Rapid Communication

Comparison of $DiOC_6(3)$ Uptake and Annexin V Labeling for Quantification of Apoptosis in Leukemia Cells and Non-Malignant T Lymphocytes From Children

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Early during apoptosis, there is a reduction in mitochondrial transmembrane potential (MTP) and externalization of phosphatidylserine (PS) in cell membrane prior to eventual cell death. Flow cytometric detection techniques targeting these changes, reduction of $DiOC_6(3)$ uptake upon the collapse of MTP and annexin V binding to PS have been successfully used to detect apoptotic cells. These methods have given comparable results when cell lines were used.

We compared the two different techniques, $DiOC_6(3)$ uptake and Annexin V-propidium iodide co-labeling in the quantification of cytarabine, vincristine and daunorubicin induced apoptosis on three leukemia cell lines (HL-60, CEM, U937), and bone marrow blasts from 26 children with acute myeloid leukemia, 14 with T cell acute lymphoblastic leukemia. Anti-Fas-induced apoptosis in culture-grown peripheral blood T lymphocytes on 18 samples from 9 children with non-malignant conditions were also studied by these techniques.

Our results showed that there is a correlation ($P < 0.05$) between the apoptosis rates measured by these two techniques for drug-induced apoptosis in myeloid and lymphoid blasts, and for anti-Fas mAb-induced apoptosis in T lymphocytes.

This data suggests that reduction of the MTP and PS externalization may be common to many apoptotic pathways and techniques targeting either of these changes may be used in quantification of apoptosis in different clinical samples. Cytometry (Comm. Clin. Cytometry) 42:74–78, 2000.

Key terms: apoptosis; flow cytometry; annexin V; $DiOC_6(3)$; leukemia; Fas

Several apoptotic pathways are operational in different cell types and with different apoptosis-inducing stimulus in a cell type. Various biochemical and morphologic alterations may be observed during apoptosis and it is possible that certain changes are related to the specific pathways utilized. Reduction of mitochondrial transmembrane potential (MTP) is among the changes encountered during early reversible stages of apoptosis and is preceded by cytochrome-c release in several cell types (1,2). 3.3′ dihexyloxacarbocyanine iodide ($DiOC_6(3)$) is a cationic dye, which strongly labels mitochondria. A decrease in MTP in apoptotic cells is associated with a reduction of $DiOC_6(3)$ uptake as demonstrated on flow cytometric analysis (3–5).

Externalization of PS in cell membrane due to flip-flop of lipids, is another early event of apoptotic process and marks irreversible commitment to cell death (6). Annexin V (Ann V) is a plasma protein, which binds to PS and fluorescence-conjugated Ann V was used to detect cells undergoing apoptosis by flow cytometry (7). Propidium iodide (PI) is a membrane impermeable nucleic acid dye which enters the cells more readily during late apoptosis and upon cell death due to loss of membrane integrity (8). Thus, co-labeling with Ann V and PI has enabled the recognition of viable, early, and late apoptotic/dead cells (9–10).

Since comparable results have been obtained by these methods particularly in cell line studies, in this study we compared apoptosis rates measured by Ann V-PI co-labeling and $DiOC_6(3)$ uptake, in clinical samples, e.g.

