Detection and Characterization of Apoptotic Peripheral Blood Lymphocytes in Human Immunodeficiency Virus Infection and Cancer Chemotherapy by a Novel Flow Immunocytometric Method

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We have developed a quantitative and sensitive flow cytometric method for the detection of human apoptotic lymphocytes that, unlike previously described assays, allows their identification in mixed populations of peripheral blood leukocytes as well as their immunophenotyping. Apoptotic lymphocytes are identified on the basis of peculiar light scatter changes, reflecting their smaller size and their modified nucleus/cytoplasm organization, and of the decreased expression of surface CD45 molecules. Based on these criteria, apoptotic lymphocytes generated by exposure to ionizing radiation can be easily distinguished from viable cells and from necrotic lymphocytes generated by treatment with antibody and complement. Using this assay, we reappraised the phenomenon of the in vitro apoptosis of lymphocytes from patients with human immunodeficiency virus (HIV) infection. Lymphocytes from HIV patients, unlike those from normal HIV-negative subjects, undergo apoptosis upon simple in vitro culture. We found that the percentages of lymphocytes undergoing apoptosis were significantly higher in patients with low CD4 cell counts (<400/μL) than in patients at earlier stages (>400 CD4 cells/μL). However, phenotypic analysis disclosed that apoptotic lymphocytes generated in these cultures were mostly CD8+ T cells and CD19+ B cells. Thus, in contrast to what has been previously suggested, the phenomenon of in vitro lymphocyte apoptosis might not be pathogenetically related to the depletion of CD4+ T cells in acquired immunodeficiency syndrome. Nevertheless, it might represent an useful marker of disease progression. Our assay allows the analysis of unfraccionated peripheral blood leukocytes and thus the identification of apoptotic lymphocytes circulating in vivo. Apoptotic lymphocytes could indeed be detected in the circulation of a patient with cancer shortly after high-dose cytotoxic chemotherapy. By contrast, no apoptotic lymphocytes could be detected in vivo in patients with early or advanced HIV infection.

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APOTOPSIS, or “programmed cell death,” is a form of cell suicide occurring in morphogenesis and normal cell turnover as well as in some pathologic conditions. Apoptosis is an active biochemical process consisting of the loss of water with nuclear and cytoplasmic condensation, followed by the production of a set of enzymes that eventually cleave DNA at internucleosomal sites (endonuclease activity) and fix the cell membrane (tissue transglutaminase activity). Apoptosis is distinct from the other way of cell death, necrosis, which is a passive phenomenon caused by the loss of homeostasis and is characterized by mitochondrial damage, followed by swelling and lysis of the cell and shedding of its content into the extracellular space.

The most widely used method for assessing apoptosis is the qualitative analysis of DNA fragmentation at internucleosomal sites by gel electrophoresis, combined with the measurement of low molecular weight DNA extractable from cells after mild lysis. However, this approach suffers from important limitations such as its relatively low sensitivity and its inability to discriminate which cell types undergo apoptosis in mixed populations. Several alternative methods are based on the flow cytometric analysis of cellular light scattering and of the uptake of DNA-binding fluorescent dyes such as propidium iodide, Hoechst 33342, Ethidium bromide, 7-amino-actinomycin D (7-AAD), or other dyes. However, these methods either cannot identify the phenotype of dead cells or cannot discriminate between apoptosis and necrosis. Furthermore, the permeability to propidium iodide and 7-AAD varies from the early to the late phases of cell death.

The recent interest in the pathogenetic role of apoptosis in acquired immunodeficiency syndrome (AIDS) and cancer has given impetus to its evaluation in clinical research. However, some inherent limitations of the available methods of analysis, and the fact that they all require specifically dedicated and relatively cumbersome experiments, have limited their use in clinical studies. We describe here a simple flow immunocytometric method for the detection of apoptotic lymphocytes in human peripheral blood, which can be used for large scale clinical studies.

