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METHOD

Detection of Drug-induced Apoptosis by Flow Cytometry after Alkaline Extraction of Ethanol Fixed Cells*

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A new flow cytometric method was developed to detect apoptotic cells with fragmented DNA and to determine cell cycle distribution of viable cells, in the same sample, by propidium iodide staining. Apoptosis, in HT58 human B lymphoma cells, was induced by etoposide and/or by staurosporine. Using appropriate alkaline solutions (between 1-10 mN NaOH in 150 mM saline) followed by neutralization with buffer solution, the fragmented DNA can be extracted quantitatively from ethanol fixed cells. Further, good resolution of the cell cycle distribution can be obtained in unimpaired cells without RNase treatment. Furthermore, unlike the widely used hypotonic-detergent extraction of unfixed cells, the suggested extraction method can prevent drug-induced disintegration of dead cells when karyorrhexis occurs. (Pathology Oncology Research Vol 2, No1–2, 78–83, 1996)

Key words: apoptosis; cell cycle; drug-induced; DNA extraction; flow cytometry

Introduction

Apoptosis, a mode of cell death, is usually identified by morphological criteria. Currently, however, there is a need for more reliable and easy-to-use assays to recognize apoptotic cells, especially by analytical methods.

Apoptosis is frequently accompanied by the oligonucleosomal fragmentation of DNA,[1,20] though apoptosis can occur without it.[7] When extracting DNA fragments from apoptotic cells a subpopulation with hypo-Gl-ploidy (sub-Gl DNA content) can be detected on DNA histograms by FCM. Although loss of some DNA from ethanol-fixed apoptotic cells can happen even in an isotonic solution at neutral pH,[7] usually a more efficient DNA extraction is necessary to discriminate sub-G1 apoptotic cells from cycling viable cells. From cells arrested in G2/M phase, more than 50% of the DNA should be extracted. This is the reason why hypotonic DNA extraction is usually used, either on unfixed cells lysed by detergents,[17] or on ethanol-fixed cells[11] to identify the apoptotic cell population. Other types of extractions, e.g. acidic[6] or heat treatment,[10] have also been proposed.

Depending on the system used (cell type and form of apoptosis), the hypotonic extraction may be either too hard, in the case of unfixed cells,[3] or too weak, in the case of fixed cells[8], resulting in poor distinction of the apoptotic cell population either from the debris (including apoptotic bodies) or from the viable (unimpaired) population, respectively, on FCM-made DNA cytograms. A common disadvantage of the known DNA extraction methods is that they are not flexible enough to extract the appropriate amount of fragmented DNA from certain types of apoptotic cells, therefore their use is limited.

Our aim was to develop a reliable analytical method for the detection of apoptosis by FCM. Here, we describe an isotonic, alkaline extraction method for ethanol-fixed cells that allows optimization of the amount of DNA extracted from apoptotic cells by changing the alkaline strength (pH) of the extraction solution. Furthermore, ethanol

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Abbreviations: ETO: etoposide; FCM: flow cytometry; FLS: forward light scatter; EOH: alkaline extraction of ethanol fixed cells; HTC: hypotonic, detergent extraction of unfixed cells; STA: staurosporine

fixation and alkaline extraction prevent the cell loss that can occur when hypotonic detergent extraction is used with unfixed cells.

Materials and Methods

Cells, cultures and treatments

HT58 human B-lymphoma cell line[14] was maintained in RPMI 1640 (Sigma), supplemented with 10% FCS (Seback), 2 mM L-glutamine, 50 μg/ml streptomycin and 50 IU penicillin. Apoptosis was induced in 50 ml flasks or 24 well plates (Greiner) by various combinations of etoposide and staurosporine (Sigma) at $2x10^5$ cells/ml density to achieve a high apoptotic rate within a short period of time. The occurrence of apoptosis was checked by hematoxylin-eosin staining on cytospins and by DNA ladder formation in agarose gel.

Light microscopy

Cytospins were made routinely, stained with hematoxylin-eosine, and 1000 cells per sample were counted. Apoptosis was assumed when nuclei were segmented and appeared as darks spots or were pycnotic within a cell with a well preserved cytoplasmic membrane *(Fig. 1)*.

