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REVIEW ARTICLE

Features of Apoptotic Cells Measured by Flow Cytometry

Z. Darzynkiewicz[1], S. Bruno, G. Del Bino, W. Gorczyca, M.A. Hotz, P. Lassota, and F. Traganos

The Cancer Research Institute, New York Medical College, Valhalla, New York 10595

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The present review describes several methods to characterize and differentiate between two different mechanisms of cell death, apoptosis and necrosis. Most of these methods were applied to studies of apoptosis triggered in the human leukemic HL-60 cell line by DNA topoisomerase I or II inhibitors, and in rat thymocytes by either topoisomerase inhibitors or prednisolone. In most cases, apoptosis was selective to cells in a particular phase of the cell cycle: only S-phase HL-60 cells and G_0 thymocytes were mainly affected. Necrosis was induced by excessively high concentrations of these drugs. The following cell features were found useful to characterize the mode of cell death: a) Activation of an endonuclease in apoptotic cells resulted in extraction of the low molecular weight DNA following cell permeabilization, which, in turn, led to their decreased stainability with DNA-specific fluorochromes. Measurements of DNA content made it possible to identify apoptotic cells and to recognize the cell cycle phase specificity of the apoptotic process. b) Plasma membrane integrity, which is lost in necrotic but not in apoptotic cells, was probed by the exclusion of propidium iodide (PI). The combination of PI followed by Hoechst 33342 proved to be an excellent probe to distinguish live, necrotic, early- and late-apoptotic cells. c) Mitochondrial transmembrane potential, assayed by retention of rhodamine 123 was preserved in apoptotic but not necrotic cells. d) The ATP-dependent lysosomal proton pump, tested by the supravital uptake of acridine orange (AO) was also preserved in apoptotic but not necrotic cells. e) Bivariate analysis of cells stained for DNA and protein revealed markedly diminished protein content in apoptotic cells, most likely due to activation of endogenous proteases. Necrotic cells, having leaky membranes, had minimal protein content. f) Staining of RNA allowed for the discrimination of G_0 from G_1 cells and thus made it possible to reveal that apoptosis was selective to G_0 thymocytes. g) The decrease in forward light scatter, paralleled either by no change (HL-60 cells) or an increase (thymocytes) of right angle scatter, were early changes during apoptosis. h) The sensitivity of DNA in situ to denaturation, was increased in apoptotic and necrotic cells. This feature, probed by staining with AO at low pH, provided a sensitive and early assay to discriminate between live, apoptotic and necrotic cells, and to evaluate the cell cycle phase specificity of these processes. i) The in situ nick translation assay employing labeled triphosphonucleotides can be used to reveal DNA strand breaks, to detect the very early stages of apoptosis. The data presented indicate that flow cytometry can be applied in basic research on molecular and biochemical mechanisms of apoptosis, as well as in the clinic, where the ability to monitor early signs of apoptosis in samples from patients' tu-

[1]Address reprint requests to Dr. Zbigniew Darzynkiewicz, The Cancer Research Institute, New York Medical College, 100 Grasslands Road, Elmsford, NY 10523.

mors may be predictive of the outcome of some treatment protocols.

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I. APOPTOSIS, PROGRAMMED CELL DEATH, AND NECROSIS

The term *apoptosis* is used to describe the characteristic mode of cell death common to various cell types and often triggered by diverse environmental stimuli (2,36,37,59,60). During this process, a cascade of specific biochemical events, the most prominent of which is the activation of an endogenous endonuclease that has affinity for internucleosomal (spacer) DNA regions, is paralleled by specific morphological changes in both the cell nucleus and cytoplasm (59,60). Extensive literature exists regarding morphological, biochemical and molecular changes that occur during apoptosis (reviews 2,25,36,53,59,60).

The term *programmed cell death* is often used interchangeably with apoptosis. Mechanisms involved in programmed cell death and apoptosis, although similar in many respects, are not identical, and the terms are not synonymous (reviewed in 41). The interest in these mechanisms stems from the fact that they play a key role in tissue and organ development during embryogenesis (30,41), regulation of immune responses (27,43), or natural death of differentiated cells at the end of their lifespan (41,42,47).

In oncology, the interest in apoptosis originates from the observations that ionizing radiation or interactions of antitumor drugs with their respective intracellular targets often result in this mode of cell death, and that the efficacy of several antitumor drugs correlates with their ability to induce apoptosis (5,25,35,38,50,55). The possibility, therefore, of modulating this process can open new strategies for improving chemotherapy. Also, the ability to monitor early signs of apoptosis in samples from patient tumors may be predictive of the outcome of treatment protocols.

In contrast to apoptosis, or programmed cell death, *necrosis* is a nonspecific mode of cell death, often caused by excessively high concentrations of toxic agents (36,40,46,53). Necrosis, occurring as the result of rapid cell poisoning, is characterized by swelling of mitochondria and immediate loss of plasma membrane integrity.

Several attempts to distinguish apoptotic cells by flow cytometry were reported (1,10,11,26,44,49,51,52, 56,58). In nearly all these studies, decreased DNA stainability with different fluorochromes has been proposed as a marker of apoptotic cells. In the most exhaustive studies, Telford et al. (51) compared several DNA fluorochromes with respect to their ability to stain DNA in apoptotic cells. They observed that the stainability of apoptotic cells with each of the dyes studied was diminished to a similar degree, compared to normal cells, regardless of the mechanism of binding of the dye to DNA (intercalation, involving different unwinding angles or binding to the minor grove of the double helix).

In the course of our studies on the mechanism of action of DNA topoisomerase type I or type II inhibitors, we observed that the DNA topoisomerase I inhibitor camptothecin (CAM) or certain inhibitors of DNA topoisomerase II (teniposide, TN; *m*-AMSA; Fostriecin, FST; Genistein, GEN), induce apoptosis of cells of human myelogenous leukemic HL-60 or KG1 lines (20–24,31,32). We have also investigated apoptosis of rat thymocytes triggered by topoisomerase inhibitors as well as by the glucocorticoid hormone prednisolone (6,7). In both cases, we observed that apoptosis is selective to cells in particular phases of the cell cycle, namely to S phase cells in the case of myelogenous leukemias (21) and to G_0 cells in the case of thymocytes (7). By applying a variety of flow cytometric techniques designed to analyze diverse metabolic features of the cell, we were able to characterize the changes that accompany this process. In this article, we review these results and provide additional observations related to this subject. We also compare our results with observations of others, on other cell systems. Most data pertaining to materials and methods were presented in our recent publications (6,7,20–24,31,32), although some details are presently included in the legends to figures.

II. INDUCTION OF APOPTOSIS OR NECROSIS

Exponentially growing HL-60 cells were exposed to a wide range of concentrations of CAM, TN, *m*-AMSA, FST, or GEN, as described in detail elsewhere (6,7,20–24,31,32). At certain concentrations of CAM, TN, or m-AMSA, a selective decrease of DNA stainability in S-phase cells was observed as early as 2 h after addition of the drugs; the drugs had no significant effect on cells in G_1, G_2, or M phases of the cell cycle (20,22). On the other hand FST and GEN caused apoptosis of HL-60 cells in all phases of the cycle (32,54). The same cells (HL-60), as well as the human lymphocytic leukemic MOLT-4 cell line, treated with excessively high concentrations of the drugs, served as a model of necrotic death (31,32). Another model of apoptosis were thymocytes isolated from the thymus glands of 2–4 weeks old Sprague-Dawley rats, treated in culture with 5 μM prednisolone, 0.15 μM CAM or 1 μM TN. In this cell system, the decrease of DNA stainability was also observed after 2 h of treatment, though the treatment was selective for G_0 cells (7). In both cases, the endonucleolytic activity triggered by the drugs resulted in typical fragmentation of DNA into sections of the size

