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Novel Fluorescence Assay Using Calcein-AM for the Determination of Human Erythrocyte Viability and Aging

Daniela Bratosin,[1]* Laura Mitrofan,[2] Carmen Palii,[3] Jérôme Estaquier,[4] and Jean Montreuil[5]

[1]Institutul National de Cercetare-Dezvoltare pentru Stiinte Biologice, Bucuresti, Romania
[2]Institutul de Biologie al Academiei Române, Bucuresti, Romania
[3]Universitatea Al. I. Cuza, Facultatea de Biologie, Iasi, Romania
[4]Institut Pasteur, Unité de Physiopathologie des Infections Lentivirales, Paris, France
[5]Université des Sciences et Technologies de Lille 1, Laboratoire de Chimie Biologique, Villeneuve d'Ascq, France

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Background: A highly sensitive, fast, and simple flow cytometric assay to assess human red blood cell (RBCs) viability and aging is reported.
Methods: The assay described in this report is based on the use of acetoxymethyl ester of calcein (calcein-AM), a fluorescein derivative and nonfluorescent vital dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein, which is retained by cells with intact membranes and inactive multidrug resistance protein. The loss of calcein can be easily determined by flow cytometry, and the cytosolic localization of esterases was demonstrated by spectrofluorometric analyses.
Results: We found that RBCs incubated with Ca^{2+}, which induces a rapid and modulated self-death that shares several features with apoptosis (Bratosin et al., Cell Death Differ 2001;8:1143–1156), externalized phosphatidylserine and lost calcein staining and cytosolic adenosine triphosphate content. Double labeling using phycoerythrin-labeled annexin-V and calcein-AM showed that the decrease of esterase activity is an early event that precedes the externalization of phosphatidylserine residues. In addition, this assay allowed us to distinguish young and aged RBCs isolated by ultracentrifugation in a self-forming Percoll gradient and can be considered as a reliable marker of RBC aging.
Conclusions: Calcein-AM assay may represent a wide application for assessing RBC viability, particularly in blood banks. © 2005 Wiley-Liss, Inc.

Key terms: red blood cell; flow cytometry; calcein-AM; cell death; viability; aging; blood storage

Pioneered by the early works of Brunning et al. (1), numerous methods using fluorescent dyes to measure cell viability and cytotoxicity have been described and the number of these probes is continually increasing (2). Among the dyes we have used, acetoxymethyl diacetylester of calcein (calcein-AM), a derivative of fluorescein, stands out as the first indicator of cell viability and cellular cytotoxicity due to its superior cell retention and relative insensitivity of its fluorescence to pH in the physiologic range (2,3). Calcein-AM is a highly lipophilic vital dye that rapidly enters viable cells, is converted by intracellular esterases to calcein that produces an intense green (530-nm) signal (3), and is retained by cells with intact plasma membrane. From dying or damaged cells with compromised membrane integrity or from cells expressing multidrug resistance protein (MRP) (4,5), unhydrolyzed substrates and their fluorescent products are rapidly extruded from cells. In the past decade, the calcein-AM assay has replaced the conventional ^{51}Cr-release method for assessment of cell viability (6–12) and cytotoxicity (13–18) and has been applied to quantitate apoptosis (19–24).

The occurrence of MRP in the human red blood cell (RBC) membrane demonstrated by Pulaski et al. (25) makes this cell potentially useful when studying the function of MRP and the efficiency of potential MRP inhibitors. For monitoring the efflux activity of MRP human RBCs, numerous investigators have used fluorescent probes,

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*Correspondence to: Dr. Daniela Bratosin, Université des Sciences et Technologies de Lille 1, Laboratoire de Chimie Biologique, UMR USTL/CNRS 8576, 59655 Villeneuve d'Ascq Cedex, France.
E-mail: dbratosin@hotmail.com

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among them fluorescein derivatives such as 2,′7′-bis-(carboxypropyl)-5/6-carboxyfluorescein, acetoxymethyl ester (26), 2′,7′-bis-(carboxyethyl)-5/6-carboxyfluorescein, or calcein-AM (27). However, to our knowledge, the calcein-AM assay has not been reported to assess RBC viability.

