Dipropylcyclopentylxanthine triggers apoptosis in Jurkat T cells by a receptor-independent mechanism

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Abstract

1,3-Dipropyl-8-cyclopentylxanthine (DPCPX), a xanthine analog used as selective antagonist of adenosine receptors, caused apoptosis in a human leukemia T cell line. Jurkat cells treated with DPCPX underwent apoptosis as demonstrated by flow cytometry, by DNA fragmentation and by accumulation of histones, H2A, H2B, H3 and H4, in the nucleoplasm of cells. Cell cycle and cell sorting analyses indicated an arrest of cells in G_2/M followed by the appearance of apoptotic cells in G_1 and G_2/M phases. The mechanism of programmed cell death does not seem to be mediated by signal transduction events at the plasma membrane since it did not involve activation of cell membrane receptors and modification of the intracellular levels of Ca^{2+} or cAMP. Apoptosis by incorporation into DNA of a derivative of DPCPX is suggested in basis of the presence of radioactivity label in the DNA obtained from cells preincubated with [^{3}H]DPCPX.

Keywords: apoptosis; 1,3-Dipropyl-8-cyclopentylxanthine; T lymphocytes; leukemia; therapy

Abbreviations: BSA, bovine serum albumin; DPCPX, 1,3-Dipropyl-8-cyclopentylxanthine

Introduction

Apoptosis (programmed cell death) and necrosis are the two mechanisms by which nucleated eukaryotic cells die (Duvall and Wyllie, 1986). Necrosis is considered as a pathological event in response to major perturbations in the cellular environment. Necrotic cells swell leading to rupture of plasma and organelle membranes, release of cell content and loss of organized structure (Eastman et al, 1994). On the other hand, apoptosis is considered as a physiological process taking part in homeostatic regulation; in this way death is necessary in normal tissue turnover (Duvall and Wyllie, 1986; McConkey et al, 1989; Rothenberg, 1990). The mechanism(s) of apoptosis may differ from one system to another. In most cases,

apoptosis follows an increase in $[Ca^{2+}]_i$, requires protein synthesis, and is manifested by endogenous nuclease activation which results in DNA fragmentation. At the optical microscopy level, apoptosis can be suspected in cells presenting a nucleus with condensed chromatin (reviewed in McConkey et al, 1990; Cohen, 1993).

Purine compounds have been widely used in the therapy of proliferative diseases. 6-mercaptopurine and 6-thioguanine were among the first to be used (Hitchings, 1991). These compounds are metobolized in the cell and some of the resulting products interfere with DNA synthesis. Thus, replication of DNA in cancer cells does not occur and tumor cell proliferation is blocked. Recent studies have demonstrated that several purine analogs used as anticancer drugs are cytotoxic because they induce apoptosis in proliferating cells. Thus, 2-chloro-2′-deoxyadenosine and 9-β-D-arabinosyl-2-fluoroadenine are potent inducers of apoptotic death in lymphocytes from patients with chronic lymphocytic leukemia (Carrera et al, 1991; Robertson et al, 1993). The molecular mechanism of death induction is not known but Hentosh and Grippo (1994) have presented evidence that 2-chloroadenine incorporated into DNA may inhibit daughter strand synthesis thus contributing to the cytotoxic effects of 2-chloro-2′-deoxyadenosine and its triphosphate derivative (cladribine), which is also used as an antileukemic agent.

The purine nucleoside adenosine is able to cause apoptosis in human thymocytes by a mechanism that involves interaction with specific adenosine receptors located on the cell surface and which are coupled to heterotrimeric G proteins. Agonists to these receptors produce variations in the intracellular level of second messengers, cAMP and Ca^{2+}, that seem responsible for the activation of the apoptotic events (Kizaki et al, 1990; Szondy, 1994). In contrast to these findings in non malignant human T cells, adenosine triggers apoptosis in human leukemia HL-60 cells by a receptor-independent mechanism that requires the entry of the nucleoside to the cell (Tanaka et al, 1994).

In this paper we present evidence that a xanthine derivative, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), activates the apoptotic machinery in Jurkat cells, which derive from a human acute T leukemia.

Results

DPCPX-induced apoptosis in Jurkat cells

The xanthine derivative, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), is used as selective antagonist of A_1 and A_{2b} adenosine receptors present on the surface of cells. In T lymphocytes, the most abundant adenosine receptor is the A_{2b} subtype, which mediates increases in cAMP, further potentiated by activation via the receptor for the antigen (Fredholm and Sandberg, 1983; Kvanta and Fredholm, 1991).