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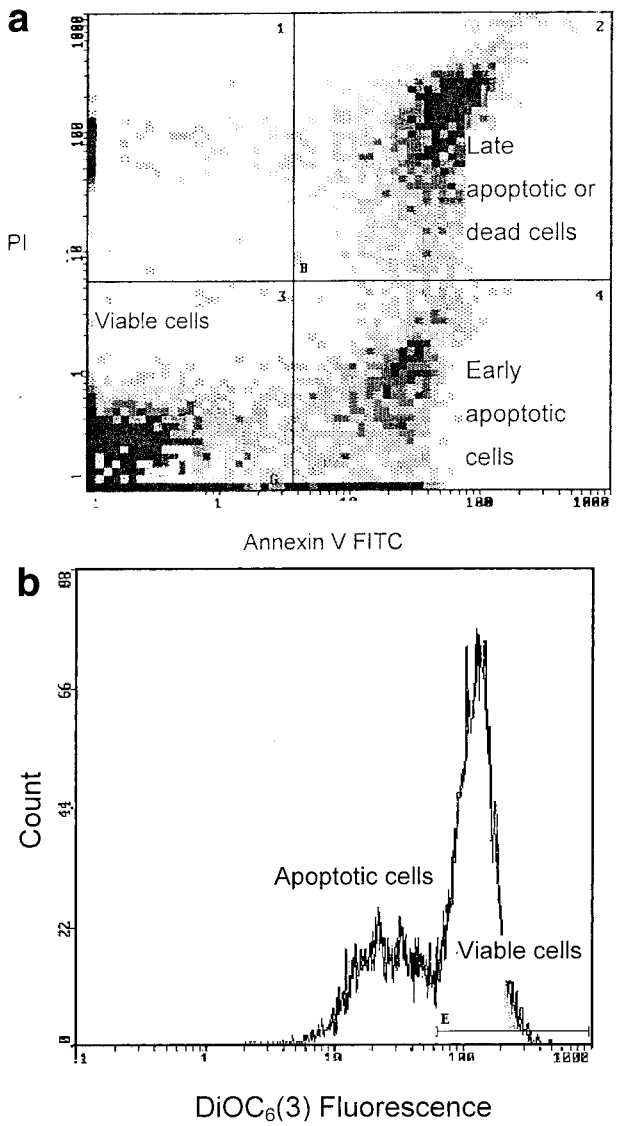

FIG. 1. Detection of apoptotic cells by Annexin V-PI co-labeling (**a**) and $DiOC_6$ (**b**) methods in a T-ALL sample without gating.

human leukemia cells and activated T cells, induced by various stimuli.

MATERIALS AND METHODS

Chemicals and Cells

$DiOC_6(3)$, RPMI 1640, L-glutamine, and histopaque were purchased from Sigma (St Louis, MO), IgM anti-Fas monoclonal antibody (mAb) from Kamiya (Seattle, WA), Annexin V-PI apoptosis detection kit and interleukin 2 (IL-2) from R & D Systems (Minneapolis, MN), anti-CD45 mAb from Becton Dickinson (Mountain View, CA) and phytohemagglutinin (PHA) from Caltag (Burlingame, CA). Heparinized blood or bone marrow samples of 26 children with AML and 14 with T-ALL were obtained from Pediatric Oncology Group. Peripheral blood samples were collected from 9 children with non-malignant conditions (hemophilia, ITP, etc.) in our clinic after an informed consent was obtained.

Cell Culture

1×10^6 Ficoll-seperated fresh blood or bone marrow mononuclear cells from patient samples with leukemia and leukemia cell lines (CEM, HL-60, U937) were incubated with cytarabine (Ara-C) (10 μM), daunorubicin (DNR) (0.5 μM), and vincristine (VCR) (0.4 μM) in 1 ml dialyzed RPMI containing 15% fetal calf serum, ITS with either 20% supernatant from human bladder carcinoma cell line 5637 as a source of myeloid growth factors or 20% supernatant from mixed lymphocyte culture in a CO_2 incubator at 37°C for 15–16 hr. Peripheral blood lymphocytes were cultured in RPMI with 10% fetal bovine serum (FBS), initially activated with PHA (1 μg/mL), then maintained in two different concentrations of IL-2 (60 and 300 IU/mL). At day ten, 1×10^6 activated T lymphocytes were cultured with 1 μg anti-Fas mAb in 1 mL RPMI with 10% FBS for 15–16 h before analysis. Cells incubated in the absence of drugs or anti-Fas mAb served as controls.

