Accurate Apoptosis Measurement Requires Quantification of Loss of Expression of Surface Antigens and Cell Fragmentation

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Background: The use of ratiometric cell enumeration methods emerges as a more accurate method of measurement of the occurrence of apoptosis in cell cultures. These new flow cytometry methods were used to quantify the impact of cell fragmentation and loss of lineage antigen (LAG) expression on measurement of apoptosis.

Methods: Highly purified human lymphocyte populations were negatively sorted and cultured for 24 h. Apoptotic cells were identified using annexin V, 7-amino-actinomycin D and their LAGs were stained with antibodies. A new indicator, the apoptotic rate, was used to determine apoptosis occurrence and its validity compared with the widely accepted percentage of apoptotic cells (apoptotic index, AI).

Results: Loss of LAG expression and cell fragmentation were observed under all conditions assayed and for all cell populations studied.

Conclusions: Current methods for quantifying of apoptosis involving AI systematically underestimate apoptosis occurrence in all populations and conditions, especially among cells undergoing spontaneous apoptosis. © 2006 International Society for Analytical Cytology

Key terms: apoptosis; apoptotic index; apoptotic rate; cell enumeration; accurate apoptosis measurement; microbeads; annexin V; antigen loss; cell fragmentation

The initial methods developed for the in vitro quantification of apoptosis (such as the assessment of nucleosomal DNA fragmentation after gel electrophoresis) measured phenomena associated with apoptosis in cultures at the population level (1–3). However, it soon became clear that individual cells undergo apoptosis in a heterogeneous and asynchronous manner (4). It was therefore realized that the accurate measurement of apoptosis required methods that could identify apoptosis events at the single-cell level (5–11). These methods revealed the heterogeneity of the apoptotic process to be correlated with cell phenotype at least to a certain extent (12). The ongoing development of flow cytometric techniques eventually made it possible to simultaneously identify and quantify apoptotic cells phenotypically defined by the expression of their surface lineage antigens (LAG).

Currently, most authors quantify the frequency of phenotypically defined apoptotic cells after calculating the apoptotic index (AI), i.e., the percentage of apoptotic cells displaying a specific LAG within a population of cells that remain unfragmented and retain the expression of the LAG (13–15). However, this approach has two major limitations.

First, apoptotic cells fragment into apoptotic bodies that disintegrate later. This leads to an underestimation of the percentage of apoptotic cells if the debris is excluded from the gates for cell analyses, or, alternatively, to the overestimation of apoptosis if several apoptotic bodies derived from a single cell are misinterpreted as individual apoptotic cells (16). Second, apoptotic cells frequently lose, partially or even completely, the cell surface expression of the LAG.
used for the identification of specific cell subsets (17–19); this means that the supposedly defined cell subsets can no longer be identified as targets in the apoptosis quantification, which leads to miscalculations (19).

The present study address the potential advantages of the combined use of two flow cytometry techniques recently developed to improve the accurate measurement of apoptosis in cultured cells: the determination of the apoptotic rate (AR) (16) and the ratiometric enumeration of apoptotic cells (20). Herein, the use of highly pure cell populations showed the combination of these techniques to accurately quantify both the number of apoptotic cells that have lost the expression of their LAGs as well as the absolute number of apoptotic, nonapoptotic, and fragmented cells. The application of proposed ratiometric method of enumeration of apoptotic cells in culture showed that loss of antigen expression occurs at different stages of apoptosis and that it takes place before cell fragmentation. This finding uncovered that the estimation of apoptosis occurrence by standard methods, involving AI measurements in cell subsets, can be seriously biased.

**MATERIALS AND METHODS**

**Isolation of Peripheral Blood Mononuclear Cells**

Peripheral blood mononuclear cells (PBMC) from six healthy control subjects were obtained by Ficoll-Hypaque (Lymphoprep™, Axis-Shield, Oslo, Norway) gradient centrifugation as previously described (21). Purified PBMCs were resuspended in RPMI 1640 (Biowhittaker Products, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum, 25 mM HEPES (Biowhittaker Products), and 1% penicillin-streptomycin (Biowhittaker Products).

