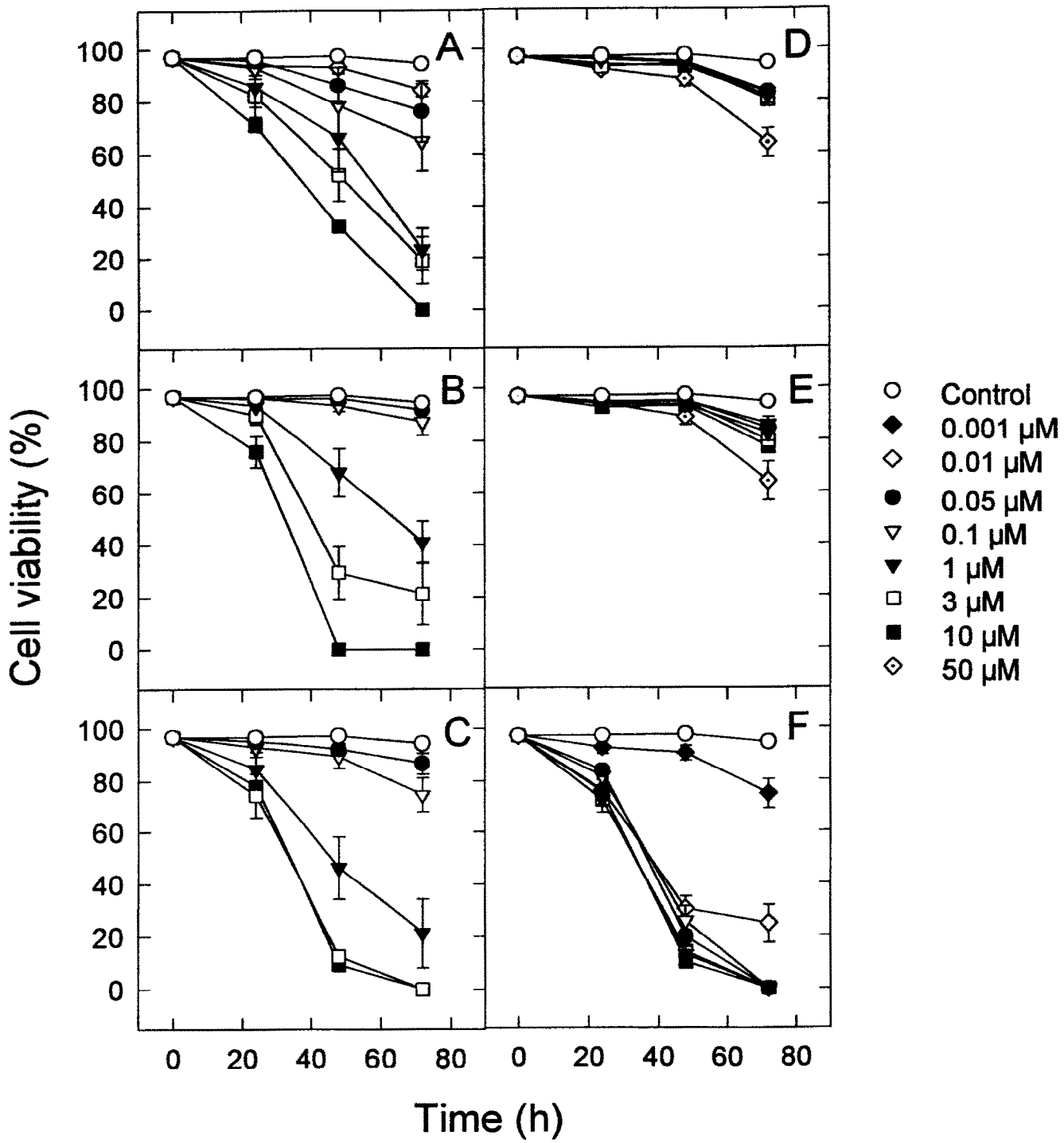

FIG. 4. Time- and dose-dependent cytotoxic effects of anticancer drugs on Jurkat T-lymphoblasts. Cells (3×10^5 cells/mL) were incubated for 24–72 hr with the anticancer drugs, in the concentrations indicated. Cell number and cell viability were measured every 24 hr as described in Materials and Methods and in the legend to Fig. 1. Results are given as mean ± SE of 4–5 independent experiments performed in duplicate. (A) MTX; (B) ADR; (C) DNR; (D) 6MP; (E) PRD; (F) VCR.