DNA gel electrophoresis

The assay was performed according to Swat *et al.*,[18] with modifications. Ethanol preserved samples of $2x10^5$ cells were washed in NaCl (150 mM) and resuspended in 1 ml saline containing 0.1 mg/ml RNase for 30 min at room temperature. Cells were then pelleted, resuspended in 20 μl aliquots of DNA buffer (10 mM EDTA, 50 mM Tris, pH 8.0, containing SDS 0.5% w/v and proteinase K 0.5 mg/ml), and incubated at 50°C for 30 minutes or at 37°C overnight. Electrophoresis was carried out in 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

Sample preparations for flow cytometry

Termination of apoptosis-induction was achieved by either lysing the unfixed cells in hypotonic lysing solution or fixing them in ethanol.

Method 1 – (HTC): Fresh cells were lysed as described by Nicoletti *et al.*[17] One ml cultured cells were centrifuged (350 g, 5 min.). The pellet was mixed with 1 ml lysing solution containing 0.1% (v/v) Triton-X-100, 0.1% (w/v) Na-citrate and 10 μg/ml propidium iodide (PI) (Sigma) in distilled water (HTC lysing solution). The samples were incubated for 30 minutes at room temperature or stored at 4°C until next day. The centrifugation can be omitted if a 100 μl of cultured sample is lysed by 400 μl HTC lysing solution. This preparation gave comparable results with the original assay.

Method 2 – (EOH): The ethanol (70 % v/v in distilled water, –20°C) fixed cells were stored at –20°C, usually overnight, and for another 30 minutes at 4°C before extraction. Cells were centrifuged (600g, 3 min), pellets were resuspended in 950 μl NaCl solution containing 0.3-10.0 (usually 2.5-5.0) mN NaOH for 1 min at room temperature, then neutralized with 50 μl, 200 mM, Na_2HPO_4-citric-acid buffer, pH 6.5, containing 200 μg/ml PI (finally 10 μg/ml). The samples were incubated at room temperature or stored at 4°C until the next day. The efficiency of the extraction was dependent on the cell number and on the extraction time. In the case of too high cell count ($>5x10^5$), the extracted DNA caused cell clumping.

Flow cytometry

Measurements were carried out on a Cytoron-Absolute cytometer (Ortho). Data were collected by DS-2 software (Ortho), while analysis was performed with Winlist (Verity Software House). For determination of apoptosis, forward light scatter (FLS) and the red fluorescence channel (PI fluorescence, DNA content) were set to logarithmic gain, while cell cycle distribution was evaluated by linear gains with Cell-Cycle software (Ortho). Adjustments of the cytometer were stored as a standard protocol, therefore the

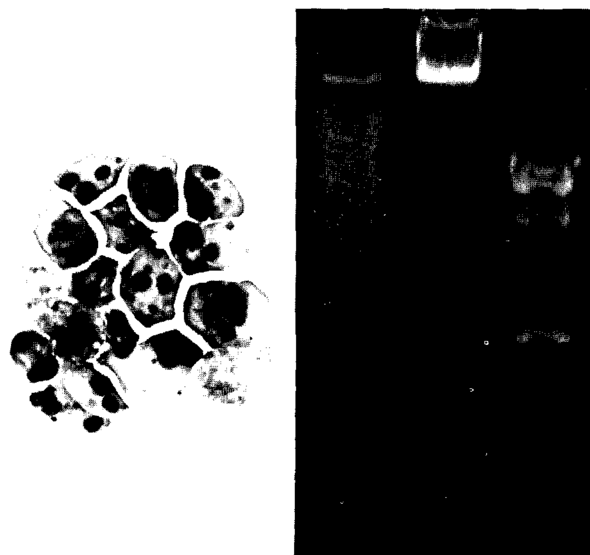

Figure 1. *Morphology and DNA ladder of apoptotic HT58 lymphoma cells. Cells were exposed to STA (500 nM) and ETO (10 μM) or DMSO as control for 3 hr. (A): Hematoxylin-eosin stained cytospin sample of drug-treated cells. Note the numerous dark, rounded nuclear fragments, characteristic of karyorrhexis. (B): Oligonucleosomal DNA fragmentation occurred in drug treated samples. Lane 1: ETO+STA treated sample; lane 2: control sample, lane 3: DNA marker (Gibco).*

G1 peak of lymphoma cells set a standard channel number on DNA histograms. Cell recovery was determined by absolute cell counting with FCM. Here, we suggest that measuring for constant time instead of constant cell number is preferable on any cytometer, allowing comparison of the cell concentration in different samples. Gating on apoptotic samples was performed as indicated in *Figs.2 and 3*. Using FLS and PI fluorescence cytograms (two parameters dot plot), gates were set on well-separated, selected cell populations to leave out debris and cell fragments. This procedure was arbitrary in the case of the HTC method (see below).