Initial cell enumeration was performed by conventional light microscopy using a Neubauer chamber and following trypan-blue dead cell exclusion criteria, and by flow cytometry (FACScalibur, Becton Dickinson Biosciences, San José, CA) as previously described (20). The viability of fresh PBMCs was checked by both trypan blue (light microscopy) and 7-aminoactinomycin D (7-AAD) (flow cytometry) exclusion (11); viability was always greater than 95%. The final cell concentration was adjusted to 0.5 × 10^6 cells/ml. In all cultures, cells were plated at a density of 2.5 × 10^5 cells/ml.

**Fluorescence-Activated Cell Sorting**

Lymphocyte subsets were purified by negative selection using fluorescence-activated cell sorting (FACS). Freshly isolated PBMCs were incubated with 3- and 4-color combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein conjugate (PerCP), and allophycocyanin (APC)-labeled monoclonal antibodies (MAb). To purify CD4+CD28−, CD3+CD8− and CD3+CD5+TCRβ− T cells, B cells, and NK cells, PBMCs were depleted of unwanted cell subsets using the following MAb combinations (FITC/PE/PerCP/APC): TCRγδ/ CD14+CD16-CD56/CD8/CD19, TCRγδ/CD14+CD16+CD56/CD4/CD19, TCRγδ/CD14+CD16+CD56//−/CD19, −/CD14+CD16-CD56/CD3/−, and −/CD14/CD3/CD19 respectively. Anti-CD19-APC, anti-CD56-PE, anti-CD8-PerCP, anti-CD4-PerCP, anti-CD3-PerCP, and anti-TCRγδ-FITC were obtained from Becton Dickinson Biosciences, and anti-CD14-PE, anti-CD16-PE, and anti-CD45-FITC from Caltag Laboratories (San Francisco, CA).

All sorting experiments were performed using a FACSTARplus flow cytometer running CELLQUEST software (Becton Dickinson Biosciences). An electronic gate was drawn on a forward light scatter (FSC) versus sideways light scatter (SSC) bivariate dot plot to select lymphocyte populations with low FSC and SSC characteristics. Further sorting criteria included the absence of expression of the antigens specifically stained in each combination. The purities of the negatively sorted lymphocyte populations were as follows: CD4+CD28− T cells >98% for CD4+ cells (>96% of these cells being CD28−), CD3+CD8− T cells >98% for CD3+ cells (>96% of these cells being CD8−), CD3+CD5+ T cells >98% for CD3+ cells (>98% of these cells being CD5+), and NK cells >97% for CD3−CD56− cells. Since the cocktails for the negative sorting of CD19+ B cells from healthy controls were of relatively low purity (>80%), B lymphocyte experiments were also performed with positively sorted CD19+ cells (purity >98%).

**Cell Cultures for the Induction of Apoptosis in Sorted Cell Populations**

The sorted lymphocyte subsets (5 × 10^4 cells/well) were cultured for 24 h in triplicate in complete medium in 96 flat-bottom culture plates. Spontaneous apoptosis was studied by culturing cells in complete medium, in the absence of any inducers. Phytohemagglutinin (PHA, 2 μg/ml, Difco Lab, Detroit, MI, USA) or staurosporin (ST, 0.5 μM, Sigma St. Louis, MO) was used to induce apoptosis in other experiments, as previously described (16). Optimal doses of both PHA and ST were previously determined through dose/response titrations (data not shown). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. The cell cycle distribution of cultured cells was analyzed as previously described (22) to determine the percentage of cells in the S, G2, and M phases. The uptake of tritiated thymidine was also determined to demonstrate the absence of DNA synthesis in cultures.

**Analysis of Apoptotic Cells**

Fresh or cultured lymphocytes were incubated and labeled with MAb (PE/APC) for 20 min at 4°C. These lymphocytes were then washed in 2 ml of fresh complete medium by centrifuging them for 5 min at 300g (4°C) and resuspending them in an annexin V binding buffer containing Ca^{2+} (Hepes, 10 mM; NaCl, 150 mM; MgCl2, 1 mM; CaCl2, 1.8 mM; and KCl, 5 mM; pH adjusted to 7.4; Sigma). Cells were then sequentially incubated with a solution containing annexin V–FITC (Bender MedSystem, Vienna, Austria) in Ca^{2+}-binding buffer (10 min at 4°C) followed by a 3 min incubation with a 7-AAD solution (Sigma; final concentration 2.5 μg/ml) in the same buffer to identify early and late apoptotic cells respectively. Finally, 100 μl of a 1/100 (v/v) dilution of 6 μm CALIBRITE microbeads (Becton Dickinson) in Ca^{2+}-binding buffer, plus 0.05%
(w/v) gelatin (Sigma), was added to the cell suspension prior to flow cytometry.

