

# Measurement of Phosphatidylserine Exposure in Leukocytes and Platelets by Whole-Blood Flow Cytometry with Annexin V

Submitted 07/12/99; revised 8/20/99

(communicated by Brian S. Bull, M.D., 08/27/99)

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**Abstract:** Phosphatidylserine (PS) exposure serves as a procoagulant stimulus and a signal for phagocytic clearance of apoptotic cells. In order to measure PS exposure in blood cells, we developed a flow-cytometric procedure to measure annexin V binding to leukocytes and platelets in whole-blood samples. Leukocytes were identified by CD45 and side-scatter gating, and platelets by CD61 and side-scatter gating. The absolute number of annexin V molecules bound per cell was determined from an independent calibration procedure. Normal populations had the following levels of annexin V binding (in molecules per cell): lymphocytes,  $0.53 \times 10^3$ ; neutrophils,  $1.75 \times 10^3$ ; monocytes,  $2.45 \times 10^3$ ; platelets,  $0.14 \times 10^3$ . These levels represent  $\leq 0.1\%$  of the values obtained after maximal stimulation of PS exposure with calcium ionophore, confirming that virtually all PS is intracellular in normal circulating leukocytes and platelets. Pretreatment of whole-blood samples with ammonium chloride to lyse erythrocytes caused a 9- to 300-fold increase in annexin V binding to leukocytes, indicating that analysis of unlysed whole-blood samples is essential to avoid artifactual increases in annexin V binding to leukocytes. Comparison of annexin V with two other markers of platelet activation, CD62P and the activation-dependent epitope of glycoprotein IIb/IIIa detected by the PAC1 antibody, indicated that platelets from normal donors showed the least amount of activation with the annexin V marker. Whole-blood flow cytometry with annexin V can reliably measure the state of PS exposure in platelets and leukocytes, and the results confirm that these cell types normally circulate with extremely low levels of exposed PS.

**Keywords:** phosphatidylserine, annexin V, flow cytometry, leukocytes, platelets

## INTRODUCTION

Phosphatidylserine (PS) is distributed asymmetrically in the plasma membrane in most cell types (1,2). Increased exposure of PS has long been recognized as an integral part of the normal platelet procoagulant response during blood coagulation (2), and the same event may occur as a procoagulant mechanism in endothelial cells (3) and vascular smooth muscle cells (4) under pathological conditions. More recently, exposure of PS has been identified as an early event during apoptosis of several cell types, which may serve as a signal for their phagocytic clearance (5-9).

The phospholipid binding protein annexin V binds with very high affinity to bilayers of anionic phospholipids (10-13). It has proved to be very useful to measure cellular PS exposure both in vitro and in vivo (8,14-19). Many studies have been reported for a variety of cultured cells, or for primary cells after isolation from blood or tissue. However, methods for direct measurement of annexin V binding to peripheral-blood cells have so far been reported only for erythrocytes (17,20,21) and platelets (22). In order to assess the possible contribution of PS exposure to thrombotic disorders and the clearance of apoptotic cells in disease states, non-perturbing

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methods for measurement of annexin V binding to leukocyte and platelet populations are required.

The purpose of this study was to develop reliable methods for direct flow-cytometric measurement of the state of PS exposure in the leukocyte and platelet populations of peripheral blood. We have determined the level of PS exposure in leukocytes and platelets of normal donors. We have also developed a procedure to determine the number of annexin V molecules bound per cell in flow-cytometric measurements, which will facilitate comparison of results between different cell types and different laboratories.

## MATERIALS AND METHODS

### *Reagents, Cell Lines and Buffers*

Monoclonal CD45 antibody (clone J33) labeled with PE-Cy5, fluorescent microspheres (Flow-Set) and a stabilized human whole-blood control (4C Plus normal control) were purchased from Beckman-Coulter (Hialeah, FL). Recombinant human annexin V was prepared (21) and labeled with FITC as described (10). Calcium ionophore A23187 was from Calbiochem (La Jolla, CA). Buffer HNKGB consisted of 10 mM HEPES-Na pH 7.4, 133 mM NaCl, 5.8 mM KCl, 5 mM glucose. Buffer HNKGB consisted of buffer HNKGB plus 1 mg/ml BSA (fatty acid free, Sigma A2030). Buffer HNKGB consisted of buffer HNKGB plus 2.5 mM CaCl<sub>2</sub>.