Recently, we and others (28–30) reported that mature human RBCs can undergo a rapid self-destruction process that shares several features with apoptosis, including cell shrinkage, plasma membrane vesiculization, phosphatidylserine externalization, possible RBC disintegration, or, in the presence of macrophages, phagocytosis of these dying RBCs. Because this regulated form of programmed cell death could be induced and modulated by Ca^{2+} influx, we investigated whether the calcein-AM assay could be used to determine RBC viability.

In the present paper, we describe a rapid and sensitive calcein-AM assay for flow cytometric determination of human RBC viability.

MATERIALS AND METHODS

Chemicals

The fluorogenic dye calcein-AM, human serum albumin, phenylmethylsulfonyl fluoride, sodium azide, saponin, and adenosine 5′-triphosphate kit were purchased from Sigma-Aldrich (St. Louis, MO, USA), phycoerythrin-conjugated annexin-V (PE-annexin-V), and HEPES buffer were obtained from Pharmingen (San Diego, CA, USA), ionophore A 23187 of *Streptomyces chartreusis* was obtained from Calbiochem (La Jolla, CA, USA), and Percoll from Pharmacia-Biotech AB (Uppsala, Sweden).

Blood Samples

Human blood type O Rh^+ erythrocytes collected in heparin were kindly supplied by the Etablissement Régional de Transfusion Sanguine de Lille, France. After blood centrifugation (2,000*g* at 4°C for 5 min), plasma, platelets, and leukocytes were removed by pipetting, and erythrocytes were washed three times in Dulbecco's phosphate buffered saline solution (PBS; pH 7.4; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). RBCs were resuspended (10^7 cells/ml) in HEPES buffer (10 mM HEPES and 140 mM NaCl), pH 7.4, supplemented with 0.1% human serum albumin in the absence or presence of Ca^{2+} (2.5 mM $CaCl_2$) and ionophore A 23187 (0.5 μM), as previously described (28), and incubated at 37°C for 3 and 20 h under 5% CO_2 atmosphere. Young and old erythrocytes were separated by ultracentrifugation of blood in a self-forming Percoll gradient according to the procedure of Lutz et al. (31) modified as previously described (32). Both fractions were washed three times with PBS buffer, pH 7.4, containing 0.2 M phenylmethylsulfonyl chloride and recentrifuged in the same Percoll gradient.

Flow Cytometric Analysis

Flow cytometric analyses were performed on a FACScalibur cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest software for acquisition and analysis. The light scatter channels were set on linear gains, and the fluorescence channels were set on a logarithmic scale, with a minimum of 10,000 cells being analyzed in each condition.

Flow Cytometric Analysis of RBC Subpopulations Using Light Scatter Measurements

Variations of erythrocyte size and density were assessed using forward and side-angle scatters (FSC vs. SSC) in isotonic PBS buffer, pH 7.4, osmolality 320 to 330 mosmol/kg according to Bratosin et al. (32).

