Biochemical and Biophysical Research Communications **258,** 199–203 (1999)
Article ID bbrc.1999.0620, available online at http://www.idealibrary.com on IDEAL®

Detection of Phosphatidylserine Surface Exposure on Human Erythrocytes Using Annexin V-Ferrofluid

Danielle Geldwerth,*[,1] Dominique Helley,† Kitty de Jong,‡ Domagoj Sabolovic',§ Claude Sestier,§ Jacky Roger,¶ Jean-Noel Pons,¶ Jean-Marie Freyssinet,‖ Philippe F. Devaux,* and Frans A. Kuypers‡

**IBPC, 13 rue Pierre et Marie Curie, 75005 Paris, France; †Laboratoire de Recherche sur l'Hémostase et la Thrombose, Faculté de Médecine Xavier Bichat, BP 416, 75870 Paris cedex 18, France; ‡Children's Hospital Oakland Research Institute, 747 52nd Street, Oakland, California 94609; §INSERM U511, CHU Pitié-Salpêtrière, 91 bd de l'Hôpital, Paris cedex 13, France; ¶Laboratoire des Liquides Ioniques et Interfaces Chargés, Université Pierre et Marie Curie, 4 place Jussieu, 75252 Paris cedex 05, France; and ‖Institut d'Hématologie et d'Immunologie, Faculté de Médecine, 4 rue Kirschleger, 67085 Strasbourg, France*

Received March 29, 1999

The asymmetric transbilayer distribution of phospholipids in the plasma membrane and the regulation of phosphatidylserine (PS) exposure at the cell surface of animal cells are of high physiological significance. It has been shown previously that annexin V is one of the most sensitive tools with which the presence of small amounts of PS on the outer surface of eukaryotic cells can be detected. We present here the covalent coupling of annexin V molecules to magnetic nanoparticles of maghemite. The resulting annexin V-ferrofluid is used in the magnetic separation of PS exposing cells, as illustrated for human erythrocytes modified in their phospholipid transbilayer asymmetry by the use of a calcium ionophore. Results on stored human erythrocytes and comparison with results obtained using iodinated and fluorescein-labeled annexin V are also presented.

The asymmetric distribution of membrane phospholipids in eukaryotic cells was first described more than two decades ago (1). It has been shown in particular that in the normal state, PS is localized exclusively in the cytosolic leaflet of the plasma membrane. The loss of phospholipid asymmetry on the cell outer surface is associated with many physiological and pathological phenomena (2-5). For example in the case of aged cells or during the course of apoptosis phosphatidylserine (PS) appears on the outer leaflet. The exposure of PS on the outer surface of platelets is also a fundamental step during the process of blood coagulation. An important effort has been carried out to identify the various molecular mechanisms involved in the maintenance and loss of this asymmetry. Understanding the mechanisms that govern membrane lipid sidedness, as well as the disease states in which unwanted PS exposure or lack of PS exposure is observed, relies on the availability of specific and sensitive tools for the detection of PS at the membrane surface. The calcium-dependent binding of annexin V to PS containing membranes (6) has been widely used, and several derivatives of the annexin V molecule, that is fluorescent, radioactive or biotinylated (7-9), have been developed in the last years. We describe in this paper a new annexin V derivative, annexin V-ferrofluid (AnxV-FF), and the use of this probe, in which annexin V molecules are covalently linked to magnetic nanoparticles, for the detection of cell surface exposure of PS. Preliminary reports were given in ref 13 and 14.

[1] To whom correspondence should be addressed. Fax: 00 33 1 40 51 83 17. E-mail: geldwerth@ibpc.fr.

MATERIALS AND METHODS

Material. Human annexin V was either purified from human placenta as described by Funakoshi *et al.* (10), from a recombinant *E. coli* strain (11) or purchased from Bender (Wien, Austria). Human erythrocytes were obtained from the Centre National de la Transfusion Sanguine and stored at 4°C as globular concentrates in SAG-mannitol (SAG-M) (anhydrous NaCl: 8.77 g/l, anhydrous adenine base: 0.211 g/l, monohydrated glucose: 11.25 g/l, mannitol: 6.56 g/l).

Calcium and ionophore treatment of erythrocytes. Calcium and A 23187 ionophore treatment of erythrocytes was as described in reference 11.

