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Quantitation of Apoptosis Induction by Etoposide or Hydroxyurea in Mouse Interleukin 3-dependent Lymphoma Cells

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Abstract. *Background: The apoptosis induction by etoposide or hydroxyurea in mouse interleukin 3-dependent lymphoma cells (DA-1) was studied. Treatments with 1.25 mM hydroxyurea and 100 μM etoposide for 17 hours were considered appropriate concentrations for such studies. Materials and Methods: The viability of the cells was studied by propidium iodide exclusion. The presence of apoptotic bodies was observed, and DNA fragmentation of apoptotic cells was asessed by the observation of ladder patterns in agarose electrophoresis gels and an increase in the proportion of cells with subdiploid DNA content in cytometric studies. Results: Alterations of membrane properties, DNA degradation and chromatin condensation highlighted a possible apoptotic process induced by these two antitumor agents. Conclusion: These studies have established conditions for examining apoptotic processes in interleukin 3-dependent lymphoma cells. This is of great interest for deeper analysis of the mechanisms involved in the apoptosis of these cells after interleukin 3 depletion or treatment with cytotoxic antitumor compounds.*

We analyzed the apoptotic process induced by either etoposide or hydroxyurea in DA-1 lymphoma cells. The study of the apoptotic process in this murine cell line is of interest because of its sensitivity to the action of antitumor and toxic compounds. Also, the influence of interleukin 3 (IL-3) on apoptosis of lymphoid cells can be studied in this IL-3-dependent lymphoid cell line.

Abbreviations: IL-3, Interleukin 3; FITC, fluorescein isothiocyanate; PCD, programmed cell death.

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Programmed cell death (PCD) is a physiologically regulated process that culminates in irreversible cessation of biological activity. Although PCD usually occurs *via* apoptosis, there are also other atypical patterns of PCD that are different from apoptosis. Apoptosis is a form of active cell death characterised by the concurrence of several features such as changes in cell morphology, DNA degradation and alterations in plasma membrane structure and transport function. The assessment of several of these features allows definition of the apoptotic nature of the process of cell death by excluding processes of accidental cell death (necrosis) and other processes of atypical programmed cell death.

Simultaneous use of several methods is needed to demonstrate cell apoptosis in physiological and pathological conditions. Basically, microscopy analyses revealing nuclear fragmentation, fluorescent methods to demonstrate alterations in cell membrane permeability and composition and methods to study DNA fragmentation should be performed to reveal the apoptotic mechanism of the cell death process.

Lack of growth factors and cytokines can induce apoptosis in different cell lines. In principle, they exert a protective effect against the apoptosis induced by antitumor compounds in bone marrow, leukemic cells, *etc.* (1,2). IL-3 has colony-stimulating activity on a variety of hemopoietic cell lineages (3-6). Induction of apoptosis in tumor cells is the main mechanism of action of several chemotherapeutic agents (7-10). Etoposide belongs to the group of podophylotoxin molecules and it is claimed to inhibit the action of topoisomerase II (11,12). A different regulation of apoptosis induction by etoposide in different cell types has been considered (12).

Hydroxyurea is a compound that acts on ribonucleoside diphosphatereductase and inhibits DNA synthesis. This reagent is used in therapy for treatment of some kinds of leukemia cells (13). At high concentration, hydroxyurea produces toxic effects promoting cell death (14, 15). The mechanism through which hydroxyurea mediates apoptosis is not clearly known.

We analyzed the induction of apoptosis by cytotoxic compounds such as etoposide or hydroxyurea in IL-3-

dependent lymphoma cells. We established the conditions (time dependence, drug concentrations, *etc.*) to study apoptosis induction. Also, the effects subsequent to IL-3 depletion were studied.

Materials and Methods

Cell lines. Two cell lines were used: Mouse interleukin 3-dependent lymphoma DA-1 were kept at 37˚C and 5% CO_2, in Iscove's MDM medium containing 10 % fetal calf serum, 2 mM L-glutamine and 2.5 x 10^{-5} M β-mercaptoethanol. A cell density of 2 x 10^5 cell/ml was used. The WEHI-3B cell line was used as a source for production of conditioned medium containing IL-3 that is required for the survival of DA-1 cells. IL-3-containing conditioned medium was obtained by centrifugation at 1200 rpm for 5 min and subsequent filtration. DA-1 cells were grown in a medium containing 5% of this conditioned medium.

Cell viability. The cell viability of DA-1 cells incubated in the presence and absence of IL-3 and treated with etoposide or hydroxyurea was determined, using exclusion of Trypan blue in an hemocytometer.

Cell treatments with etoposide or hydroxyurea. DA-1 cells were incubated at a density of 2x10^5 cells/ml for 17 h at 37˚C, with 5% CO_2, under the following conditions: DA-1 cells grown in the presence of 5% of conditioned medium containing IL-3, DA-1 cells grown in the absence of IL-3-conditioned medium, DA-1 cells grown in culture with 5% IL-3-conditioned medium and 1.25 mM hydroxyurea, DA-1 cells grown in the absence of IL-3-conditioned medium and 1.25 mM hydroxyurea, DA-1 cells grown in 5% IL-3 medium and 100 μM etoposide, DA-1 cells grown in the absence of conditioned medium and 100 μM etoposide.

