

Identification of Nuclei From Apoptotic, Necrotic, and Viable Lymphoid Cells by Using Multiparameter Flow Cytometry

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Background: Methods widely used to detect apoptosis do not allow us to easily distinguish between nuclei from viable or necrotic cells. Even if apoptosis and necrosis seem to occur as alternatives at the single cell level, they could be present simultaneously in a cell population much more frequently than expected. For this reason, attention was focused on attempting to recognize, by multiparameter flow cytometry, the characteristics of viable cells and of apoptotic or necrotic dead cells.

Methods: Apoptosis and necrosis were induced *in vitro* in murine thymocytes and lymphocytes from adult peripheral blood by using dexamethasone or prostaglandin E2 treatment and heat shock at 60°C or hydrogen peroxide, respectively. Traditional methods, such as DNA gel electrophoresis and propidium iodide staining followed by single-fluorescence analysis or annexin-V-fluorescein isothiocyanate plus propidium iodide staining by using flow cytometry, were compared with a new method. This method consisted of combined light-scatter and red fluores-

cence analysis by flow cytometry after isolation of nuclei by hypotonic solution as well as high-dose detergent treatment and DNA staining with propidium iodide.

Results: Results showed that, although traditional methods such as DNA-gel electrophoresis and single-parameter fluorescence flow cytometry analysis were unable, as expected, to discriminate among viability, apoptosis, and necrosis, our new method has enabled us to easily identify nuclei from viable, apoptotic, and necrotic cells. Results obtained by using our method were comparable to those obtained by using two-color analysis of cells after propidium iodide/annexin V staining.

Conclusions: A highly reproducible, inexpensive, rapid, and easily accessible method of analysis has been developed for simultaneously detecting apoptosis and necrosis. Cytometry 35:145–153, 1999. © 1999 Wiley-Liss, Inc.

Key terms: apoptosis; necrosis; flow cytometry; lymphoid cells; propidium iodide

Apoptosis and necrosis are two types of cell death that have been distinguished in terms of morphology, biological significance, and biochemical and molecular mechanisms [reviewed in Arends et al., (1) Darzynkiewicz et al., (2) Majino and Joris, (3) and Tsujimoto (4)]. Apoptosis is usually considered a common phenomenon involved in the physiological control of cell growth in tissues and organs. It is considered to be the result of an ordered sequence of cellular events, initiated by a variety of stimuli and genetically controlled [reviewed in Baker and Reddy (5), Cohen (6), Nagata (7), Nakano (8), and Yang and Korsmeyer (9)]. The main features of apoptosis consist of chromatin condensation and margination, cell shrinkage and blebbing, fragmentation of nuclei, and formation of apoptotic bodies (2). Conversely, necrosis principally occurs as a consequence of harmful events associated with pathological conditions. In contrast with apoptosis, it is mainly characterized by an early increase in volume of subcellular organelles and *in toto* cells. Cell destruction caused by lytic enzymes and associated late nuclear

changes follows. Even if apoptosis and necrosis seem to occur as alternatives at the single cell level, the line between apoptosis and necrosis is not always defined sharply. As a consequence, natural and experimental conditions in which they could be present simultaneously could be much more frequent than expected. For example, results recently obtained by us and other investigators indicate that the cytopathic effect, recognized as the main pathogenetic event during viral infectious processes, can be correlated to both cell death models during herpes virus infections (10,11). Also, a high degree of oxidative stress can cause necrosis, whereas a lower level can induce apoptosis (12,13).

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During recent years, different methods have been developed to investigate cell death phenomena (reviewed in Darzynkiewica et al. (14) and Gorman et al. (15)). Among these methods, analysis of DNA fragmentation by gel electrophoresis or single-parameter flow cytometry analysis of fluorescence after DNA-binding dye staining is widely used. However, these techniques do not allow us to distinguish between nuclei from viable or necrotic cells. For this reason, in the past, other investigators were interested in developing new methods able to detect apoptosis and necrosis within a single sample (16–18). In the present study, we have focused our attention on setting up the optimal conditions to discriminate by multiparameter flow cytometry the characteristics of viable cells and apoptotic or necrotic dead cells by using a highly reproducible, rapid and easily accessible method. To this end, we have modified and adapted a method to our purpose, based on the isolation of nuclei by hypotonic solution and detergent treatment and DNA staining with propidium iodide (PI). Similar methods are currently widely used to determine the presence of apoptosis, but not necrosis, in lymphoid cell suspensions (19,20). Our results show that it is possible to easily distinguish between viability, apoptosis, and necrosis by using a single run of samples. Our method of analysis has been applied successfully to nuclei obtained from murine and human lymphoid cells after treatment able to cause apoptotic and/or necrotic cell death.

MATERIALS AND METHODS

Cell Preparation and Culture

Murine thymocytes from 6-week-old C57BL/6NCrIBR mice (Charles River Italia, Calco, Como, Italy) and human peripheral blood mononuclear cells from healthy donors, separated by density gradient according to the standard Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) technique, were used. In some experiments, highly enriched peripheral blood lymphocytes were obtained from peripheral blood mononuclear cells by countercurrent centrifugal elutriation, as described previously (21). Apoptosis was induced by dexamethasone (DEX) (Sigma Chemical Co., St. Louis, MO) at concentrations from 10^{-5} to 10^{-8} M or by prostaglandin (PG) E2 (Cayman Chemical Co., Ann Arbor, MI) at concentrations from 10^{-4} to 10^{-7} M. To induce necrosis, cells were treated at 60°C for 1 h and 30 min in a water bath or with 100 mM hydrogen peroxide (H_2O_2) for 30 min and washed in phosphate-buffered saline before successive incubation. Untreated or treated cells were incubated at 37°C at a density of 10^6 cell/ml for varying times in a humidified 5% CO_2 atmosphere in RPMI1640 (Life Technologies, Paisley, Scotland) supplemented with 10 (fetal bovine serum (Life Technologies), 2 mM glutamine (Hyclone, Cramlington, England), 50 U/ml penicillin, and 50 U/ml streptomycin (Hyclone).