Flow Cytometry

At the end of the incubation period, cells were washed with PBS and stained with Ann V/PI and $DiOC_6(3)$ separately described as previously (9,11), then analyzed on a Coulter XL Epics Flow Cytometer (Coulter Corp., Miami, FL). Briefly, following washing with PBS, 1×10^5 Ficoll-separated mononuclear cells were diluted in 100 μL of buffer solution and 10 μL Ann V and 10 μL PI were added as instructed by the producer of the apoptosis kit. Cell suspension was incubated at room temperature in the dark for 20 minutes, then 400 μL of buffer solution was added before flow cytometric analysis. For $DiOC_6(3)$ staining 1×10^5 cells were resuspended in 500 μL of normal saline following cell wash with PBS, and 10 μL of $DiOC_6(3)$ (40 nM, dissolved in DMSO) was added. The suspension was incubated in the dark at room temperature for 30 minutes before flow cytometric analysis. Flow cytometric analysis was based on the gated blast cell population by side scatter (SS) and forward scatter (FS) characteristics determined on the control sample in patient samples with leukemia for both staining methods. Since they were homogenous, entire cell population was included in the analysis when cell lines or culture-grown T cells were studied. Blast population of individual samples were determined by SS / CD45 analysis. Adjustments were done for daunorubicin autofluorescence.

Statistical Analysis

Pearson's correlation test and paired *t*-test were used for statistical analysis.

RESULTS

All three drugs utilized in the study and anti-Fas mAb induced varying degrees of apoptosis in leukemia cell lines, human leukemia samples, and culture-grown, activated T cells, respectively. In Ann V-PI co-labeling method, Ann V-ve/PI-ve fraction represented viable cells;