Flow Cytometric Assay of Cell Viability Using Calcein-AM

To assess cell viability, the membrane-permeable dye calcein-AM was prepared as a stock solution of 10 mM in dimethylsulfoxide stored at −20°C and as a working solution of 100 μM in PBS buffer, pH 7.4. RBCs, 4×10^5 in 200 μl PBS buffer, were incubated for different times (0 to 135 min at 15-min intervals) with 10 μl of calcein-AM working solution (final concentration in calcein-AM: 5 μM) at 37°C in the dark and then diluted in 0.5 ml of PBS buffer for immediate flow cytometric analysis of calcein fluorescence retention in cells. All studies were performed at least three times, with three replicates each time. RBC experimental permeabilization as a control of dying cells was readily made with saponin according to Jacob et al. (33). Cells, 2×10^7, were incubated for 15 min in 1 ml of a 1% saponin solution in PBS buffer, pH 7.4, containing 0.05% sodium azide. Due to the reversible nature of cell membrane permeabilization, saponin must be included in all buffers used for further staining and washing steps. After saponin permeabilization, 4×10^5 RBCs in suspension in PBS buffer containing 0.1% saponin and 0.05% sodium azide were incubated (37°C in the dark for 45 min) with calcein-AM to a final concentration of 5 μM, washed three times with the same PBS buffer containing 0.1% saponin and 0.05% sodium azide, and analyzed by flow cytometry as described above. Experiments were carried out in triplicate.

PE-Annexin-V and Calcein-AM Double-Labeling Flow Cytometric Analysis

RBCs were first incubated (37°C for 45 min) with calcein-AM (0.5 μM) as described above. Thereafter, cells were isolated by centrifugation (2,000*g* at 4°C for 5 min), resuspended in 100 μl HEPES binding buffer, pH 7.4, containing 2.5 mM calcium chloride and incubated with 10 μl PE-annexin-V for 15 min at room temperature in the dark. After addition of 400 μl binding buffer, cells were analyzed for biparametric histograms FL1 (calcein) versus FL2 (PE-annexin-V). Experiments were carried out in triplicate.

Fluorometric Measurement of Cytosolic Esterase Activity

Two types of measurements of cytosolic esterase activity were performed. In the first type RBCs were pretreated

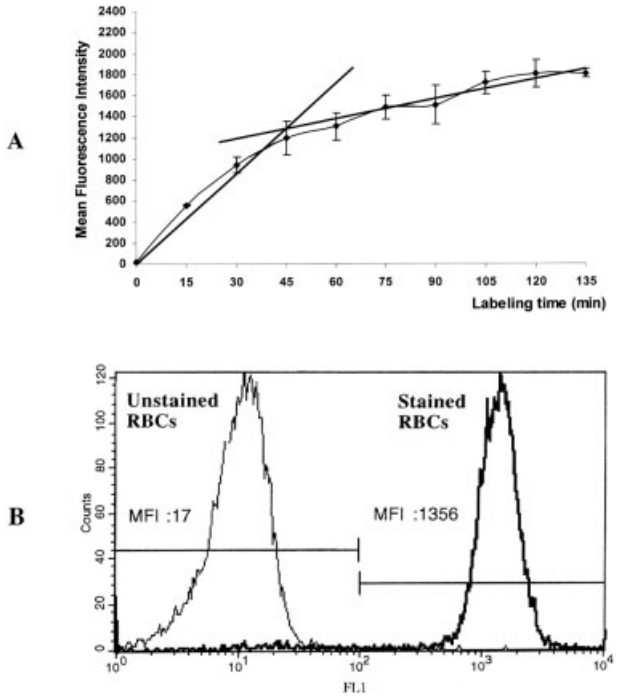

FIG. 1. Flow cytometric analysis of RBC esterase activity. **A:** Effect of incubation time on human RBC cytosolic activity toward calcein-AM. Freshly isolated RBCs were incubated at 37°C in the presence of 5 μM calcein-AM and flow cytometric analysis was performed at different time points. Data are expressed as mean fluorescence intensity ± standard deviation of three independent experiments. **B:** Overlay (single parameter) of flow cytometric analysis of cell esterase activity of human RBCs incubated in presence of 5 μM calcein-AM for 0 min (unstained RBCs) and 45 min (stained RBCs). Fluorescence mean values are expressed as MFI. Abcissae: log scale green fluorescence intensity (FL1). Ordinates: relative cell number. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

(37°C for 45 min) with calcein-AM (final concentration: 5 μM) as described above and a suspension of 10^6 RBCs was hemolyzed with 300 μl of cold water by stirring with a Vortex for a few minutes. After removal of cell membranes by ultracentrifugation (10,000*g* at 4°C for 20 min) fluorescence intensity of the cytosol was determined using a Microplate Fluorescence Reader FL×800 (Bio-Tek Instruments, Winooski, VT, USA) with a KC Junior program under the following conditions: 485-nm excitation, 528-nm emission, 20 bandwidth, and 100% sensitivity. Results were expressed as "means of fluorescence units." Blanks were examined under the same conditions in the absence of calcein-AM.