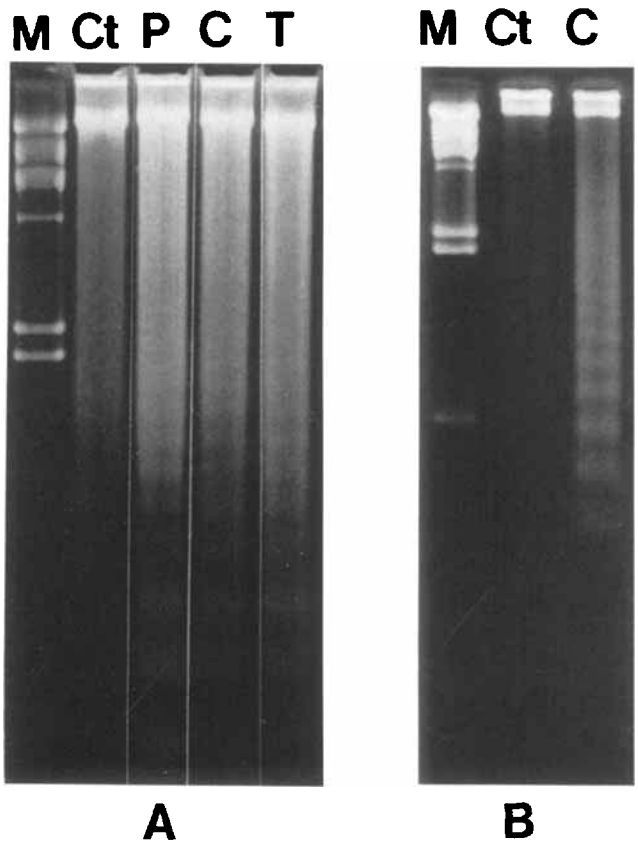

FIG 1. Agarose gel electrophoresis of DNA extracted from rat thymocytes (**A**) or human myelogenous leukemic HL-60 cells (**B**). A: thymocytes were incubated in cultures either in the absence of any drugs (Control, Ct), or in the presence of 5 μM prednisolone (P), 0.15 μM camptothecin (C), or 1 μM teniposide (T) for 6 h. The three lowest bands on the molecular weight marker lane (M) represent DNA of MW equivalent of 4,631, 2,322, and 2,027 base pairs, respectively (for details, see 7). B: HL-60 cells, untreated, growing exponentially (Control, Ct), or treated for 4 h with 0.15 μM camptothecin (C). The three lowest bands on the M lane represent DNA of MW equivalent of 2,322, 2,027, and 564 base pairs, respectively. For details, see (22). Note the typical "ladder" pattern of DNA extracted from the drug treated cells, which indicates the presence of DNA fragments of the size equivalent to single nucleosomes or oligonucleosomes. This pattern is characteristic of the DNA of apoptotic cells.

equivalent of individual nucleosomes or oligonucleosomes (Fig. 1).

III. DNA CONTENT (STAINABILITY)

It has been recognized independently in several laboratories that apoptotic cells have reduced DNA stainability following staining with a variety of fluorochromes (1,10,11,44). Therefore, the appearance of cells with low DNA stainability, lower than that of G_1 cells ("sub-G_1 peak," "A_0 cells") in cultures treated with various drugs, has been considered to be the marker of cell death by apoptosis. It was unresolved, however, whether the reduced DNA stainability represented a decrease in DNA content (as a result of activation of the apoptosis-specific endonuclease) or a change in DNA conformation and accessibility to the dyes. In nearly all these studies, the authors interpreted the observed decrease in DNA stainability as representing a change in *DNA accessibility* to fluorochromes rather than loss of cellular DNA.

However, several observations, as summarized below, indicate that the observed reduced DNA stainability in apoptotic cells is a consequence of *partial loss of DNA* from these cells due to activation of an endogenous endonuclease and subsequent diffusion of the low molecular weight DNA products from the cell, prior to cell measurement:

1. The change in DNA accessibility to the dyes as a result of the change in structure of nuclear chromatin of apoptotic cells is expected to be reflected in differences in the accessibility of particular dyes, depending on their mechanism of binding to DNA, as has been observed in other cell systems (17,29). Yet, there is striking concordance in the degree of reduction of DNA stainability in apoptotic cells with different fluorochromes (51). Furthermore, the chromatin structure of the fragmented nuclei of apoptotic cells (i.e., the presence of all histones, HMG proteins, etc.) is not much changed compared to that of the cells prior to apoptosis (2).
2. In the most detailed biochemical studies on chromatin of apoptotic cells, Arends et al., (2) have observed that whereas the DNA content of unfixed apoptotic cells is unchanged compared to normal cells, cell lysis (detergent treatment) released low molecular weight DNA, some of it associated with free mononucleosomes or oligonucleosomes. Over 30% of DNA in apoptotic cells was not associated with the nuclear fraction and could not be sedimented by centrifugation at 100 g but sedimented at 27,000*g* (2). A large portion of DNA in apoptotic cells, thus, is of a low molecular weight and is expected to dissociate from cells which are permeabilized by detergents or fixed by ethanol, prior to staining and measurement by flow cytometry.
3. In our studies on either HL-60 cells or thymocytes, we noticed that the decrease in DNA stainability of apoptotic cells was markedly enhanced if, following fixation or permeabilization by detergent, and prior to cell measurement, the cells were left in suspension for an extended period of time. Thus, for example, when the apoptotic cells were fixed in cold ethanol (−20°C), centrifuged, immediately stained (at 0–4°C) with DAPI, and their fluorescence measured shortly after staining, the difference in DNA stainability of normal and apoptotic cells was minimal, and often undetected. However, if the same cells were kept suspended in PBS at 24–37°C, for longer than 5 min prior to their measurement, a decrease in the fluorescence of apoptotic cells stained with DAPI was clearly evident (Fig. 2). The decrease was more pronounced when the cells were maintained in PBS prior to staining for longer periods of time, or were exposed to higher temperatures. Fur-

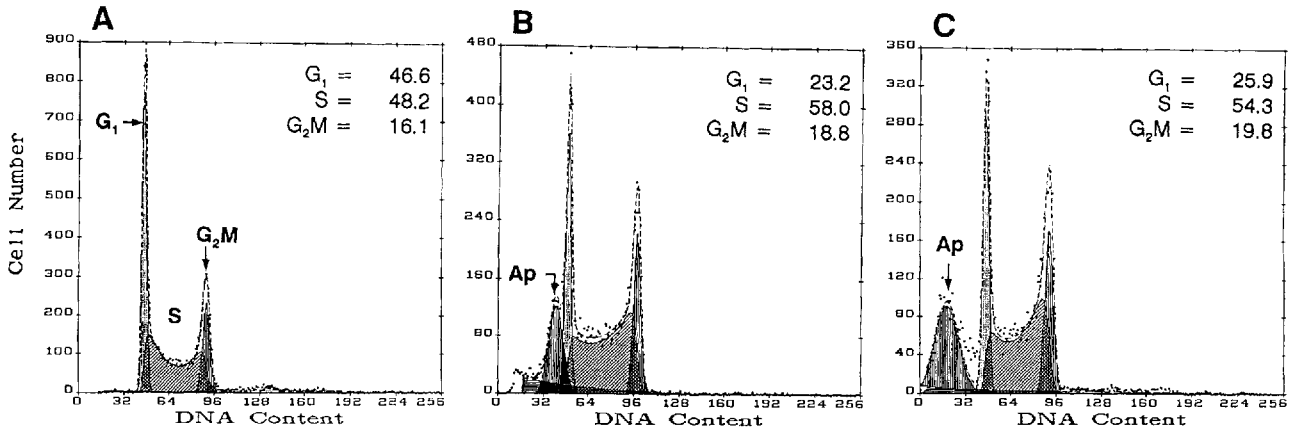

FIG. 2. DNA content frequency histograms of HL-60 cells, untreated (**A**) and treated with 100 μg/ml of Genistein (GEN) for 4 h (**B** and **C**). The cells were either stained immediately following fixation at 0°C with DAPI (19,22), and measured by flow cytometry (A and B); or, after fixation, were suspended in Hanks' buffered saline and maintained at 37°C for 20 min prior to staining and measurement (C). GEN induces apoptosis of HL-60 cells (54). Note that the position of apoptotic cells (Ap) was shifted to lower DNA content values when the cells were maintained in suspension prior to measurement, suggestive of DNA extraction from these cells.

thermore, the presence of low molecular DNA, of the size equivalent of mononucleosomes, was detected in the PBS in which the apoptotic cells were suspended following fixation in ethanol or permeabilization with Triton X-100 (Lassota et al., in preparation).