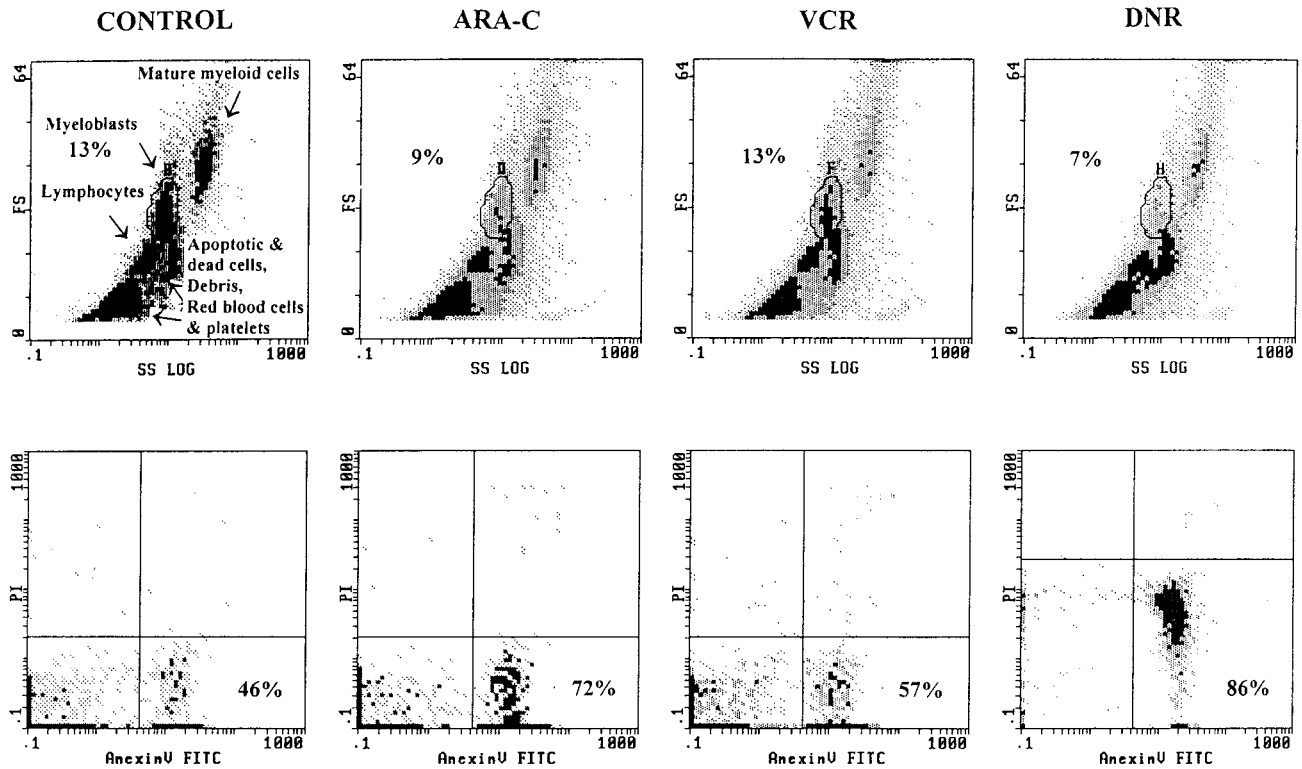

FIG. 2. Gating for the blast cells and Ann V-PI staining patterns in an AML sample treated with different drugs.

Ann V + ve/PI-ve fraction early apoptotic cells; Ann V + ve/PI + ve fraction late apoptotic and dead cells (Fig. 1a). In $DiOC_6(3)$ uptake method, $DiOC_6(3)$-positive fraction represented viable cells, $DiOC_6(3)$-negative fraction apoptotic and/or dead cells (Fig. 1b). Gating done in leukemia samples from children aimed to exclude mature cells as well as to minimize the influence of subjective selection of populations upon drug treatment with varying shift patterns secondary to apoptotic process (Fig. 2). Due to the gating a very small proportion of Ann V + ve/PI + ve cells has been encountered in the analysis of leukemia samples.

We have observed a statistically significant correlation between the percentage of $DiOC_6(3)$-negative cells and the percentage of Ann V + ve cells in AML samples during the assessment of spontaneous (apoptosis rates observed in control tubes) and drug-induced apoptosis with all three drugs ($P < 0.01$) and in drug-treated human leukemia cell lines, U937 and HL-60 ($P < 0.05$) (Table 1). However, Ann V + ve cell fractions were higher than with $DiOC_6(3)$-negative cell fractions, more pronounced in samples treated with Ara-C and DNR (the difference was not statistically significant by paired *t*-test—data not shown).

In T-ALL samples, a positive correlation between the two methods was also seen for spontaneous and drug-induced apoptosis rates ($P < 0.01$) (Table 1, Fig. 3). There

Table 1
*Apoptosis Rates Measured by $DiOC_6(3)$ Uptake and Ann V-PI Co-labeling Methods Given as Mean Values With 2 SD (Standard Deviation) and Comparison of These Values**

Sample (*n*)	Treatment	$DiOC_6(3)$-negative cells (%)	Ann V + ve cells (%)	*r* value	*P* value
AML (26)	Control	14 ± 15	15 ± 16	0.513	<0.01
	Ara-C	24 ± 18	35 ± 21	0.565	<0.01
	VCR	21 ± 18	22 ± 21	0.528	<0.01
	DNR	34 ± 24	49 ± 26	0.509	<0.01
T-ALL (14)	Control	23 ± 16	15 ± 14	0.820	<0.01
	Ara-C	29 ± 20	22 ± 18	0.812	<0.01
	VCR	31 ± 21	18 ± 15	0.748	<0.01
	DNR	54 ± 28	59 ± 26	0.584	<0.05
T CELL (18)	Control	8 ± 6	5 ± 5	0.010	NS
	Anti-Fas	33 ± 20	27 ± 21	0.752	<0.01

*NS, not significant.