In the second type of measurement, cytosolic fraction was prepared by stirring 500 μl of RBCs in 1 ml of cold water and cell membranes were removed from the lysate by ultracentrifugation (10,000*g* at 4°C for 20 min). To 250 μl of the supernatant was added 15 μl of the working solution of 100 μM calcein-AM in 96-well plastic black microtiter plates (Nunc, Naperville, IL, USA) and the mixture was maintained at 37°C for different time points (0 to 50 min at 5-min intervals). Blanks were examined under the same conditions in the absence of calcein-AM and fluorescence intensity was determined using a Microplate Fluorescence Reader FL×800 as described above. All experiments were performed at least three times with three replicates each time.

Determination of Adenosine Triphosphate Level in RBCs

Adenosine triphosphate (ATP) level was determined using the Sigma-Aldrich adenosine 5′-triphosphate kit and expressed in micromoles of ATP per deciliter of blood. Experiments were carried out in triplicate.

RESULTS

Influence of Incubation Time on Labeling Efficiency Using Calcein-AM

As shown in Figure 1A, calcein-AM labeled RBCs and the rate of calcein-AM hydrolysis by RBC esterases rapidly increased within 45 min of incubation. The mean fluorescence intensity did not greatly increase thereafter, perhaps due to the concomitant spontaneous release of calcein resulting from the cell membrane permeabilization during long periods of incubation. Based on the slope of calcein-AM staining, we adopted an incubation time of 45 min. Figure 1B shows that, under these experimental conditions, regions of unstained and stained RBCs were clearly defined.

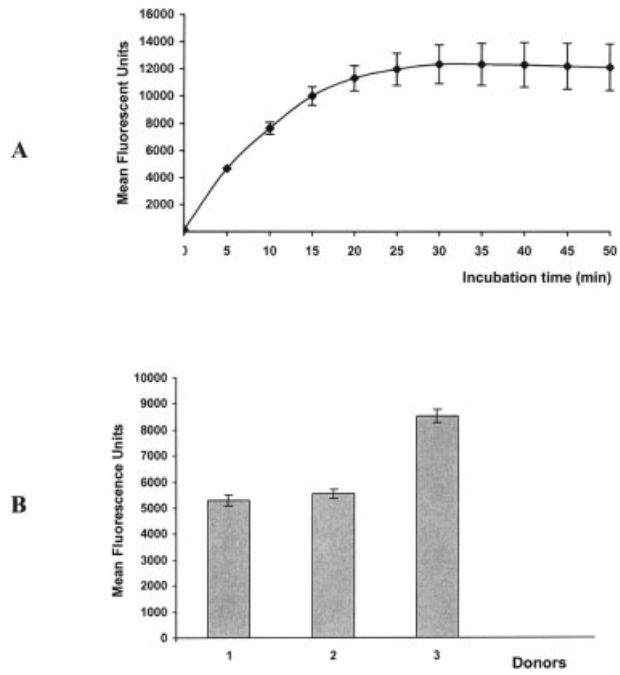

FIG. 2. Fluorometric measurement of esterase activity in cytosolic fraction of human RBCs using a Microplate Fluorescence Reader. **A:** Kinetics of esterase activity: freshly isolated human RBCs were hemolyzed and, after removal of membranes by ultracentrifugation, the cytosolic fraction was incubated at 37°C in the presence of 5 μM calcein-AM and its fluorescence was determined at different time points as described in Materials and Methods. Data represent means ± standard deviation of three independent experiments. **B:** Fluorescence intensity of the cytosol of human RBCs that were preincubated (37°C for 45 min) with calcein-AM before lysing. The obtained cytosolic fraction of three different donors was analyzed and data represent mean ± standard deviation of three independent experiments.