Results and Discussion

Etoposide (ETO), a topoisomerase II inhibitor,[15] and staurosporine (STA), a protein kinase inhibitor,[5] are well known inducers of apoptosis.[12] Previously we have shown that in HT58 lymphoma cells the action of ETO and STA can be oppositely modulated by phorbol ester[16] and that ETO-induced apoptosis is effectively enhanced by STA in short term (4-6 hr) cultures of HT58 lymphoma cells. This combination along with single drug exposure was used to study the effectiveness of various analytical methods in detecting apoptotic cells by FCM. Parallel with FCM, the appearance of apoptosis induced by these drugs was proved morphologically, as well as biochemically, including DNA fragmentation *(Fig.1)*.

Alkaline extraction of apoptotic tumor cells

According to Berezney and Coffey,[2] several DNA extraction procedures were tried. By changing the salt concentrations and pH values, we found that a broad range of NaCl concentrations (50-600 mM) can be used to extract large amounts of DNA from ethanol fixed thymocytes, but not from tumor cell lines (data not shown). However, changing the pH of the isotonic solution, 1.0-10.0 mN concentrations of NaOH optimized the extraction of fragmented DNA from cell lines. In HT58 lymphoma cells, 2.5 mN NaOH provided the best extraction of DNA from ETO- and STA-induced apoptotic cells. *Fig.2.* shows that increasing alkaline con-