4. Observation of apoptotic cells stained with DAPI and sulforhodamine 101 under the U.V. light microscope revealed that DNA was in two compartments (Fig. 3B). One of these compartments were fragmented nuclei, appearing as granules of different sizes, strongly and uniformly stainable with DAPI. In contrast to normal nuclei, no distinct chromatin structure in these granules was evident. The chromatin in these granules was highly condensed and the DNA, when stained with hematoxylin, showed hyperchromicity (not shown). DNA in the second compartment appeared in a dispersed form, outside the granules. Its presence could be inferred from the blue fluorescence component of the cytoplasm, which was evident in apoptotic cells outside the nuclear fragments, and was not apparent in normal cells (Fig. 3A). The uniformity of the blue hue of the cytoplasm of these cells suggested that this DNA was evenly dispersed outside of the granules. When apoptotic cells fixed on slides were subsequently rinsed for over 10 min in PBS prior to staining with DAPI and sulforhodamine 101, the blue fluorescence component in the cytoplasm was no longer apparent (Fig. 3C).

5. It has been shown that at later stages of apoptosis, so called apoptotic bodies (fragments of the cell interior, including DNA, enveloped in plasma membrane) detach from the cell (36,37). Their release is thus expected to reduce cellular DNA content as well.

Considering the above observations, the most likely explanation for the reduced stainability of apoptotic cells with DNA-specific dyes is not the change in DNA accessibility to the dyes, but its progressive loss from these cells. At early stages of apoptosis, this loss occurs predominantly following cell fixation, or during permeabilization with detergents. The course of events leading to DNA extraction from apoptotic cells can thus be visualized as follows: Initially, activation of an endonuclease in cells undergoing apoptosis cleaves DNA between nucleosomes. Free, single nucleosomes or small oligonucleosomes may then diffuse outside the nucleus or nuclear fragments (chromatin granules), but cannot leave the cell because the integrity of the cell membrane is still preserved. These nucleosomes cannot be irreversibly immobilized by fixation (e.g., in ethanol), or retained in the cells after their permeabilization with detergents. Following fixation, when the cells are rinsed, these free nucleosomes diffuse out of the cell. The portion of DNA remaining in apoptotic cells after their permeabilization and rinsing most likely represents fragments of DNA loops still attached to the nuclear matrix and long stretches of nucleosomes which cannot be extracted under these conditions.

Thus, apoptotic cells can be recognized by their diminished stainability with DNA specific fluorochromes, such as propidium iodide (PI), DAPI, acridine orange (AO), or Hoechst dyes, due to DNA degradation and its subsequent leakage from the cell. Because the degree of DNA leakage can be manipulated by the extent of cell washing after fixation (or permeabilization), it is possible to adjust DNA content of apoptotic cells (position of the "sub-G_1 peak" on the DNA frequency histograms) so that their overlap with cells that do not undergo apoptosis is minimal and separation between these two populations is adequate (e.g., Fig. 2C vs. 2B).

In contrast to apoptotic cells, necrotic cells generally do not show an immediate reduction in DNA stainability (Fig. 4). Thus, the discrimination between normal

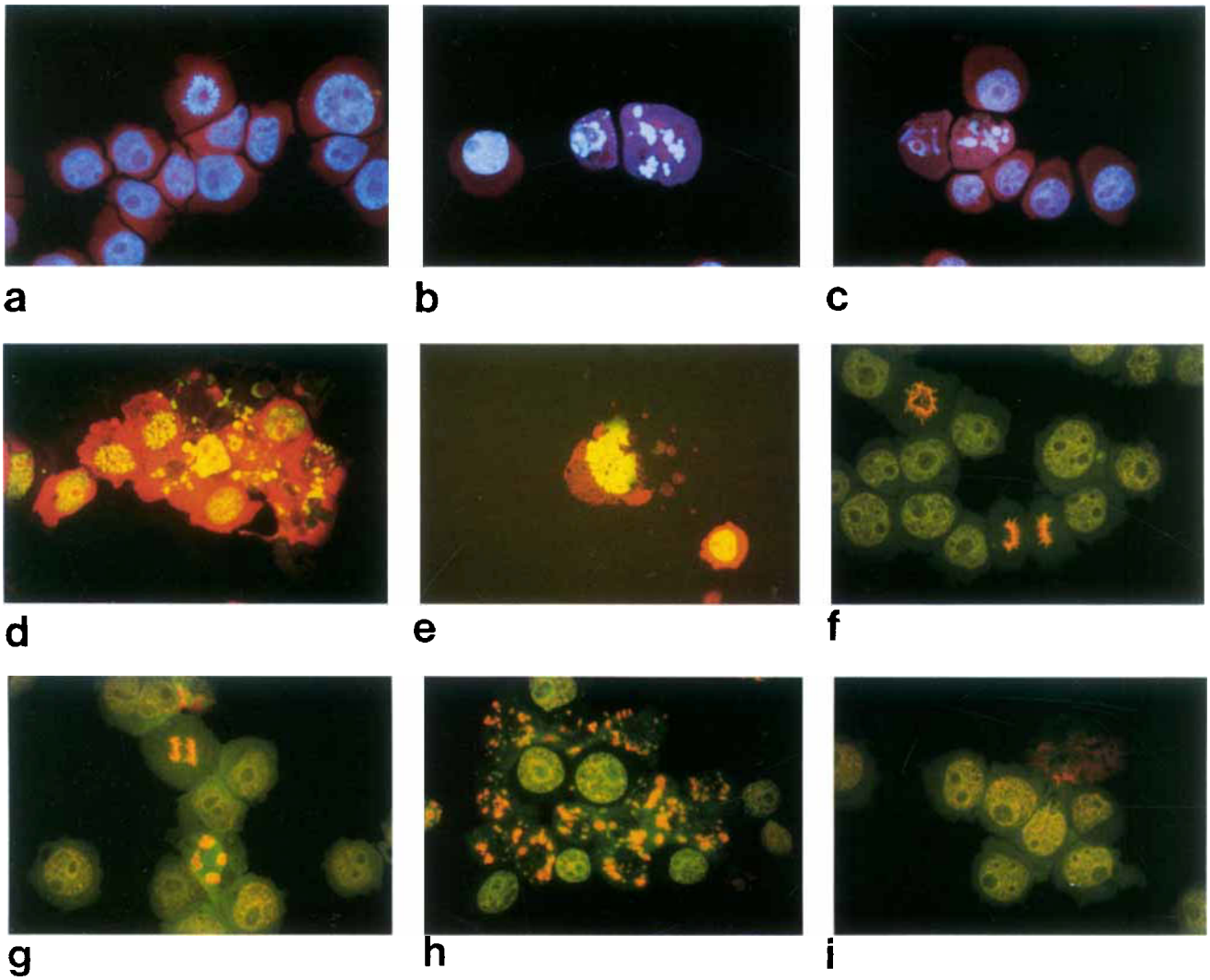

FIG. 3. U.V. light photography of HL-60 cells stained with DAPI and sulforhodamine 101 or AO. **a:** Untreated cells were cytocentrifuged, fixed in 70% ethanol, air dried, and stained with DAPI and sulforhodamine 101 to differentially stain DNA and protein (22). **b:** Cells treated with 0.15 μM of camptothecin for 4 h and stained as a. **c:** Cells treated with camptothecin (as b) and stained as a and b, except that following fixation and prior to staining the cells were suspended in HBSS for 20 min. Note the loss of the blue component of the cytoplasmic fluorescence of apoptotic cells in c, compared to b, which suggests that DNA that is evenly dispersed in the cytoplasm of these cells is extracted with HBSS. **d:** The cells treated with 0.15 μM of camptothecin for 4 h had their DNA and RNA differentially stained with AO (13); DNA (double stranded) stains green, RNA and single stranded DNA, red; overlap of the cytoplasm (RNA) over and under nucleoplasm (DNA) contributes to the nuclear yellow fluorescence. Some apoptotic cells (with fragmented nuclei) appear to have unchanged RNA content. **e:** The cells were treated with 1 mM fostriecin for 4 h (i.e., under conditions that induce necrosis) and stained with AO (as in d); a typical necrotic cell with damaged plasma membrane is in the center. **f, g:** Cells from untreated cultures, with their DNA partially denatured by acid and stained with AO to differentially stain double stranded (green) and denatured (red) DNA. Note that DNA of mitotic cells (metaphase and telophase) stains more intensely red and has lower green fluorescence than that of the interphase cells. Spontaneous apoptosis occasionally occurs in control HL-60 cultures, the nucleus of such a cell is seen in g; initial chromatin condensation on the periphery of the nucleus is then followed by nuclear fragmentation. **h:** The cells were treated with 0.15 μM camptothecin for 4 h and stained as in f and g. Note more extensive DNA denaturation in fragmented nuclei of the apoptotic cells than in the normal interphase nuclei. **i:** The cells were stained as in f and g; note the increased DNA denaturation (red luminescence) in the typical necrotic cell.

live- and necrotic- cells is impossible based on single-parameter DNA content analysis alone.