Permeability of treated cells to propidium iodide. 10^6 treated cells were harvested and washed twice in PBS, centrifuged at 1200 rpm for 5 min, resuspended in 500 μl of PBS, stained with propidium iodide (0.1 mg/ml) and incubated for 5-10 min in the dark. The samples were analysed in a FACScan cytometer.

Fluorescence microscopy analyses. Cells treated as indicated above were centrifuged at 1200 rpm for 5 min. Pelleted cells were resuspended in PBS containing Hoescht 33342 (1 μg/ml) and incubated for 15 min at 37˚C and 5% CO_2 in the dark. After that incubation, the cells were examined by fluorescence microscopy and phase contrast light microscopy at an amplification of 400 x.

DNA fragmentation analyses. 10^7 cells were used per assay. After treatment of DA-1 cells, they were washed with PBS and centrifuged at 1500 rpm for 5 min. The pellet was resuspended in 250 ml of PBS and 250 ml of lysis buffer containing 20 mM EDTA, 0.5% Triton X-100, 5mM Tris pH 8.0. The mixture was incubated for 15 min at 4˚C, which allows cell membrane lysis and the release of the cytoplasmic content. Afterwards, it was centrifuged at 1000 rpm for 10 min. The supernatant was collected and used for DNA purification. The pellet was centrifuged again at 13000 rpm for 30 min. This final pellet contained remnant intact nuclei.

The supernatant used for DNA purification was treated with 2-2.5 volumes of absolute ethanol (kept at –80˚C) for 1-2 h. It was centrifuged at 13000 rpm at 4˚C for 15 min. The supernatant was discarded and the pellet was resuspended in 200 μl of TE buffer (1 mM EDTA, 10 mM Tris pH 8.0). The mixture was first incubated at 37˚C for 30 min with RNAse A 0.1 mg/ml and subsequently at 37˚C for 2-3 h with Proteinase K 0.25 mg/ml. To this mixture an equal volume of phenol pH 8.0 was added. The sample was gently mixed and centrifuged at 12000 rpm for 2 min. To the collected upper phase, an equal volume of a mixture 1:1 of phenol/chloroform-isoamilic alcohol (24:1) was added. The sample was mixed gently for 1 min and centrifuged at 12000 rpm for 2 min. The aqueous phase was recovered and treated again as indicated above. The obtained aqueous phase was recovered and treated with chloroform-isoamilic alcohol (24:1) for 1 min and centrifuged at 12000 rpm for 2 min. The final aqueous phase was used for DNA precipitation by addition of 1/10 volumes of 3 M sodium acetate pH 5.3 and 2.5 volumes of cold absolute ethanol. The samples were kept at –80˚C for 1-2 h and centrifuged at 12000 rpm for 15 min. The supernatant was discarded and the pellet was dried and resuspended in 20 ml of TE buffer. The total amount of DNA was determined spectrophotometrically.

DNA (10 mg/sample) was subjected to electrophoresis DNA in 1.5% agarose-gels in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.0) using molecular weight markers (100 bp ez Load Molecular Ruler 170-8352 from Bio-Rad Laboratories, Inc. Hercules, CA, USA). Electrophoresis was run at 100 volts for 1 h in TAE buffer. After electrophoresis, the gel was stained with ethidium bromide, the bands were observed using UV light and the gel was photographed.

Analyses of phosphatidylserine residues in cell membrane. 10^6 treated cells were washed with PBS and resuspended in Ca^{2+} binding Hepes buffer (10mM Hepes, 150 nM NaCl, 1mM $MgCl_2$, 1.8mM $CaCl_2$, 5mM KCl, pH=7.4). Cells were labelled with Annexin V-FITC in Hepes buffer. Subsequently, the cells were labelled with a solution of Annexin V-FITC diluted in Hepes buffer and incubated for 10 min to identify apoptotic cells. Finally, to the cell suspension 100 μl of a dilution 1/100 (vol/vol) of microparticle of 6 mm (CALIBRITE microbeads) in Ca^{2+} binding buffer plus 0.05% (w/v) gelatine was added and it was analyzed by flow cytometry.

Flow cytometry analyses. The acquisition by flow cytometry was done using FACScan or FACScalibur (Becton Dickinson, San Diego, CA, USA). For each sample containing microparticles, the acquisition was finished when 2000 particles had been counted. For data analyses, the program CellQUEST was used. In sample analyses, cell fragments were discriminated from the nonviable cells with dot plot FSC/FL-2-H where FL-2-H corresponds to the fluorescence associated to propidium iodide. In experiments of cell permeability to propidium iodide, the number of cells was represented in correspondence to the fluorescence of propidium iodide. To establish the total number of events that corresponds to each cell state, the cell population was delimited considering the fluorescence of propidium iodide in comparison to that of annexin V-FITC.

Cytometric analysis of cells with subdiploid DNA content. Apoptotic cells were counted on the basis of low molecular weight DNA, as described by Nicoletti *et al.* (16) and modified by Prieto *et al.* (17).