Apoptosis is accompanied by water loss, shrinkage of the cell, and enzymatic fixation of the membrane, whereas necrosis is not. Necrotic and apoptotic cells differ, therefore, both in differential light scattering and, presumably, in the antigenic make-up of the cell membrane. We exploited these characteristics to develop our method, which is based on the evaluation of cell size, internal structure, and surface details by accurate differential light scattering analysis and on the identification of specific changes of the apparent density of some surface antigens by staining with monoclonal antibodies (MoAbs). This technique allows the discrimination between viable, apoptotic, and necrotic lymphocytes and the determination of their phenotype: is quantitative and sensitive; and can be performed with commonly used MoAbs directly in the context of lymphocyte immunophenotyping for clinical purposes. Its value for studying human pathologic conditions is outlined by our results in patients.
with human immunodeficiency virus (HIV) infection and in a patient undergoing high-dose cytotoxic chemotherapy.

**PATIENTS AND METHODS**

**Patients.** We studied 13 individuals with HIV infection, 10 males and 3 females aged between 21 and 36 years (median, 28), who were clinically classified as Centers for Disease Control (CDC) stage III. The absolute numbers of CD4+ T cells were greater than 400/μL in 9 patients and less than that value in 4 patients. One patient was examined twice: once when he had greater than 400 CD4 cells/μL and, 2 weeks later, when he had less than this value. None of the patients was receiving antiretroviral drugs or was affected by systemic opportunistic infections.

We also studied 3 patients treated with high-dose cytotoxic drugs. All these patients had gastrointestinal tract cancer and were treated with mitomycin (20 mg/m²) and carmustine (600 mg/m²) administered by rapid intravenous (IV) infusion, followed 24 hours later by autologous bone marrow transplantation. Blood samples were obtained before and 16 hours after the end of chemotherapy and were immediately processed. In addition, we studied a child aged 4 months who was referred to us with a diagnosis of severe combined immunodeficiency (SCID), variant with B cells.**

**Blood samples from healthy subjects were used for the in vitro experiments with apoptosis- and necrosis-inducing agents and as controls for the experiments with cultured cells from HIV-infected patients.**

**Cell preparation and treatments.** For the immunocytometric analysis of unseparated lymphocytes, EDTA-treated peripheral blood samples were made devoid of erythrocytes with a lysing solution (Ortho Diagnostic Systems, Raritan, NJ) according to the manufacturer's instructions, and nucleated cells were stained with MoAbs or DNA dyes as described below.

For cell cultures, mononuclear cells from blood samples were isolated by centrifugation (400g for 30 minutes at 18°C) onto a Ficoll-Hypaque (1.077 mg/mL) density gradient (Lymphoprep; Nycomed AS, Oslo, Norway), washed, and suspended at 1 to 5 X 10^6/mL in RPMI 1640 (GIBCO, Grand Island, NY) with 10% fetal calf serum (FCS; Biochrom KG, Berlin, Germany) and antibiotics (complete medium). Irradiated (6 Gy from a 60Co source) and unirradiated cells were cultured for 24 hours in 24-well plates at 37°C in 5% CO₂. Sodium azide (NaN₃)-induced death was obtained by incubating cells in complete medium containing 0.2% NaN₃ for 30 minutes at 16 hours to 37°C in 5% CO₂. Complement (C)₃-dependent lysis was obtained by treating 2 to 5 X 10^6 cells/mL with NaN₃-free anti-CD3 MoAb (OKT3; Ortho Diagnostic Systems) at 5 μg/mL and 20% fresh autologous serum as a source of C for 30 to 90 minutes at 37°C. After treatments, cells were split and either stained and analyzed by flow cytometry or lysed for DNA gel electrophoresis.

**Staining with MoAbs and dyes and flow cytometry.** For each determination, 5 x 10^5 cells in 50 μL of complete medium were double-stained with different mixtures of fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated MoAbs to the following lymphocyte surface molecules: CD3 (Leu4), CD4 (Leu3a), CD8 (Leu2a), CD19 (Leu12), CD45 (antileukocyte), CD56 (Leu19), TcR α/β, HLA-DR (all from Becton Dickinson, Mountain View, CA), LFA-1, and ICAM-1 (from Immunotech, Marseille, France). Stainings were performed at 4°C for 30 minutes using saturating amounts of antibody (experimentally determined for each MoAb). After one wash, cells were resuspended in complete medium and analyzed by flow cytometry.