After incubation, cells were washed three times with phosphate-buffered saline (Hyclone) before successive manipulations.

Flow Cytometry Analysis of Nuclei

To stain isolated nuclei, the cells were treated with a solution of 25 μ g/ml PI (Sigma Chemical Co.) plus 0.05% sodium citrate (Fluka Chemia AG, Buchs, Switzerland) and with detergent at a high concentration (2% Triton X-100; Sigma Chemical Co.). A high concentration of detergent was found to be a critical point in our assay. When a lower concentration of detergent was used, the resolution among the three clusters of nuclei from apoptotic, necrotic, or living cells, as they could be discriminated by two-parameter forward-scatter (FSC) versus side-scatter (SSC) analysis, was decreased. To avoid bubbling and foaming caused by the high concentration of detergent, solutions were manipulated very carefully without pipetting. For this reason, samples were prepared as follows. A pellet containing 1×10^6 cells was resuspended in 50 μ l RPMI by vortexing in 15-ml polypropylene tubes (Falcon, Becton Dickinson & Co., Lincoln Park, NJ). A volume of 0.9 ml of the staining solution and, soon after, 0.1 ml of Triton 20% in distilled water were then added slowly to the tube at room temperature without pipetting. After 30 min of gentle mixing by a rotating apparatus, the tubes were placed at 4°C in the dark overnight before analysis. Just before analysis, samples were filtered through a 100-mesh nylon textile and transferred to 12×75 -mm polystyrene tubes (Falcon). Isolated nuclei were then analyzed by using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Fluorescence data were collected by using 488-nm excitation from a 15 mW air-cooled argon-ion laser. The emission was collected through a 585/42-nm band-pass standard filter. FSC and SSC were acquired in linear mode. Fluorescence of PI was acquired in log mode on channel 2. Detectors and amplifier gains for FSC and SSC were adequately selected to simultaneously detect nuclei from viable, apoptotic, and necrotic cells. Events were gated on FSC versus SSC in such a way that degraded DNA from cell debris or from doublets was excluded, and nuclei from viable, apoptotic, and necrotic cells were assayed. A minimum of 5,000 events were collected on each sample and acquired in list mode by a Hewlett-Packard 9000 computer (Fort Collins, CO) and FACScan[™] or Paint-a-Gate[™] software (Becton Dickinson).

Assessment of Apoptosis by Gel Electrophoresis

The analysis of DNA fragmentation by gel electrophoresis was performed as follows. Cells (8×10^6) were lysed in 0.2 ml lysis buffer containing 10 mM Tris, pH 8.0; 1.5 mM $MgCl_2$; 140 mM NaCl; and 0.5% Igepal CA-630. After a 5-min incubation performed at ice temperature, samples were collected at 12,000g for 90 s in a refrigerated centrifuge. Supernatants were then transferred into new tubes, 10 μ g/ml ribonuclease A was added, and the samples were incubated at 37°C. After 1 h, 200 μ l of a $2 \times$ proteinase K buffer (200 mM Tris, pH 8.0; 25 mM ethylenediaminetetraacetic acid, pH 8.0; 0.3 mol/L NaCl; and 2% sodium dodecyl sulfate) containing 50 μ g/ml proteinase K was added. At the end of a 30-min incubation performed at 37°C, DNA was extracted by adding an equal volume of buffered and saturated phenol/chloroform/

isoamyl alcohol (25:24:1). The aqueous phase was collected by a brief centrifugation at 12,000g and transferred into a new tube. Total DNA was precipitated by addition of 1/10 vol of 3 M sodium acetate (pH 5.2) and 2 vol of absolute ethanol and incubation overnight at -20°C . DNA pellet, collected by centrifuging at 12,000g for 30 min at 4°C , was washed once with 70% ethanol and resuspended in 50 μl 10 mM Tris (pH 8.0) and 0.1 mM ethylenediaminetetraacetic acid (TE). After a preincubation at 65°C for 5 min, the samples were separated by overnight horizontal electrophoresis (2 V/cm) in 1.0% agarose gel with 90 mM Tris, 90 mM boric acid and 2 mM ethylenediaminetetraacetic acid, pH 8.0 Tris-borate-EDTA (TBE), as running buffer; stained with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide; and visualized under UV light. A DNA molecular size standard (GIBCO BRL, Gaithersburg, MD) was included in each gel.