IV. INTEGRITY OF THE PLASMA MEMBRANE

The integrity of the plasma membrane of cells undergoing apoptosis is preserved and most functions of the membrane remain unchanged (2,37,59,60). Apoptotic cells, thus, exclude "viability assay" dyes such as trypan blue or PI (22). This is in contrast to cell necrosis, where one of the earliest changes is loss of membrane function and its structural integrity (40). By their ability to exclude PI, apoptotic cells can be erroneously classified by flow cytometry as viable cells.

The assay of cell viability based on simultaneous cell

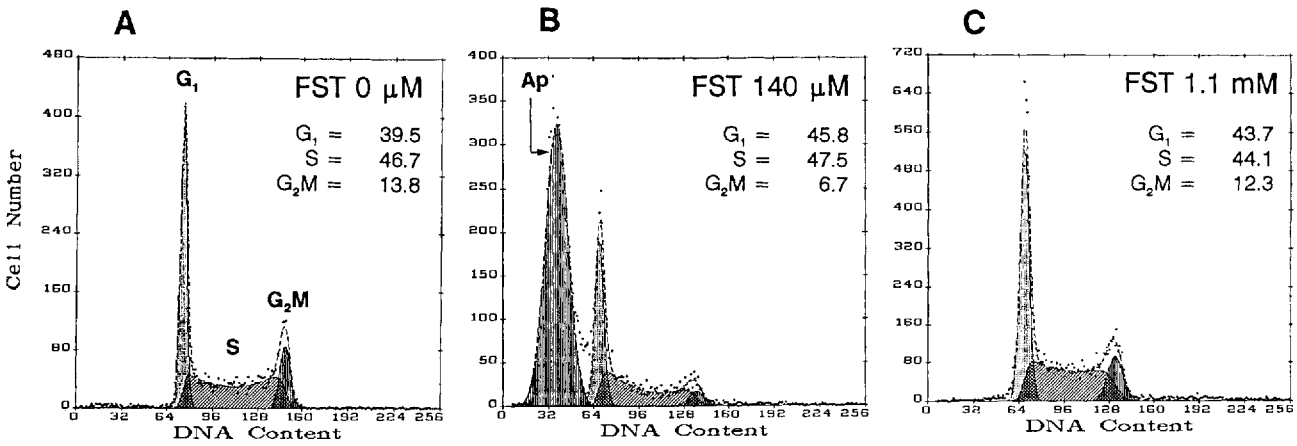

FIG. 4. DNA content of apoptotic and necrotic cells. DNA frequency histograms (after staining with DAPI) of HL-60 cells, untreated (**A**), treated with 140 μM fostriecin for 6 h, which induces apoptosis (**B**) and 1.1 mM fostriecin, a concentration at which the cells rapidly become necrotic (**C**). Note no change in DNA content of necrotic cells, compared to control, which contrasts to apoptotic (Ap) cells (for details, see 31,32).

staining with *PI and Hoechst 33342* (HO 342), e.g., as proposed by Pollack and Ciancio (45), provides a means to discriminate between live versus apoptotic versus necrotic cells (Fig. 5). In this method, the cultured cells are first exposed to PI. This dye is excluded by the cells that have their plasma membrane integrity preserved, but it stains DNA in the cells that have damaged membranes. Subsequent staining of cells with HO 342 results in a situation in which the red fluorescence (PI) of the dead cells is higher than that of live cells, and conversely, the blue fluorescence (HO 342) of live cells is higher than that of dead cells. DNA stainability with each of these dyes, respectively, is proportional to DNA content, which allows one to determine the cell cycle positions of cells in both the live and dead cell populations (45).

When this method was applied by us to the analysis of apoptotic cells (22) it became apparent that HO 342 DNA stainability in these cells was reduced, reflecting, as already discussed, endonuclease-triggered DNA loss (Fig. 5). Yet, for a significant period of time (4–6 h) the apoptotic cells were still able to exclude PI and therefore their red fluorescence was minimal, i.e., similar to that of DNA in live cells. With time, DNA in apoptotic cells started to stain with PI, though, as with their blue fluorescence, the red fluorescence was also reduced compared to truly viable cells. Necrotic cells showed reduced HO 342 stainability though their PI fluorescence was not reduced as in the case of apoptotic cells. Thus, this method offers an attractive assay to differentiate between live, apoptotic and necrotic cells, and as well as to recognize early and late stages of apoptosis based on the difference in membrane permeability to PI (22).

Kubbies (39) applied this method to human lymphocyte cultures treated with X-rays, mitomycin C, or bleomycin. In his studies, the stainability of apoptotic lymphocytes with HO 342 was similar to that of HL-60 treated with DNA topoisomerase inhibitors (22; Fig. 5). In contrast to apoptotic cells, the intensity of red fluorescence of necrotic cells was high, reflecting unchanged DNA content in these cells (39). Most recently, Dive et al. (26), using similar methodology, studied apoptosis of rat thymocytes, Burkitt lymphoma and rat hepatoma cells treated with methylprednisolone, anti-immunoglobulin, or cis-platin. The authors (26) were able to identify live, apoptotic and necrotic cells within all three cell types, based on the differences in their stainability with HO 342 and PI.

Because of the difference between apoptotic and necrotic cells in the integrity of their plasma membrane, preincubation with a mixture of trypsin (0.25%) and DNase I (200 μg/ml) results in total dissolution and loss, from the cell suspension, of necrotic and late apoptotic cells, which cannot exclude these enzymes (19). In contrast, live and early apoptotic cells exclude trypsin and DNase I and remain in the suspension following incubation with these enzymes. Such treatment, therefore, applied prior to cell fixation and measurements by flow cytometry, by eliminating the necrotic and late apoptotic cells, can ensure that only live and early apoptotic cells are analyzed.

V. MITOCHONDRIAL TRANSMEMBRANE POTENTIAL

No marked changes in energy metabolism were observed in apoptotic cells (36,37). Actually, apoptosis is an energy requiring process, and in many instances de novo protein synthesis is needed for apoptosis to occur (8,9). The morphology of mitochondria of apoptotic cells also remains unchanged (36,37). This is in contrast to necrosis, where mitochondrial swelling is one of the earliest changes (36,40).