Membrane permeability, as an indicator of late apoptosis and cell death, was evaluated by staining with propidium iodide (PI) or 7-AAD (Sigma Chemical Co, St Louis, MO) at 1 μg/mL for 30 minutes at 4°C. Stained cells were analyzed by flow cytometry without further washings.

Flow cytometric analyses were performed using either a Cytoron Absolute (Ortho Diagnostic Systems) with 256-channel resolution or a FACScan (Becton Dickinson) with 1024-channel resolution. The differential light scatter detection parameters were set to specifically focus on the lymphocyte region, 19 partially sacrificing the contemporaneous vision of granulocytes and monocytes. This was achieved by incrementing the amplification gain of the forward scatter (FW-SC) photodiode and both the amplification gain and the excitation voltage of the right scatter (RT-SC) photomultiplier, so as to delimit the lymphocyte region (region A), as shown in Fig 1a.

**DNA fragmentation assay.** The DNA fragmentation analysis was performed as a minor modification of the method of Sellins and Cohen. Two million cells, either freshly isolated or cultured, were washed once in RPMI-1640 and pelleted in a microfuge tube. Cells were gently resuspended in 400 μL of hypotonic lysing buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 7.5, 0.2% Triton X-100) and slowly rolled for 30 minutes at 4°C. Thereafter, nuclei were pelleted by centrifugation at 12,000 g for 15 minutes and supernatants were transferred to a new microfuge tube. DNA was precipitated from supernatants in 50% isopropanol and 0.5 mmol/L NaCl at −20°C overnight, pelleted at 12,000 g for 15 minutes, dried, and resuspended in 100 μL of loading buffer (3 mmol/L EDTA, 0.4% sodium dodecyl sulfate [SDS], 10% glycerol, 0.1% bromophenol blue) containing 100 μg/mL RNAse (Sigma Chemical Co). Samples were heated at 37°C for 30 minutes, followed by heating at 65°C for 10 minutes, loaded on an 1.5% agarose gel, and run at 30 V overnight. DNA was visualized with ethidium bromide.

**RESULTS**

**Discrimination between viable, apoptotic, and necrotic human lymphocytes.** A fraction of γ-irradiated (6 Gy) normal human peripheral blood lymphocytes showed, after 24 hours of in vitro culture, characteristic modifications of differential light scattering and accumulated in a restricted region of the scattergram (region C of Fig 1a). The radiation dose used is optimal for inducing apoptosis of lymphocytes, which was shown in these experiments by the massive DNA ladder caused by internucleosomal fragmentation (Fig 1). Irradiated cells in the scatter region C had lower forward scatters and higher side scatters than viable cells in region A, reflecting the smaller size and the different nucleus/ cytoplasm organization typical of apoptotic cells. These changes were clearly evident only when the light-scatter detection parameters were specifically set to focus on the lymphocyte region (see Patients and Methods). In region C, 60% to 70% of the cells were stained by propidium iodide, whereas less than 2% of cells in region A were permeable to this dye (Fig 1b); about 40% of cell in region C were stained by 7-AAD (data not shown).

The pattern of surface antigen expression by viable (gated in region A) and apoptotic (gated in region C) cells was examined by dual staining with anti-CD45 and fluorochrome-conjugated MoAbs directed to the molecules listed in Patients and Methods. Cells in region C were mostly T lymphocytes, as determined by staining with an anti-CD3 MoAb. The percentages of cells positive for CD4+ and CD8+ and for the other surface markers tested were roughly comparable to those of the viable lymphocytes in region A, with the exception of HLA-DR. In fact, HLA-DR determinants were expressed by a larger proportion of apoptotic (about 30% positive) than viable (<2% positive) T lympho-
Fig 1.  (Top) Differential light scattering (left panels), propidium iodide uptake, and CD45 expression (right panels) by viable, apoptotic, and necrotic lymphocytes.  (a) Left, setting of the light scatter analysis regions A (viable lymphocytes) and C (dead lymphocytes); right, scattergram of untreated cells after 24 hours of culture.  (b) γ-Irradiated lymphocytes: the left panel shows the accumulation of cells in region C of the scattergram; the right panel shows the permeability to propidium iodide of cells in regions A and C analyzed separately.  (c) γ-Irradiated lymphocytes: the left panel is as described above; the right panel shows the expression of CD45 by cells in a scatter region encompassing regions A and C.  (d) Same as in (c), except that regions A and C are analyzed separately for CD45 expression.  (e) NaN₃-treated cells: data as in (d).  (f) Cells treated with antibody C: data as in (d).  (Bottom) Gel electrophoresis of DNA extracted from lymphocytes untreated (lanes 1 and 5) or treated with antibody + C' (lanes 2 and 6), NaN₃ (lanes 3 and 7), γ-irradiation (lanes 4 and 8).  Lane 9: DNA from γ-irradiated rat thymocytes. Lymphocytes from two different normal donors were used for each treatment. The data show that apoptosis induced by γ-irradiation or NaN₃ is accompanied by an apparent reduction of CD45 on the cell surface, whereas necrosis induced by C' is not. A DNA ladder indicative of apoptosis is generated in γ-irradiated and NaN₃-treated cells, but not in C'-treated cells.