DISCUSSION

In the present work, we investigated the role of apoptosis as a common mechanism of cell death induced by several anticancer drugs on two human leukemic T-cell lines. Several morphological and biochemical changes, generally regarded as typical of cells undergoing apoptosis [7, 8, 12], were identified in CEM and Jurkat T-lymphocytes following exposure to the compounds MTX, ADR, DNR, and VCR. Significant effects of these anticancer drugs on cell morphology were detected early in the course of treatment and were essentially manifested by condensation of chro-

TABLE 1. Percentage of cells with a DNA content <2 N (apoptotic bodies) in CEM and Jurkat cultures, following treatment with anticancer drugs

	Apoptotic bodies (%)			
	CEM		Jurkat	
Drug	24 hr	48 hr	24 hr	48 hr
–	0	0.4	3.5	3.0
MTX	6.5	7.4	25.5	28.5
ADR	4.6	6.3	22.6	34.5
DNR	6.6	9.6	37.0	47.3
VCR	20.4	24.9	37.5	47.5
6MP	0.3	0.1	3.6	2.5
PRD	0.5	0.3	5.5	2.6

Cells were incubated with the drugs, in a concentration of 1 μM, for the times indicated. Cellular DNA content was analysed by flow cytometry after staining with propodium iodide, as described in Materials and Methods. The percentage of cells with a DNA content <2 N was calculated on the basis of the total number of cells measured by flow cytometry.

matin, nuclear fragmentation, blebbing of the plasma membrane, and formation of apoptotic bodies.

One of the well-documented characteristics of apoptosis is the fragmentation of DNA into multimers of approximately 200 base pairs, due to activation of an endonuclease [7, 14, 18]. In the present study, agarose gel electrophoresis of DNA from MTX-, ADR-, DNR-, and VCR-treated CEM and Jurkat cells demonstrated the multimeric pattern characteristic of internucleosomal cleavage of the genomic DNA. The appearance of this nucleosomal ladder of DNA fragments was dependent on the dose and time of exposure to the drugs. Hence, no DNA degradation was detected when both CEM and Jurkat T-cells were exposed to these compounds in concentrations of 50 nM, even after prolonged periods of incubation (24 hr). However, at the highest drug concentrations tested (1 μM), distinct patterns of fragmented DNA were obtained for CEM cells treated with each of the four compounds mentioned above, both at 8 and at 24 hr. In the case of Jurkat lymphocytes, after short-term exposure (8 hr) to the same anticancer drugs (1 μM), small amounts of fragmented DNA could only be detected in MTX- and DNR-treated cell samples, whereas, for longer periods of incubation (24 hr), a typical ladder of DNA fragments was also produced with the other compounds. However, in the case of MTX and DNR, the intensity of the DNA nucleosomal ladder also increased with time. Taken together, these data clearly demonstrate that MTX, ADR, DNR, and VCR induce apoptosis in CEM and Jurkat T-lymphocytes. In contrast, under the experimental conditions used, no apoptosis-related events were observed in cell samples treated with the other anticancer drugs tested, namely 6MP and PRD.

Some of these chemotherapeutic agents, particularly MTX and ADR, have already been reported to induce apoptosis in Chinese hamster ovary cells [10] and HL-60 and MOLT-4 cell lines [19], respectively. Our results, demonstrating a failure to detect signs of apoptosis in cells exposed to PRD, are supported by the previous work of Distelhorst [20], which shows a lack of DNA fragmentation in lymphocytes from patients with acute lymphoblastic leukemia exposed to this agent.

In the present study, detection of particles with a sub-G1 DNA content (<2N) by flow cytometry, in cell samples treated with MTX, ADR, DNR, and VCR, correlated with formation of apoptotic bodies observed by light and electron microscopy. Similar to what has been reported by others [17, 19, 21], the heterogeneity of DNA content, as well as forward angle light scatter, of the sub-G1 populations observed in our work suggest that apoptotic bodies are present among such particles.

The apoptotic changes induced by drug treatment of CEM and Jurkat lymphocytes were compared with the cytotoxic effects of these anticancer agents. Drug-induced cell death was initially evaluated in terms of loss of membrane integrity, as assessed by Trypan blue uptake. Our results indicated that the cytotoxic effects of these compounds were both dose- and time-dependent. Although Jurkat lymphocytes showed a much higher sensitivity to VCR than CEM cells, the cytotoxic effects of MTX, DNR, and ADR were very similar for both cell lines; the order of relative cytotoxic potencies of these three compounds being MTX = DNR > ADR. On the other hand, little or no cell death was induced in CEM and Jurkat T-cells by the compounds 6MP and PRD. These results were in good agreement with the data on drug-induced morphological changes, because MTX and DNR, on one hand, and VCR and DNR, on the other hand, were the drugs that caused the highest degrees of morphological changes in CEM and in Jurkat cells, respectively.

