Detection of Altered Membrane Phospholipid Asymmetry in Subpopulations of Human Red Blood Cells Using Fluorescently Labeled Annexin V

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The phospholipids of the human red cell are distributed asymmetrically in the bilayer of the red cell membrane. In certain pathologic states, such as sickle cell anemia, phospholipid asymmetry is altered. Although several methods can be used to measure phospholipid organization, small organizational changes have been very difficult to assess. Moreover, these methods fail to identify subpopulations of cells that have lost their normal phospholipid asymmetry. Using fluorescently labeled annexin V in flow cytometry and fluorescent microscopy, we were able to identify and quantify red cells that had lost their phospholipid asymmetry in populations as small as 1 million cells. Moreover, loss of phospholipid organization in subpopulations as small as 0.1% of the total population could be identified, and individual cells could be studied by fluorescent microscopy. An excellent correlation was found between fluorescence-activated cell sorter (FACS) analysis results using annexin V to detect red cells with phosphatidylserine (PS) on their surface and a PS-requiring prothrombinase assay using similar red cells. Cells that bound fluorescein isothiocyanate (FITC)-labeled annexin V could be isolated from the population using magnetic beads covered with an anti-FITC antibody. Evaluation of blood samples from patients with sickle cell anemia under oxygenated conditions demonstrated the presence of subpopulations of cells that had lost phospholipid asymmetry. While only a few red cells were labeled in normal control samples (0.21% ± 0.12%, n = 8), significantly increased (P < .001) annexin V labeling was observed in samples from patients with sickle cell anemia (2.18% ± 1.21%, n = 13). We conclude that loss of phospholipid asymmetry may occur in small subpopulations of red cells and that fluorescently labeled annexin V can be used to quantify and isolate these cells.

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The asymmetric distribution of phospholipids in membranes was postulated 20 years ago by Bretscher.1-3 Phospholipid asymmetry has since been demonstrated in a wide variety of cell types. Techniques used to distinguish between the two membrane leaflets and between headgroups of the different membrane phospholipid molecular species include the use of chemical reagents or phospholipases to specifically alter the endogenous phospholipids of the outer monolayer, strategies to monitor the redistribution of exogenously added phospholipid probe molecules across the bilayer, and the activation of hemostatic processes such as the phosphatidylserine (PS)-mediated activation of prothrombin to thrombin by prothrombinase.1-6

Application of these techniques to normal erythrocyte membranes shows that the cholinerich containing phospholipids phosphatidylcholine (PC) and sphingomyelin (SM) are localized mainly in the outer monolayer of the erythrocyte membrane, whereas the aminophospholipids phosphatidyl-ethanolamine (PE) and PS are mainly PE or exclusively PS found in the inner monolayer. Although phospholipid asymmetry is essentially conserved throughout the life span of the cell, phospholipids are actually in a state of dynamic equilibrium between the two monolayers. The net equilibrium is a balance of two mechanisms. First, all phospholipids diffuse passively across the bilayer at a relatively slow rate. Second, aminophospholipids are actively transported from the outer monolayer to the inner monolayer by a Mg2+-adenosine triphosphate (ATP)-dependent aminophospholipid translocase, and their distribution at equilibrium affects that of the other phospholipids.6

The rate of active transport of aminophospholipids from outer to inner monolayer (flip) is significantly higher than the rate from inner to outer monolayer (flop). It appears, in particular, that PS present in the outer monolayer is rapidly transported to the inner monolayer by the aminophospholipid translocase. Although an inactivated aminophospholipid translocase could be expected to lead to a loss of asymmetry due to passive diffusion, driven by a concentration gradient, this is apparently a slow process. Even under conditions where the aminophospholipid translocase is severely impaired, such as during storage of red blood cells (RBC), endogenous PS molecular species move only very slowly from the inner to the outer leaflet, and no equilibration across the bilayer is observed. Loss of asymmetry of endogenous PS becomes apparent only when the aminophospholipid translocase activity is impaired and the membrane phospholipid organization is scrambled by other means as well, as is seen when RBCs are incubated with calcium and ionophore.10