Assessment of Cell Death by Detection of Phosphatidylserine Exposure and Loss of Membrane Integrity

Phosphatidylserine exposure in thymocytes, as a marker of cell death, was measured by the binding of annexin V-fluorescein isothiocyanate (FITC), by using Annexin-V-FLUOS (Boehringer Mannheim, Mannheim, Germany) according to the instructions of the manufacturer. For differentiation of apoptosis and necrosis, cells were also stained with 100 mg/ml PI to detect membrane integrity and were immediately analyzed with a FACScan flow cytometer (Becton Dickinson). FSC and SSC were acquired in linear mode. Fluorescence of annexin V-FITC and PI were acquired in log mode on channels 1 and 2, respectively. Detectors and amplifier gains for FSC and SSC were adequately selected to simultaneously detect presumably viable, apoptotic, and necrotic cells. Events were gated on FSC versus SSC in such a way that cell debris or doublets were excluded and viable, apoptotic, and necrotic cells were assayed. A minimum of 5,000 events were collected on each sample and acquired in list mode by a Hewlett-Packard 9000 computer. A two-color analysis was performed by using the FACScan.

RESULTS

DNA Gel Electrophoresis and Single-Parameter Fluorescence Flow Cytometry Analysis Cannot Discriminate Among Viability, Necrosis, and Apoptosis

Apoptosis and necrosis were induced in vitro in murine thymocytes by using DEX or PGE2 treatment and heat shock at 60°C , respectively. After 20 h of culture, DNA was extracted, and the gel electrophoresis technique was applied to untreated, heat-shocked, DEX-treated, and PGE2-treated cultured cells. The analysis showed DNA fragmentation, revealed by the appearance of the characteristic apoptosis ladder, in all of the above-mentioned samples (Fig. 1, lanes 1, 3, and 4) with the exception of the heat-shocked necrotic thymocytes (Fig. 1, lane 2). Other samples were prepared by mixing, before DNA extraction, equal numbers of thymocytes that were untreated or that had undergone different treatment. In these samples,

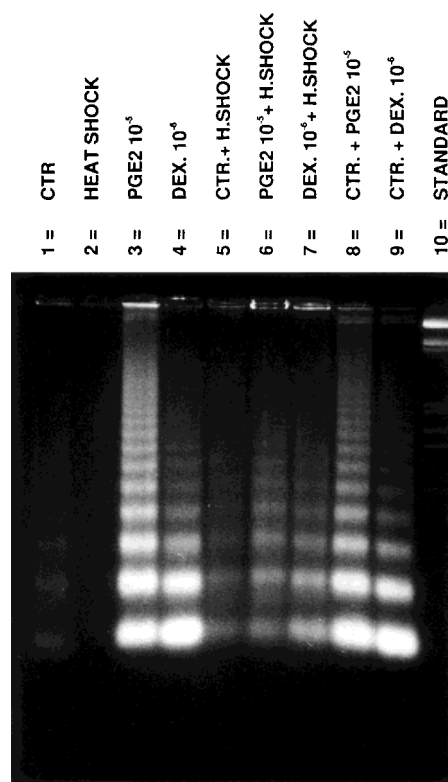


Fig. 1. Representative analysis of DNA fragmentation by agarose gel electrophoresis from murine thymocytes at different status of cell death. Thymocytes were left either untreated (CTR, lane 1), heat shocked at 60°C for 1 h (lane 2), treated with 10^{-5} M PGE2 (lane 3), or treated with 10^{-6} M DEX (lane 4). After 20 h at 37°C in a CO_2 incubator, part of the thymocytes was used for preparing samples by mixing equal numbers of cells (CTR+ H.SHOCK, lane 5; PGE2+H.SHOCK, lane 6; DEX+ H.SHOCK, lane 7; CTR+PGE2, lane 8; and CTR+DEX, lane 9). Cells from all samples were then collected, and DNA was extracted, electrophoresed on 1.0% agarose gel, and visualized by staining with ethidium bromide and by UV light transillumination. Lane 10, molecular-weight marker, 1 DNA/*Hind*III fragments. The upper band represents DNA of molecular weight equivalent of 23,130 base pairs, whereas the four lowest bands represent DNA of molecular weight equivalent of 2,322; 2,027; 564; and 125 base pairs, respectively.

apoptotic, necrotic, or living thymocytes would be expected to be simultaneously present at different ratios. However, the ladder appeared in all these samples, and we were unable, as expected, to distinguish the DNA molecules belonging to cell populations at different status of cell death (Fig. 1, lanes 5–9).

In other experiments, murine thymocytes underwent similar treatment by using DEX or PGE2 and heat shock at 60°C to induce apoptosis and necrosis, respectively. After 20 h of culture, also in this case, part of the untreated cells and part of the apoptotic- and necrotic-enriched cells were mixed in equal number to obtain samples containing living, apoptotic, and necrotic cells together. Samples were then treated with detergent solution and stained with PI before being analyzed by flow cytometry. As shown in Figure 2A in the single-fluorescence FL2 diagram, as expected, nuclei from control thymocytes cultured for 20 h presented two different populations: the greater part were diploid nuclei (FL2 channel of >700) from viable