The ability of mitochondria to maintain their trans-

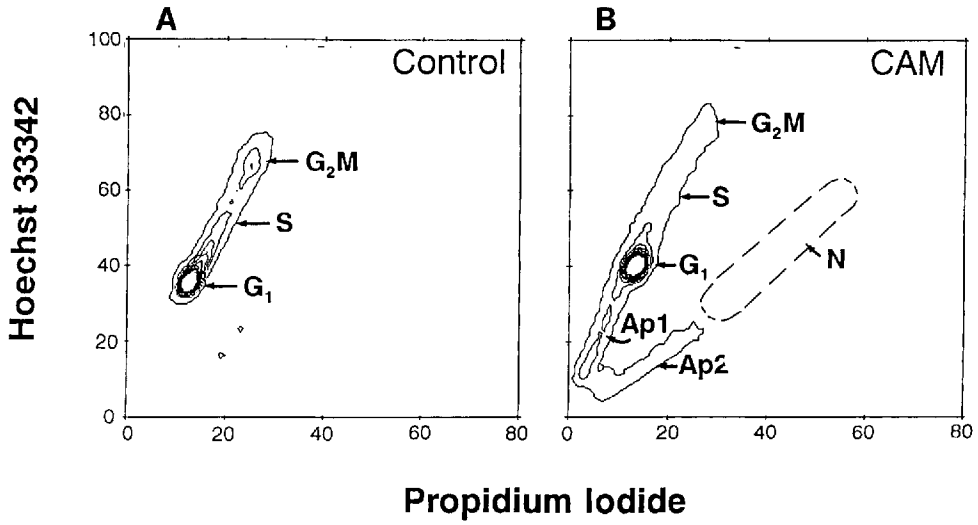

FIG. 5. Stainability of untreated (A) and CAM-treated (B) cells with Hoechst 33342 and PI. The cells were first exposed to PI, then permeabilized and stained with Hoechst 33342 (HO 342; 22,45). In this assay, the cells with undamaged plasma membranes exclude PI and stain predominantly blue with HO 342, whereas cells that cannot exclude PI have more intense red fluorescence and proportionally lower blue fluorescence (45). Note that live cells from the untreated cultures (A) stain strongly with HO 342, in proportion to their DNA content, and have low PI fluorescence. The cells that undergo apoptosis (6 h treatment with 0.15 μM camptothecin), at first (during the initial 2–6 h), show diminished HO 342 fluorescence and low PI fluorescence (Ap1), indicating that they still exclude PI. With time (after 6 h), apoptotic cells stain more with PI and less intensely with HO 342 (Ap2). Necrotic cells (N) have unchanged DNA content and would stain more intensely with PI than with HO 342 (not shown, but their position is indicated by the broken outline).

membrane potential (electronegativity of the mitochondrial interior), which can be assayed by retention of the cationic probe, rhodamine 123 (R 123) (15,33,57) is preserved in apoptotic cells (22). Thus, this dye can be used to discriminate between cells dying by apoptosis versus necrosis (Fig. 6). Simultaneous staining with R 123 and PI makes it possible to distinguish cells excluding PI from cells that have a damaged plasma membrane, and thus to evaluate the mitochondrial transmembrane potential of the former.

VI. LYSOSOMAL PROTON PUMP

Supravital cell stainability with AO observed at low concentration of this dye (1–5 μM) and appearing as red luminescence, is a reflection of the activity of the proton pump of lysosomes (reviewed in 13). The uptake of AO, which is a weak base, is due to the low pH inside these organelles. Namely, the cell membrane is permeable to the uncharged form of AO and the dye can penetrate into lysosomes. Once inside, the dye becomes protonated as a result of the high proton concentration, and therefore entrapped in these organelles (13). Accumulation of AO is predominantly determined by the pH gradient across the membrane of these lysosomes, which is maintained by the ATP-dependent proton pump (13).

The ability of apoptotic cells to accumulate AO in lysosomes is not markedly changed (22; see Fig. 7). This would indicate that, during apoptosis, the ATP-dependent proton pump is not significantly affected. Necrotic cells, on the other hand, tend to lose their ability to concentrate AO in lysosomes resulting in minimal AO red luminescence (Fig. 7).

VII. PROTEIN AND RNA CONTENT

Differential staining of DNA and protein with DAPI and sulforhodamine 101 reveals that apoptotic cells have, in addition to lowered DNA content, markedly diminished protein content (Fig. 8). When apoptosis was induced in HL-60 cells or thymocytes by various drugs, including DNA topoisomerase I or II inhibitors, the decrease in protein content was observed to occur simultaneously with a decrease in DNA content (21–24). Thus, during apoptosis the endonucleolytic activity was paralleled by proteolysis which was suggestive that these two processes may be coupled. Indeed, in subsequent studies, we observed that suppression of intracellular proteolysis by small molecular weight inhibitors of serine proteinase(s) prevented DNA degradation and apoptosis in HL-60 cells (6) and rat thymocytes (7). These results indicated that proteolysis is necessary for DNA degradation to progress, and provided evidence that the nucleolytic and proteolytic steps may indeed be coupled (6,7). Simultaneous staining of DNA and protein, therefore, by revealing a decrease in the content of both these constituents during apoptosis, made it possible to investigate a correlation between these processes (6,7).

Because the plasma membrane of necrotic cells is leaky, the protein content of these cells is also reduced (data not shown). However, since the DNA content of necrotic cells is not markedly altered compared to live

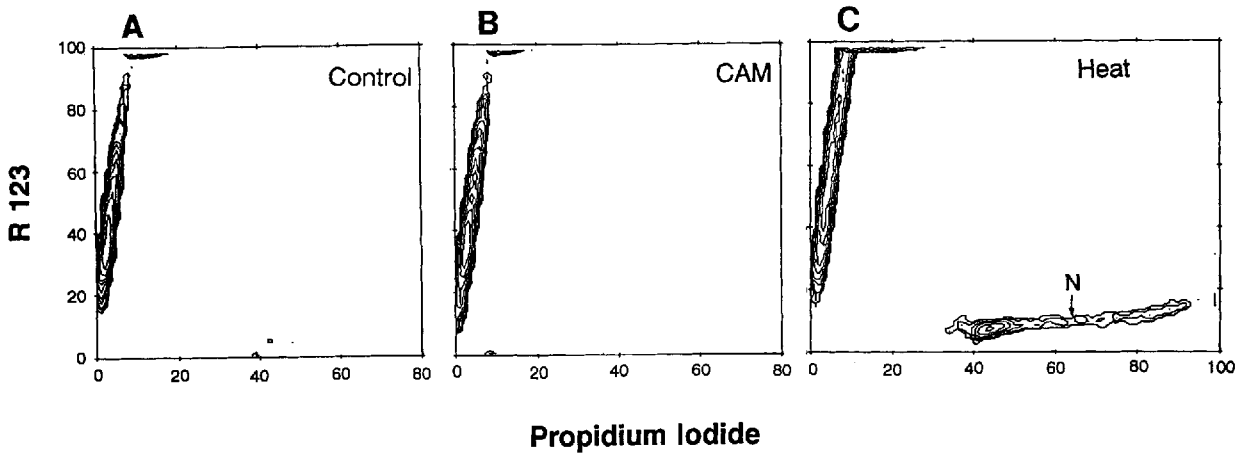

FIG. 6. Retention of R 123 and exclusion of PI by apoptotic cells. Cultures of HL-60 cells, untreated (**A**), treated with 0.15 μM camptothecin for 4 h (**B**) or heated at 45°C for 2 h (**C**), were exposed to 10 μg/ml of R 123 for 30 min. The cells were then rinsed and suspended in HBSS solution containing 20 μg/ml of PI (22). The green fluorescence (R 123) reflects the cells' ability to retain R 123, due to their preserved mitochondrial transmembrane potential (15,33) while the lack of red fluorescence (PI exclusion) indicates that plasma membrane integrity is preserved. Apoptotic cells that are numerous (~40%) in the camptothecin treated cultures, exclude PI and retain R 123. Necrotic cells (N) present in the heated culture lose their ability to retain R 123 and stain with PI.

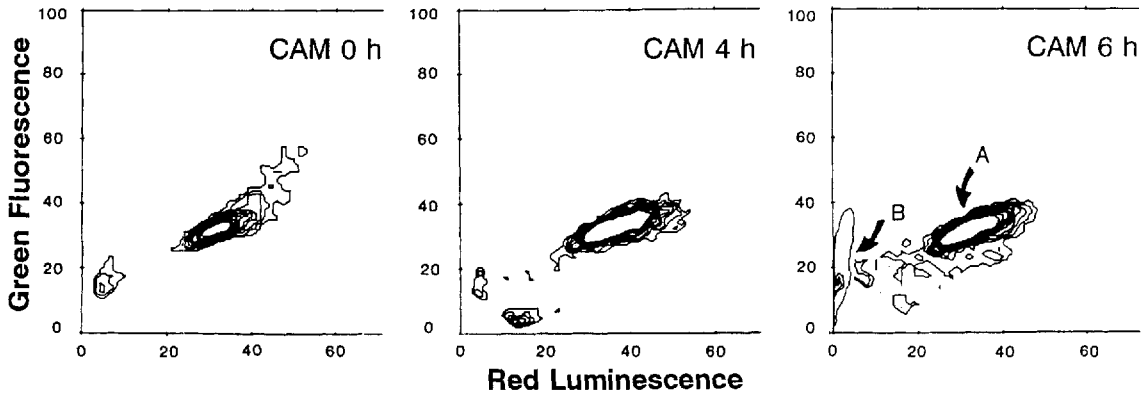

FIG. 7. Supravital uptake of AO by HL-60 cells, untreated (CAM 0 h) or treated with 0.15 μM camptothecin (CAM) for 4 and 6 h. The red luminescence represents the accumulation of AO in lysosomes due to an active proton pump (13,22). Treatment with camptothecin, which induces extensive apoptosis, has little effect on the uptake of AO; over 95% of the cells remain within population A following 4 or 6 h exposure to the drug, with only a few cells losing the ability to accumulate the dye in lysosomes (population B) (for details see 22).

cells, the DNA/protein ratio of necrotic cells is higher than that of live, or apoptotic cells. This ratio, therefore, in combination with total DNA content, may also be used as a marker discriminating between live, necrotic and apoptotic cells.