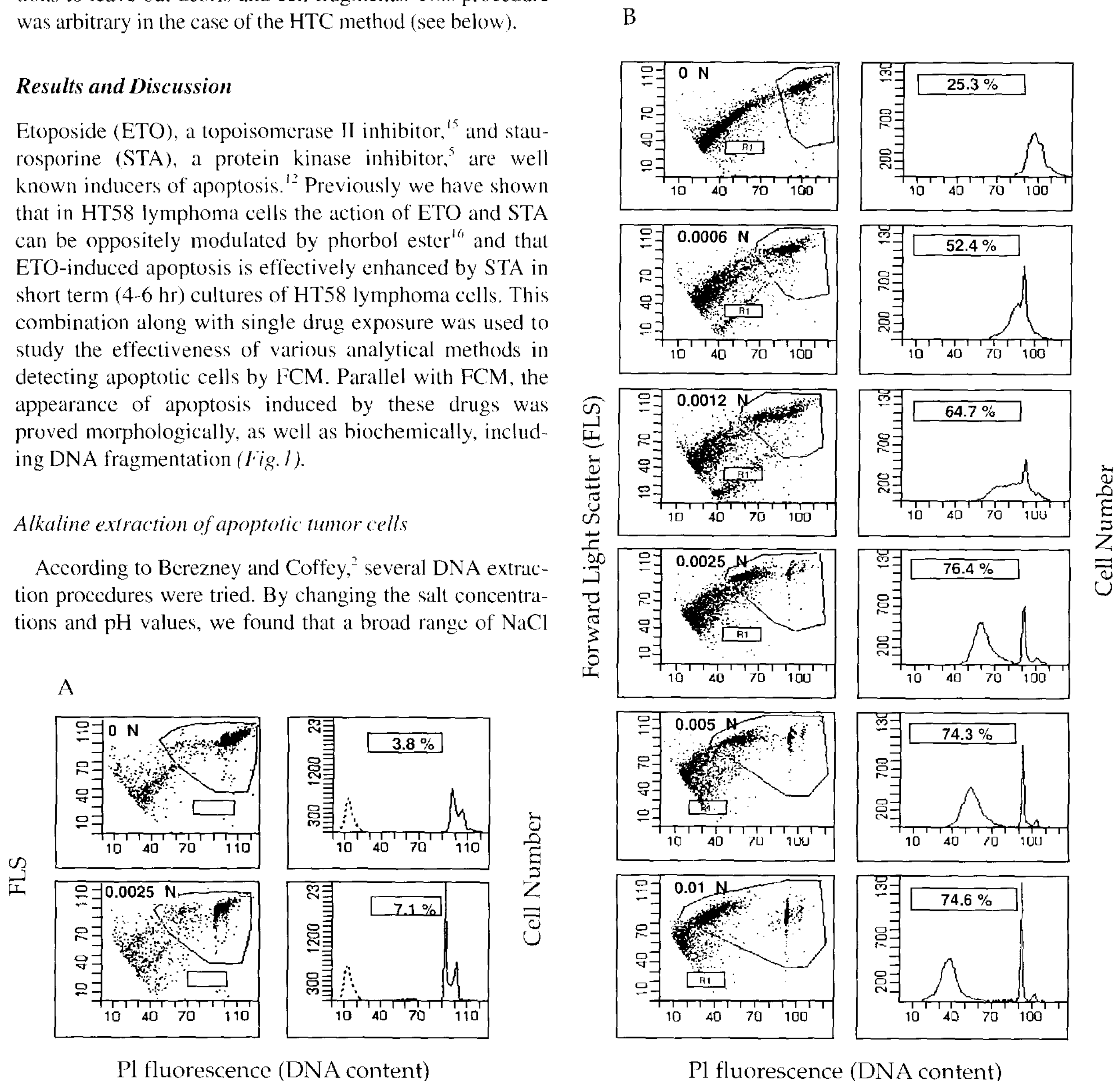

Figure 2. *Extraction of fragmented DNA at alkaline pH. HT58 lymphoma cells were treated with 10 μM ETO + 500 nM STA (to induce apoptosis) or with the solvent (DMSO) alone (control) for 3 hr. (A): Control sample with (continuous line) and without (dashed line) PI staining after extraction with the indicated concentration of NaOH in saline. (B): ETO + STA treated samples extracted with increasing concentration of NaOH. Inserted values in cytograms (dot plot) indicate the concentration of NaOH and in the histograms the determined percentage of the apoptotic cells in the regions marked out by the rectangles on the PI fluorescence axis. The populations gated on cytograms (left) are presented on the next DNA histograms (right).*