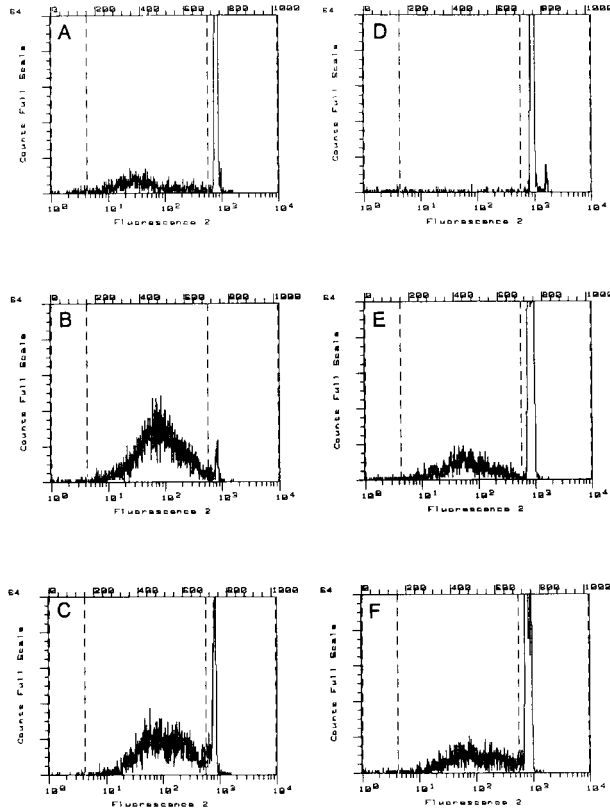


FIG. 2. Representative analysis by single-fluorescence flow cytometry of nuclei from murine thymocytes at different status of cell death after treatment with detergent solution and staining with PI. Thymocytes were left either (A) untreated, (B) treated with 10^{-5} M DEX, (C) treated with 10^{-5} M PGE2, or (D) heat shocked at 60°C for 1 h. After 20 h at 37°C in a CO_2 incubator, part of the thymocytes was used for preparing samples by mixing equal numbers of cells (E: DEX-treated plus heat shocked; F: PGE2-treated plus heat shocked). Cells from all samples were then collected, treated with a detergent/PI solution, and analyzed for red fluorescence emission. The dashed lines indicate the boundaries among the peaks of diploid and hypodiploid nuclei and of debris that were arbitrarily set on untreated samples and maintained for all other samples. Fluorescence 2 in PI fluorescence intensity (arbitrary units). The upper scale in each frame indicates channel numbers on the histogram of log FL2.

cells, whereas a minority of signals decreased into the 200–700-channel range, corresponding to the typical emission of hypodiploid nuclei. These signals corresponded to basal apoptosis of thymocytes. Figure 2B indicates that most of the nuclei from cells treated with 10^{-6} M DEX presented the typical emission of hypodiploid nuclei, as expected from apoptotic cells. Analogous results were obtained in cells after 20-h incubation with 10^{-5} M PGE2 (Fig. 2C). Conversely, cells heat shocked at 60°C for 1 h and cultured for 20 h, i.e., cells in which necrosis was induced, produced nuclei that decreased into the same emission channels of the diploid nuclei from viable cells (Fig. 2D). Single FL2 analysis of the two samples obtained after the mixing of untreated, heat-shocked, and DEX- or PGE2-treated apoptotic cells are shown in Figure 2E and F, respectively. In both FL2 diagrams, it is clear that, although it was possible to discriminate the nuclei from apoptotic (hypodiploid) cells from those obtained from the other

cells, we were unable to distinguish nuclei from living and necrotic cells.

Two-Parameter FSC/SSC Flow Cytometry Analysis After Detergent Treatment and PI Staining Allows Three Clusters of Nuclei to Resolve, Presumably Corresponding to Nuclei From Viable, Apoptotic, and Necrotic Cells

The same samples analyzed for red fluorescence were also assayed by a two-parameter FSC/SSC analysis after an adequate setting of the instrument. In the sample derived from untreated thymocytes consisting of viable (the greater part) and basal apoptotic (a minority) cells, two populations were visible at the FSC/SSC analysis. Most of the nuclei were positioned at medium FSC and low SSC values, whereas a low percentage was positioned in the left part of the FSC-SSC cytograph, corresponding to small nuclei of medium granularity (Fig. 3A). The latter cluster of nuclei, positioned in the left section of the FSC-SSC cytograph, was highly present in samples in which apoptosis was induced by DEX (Fig. 3B) or PGE2 (Fig. 3C), indicating that it was mainly attributed to nuclei derived from apoptotic cells. Nuclei derived from thymocytes in which necrosis was induced were remarkably different from nuclei derived from viable or apoptotic cells by using FSC/SSC analysis. In fact, they showed higher FSC and SSC values, corresponding to a wider dimension and higher granularity (Fig. 3D). The same nuclei were indistinguishable from nuclei from viable cells by using FL2 single-parameter analysis. Regarding the two samples obtained after the mixing of untreated, heat-shocked, and DEX- or PGE2-treated cells, Figure 3E and F shows that two-parameter FSC/SSC analysis allowed us to clearly distinguish three different clusters of nuclei, positioned in the left/medium, central/low, and right/upper regions of the cytograph and, according to results obtained in nonmixed samples, corresponding to nuclei from presumably apoptotic, viable, and necrotic cells, respectively.