cytes (data not shown). These findings are in agreement with the report of Malinowski et al on the enhanced expression of HLA-DR by γ-irradiated lymphocytes.

The apparent per-cell density of all the surface antigens tested, again with the exception of HLA-DR, was lower on apoptotic than on viable lymphocytes (data not shown). Among these antigens, the most striking differences were observed for CD45, the “leukocyte common antigen” expressed by all cells of hematopoietic origin. The remarkable homogeneity of expression of CD45 on viable lymphocytes allowed to clearly distinguish two peaks of dim and bright fluorescence when scatter-gating included both the
"apoptotic" and the "viable" regions (Fig 1c); the CD45 population was exclusively contributed by the cells in region C (Fig 1d). To test whether the reduced expression of CD45 and of other surface molecules is a specific indicator of apoptosis, we treated lymphocytes with agents presumed to induce necrosis, such as Ab plus C and NaN₃. Both types of treatment conveyed a large fraction of lymphocytes into the "apoptotic" scatter region (Fig 1e and f). In the case of Ab plus C, accumulation in this region occurred within 30 minutes of treatment and it was rapidly followed by a wide dispersion of dot plots throughout the scattergram. By contrast, most NaN₃-treated cells that entered the "apoptotic" scatter region remained there for at least 24 hours. The expression of CD45 on cells in the "apoptotic" region, compared with that on cells in the "viable" region, decreased in NaN₃-treated samples to an extent similar to that observed after γ-irradiation (Fig 1e), whereas it was unmodified in cells killed by Ab plus C (Fig 1f). These data suggested that Ab plus C was actually inducing cell death by necrosis, whereas NaN₃ was inducing apoptosis. This was confirmed by DNA analysis, which showed a typical internucleosomal fragmentation pattern in NaN₃-treated but not in Ab plus C-treated lymphocytes (Fig 1).

To confirm that lymphocytes in the light scatter region C displaying a CD45 population were indeed apoptotic, we enriched these cells by density gradient centrifugation. Irradiated lymphocytes were cultured for 24 hours and then centrifuged onto a Ficoll-Hypaque gradient. Dense cells accumulating at the bottom of the gradient fell in region C by light scatter analysis and had a CD45 population (Fig 2A). These cells displayed the morphologic features and the in-
ternucleosomal DNA degradation pattern typical of apoptotic lymphocytes (Fig 2B and C). By contrast, the light cells at the top of the gradient occupied region A of the scattergram and had the features of viable lymphocytes according to the latter criteria (Fig 2).

The results of several consecutive experiments with γ-irradiation, NaN₃, or C'(Fig 3) clearly indicate that the combination of a peculiar light scatter pattern and of a CD45<sup>dim</sup> phenotype is highly distinctive of apoptotic lymphocytes, and allows the distinction of these cells from viable and necrotic lymphocytes. The variation of the mean fluorescence intensity between “apoptotic” and “viable” cells corresponded to an apparent decrease of the density of surface CD45 epitopes of about 50% (Fig 3).

The apparent decrease of the density of CD45 and of other surface antigens on apoptotic lymphocytes could be caused by the suppression of their expression at the transcriptional or posttranscriptional levels or, perhaps more likely, to their chemical modification. In fact, surface molecules on apoptotic cells are “fixed” by the enzyme tissue transglutaminase; therefore, because these molecules are at least partially denatured by enzymatic fixation, there could be a decreased availability of antigenic determinants recognized by MoAbs directed to native epitopes.