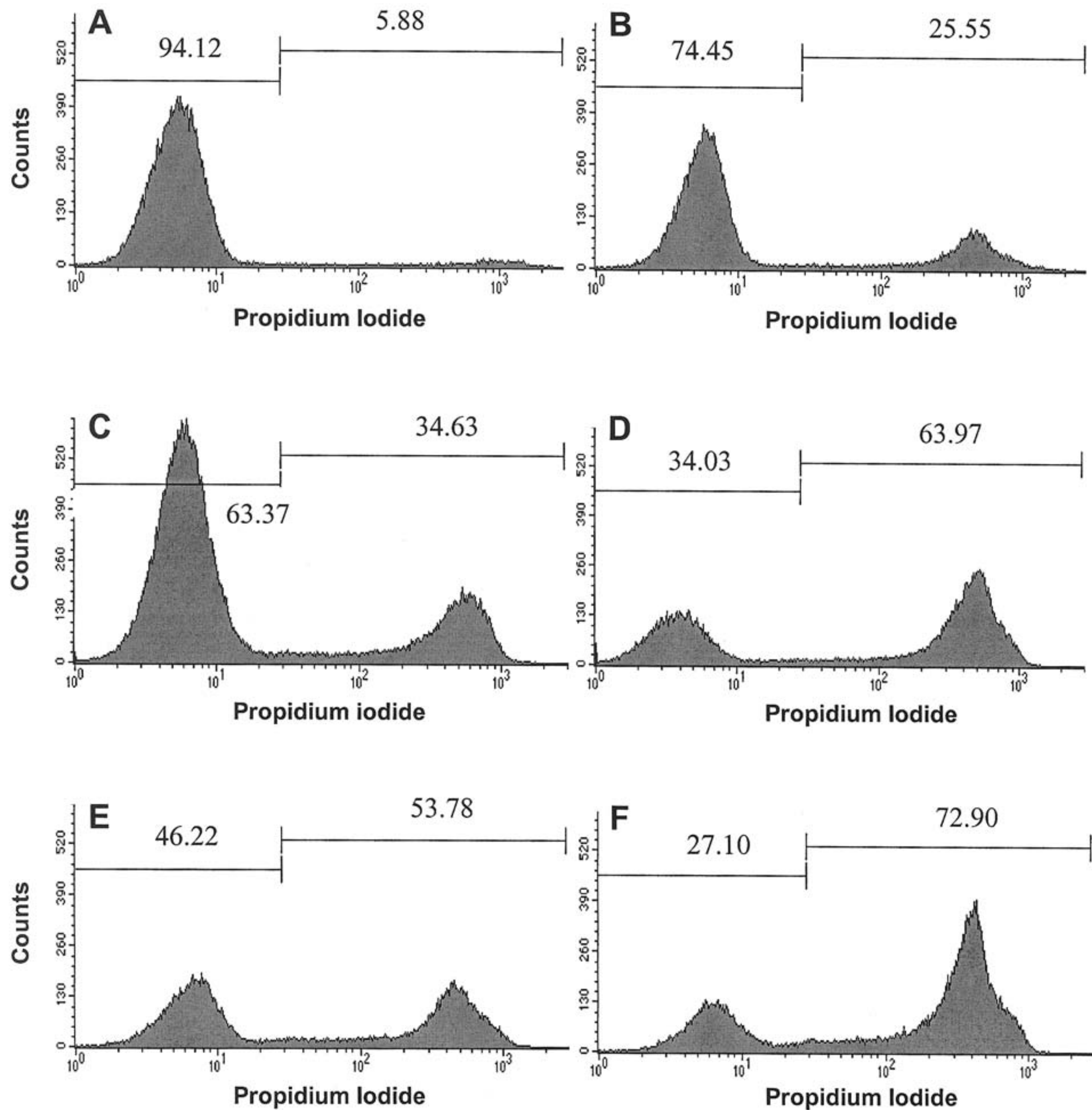

Figure 1. *Cell viability measured as cell permeability to propidium iodide. Percentage of living cells was estimated in DA-1 cells incubated for 17 hours in different conditions: A) in the presence of IL-3; B) in the absence of IL-3; C) in the presence of 1.25 mM hydroxyurea and IL-3; D) in the presence of 1.25 mM hydroxyurea without IL-3; E) in the presence 100 μM etoposide and IL-3; F) in the presence of 100 μM etoposide without IL-3. The percentages of both viable and non viable cells are shown in each histogram. The curve on the left side of the panels represents viable cells. The curve on the right side of the panels represents non viable cells.*

After treatments with etoposide or hydroxyurea, $5x10^5$ cells were collected and washed twice with PBS. The pellet was resuspended in 700 μl of a solution containing 0.1% sodium citrate, 0.1 % (v/v) Triton X-100 in order to isolate low molecular weight DNA from apoptotic cell nuclei. The remnant DNA in cells was stained with 5 μg/ml of propidium iodide. The samples were incubated at 4˚C for 1 hour in the dark.

Immediately before measuring the fluorescence in the cytometer, 100 μl of a solution of microparticles (dilution 1/100 (v/v) of CALIBRITE microbeads in 0.9% NaCl and 0.05% gelatine were added.

Cells with hypodiploid DNA content (apoptotic cells) were distinguished from those containing diploid DNA (non apoptotic cells) on the basis of the lower fluorescence intensity of the propidium iodide bound to their DNA.