We were interested in determining whether agonists or antagonists of adenosine receptors modulate T cell activation. We found that N-ethylcarboxamido adenosine (up to 250 μM, 24 – 72 h period), an agonist of A_{2b} receptors, did not modify the proliferative capacity of Jurkat cells whereas DPCPX had an antiproliferative effect. In fact, concentrations of 10 μM DPCPX or higher led to a marked decrease in the number of cells and in the [^{3}H]thymidine incorporation. The percentage of reduction in the number of cells in chronic treatments (up to 144 h) with 25 μM DPCPX approached 90%. This was not due to a slower rate of proliferation but to cytotoxicity since it was accompanied by a reduction of the viability of the cells. These effects of DPCPX were receptor-independent since they were not antagonized by N-ethylcarboxamido adenosine.

The presence of apoptotic bodies in cells treated for 48 h with 25 μM DPCPX led to the suspicion that the reagent initiated a death cell program in Jurkat T cells (Figure 1). The laser-beam flow cytometric analysis of untreated cells indicated a single population whereas treated cells presented two populations, one similar to that of untreated cells and the other displaying decreased forward light scattering (FSC) and increased side light scattering (SSC) (Figure 1). The combination of high side scattering and low forward scattering is an indication of apoptotic cells (Darzynkiewicz *et al*, 1992; Dive *et al*, 1992).
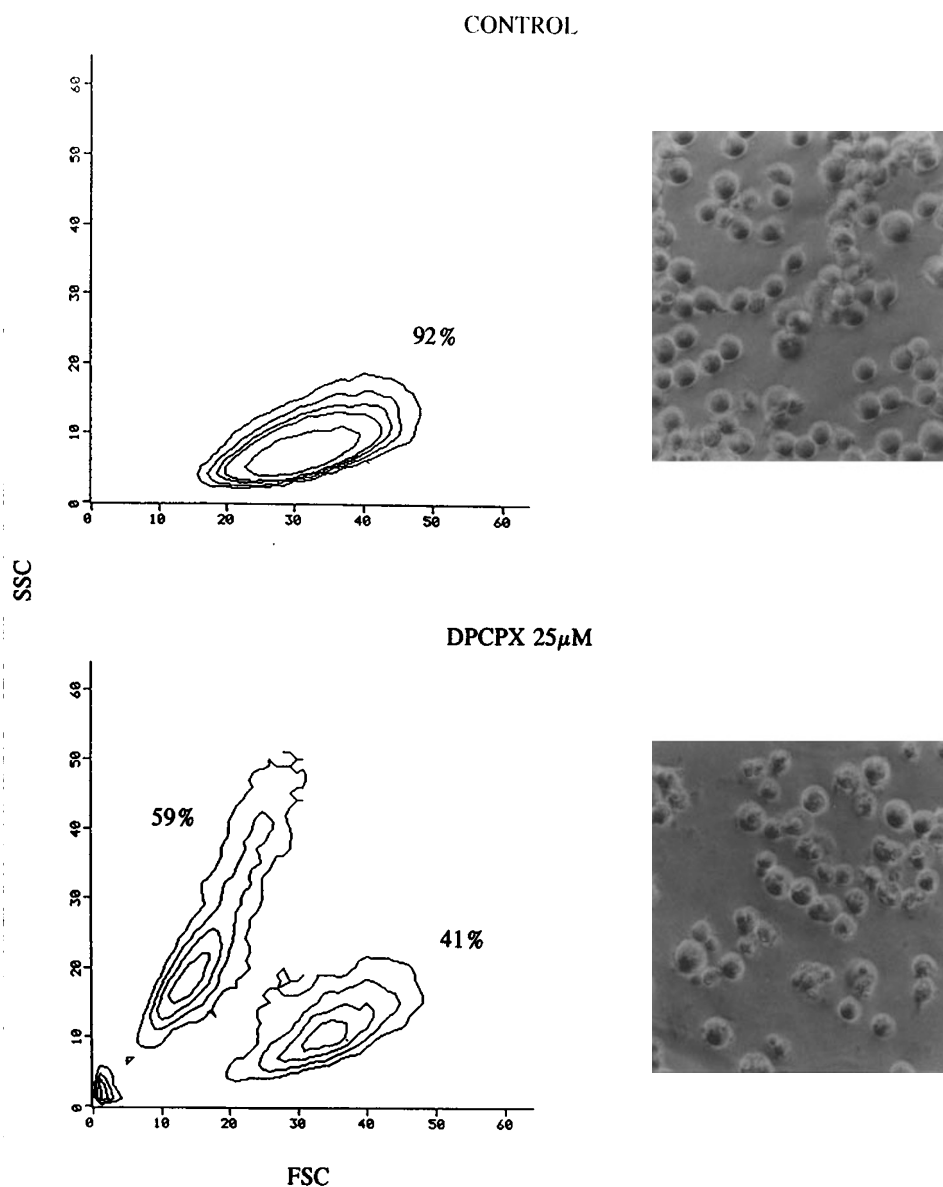
Figure 1 Morphology of DPCPX-treated Jurkat cells. The forward (cell size) versus side scatter (properties of cytoplasmic and nuclear components) distribution of naïve Jurkat cells (upper part) or cells treated with 25 μM DPCPX for 48 h (lower part). The percentage of cells in each population respect to the total amount of cells analyzed by flow cytometry is given. Inserts: micrographs representing the total population (300 ×)