Detection and characterization of apoptotic lymphocytes
in cultures of peripheral blood mononuclear cells from HIV-infected patients. Peripheral blood lymphocytes from HIV-infected patients have been reported to undergo apoptosis when cultured in vitro either in the absence or in the presence of activating stimuli. These findings have raised interest in the possible role of apoptosis in the pathogenesis of AIDS. We used our assay to provide additional information on lymphocyte apoptosis in HIV infection.

In agreement with previous reports, we found that lymphocytes from HIV patients, but not those from normal HIV-negative subjects, generated a DNA ladder characteristic of apoptosis when cultured in vitro for 24 hours in the absence of exogenous stimuli (Fig 4). Significant populations of cells with the light scatter characteristics of apoptotic lymphocytes (region C cells) were also generated in cultures from HIV-infected but not from uninfected subjects; these cells were found to be denser than a standard Ficoll-Hypaque gradient and were permeable to propidium iodide (data not shown). Neither a DNA ladder nor cells with region C-type light scatters could be detected in patients’ lymphocyte samples immediately after isolation onto Ficoll-Hypaque.

The cultured cells were analyzed by our assay for apoptosis according to the protocol outlined in the representative experiment shown in Fig 5, which illustrates the quantitative evaluation of apoptotic lymphocytes belonging to the CD3+ subpopulation. Cells were analyzed by three-color immunocytometry using anti-CD45 in combination with antisubset antibodies, and those cells from scatter regions A and C coexpressing CD45 and a given subset marker (CD3 in the case of Fig 5) were electronically gated. The percentages of apoptotic cells were calculated on the basis of the number of CD45dim cells expressing a given marker in region A versus the total number of CD45+ cells expressing that marker present in the two regions. An objective boundary between CD45dim and CD45bright cells was determined using Kolmogorov-Smirnov statistics to compare the CD45 fluorescence intensities of cells expressing a given marker in regions A and C, and by taking as the cut-off point the fluorescence channel with maximum difference between the two regions. This approach allows the identification of CD45dim and CD45bright cells belonging to defined subsets; its sensitivity is outlined by the data reported in Table 1 showing the mean percentages of CD3+CD45dim or CD19+CD45dim cells in regions A and C in different patient populations. These data illustrate that the contaminations by CD45dim cells in region A and by CD45bright cells in region C are not significantly different between the populations of individuals studied. This finding supports the concept that the observed changes of flow cytometric parameters actually reflect a reproducible biologic phenomenon. Furthermore, the finding of relatively high proportions (20% to 30%) of CD45bright cells in region C suggests that water loss and shrinkage of the cell precede the downregulation of surface molecules.

Table 1 shows that cultures from HIV-infected patients with greater than 400 CD4 cells/μL contained significantly more apoptotic CD3+ lymphocytes than did cultures from
normal subjects. Furthermore, patients with less than 400 CD4 cells/μL had significantly higher proportions of apoptotic CD3⁺ cells than did patients with greater than 400 CD4/μL. The levels of apoptosis for CD19⁻ cells were also found to be significantly increased in HIV-infected subjects than in controls, but there were no differences between patients at early or advanced stages (Table 1). A relationship between the extent of in vitro T-cell apoptosis and disease progression is suggested by the observation that, among the HIV-infected patients with CD4 cell counts greater than 400/μL, the one with the highest rate of in vitro apoptosis had a strikingly rapid decline of CD4 cells. In fact, in this patient, CD4 cell counts decreased from 492/μL at the time of the initial study, when the percentage of apoptosis for CD3⁺ cells was 12.3%, to a value of 324/μL observed only 2 weeks later, at which time T-cell apoptosis reached 20%. The decrease of CD4 cells in this patient was particularly remarkable in view of the fact that they had remained greater than 700/μL for more than 2 years.