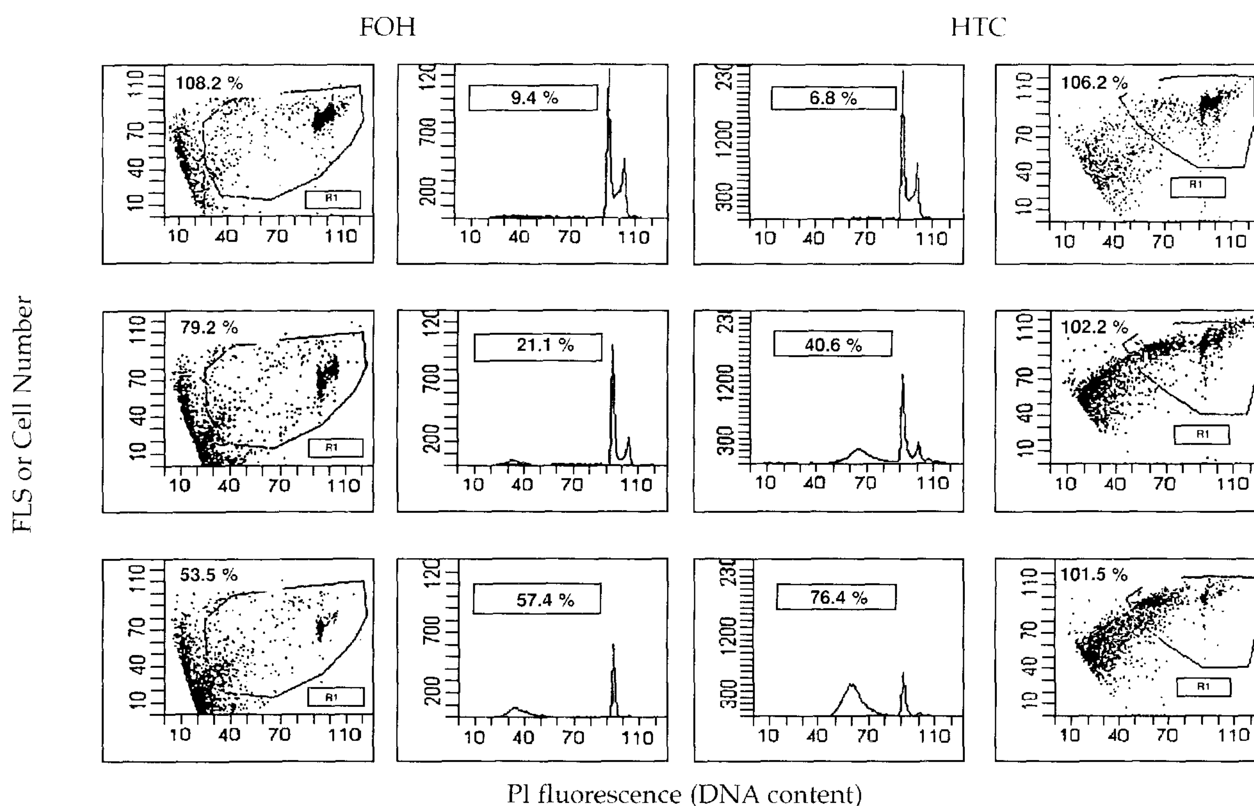

Figure 3. Detection of drug-induced apoptosis by HTC and FOH methods. Both methods were applied to samples of HT58 lymphoid cells treated for 3 hr with solvent (DMSO) (line 1; upper boxes); with ETO 30 μM + STA 100 nM (line 2; middle boxes); with ETO 10 μM + STA 500 nM (line 3; lower boxes). NaOH concentration was 2.5 mN in all extractions. Measurements were carried out on a Cytoron Absolute cytometer at standard time set for the measurement end condition. Inserted data on cytograms (dot plots) are the numbers of relative gated events (in percentage related to initial cell number) and on histograms the percentage of apoptotic cells.

centration shifted the sub-G1 cells more and more to the left (i.e. showing lower and lower DNA content) due to the extraction of increasing amount of DNA. The peak for apoptotic cells was separated clearly from viable cells and from cell debris. Note the ungated comet-events on the FLS-PI dot plots, which may represent apoptotic bodies and cell fragments *(Fig.2)*. Alkaline extraction by itself did not increase this population. Similar alkaline extraction was used in gel-embedded cells to measure DNA fragmentation at the cellular level.[18] In a separate experiment, we compared the rate of apoptosis detected by FCM after alkaline extraction with microscopic counting. As *Table 1* indicates, the two methods provided similar results.

Table 1. Percentage of apoptotic cells counted by FCM or light microscopy

	*FCM**		*Microscope**
	16 hr	*2 wk*	
Control	3.2 ± 0.6	2.5 ± 0.8	0.8 ± 0.4
STA	48.2 ± 2.2	49.3 ± 2.6	46.1 ± 4.2

* Cells were exposed to STA (500 nM for 3 hr), preserved for the indicated time in 70% ethanol at -20 °C, and prepared according to FOH. Results are mean ± SD of triplicate samples
** Cytospins were prepared and counted as described in Material and Methods

Comparison of extraction methods

Our method (FOH) was compared to the widely used method of Nicoletti *et al.* (HTC).[17] In HT58 lymphoma, it was rather common that less drug-induced apoptotic cells were observed with HTC than with FOH (*Fig.3*), especially after short term exposure to drugs. Comparing the absolute cell number per sample (inserts in FLS-PI dot plots on *Fig.3*), it is obvious that while the obtained number of viable cells is usually very similar with both methods, the whole cell number in gates was significantly reduced with HTC extraction due to the difficulties in separating apoptotic cells from debris. This explains the lower apoptotic percentage with HTC in HT58 (*Fig.3*). It is noteworthy that due to arbitrary gating, we could choose gates for higher cell numbers as well. But, population gating on the cytograms of FOH extracted samples was much less arbitrary. Zamai *et al.*[21] suggested that, in cell types with a relatively high cytoplasm/nucleus ratio, the apoptotic cell fragments could

make the separation of dead cells from the viable cells very difficult using the FLS parameter in FCM. The debris obtained with the HTC method was the result of the morphologically visible phenomenon of drug-induced karyorrhexis (*Fig.1*). In this form of apoptosis, the transglutaminase activity, which would prevent the detachment of cytoplasmic components from the fragmented nuclei,[9] can be rather low, allowing the disintegration of apoptotic cells and nuclei when the HTC method was used. This type of deterioration was prevented by ethanol fixation (FOH), which, in contrast to paraformaldehyde, does not influence the extractability of DNA.