To confirm the presence of DPCPX-induced apoptosis in Jurkat cells, a set of complementary experiments was carried out. First, the effect of DPCPX upon Jurkat's cell cycle was analyzed. For this purpose the DNA of the cells (untreated or treated) was labeled with Hoechst 33342 (see Materials and Methods). In control cells the pattern did not change significantly over time; the percentage of cells in G_0/G_1 phase was $37-44$ whereas the percentage in S phase was $41-44$. Treatment of cells with DPCPX led to a progressive accumulation of fluorescence near the Y-axis due to DNA fragments released from dead cells. After 8 h of treatment, DPCPX led to the accumulation of cells in the G_2/M phase (Figure 2). At a longer time (24 h) a large proportion of DPCPX-treated cells was found in a peak previous to that denoting the G_1 phase (Figure 2). This peak, which is characteristic of apoptotic cells, corresponds to cells with fragmented DNA (Gorzyca *et al*, 1993; Belloc *et al*, 1994; Zhu and Anasetti, 1995). At 48 h of treatment, the peak corresponding to cells with fragmented DNA was also present. A fourth peak prior to that representing the G_2/M phase appeared after 24 or 48 h of treatment. Thus, the xanthine analog induced the appearance of a double cell cycle with the two standard peaks representing G_1 and G_2/M phases and two extra peaks. It should be noted that similar results were obtained when, instead of Hoechst 33342, either 4,6-diamidino-2-phenylindole (DAPI) or propidium iodide were added to fixed Jurkat cells.

As described by Belloc *et al* (1994), the flow cytometric method using Hoechst 33342 is useful for simultaneous cell cycle analysis and apoptosis determination. Fluorescence emission at 395 nm and 675 nm in control cells (Figure 3A top) corresponds to cells in phases G_1 (population B2), G_2/M (population B1) and S. An image of these untreated cells labeled with both Hoechst 33342 and propidium iodide indicates no sign of cell death (Figure 3A). The image of DPCPX-treated cells labeled in the same manner indicates the presence of apoptotic cells, some already presenting

highly permeable plasma membranes. The presence of cells with a pycnotic nucleus but with an intact plasma membrane, i.e. not labeled with propidium iodide, is an indication that death comes as a consequence of apoptosis. Analysis of fluorescence at 395 nm and 675 nm in treated cells revealed B1, B2, B1′ and B2′ populations (See Figure 3A bottom); two were similar to those found in control cells (B1 and B2) whereas the other two (B1′ and B2′) emitted more fluorescence at 675 nm. A fifth population corresponding to small DNA fragments in apoptotic bodies or released to the culture medium was located near the axis origin and was not further analyzed. A cell sorter permitted the isolation of the four different populations, which were then observed under an epifluorescence microscope. All cells among B1 and B2 populations had a normal appearance. In contrast, all cells of B1′ and B2′ populations were apoptotic, as indicated by the pycnotic nucleus (Figure 3B). Thus, DPCPX produced apoptotic B1′ cells that probably derive from cells initially in the G_1 phase and apoptotic B2′ cells that probably derive from cells initially in G_2/M phase. The number of cells having a pycnotic nucleus increased with DPCPX treatment in a dose-dependent fashion and was evident already at 5 μM (at 24 or 48 h of treatment).

Apoptosis was further confirmed by the DPCPX-induced appearance of the fragmented DNA ladder and of nucleosomal histones (H2A, H2B, H3 and H4) in the nucleoplasm of treated cells (Figure 4).