Calcein-AM Assay Is Specific to Esterase Activity of RBC Cytosol

To demonstrate that the calcein-AM assay was specific to cytosol and not to membrane esterases such as acetylcholinesterase, an extracellular enzyme of human RBCs whose activity significantly decreases with RBC aging (34), calcein-AM was added to the cytosolic fraction of RBCs devoid of plasma membranes. As shown in Figure 2A, spectrofluorometric analysis, using a Microplate Fluorescence Reader, demonstrated unambiguously that RBC cytosol contains active esterases and that the plateau is reached in less than 30 min due to the absence of the membrane barrier.

This result was confirmed by the fluorescence intensity measured with a Microplate Fluorescence Reader on the cytosolic fractions of RBCs preincubated (37°C for 45 min) with calcein-AM (5 μM) before lysing. As shown in Figure 2B, three separate experiments carried out each in triplicate with RBCs from three different donors showed an intense fluorescence that varied across individuals.

Calcein-AM Assay for Human RBC Membrane Integrity

To assess whether the calcein-AM assay would be suitable for testing membrane permeabilization as a control of cell death, we treated RBCs in the absence or presence of saponin. Figure 3 shows a loss of calcein fluorescence in saponin-treated RBCs (mean fluorescence intensity [MFI]: 25) compared with untreated RBCs (MFI: 1418). This result allowed us to conclude that the unstained region of histogram represents the dead or dying cells with damaged membranes, thus explaining the release of calcein fluorescence.

Calcein-AM as a Marker for Human RBC Viability

We and others (28–30) previously reported that a regulated form of programmed cell death, which shares several features with apoptosis including cell shrinkage and phosphatidylserine externalization, can be induced in mature human RBCs by calcium influx. We then determined whether the calcein-AM assay could be used to assess human RBC viability after incubation (37°C for 3 and 20 h) in the presence of Ca^{2+} (2.5 mM) and ionophore A 23187 (0.5 μM). As we previously described (28) and as shown in Figure 4A, flow cytometric analysis using light scatter measurements indicated dramatic morphologic changes associated with cell shrinkage (decreased forward scatter and increased side scatter), one of the characteristic features of RBC death. In Figure 4B, double-color flow cytometric analysis (calcein-AM vs. annexin-V) indicates (i) that dying RBCs display progressive externalization of phosphatidylserine residues after Ca^{2+}-ionophore treatment (35–37) and (ii) that annexin-V–negative RBCs nevertheless display a decrease in calcein-AM staining. Thus, in the experiment shown, after 3 h of incubation with Ca^{2+}, 35% of RBCs were annexin-V positive, whereas esterase activity decreased by two thirds (387 MFI at 3 h vs. 1.210 MFI at 0 h). After 20 h of incubation, most cells were dead (92% were annexin-V positive) and the fluorescence staining of the calcein completely disappeared (8 MFI) due to the loss of cell esterase activity and membrane permeabilization. Consequently, the histogram of calcein-AM staining shows no bimodal peak. Altogether, these data support the idea that loss of RBC esterase activity precedes phosphatidylserine exposure. We also found that loss of calcein fluorescence is associated with a decreased amount of ATP (Fig. 4D), another marker of RBC senescence and apoptosis that is classically considered an indicator of viability for RBCs stored in blood banks.