To compare our results with those obtained with another technique that is supposed to distinguish living, apoptotic, and necrotic cells, we used a two-color flow cytometry analysis after binding with annexin V-FITC and staining with PI. Murine thymocytes were treated with PGE2 or heat shocked at 60°C as described above to induce apoptosis and necrosis, respectively. After 20 h of culture, some of the cells were treated with detergent solution and stained with PI, according to our technique, and some were stained with annexin V-FITC and PI before being analyzed by flow cytometry. Fresh thymocytes were also used as control living cells. Results obtained in a representative experiment are shown in Figure 4. Dual-parameter analysis after staining with annexin V-FITC and PI showed the characteristic double-negative living cells in fresh thymocyte samples (Fig. 4D). After detergent treatment and PI staining, FSC/SSC analysis of nuclei from the same cells confirms the characteristics described in Figure 3A for living cells (Fig. 4A). PGE2-treated cells showed the simultaneous presence of living and apoptotic cells when analyzed by using the PI/annexin V technique (Fig. 4E), as

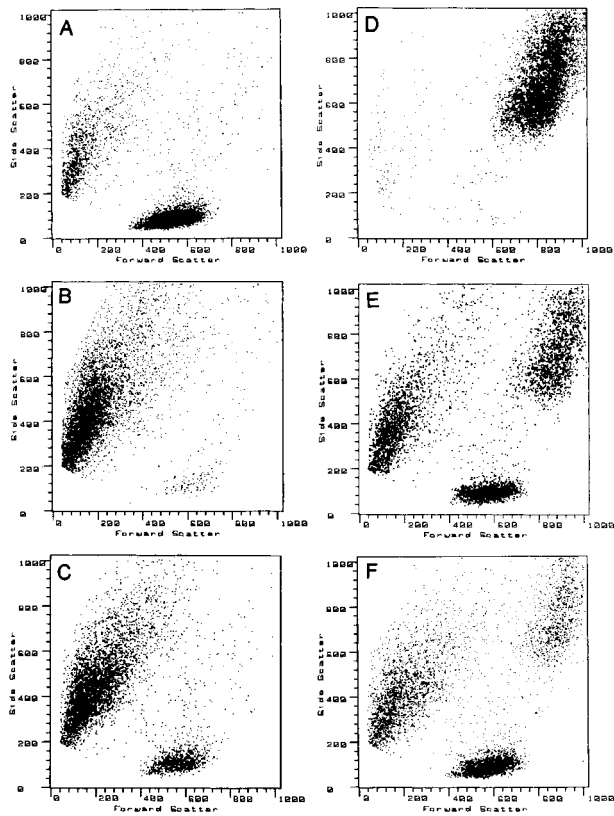


FIG. 3. Representative analysis by two-parameter FSC/SSC flow cytometry of nuclei from murine thymocytes at different modes of cell death after treatment with detergent solution and staining with PI. Thymocytes were treated as described in Figure 2, and nuclei were analyzed for their FSC and SSC characteristics. Three clusters of nuclei in the cytographs can be identified, corresponding to FSC and SSC patterns of nuclei from viable (low/central), apoptotic (medium/left), or necrotic (upper/right) cells. A: Untreated thymocytes. B: Thymocytes treated with 10^{-5} M DEX. C: Thymocytes treated with 10^{-5} M PGE2. D: Thymocytes heat shocked at 60°C for 1 h. E: DEX-treated plus heat-shocked. F: PGE2-treated plus heat-shocked.

well as the corresponding FSC/SSC profile described in Figure 3C, when analyzed according to our technique (Fig. 4B). Similarly, heat-shocked cells showed the unequivocal characteristics of necrotic double-positive cells by using the PI/annexin V method, whereas their nuclei showed a typically high FSC and SSC pattern (Fig. 4C) when analyzed by our method, as described above for this kind of cell death in Figure 3D. Assessment of cell death by detection of phosphatidylserine exposure and loss of membrane integrity, by using annexin V and PI, thus confirmed the capacity of our method to resolve three clusters of nuclei by using FSC/SSC analysis, presumably corresponding to nuclei from viable, apoptotic, and necrotic cells.

Identification of Nuclei From Viable, Apoptotic, and Necrotic Cells by Multiparameter Flow Cytometry Analysis

To further confirm the identification of nuclei from viable, apoptotic, and necrotic cells obtained by two-parameter FSC/SSC analysis, a combined analysis for FSC/