Loss of normal phospholipid asymmetry has been implicated in red cell pathology in diseases such as sickle cell anemia.11 Impaired aminophospholipid translocase activity can be measured in sickle cells using exogenous phospholipid probe molecules,12 and in vitro manipulations of these cells indicate loss of endogenous phospholipid asymmetry.13 However, it has been difficult to unambiguously show the loss of phospholipid asymmetry in vivo. This difficulty could be in part due to the methods used to measure endogenous phospholipid organization. With the exception of a PS-in-

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duced prothrombinase activity assay or the use of radioactively labeled annexin V, current assays to measure phospholipid sidedness do not permit assessment of small changes in asymmetry. The use of most traditional techniques for measurement of PS asymmetry is also limited by hemolysis. Even limited levels of hemolysis will greatly interfere with the interpretation of the data, because the resulting exposure of the inner monolayer to the probes will affect the data collected. Furthermore, as all of these measurements describe the average phospholipid organization in a population of red cells, none of them examines the phospholipid organization in a population of red cells, none of them examines the phospholipid asymmetry in small subpopulations of a red cell sample. Even a complete loss of asymmetry in small subpopulations of a sample might not be detected using the methods described to date. This would, however, be feasible with a probe that can assess phospholipid organization in individual cells.

Annexins are a family of proteins that bind to acidic phospholipids, particularly PS. These proteins are found in many organisms, and although extensive in vitro studies have described their calcium-dependent binding to phospholipids, the exact biologic function of the annexins is not yet known. Fluorescently labeled annexins have been used to identify PS on the surface of activated platelets under physiologic conditions in vitro as well as on the surface of vesicles released from activated platelets and apoptotic cells. Radiolabeled annexin V was used to identify PS exposure in red cells. However, binding of radiolabeled annexin V only indicates the loss of phospholipid asymmetry in the average cell and does not allow analysis of the red cell population. In the present study, we show that fluorescently labeled annexin V used in flow cytometry and fluorescent microscopy offers an excellent method for the determination of loss of normal phospholipid asymmetry in subpopulations or individual red cells. Using this technique, we have demonstrated that under certain in vitro conditions known to scramble membrane phospholipids, or in pathologic states such as sickle cell anemia, loss of phospholipid asymmetry is heterogeneous and may be confined to subpopulations of red cells.

MATERIALS AND METHODS

Erythrocytes. Human erythrocyte suspensions were prepared from fresh human venous blood collected in heparin or EDTA after informed consent was obtained from laboratory volunteers or patients with sickle cell anemia. Erythrocytes were pelleted by centrifugation, washed twice with 0.9% NaCl and once with incubation buffer, and finally diluted in incubation buffer to the appropriate concentration. RBCs at a 16% hematocrit were equilibrated in incubation buffer with 1 mmol/L calcium for 3 minutes at 37°C. Subsequently, calcium ionophore A23187 was added to the RBC suspension to a final concentration of 4 μmol/L, unless otherwise noted. This final mixture was incubated for 1 hour. Because incubation of RBCs with calcium ionophore will result in a number of cellular changes, including ATP depletion and vesiculation, cells will ultimately hemolyze under these conditions. The process was stopped by a wash with 2.5 mmol/L EDTA to remove calcium. Subsequently, the cells were washed three times in buffer containing 1% bovine serum albumin (BSA) to remove the ionophore and were resuspended in buffer without BSA to prevent calcium uptake during annexin V-labeling of the cells (see below). This removal of ionophore was crucial, as omission and subsequent incubation in the 1.2 mmol/L calcium buffer used for annexin V labeling led to massive hemolysis. In some experiments, aliquots of the suspension were removed at designated time points and treated with EDTA and BSA as indicated above.

Annexin V purification and fluorescein isothiocyanate (FITC) labeling. Annexin V was initially purified from fresh human plasma using a modification of the method of Haigler et al. Currently, recombinant annexin V is purified from an Escherichia coli expression system by phospholipid affinity chromatography. Both preparations give similar results.