The following combinations of MAb (PE/APC) were used to identify cell subsets: CD28/CD4, CD3/CD8, CD5/CD3, NSC6/—, and CD5/CD19. Control studies involving unstained cells and cells incubated with isotype-matched irrelevant PE- and APC-labeled MAb were performed in parallel with each experiment. For these procedures, anti-CD28-PE, anti-CD56-PE, anti-CD3-APC, anti-CD19-APC, and anti-CD4-APC MAb were obtained from Becton Dickinson Biosciences and anti-CD5-PE, anti-CD8-APC, and anti-CD3-PE were purchased from Caltag Laboratories. In all cases, data acquisition and analysis were performed using a FACScalibur flow cytometer (Becton Dickinson Biosciences) running CellQuest software.

**Calculation of the AI and the AR**

The AI within a phenotypically defined cell subset was calculated by dividing the percentage of antigen positive apoptotic cells (annexin V+) by the total percentage of antigen positive cells in the sample [apoptotic (annexin V+) plus nonapoptotic cells (annexin V-)]:

$$AI = \frac{A^+}{(A^+ + A^-)}$$  \hspace{1cm} (1)

where $A^+$ is the percentage of apoptotic cells (annexin V+ positive cells) and $A^-$ is the percentage of nonapoptotic cells (annexin V negative cells).

The AR was calculated in two steps. The number of cells that completed apoptosis and underwent fragmentation into apoptotic bodies was determined using the following formula:

$$NFC = NSC - NRC$$  \hspace{1cm} (2)

where NFC is the number of fragmented cells, NSC the number of seeded cells, and NRC the number of remaining cells (including annexin V+, annexin V-LAg−, and annexin V-LAg− cells). In the second step, the AR was calculated using the following equation:

$$AR = \frac{(NApoLAg^+ + NApoLAg^- + NFC)}{NSC}$$  \hspace{1cm} (3)

where $NApoLAg^+$ is the number of apoptotic cells that retained their LAg expression, and $NApoLAg^-$ is the number of apoptotic cells that lost this expression.

The percentage of apoptotic cells that completely lost the expression of their LAg—and which would be ignored by conventional flow cytometry methods—was calculated using the following equation:

$$\%ApoLAg^{loss} = 100 \times \frac{\%ApoLAg^-}{(\%ApoLAg^- + \%ApoLAg^+)}$$  \hspace{1cm} (4)

where $\%ApoLAg^{loss}$ is the percentage of apoptotic cells that lost the expression of their LAg, $\%ApoLAg^-$ the percentage of apoptotic cells that lost the expression of their LAg over the number of cells that remained in culture, and $\%ApoLAg^+$ the percentage of apoptotic cells that retained the expression of their LAg among the cells that remained in culture.

The percentage of apoptotic cells that fragmented into apoptotic bodies—which would be ignored in conventional calculations of apoptosis—was determined using the following equation:

$$\%FC = 100\frac{(NSC - NRC)}{NSC}$$  \hspace{1cm} (5)

where $\%FC$ is the percentage of fragmented cells, NSC the number of seeded cells, and NRC the number of cells remaining in culture.

The percentage underestimation of apoptosis by AI ($\Delta AI$) was calculated to determine the magnitude of the error according to the following equation:

$$\Delta AI = 100\frac{(AR - AI)}{AR}$$  \hspace{1cm} (6)

**Statistical Methods**

The differences between the AI and the AR for two or more populations of cells were evaluated by the Mann-Whitney $U$ test and the Wilcoxon signed ranks test. All calculations were performed using the Statistical Package for the Social Sciences (SPSS, version 11.0, Chicago, IL). Significance was set at $P < 0.05$.

**RESULTS**

**The Loss of Surface Expression of LAg is an Inherent Characteristic of Apoptosis That Precedes the Fragmentation of Apoptotic Cells into Apoptotic Bodies**

Apoptosis led to the fragmentation of apoptotic cells into apoptotic bodies under different culture conditions (Fig. 1). Compared to whole cells, apoptotic bodies are smaller, consistent with the notion that one cell generates several apoptotic bodies. The inclusion of the latter in the cell analysis gate and their subsequent consideration as apoptotic cells results in an overestimation of the frequency of apoptosis.