Mechanism of DPCPX-induced apoptosis

To determine whether the DPCPX-induced apoptosis was mediated by signal transduction events, the intracellular level of two second messengers, Ca^{2+} and cAMP, was measured in control and treated cells. The xanthine analog (up to 25 μM) did not produce any increase in the intracellular concentration of Ca^{2+} or any variation in the
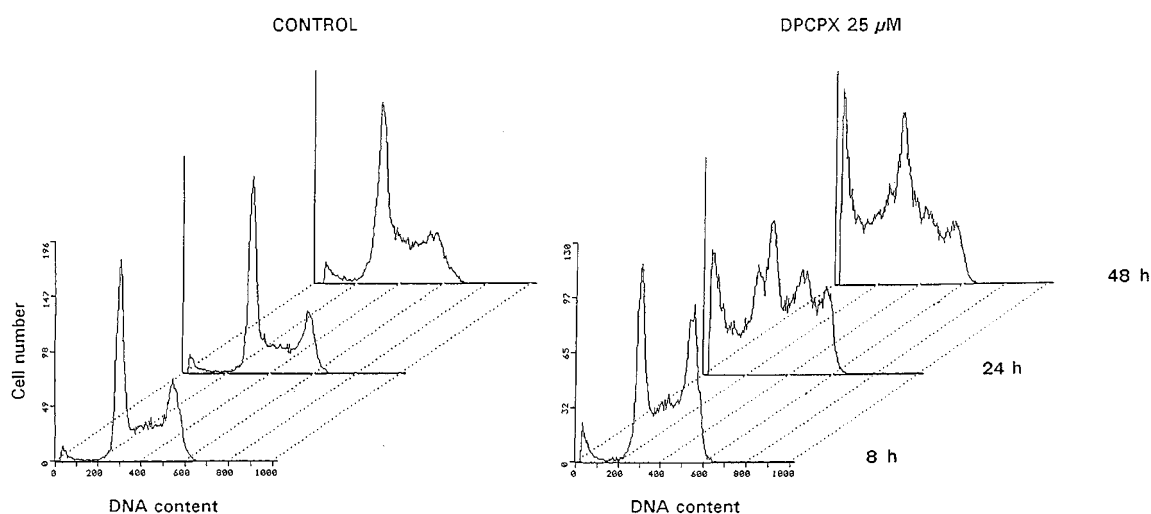

Figure 2 Cell cycle analysis of untreated or DPCPX-treated cells. Cells were treated with DPCPX for the indicated times and DNA was stained with Hoechst 33342 as described in Materials and Methods

level of cAMP. Since xanthines are often used as inhibitors of cAMP phosphodiesterases, we hypothesized that DPCPX could activate the apoptotic machinery by inhibiting phosphodiesterases IV and/or VII, which are present in Jurkat cells. To confirm this hypothesis we tested two phosphodiesterase inhibitors (500 μM rolipram or 500 μM 1,4-dihydro-2-methyl-6-oxo-3,4-bipyridine-5-carbonitrile-milrinone-) as possible inducers of programmed cell death.
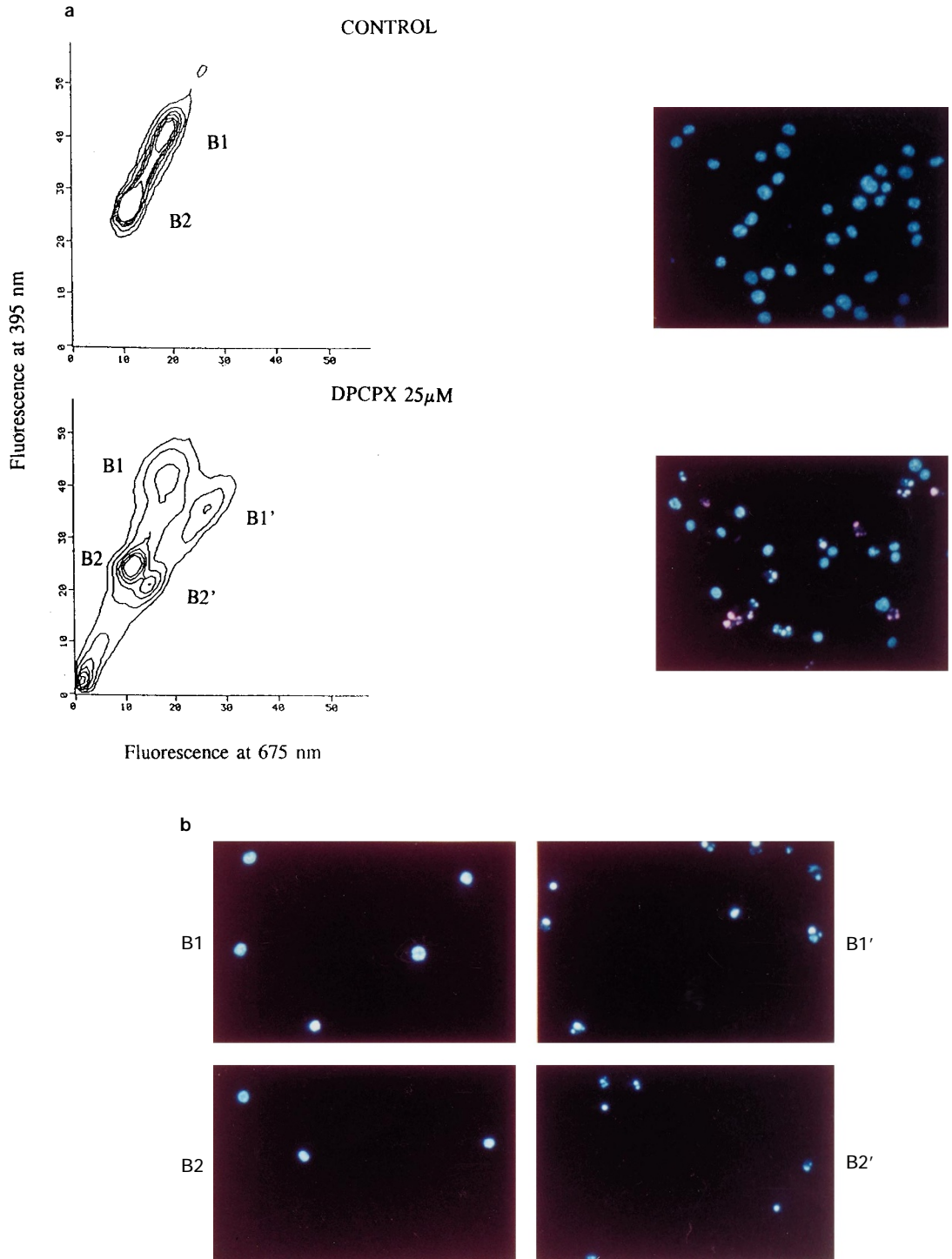
Figure 3 Nuclear morphology in DPCPX-treated cells. (**A**) Double fluorescence at 395 and 675 nm of cells (untreated or treated with 25 μM DPCPX for 24 h) labeled with Hoechst 33342 was analyzed. Insert: Image corresponding to the total cell population further labeled with propidium iodide. (**B**). Micrographs of sorted cells from populations B1, B1′, B2 and B2′ (See panel A). In this case analysed cells were not further labeled with propidium iodine