Purified annexin V is incubated in 50 mmol/L Borate buffer, pH 9.0, at 4°C for 16 hours in the dark at a final concentration of 1 mg annexin V per milliliter in the presence of 20 molar equivalents of FITC (Molecular Probes, Eugene, OR). Subsequently, unreacted FITC is removed by incubation with 1.2 mol/L Tris/HCl, pH 8.0, and filtration on a PD-10 Sephadex G-25 column (Pharmacia, Uppsala, Sweden). The heterogeneously labeled annexin V species are separated by FPLC, and the brightly labeled fractions are collected. The preparation used in our studies had an average of three FITC molecules per protein molecule. The total fluorescence in solutions containing FITC-annexin V was measured in a Perkin Elmer LS5B Luminescence spectrometer (Perkin Elmer, Beaconsfield, Buckinghamshire, UK).

Annexin V labeling of RBCs. RBCs were suspended in buffer to a final concentration of 4 × 10^6/mL. Unless noted otherwise, 4 μL of a 500 μmol/L FITC-labeled annexin V solution was added to 0.5 mL of this suspension in the presence of 1.2 mmol/L Ca^{2+}. The samples were incubated for 30 minutes at room temperature and subsequently washed with buffer to remove unbound annexin V. The labeled cells were resuspended to approximately 10^10 cells per 250 μL in buffer with 1.2 mmol/L calcium for flow cytometric and microscopic analysis.

Magnetic cell separation. Magnetic beads (average size, 15 nm) covered with an anti-FITC antibody were supplied by Miltenyi Biotech Inc (Auburn, CA). The stock solution of beads was fivefold diluted in annexin V labeling buffer. Red cells labeled with FITC-annexin V were washed, and 6 × 10^7 cells were taken up in 80 μL of buffer. To the cell suspension, 20 μL of the diluted beads was added. After a 10-minute incubation at room temperature, the cells were separated in a magnetic separation setup (Minima; Miltenyi Biotec Inc) according to the standard protocol supplied by the manufacturer.

Flow cytometry. Samples were analyzed on a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA). Acquisition and data analysis were performed using LYSYS II software (Becton Dickinson). Ten thousand events per sample were acquired to ensure adequate mean fluorescence levels. The light scatter and fluorescence channels were set at a logarithmic gain. The forward angle light scatter setting was E-1 with a threshold of 36. The red
cell population was defined by size in forward and side scatter plots. Events that correlated with intact RBCs were analyzed for fluorescence intensity. Mean fluorescence intensities were expressed in linear mode, and positive fluorescence was defined by comparison with unlabeled control samples.

The treatment of RBCs with NEM followed by incubation with calcium and ionophore slightly changed the forward scatter characteristics of the population in the flow cytometer as compared with control RBCs (not shown). Gated regions were adjusted accordingly. This shift in characteristics can be expected because this treatment will result in a number of changes in the cell, including the severe alterations of morphology and loss of membrane material by vesiculation.23 As expected, RBCs do not show any appreciable fluorescence in fluorescence-activated cell sorter (FACS) analysis in the absence of an added fluorophore (not shown). Incubation of normal cells with fluorescently labeled anti-glycophorin A antibody (Becton Dickinson) showed a clear labeling of the population (Fig 1A). In contrast, these cells were not labeled by FITC-annexin V in the presence of 1.2 mmol/L calcium (Fig 1B). Treatment of RBCs with NEM followed by incubation with calcium and ionophore resulted in the binding of FITC-labeled annexin V to the cells (Fig 1D). A negative control, such as that shown in Fig 1B, was used to define background fluorescence for each assay. The population of cells significantly labeled with annexin V above background is indicated by marker M1 (Fig 1). For example, 98% of the cells as shown in Fig 1D meet this criterion.

Fluorescence microscopy. Cells were examined under 300 × optical magnification using a liquid N₂-cooled CCD camera as described.25 This system allowed accurate generation of digital images of cells at low fluorescence levels with a minimum of background noise. The microscope objective is used with a fluorescein filter set (excitation, 480 ± 15 nm; emission, 535 ± 20 nm). Bright fields (413 ± 5 nm) were exposed for 10 msec; fluorescent fields, for 600 msec.