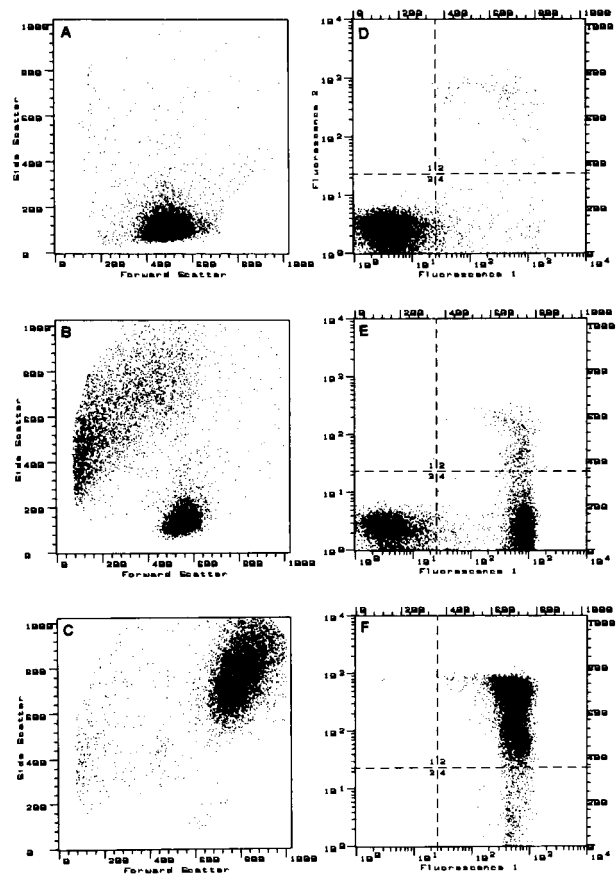


FIG. 4. Representative comparison between two-parameter FSC/SSC flow cytometry analysis of nuclei from murine thymocytes at different modes of cell death after treatment with detergent solution and staining with PI and two-parameter FL1/FL2 flow cytometry analysis of murine thymocytes from the same cultures after staining with annexin V-FITC (in FL1) plus PI (in FL2). Thymocytes were (B,E) treated with 10^{-5} M PGE2 or (C,F) heat shocked at 60°C for 1 h and cultured for 20 h at 37°C in a CO_2 incubator. A,D: Freshly isolated thymocytes were used as control living cells. Part of the cells from the different cultures was used in the preparation of samples for analysis of isolated nuclei after detergent treatment (A-C) and part was used for the PI/Annexin V technique (D-F).

SSC and red fluorescence was performed. Figure 5 shows the results obtained in samples in which untreated, heat-shocked, and DEX- (Fig. 5A,B) or PGE2-treated (Fig. 5C,D) murine thymocytes were mixed. The differential coloration of three populations distinguishable at the FSC/SSC analysis (Fig. 5A,C) obtained by using the Paint-a-Gate software allows us to determine how they were distributed in the red fluorescence histograms (Fig. 5B,D). It is evident that nuclei, presumably from necrotic and viable cells by FSC/SSC analysis, fell into the same fluorescence emission peak. Conversely, nuclei presumably from apoptotic cells, with reference to FSC/SSC analysis, were strictly located in the hypodiploid area of the red fluorescence histograms. Thus, their apoptotic nature was confirmed.

The same method was also applied to mature lymphoid cells from humans. Figure 6 shows the results obtained in human peripheral blood lymphocytes that were either

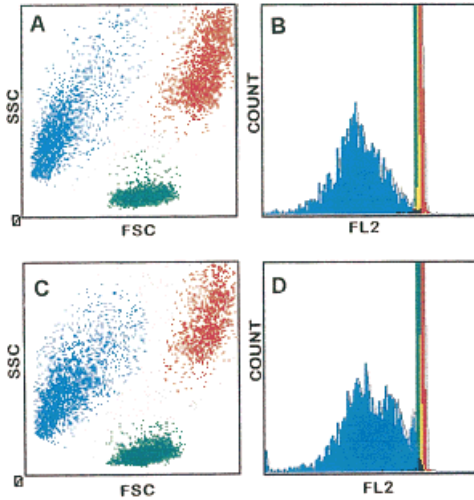


FIG. 5. Representative combined flow cytometry analysis by two-parameter FSC/SSC analysis and red fluorescence (FL2) of nuclei from murine thymocytes at different status of cell death after treatment with detergent solution and staining with PI. Thymocytes were left either untreated, treated with 10^{-5} M DEX, treated with 10^{-5} M PGE₂, or heat shocked at 60°C for 1 h. After 20 h at 37°C in a CO_2 incubator, cells from different cultures were used in the preparation of samples by mixing equal numbers of (A,B) untreated plus DEX-treated plus heat-shocked cells or (C,D) untreated plus PGE₂-treated plus heat-shocked cells. Cells were then treated with a detergent/PI solution and analyzed by using the Paint-a-Gate software. Nuclei belonging to three distinct clusters at two-parameter FSC/SSC analysis were assigned three different colors, corresponding to nuclei from viable (green), apoptotic (light blue), or necrotic (red) cells respectively (A and C). Single red fluorescence analysis of the nuclei corresponding to the three different FSC/SSC clusters, distinguished by different colors, is shown in B and D.

untreated (Fig. 6A,D), treated with 10^{-5} M DEX (Fig. 6B,E), or heat shocked for 1 h at 60°C (Fig. 6C,F) after 72 h culture in complete medium without additional stimuli or growth factors. The instrument was set as described for thymocyte samples. Particularly, adequate values of FSC and SSC were chosen to simultaneously view nuclei from apoptotic and necrotic samples on the screen. In these conditions, as described for thymocyte samples, nuclei from untreated cells or from cells submitted to apoptosis- or necrosis-inducing treatments were easily distinguished by using the combined analysis of their different pattern of FSC and SSC and of red fluorescence signals. Also in this case, a strict correlation between emission peaks of red fluorescence and areas in which the events were located at the FSC/SSC two-parameter analysis was observed.

To verify whether our method could be applicable to cells exposed to different and less unambiguous inducers of necrosis, we then compared the effects of heat-shock treatment at 60°C with those obtained by using oxidative stress caused by H_2O_2 . Murine thymocytes were exposed to H_2O_2 or heat shock at 60°C for 30 min and then cultured for 20 h before detection of cell death by using our method. Fresh thymocytes were used as control living cells. Results of a representative experiment, in which H_2O_2 was used at the concentration of 100 mM, are reported in Figure 7. Single-fluorescence analysis of nuclei from cells treated with H_2O_2 at this concentration (Fig. 7B)