### *Analysis of Leukocytes in Whole Blood*

Blood was drawn by venipuncture from healthy adult donors under conditions that minimized possible platelet activation. A 21-gauge needle was inserted, the tourniquet released, and 5 ml of blood drawn and discarded. A 5-ml blood specimen was then drawn directly into one-tenth volume of 109 mM sodium citrate pH 6.5, 1 μM prostaglandin E<sub>1</sub>. The assay was then set up in 12 × 75 mm polypropylene tubes in HNKGB buffer. FITC-annexin V was added to a final concentration of 100 nM, and anti-CD45-

PE-Cy5 was added to a final dilution of 1:40 from the manufacturer's stock solution. Whole blood (40 μl) was then added to give a final assay volume of 800 μl. After a 15-min incubation on ice, the sample was washed twice by centrifugation (5 min at 500 × g) in 400 μl of HNKGB buffer, resuspended in 2000 μl of HNKGB buffer, and kept on ice until analyzed. On the flow cytometer lymphocytes, monocytes and neutrophils were separated by gating on CD45-PE-Cy5 and side scatter. A minimum of 700 events were collected for monocytes, and a minimum of 2000 events for lymphocytes and neutrophils. Coincident-cell events were excluded by gating on the ratio of peak forward-scatter intensity to integrated forward-scatter intensity. For determination of molecules of FITC-annexin V per cell, appropriate blanks were subtracted to remove the contribution of cellular autofluorescence.

### *Analysis of Platelets in Whole Blood*

Blood was drawn as described above, and blood samples were analyzed within 45 min of phlebotomy. For annexin V measurements, duplicate 12 × 75 mm polypropylene tubes were prepared at room temperature to a total volume of 85 μl containing: 5 μl of whole blood, 49 μl of assay buffer HNKGB, 2.5 μl of 85 mM CaCl<sub>2</sub>, ± 2.5 μl 170 mM EDTA (as a negative control to prevent annexin V binding), 8.5 μl of 1 μM FITC-annexin V, and 20 μl of anti-CD61-PerCP (Becton-Dickinson). After gentle mixing, the sample was incubated for 15 min at room temperature in the dark and then diluted to 1 ml with HNKGB. Flow cytometry was then performed with side-scatter and CD61 gates set to include only individual platelets. A minimum of 5000 events were collected. Tubes were prepared in the same fashion with FITC-PAC1 and phycoerythrin-anti-CD62P (both from Becton-Dickinson), except calcium was omitted and the negative control consisted of a tube with 0.6 mg/ml peptide Arg-Gly-Asp-Ser (for PAC1) and an irrelevant phycoerythrin-labeled antibody (for

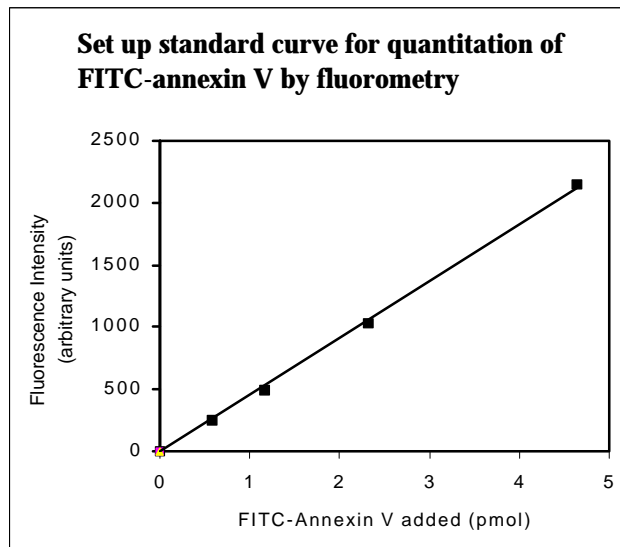
anti-CD62P). For comparative analysis of the three markers, the threshold for marker-positive platelets was set individually for each patient sample to exclude 99% of the events that occurred in the corresponding negative-control tube.