Prothrombinase assay. The activation of prothrombin to thrombin is a measure of the amount of PS available to the prothrombinase complex.6 To measure the availability of PS in the outer monolayer of the membrane, RBCs were suspended in buffer to a final concentration of 10⁷/mL in Tris buffer (50 mmol/L Tris-HCl, 120 mmol/L NaCl, 6 mmol/L CaCl₂, pH 7.4). The mixture was preincubated at 37°C, and 0.33 U/mL of bovine factor V and bovine factor Xa (Enzyme Research Laboratories, South Lafayette, IN) were added, followed by 0.13 mg of bovine prothrombin (Enzyme Research Laboratories). EDTA (to a final concentration of 15 mmol/L) was added to stop the reaction after 4 minutes. The RBCs were pelleted by centrifugation, and the amount of thrombin formed was determined by adding an aliquot (150 μL) of the supernatant to 2 mL of a 100 μmol/L solution of the chromogenic thrombin substrate S-2238 (Chromogenix, Mölndal, Sweden). The increase in absorbance at 405 nm, 37°C, was determined in a Spectronic 3000 Diode Array Spectrometer (SLM Instruments, Rochester, NY) equipped with enzyme kinetics software. The conversion rate of the chromogenic thrombin substrate was used to determine the amount of thrombin formed.

RESULTS

A recent study demonstrated that radioactive annexin V can be used as a probe to detect red cells that have lost phospholipid asymmetry.13 However, neither this study nor any of the other methods used to date have been able to determine if loss of phospholipid asymmetry occurs in all cells or if it is localized to subpopulations of cells. Given the observation that activated platelets that contain PS on their surface can be identified and quantitated in vivo using fluorescently labeled annexin V,16 we determined if this method could also be used to identify subpopulations of red cells that had lost phospholipid asymmetry by flow cytometry (FACS). The red cell population was defined by size in

![Fig 1. Flow cytometric analysis of (A) RBCs incubated with fluorescently labeled anti-glycophorin A, (B) RBCs incubated with FITC-labeled annexin V, (C) RBCs incubated with FITC-labeled annexin V after treatment with NEM, and (D) RBCs incubated with FITC-labeled annexin V after treatment with NEM and subsequent incubation for 1 hour in 1 mmol/L calcium in the presence of 4 μmol/L ionophore A23187. RBCs were selected by their light scattering properties. The number of cells with fluorescence above background was defined by gate M1.](image-url)
forward and side scatter plots. Events that correlated with intact RBCs were analyzed for fluorescence intensity. RBCs do not show any appreciable fluorescence in FACS analysis in the absence of an added fluorophore (not shown). Incubation of normal red cells with fluorescently labeled anti-glycophorin A antibody (Becton Dickinson) showed a clear labeling of the population (Fig 1A). In contrast, these cells were not labeled by FITC-annexin V in the presence of 1.2 mmol/L calcium (Fig 1B). We hypothesized that loss of the asymmetric distribution of RBC membrane phospholipids would lead to the binding of FITC-annexin V to these RBCs. As a first step, to characterize the binding properties of annexin V to red cell membranes that have lost their asymmetric phospholipid distribution, we combined inhibition of the aminophospholipid translocase with acceleration of the movement of aminophospholipids from inner to outer monolayer. Treatment of RBCs with 10 mmol/L NEM, which blocks the activity of the aminophospholipid translocase, did not lead to labeling of the cells with annexin V (Fig 1C). Moreover, when NEM-treated cells were incubated for 20 hours at 37°C, only 2% of the cells labeled above background (not shown). Incubation of NEM-treated RBCs with ionophore in the absence of calcium also did not result in any annexin V binding (not shown). In contrast, treatment of RBCs with NEM followed by incubation with calcium and ionophore resulted in the binding of FITC-labeled annexin V to the cells (Fig 1D). These conditions were chosen as they had previously been reported to cause loss of phospholipid asymmetry. The frequency distribution curves of the flow cytometric data contain information on the number of cells labeled above background and average fluorescence per cell.

The presence of a subpopulation of annexin-binding cells due to their loss of normal phospholipid asymmetry, can be determined by comparison with control. As an example, the population of cells labeled with annexin V above background in Fig 1, indicated by the M1 marker, represents 98% of the cells.

As elevated intracellular calcium can inhibit the aminophospholipid translocase as well as scramble membrane phospholipids, we investigated the effects of calcium loading on phospholipid asymmetry over a 1-hour time period (Fig 2). The scrambling effect of calcium and ionophore on phospholipid asymmetry is a gradual process. At 1 hour, a plateau seems to be reached, as only a few more cells were labeled. Also, the average fluorescence of the cells did not significantly increase (not shown). Prolonged incubation, however, led to substantial hemolysis. Data from experiments described above with NEM-treated cells were obtained after 1 hour with calcium and ionophore. A substantial decrease of this time resulted in a lower level of annexin V binding.