a

b
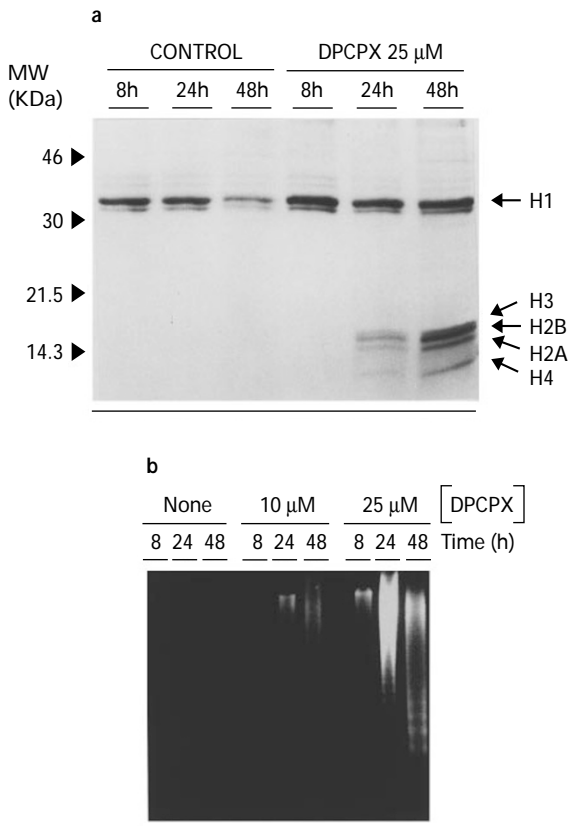
Figure 4 Biochemical changes in DPCPX-treated cells. (**A**) Polyacrylamide gel electrophoresis of nucleoplasm extracts from 5 million untreated or DPCPX-treated Jurkat cells. Histones H1, H2A, H2B, H3 and H4 are identified in the figure. (**B**) Agarose gel electrophoresis of low-molecular weight DNA extracted from 3 million untreated or DPCPX-treated Jurkat cells
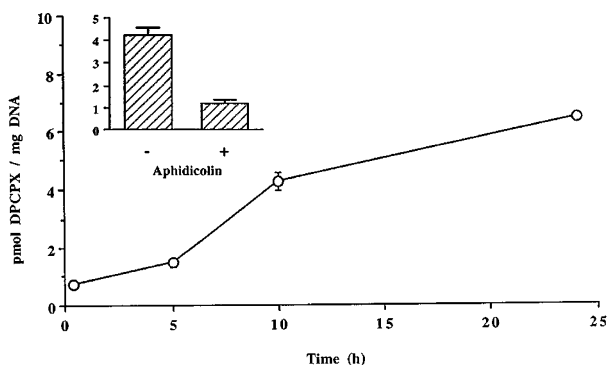
Figure 5 Accumulation of tritium from [^{3}H]DPCPX into nascent DNA. Radioactivity incorporated into DNA after incubation of cells (5 million/ml) with 22 μM DPCPX and 50 nM [^{3}H]DPCPX. Insert: Radioactivity incorporated into DNA after incubation with [^{3}H]DPCPX for 10 h in the absence or in the presence of 10 μg/ml aphidicolin