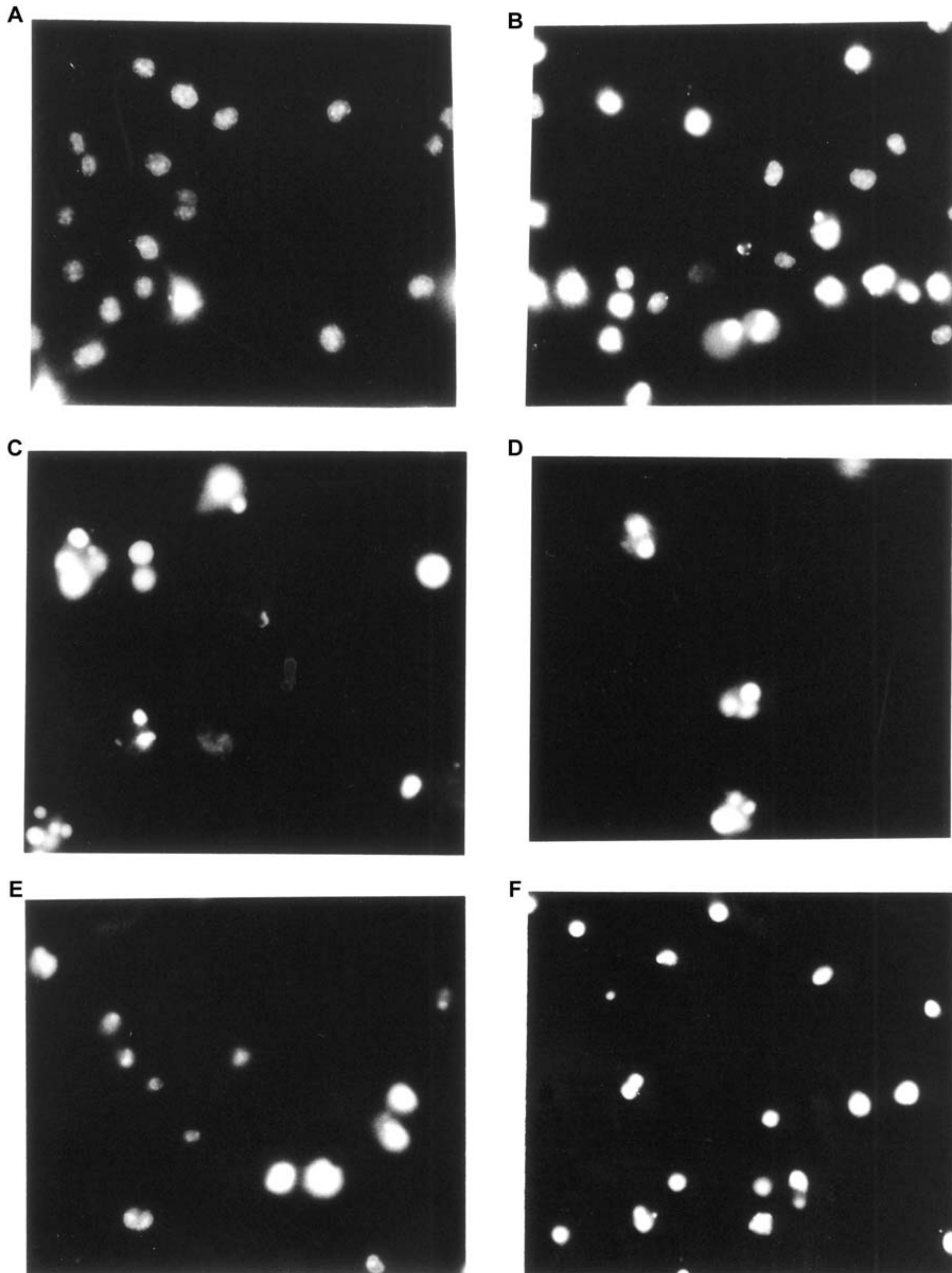

Figure 2. *Fluorescent microscopy analyses of DA-1 cells grown in different conditions. A) in the presence of IL-3; B) in the absence of IL-3; C) in the presence of 1.25 mM hydroxyurea and IL-3; D) in the presence of 1.25 mM hydroxyurea without IL-3; E) in the presence 100 μM etoposide and IL-3; F) in the presence of 100 μM etoposide without IL-3. Hoescht was used as fluorescent dye and nuclear fragmentation was visualized by the presence of apoptotic bodies.*

Results

Cell survival. The viability of DA-1 cells was determined using trypan blue staining and propidium iodide permeability. Removal of IL-3 from the medium reduced cell survival as expected. We chose 17 h of cell treatment with 1.25 mM for hydroxyurea and 100 µM for etoposide as appropriate conditions to study apoptosis in DA-1 cells since approximately a 50% cell survival was obtained in both cases.

Figure 1 shows the number of viable cells as a function of the fluorescence of propidium iodide. It reveals an increment of cell permeability to propidium iodide as tested by its fluorescence in cells subjected to hydroxyurea or etoposide treatment, confirming the data observed by microscopy in cells stained with trypan blue. Under the conditions used, etoposide treatments (Figure 1 E and 1 F) produced more dead cells than hydroxyurea (Figure 1 C and 1 D). Also, a different extent of propidium iodide permeability was observed depending on the presence or absence of IL-3 in the medium for hydroxyurea and for etoposide treatments.

Cytometric analyses of the presence of phosphatidylserine residues in the outer side of the cell membrane. Phosphatidylserine residues in the outer surface of the cell membrane were detected using annexin V-FITC, whose fluorescence allows one to identify the presence of such residues (not shown). Apoptotic cells can be detected. Cell viability accounted for 50% and 27% in samples treated with hydroxyurea for 17 h in the presence and absence of IL-3, respectively, and for 22% and 4% in samples treated with etoposide for 17 h in the presence or absence of IL-3, respectively.