To ensure optimal labeling of the cells with annexin V, we incubated the cells with different amounts of annexin V and calcium. The same sample of scrambled cells showed an increase in labeling dependent on the annexin V concentration (Fig 3). At the concentrations routinely used, a plateau is reached in the percentage of fluorescently labeled cells (Fig 3A). Moreover, the average fluorescence per cell did not increase, indicating that individual cells do not label more heavily with annexin V at higher concentrations. Maximum labeling was reached within 10 minutes after the addition of annexin V. Prolonged incubation with an excess of fluorescently labeled annexin V for up to 12 hours at 4°C did not change the labeling pattern. For practical reasons, a 30-minute incubation time was chosen to standardize labeling conditions and ensure optimal labeling. The binding of FITC-annexin V to scrambled cells was further monitored by measuring the fluorescence in the supernatant before and after the labeling incubation. Under routine conditions at 2 μmol/L FITC-annexin V, more than 95% of the label can be recovered in the medium after labeling of 2 × 10⁶ red cells (Fig 3B), indicating an excess of label. Only when the concentration of the cells is substantially increased can a decrease in free, unbound annexin V be observed. Based on the number of cells in the labeling mixture and the concentration of annexin V in the supernatant after labeling, an estimate can be made for the number of annexin V molecules

Fig 2. The binding of annexin V to cells incubated with calcium and ionophore A23187. RBCs were incubated in 1 mmol/L calcium in the presence of 4 μmol/L ionophore A23187. The percentage of cells that bind FITC-labeled annexin V is plotted against time.

Fig 3. Binding of FITC-labeled annexin V to RBCs, scrambled with NEM, calcium, and ionophore A23187, in relation to the concentration of FITC-labeled annexin V present. (A) The percentage of 2 × 10⁶ cells fluorescently labeled by incubation in 250 μL with various concentrations of annexin V. (B) Percentage of free FITC-labeled annexin V in the supernatant when either 2 × 10⁶ or 1.6 × 10⁶ RBCs were labeled in 250 μL in the presence of 1 μmol/L annexin V.
RBC PS ASYMMETRY AND FLUORESCENT ANNEXIN V

![Fluorescence-Scrambled Cells](image)

**Fig 4.** Flow cytometric analysis of FITC-labeled annexin V binding to a mixture of cells in which part of the population exhibited a scrambled membrane induced by treatment with NEM and incubation for 1 hour in 1 mmol/L calcium in the presence of 4 μmol/L ionophore A23187. (A) A typical analysis of such a population in which 10% of the cells showed fluorescence above background, as determined by the percentage of cells gated by M1. (B) Relationship between the percentage of the population treated with calcium and ionophore and the percentage of red cells that bind FITC-labeled annexin V (■), as determined by flow cytometry as indicated in panel A, or the prothrombin-converting activity (○) of the red cells expressed as percentage of thrombin formed per minute relative to scrambled cells incubated at 37°C in buffer containing calcium, purified bovine prothrombin, and factors V and Xa. Both data indicate the average and standard deviation of four separate experiments.

bound per cell. For the average cell after ionophore treatment, approximately 300,000 molecules of FITC-annexin V are bound per cell.

The concentration of calcium in the labeling buffer affected the binding of annexin V. Labeling was tested on fresh control RBCs as well as a cell population in which 94% of the cells could be labeled with annexin V under conditions as indicated in Fig 3 in the presence of 1.2 mmol/L calcium. At calcium concentrations below 0.5 mmol/L, the number of cells labeled with annexin V significantly decreased below 90% (not shown). From 1 to 2 mmol/L of calcium, the number of cells or the average fluorescence per cell did not increase. Also, up to 2 mmol/L calcium, no increase in the number of control RBCs that were labeled with annexin V was found.

To show that annexin V labeling could be used to selectively identify those RBCs in a population that have lost their normal phospholipid asymmetry, we mixed normal RBCs with NEM and calcium/ionophore-treated RBCs before the addition of fluorescent annexin V. Figure 4A shows a typical FACScan of a population in which 10% of the cells have a scrambled membrane phospholipid organization. A linear relationship was found between the fraction of scrambled cells in the mixture and the percentage identified as such by FACScan (Fig 4B). We found that subpopulations of abnormal cells as small as 0.1% of the total population were detectable with annexin V labeling.