Binding of erythrocytes to iodinated annexin V. Annexin V purified from human placenta was iodinated to a specific radioactivity of 2500 cpm/ng using the Iodogen method as described in reference (8). Red cells were incubated in 10 mM Hepes, 70 mM NaCl and 70 mM KCl, pH 7.4 with varying amounts of iodinated annexin V (125I-AnxV) up to 100 nM total annexin concentration, in the presence of 2.5 mM $CaCl_2$ and 0.1% BSA. The final hematocrit was between 1 and 11% depending on the sample. Red cells and unbound radioac-

0006-291X/99 $30.00

tive annexin V were separated by centrifugation through a silicone oil layer, as described elsewhere (12). Non-specific labeling was measured with the same conditions, except for the absence of calcium and presence of 0.5 mM EDTA, and deducted from the binding observed in the presence of calcium. Fitting the experimental saturation curves allowed an estimation of the average number of 125I-AnxV specific sites per cell and their corresponding affinity.

Binding of erythrocytes to FITC-labelled annexin V. Fluorescein isothiocyanate (FITC) labeling of recombinant human annexin V was as described in Kuypers *et al.* (11). Binding of erythrocytes to FITC-labelled annexin (FITC-AnxV), and flow cytometric analysis of the labeled population were as reported (11).

Synthesis of annexin V-ferrofluid. AnxV-FF was prepared by covalently linking annexin V molecules to nanoparticles of maghemite, as schematically illustrated in Fig. 1 (14). The magnetic nanoparticles were synthesized as described previously (15). Briefly, this involves coprecipitating a mixture of ferrous and ferric chlorides in an ammoniacal solution, and oxidation of the resulting magnetite with ferric nitrate at 90°C to yield maghemite (γFe_2O_3). A cationic ferrofluid with an isoelectric point at pH 7.5 is obtained by dispersing the particles in nitric acid. The formation of a complex with meso 2,3-dimercaptosuccinic acid (DMSA) in an acidic medium, followed by peptization of the flocculate in an alkaline medium and neutralization, yields to a ferrofluid that is stable at physiological pH and ionic conditions (16). Binding of annexin V molecules, directly to the particle core through DMSA via a S-S bridge, using the heterobifunctional reagent N-succinimidyl 3-(2pyridyl dithio)propionate (SPDP), is followed by masking excess reactive groups at the particle surface. The molar ratio is one annexin V molecule per nanoparticle.

Erythrocytes binding to annexin V-ferrofluid. Red cells were incubated with excess AnxV-FF in Tris-buffered saline pH 7.4, containing 2.5 mM $CaCl_2$ and 0.1% bovine serum albumin (BSA) (final hematocrit 0.3%; 3 10^{15} particles/ml). The incubation mixture was diluted and passed through a tubing (external and internal diameter respectively 4 and 2 mm; length: 80 cm) in a magnetic field (magnetic field 1000G; field gradient 100 G/mm) at a flow rate of 1.2 cm^3 mn^{-1} to separate annexin-bound cells from non-retained cells. Cellular counting of the initial unfractionated, bound and non-retained populations was performed under a microscope, and yields to the percentage of annexin V binding cells in the population. Under these flow conditions, no AnxV-FF binding is observed when $CaCl_2$ is omitted from the washing solution. A low percentage of normal erythrocytes is retained on AnxV-FF in the presence of calcium chloride at 2.5 mM: an average of 10.7 +/− 5.9% was measured for a total of 13 independant samples of normal erythrocytes assayed up to three days after collection (13).

Measurement of erythrocyte hemolysis. Erythrocyte hemolysis during storage was measured by comparing the amount of hemoglobin recovered in the first low speed centrifugation supernatant to its total amount in the sample. Hemoglobin concentration was estimated from optical density at 405 nm.

RESULTS AND DISCUSSION

Magnetic Sorting of Erythrocytes That Bind Annexin V-Ferrofluid

Physical separation of the erythrocytes binding annexin V-bearing nanoparticles relies on the sequestration of the cells in a plastic tubing under flow conditions in the presence of a magnetic field gradient. Several parameters influence the conditions of cell sequestration, such as the intensity of the magnetic field gradient, the diameter of the tubing, the flow velocity and the size of the cells submitted to sorting. The