The CD4⁴/CD8⁺ ratios in regions A and C were taken as a measure of the relative involvement of these T-cell subsets in the apoptotic process. For this analysis, only CD4⁴ dim cells in region C and CD4⁴ bright cells in region A were considered, according to the protocol outlined in the previous paragraph for defining viable and apoptotic cells within a given subset. In normal subjects, the CD4/CD8 ratios were similar within viable and apoptotic cells, indicating a lack of selectivity for cell death. By contrast, the striking differences between the CD4/CD8 ratios in the apoptotic and viable populations in the patients, irrespective of the disease stage, point to the surprising fact that the phenomenon of in vitro T-cell apoptosis in HIV infection is neither random nor selective. However, the CD4/CD8 ratio was not determined in all patients, and the results obtained in the remaining patients should be regarded as indicative, rather than definitive.

**Detection of apoptotic lymphocytes circulating in vivo.** We assessed the possibility of using our assay to detect apoptotic lymphocytes circulating in vivo in HIV patients. To this end, leukocytes were examined immediately after blood collection, and immunocytometric analyses were performed by the erythrocyte lysis method avoiding a density gradient centrifugation step that would remove apoptotic cells. We could not obtain evidence by immunocytometry for the presence of apoptotic lymphocytes circulating in vivo neither in patients with more (n = 6) nor in those with less (n = 4) than 400 CD4 cells/μL.

Many cytotoxic drugs induce cell death by apoptosis. We therefore used the same approach using unseparated blood leukocytes to look for circulating apoptotic lymphocytes in 3 patients with cancer of the gastrointestinal tract shortly after treatment with high-dose cytotoxic drugs. Leukocytes were examined immediately after blood collection before and 16 hours after therapy. In 1 patient we could observe, in the posttherapy sample, a flow cytometric pattern suggesting the presence of apoptotic lymphocytes. In fact, about 25% of this patient's T lymphocytes, as identified by dual staining with anti-CD3 and anti-CD45, were found in the scatter region C and had a CD4⁴ dim phenotype (Fig 6a). Gel electrophoresis of DNA from density gradient-purified mononuclear cells from the same blood sample showed a significant amount of internucleosomal degradation (Fig 6).

These results show that apoptotic lymphocytes present in vivo can be detected and enumerated on the basis of their immunophenotype. Confirmation for the specificity of our assay was provided by the findings in one child with SCID. During standard lymphocyte phenotyping, it was found that a proportion of this patient's peripheral blood lymphocytes displayed a differential light scatter pattern similar to that of apoptotic lymphocytes, although they appeared to be somewhat larger as indicated by their FW-SC (Fig 6b). However, these cells expressed as much CD45 as lymphocytes residing in region A of the scattergram (Fig 6b). That these cells were indeed not apoptotic was confirmed by the absence of internucleosomal DNA fragmentation (Fig 6). Upon further characterization, these unusual lymphocytes turned out to be a subpopulation of viable B cells, which in general are slightly smaller by FW-SC as compared with T lymphocytes.

**DISCUSSION**

Our approach allows the unambiguous identification and the characterization of apoptotic lymphocytes in heterogeneous populations of human peripheral blood leukocytes. Apoptotic lymphocytes are identified on the basis of their typical light scattering and by the low expression of CD45 antigenic determinants and can be characterized by contemporaneous staining with other MoAbs. Besides its simplicity, this method offers two major advantages over the currently used techniques. First, the subset of lymphocytes undergoing apoptosis can be precisely determined. Second, apoptotic lymphocytes can be identified even in the presence of other contaminating apoptotic cells, such as granulocytes, thus avoiding the need for cell fractionation steps that could bias the results.

Concerning the first point, the phenotype of apoptotic lymphocytes in mixed cell populations cannot be determined either by the DNA fragmentation assay or by the contemporaneous staining with nonvital DNA dyes and fluochrome-conjugated antibodies. In fact, the former approach does not give any indication on the cell type undergoing apoptosis, whereas the latter methods do not discriminate apoptotic from necrotic lymphocytes. Furthermore, permeability to propidium iodide and 7-AAD occurs only in the late phases of apoptosis. The direct determination of the phenotype of lymphocytes undergoing apoptosis is of particular importance in studying pathologic conditions in which specific subsets of lymphocytes are presumed to die, as in the case of CD4⁺ T cells in HIV infection. The second advantage of our method is that apoptotic lymphocytes circulating in vivo can be identified without the need for cell fractionation steps. The classical technique for detecting apoptosis by gel electrophoresis of DNA requires the separation of lymphocytes from granulocytes by density gradient centrifugation, because degraded DNA from the latter cells, which undergo apoptosis, interferes with the assay. However, this largely precludes the quantitation of apoptotic lymphocytes in freshly isolated blood samples, because many of these cells go to the bottom of a standard Ficoll-Hypaque gradient because of their increased density. Our results illustrate the value of our
method for studying lymphocyte apoptosis in human pathologic conditions and particularly in HIV infection.