To determine if annexin V binding results correlated with another measure of loss of phospholipid asymmetry, we compared the annexin V labeling of these cell mixtures with the activation of the prothrombinase complex, a well-known assay for the presence of PS in a membrane surface. In this case, PS was provided by the red cell membrane. More thrombin was formed as a function of the number of scrambled cells in the mixture (Fig 4B). This increase in prothrombinase activity indicates, independently of the annexin V labeling, the presence of PS in the outer monolayer of the red cell. An excellent correlation between the number of cells detected by annexin V in the mixture and the procoagulant activity of the mixture was observed. To illustrate the power of the annexin-based assay, the interpretation of the results using the procoagulant measurement would be that a 10% loss of asymmetry in the red cell mixture was noted. However, using fluorescently labeled annexin V, one can determine that 10% of the cells have completely lost asymmetry, while the rest are normal.

To confirm the labeling of red cells by annexin and demonstrate that labeling occurred on the surface of the cells, we analyzed the fluorescently labeled cells by fluorescence microscopy. A typical example is shown in Fig 5. In this case, a population in which 64% of the cells tested positive as the result of calcium and ionophore treatment (Fig 5A) showed a heterogeneous labeling by fluorescence microscopy (Fig 5B and C). A field was chosen in which an RBC ghost was present (indicated by arrow): the bright labeling of the ghost indicates that the inner monolayer was also available for labeling with annexin V. The remainder of the cells that bind annexin V appear to be labeled at their membrane surface. The number of cells detected by fluorescent microscopy is identical to that measured by flow cytometry.

We next determined if the red cells that had lost their phospholipid asymmetry could be isolated from the cells that
fluorescence had not undergone this rearrangement, using a preparation of small magnetic beads covered with an FITC antibody. A typical example is shown in Fig 6. Approximately 10% of the cells in this sample exhibited an abnormal phospholipid organization. Figure 6A shows the flow cytometric analysis of this sample after annexin V labeling and subsequent incubation for 10 minutes with FITC antibody-covered beads. The presence of these very small beads on the surface of the cells did not affect the flow cytometric analysis. The population was separated on a column placed in a magnetic field, according to standard protocols supplied with the beads (Miltenyi Biotec Inc). The approximately 10% fluorescent cells in the population labeled with annexin V and magnetic beads were retained on the column. A simple wash with buffer removed the rest (unlabeled portion) of the cells. Subsequently, the column was removed from the magnetic field and the annexin V-labeled cells, now no longer retained, were collected and analyzed by flow cytometry (Fig 6B). All these steps were performed in our annexin V labeling buffer containing 1.2 mmol/L calcium. When the purified subpopulation was washed in buffer without calcium, FITC-annexin V together with the beads was removed from the cells (Fig 6C). Subsequent incubation of cells in buffer containing 1.2 mmol/L calcium and FITC-labeled annexin V resulted in renewed labeling of the cells (Fig 6D).

Loss of phospholipid asymmetry has been implicated in sickle cell anemia. Although changes in membrane phospholipid organization can be induced in RBCs in vitro by deoxygenation, it has been difficult to show loss of phospholipid asymmetry in RBCs in vivo. Hence, we applied our new method to peripheral blood collected from patients with sickle cell disease and compared the results with normal control cells. In contrast with normal cells from eight volunteers, in which 0.21% of the cells labeled with annexin (Fig
phospholipid as compared with the total phospholipid pool in the outer monolayer is less than 1 mol outer monolayer, but the relative amount of this minor lipid in this monolayer. Of PI, 20% is also present in the presence of calcium.29 In the normal red cell, approximately 20% of all PE can be found in the outer monolayer, which also contains PE and phosphatidylinositol (PI), phospholipids that do bind annexin V in a vesicle system in the presence of calcium.28 This indicates that the outer monolayer of normal RBC, which lacks the presence of PS, does not bind annexin V. Research on the binding specificities of annexin V in vesicle systems has shown that SM and PC do not bind annexin V in the presence of calcium.29 Thus, the presence of SM and PC on the outer monolayer is not expected to contribute to annexin V binding. However, the outer monolayer also contains PE and phosphatidylinositol (PI), phospholipids that do bind annexin V in a vesicle system in the presence of calcium.28 In the normal red cell, approximately 20% of all PE can be found in the outer monolayer, which would account for approximately 11 mol% of all phospholipid in this monolayer. Of PI, 20% is also present in the outer monolayer,30 but the relative amount of this minor phospholipid as compared with the total phospholipid pool in the outer monolayer is less than 1 mol%. The lack of fluorescent labeling of normal cells indicates that normal levels (in the outer monolayer) of PE and PI do not act to bind annexin V in the presence of relatively low concentrations (1.2 mmol/L) of calcium used in our experiments.