Detection of cell cycle distribution of viable cells

Another advantage of alkaline extraction is the good cell cycle resolution available in the same sample for the non-apoptotic, viable cells. Inserted data on *Fig.4.* demonstrate that cell cycle distribution obtained with FOH and HTC were quite similar (the CV values were 3.5 ± 0.2 and 2.6 ± 0.15, respectively).

In conclusion, we have shown that weak alkaline extraction of ethanol-fixed tumor cells provides an inexpensive, reliable, simple analytical tool for the detection of apoptotic cells with fragmented DNA, and furthermore, for the determination of cell cycle distribution of viable cells without RNase digestion. It was also evident that our method was superior to the method of hypotonic, detergent lysis of unfixed cells in the case of early detection of drug-induced apoptotic cells where karyorrhexis is incidental to apoptosis.

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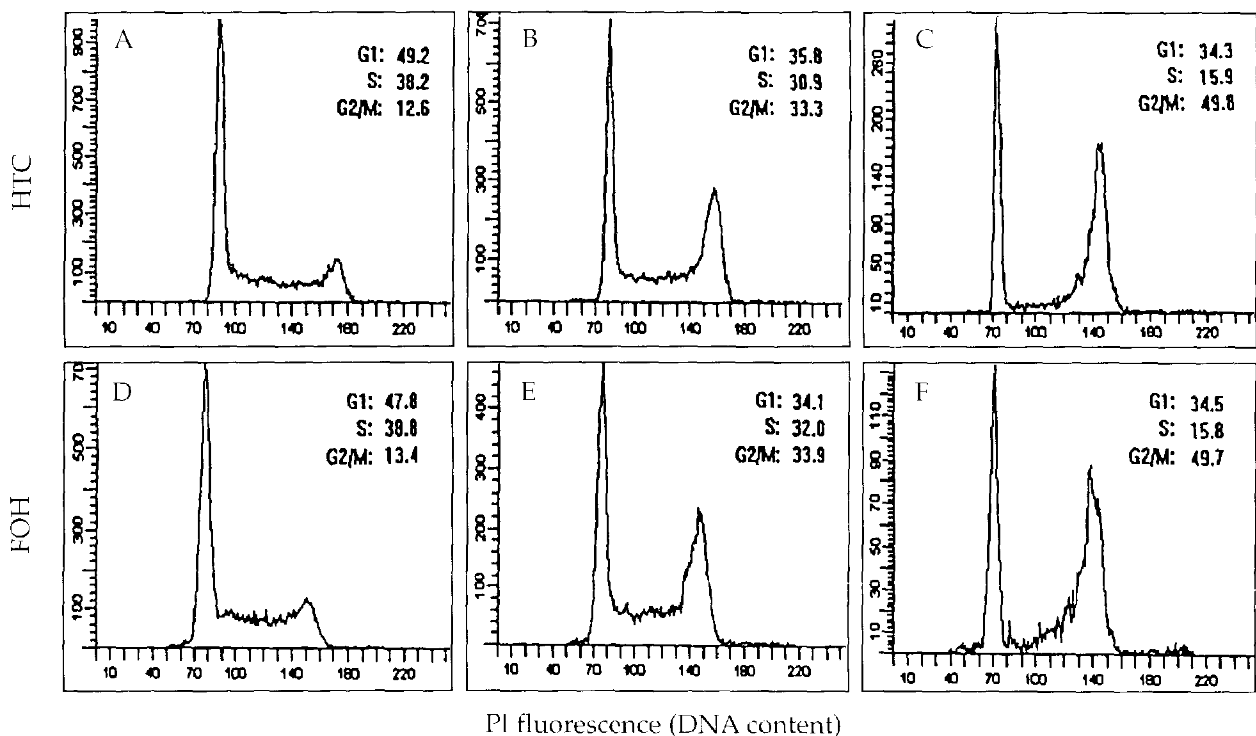

Figure 4. Cell cycle distribution of HT58 cells determined after HTC or FOH extraction. HT58 lymphoma cells were treated with STA (200 nM) for various times to get different cell cycle distributions. In these extractions 0.005 N NaOH was used. Time of sampling: A and D at 0 hr; B and E at 6 hr; C and F at 24 hr. Using either method, histograms were recorded by linear amplification gain after 30 min PI staining.

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