

Increased Erythrocyte Phosphatidylserine Exposure in Chronic Renal Failure

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Abstract. The appearance of phosphatidylserine, an aminophospholipid normally confined to the inner monolayer, at the outer leaflet of red cell membrane may have several pathophysiologic implications. This study examines erythrocyte phosphatidylserine exposure in chronic renal failure (CRF) patients on conservative treatment or on dialysis, to assess possible alterations to phospholipid asymmetry in a condition associated with a state of deranged red cell function. A significant increase in phosphatidylserine-expressing erythrocytes was found in undialyzed patients with CRF (2.32%) and patients on hemodialysis (3.06%) and on peritoneal dialysis (2.14%) compared with control subjects (0.68%). In undialyzed CRF patients, a strong correlation ($r = 0.903$) was found between the percentage of phosphatidylserine-expressing red cells and the serum creatinine concentration. The increased exposure of phosphatidylserine in uremic erythrocytes may be

due to inhibition of phosphatidylserine transport from the outer to the inner leaflet of plasma membrane and may promote an increased erythrophagocytosis. In reconstitution experiments, normal erythrocytes showed an increase in phosphatidylserine-expressing cells when incubated in uremic plasma (3.2% after 2 h *versus* 1.1% at beginning of incubation), whereas phosphatidylserine-positive uremic erythrocytes decreased when resuspended in normal plasma (2.03% after 2 h and 1.65% after 8 h *versus* 2.9% at beginning of incubation). Preliminary characterization of the putative uremic compound(s) indicates a molecular weight between 10,000 and 20,000, as well as heat instability. These findings show an impairment of erythrocyte membrane phospholipid asymmetry in CRF patients, regardless of the dialysis treatment. Such abnormality seems related to the uremic state and could contribute to the red cell pathology present in CRF.

Chronic renal insufficiency may affect the red blood cell (RBC) mass by interfering with both red cell production and the red cell life span (1). Although depressed erythropoiesis is the predominant defect responsible for the anemia in patients with renal failure, a reduced red cell life span was also found to contribute (2–4). The accelerated destruction of RBC in uremia appears to be extracorporeal and has been related to the retention of one or more uremic compounds (1,5). However, although the shortened RBC life span in chronic renal failure (CRF) has a well-documented toxic origin, the pathophysiologic link between this defect and uremic compounds has not been thoroughly elucidated at a molecular level.

The phospholipids of the human RBC are distributed asymmetrically in the bilayer of the red cell membrane (6). Maintaining plasma membrane asymmetry, even at the expense of energy consumption (7), is of critical importance for cells. Indeed, loss of normal phospholipid asymmetry, especially the appearance of PS (an aminophospholipid normally confined to the membrane's inner leaflet) inside the outer leaflet of the

erythrocyte membrane, may have several pathophysiologic implications (reviewed in references (7) and (8)). In particular, exposure of PS promotes macrophage recognition and splenic clearance of aged or abnormal erythrocytes (9–11). Patients with sickle cell disease have an increased PS exposure in erythrocytes (12–15), and surface-exposed PS has been implicated in the decreased RBC survival time characteristic of the disorder (11,15).

A number of abnormalities in the structure and function of the erythrocyte membrane have been found in patients with CRF (16–21). It is therefore possible to hypothesize that changes in PS exposure at the extracellular face of the RBC membrane may occur in CRF. The present study was undertaken to examine the exposure of PS in erythrocytes from undialyzed patients with CRF, patients on peritoneal dialysis, and those on hemodialysis.

Materials and Methods

Patients

Erythrocyte PS exposure was measured in 15 healthy control subjects (seven women, eight men; mean age 55.8 ± 2.2 yr; range, 41 to 71), 30 undialyzed patients with differing degrees of CRF and on conservative treatment (13 women, 17 men; mean age 57.2 ± 1.4 yr; range, 43 to 72), 30 stable patients on chronic maintenance hemodialysis (HD) (15 women, 15 men; mean age 58 ± 1.7 yr; range, 31 to 70; mean time on dialysis 54 ± 8 mo), and 24 stable patients on continuous ambulatory peritoneal dialysis (CAPD) (12 women, 12 men; mean age 54.8 ± 2.1 yr; range, 37 to 70; mean time on dialysis