Neither compound was able to mimic the apoptosis provoked by DPCPX in Jurkat cells. On the other hand, 250 μM N-ethylcarboxamido adenosine, a nonselective agonist of adenosine receptors, did not modify the

percentage of cells with pycnotic nucleus after treatment with 25 μM DPCPX for 24–72 h; this indicates that adenosine receptors were not involved in the process.

If DPCPX does not act at the cell surface level there is a possibility that it enters the cell and affects DNA replication in a direct manner. Indeed, when tritiated DPCPX was added to Jurkat cells, there was a progressive incorporation of the radioactive label in the fraction of purified DNA (Figure 5). After 10 h of treatment, this incorporation was prevented by aphidicolin (10 μg/ml of cell culture), a specific inhibitor of replicative DNA polymerases.

Discussion

The fundamental unit of chromatin in all eukaryotic cells is the nucleosome, which is constituted of approximately 200 bp DNA. Histones H2A, H2B, H3 and H4 constitute the core of the particle and histone H1 is an extranucleosomal molecule that stabilizes DNA structure (Pederson *et al*, 1986). Adjacent nucleosomes are connected by the linker DNA which is an endonuclease-sensitive site. Programmed cell death activates nucleases, which produce DNA fragmentation and release of histones to the nucleoplasm (Laurent-Crawford *et al*, 1991). In this paper the DNA fragmentation and the release of histones induced in Jurkat cells clearly demonstrate that the xanthine analog, DPCPX, triggers apoptosis in cells from acute T leukemia. We first hypothesized that signal transduction mechanisms at the plasma membrane level were involved in the activation of the death program. The lack of involvement of cell surface adenosine receptors was demonstrated by the absence of reversion of DPCPX-induced apoptosis by the nonselective agonist N-ethylcarboxamido adenosine. It was still possible that DPCPX interacted with other molecules at the cell surface level and produced a modification in the intracellular concentration of second messengers. Two of them, Ca^{2+} and cAMP, were investigated and their levels did not change in Jurkat cells upon incubation with the xanthine analog. The possibility that apoptosis was mediated by an inhibition of phosphodiesterases was ruled out after showing that other phosphodiesterase inhibitors, rolipram or milrinone, did not reproduce the apoptotic effects of DPCPX.

A possible indication of which was the mechanism of apoptosis was given by inspecting the evolution of the cell cycle pattern in DPCPX-treated cells which first suffered an arrest in G_2/M. It is not until 24 or 48 h that the apoptotic peak, previous to that of G_0/G_1, is clearly present. A plausible interpretation of these results is that the reagent produces cell damage by a modification in the DNA. Subsequently, the cell activates the mechanisms of repair and upon their failure, it develops the death program. This interpretation is sustained by the incorporation of the radioactivity of [^{3}H]DPCPX into the purified DNA fraction. This incorporation was prevented by aphidicolin, which blocks DNA replication, and thus it is likely that a DPCPX-derived nucleotide is incorporated into nascent DNA. Replication of DNA requires deoxyribonucleotide triphosphates and thus DPCPX should first incorporate the ribose-5′-phosphate moiety probably in a phosphoribosyl pyrophosphate-dependent way, i.e. by the purine salvage

pathway. We tested hypoxanthine-guanine phosphoribosyl transferase as the activity that converts DPCPX into its ribose-phosphate derivative. The absence of inhibition by DPCPX of a commercial preparation from yeast (Sigma) and the lack of reversion of the apoptosis by hypoxanthine (which by itself had no effect, data not shown) indicate that this enzyme is not responsible for the reaction. Irrespective of the way DPCPX is incorporated into the nucleotide pool, it is not likely that a deoxynucleotide triphosphate version of DPCPX could enter into a *de novo* DNA strand. Thus, it would be possible that at some point of the intracellular metabolism of DPCPX the purine ring suffers some modification. One possibility is the incorporation of an amino group at position 2 of the purine ring, which would yield the 1,3-dipropyl-8-cyclopentyl-derivative of guanine. The deoxyribonucleotide triphosphate version of this guanine derivative would then be the compound that actually participates in DNA replication.