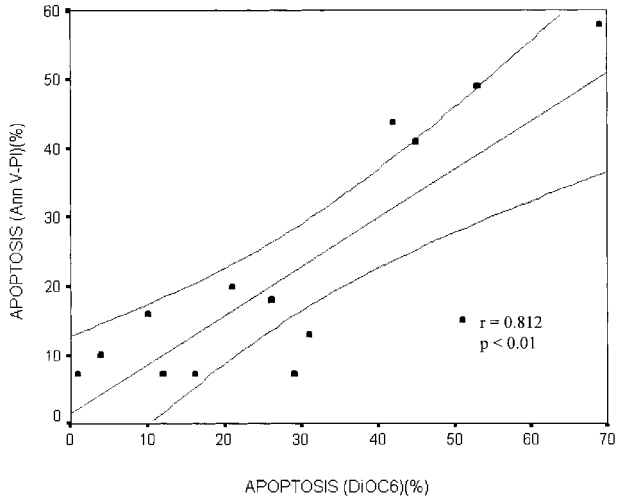

FIG. 3. Comparison of Ara-C-induced apoptosis rates determined by Annexin V-PI co-labeling and $DiOC_6(3)$ methods in T-ALL samples. Curved lines indicate 95% confidence intervals.

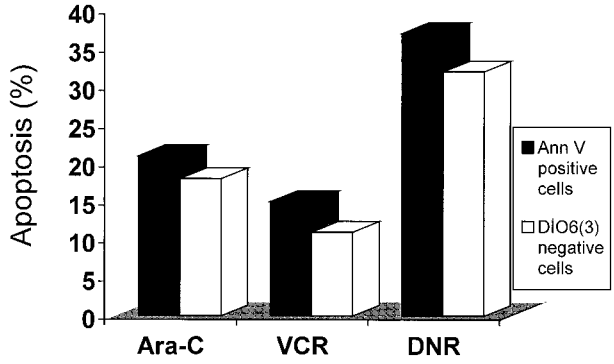

FIG. 4. Drug-induced apoptosis rates measured by $DiOC_6(3)$ uptake and Ann V-PI co-labeling methods in CEM cell line.

was also a positive correlation between percent apoptotic cells detected by these two methods in anti-Fas mAb-induced apoptosis of activated T lymphocytes ($P < 0.01$) (Table 1). However, unlike in AML samples, in experiments where malignant or non-malignant T cells were used, apoptotic cell population detected by $DiOC_6(3)$ technique exceeded the rates determined by Ann V-PI co-labeling method, except in DNR-treated T-ALL samples and CEM cell line (Table 1, Fig. 4).

DISCUSSION

Flow cytometry has been successfully used for the detection and quantification of apoptotic cells of different origin (12). $DiOC_6(3)$ uptake and Ann V-PI co-labeling techniques target different compartmental changes for detection of apoptotic cells (9,13). $DiOC_6(3)$ appears to be one of the best fluorochromes for the quantification of different mitochondrial changes occurring during early apoptosis (14). Externalization of PS in the cell membrane (detected by Ann V binding) occurs prior to nuclear condensation and DNA fragmentation, but it is probably a downstream event to the reduction of MTP in the apoptotic process (15,16).

In our study, the percentage of apoptotic cells detected by $DiOC_6(3)$ technique were higher than the rates determined by Ann V-PI method in T-ALL cells treated with two different drugs and activated peripheral blood T cells treated with anti-Fas mAb. Similar results were reported by Hertveldt et al who utilized radiation for induction of apoptosis (11). However, higher apoptosis rates were obtained by Ann V-PI co-labeling in control and drug-treated AML cells; it was more pronounced with Ara-C or DNR treatment.

These varying results in T-ALL and AML samples, though statistically not significant may suggest an inherent difference between lymphoid cells and myeloblasts to undergo apoptosis or may point to a mechanistic variation in response to different stimuli. The results in malignant and non-malignant T cell samples from patients are consistent with the generally accepted notion that reduction in MTP precedes cell membrane phospholipid flip-flop and that with time $DiOC_6(3)$-negative cells complete apoptotic process (15). Alternatively, the lower apoptosis rates detected by Ann V-PI co-labeling may indicate intervening repair and recovery, at least in some cells. The opposite results in AML cases would suggest such repair mechanisms are less efficient in myeloid cells. Thus these differences may be reflective of the shorter life span of normal myeloid cells compared to that of T cells.