In addition, the electrophoretic analysis of drug-induced DNA fragmentation and, in particular, the information obtained on the time and concentration dependency of this process, also showed some correlations with cytotoxicity data. In fact, at low and nontoxic concentrations of the drugs (50 nM, 24 hr), no DNA degradation was detected. In contrast, fragmentation of DNA was generally seen in those samples from cells exposed to MTX, ADR, DNR, or VCR, in concentrations that were also found to induce extensive loss of cell viability (above 1 μM). In terms of DNA fragmentation, the effects of VCR on Jurkat cells were, however, unexpected. In contrast to the results obtained, and taking into account the highly cytotoxic activity of this compound, some degree of DNA fragmentation should have, in principle, been observed for the lowest drug concentrations tested. However, some recent studies have demonstrated that DNA fragmentation is not an essential feature of apoptosis [22, 23]. In those studies, evidence has been presented that key morphological changes in apoptosis precede and can be experimentally dissociated from the internucleosomal cleavage of DNA produced by endonucleases, which in itself is not an essential step in the apoptotic process.

It is important to note that Trypan blue uptake, which is due to loss of membrane integrity and, thus, cell death,

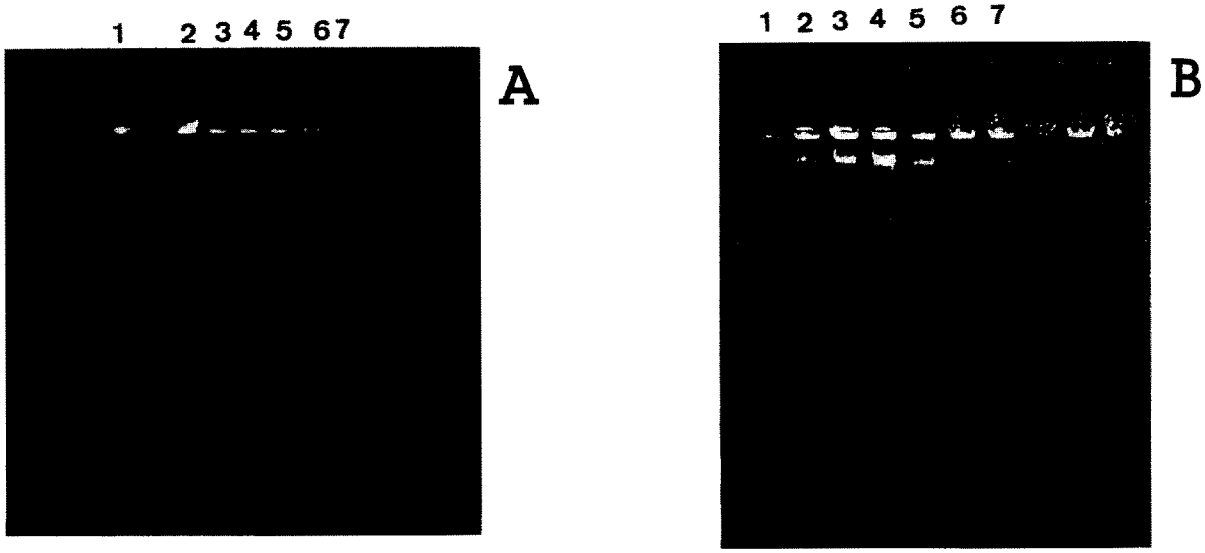

FIG. 5. Agarose gel analysis of DNA following treatment of CEM and Jurkat T-cells with anticancer drugs. CEM cells (A) and Jurkat cells (B) were continuously exposed to a concentration of 1 μM of the anticancer drugs for 24 hr. At the end of the incubation period, cells were harvested by centrifugation and processed for the analysis of DNA fragmentation as described under Methods. Electrophoresis of DNA samples was carried out in 1.5% agarose gels, as detailed in the experimental section. Lane 1, control; lane 2, MTX; lane 3, ADR; lane 4, DNR; lane 5, VCR; lane 6, 6MP; lane 7, PRD.

always occurred after DNA degradation had been detected. In most cases, considerable loss of membrane permeability occurred at least 24 hr after distinct internucleosomal fragmentation of DNA was observed. As an example, exposure of both cell lines to MTX for 8 hr, in a concentration of 1 μM, induced fragmentation of the DNA although, at this drug concentration, no significant loss of cell viability could be detected before 48 hr of incubation. Therefore, in agreement with reports from other groups [10, 18, 24], these observations confirm that DNA breakage is an early event in cell death and occurs long before loss of plasma membrane integrity.