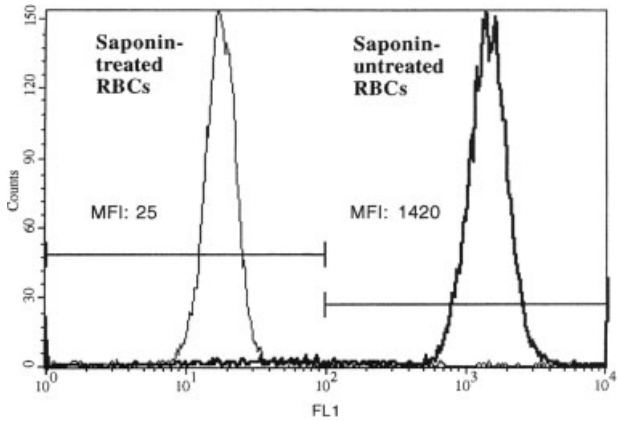

FIG. 3. Flow cytometric analysis of human RBCs treated with saponin. Overlay (single parameter) of flow cytometric analysis of calcein-AM staining of saponin-untreated and saponin-treated human RBCs. MFI: fluorescence mean values expressed as mean fluorescence intensity. Abscissae: log scale green fluorescence intensity (FL1). Ordinates: relative cell number. Number of counted cells: 10,000. Results are from one representative experiment of three performed.

Calcein-AM Assay as a Marker for Human RBC Aging

We then assessed whether this assay could be used to distinguish young from old RBCs isolated by ultracentrifugation of freshly collected human blood in a self-forming Percoll gradient (31). This method furnishes highly enriched populations of young and old erythrocytes with few contaminant cells. The percentage of isolated cells was estimated to 1.0 ± 0.2% for both populations. These values are similar to those obtained by Sorette et al. (38) and Bratosin et al. (32).

As shown in Figure 5A, calcein fluorescence in old RBCs was lower than that in young RBCs due to decreased esterase activity. Similarly, we observed a good correlation with ATP content, which was lower in old RBCs than in young ones (Fig. 5B). As shown in Figure 4B, the decreased calcein fluorescence intensity of old RBCs was not associated with annexin-V staining (data not shown), suggesting that decreased esterase activity precedes phosphatidylserine exposure. As reported by Bratosin et al. (35), only the senescent RBCs that were phagocytized by macrophages reacted with annexin-V. This finding shows, as previously reported by Gatti et al. (20) using adherent cell lines PC12 and NIH3T3, that annexin-V might be less sensitive than calcein-AM for early apoptosis detection. These results