Belloc *et al* (1994) reported a red shift in the fluorescence emitted from apoptotic cells labeled with Hoechst 33342. When the fluorescence at 395 and 675 nm was analyzed in DPCPX-treated Jurkat cells, B1' and B2' populations appeared which were different from those found in control cells (Figure 3). These two cell populations presented a red shift and contained apoptotic cells (Figure 3). Similar red shifts in Hoechst 33342 fluorescence have been described in apoptotic rat thymocytes (Ormerod *et al*, 1993) and in antitumoral-induced apoptotic human polymorphonuclear cells (Belloc *et al*, 1994). The red shift has been used to detect 'early damaged' cells in murine cell populations and the differences in red fluorescence of intact and damaged cells have been attributed to differences in chromatin condensation (Ellwart and Dörmer, 1990).

Eastman *et al* (1994) have established the differences between physiological cell death and induced cell death. Activation of a glucocorticoid receptor or removal of a growth factor leads to 'physiological' apoptosis at the G_0/G_1 phase. In contrast, induced apoptosis can result from damage elicited at any phase of the cell cycle (Gorczyca *et al*, 1993; Eastman *et al*, 1994). The topoisomerase inhibitor, etoposide, causes an initial and rapid apoptosis in cells in phase S and a slower apoptosis in cells progressing to G_2. After 8 h DPCPX induce an arrest in G_2/M in Jurkat cells; afterwards, there is a progression towards apoptosis that seems to be irrespective of the cell cycle. Accumulation of apoptotic cells in two peaks, one previous to that of G_0/G_1 an the other previous to that of G_2/M, supports this hypothesis. These results are similar to those obtained in HL-60 cells treated with the protein kinase C inhibitor, H7, or γ-radiation, since they underwent apoptosis in G_2/M (Gorczyca *et al*, 1993). Of course, the possibility that the peak prior to that denoting the G_2/M phase would correspond to an arrest in phase S cannot be ruled out. In such a situation the results presented indicate that the apoptosis would occur at least in cells in phase G_0/G_1 and in cells in phase S. Accumulation of cells between the G_0/G_1 phase and the G_2 phase has been reported in human leukemic U937 cells treated for 13 h with idarubicine and it has been

interpreted as a block in the middle of the S phase. It should be noted that the same cells, when treated with other chemotherapeutic drugs that induce apoptosis (camptothecin or aracytine), suffered a block in the early S phase and accumulated in G_0/G_1 (Belloc *et al*, 1994). The cell death program triggered by DPCPX in leukemia-derived cells suggests that this reagent, like other apoptotic-inducing drugs, may be useful in the therapy of lymphoproliferative diseases of T origin.

Materials and Methods

Materials

1,3-dipropyl-8-cyclopentylxanthine (DPCPX) was from Research Biochemicals International (Natick, MA, USA). 2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole (Hoechst 33342), 4,6-diamidino-2-phenylindole (DAPI), propidium iodide, ethidium bromide, bovine serum albumin (BSA), N-ethylcarboxamido adenosine, Triton X-100 and RNase were from Sigma Co. (St. Louis, MO, USA) and proteinase K was obtained from Boehringer Mannheim (Barcelona, Spain). All other products were of the best grade available and were purchased from Merck (Darmstad, Germany) and Sigma. Deionized water further purified with a Millipore Milli-Q system was used throughout.

Cells and culture conditions

Jurkat cells were maintained in RPMI 1640 medium (GIBCO; Grand Island, NY, USA) supplemented with 10% (v : v) fetal calf serum (Boehringer Mannheim; Barcelona, Spain) 2 mM L-glutamine and antibiotics (GIBCO; 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone) at 37°C in a humid atmosphere of 5% CO_2. Cells were split to 5×10^5 cells/ml to begin the experiments.

Viability and proliferation assays

Plasma membrane integrity was assessed in triplicate by trypan blue or propodium iodide exclusion and cell density was determined with a Coulter Multisizer particle counter (Coulter Corporation). [³H]thymidine incorporation assays were carried out in 96-well microtiter plates with flat-bottomed wells (Corning) in a total volume of 200 μl. Jurkat cells were incubated in triplicate with different concentrations of DPCPX for 24 h at 37°C in a 5% CO_2 humified atmosphere. At those times, 1 μCi of [³H]thymidine (24 Ci/mmol; Amersham) was added and cells were further incubated for 8 h before harvesting onto fiberglass filters. The amount of tritium incorporated into cellular DNA was assessed by scintillation counting using a Packard model TRI-CARB 1600 TR liquid scintillation counter.

Assessment of apoptosis by biochemical techniques

For analysis of internucleosomal DNA damage, cells (untreated or treated) were centrifuged at 200 × g for 15 min at 4°C. Cell pellets were then treated with TTE solution (10 mM Tris/HCl pH 7.4, 1 mM EDTA and 0.2% Triton X-100), vigorously vortexed and centrifuged at 13 000 × g for 10 min at 4°C in order to separate fragmented DNA from intact chromatin. 0.1 M NaCl was then added to supernatants, which contained low molecular weight DNA, and precipitation was carried out overnight at −20°C with isopropanol.