As shown in Figure 1, the discrimination between either viable and apoptotic whole cells or cell fragments was achieved by the analyses of both their bivariate profiles of size (FSC)/DNA-staining with 7-AAD (left panels) and their FSC/ granularity (SSC) distribution (right panels). Contour plots in the top panels show that freshly purified CD19+ lymphocytes formed a homogeneously sized population of viable cells that uniformly excluded 7-AAD. After 24 h of culture (bottom panels), both apoptotic cells and apoptotic bodies emerged but the remaining subset of viable B cells maintain the characteristics from the original fresh B lymphocytes, since they shared their FSC/SSC features (panel D) and did not take up 7-AAD (contour levels under viable cell arrows in the panels C and D). In contrast, apoptotic cells showed a reduced FSC and a slightly increased SSC (panel D) that was coincident with variable 7-AAD staining related to progression into late apoptosis (panel C). Finally, apoptotic bodies showed markedly smaller FCS and SSC signals than did live or apoptotic whole cells independently of their occasionally weak 7-AAD staining (panels C and D). Therefore, an event was considered to correspond to a whole cell when it provided an FSC signal that was similar to the FSC value of a typical cell (panel D).
greater than the lower limit of 7-AAD+ apoptotic cells (insert of continuous line boxes in bottom panels). Using these criteria, whole apoptotic cells were clearly distinguishable from apoptotic bodies (inserts of discontinuous line boxes).

It has been argued that the loss of expression of LAg by unfragmented whole cells is also important for the accurate estimation of the frequency of apoptotic cells (19). In a heterogeneous cell culture, each cell type is routinely distinguished from other lineages by the expression of one or more LAgS. If a cell completely loses the expression of its LAg (i.e., to below detection level), it can no longer be identified as a member of its corresponding phenotypically defined population. We reasoned that if the loss of LAg expression were significant in apoptotic whole cells before disintegration, then the frequency of apoptosis recorded for the LAg+ population would be routinely underestimated. To address this, negative fluorescence-activated cell sorting was used to obtain a fresh homogeneous CD19+ annexin V− B lymphocyte population (Fig. 2, panel A, B). When the CD19+ B cells were cultured in complete medium for 24 h, the viable B cell subset showed fresh lymphocyte-like size, annexin V−, retained their CD19 original expression, and did not take up 7-AAD (R5 in panel D and panel E). However, the experiment showed that a large number of annexin V+ apoptotic B cells appears (Fig. 2, panels D, E, G) within the whole cell gate after 24 h of culture (Fig. 2, panel C). As shown in Figure 2D, the spontaneously

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**Fig. 1.** Flow cytometry approach used to discriminate whole cells from apoptotic bodies by gating in 7-AAD/FSC bivariate dot-plots. Freshly purified CD19+ lymphocytes were labeled with CD19-APC, annexin V-FITC, and 7-AAD. Flow cytometry analysis was performed prior to and after 24 h of culture. The experiment was repeated six times. Panels A and B show 7-AAD/FSC and SSC/FSC bivariate contour plots of freshly purified B cells. Panels C and D show how whole cells (R1) were differentiated from apoptotic bodies (R2, 7-AAD+ and lower FSC signal than the lower limit of the 7-AAD+ apoptotic cells) through combined analysis of the FSC/SSC/7-AAD characteristic of the events measured.
apoptotic (annexin V+) CD19+ cells showed partial or complete loss of CD19 antigen expression. Notably, the reduction and loss of CD19 B-lineage marker expression preceded not only the whole cell fragmentation in apoptotic bodies (Fig. 2C) but also the reduction in FSC and the uptake of 7-AAD in many cells (panels D and G). Therefore, LAg loss is an early event in apoptosis.

**Apoptotic Cells Fragmented into Apoptotic Bodies Escape from Cell Gates**

To determine the frequency of cell fragmentation, the percentage of apoptotic cells that fragmented into apoptotic bodies was calculated (Table 1). Fragmentation into apoptotic bodies was a common feature of different lymphocyte subsets. However, the percentages of B lymphocytes

**Table 1**

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Lymphocyte subset</th>
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<tbody>
<tr>
<td></td>
<td>CD5+ T-cells</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>12.3 ± 1.2</td>
</tr>
<tr>
<td>PHA</td>
<td>31.5 ± 2.4</td>
</tr>
<tr>
<td>ST</td>
<td>15.3 ± 1.6</td>
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</table>