It is difficult to estimate RNA content of apoptotic cells because simultaneously with the possible loss of RNA, there is a change in the conformation of DNA. Since both RNA probes, AO (18) as well as pyronine Y (PY), cannot easily discriminate between RNA and single stranded DNA (14), an increase in the proportion of denatured (single-stranded) DNA can compensate for loss of RNA. The data presented in Fig. 9 indicate that, indeed, the proportion of denatured DNA is increased in apoptotic cells. Namely, G_0 thymocytes, prior to apoptosis, have minimal RNA content and thus low red luminescence. During apoptosis, these cells are characterized by increasing red luminescence in proportion to a decrease in their green fluorescence (Fig. 9; see the negative correlation between green and red luminescence of apoptotic cells). The increased red luminescence of DNA stained with AO in fragmented nuclei of apoptotic cells, compared to intact nuclei, is also evident in the micrograph shown in Fig. 3D. Some denaturation of DNA in apoptotic cells, thus, is apparent. Therefore, during apoptosis, the increase in the proportion of single stranded DNA can obscure the possible loss of RNA since both stain similarly (red) with AO.

These data appear to be at odds with observations of Arends et al., (92) who reported that the degree of DNA denaturation in apoptotic cells is minimal. However, the dyes used to stain RNA (48) or single stranded

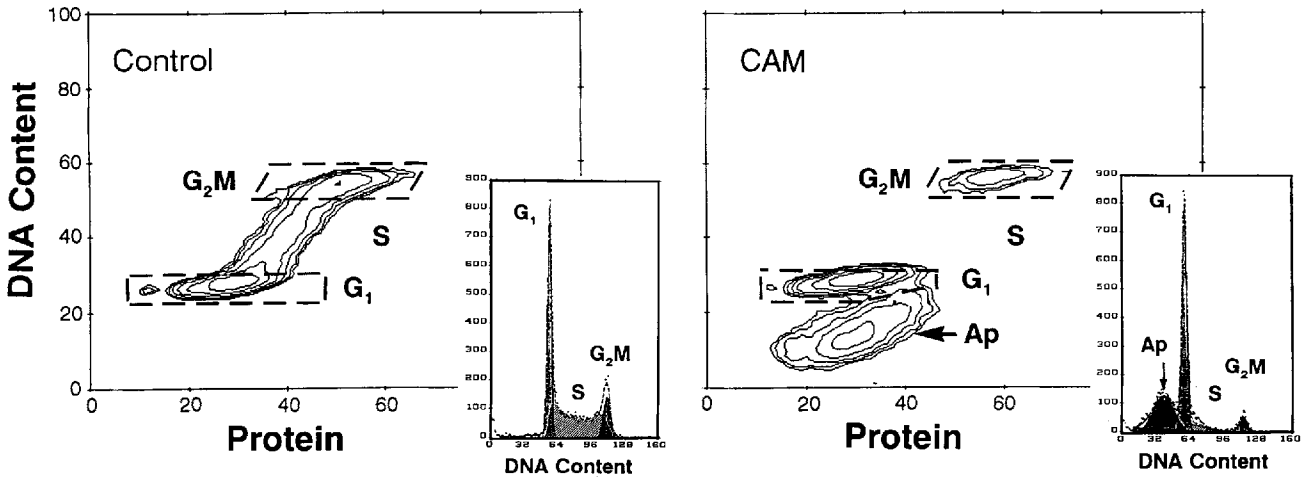

FIG. 8. Bivariate, DNA, and protein content distribution of HL-60 cells, untreated (Control) and treated with 0.15 μM camptothecin for 4 h (CAM). The cells were stained with DAPI and sulforhodamine 101; their DNA frequency histograms are included in the respective inserts. Note that the appearance of apoptotic cells (Ap) coincides with a loss of cells from S phase of the cell cycle, which indicates that S phase cells selectively undergo apoptosis (22). A decrease in DNA content in these cells is paralleled by a decrease in their protein content.

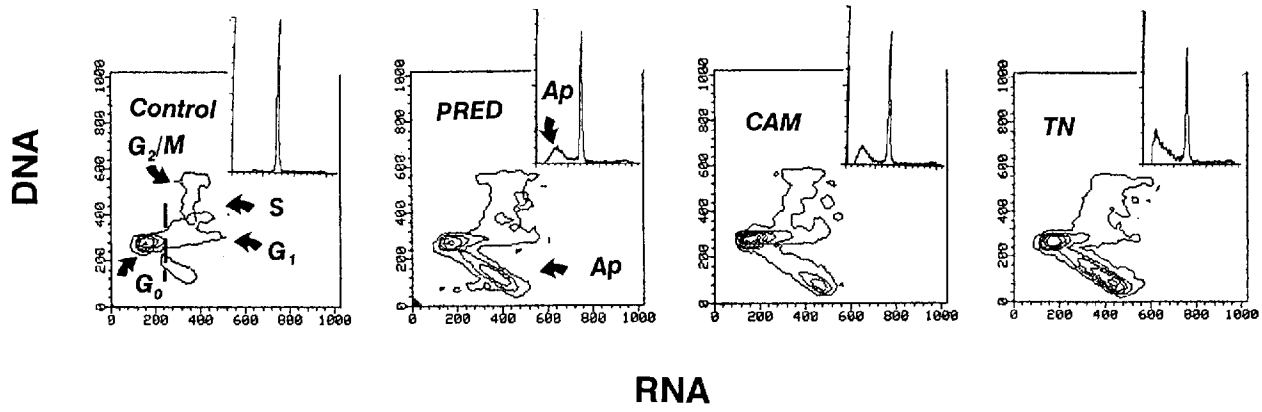

FIG. 9. Apoptosis of rat thymocytes induced by prednisolone (PRED), camptothecin (CAM) or teniposide (TN). Freshly isolated rat thymocytes were incubated either with 5 μM prednisolone, 0.15 μM camptothecin or 3 μM teniposide for 4 h. Their DNA and RNA was then differentially stained with AO (6,7,18). Based on differences in RNA content it is possible to discriminate between G_0 and G_1 cells. The appearance of apoptotic cells with reduced DNA content (Ap) coincides with the selective loss of G_0 cells. This indicates that, unlike in the case of HL-60 cells where S phase cells selectively undergo apoptosis, apoptosis of thymocytes regardless of the inducer (PRED, CAM or TN) was preferential to G_0 cells (7).

DNA (AO or PY), by virtue of their high affinity to single stranded regions, are themselves capable of potentiating the degree of DNA denaturation (14). This staining pattern, therefore, may reflect the increased sensitivity of DNA to denaturation (see further) rather than the mere presence of single stranded DNA sections.

As with protein content, RNA content of necrotic cells is minimal, due to the efflux of RNA resulting from the loss of plasma membrane integrity (not shown).

Simultaneous measurement of cellular DNA and RNA, when applied in studies of apoptosis, offers one significant advantage. Namely, RNA content allows one to discriminate G_0 cells from G_1 cells (16,18). Therefore, the method can be used to reveal whether G_0 or G_1 cells are selectively affected. An illustration of this approach is presented in Fig. 9. The data shown in this figure, (and published in greater detail elsewhere; 7) clearly indicate that when thymocytes are treated with prednisolone or CAM, apoptosis is selective to G_0 cells; G_1, S and G_2 cells remain unaffected by the treatment. Because RNA content is measured prior to apoptosis or in those cells that do not undergo apoptosis, the problems with denatured DNA stainability, as discussed above, do not apply in this case. The method, thus, can be very useful to elucidate the cell cycle specificity of the drugs that induce apoptosis.