#### *Analysis of Leukocytes after Erythrocyte Lysis*

Blood was drawn by venipuncture into evacuated tubes containing EDTA anticoagulant. The erythrocytes were lysed by adding 0.5 ml of blood to 13.5 ml of 0.15 M  $\text{NH}_4\text{Cl}$ , 0.01 M  $\text{NaHCO}_3$ , 1 mM EDTA and incubating for 5 min at room temperature. The sample was then centrifuged for 5 min at  $800 \times g$ , washed once with 14 ml of room temperature phosphate-buffered saline, and once with 14 ml of room temperature HNKGB buffer. The washed white blood cells were then resuspended in 0.5 ml of HNKGB buffer. The assay was performed on  $3 \times 10^5$ – $1 \times 10^6$  white blood cells in a total volume of 200–600  $\mu\text{l}$  as described above. Stimulation with A23187 was performed by adding 5  $\mu\text{M}$  A23187 during the 15-min assay incubation step.

#### *Determination of Molecules of FITC-Annexin V Bound per Cell*

The flow-cytometric measurements of mean fluorescence intensity were converted to molecules of FITC-annexin V bound per cell by the following method (Figure 1). First, a stock solution of FITC-annexin V was quantitated by colorimetric protein assay (BCA Assay, Pierce Chemical, Rockford, IL) using BSA as standard. The FITC-annexin V was then serially diluted in HNKGB plus 5 mM EDTA and a standard curve of fluorescence intensity versus FITC-annexin V concentration was measured on a fluorometer (TDx Analyzer, Abbott Laboratories, North Chicago, IL) over the range 0.5–4 pmol/ml (Figure 1, inset). Second, assay tubes were prepared with 1 ml of HNKGB;  $2 \times 10^7$  fixed human red blood cells (4CPlus normal control); and 0, 4, 10, 50, or 100 nM FITC-annexin V. After a 15-min incubation on ice, the cells were



**Set up cell binding assay over a range of concentrations of FITC-annexin V to give different levels of bound FITC-annexin V**

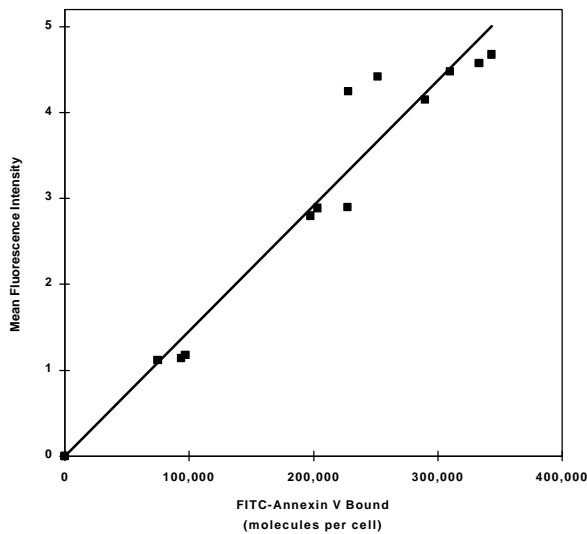
**Elute bound FITC-annexin V and quantitate by fluorometry**  
**Quantitate cell number by cell counting**  
**Calculate molecules of FITC-annexin V bound per cell**

**Measure parallel set of samples on flow cytometer to determine mean fluorescence intensity of bound FITC-annexin V**

**Determine relationship between mean fluorescence intensity on flow cytometer and molecules of FITC-annexin V bound per cell**