Results are expressed as mean percentage ± standard deviation of apoptotic cells fragmented into apoptotic bodies, calculated from replicated samples according to Eq. (4) (see Material and Methods). One representative experiment from six is shown.
Large Percentages of Apoptotic Lymphocytes Completely Lost Their Expression of LAg

Once demonstrated that a significant percentage of apoptotic cells partially or completely lose their ability to express their characteristic LAg (Fig. 2), we tried to understand the impact of such loss on the quantification of apoptosis. To achieve this goal, it was crucial to determine the proportion of apoptotic cells that completely lost the expression of their LAg (Table 2). This was greater than 20% for most culture conditions and antigens studied. The greatest percentage of cells that completely lost its LAg was found in NK cells for the expression of CD56 marker and the lowest was found in B cells for the expression of CD19 marker. The results in Table 2 show that the loss of LAg is a feature of spontaneous apoptosis and that this phenomenon is not an effect of exogenous inducers.

The Accurate Quantification of Apoptosis in Phenotypically Defined Populations Must Concurrently Take into Account for Loss of Antigen Expression and Cell Fragmentation

Phenotypically pure subpopulations of lymphocytes were cultured under different conditions to determine the frequency of apoptosis (either spontaneous/natural or PHA/ST induced) in the indicated populations. The absence of proliferation in the first 24 h of culture was assessed and corroborated by the absence of DNA synthesis as indicated by flow cytometric cell cycle analysis and tritiated thymidine uptake (data not shown). Under all the experimental conditions assayed, significant proportions of the cells from all phenotypically defined cell populations completely lost the expression of their LAg (Table 2) or suffered cell fragmentation (Table 1). PHA-induced apoptosis increased in all lymphocyte subsets the percentage of apoptotic cells that completely lost their LAg expression or that suffered cell fragmentation. Incubation with ST led to the loss of expression of CD5, CD8, and CD28, but not of the other markers studied. For all cell populations, loss of LAg expression and cell fragmentation were greatest when apoptosis was induced by PHA with respect to all experimental condition studied, except for the CD28 antigen that showed weaker expression after incubation with ST. The frequency of cell fragmentation was similar for all T cell subsets under all culture conditions tested. NK cells and B lymphocytes showed a significantly lower frequency of cell fragmentation than did T cells.

Overall, these results show that an important fraction of apoptotic cells completely lost the expression of LAg. These LAg negative apoptotic cells and also fragmented ones are neglected by conventional flow cytometry apoptosis protocols (AI) and therefore led to an inaccuracy of the estimation of the proportion of cells of a given antigen-defined population that suffered apoptosis. This underestimation of apoptosis is evident in T cell subsets undergoing spontaneous apoptosis. This point is clearly illustrated by the differences in estimation of apoptosis by AI and AR in a culture of sorted CD28+ T helper cells (Figure 3).

Table 2

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>CD5+ T-cells</th>
<th>CD3+ T-cells</th>
<th>CD8+ T-cells</th>
<th>CD4+ T-cells</th>
<th>CD28+ T-cells</th>
<th>CD56+ NK-cells</th>
<th>CD19+ B-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous</td>
<td>24.8 ± 1.4</td>
<td>23.8 ± 1.6</td>
<td>51.3 ± 4.3</td>
<td>21.7 ± 2.5</td>
<td>42.4 ± 3.7</td>
<td>77.1 ± 4.7</td>
<td>12.3 ± 0.9</td>
</tr>
<tr>
<td>PHA</td>
<td>23.5 ± 2.1</td>
<td>23.9 ± 2.0</td>
<td>31.5 ± 2.4</td>
<td>40.9 ± 3.3</td>
<td>45.5 ± 4.2</td>
<td>86.2 ± 3.5</td>
<td>16.3 ± 1.2</td>
</tr>
<tr>
<td>ST</td>
<td>21.3 ± 1.1</td>
<td>22.0 ± 0.2</td>
<td>35.5 ± 2.2</td>
<td>10.6 ± 1.6</td>
<td>69.5 ± 4.2</td>
<td>53.4 ± 4.6</td>
<td>9.8 ± 0.9</td>
</tr>
</tbody>
</table>

Results are expressed as mean percentage ± standard deviation of replicated samples of apoptotic cells that completely lost their corresponding LAg, calculated according to Eq. (3) (see Material and Methods). One representative experiment from six is shown.

and NK cells that fragmented into apoptotic bodies were markedly lower than those shown by different subsets of T cells. The frequency of cell fragmentation was always higher when apoptosis was induced by PHA and lower when apoptosis was spontaneous.