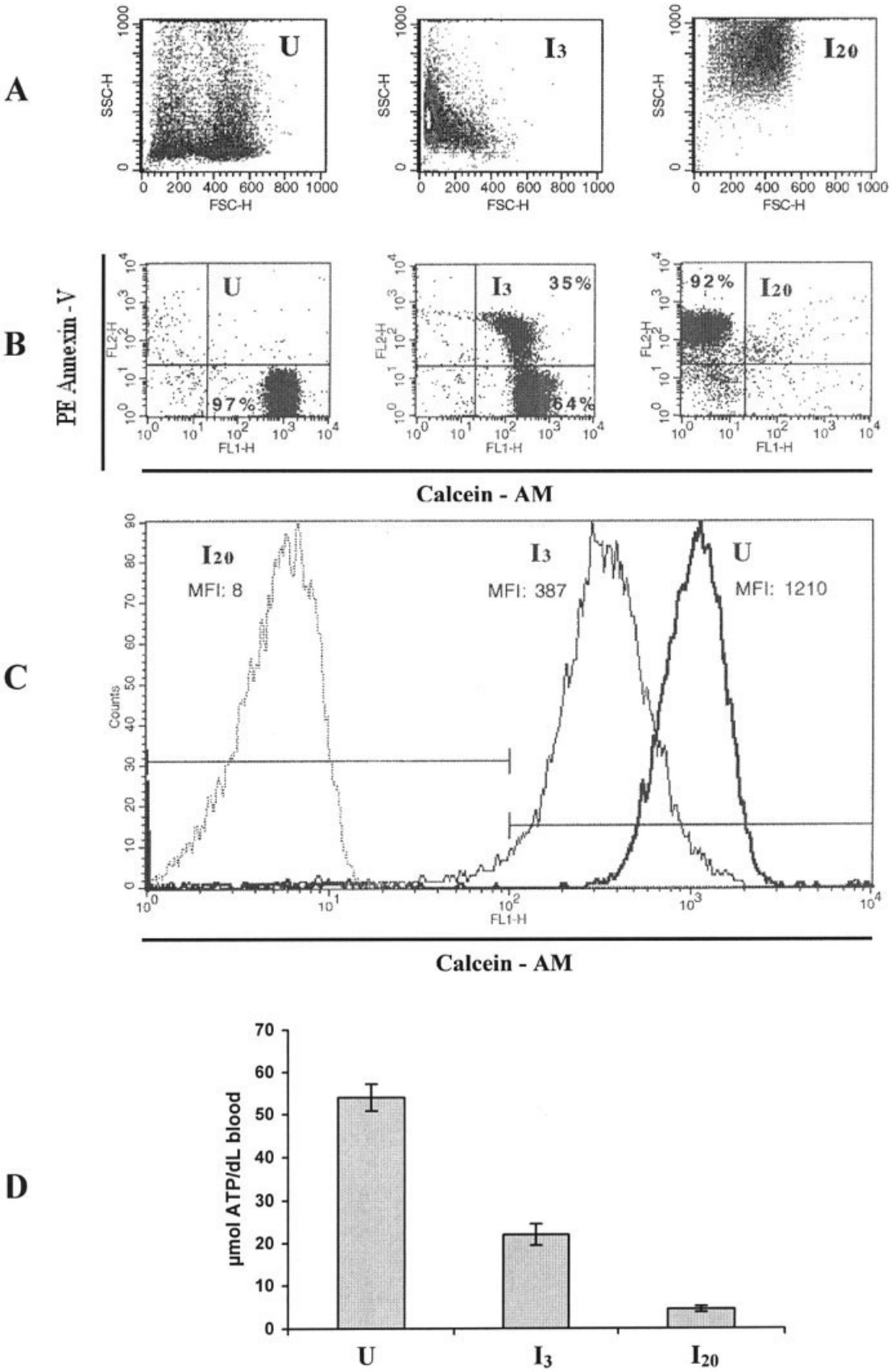

FIG. 4

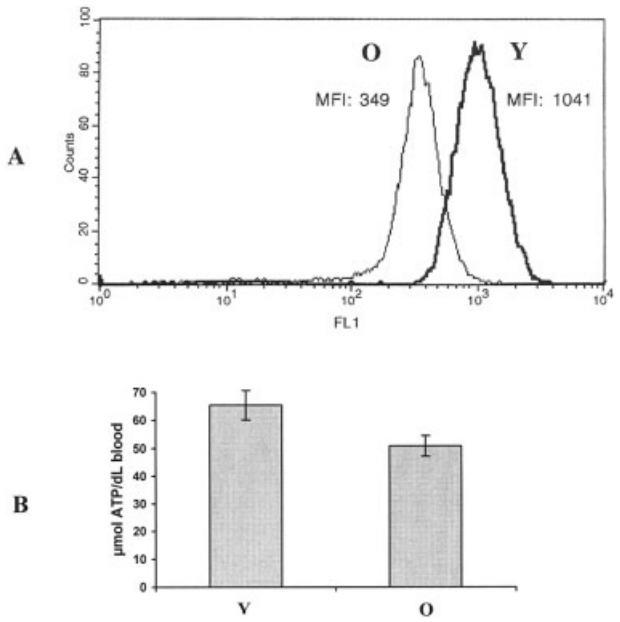

FIG. 5. **A:** Overlay (single parameter) of flow cytometric analysis of cell esterase activity of young (Y) and old (O) human RBCs isolated by ultracentrifugation in a Percoll self-forming gradient according to the method of Lutz et al. (31) as modified by Bratosin et al. (32). Numbers represent fluorescence mean values (MFI). Abscissae: log scale green fluorescence intensity (FL1). Ordinates: relative cell number. Number of counted cells: 10,000. Results presented are from one representative experiment of four performed. **B:** Evolution of ATP content in young (Y) and old (O) RBCs. Data represent mean ± standard deviation of three independent experiments.

lead us to propose that the calcein-AM assay might be used as a marker for human RBC aging.