**Figure 1.** Flowchart for method to determine molecules of FITC-annexin V per cell. The inset shows a representative standard curve for the quantitation of FITC-annexin V by fluorometry in Step 1 of the procedure.

centrifuged and the supernatant discarded; the cells were resuspended in buffer HNKGB, centrifuged, and the supernatant discarded. The



**Figure 2.** Determination of molecules of annexin V bound per cell. Measurements were made on erythrocytes with exposed PS incubated with different amounts of FITC-annexin V as described under Methods. The ordinate gives the mean fluorescence of each sample as determined by flow cytometry, and the abscissa gives the number of annexin V molecules bound per cell as determined by fluorometry and cell counting. The line was fitted by linear regression analysis, yielding a correlation coefficient of 0.96.

cells were resuspended in HNKGBC and this suspension was divided into two equal aliquots. Third, one of the two aliquots was centrifuged, the supernatant discarded, and the cells resuspended in HNKGB plus 5 mM EDTA to release the bound FITC-annexin V. The cells were removed by centrifugation and the amount of released FITC-annexin V in the supernate was measured on the fluorometer. The number of cells present was determined by resuspending the cells in buffer HNKGBC and counting them with a hemacytometer or an automated cell counter. Fourth, the other aliquot of cells from Step 2 was resuspended in HNKGBC and analyzed on the flow cytometer to determine the mean fluorescence intensity due to bound FITC-annexin V. Fifth, the relationship between mean fluorescence intensity and molecules of FITC-annexin V bound per cell was determined by linear-regression analysis (Figure 2).

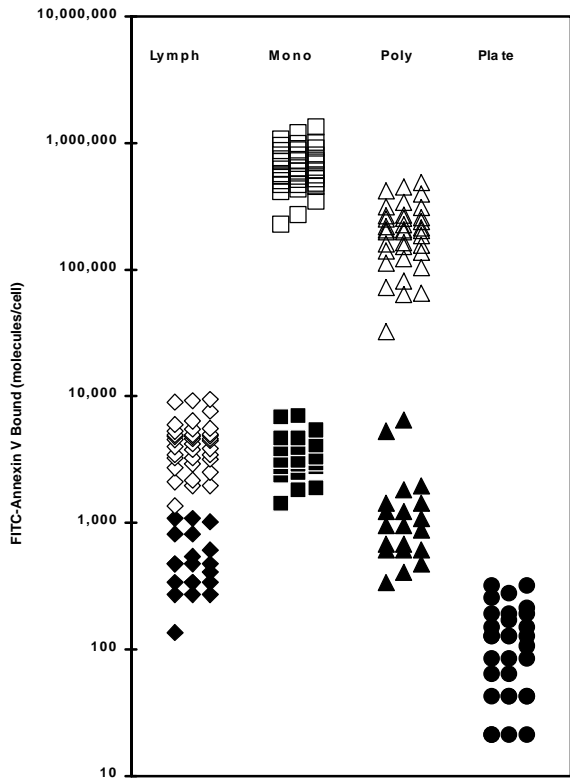
### *Standardization of Flow Cytometer*

All measurements were made on a Coulter Epics model XL-MCL flow cytometer. Color compensation settings were determined by running positive control samples with a range of concentrations of antiCD45-PE-Cy5 or FITC-annexin V, and then determining the average spillover into each fluorescence channel. Each day the fluidic and optical systems were evaluated by analysis of Flow-Check fluorospheres (Coulter) and half peak coefficient of variation values were verified to be <2% for each fluorescence channel, <2% for forward scatter and <6% for side scatter. A stabilized human blood control (4C Plus normal control) with annexin-positive red blood cells was used as a positive control for the assay; it was assayed under the same conditions as the white blood cells except that CD45-PE-Cy5 was omitted and  $4 \times 10^6$  red blood cells were added. The between-day coefficient of variation of the mean fluorescence intensity for this control was 11.6% ( $n = 52$ ).