**Figure 3.** Impact of loss of LAg expression and cell fragmentation on the accurate measurement of apoptosis by AI and AR. Purified T cell subsets of lymphocytes were cultured for 24 h without apoptosis inducers (spontaneous apoptosis). For each T cell subset, the left column shows the percentage of LAg+ apoptotic cells with respect to the number of remaining LAg+ cells (AI). The right column (stacked bars) shows the percentages of apoptotic cells with respect to the number of cells originally seeded (AR). The different stages of apoptotic cells are represented with different colors: apoptotic cells whose LAg remained detectable ( ), apoptotic cells that completely lost the expression of their lineage antigen ( ), and fragmented apoptotic cells ( ).
The underestimation of the true frequency of apoptosis varied depending on the LAg in question and the culture conditions. Accordingly, two clusters of cell populations were distinguished in cultures treated with ST: those showing low frequency LAg loss (CD5\(^1\), CD3\(^1\), CD4\(^1\), and CD19\(^1\) subsets) and those showing high frequency LAg loss (CD8\(^1\), CD28\(^1\), and CD56\(^1\) subsets). Interestingly, the differences between AI and AR were significantly greater for the subsets with higher percentages of cells that completely lost the expression of their LAg. The differences between AI and AR were significant for all populations and under all conditions of apoptosis induction.

**The AI Significantly Underestimates the Frequency of Apoptosis**

For all T cell markers, the maximum underestimation of apoptosis by AI was observed under conditions of spontaneous apoptosis. This was greater than 50% for all cell subsets except for CD4\(^+\) T cells (Table 3). When apoptosis was induced by PHA, this percentage was about 20%. Lower values were obtained in ST-induced apoptosis for all T cell markers except CD28. The less serious underestimation observed when apoptosis was induced with ST is explained by the lower percentages of both fragmented and LAg\(^-\) apoptotic cells.

Given that the greatest differences between AR and AI were found under conditions of spontaneous and PHA-induced apoptosis, these scenarios were used to compare the kinetics of the differences in their sensitivity in PBMC cultures. Figure 4 shows that AR was significantly more sensitive than AI at quantifying apoptotic cells in all the lymphocyte populations studied and at all kinetic points. The difference between AR and AI increased progressively, since AR increased with time, reflecting the fact that new

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Table 3

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>CD5(^+)T-cells</th>
<th>CD3(^+)T-cells</th>
<th>CD8(^+)T-cells</th>
<th>CD4(^+)T-cells</th>
<th>CD28(^+)T-cells</th>
<th>CD56(^-)NK-cells</th>
<th>CD19(^+)B-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous</td>
<td>52.5 ± 3.9</td>
<td>53.2 ± 4.0</td>
<td>58.0 ± 4.1</td>
<td>48.5 ± 4.7</td>
<td>62.4 ± 4.5</td>
<td>15.0 ± 1.3</td>
<td>10.0 ± 1.1</td>
</tr>
<tr>
<td>PHA</td>
<td>17.5 ± 1.6</td>
<td>18.7 ± 1.6</td>
<td>20.2 ± 1.7</td>
<td>25.4 ± 2.4</td>
<td>23.7 ± 2.8</td>
<td>44.3 ± 4.6</td>
<td>17.6 ± 1.5</td>
</tr>
<tr>
<td>ST</td>
<td>13.2 ± 1.2</td>
<td>11.8 ± 1.1</td>
<td>25.3 ± 1.9</td>
<td>15.4 ± 1.1</td>
<td>55.4 ± 3.9</td>
<td>5.7 ± 1.2</td>
<td>2.4 ± 0.8</td>
</tr>
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</table>

Results are expressed as mean percentage ± standard deviation of replicated samples of apoptotic cells underestimated by the AI (compared to the AR), calculated according to Eq. (5) (see Material and Methods). One representative experiment of six is shown.