Statistically significant correlation between $DiOC_6(3)$-negative and Ann V + ve populations was demonstrated in both cell lines and clinical samples in this study. Although gating for the blast population by side and forward scatter characteristics of control sample in leukemia samples may result in exclusion of some apoptotic cells, such gating is necessary in clinical samples that usually contain mixed population of cells. This approach also provides a relatively more objective way of evaluating the apoptotic response occurring at the time of testing because of the significant variance in the shift patterns in size and granularity observed during apoptosis in individual samples, which hinders the precise selection of the target population. Moreover, the above approach minimizes the number of apoptotic bodies or large cell fragments as well as contaminating red blood cells and platelets counted as events, which confound the analysis.

In conclusion, Ann V-PI co-labeling and $DiOD_6(3)$ uptake techniques are useful and give comparable results for the quantification of apoptotic cells of different type and apoptosis induced by different stimuli including drugs in clinical samples.

LITERATURE CITED

1. Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 1997;91:627–637.
2. Chen Q, Takeyama N, Brady G, Watson AJM, Dive C. Blood cells with reduced mitochondrial membrane potential and cytosolic cyto-

chrome c can survive and maintain clonogenity given appropriate signals to suppress apoptosis. Blood 1998;92:4545–4553.
3. Petit PX, Lecoeur H, Zorn E, Dauguet C, Mignotte B, Gougeon ML. Alteration in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. J Cell Biol 1995;130: 157–167.
4. Korchak HM, Rich AM, Wilkenfeld C. A carbocyanine dye, DiOC6, acts as a mitochondrial probe in human neutrophils. Biochem Biophys Res Com 1982;108:1495–1501.
5. Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, et al. Reduction in mitochondrial potential constitutes irreversible step programmed lymphocyte death in vivo. J Exp Med 1995;181:1661–1672.
6. Fadok VA, Voelker DR, Cammpbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992;148:2207–2216.
7. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 1994;84:1415–1420.
8. Ormerod MG, Sun X-M, Snowden RT, Davies R, Fearnhead H, Cohen GM. Increased membrane permeability of apoptotic thymocytes: a flow cytometric study. Cytometry 1993;14:595–602.
9. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled annexin V. J Immunol Methods 1995;184:39–51.
10. Van Engeland M, Nieland LJW, Ramaekers FCS, Schutte B, Reutelingsperger CPM. Annexin V-affinity assay: based on phosphatidylserine exposure. Cytometry 1998;31:1–9.
11. Hertveldt K, Philippe J, Thierens H, Cornelissen M, Vral A, Ridder L. Flowcytometry as a quantitative and sensitive method to evaluate low dose radiation induced apoptosis in vitro in human peripheral blood lymphocytes. Int J Radiat Biol 1997;71:429–433.
12. Ormerod MG. The study of apoptotic cells by flow cytometry. Leukemia 1998;12:1013–1025.
13. Macho A, Decaudin D, Castedo M, Hirsch T, Susin SA, Zamzami N, et al. Chloromethyl-X-rosamine is an aldehyde-fixable potential-sensitive flourochrome for the detection of early apoptosis. Cytometry 1996; 25:333–340.
14. Metivier D, Dallaporta B, Zamzami N, Larochette N, Susin SA, Marzo I, Kroemer G. Cytofluorometric detection of mitochondrial alterations in early CD95/Fas/APO-1-triggered apoptosis of Jurkat lymphoma cells: comparison of seven mitochondrion-specific fluorochromes. Immunol Let 1998;61:157–163.
15. Castedo M, Hirsch T, Susin SA, Zamzami N, Marchetti P, Macho A, Kroemer G. Sequental acquision of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. J Immunol 1996;157: 512–521.
16. Zamzami N, Susin SA, Marchetti P, Hirsch T, Montgomery IG, Castedo M, Kroemer G. Mitochondrial control of nuclear apoptosis. J Exp Med 1996;183:1533–1544.