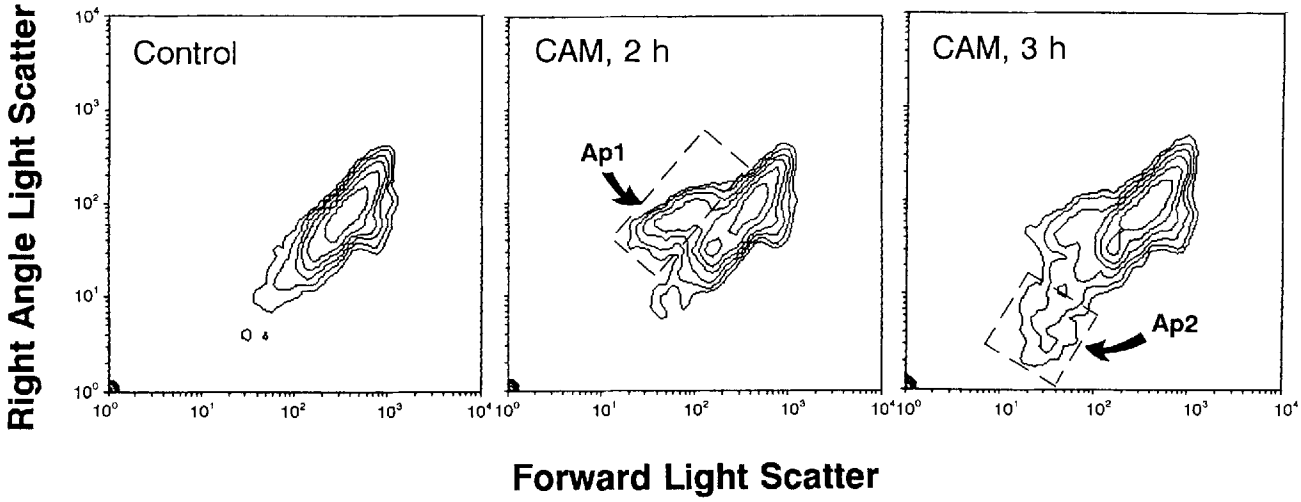

FIG. 10. Changes in light scatter properties of HL-60 cells treated with 0.15 μM camptothecin for 2 and 3 h. Treatment with camptothecin induces apoptosis in approximately 35–40% of the cells (S-phase cells). The initial decrease in forward light scatter of the cells which undergo apoptosis after 2 h of the treatment (Ap1), is followed by an even more dramatic change in their ability to scatter light at right angles after 4 h (Ap2).

VIII. LIGHT SCATTER

Changes in the morphology of cells undergoing apoptosis affect their light scattering properties (Fig. 10). In the case of HL-60 cells, the earliest change was observed 2 h after exposure to CAM, i.e. prior to a change in DNA content (Gorczyca et al., in preparation). Initially, a decrease in forward light scatter was apparent, with no change in the intensity of the right angle scatter signal (Ap1 population). After 3 h, a decrease in both forward and right angle scatter were evident (Ap2). Cell death by necrosis results in an immediate decrease of the forward and right angle scatter signals (Gorczyca et al., in preparation).

The decrease in forward light scatter was also observed by Swat et al., (49) in the case of thymocytes triggered to undergo apoptosis by prednisolone. In the latter cell system, this decrease was paralleled by an increase in right angle scatter, most likely reflecting condensation of chromatin and fragmentation of nuclei (49). These authors have also observed the appearance of apoptotic bodies as separate particles with low light scatter properties (49). Most recently, Dive et al., (26) have combined cell staining with HO 342 and PI, as described above, with light scatter measurements of the same cells. This allowed them to correlate the light scatter changes and stainability with PI and HO 342 of thymocytes, Burkitt lymphoma, and hepatoma cells undergoing apoptosis. The decrease in forward light scatter and a minor increase in right angle scatter was typical of cells which had reduced stainability with HO 342 (26).

The major advantage of the detection of apoptotic cells by their altered light scatter properties is the possibility of combining this method with their surface immunofluorescence analysis. As demonstrated by Swat et al., (49) this approach allows one to identify subtypes of lymphocytes that selectively undergo apoptosis following specific treatments. This analysis can also be combined with live cell sorting.

IX. SENSITIVITY OF DNA IN SITU TO DENATURATION

The metachromatic dye AO can differentially stain double stranded and denatured DNA in cells or cell nuclei (14). The dye intercalates into double stranded DNA and, when bound in this form, fluoresces green. AO can also condense the denatured sections of DNA and in the condensed form AO luminesces red (14). The ratio of red to total (red plus green) cell luminescence (α_t; see ref. 12) represents the proportion of denatured DNA in the cell. When the measurements are standardized, the sum of intensities of green and red luminescence (L_{tot}) is proportional to total DNA content (12). This method, therefore, can be applied to evaluate the sensitivity of DNA to denaturation. The cells are fixed, treated with RNase to remove any RNA which can obscure DNA stainability, treated with heat or acid to partially denature DNA, and stained with AO, as described in detail elsewhere (12). In earlier studies, we (12) and others (4) have observed that DNA in condensed chromatin, such as in the case of mitotic or G_0 cells, is much more sensitive to denaturation than is DNA in interphase cells (see Fig. 3F,G). Therefore, the method can be used to identify these cells by flow cytometry.

The DNA of apoptotic and necrotic cells shows markedly increased sensitivity to denaturation, higher even than the DNA of mitotic cells (32; Figs. 3E–I, 11). Ac-