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The underestimation of the true frequency of apoptosis varied depending on the LAg in question and the culture conditions. Accordingly, two clusters of cell populations were distinguished in cultures treated with ST: those showing low frequency LAg loss (CD5\(^+\), CD3\(^+\), CD4\(^+\), and CD19\(^+\) subsets) and those showing high frequency LAg loss (CD8\(^+\), CD28\(^+\), and CD56\(^+\) subsets). Interestingly, the differences between AI and AR were significantly greater for the subsets with higher percentages of cells that completely lost the expression of their LAg. The differences between AI and AR were significant for all populations and under all conditions of apoptosis induction.

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**Fig. 4.** Comparison of the AR and AI for the kinetic quantification of occurrence of apoptosis in cultured PBMC. PBMCs were cultured for 3, 6, 18, and 24 h in the absence (panel A) or presence of PHA (panel B). Apoptosis was determined in several populations, defined by the expression of different surface antigens: CD3\(^+\) (T cells), CD3\(^+\)CD4\(^+\) (helper T cells), CD3\(^+\)CD8\(^+\) (cytotoxic T cells), CD3\(^+\)CD56\(^-\) (NK cells) and CD19\(^+\) (B cells). Apoptosis was measured by two indicators: the AI (●) and the AR (○). Results are expressed as mean ± standard deviation of replicated samples. One representative experiment from four is shown.

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cells are undergoing apoptosis. This progressive increase in the proportion of apoptotic cells was not detected by AI—the value of which was nearly constant over time. This suggests that the proportion of cells undergoing apoptosis does not include the significant fraction that entered the apoptosis program but suffered LAg loss or cell fragmentation (and were therefore no longer identifiable as LAg cells). Noteworthy, the neglected apoptotic cells can even cause decrease of AI over time as can be observed for CD5+CD8+ lymphocytes.

**DISCUSSION**

This study shows that the standard flow cytometry criterion for the quantification of apoptosis in cell cultures—AI—underestimates the phenomenon in antigen-defined cell subsets. This error is overcome by the measurement of the AR. The accuracy of the AR is owed to the concurrent measurement of two biological phenomena that occur with great frequency in apoptotic cells: the loss of LAg expression and fragmentation into apoptotic bodies. Neither process is taken into account in current routine calculations, leading to the systematic underestimation of the frequencies of apoptotic cells in many usual conditions.

The comparison of AI and AR in short-term lymphocyte cultures of different pure populations confirmed that the loss of LAg expression and cell fragmentation lead to the underestimation of apoptosis by AI. It should be noted that cell fragmentation was more homogeneous in the different T cell subsets, whereas the loss of expression of LAg was more varied. It might therefore be concluded that the inaccuracy inherent in the AI due to cell fragmentation may be the same for different lymphocyte populations but can vary due to different patterns of LAg loss. Additionally, variations in the culture conditions appear to have a negative impact on the accuracy of estimations: the different apoptosis-inducing conditions studied in this work had different effects on the expression of LAg by apoptotic cells. More importantly, the differences between AR and AI indicate that the relative underestimation of apoptosis by the latter is more pronounced when apoptosis is spontaneous than when apoptosis-inducing agents are used. This finding may be related to the fact that apoptosis is massive and synchronously induced by PHA and ST, and that despite the loss of LAg or cell fragmentation, AI still detects a significant fraction of the apoptotic cells. In contrast, spontaneous apoptosis occurs in a nonsynchronous fashion and at any given moment affects a smaller proportion of cells. In any event, it is in these low intensity spontaneous or natural apoptotic scenarios (the most similar to physiological conditions) the AR can be a very much more accurate and valid apoptosis indicator than the available standard, AI.

Together, the present results show that apoptosis cannot be accurately quantified by simply taking into account the percentage of cells that show apoptotic lesions. Single-cell approaches must therefore be used with care if occurrence of apoptosis is to be accurately evaluated, and should take into account absolute cell enumeration via the use of an internal microbead standard. This change of paradigm suggests that we may need to reassess the results reported in many articles (6–8, 11, 23).

Owing to the loss of LAg expression and cell fragmentation, the AR should be considered the best standard for measuring the true occurrence of apoptosis within phenotypically defined cell subsets. However, the AR has limitations in the quantification of apoptosis in cultures of proliferating cells but then the enumeration of proliferating tracked cells can be a useful tool. Further research is required to combine cell enumeration with the identification of apoptosing and proliferating cells.

**LITERATURE CITED**

18. Philipp J, Louage H, Thiersen H, Vral A, Cornillussen M, De Ridder L. Quantification of apoptosis in lymphocyte subsets and effect of apo...