DISCUSSION

The fundamental mechanisms of cell lesions related to senescence and apoptosis of nucleated cells, including nucleated erythrocytes (39), are well known and led to the definition of cell viability criteria such as mitochondrial membrane potential ($\Delta\Psi m$) loss, cysteine protease activation, chromatin condensation and fragmentation, and propidium iodide uptake. These criteria are not applicable to human RBCs due to their lack of nuclei, mitochondria, and other organelles. Therefore, one of the main challenges is to determine early events involved in the death of RBCs. In the present study, we devised a new flow cytometric assay for the measurement of viability and aging of human RBCs using calcein-AM. This assay is rapid and simple and requires very small quantities of cells. It can be easily performed on a single-laser flow cytometer. Analyses showed that two regions could be clearly and unambiguously defined: (i) the region of fluorescent RBCs with intact membranes that is related to intracellular esterase activity and strongly correlated with the number of living cells, including aging or dying cells; and (ii) the region of nonfluorescent dead cells with damaged cell membranes. In addition, we observed that the loss of esterase activity was an early event that occurred before phosphatidylserine exposure.

The safe and sensitive calcein-AM assay we have developed may represent a valuable test of aging and viability of RBCs stored in blood banks. In this regard, it is interesting to refer to an article published by Beutler (40) entitled "Back to the Future in RBC Preservation" that is devoted to the formidable problem of prolonging the time that RBCs can be stored in the liquid state. Beutler wrote: "Progress has been slow for a number of reasons. First of all, the fundamental nature of the storage lesion remains unknown. Second, no good surrogate test has ever been found for the performance of viability studies in human volunteers." The calcein-AM assay we have described may contribute to solve the second problem evoked by Beutler.

FIG. 4. Comparative analysis of morphologic changes, phosphatidylserine exposure, cell esterase activity, and ATP content of human RBCs incubated in the presence of ionophore A 23187 and Ca^{2+} for 3 and 20 h. Freshly isolated human RBCs (U) and RBCs were incubated in the presence of ionophore A 23187 (0.5 µM) and Ca^{2+} (2.5 mM) for 3 h (I_3) and 20 h (I_{20}). **A:** Dot-plot analysis of RBC shape changes. Abscissae: forward scatter (cell size); ordinates: side scatter (cell density). The upper left region of dot plots corresponds to shrunken erythrocytes with smaller volume and greater density. **B:** Flow cytometric quadrant analysis of calcein-AM (abscissae) and PE-annexin-V (ordinates) double-stained RBCs. Lower right quadrant: region of calcein-positive and annexin-V-negative cells; upper right quadrant: calcein and annexin-V–positive cells; upper left quadrant: calcein-negative and annexin-V–positive cells. Numbering refers to the percentage of each cell population in each quadrant. Number of counted cells: 10,000. **C:** Overlay (single parameter) of flow cytometric analysis of calcein-AM–labeled RBCs after 3 and 20 h of incubation in the absence (U) or presence of ionophore A 23187 and Ca^{2+} (I_3 and I_{20}). Numbers represent fluorescent mean values expressed as MFI. Abscissae: log scale green fluorescence intensity of calcein (FL1). Ordinates: relative cell number. Number of counted cells: 10,000. A–C show one representative experiment of three performed giving similar results. **D:** Histogram of the evolution of ATP content in untreated (U) and Ca^{2+}-ionophore–treated (I_3 and I_{20}) RBCs. Data represent mean ± standard deviation of three independent experiments.

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