Based on the observation that lymphocytes from HIV-infected patients die spontaneously upon in vitro culture,\textsuperscript{22,23} it has been suggested that apoptosis is an important pathogenetic mechanism in AIDS. Previous studies have shown that, in addition to CD4 cells, significant proportions of CD8 T cells also undergo apoptosis in vitro.\textsuperscript{22,23} We have extended these results by showing that the death of CD8 cells not only occurs but rather predominates over that of CD4 cells. In fact, the populations of apoptotic lymphocytes in cultures from HIV patients contained a large excess of CD8 T cells in comparison to the populations of T cells remaining viable. In addition, we observed that also B cells from HIV patients appear to be programmed to exaggerated culture-induced apoptosis, suggesting a bystander killing process. These data are not readily reconciled with the selective depletion of CD4 cells that occurs in AIDS. Nevertheless, one can hypothesize that an HIV-related noxious factor(s) affects preferentially, but not exclusively, CD4 T cells and programs them to apoptosis. The apparently scanty death of these cells in cultures of peripheral blood lymphocytes might depend on the fact that, in vitro, CD4 cells undergo an accelerated apoptotic process, and are therefore rapidly removed by monocytes or become unidentifiable by immunophenotyping. Alternatively, CD4 cells programmed in vivo to apoptosis might be sequestered and die within specific environments such as the lymph nodes, where massive accumulation of HIV occurs.\textsuperscript{28} However, the relevance of the “spontaneous” in vitro lymphocyte apoptosis to the pathogenesis of AIDS is further questioned by the fact that the same phenomenon is observed in Epstein-Barr virus-induced infectious mononucleosis.\textsuperscript{29} Despite these caveats, our findings of a significantly higher rate of cell death in patients with CD4 cells less than 400/\mu L indicate that apoptosis is associated with advanced disease. This suggestion is strengthened and extended by our observation that 1 patient with a strikingly high rate of in vitro T-cell apoptosis had a very rapid decline of CD4 cells. Although preliminary, these data suggest that a high in vitro lymphocyte apoptosis may represent a marker of accelerated disease progression.

Our findings in a patient treated with high-dose cytotoxic drugs illustrate how our technique can be used for detecting and characterizing apoptotic lymphocytes present in vivo in peripheral blood. The fact that we could detect circulating apoptotic lymphocytes only in 1 of 3 patients treated with the same cytotoxic regimen is probably caused by differences in the kinetics of generation and clearance of the apoptotic cells. More extensive studies are needed to clarify whether this assay could be used to monitor the effects of cytotoxic therapies.

Even the most recently described techniques for detecting apoptosis suffer from some inherent limitations; they do not allow the determination of the phenotype of the apoptotic cells, or the discrimination between apoptotic and necrotic cells, or the detection of the early phases of apoptosis.\textsuperscript{3,13-15} Furthermore, these methods have been tested only using thymocytes or cell lines,\textsuperscript{9} and require specifically dedicated and often cumbersome experiments. On the contrary, our assay is very simple, works well with human peripheral blood lymphocytes, and relies on an immunologic reagent, anti-CD45 MoAb, largely used in routine immunocytometry. In fact, the so-called “immunogating” of lymphocytes with a combination of anti-CD45 and anti-CD14 antibodies is recommended by Ault et al\textsuperscript{30} and manufacturers (Leukogate; Monoclonal Antibody Source Book; Becton Dickinson). The technique described here is, therefore, of

### Table 1. Spontaneous In Vitro Apoptosis of Lymphocytes From HIV-Infected Patients: Quantitative and Phenotypic Analysis of Apoptotic Cells