Formation of apoptotic bodies. Treatments with 1.25 mM hydroxyurea or 100 µM etoposide for 17 h also produced changes in the morphology of the nuclei. To observe chromatin fragmentation, cells were stained with Hoescht and observed by fluorescent microscopy.

Figure 2 shows DA-1 cells grown in different conditions. IL-3 withdrawal gave rise to fragmentation of nuclei. Nuclear fragments were quickly visible after incubation with Hoescht. Nuclei appeared very bright and fragmented. Also, a modification of the morphology of the cell membrane was observed by phase contrast microscopy (see also Figure 2). Either etoposide or hydroxyurea cell treatments are a cause of nuclear fragmentation and changes in cell morphology characteristic of an apoptotic mode of cell death (Figure 2).

DNA fragmentation. DNA from DA-1 cells treated with etoposide or hydroxyurea was extracted. Subsequently, it was analyzed by agarose (1.5 %) gel electrophoresis (Figure 3). DNA ladder formation was observed under the conditions studied, *i.e.* etoposide or hydroxyurea treatments or depletion of IL-3. Either IL-3 depletion or treatments with either of the two compounds used in this study produced significant ladder fragmentation (Figure 4).

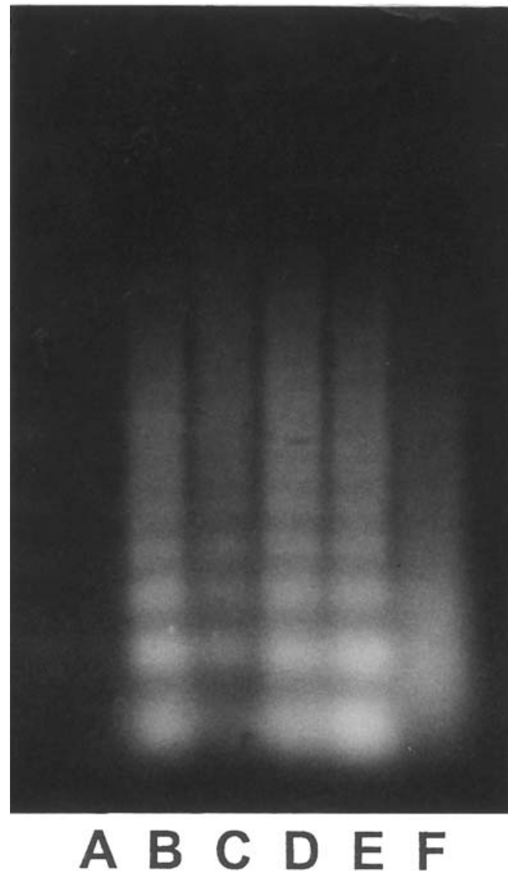

Figure 3. *Electrophoresis analyses of DNA fragmentation of DA-1 cells treated with etoposide or hydroxyurea. DNA was extracted and analyzed by agarose (1.5 %) gel electrophoresis loading 10 µg of DNA per well. DNA ladder formation was observed in the conditions studied: A) in the presence of IL-3; B) in the absence of IL-3; C) in the presence of 1.25 mM hydroxyurea and IL-3; D) in the presence of 1.25 mM hydroxyurea without IL-3; E) in the presence 100 µM etoposide and IL-3; F) in the presence of 100 µM etoposide without IL-3.*

Cytometric analyses of apoptotic cells: proportion of cells with hypodiploid DNA content. Apoptotic cells were analyzed by flow cytometry to measure the number of cells containing diploid or hypodiploid DNA content. A higher proportion of cells with hypodiploid DNA contents correspond to higher levels of apoptosis

The effects of hydroxyurea (1.25 mM) or etoposide (100 µM) for different times (4, 8 and 17 h) were studied (Figure 4). In the case of hydroxyurea, this apoptotic effect was clearly lower than in the case of etoposide (See also Figure 4). Incubation for 4 h in the presence of hydroxyurea or etoposide showed maintenance of DNA contents (Figure 4 a). However, at 8 h of drug treatment, differences between the action of etoposide or