With loss of asymmetric distribution of RBC membrane phospholipids, there is a significant increase in the exposure of PS on the outer monolayer, and binding of annexin V to these RBCs can be expected. Incubation of RBCs with NEM results in the inhibition of the aminophospholipid translocase,4 and additional treatment of RBCs with calcium and ionophore will lead to a scrambling of the normal membrane phospholipid organization by effectively accelerating the flop of aminophospholipids from inner to outer monolayer.10 When these cells were incubated with FITC-labeled annexin V, a very significant binding of annexin V to virtually all cells was observed (Fig 1D). Although these results do not prove that annexin V binds specifically to PS in human red cells, the excellent correlation noted between the binding of fluorescently labeled annexin V and the procoagulant properties of red cells in which phospholipid asymmetry was disrupted (Fig 4) supports an important role for PS in this process.

Our results clearly indicated that both impairment of the aminophospholipid translocase as well as an additional scrambling of the phospholipid organization was important for high levels of labeling with annexin V. The inhibition of the aminophospholipid translocase by NEM, without subsequent incubation in calcium and ionophore, did not result in labeling of RBC with annexin V (Fig 1C). Even prolonged incubations resulted in only low levels of annexin V binding. While virtually all cells incubated with calcium and ionophore were labeled after NEM pretreatment (Fig 1D), only 60% of the cells are labeled when this treatment was omitted (Fig 2). Aminophospholipid translocase activity is inhibited by increasing cytosolic calcium to 1 mmol/L,31,32 conditions that exist during calcium and ionophore treatment. Unlike NEM treatment, however, calcium-induced inhibition of the translocase seems to be at least partially reversible: when calcium and ionophore are removed by a wash with EDTA and BSA, restored translocase activity can be expected to reverse some or all of the scrambling that occurred during incubation, resulting in a (partial) restoration of the phospholipid organization. NEM treatment effectively prevents any reversal of membrane scrambling. Collectively, these data indicate that for maximal labeling of cells with annexin V, both an inhibited flip (deactivated aminophospholipid translocase) and increased flop are required.

The binding of approximately 300,000 molecules of FITC annexin V to cells, as deduced from the removal of FITC from the medium, compares favorably with similar data obtained using radioactively labeled annexin V.11 However, this number has to be treated cautiously, given the heterogeneity of the labeling, and is merely a rough approximation of the number of binding sites per cell. Experiments in which red cells with PS on their surface were mixed with normal red cells illustrated the power of FITC-labeled annexin V and flow cytometry to detect small, subpopulations of red cells with abnormal phospholipid asymmetry (Fig 4). This approach not only facilitates analy...
sis of very small red cell samples (10^6 cells or 0.1 μL of packed RBCs) but is capable of detecting loss of normal phospholipid organization in less than 1% of a red cell mixture. Individual cells that bind annexin V can be studied using fluorescence microscopy (Fig 5) and can be separated from the population using magnetic beads (Fig 6). The simple purification from the population combined with the removal of annexin V and beads from the cell surface by removing calcium from the buffer is a very gentle way to prepare these cells for further analysis.