<table>
<thead>
<tr>
<th>CD3</th>
<th>% CD45&lt;sup&gt;dim&lt;/sup&gt;</th>
<th>Region A</th>
<th>Region C</th>
<th>% Apoptosis&lt;sup&gt;†&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt;/CD8&lt;sup&gt;+&lt;/sup&gt; Ratio</th>
<th>Viable</th>
<th>Apoptotic</th>
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<tr>
<td>HIV&lt;sup&gt;+&lt;/sup&gt; (n = 5)</td>
<td>&gt;400 CD4/\mu L (n = 9)</td>
<td>&lt;400 CD4/\mu L (n = 6)</td>
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<td>CD3</td>
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<tr>
<td>% CD45&lt;sup&gt;dim&lt;/sup&gt;</td>
<td>Region A</td>
<td>6.5 ± 2.5</td>
<td>6.5 ± 2.5</td>
<td>7 ± 5</td>
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<tr>
<td>Region C</td>
<td>79 ± 2.3</td>
<td>79 ± 2.3</td>
<td>69 ± 5.8</td>
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<tr>
<td>% Apoptosis&lt;sup&gt;†&lt;/sup&gt;</td>
<td>2.1 ± 0.5</td>
<td>5.5 ± 2.7</td>
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<tr>
<td>CD19</td>
<td>% CD45&lt;sup&gt;dim&lt;/sup&gt;</td>
<td>Region A</td>
<td>Region C</td>
<td>% Apoptosis&lt;sup&gt;†&lt;/sup&gt;</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;/CD8&lt;sup&gt;+&lt;/sup&gt; Ratio</td>
<td>Viable</td>
<td>Apoptotic</td>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;/CD8&lt;sup&gt;+&lt;/sup&gt; Ratio</td>
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<td></td>
<td>Apoptotic</td>
<td>2.9 ± 3.27</td>
<td>0.4 ± 0.1</td>
<td>0.19 ± 0.14</td>
<td></td>
<td></td>
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</table>

All data are expressed as the mean ± 1 SD in the populations examined.

* The cut-off between CD45<sup>dim</sup> and CD45<sup>prop</sup> (for CD3<sup>+</sup> or CD19<sup>+</sup> cells) was calculated as described in Fig 5. These data provide information on the sensitivity of the assay and on its reproducibility in the different subject populations.

† Calculated for either CD3<sup>+</sup> or CD19<sup>+</sup> cells as described in Fig 5.

‡ Ratios between the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> cells among CD45<sup>prop</sup>/region A cells (viable) and CD45<sup>dim</sup>/region C cells (apoptotic). Statistic analyses were performed by the Student's t-test.
Fig 6. Apoptotic lymphocytes circulating in vivo after high-dose chemotherapy (a) can be detected by light scatter (left) and CD45 expression (right) analysis of freshly isolated whole blood leukocytes. Apoptotic cells (mostly CD3⁺ T lymphocytes) are about 25% of total lymphocytes. In 1 patient with SCID (b), about 50% of circulating lymphocytes (virtually all B cells) have abnormal light scatters and fall in region C, but express CD45 as intensely as viable lymphocytes in region A. Gel electrophoresis of DNA from gradient-purified mononuclear cells shows a DNA ladder in cells from the chemotherapy patient (lane 3) but not in cells from the SCID patient (lane 2). Lane 1, molecular size markers (itesse RE/HaeIII fragments); lane 4, DNA from γ-irradiated rat thymocytes.

potential value for large-scale clinical studies on lymphocyte apoptosis in AIDS, in oncology, and in other human diseases. To this end, it is sufficient to adopt for routine immunophenotyping appropriate scatter-gate settings encompassing regions A and C as defined by our parameters, and to include in the analyses both CD45dim and CD45bright lymphocytes. It is clearly essential to confirm, in this type of studies, that cells in the “apoptotic” scatter region expressing dim CD45 fluorescence are indeed lymphocytes, because other cells, including degranulated granulocytes and immature leukocytes, might have these characteristics. This can be easily performed by two-color staining with anti-CD45 and antibodies to specific lymphocyte markers.

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Detection and characterization of apoptotic peripheral blood lymphocytes in human immunodeficiency virus infection and cancer chemotherapy by a novel flow immunocytometric method

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