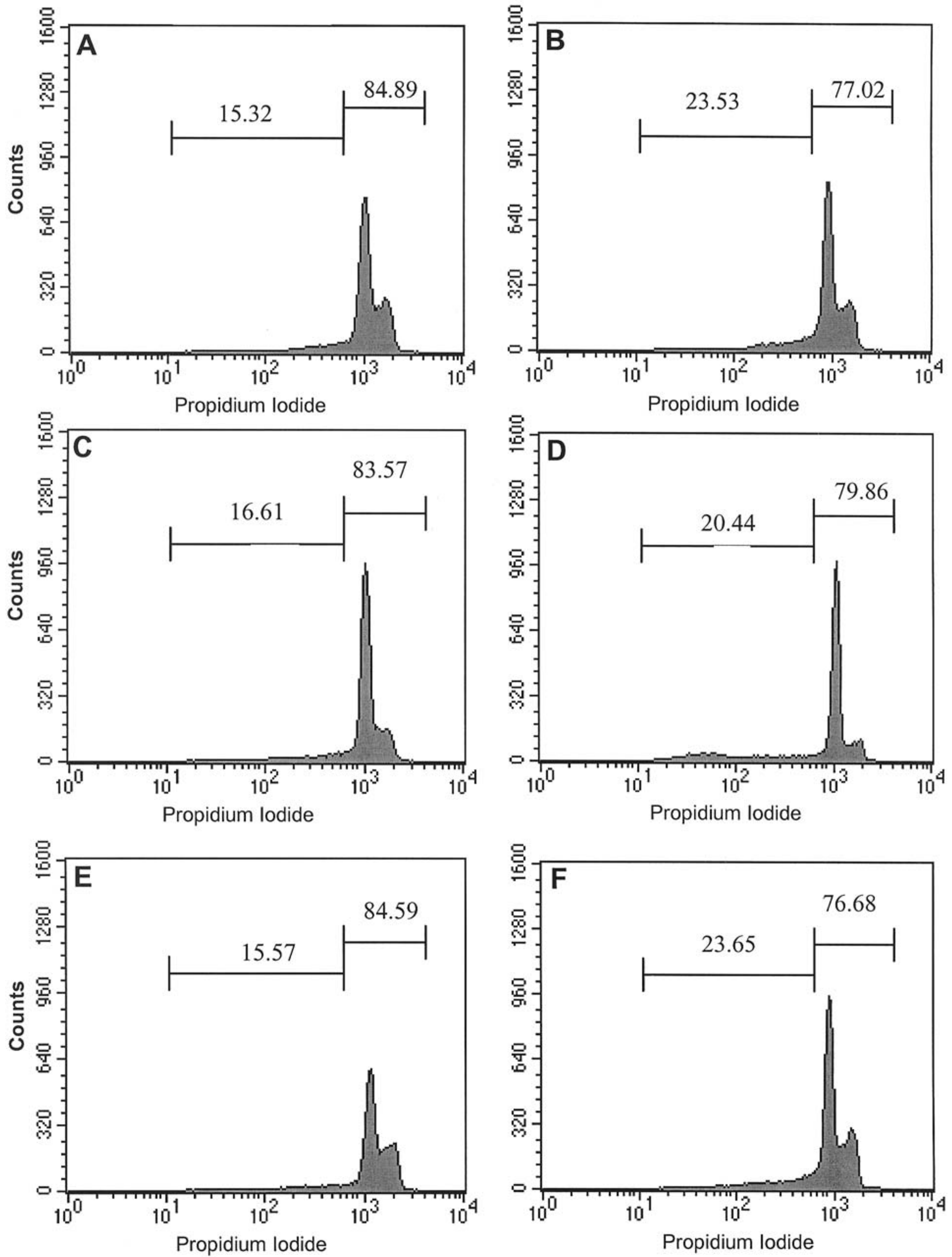

Figure 4a

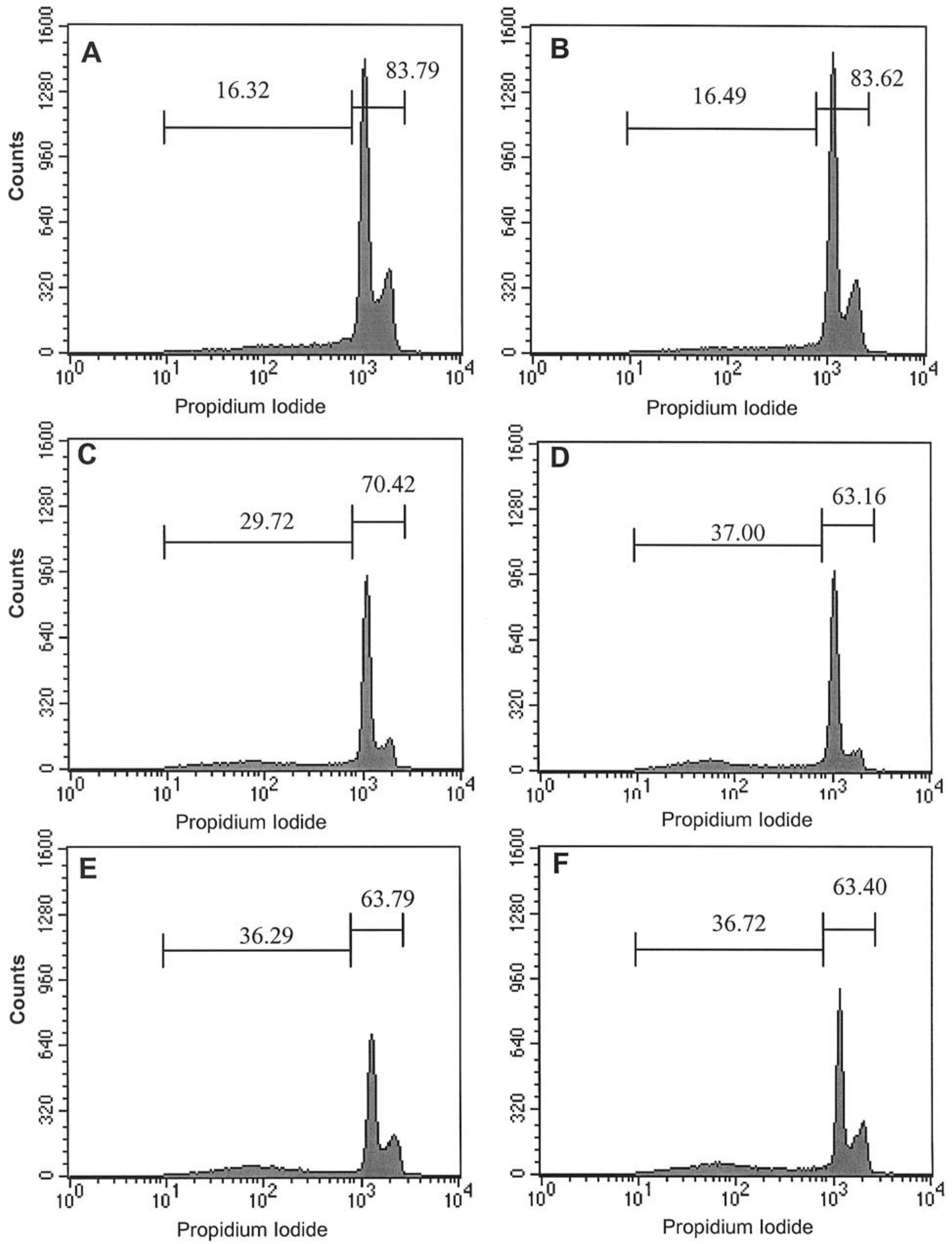

Figure 4b

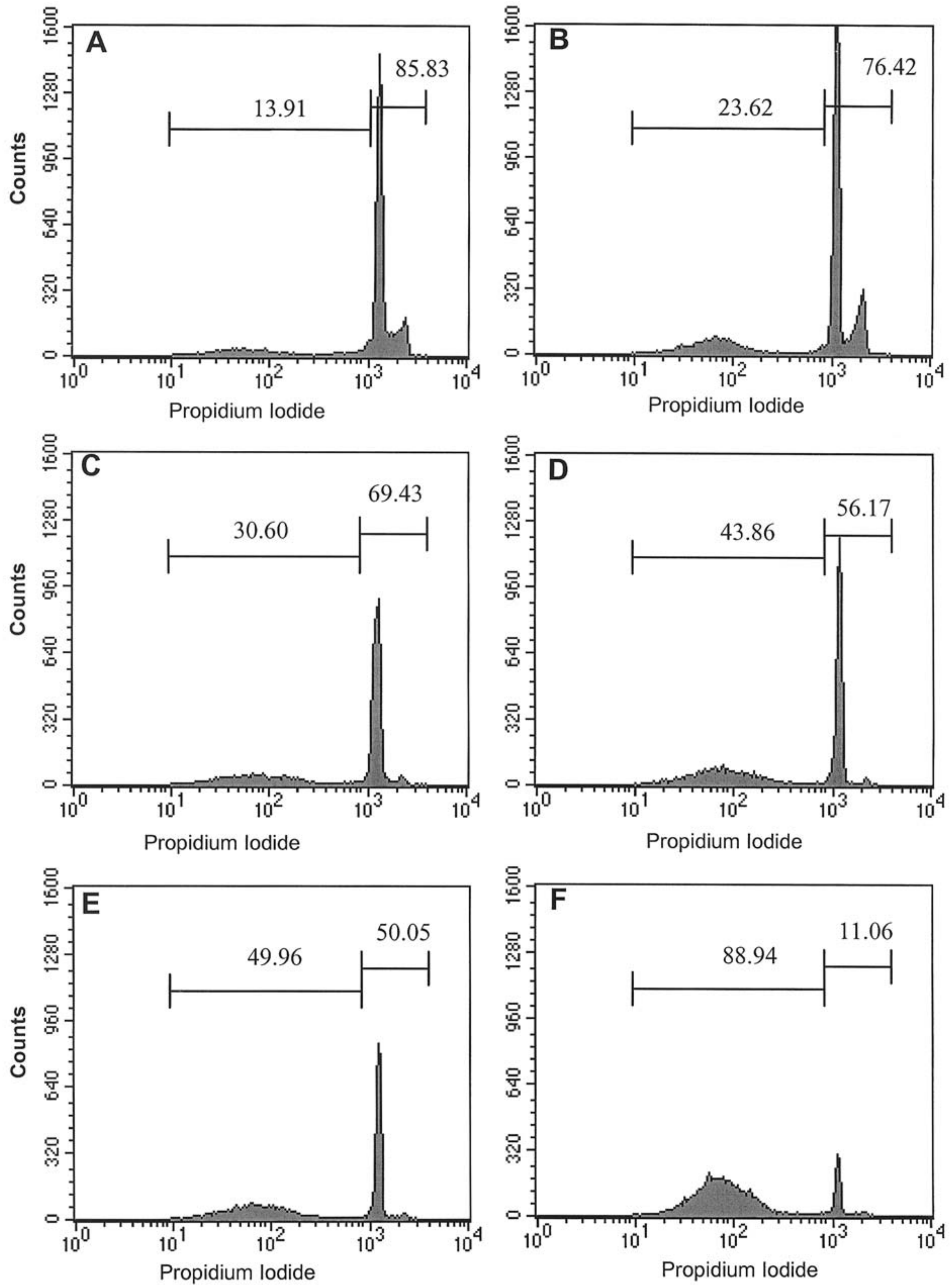

Figure 4c

hydroxyurea were observed (Figure 4 b). The proportion of cells with diploid DNA content represents 63% of the total cellular DNA in samples treated with hydroxyurea in the absence of IL-3 (Figure 4 b D). This value is lower than the value obtained for DA-1 cells treated with hydroxyurea in the presence of IL-3 (70.4%). In contrast, cells treated with etoposide in the presence or absence of IL-3 for 8 h showed the same proportion of cells with diploid DNA content (around 63.5%) (Figure 4 b E and F). The relative proportion of the peak corresponding to cells with hypodiploid DNA content increased as longer times were used. This effect was clearly visible after treatment with etoposide for 17 h (see Figure 4c). At this time of incubation, the proportion of cells with diploid DNA content decreased up to 11% for etoposide treatments in the absence of IL-3 (Figure 4c F). The presence of IL-3 in the medium increased this value up to 50% (Figure 4c E). Also, differences were observed between cells treated with hydroxyurea in the presence or absence of IL-3 (Figure 4 c C and D) but, in this case, the difference between these values was lower than in the case of etoposide.