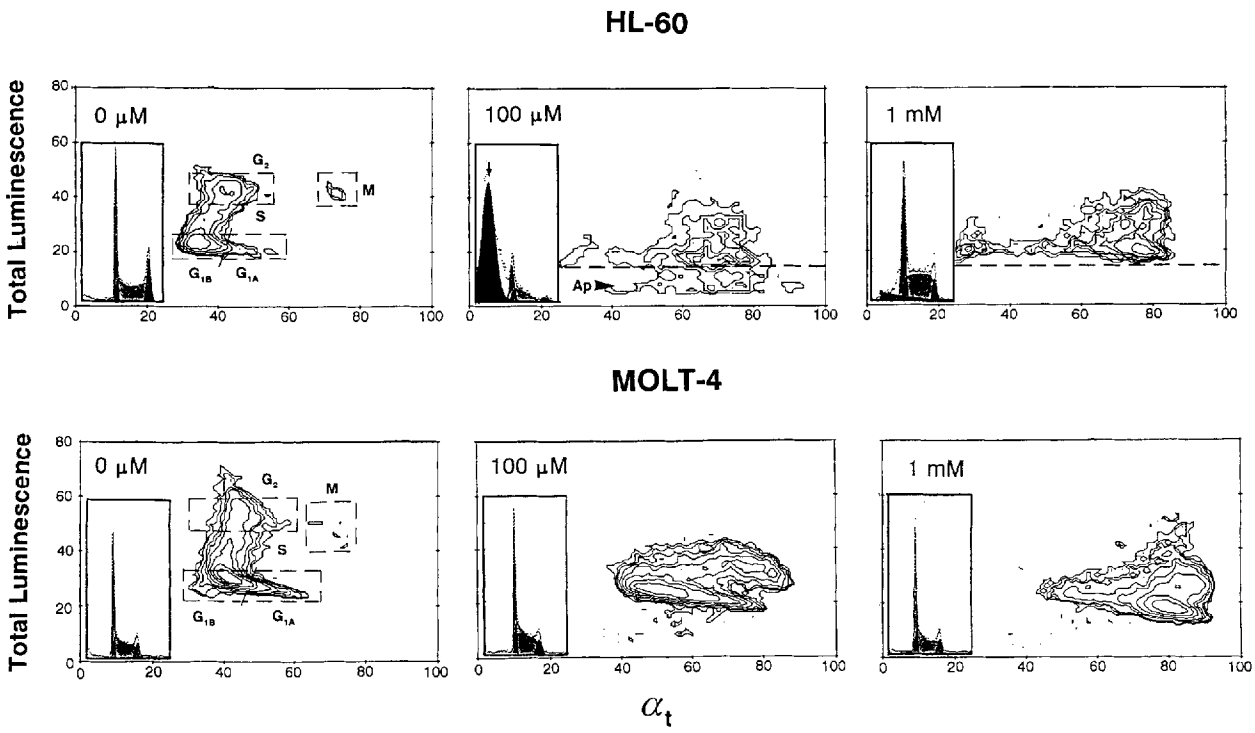

FIG. 11. Sensitivity of DNA in situ to denaturation in apoptotic or necrotic HL-60 and MOLT-4 cells. HL-60 cells were treated with 100 μM fostriecin, which induces apoptosis, or 1 mM fostriecin, which causes necrosis (31,32). MOLT-4 cells when treated with either 100 μM or 1 mM fostriecin undergo necrosis (32). DNA sensitivity to denaturation was assayed by exposure of the RNase-treated cells to 0.1 M HCl followed by staining with AO at pH 2.6. Under these conditions double stranded DNA sections stain green whereas denatured DNA stains red. Total cellular luminescence (green plus red) is proportional to total DNA content; α_t, the ratio of red to total luminescence, represents the fraction of denatured DNA (12). DNA frequency histograms (after staining with DAPI) of the cells from respective cultures are included in the insets. Apoptotic cells can be distinguished by their increased α_t values and reduced total luminescence. Necrotic cells have increased α_t and only slightly lowered total luminescence. This method allows one to correlate sensitivity of DNA to denaturation with cell position in the cycle. For example, the increase in α_t of MOLT-4 cells observed at 100 μM FST is preferential to G_2 and late S indicating that these cells are more sensitive to FST (32).

tually, the data, when plotted as bivariate distributions of total cell luminescence (L_{tot}) versus α_t reveal both the total DNA content and the fraction of DNA that is denatured. This approach, thus, provides a sensitive and early assay to discriminate between viable, apoptotic and necrotic cells. Viable interphase cells have low α_t values and high L_{tot} (proportional to their DNA content). Apoptotic cells are characterized by very high α_t and diminished L_{tot}, reflecting DNA degradation. Necrotic cells also have highly increased α_t values, but their L_{tot} is higher than that of apoptotic cells (32). Necrotic cells, however, overlap, in their α_t and L_{tot} values with mitotic cells.

The assay of DNA in situ sensitivity to denaturation allows one not only to identify apoptotic or necrotic cells, but also to correlate these processes with the position of cells in the cell cycle, as illustrated in Figure 11.

X. IN SITU NICK TRANSLATION

Jonker et al. (34) have recently reported that a nonradioactive in situ nick translation assay (3) can be applied to detect DNA strand breaks in apoptotic cells. In this assay, DNA breaks are labeled in a cytochemical reaction employing exogenous DNA polymerase and a mixture of triphosphonucleotides, one of which is biotinylated. By combining this reaction with cellular DNA staining with PI, the authors (34) were able to reveal the cell cycle phase specificity of DNA breaks. This method, when applied to thymus lymphocytes, was sensitive enough to detect effects of 0.25 Gy irradiation (34). We have applied their method to detect apoptosis in CAM-treated HL-60 cells (Fig. 12; Gorczyca et al., in preparation). The data clearly show the utility of this approach in the identification of the origin of the cells with DNA breaks (incorporating biotinylated dUTP) with respect to their cell cycle position.

The major advantage of the nick translation assay appears to be its ability to reveal early DNA breaks during apoptosis, prior to the loss of any significant DNA content or nuclear fragmentation. The method, thus, is well suited to identify cells at the very early stages of apoptosis. However, since the presence of DNA strand breaks is not unique to apoptosis, this assay should be run together with other methods that can confirm the apoptotic mode of cell death.

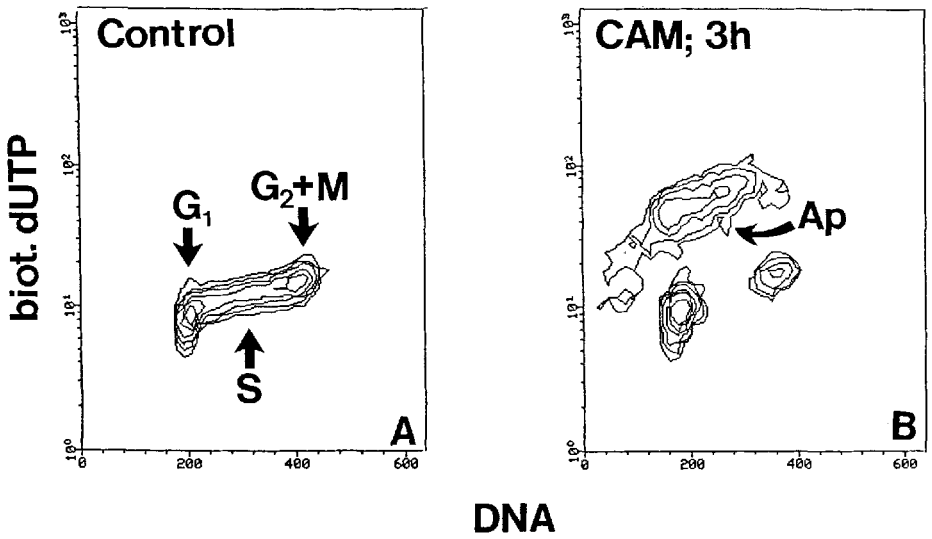

FIG. 12. Detection of apoptotic cells by the in situ nick translation. HL-60 cells, untreated (control) or exposed to 0.15 μM CAM for 3 h were subjected to the nick translation reaction, as described by Jonkers et al. (34). Incorporation of the biotinylated dUTP (note the exponential scale) is selective to the S-phase cells, which undergo apoptosis when treated with CAM.

XI. FURTHER DEVELOPMENTS

Apoptotic cells are characterized by a very specific pattern of metabolic, biochemical and molecular features which, as discussed, can be probed by flow cytometry. Reduction of cellular DNA content was the most common feature measured thus far. It should be mentioned at this point, however, that detection of any single feature which characterizes apoptotic cells, including loss of DNA, cannot be considered, per se as evidence of apoptosis. Other features, such as preservation of the integrity of the plasma membrane, or positive "viability" tests (retention of R 123, activity of the lysosomal proton pump) provide further information on the mechanism of cell death. Activation of the endogenous endonuclease which has preferential affinity to internucleosomal (linker) sections of DNA is a very characteristic feature and is generally accepted as an evidence of apoptosis; this evidence can be obtained by gel electrophoresis of DNA extracted from the cells (Fig. 1).

One can visualize that future applications of flow cytometry in studies of apoptosis can develop in two directions. The first direction will be in the field of cell- and molecular biology and pharmacology. It will involve further exploration of various metabolic properties of cells undergoing apoptosis, to better understand the mechanisms of this process. A variety of markers can be used to reveal molecular changes that occur in the sequence of events that lead to DNA degradation and cell death. Particularly important will be to understand the nature of the phenotype- or cell cycle specific factors that are responsible for the observation that certain cells selectively respond to different treatments or drugs by apoptosis, while other cells resist such treatments. With knowledge of these mechanisms it may be possible to modulate the apoptotic process, with the ultimate aim of enhancing the efficiency of chemotherapy in the clinic. Recent findings that implicate the expression of c-myc in induction of cell sensitivity to undergo apoptosis (28) are of extreme interest and of potential importance, especially in the field of oncology.

The second area in which flow cytometry will prove useful is more related to the clinic. This methodology offers a promise for early detection of apoptotic cells in tumors during treatment. Sampling of tumors (e.g., by needle biopsy) for apoptotic cells may offer a very early marker of tumor response, predictive of the overall sensitivity of the tumor to the particular treatment regimen. Development of new probes of apoptotic cells which can detect specific changes in these cells that precede DNA degradation would be of great value in this application.

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NOTE ADDED IN PROOFS:

A method to identify apoptotic cells by flow cytometry, based on cell lysis by detergent and DNA staining with AO was recently published by Ojeda et al., (61). Sequential cell staining with Hoechst 33342 and PI was used by Sun et al., (62) and Ormerod et al., (63) to discriminate live, apoptotic and dead cells, the latter having a permeable plasma membrane.

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