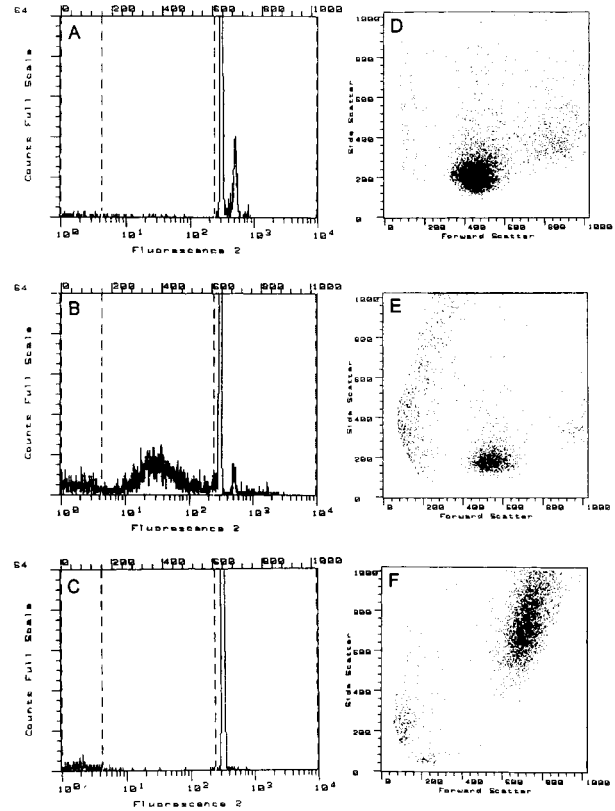


FIG. 6. Representative combined flow cytometry analysis by two-parameter FSC/SSC analysis and by red fluorescence (fluorescence 2) of nuclei from human peripheral blood lymphocytes at different status of cell death after treatment with detergent solution and staining with PI. Peripheral blood lymphocytes were left either (A,D) untreated, (B,E) treated with 10^{-5} M DEX, or (C,F) heat shocked at 60°C for 1 h. After 72-h culture in complete medium without stimulation at 37°C in a CO_2 incubator, cells were collected, treated with a detergent/PI solution, and analyzed for FSC/SSC and red fluorescence emission. The dashed lines in A-C indicate the boundaries among the peaks of diploid and hypodiploid nuclei and of debris, which were arbitrarily set on untreated samples and maintained for all other samples.

did not show differences in comparison with that of nuclei from heat-shocked cells (Fig. 7C) and was practically indistinguishable from that of nuclei from fresh thymocytes (Fig. 7A). Two-parameter FSC/SSC analysis of the same samples showed that nuclei from H_2O_2 -treated cells (Fig. 7E) were distributed similarly to those from heat-shocked cells (Fig. 7F), even if they present a lower SSC value, when analyzed in the same experimental and instrument conditions. Moreover, the H_2O_2 -treated sample showed complete absence of signals corresponding to living cells and presence of a low percentage of signals in an area of the cytograph close to that usually occupied by apoptotic nuclei when analyzed for FSC and SSC with our method. Concentrations of H_2O_2 of <100 mM produced samples showing characteristics similar to those reported in Figure 7E with a higher percentage of apoptotic-like signals (data not shown).

From these experiments, we concluded that the combined analysis of FSC and SSC and red fluorescence emission of nuclei from murine or human lymphoid cells

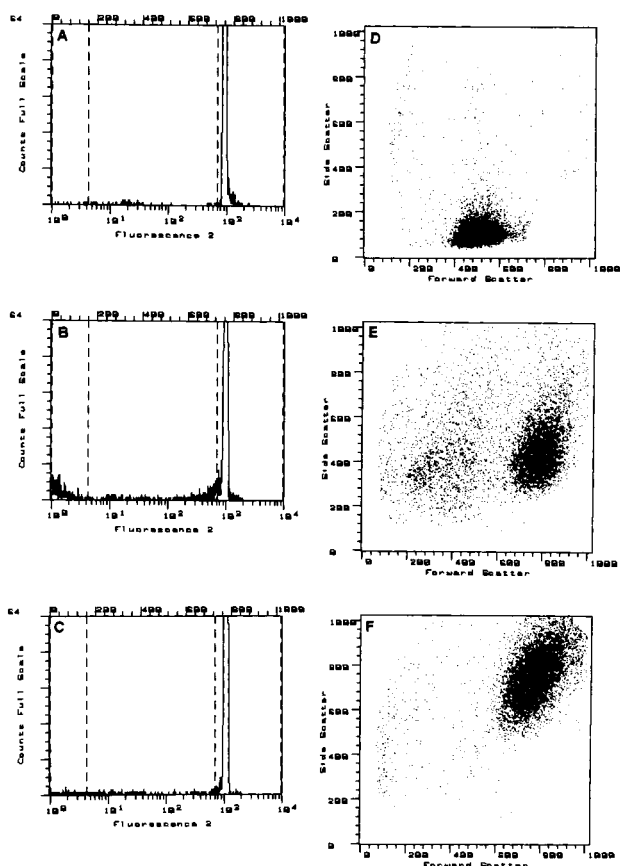


FIG. 7. Representative combined flow cytometry analysis by FSC/SSC two-parameters and by red fluorescence (fluorescence 2) of nuclei from murine thymocytes after induction of necrosis with different methods after treatment with detergent solution and staining with PI. For induction of necrosis, murine thymocytes were (B,E) treated with 100 mM H_2O_2 or (C,F) heat shocked at $60^\circ C$ for 30 min. After 20-h culture in complete medium at $37^\circ C$ in a CO_2 incubator, cells were collected, treated with a detergent/PI solution, and analyzed for red fluorescence emission (B,C) and FSC/SSC (E,F). A,D: Freshly isolated thymocytes were used as control living cells. The dashed lines in A-C indicate the boundaries among the peaks of diploid and hypodiploid nuclei and of debris, which were arbitrarily set on untreated samples and maintained for all other samples. Note that nuclei from H_2O_2 -treated cells (E) show SSC signals slightly lower than those from heat-shocked cells (F).

after PI staining and detergent treatment allows us to identify them as specifically derived from viable, apoptotic, or necrotic cells with minor differences related to inducer agents.