Discussion

In the present work, we studied the effects of two antitumor compounds on DA-1 murine lymphoid cells that are dependent on IL-3 addition to the medium and IL-3 withdrawal can produce some apoptotic effects (18,19). The results obtained with the different methods to study DA-1 cell death processes demonstrated that apoptosis is the process responsible for the death of these cells induced by both cytotoxic agents and IL-3 deprivation.

The DA-1 cells that died in response to either serum deprivation or addition of either hydroxyurea or etoposide showed phosphatidylserine mislocation, nuclear fragmentation and DNA fragmentation, which are features of typical apoptosis.

Propidium iodide permeability measurement as a method to determine cell survival studies demonstrated that 1.25 mM hydroxyurea or 100 μM etoposide are appropiate concentrations to allow apoptosis analyses in this cell line (Figure 1). Similar concentrations of these reagents have been used in other cell systems (9,12,20). Reduction of the proportion of apoptotic cells in samples treated either with hydroxyurea or etoposide was observed when IL-3 was included in the medium in comparison with samples depleted of IL-3 (Figure 1).

Membrane alterations were also observed as changes in phosphatidylserine residues distribution in treated cells in comparison with control DA-1 cells (not shown). Fluorescence associated to Annexin V labelling demonstrated, clearly, the presence of apoptotic processes under the action of etoposide, since the percentage of cells in intermediate apoptosis after a treatment of 17 hours accounted for 62.2% in the presence of IL-3. This is a similar behavior to that shown by human cell types like Jurkat cells (21). The effect of hydroxyurea at the concentration used was lower, accounting for around 38.2% of cells in intermediate apoptosis after incubation of 17 hours in the presence of this compound and IL-3.

Apoptosis induction by cytotoxic treatments and IL-3 withdrawal was also confirmed by fluorescence microscopy (Figure 2). The formation of apoptotic bodies clearly revealed nuclear fragmentation characteristic of apoptotic processes in both cases. Activation of Ca^{+2}- and Mg^{+2}-dependent endonuclease activities correlates with DNA fragmentation in apoptosis. Drug treatments have been shown to induce DNA fragmentation in some kinds of cells (7,10,12). Although some authors have claimed that cell apoptotic markers do not correlate with cell survival levels (21), the presence of ladder DNA fragmentation patterns in agarose gel electrophoresis in all the conditions used revealed induced apoptosis processes in these lymphoma cells (Figure 3). DNA fragmentation induced by IL-3 withdrawal, etoposide or hydroxyurea treatments was clearly observed (Figure 3). DNA fragmentation can be correlated with other apoptotic changes such as activation of caspases activity, as has been demonstrated in Jurkat cells treated with etoposide and different radiations (21). A possible alteration of caspase activity seems to be likely and we are currently studying caspase involvement in such a process.

The time-dependent appearance of cells with hypodiploid DNA content after Low Molecular Weight DNA extraction correlated with DNA fragmentation analyses. Different values for diploid DNA content in DA-1 cells treated with hydroxyurea or etoposide could represent the different apoptotic action of these two compounds. The effect of these antitumor reagents in these mouse lymphoma cells is in accordance with their shown action on human lymphoma cells (22). Furthermore, different results obtained for the influence of the presence or absence of IL-3 for either hydroxyurea- or etoposide-treated cells could be due to an influence of the drug concentration or could be the expression of possible different apoptotic responses induced by these two antitumor compounds in these lymphoma cells. Since IL-3 treatments

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Figure 4. *Determination of the proportion of cells with hypodiploid DNA content. Apoptotic cells were analyzed by flow cytometry for measuring the number of cells containing diploid or hypodiploid DNA. Effects of hydroxyurea (1.25 mM) and etoposide (100 μM) for different times (4 hours: Figure 4 a ; 8 hours : Figure 4 b ; 17 hours : Figure 4 c) on DA-1 cells were studied. Different conditions for incubations were used: A) in the presence of IL-3; B) in the absence of IL-3; C) in the presence of 1.25 mM hydroxyurea and IL-3; D) in the presence of 1.25 mM hydroxyurea without IL-3; E) in the presence 100 μM etoposide and IL-3; F) in the presence of 100 μM etoposide without IL-3.*

might be applied in therapy (23), some protective effects of IL-3 on treated cells can be inferred, which can be correlated with previous data in other cell types (1,2,20).

From these results, a different mechanism in the induction of apoptosis in IL-3-dependent lymphoma by hydroxyurea or etoposide in the conditions studied can be inferred. A role for IL-3 in the intracellular modulation of the apoptotic-related action of etoposide can also be postulated. That can be useful in the prevention of selectively toxic effects of this antitumor compound when applied to lymphoma tumor cells.

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