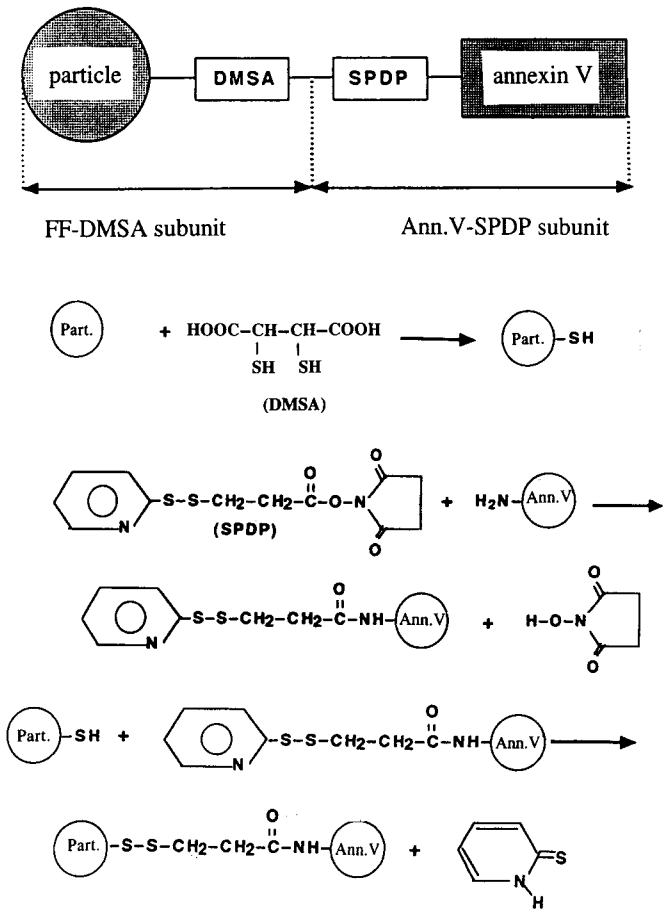

FIG. 1. Schematic representation of the AnxV-FF complex and reactions involved in the coupling of the annexin V molecules to the maghemite particle cores. DMSA: meso 2,3-dimercaptosuccinic acid; SPDP: *N*-succinimidyl 3-(2pyridyldithio)propionate; Part.: magnemite particle; Ann.V: annexin V.

annexin V-bearing nanoparticles or annexin-ferrofluid have an average size between 3 and 12 nm as measured by Electron Microscopy, and hence are too small to be by themselves retained under the magnetic field in our conditions. But when they bind to much larger objects, as erythrocytes or cells, the magnetic and inertia parameters of the nanoparticles-cell complexes allow their sequestration in the tubing, while both unbound particles and cells are washed out in the flow. Cells binding to annexin V-bearing nanoparticles, called positive cells or cells retained on annexin-ferrofluid, are recovered by simply washing the tubing while removing the magnetic field gradient. When the interactions between the magnetic particles and the cells are too weak, the cells do not get retained in the tubing under our flow conditions. This is particularly the case when calcium is absent from the medium used for the physical separation of erythrocytes after their incubation with calcium and AnxV-FF. But with the presence of calcium in the washing buffer, the affinity

of annexin V molecules to PS containing membranes and the strength of the covalent binding between annexin V molecules and the Fe atoms of the maghemite nanoparticles, are high enough to retain a significant fraction of human erythrocytes. In contrast with iodinated or fluorescent annexin V derivatives, no subtraction of non-specific binding, ie occuring in the absence of calcium, is required to quantify the binding capacity of a given erythrocyte population to the AnxV-FF. We have been able to measure such fractions from normal as well as pathological human erythrocytes with a good reproducibility having less than 10% variation for a given sample. For the present time, 5 10^6 erythrocytes are easily sorted in a few minutes using our installation. This makes the ferrofluid-based technology quite competitive compared to cellular sorting on Fluorescent Activated Cell Sorters (FACS), that cannot sort more than 5000 cells per second.