Samples were then microcentrifuged at 13 000 × g for 15 min at 4°C, and DNA pellets were washed with 70% ethanol, air dried and resuspended in TE buffer (10 mM Tris/HCl pH 7.4, 1 mM EDTA) with 100 μg/ml RNase. DNA samples were analyzed by electrophoresis on 1.4% agarose gels containing 0.5 μg/ml ethidium bromide at 40 V for 1.5 h. DNA ladder was observed under ultraviolet light. For nucleoplasm isolation and histone identification by polyacrylamide gel electrophoresis the method of Laurent-Crawford *et al* (1991) was used.

Assessment of apoptosis by flow cytometry

Cells were stained in the culture medium with 5 μg/ml Hoechst 33342. After 30 min at 37°C, cells were analyzed with an Epics Elite flow cytometer (Coulter Corporation; Hialeah, FL, USA). Hoechst 33342 was excited with the 333–364 nm lines of an argon ion laser at 25 mW and fluorescence was collected with 675 nm and 395 nm band-pass filters. For sorting experiments, cells were stained with the same procedure, and resuspended at 5×10^6 cells/ml just before analysis and separation. Sorted populations were observed under a Zeiss Axioskop epifluorescence microscope (Germany) and photographs were taken with a Kodak-Ektachrome 160 T film. Propidium iodide and DAPI were also used to analyze cell cycle distributions. In both cases, aliquots of cells were removed from control and treated cultures at appropriate times and fixed in suspension in 70% ethanol at −20°C. Following fixation, 10^6 cells were washed with PBS/BSA (PBS containing 1% (w/v) bovine serum albumin). For propidium iodide staining, fixed cells were then resuspended in 1 ml PBS/BSA containing 10 μg/ml propidium iodide and 0.1% RNase. Propidium iodide excitation was obtained with an argon ion laser adjusted to deliver 15 mW at 488 nm, and fluorescence was detected using a 675 nm band-pass filter. When DAPI was used, cells were resuspended in 0.4 ml of a solution containing 150 mM NaCl, 80 mM HCl and 0.1% Triton X-100 and kept for 10 min at 0–4°C. Then 2 ml of 180 mM Na_2HPO_4, 90 mM citric acid, 1 μg/ml DAPI, pH 7.4, were added to each sample and fluorescence was measured. A UV laser with an excitation beam of 25 mW at 333–364 nm was used, and fluorescence was collected with a 525 nm band-pass filter. 20 000 cells were counted for each histogram and cell cycle distributions were analysed with the Multicycle program (Phoenix Flow Systems, Inc.; San Diego, CA, USA).

Incorporation of [³H]DPCPX into DNA

Jurkat cells were split to 5×10^6 cells/ml in fresh medium containing 22 μM DPCPX and 50 nM [³H]DPCPX (109 Ci/mmol; New England Nuclear). At 0, 5, 10 and 24 h, triplicate 1 ml suspension samples were withdrawn for measurement of [³H]DPCPX incorporation into DNA. In parallel, cells were preincubated in the presence of 10 μg/ml aphidicolin (Sigma) for 1 h before the addition of DPCPX/[³H]DPCPX. Samples were collected after 10 h of treatment with the drug and DNA was purified as follows. Cells were centrifuged at 1400 r.p.m. for 5 min at room temperature and washed twice in 50 mM PBS pH 7.4. Cell pellets were gently resuspended in 500 μl of Hirt solution (0.6% SDS, 10 mM EDTA, 10 mM Tris/HCl pH 7.4 and 100 μg/ml proteinase K) and incubated for 1 h at 37°C. Then 1 M NaCl was added and samples were further incubated for 1 h at 0°C. The suspensions were centrifuged at 15 000 r.p.m. for 30 min at 4°C in a microfuge and the supernatants were extracted with one volume of phenol/chloroform (1:1) and one volume of chloroform. DNA was precipitated overnight at −20°C with two volumes of ethanol and 0.1 volume of 3 M sodium acetate pH 5.2. DNA pellets were then washed with 70% ethanol, allowed to dry in air and resuspended in 50 μl of TE

buffer. 40 μl of each sample was added to 10 ml of Formula 989 scintillation cocktail (New England Nuclear) and radioactivity of vials was counted. The nucleic acid content of each sample was determined spectrophotometrically by measuring absorbance at 260 nm. Results of total DPCPX incorporation into DNA were expressed as pmol/mg DNA after correction for the DPCPX/[³H]DPCPX ratio.

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