## RESULTS

### *Determination of Molecules of Annexin V Bound per Cell*

In order to allow quantitation of the amount of bound annexin V, a calibration procedure was developed to convert the observed fluorescence intensity measurements to molecules of FITC-annexin V bound per cell. Figure 2 shows a representative standard curve as determined by this method. Three independent experiments gave an average conversion factor of  $67,900 \pm 5,700$  molecules of FITC-annexin V per cell per unit of mean fluorescence intensity for the settings used to analyze leukocytes; the corresponding value for the analysis of platelets was 3.0-fold lower due to the higher gain values used on the flow cytometer.



**Figure 3.** Normal range of annexin V binding for leukocytes and platelets. Measurements were made on samples from normal donors using the whole-blood assay (closed symbols) or the ammonium-chloride lysis assay (open symbols). Results are expressed as molecules of annexin V bound per cell. Symbols: diamonds, lymphocytes; squares, monocytes; triangles, granulocytes; circles, platelets.

### *Normal Ranges for Leukocyte Populations and Platelets*

Measurements were performed on twenty normal donors with the whole-blood assay (Figure 3). Lymphocytes showed the lowest level of annexin V binding (mean of 530 sites/cell); neutrophils showed a slighter higher level (1750 sites/cell); and monocytes showed the highest level (2450 sites/cell). Measurements were also made on twenty-nine normal donors to determine the normal range for platelet binding of annexin V; a mean value of 140 annexin V molecules per

cell was determined for this population. To determine if valid measurements could also be made on white cells isolated after removal of erythrocytes, blood samples were subjected to lysis with ammonium chloride as described under Methods. However, this treatment greatly increased the amount of annexin V binding: for lymphocytes, the mean number of annexin V molecules per cell was 4500; for neutrophils, 213,000; and for monocytes, 676,000.

### *Determination of Maximal Levels of Annexin V Binding*

To determine the maximum binding of annexin V after complete loss of membrane PS asymmetry, cells isolated by the ammonium-chloride method were treated briefly with calcium ionophore A23187 (23) to activate the phospholipid scramblase (Table 1). A23187-stimulated lymphocytes bound an average of  $1.7 \times 10^6$  molecules per cell; neutrophils an average of  $1.3 \times 10^6$ ; and monocytes an average of  $3.8 \times 10^6$  molecules per cell.

### *Comparison with Other Markers of Platelet Activation*

Annexin V was also compared with two other commonly used markers of platelet activation, CD62P and the PAC1 antibody (24) (Figure 4). For this comparison, data were analyzed by setting the threshold for a marker-positive platelet at a level that excluded 99% of platelets for that donor when assayed with an appropriate negative control (i.e., EDTA for annexin V, Arg-Gly-Asp-Ser peptide for PAC1, and an irrelevant antibody for anti-CD62P). Using these criteria, annexin V showed the lowest percentage of activated platelets, with only 3% of donors exceeding a threshold of 5% marker-positive platelets. In contrast, 10% of donors exceeded this threshold for CD62P, and 70% exceeded it for PAC1.

**Table 1.** Annexin V Binding to Resting and A23187-Stimulated Cells

Cell type	Mean number of annexin V molecules per cell		Ratio resting cells/stimulated cells (%)
	Resting cells	Cells stimulated with calcium ionophore A23187	
Lymphocytes	$0.53 \pm 0.29 \times 10^3$	$1.72 \pm 0.35 \times 10^6$	0.03%
Neutrophils	$1.75 \pm 1.57 \times 10^3$	$1.25 \pm 0.16 \times 10^6$	0.14%
Monocytes	$2.45 \pm 1.99 \times 10^3$	$3.76 \pm 1.03 \times 10^6$	0.07%
Platelets	$0.14 \pm 0.09 \times 10^3$	$1.13 \pm 0.28 \times 10^6$	0.01%

*Note.* Measurements were performed as described under Methods. For resting leukocytes, results are mean values of data given in Figure 3 (for 20 donors); for ionophore-stimulated leukocytes, n = 6. For resting platelets, results are mean values of data given in Figure 3 (for 29 donors); for ionophore-stimulated platelets, n = 5.