Recognition of PS Cell Surface Exposure by Annexin V-Ferrofluid

In a preliminary report high binding of AnxV-FF to erythrocytes from patients with sickle cell anemia was measured (13). This result was in agreement with previous observations of enhanced PS exposure on these cells (11, 17, 18), and indicated that AnxV-FF recognizes and binds to PS containing membranes. To demonstrate at a more quantitative level that AnxV-FF can be used in the detection of PS exposure at the surface of erythrocytes, we prepared samples containing variable proportions of control erythrocytes and calcium/ionophore-treated erythrocytes. Calcium influx is known to destroy the normal transbilayer asymmetry of phospholipids in the erythrocyte membrane, and leads to a random distribution of PS between both leaflets with exposure of this phospholipid at the cell surface (11, 19). Normal fresh erythrocytes were submitted to calcium and ionophore treatment, long enough to ensure maximal PS exposure, as checked both by FITC-AnxV FACS analysis and measurement of prothrombinase activity. After removal of the calcium ionophore, such cells were mixed to normal untreated erythrocytes in various proportions and submitted to AnxV-FF binding and magnetic separation in the presence of calcium. 16% of untreated cells used in this experiment bound to AnxV-FF. As illustrated in Fig. 2, the fraction of cells retained on AnxV-FF in the various mixtures, after deduction from the corresponding part of normal untreated erythrocytes, equals the fraction of calcium/ionophore-treated erythrocytes in the mixtures, which validates the separation of the PS exhibiting cells in the magnetic sorting as the fraction of cells retained on AnxV-FF.

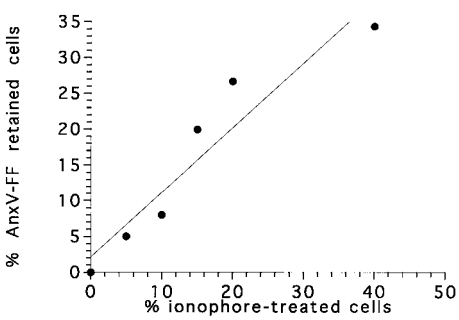

FIG. 2. AnxV-FF binding to mixtures of fresh and ionophore-treated erythrocytes. Ionophore A23187 and calcium treatment of erythrocytes and incubation with AnxV-FF were as indicated under Materials and Methods. Abscissa: proportion of ionophore-treated erythrocytes in the sample; ordinate: percent of cells retained on AnxV-FF.

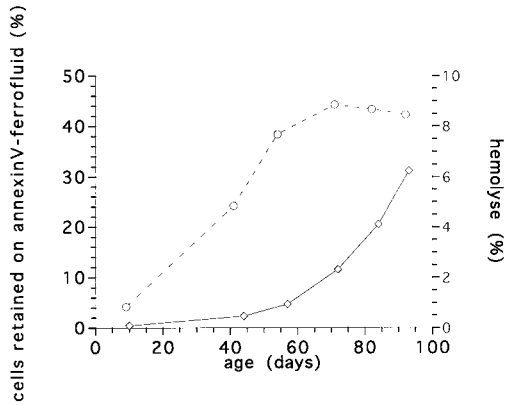

FIG. 3. Evolution of cellular hemolysis and binding to AnxV-FF during storage. Erythrocytes from a given donor were stored under blood bank conditions in SAG-M and periodically assayed over a three month period, as indicated under Materials and Methods. Abscissa: period of storage; ordinate: percent of cells retained on AnxV-FF (empty circles); cellular hemolysis (hollow diamonds).

Stored Erythrocytes

Blood storage leads to increasing PS exposure at the erythrocyte surface, as illustrated by binding to iodinated annexin V (12). Erythrocytes are collected and stored in blood banks as globular concentrates in SAG-M at 4°C up to a legal period of 45 days in France. Binding to iodinated, FITC-labelled annexin V and AnxV-FF were tested for various samples at different times of storage. As shown in Fig. 3, the fraction of cells retained on Anx-FF increases during storage, and reaches a plateau of 45% after two months of storage, when hemolysis increases beyond 1%. Labeling with iodinated annexin V shows an increase in exposure of binding sites on the red cells during prolonged storage, as presented in Fig. 4. This increase is less important than reported by Tait and Gibson (12), likely due to the absence of calcium in our storage medium. This PS exposure parallels the increase in osmotic fragility.

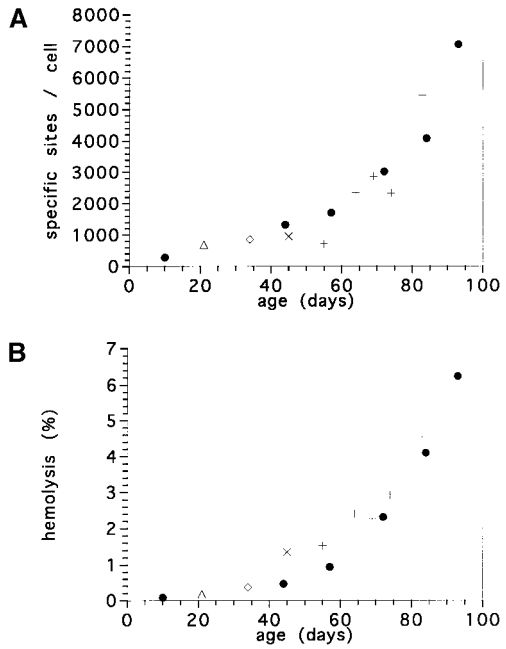

FIG. 4. Evolution of cellular hemolysis and binding to iodinated annexin V during storage. Data obtained from a given donor are represented by the same symbol (plain circle refers to samples from the donor presented in Fig. 3).