The role of calcium in mediating apoptotic cell death is still controversial [7, 14, 25, 26]. Many studies have demonstrated that sustained increases in intracellular free Ca^{2+} precede apoptosis induced by a variety of agents [26]. Observations that Ca^{2+} ionophores induce endonuclease activation and death in many types of cells [18, 26] also suggest that Ca^{2+} is an essential requirement for apoptosis. However, there are also many other documented examples of apoptosis in the absence of Ca^{2+} influx [14, 25]. In the present study, we demonstrated that only in CEM cells treated with MTX or DNR did slight increases in the free cytosolic Ca^{2+} concentration occur, although these were not the only drugs tested that induced death by apoptosis in this cell line. On the other hand, MTX and VCR were shown to reduce the resting intracellular Ca^{2+} levels in Jurkat T-cells but, again, no $[Ca^{2+}]_i$ changes could be detected with other drugs also able to trigger apoptosis. Therefore, based on all our data, no clear causal relationship between sustained changes in $[Ca^{2+}]_i$ and drug induction of apoptosis could be established. In spite of this, it is interesting to note that the ability of MTX, DNR, and VCR to induce alterations in $[Ca^{2+}]_i$ in CEM and Jurkat lymphocytes, in fact, correlates with a higher cytotoxic potency of these anticancer drugs. Therefore, the observed effects of MTX, DNR, and VCR on $[Ca^{2+}]_i$ in both cell lines may contribute to the mechanism of action of these compounds, with the changes in $[Ca^{2+}]_i$ probably representing an early step in the sequence of events leading to drug-induced cell death.

TABLE 2. Effect of anticancer drugs on the basal free cytosolic Ca^{2+} concentration in CEM and Jurkat T-lymphocytes

	$[Ca^{2+}]_i$ (nM)	
Anticancer drug	**CEM cells**	**Jurkat cells**
–	111.9 ± 8.6	154.0 ± 28.3
MTX	160.5 ± 11.1*	62.0 ± 6.0*
ADR	128.7 ± 14.0	161.6 ± 12.5
DNR	148.7 ± 8.8*	147.0 ± 32.8
VCR	135.3 ± 10.0	96.2 ± 11.5*
6MP	127.3 ± 10.9	148.1 ± 8.4
PRD	123.2 ± 9.6	135.8 ± 14.7

Cells were incubated with the drugs (in concentrations up to 10 μM) for 2 hr prior to loading with Fura-2. Fura-2 loaded cells (2–4 × 10^6 cells/2 mL) were resuspended in a Ca^{2+}-containing medium and $[Ca^{2+}]_i$ was monitored as described in Materials and Methods. Results are given as mean ± SE (n = 9–20). *$P < 0.05$.

In conclusion, the present study demonstrates that the anticancer drugs MTX, ADR, DNR, and VCR, which are generally considered to have distinct main targets in cells, induce cell death in CEM and Jurkat T-lymphocytes by a common mechanism of apoptosis. Hence, there are probably multiple steps within the apoptotic pathway at which drug effects can be manifested, thereby triggering the phenomenon. Moreover, some differences between CEM and

Jurkat cells were observed in their response to the chemotherapeutic agents, with the drug-induced apoptotic changes being, in general, more pronounced in the latter. This different behaviour of each cell line towards the same anticancer drugs also supports the current view that apoptosis is a "dynamic" process [7–9, 13], involving cell-specific factors, in the sense that the cell actively participates in the initiation of its own death.

The authors are grateful to Dr. M. Luisa Valdeira, Catarina Bernardes, and Dr. Teresa Silva for their assistance in the electron and light microscopy analysis of cell morphology. We would like to thank Prof. Dr. Santos Rosa for his help with the flow cytometric experiments and to Dr. Celeste Campos for facilities with the electron microscope. This work was supported by Junta Nacional de Investigação Científica e Tecnológica (JNICT, Portugal). C. P. da Silva was supported financially by grant N.655/90-ID, from JNICT, Portugal.

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