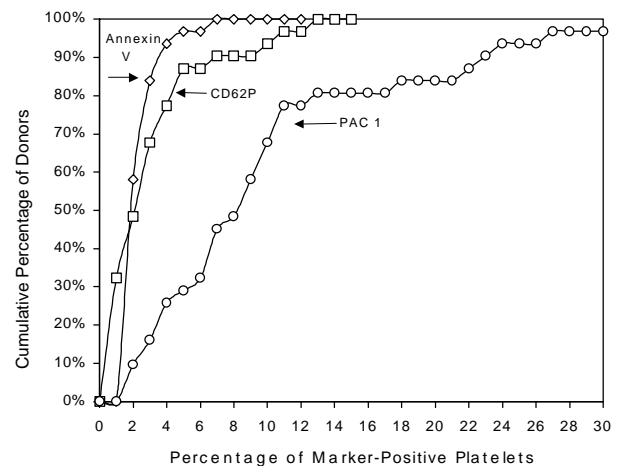
**DISCUSSION**

In this study we describe methods suitable for direct flow-cytometric measurement of the level of PS exposure in peripheral-blood leukocytes and platelets. In order to obtain the most reliable measurement of the in vivo state of these cells, we gave careful attention to variables that might cause artifactual ex vivo exposure of PS. The results show that the basal level of PS exposure in normal leukocytes is very low, ranging from about 0.03% of maximal in lymphocytes to about 0.1% of maximal in monocytes and neutrophils. Thus, it appears that all three cell types normally circulate in blood with minimal levels of exposed PS.

Similar results were obtained for platelets. The average level of PS exposure in normal donors was minuscule compared to the levels obtained after stimulation with calcium ionophore. The results obtained with annexin V are in some contrast to those obtained with two other widely used markers of platelet activation. Simultaneous assays on the same samples with anti-CD62P and PAC1 showed higher percentages of marker-positive platelets. Thus, annexin V may be a more desirable marker for clinical studies of activated platelets, since it may be less susceptible to artifactual elevation due to minor variations in sample handling and assay procedures.

It is advantageous to remove erythrocytes prior to analysis of leukocyte populations because this greatly decreases the required analysis time

on the flow cytometer. We therefore tested whether valid measurements could be made on leukocytes obtained after lysis of erythrocytes with ammonium chloride. Unfortunately, this procedure increased the amount of annexin V binding for all three leukocyte populations. The effect was modest for lymphocytes, and much more marked for neutrophils and monocytes. Thus, all three cell types are susceptible to artifactual increases in PS exposure due to in vitro handling, and reliable measurements will require direct analysis of whole-blood samples.



**Figure 4.** Comparison of annexin V with CD62P and PAC1 as markers of platelet activation. For each donor and each marker, the threshold defining an activated platelet was determined individually with a corresponding reagent blank as described under Methods. For each of the three markers, results are given as the cumulative frequency of activated platelets for a total of 29 normal donors. Symbols: diamonds, annexin V; squares, CD62P; circles, PAC1.

Development of a method to determine the number of annexin V molecules bound per cell is a novel feature of this study. This method is relatively straightforward to implement and will allow systematic comparison of studies over time and between laboratories. As a source of cells, we used a commercially available preparation of stabilized blood in which the erythrocytes have a high level of exposed PS. This preparation is convenient because it retains the same level of annexin V binding for many months. However, one could also use a cultured cell line that has a high level of exposed PS, such as the Jurkat T-cell line treated with calcium ionophore (23).

In conclusion, this study, in combination with previous studies of erythrocytes (17,20,21) shows that the level of PS exposure is very low in all the major cell populations that circulate in the blood of normal people.

## ACKNOWLEDGMENTS

We thank the staff of the Hematopathology laboratory for assistance with flow cytometry, and Donald Gibson for preparation of FITC-annexin V.

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