For an accurate determination of membrane phospholipid organization, it is important that the probe used is able to discriminate between the outer and inner monolayer of the bilayer. Either membrane permeability to the probe of the intact cell or access to the inner monolayer after hemolysis will interfere with accurate assessment of phospholipid asymmetry. It was assumed in previous studies that annexin V, a 35-kD protein, only has access to the outer monolayer of intact cells, even after calcium and ionophore treatment. The lack of calcium entry and hemoglobin loss indirectly discriminate between the outer and inner monolayer of the bilayer. Either membrane permeability to the probe of the intact cell or access to the inner monolayer after hemolysis will interfere with accurate assessment of phospholipid asymmetry. It was assumed in previous studies that annexin V, a 35-kD protein, only has access to the outer monolayer of intact cells, even after calcium and ionophore treatment. The lack of calcium entry and hemoglobin loss indirectly seems to confirm this assumption, as it is very unlikely that annexin V would be able to enter the cell while calcium can not. More convincingly, however, we show that all cells that are fluorescently labeled with annexin V can be removed from the population with 15-nm magnetic beads covered with anti-FITC antibody. The remaining population does not show any fluorescence above background. Moreover, the positive population (Fig 6B) washed with buffer without calcium loses all fluorescence (Fig 6C). We believe it is very unlikely that these 15-nm beads could enter the cells to bind to FITC-annexin possibly present on the outer monolayer and that these large complexes composed of annexin V and beads would reside on the outer monolayer and be removed from the cell by a wash in calcium-free buffer. Hence, we conclude that FITC-annexin labels the outer monolayer of the RBCs. Collectively, our data indicate that even after the relatively harsh treatment of the cell with NEM, calcium, and ionophore, annexin V cannot enter the cell and label the inner monolayer. Hemolysis during annexin V labeling was very low, excluding the binding of annexin V to the exposed inner monolayer of RBC ghosts. Moreover, even the few ghosts formed were not analyzed by flow cytometry because they did not appear in the selected RBC window, and in fluorescent microscopy, they could easily be identified (Fig 5).

Loss of normal phospholipid asymmetry has been implicated in red cell pathology in diseases such as sickle cell anemia. However, it has been difficult to unambiguously show the loss of phospholipid asymmetry in vivo. Using radioactive annexin V, 20,000 annexin V binding sites were reported in sickle cell samples, as compared with 300,000 annexin V binding sites in these cells that had lost their phospholipid asymmetry due to calcium and ionophore treatment. This could be interpreted as an approximate 3% loss of phospholipid asymmetry in the average cell. Importantly, these results can also be interpreted such that 3% of the cells have lost their asymmetry. Radioactivity measurements do not allow a distinction between these two possibilities. However, the population can be studied in detail using fluorescent annexin V. Our study shows that in the population of sickle RBCs in vivo, subpopulations exist that bind annexin V (Fig 7). These data indicate that while some cells in the sickle cell population have lost their normal phospholipid asymmetry, other cells are normal in that respect. Furthermore, a variation between patients in the numbers of these cells was noted. Hence, these results show that the use of fluorescent annexin V is distinct from the use of radiolabeled annexin V or other techniques that assess phospholipid asymmetry of the average cell in the population, and allows the identification in blood of patients of small numbers of cells that circulate with an abnormal phospholipid asymmetry, possibly of physiologic importance.

In conclusion, our data show that fluorescently labeled annexin V binds to RBCs with an altered phospholipid organization. The use of flow cytometry and fluorescent microscopy allows the detection of altered membrane lipid organization in subpopulations or individual cells. This approach allows for the measurement of very small samples (10^6 cells or 0.1 μL of packed RBCs) and is capable of measuring the loss of normal phospholipid organization in less than 0.1% of that population, and FITC antibody-containing magnetic beads allow the purification of these cells.

The use of fluorescently labeled annexin V should be a powerful probe for identification of the loss of normal phospholipid organization and will be applicable to assessment of this important characteristic in normal and pathologic erythrocytes. The ability to define loss of normal phospholipid organization in subpopulations of cells by flow cytometry, or even individual cells by fluorescence microscopy, should be very useful in studies of red cell pathology, particularly because loss of normal phospholipid organization in a small subpopulation of pathologic RBCs may be of significant physiologic importance. The selection of these cells from the population based on their ability to bind annexin V will facilitate further studies of the mechanisms that underlie the loss of phospholipid asymmetry in these cells, as well as the potential pathophysiologic properties of these cells.

REFERENCES
Detection of altered membrane phospholipid asymmetry in subpopulations of human red blood cells using fluorescently labeled annexin V

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