The corresponding binding sites to iodinated annexin nevertheless correspond to PS sites on the remnant cells: although vesicles produced during storage do exhibit prothrombinase activity and binding to annexin V (data not shown), the corresponding sites are not taken into account in our estimation because the vesicles do not cross the silicone oil layer used for the separation of bound and free iodinated annexin in our binding experiments. The nature of PS containing sites appearing on the erythrocyte surface during storage, as detected by iodinated annexin, does not vary over a three month period nor is dependent on the blood sample tested, and the corresponding K_d averages 18 ± 8 nM for 14 measurements. Only a very small fraction of these stored cells, less than 1% up to three months of storage, bind to FITC-labelled annexin V. Interestingly, some of our SAG-M erythrocyte concentrates, obtained during the legal period of storage from the blood bank, exhibited high AnxV-FF binding level, although they did not appear different from their counterparts on the basis of binding to iodinated or FITC-labelled annexin V. The fact that these samples also promoted high prothrombinase activity strongly favors the hypothesis that they indeed correspond to PS containing sites. Thus, it may well be that various PS containing sites exist at the surface of human erythrocytes, not all of them being detected similarly by the different annexin V derivatives.

Several studies carried out on artificial liposomes indicates that, although PS is preferentially detected by annexin V molecules, the binding to other phospholipids can not be ignored, especially at higher calcium concentration such as the 2.5 mM final concentration used in this study (6). In cellular plasma membranes, not only can phospholipids other than PS participate in the binding to annexin V molecules; membrane proteins may also influence the binding of annexin V molecules. Ionophore and calcium treatment of erythrocytes, presumably corresponding to phospholipid scrambling only, would lead to PS containing sites of different nature than those detected on prolonged storage of erythrocytes in SAG-M. As these last sites would be detected by AnxV-FF as well as by binding to FITC-labelled or iodinated annexin V, some binding sites to AnxV-FF also appear early during storage but remain undetectable by the iodinated or FITC-labelled annexin V derivatives. Discrepancy in the detection of PS surface exposure was also observed between FITC-AnxV binding and a prothrombinase assay on cells undergoing apoptosis (20). We cannot speculate on the exact nature of the binding sites to AnxV-FF detected in the various pathologies for which binding to AnxV-FF was reported higher than for normal control erythrocytes (13). But, the fact that this higher binding was detected in other pathologies than sickle cell anemia or thalassemia, already well identified for abnormal PS exposure, points on the potential interest of AnxV-FF binding level as a relevant parameter in the estimation of the benefit/risk ratio in blood transfusion.

Perspectives

Further studies should be aimed at a more quantitative exploration of the relation between parameters and results of the magnetic separation on the one hand and number of exposed sites at the surface of erythrocytes submitted to sorting on the other hand. In this regard, the use of partial scrambling of phospholipids should prove useful, because it results in variable extents of PS exposing cells.

The magnetic isolation of PS exposing erythrocytes has been already carried out using magnetic beads covered with an anti-FITC antibody commercialyzed by Myltenyi Biotec (11). The isolation of apoptotic human BL60 Burkitt lymphoma cells using similar beads was described recently (21). We think that a more direct isolation of such cells can be performed using our annexinV-coated nanoparticles. Due to the increased sensitivity of this annexin derivative towards PS containing sites (see above), it is possible that some PS harboring cells escape selection by the dual procedure using FITC-AnxV and anti-FITC coated particles. Moreover, the anti-FITC beads used in the previous studies are made of iron oxyde and dextran, and no covalent binding exists between the coated antibody and the core of the particle. We expect a greater sta-

bility, particularly appreciable for *in-vivo* studies, for our annexin V-coated particles compared to the dual system of FITC-AnxV and dextran anti-FITC coated particles.