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33.5 \pm 3.8 mo). Informed consent was obtained from each participant in the study. No patient had been treated or was receiving erythropoietin. Patients with diabetes were excluded, since hyperglycemia can induce a loss of phospholipid asymmetry in human erythrocytes (22). Other exclusion criteria included uncontrolled hypertension; use of drugs that might interfere with erythropoiesis (theophyllin, angiotensin-converting enzyme inhibitors); active infection; malignant or systemic disease; and iron, folic acid, and vitamin B₁₂ deficiency. In addition, none of the patients had received blood transfusion over the past 6 mo.

The CRF group consisted of eight patients with chronic glomerulonephritis, nine with nephroangiosclerosis, nine with chronic pyelonephritis, and four with autosomal dominant polycystic kidney disease. Serum creatinine concentration (mg/dl) ranged from 1.6 to 9.8 and averaged 4.4 \pm 0.4 mg/dl. The mean hematocrit (Hct) was 32.8 \pm 0.8% (range, 24 to 41.3%), and the serum hemoglobin was 10.9 \pm 0.3 g/dl (range, 8.1 to 14 g/dl). In the CAPD group, Hct (%) ranged from 25.7 to 37.1% and averaged 31.5 \pm 0.7%, and hemoglobin levels (g/dl) ranged from 8.4 to 12.2 and were on average 10.6 \pm 0.3 g/dl. Parathyroid hormone (PTH) levels were 159 \pm 29 pg/ml (range, 35 to 693; normal values 13 to 54 pg/ml). All CAPD patients had four exchanges daily of 2 L of dialysate (1.5 to 4.25% dextrose). The HD group consisted of 15 patients routinely treated with cellulosic membranes (regenerated cellulose, acetate cellulose) and 15 patients with synthetic membranes (polyacrylonitrile, polymethylmethacrylate). Dialyzers were not reused. All HD patients were on a stable anticoagulation regimen using unfractionated heparin and were being dialyzed three times a week with a 4-h dialysis session using bicarbonate dialysate. The Hct in the HD patients was between 23 and 35.9 and on average was 29.1 \pm 0.6% ($P < 0.05$ versus CRF and CAPD), while the mean hemoglobin level (g/dl) was 9.8 \pm 0.2 ($P < 0.05$ versus CRF and CAPD) and ranged from 8.0 to 11.9. PTH levels were 182 \pm 29 pg/ml (range, 28 to 673 pg/ml).

Materials

Blood was drawn into evacuated tubes containing ethylenediaminetetra-acetic acid (EDTA). Erythrocytes were isolated by centrifugation at 400 \times g for 5 min, followed by three subsequent resuspensions and washing in saline (0.9% NaCl). The buffy coat was carefully removed. Erythrocytes were then resuspended in the appropriate buffer (see below). Incubation was carried out in a shaking water bath (Shaker Bath; Lab-line Instruments, Melrose Park, IL). FITC-labeled annexin V (FITC-AnV), Hanks' buffered salt solution (HBSS), *N*-ethyl maleimide (NEM), calcium ionophore A23187, ethyleneglycol-bis(β -aminoethyl ether)-*N,N'*-tetra-acetic acid (EGTA), fatty acid-free bovine serum albumin (BSA), monoclonal mouse anti-CD71, phycoerythrin-labeled goat anti-mouse antibody, phosphate-buffered saline (PBS), heat-inactivated fetal calf serum, thrombin, and dilaurylphosphatidylserine (DLPS) were purchased from Sigma (St. Louis, MO). Purified prothrombin, factor Xa, and the chromogenic substrate for thrombin CBS 34.47 (H-D-cyclohexylglycyl- α -aminobutyryl-arginyl-paranitroanilide) were purchased from Diagnostica Stago (Asnieres, France); coagulation factor V was from Calbiochem (La Jolla, CA). Ficoll-Hypaque was from Pharmacia (Uppsala, Sweden). RPMI 1640 was purchased from Life Technologies (Grand Island, NY). All other chemicals were reagent grade.