DISCUSSION

Flow cytometry is widely used for investigating cell death of lymphoid cells. For this purpose, many different flow cytometry-based methods have been proposed in recent years, and the attention of investigators has been focused on developing simplified and nonexpensive techniques able to obtain as much information as possible on the status of cells to be analyzed (22–32). Among these, the simplest ones use the capacity of DNA-binding dyes to detect apoptosis-associated chromatin degradation (33). Similar techniques can be applied to fixed and/or permeabi-

lized cells as well as to nuclei isolated after detergent treatment. One of the major limits in using such DNA-binding, dye-based techniques was the opinion that necrotic cells could not be distinguished from living cells by using these methods. Our study confirms that neither analysis of red fluorescence after PI staining as a single parameter nor analysis of DNA fragmentation by gel electrophoresis allows us to distinguish between nuclei from viable or necrotic lymphoid cells. However, here we have shown that PI-stained nuclei from viable or necrotic cells became distinguishable when the same samples underwent a further FSC/SSC two-parameter flow cytometry analysis. Thus, when diploid nuclei were analyzed separately from the hypodiploid ones by gating according to red fluorescence emission, it was possible to clearly distinguish nuclei from viable and necrotic cells according to their different patterns of forward- and side-angle scatter signals. In fact, after the optimal staining and detergent treatment conditions described in the present report, nuclei derived from cells with a different status of viability or death were distributed in areas situated in different zones of the cytograph. Our results would therefore indicate that, in adequate conditions of analysis, flow cytometry of PI-stained, detergent-treated nuclei allows us to detect not only apoptosis, as previously shown (19,20), but also necrosis. Previously, other investigators addressed their interest to the development of flow cytometry-based methods able to discriminate between necrosis and apoptosis (16–18). To our knowledge, until now all of the methods proposed to distinguish necrosis and apoptosis by flow cytometry were based on entire cell analysis. It has been shown that, by using FSC and red fluorescence as combined parameters, bivariate analysis could be useful in distinguishing apoptotic and necrotic cells (34). Here we show that combined FSC/SSC and red fluorescence analysis can also be applied successfully to analyze isolated nuclei from apoptotic, necrotic, or living lymphoid cells.

Staining and detergent treatment procedures are very similar to those previously described by other investigators, whereas the innovation consists of the optimization of analysis. In addition to traditional red fluorescence single parameter analysis, we further analyzed nuclei by two-parameter FSC/SSC analysis to distinguish viable, apoptotic, and necrotic nuclei. A critical passage in our method of analysis is setting the instrument to simultaneously detect events corresponding to nuclei from viable, apoptotic, or necrotic nuclei by using two-parameter FSC/SSC analysis. First, events corresponding to nuclei from cells at different conditions of viability and death could fall into regions of the cytograph very distant to each other. For example, optimal setting of the instrument, relative to nuclei from viable cells, could locate events corresponding to necrotic nuclei in an area not visible on the screen. Second, debris derived from either apoptotic or necrotic cells could be located near the apoptotic nuclei at the FSC/SSC analysis, thus interfering with a correct quantitative evaluation of nuclei from cells with different viability

and death characteristics. As a consequence, after the correct setting of the FSC and SSC values, the setting of an adequate acquisition gate, including nuclei from viable, apoptotic, and necrotic cells but not debris, must precede the running of the samples. To perform this passage in optimal conditions, when analyzing samples containing unknown levels of apoptosis, necrosis, or viability, we suggest using a suitable control sample consisting of a mixture of equal numbers of three cell populations, left untreated or submitted to unambiguous apoptosis- and necrosis-inducing treatment, respectively.

Results obtained by using our method were strictly comparable with those obtained by using the two-color analysis of cells after annexin V-FITC plus PI staining, i.e., one of the few techniques usually accepted as able to distinguish among viable, apoptotic, and necrotic cells. Moreover, it has been successfully used for detecting cell death after treatment of lymphoid cells with different agents, showing only minor differences in FSC and SSC characteristics. Thus, peculiarities observed in the distribution of signals corresponding to the three clusters of nuclei from living, apoptotic, and necrotic cells are not restricted to the specificity of the inducers. When H₂O₂ was used as an inducer of necrosis, we observed in the FSC/SSC analysis the appearance of a low percentage of signals showing a distribution similar, but not identical, to that observed in nuclei from apoptotic cells. These signals could correspond to a minor population of cells triggered to apoptosis by oxidative stress but in which apoptosis was still unfinished or, alternatively, blocked as reported recently (35). It must be mentioned that these signals were indistinguishable by nuclei from living or necrotic cells when gated and analyzed at the single fluorescence analysis (data not shown). The latter result indicates the possibility of using our method together with other techniques to obtain additional information regarding phenomena of cell death at the borderline between apoptosis and necrosis, as those caused by oxidative stress (12,13,35). However, further studies are necessary to explore this possibility.

In conclusion, here we have shown a very simple and reproducible method, by multiparameter flow cytometry, for a quantitative and qualitative analysis of viable, necrotic, and apoptotic cells simultaneously present in a single sample.

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