Regarding cell and tissue imaging, the maghemite nanoparticles are easily observed in Electron Microscopy, and the superparamagnetic nature of these particles actually makes them more and more frequently used as negative staining agent in Magnetic Resonance Imaging. Detection of apoptosis *in situ* during embryogenesis was already studied using a biotinylated annexin V (22). A similar approach could be achieved using the AnxV-FF. As a complement, ferromagnetic resonance measurements are sufficently sensitive to detect ferrofluids in biological samples.

Finally, magnetocytolysis, which is the destruction of ferrofluid-bearing cells following incubation in an alternating magnetic field, may open new therapeutic approaches, and deserves more studies.

ACKNOWLEDGMENTS

We are grateful to Drs. Maurizio Sorice, Alain Zachowski, and Jean-Luc Popot for helpful discussion and to the Centre National de la Recherche Scientifique (upr 9052) for financial support. We thank Dr. Brigitte Boyer and Bernard Saliou for their help in the preparation of the iodinated annexin V.

REFERENCES

1. Bretcher, M. (1972) *Nature* **236,** 11–12.
2. Devaux, P. F. (1991) *Biochemistry* **30,** 1163–1173.
3. Diaz, C., and Schroit, A. J. (1996) *J. Membr. Biol.* **151,** 1–9.
4. Zwaal, R. F., and Schroit, A. J. (1997) *Blood* **89,** 1121–1132.
5. Kuypers, F. A. (1998) *Curr. Op. Hematol.* **5,** 122–131.
6. Walker, J. H., Boustead, C. M., Koster, J. J., Bewley, M., and Walker, D. A. (1992) *Bioch. Soc. Trans.* **20,** 828–833.
7. Dachary-Prigent, J., Freyssinet, J. M., Pasquet, J. M., Carron, J. C., and Nurden, A. T. (1993) *Blood* **81,** 2554–2565.
8. Thiagarajan, P., and Tait, J. F. (1990) *J. Biol. Chem.* **265,** 17420–17423.
9. van Engeland, M., Ramaekers, F. C., Schutte, B., and Reutelingsperger, C. P. (1996) *Cytometry* **24,** 131–139.
10. Funakoshi, T., Heimark, R. L., Hendrickson, L. E., Mac Mullen, B. A., and Fukijawa, K. (1987) *Biochem. J.* **26,** 5572–5578.
11. Kuypers, F. A., Lewis, R. A., Ernst, J. D., Discher, D., and Lubin, B. H. (1996) *Blood* **87,** 1179–1187.
12. Tait, J. F., and Gibson, D. (1994) *J. Lab. Clin. Med.* **123,** 741–748.
13. Sestier, C., Sabolovic', D., Geldwerth, D., Roger, J., Pons, J. N., and Halbreich, A. (1995) *C. R. Acad. Sci. Paris* **318,** 1141–1146.
14. Halbreich, A., Roger, J., Pons, J. N., de Fatima Da Silva, M., Hasmonay, E., Roudier, M., Boynard, M., Sestier, C., Amri, A., Geldwerth, D., Fertil, B., Bacri, J. C., and Sabolovic', D. (1997) *in* Scientific and Clinical Applications of Magnetic Carriers (Häfeli *et al.*, Eds), pp. 399–417, Plenum Press, New York.
15. Massart, R. (1982) *US Patent* **4** 329–241.
16. Fauconnier, N., Pons, J. N., Roger, J., and Bee, A. (1997) *J. Colloid Interface Sci.* **194,** 427–433.
17. Lubin, B. H., Chiu, D., Bastacky, J., Roelofsen, B., and van Deenen, L. L. (1981) *J. Clin. Invest.*, **67,** 1643–1649.
18. Wood, B. L., Gibson, D. F., and Tait, J. F. (1996) *Blood* **88,** 1873–1880.
19. Williamson, P., Kulick, A., Zachowski, A., Schlegel, R. A., and Devaux, P. F. (1992) *Biochemistry* **31,** 6355–6360.
20. Fadok, V. A., and Bratton, D. L. (1996) FASEB Summer Conference on Molecular Biophysics of Cellular Membranes, Saxtons River, VT.
21. Rickers, A., Brockstedt, E., Mapara, M. Y., Otto, A., Dörken, B., and Bommert, K. (1998) *Eur. J. Immunol.* **28,** 296–304.
22. van den Eijnde, S. M., Luijsterburg, A. J. M., Boshart, L., De Zeeuw, C. I., Hein van Dierendonck, J., Reutelingsperger, C. P. M., and Vermeij-Keers, C. (1997) *Cytometry* **29,** 313–320.