Measurement of Annexin V-Positive Red Cells

To measure the exposure of PS on the outer hemileaflet of the erythrocyte membrane, a recently developed flow-cytometric assay based on FITC-AnV was used (15). Indeed, annexin V is a member of

the annexin family of calcium-dependent phospholipid binding proteins with a high affinity for PS (23). The anticoagulated whole blood sample was diluted 1:100 (3 to 5 \times 10⁷ erythrocytes/ml) to a final volume of 0.25 ml in a buffer ("binding buffer") consisting of 10 mmol/L Hepes-Na, pH 7.4, 136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L MgCl₂, 1 mmol/L NaH₂PO₄, 5 mmol/L glucose, 5 mg/ml BSA, and 2.5 mmol/L CaCl₂. The same binding buffer and dilution factor were used to resuspend isolated RBC. FITC-AnV was added to a concentration of 100 nmol/L either to whole blood or isolated RBC, and the samples were incubated for 15 min at room temperature in the dark. After incubation, an aliquot was directly aspirated into the flow cytometer (Epics Elite; Coulter, Hialeah, FL) for analysis. Each day before analysis, the instrument was standardized with a solution of fluorescence microspheres (Immuno-Check; Epics Division, Hialeah, FL). Fifty thousand events per sample were acquired. Fluorescence parameters were collected using a three-decade logarithmic amplification. The red cell population was defined by size in forward and side scatter plots. Gated cells were counted as annexin-positive if they had a mean fluorescence of at least 1.0. This threshold value was determined in preliminary studies based on analysis of samples from healthy donors. The flow cytometer's software was used to calculate the percentage of annexin-positive cells and their mean fluorescence intensity (in arbitrary units). FITC-AnV binding to erythrocytes was given as a percentage of annexin V-positive cells, since this parameter was much more discriminative among the various clinical and experimental conditions studied than mean fluorescence intensity.

As a negative control for the assay, samples (either whole blood or RBC) were diluted 1:100 to a final volume of 0.25 ml in binding buffer without calcium (2.5 mmol/L CaCl₂). After addition of FITC-AnV (100 nmol/L) and incubation for 15 min at room temperature in the dark, samples were analyzed at flow cytometry for PS exposure as described above. In other experiments, to increase PS exposure in the outer hemileaflet, RBC were sequentially treated with NEM and A23187 as described (14). Isolated RBC were resuspended in HBSS, pH 7.4, at 30% hematocrit. RBC suspension was then incubated in the presence of 10 mmol/L NEM for 30 min at room temperature, and washed in HBSS. Subsequently, RBC at a 16% hematocrit were incubated in HBSS with 1 mmol/L calcium for 3 min at 37°C, and 4 mmol/L of the calcium ionophore A23187 was added. The sample was incubated for 1 h. At the end of the incubation, RBC were first washed with HBSS containing 2.5 mmol/L EDTA, and then washed three times in the same buffer containing 1% BSA. RBC were resuspended in incubation buffer without BSA to prevent calcium uptake during the subsequent annexin V-labeling. RBC were labeled and analyzed for PS exposure as described above.

Prothrombinase Assay

The conversion of prothrombin to thrombin induced by PS exposure in intact RBC was measured according to Wilson *et al.* (22). Isolated RBC were resuspended at 0.1% hematocrit in Tris buffer (50 mM Tris/HCl, 120 mM NaCl, pH 7.4) containing 6 mM CaCl₂, 0.33 U/ml factor V, 0.33 U/ml factor Xa, 1.3 U/ml prothrombin and incubated for 4 min at 37°C. The reaction was stopped by adding EDTA at a final concentration of 15 mM. RBC were sedimented by centrifugation, and the amount of thrombin released during the incubation was measured in an aliquot of the supernatant (200 μ l) using the chromogenic substrate CBS 34.47 (50 μ M final concentration). The amount of thrombin released was measured by reference to a standard curve of authentic thrombin.

Assessment of the Relative Age of Annexin V-Positive Cells

A two-color flow-cytometric analysis was used to assess the relative age of the annexin V-positive RBC (15). Whole blood was diluted 1:800 in the binding buffer as above but without CaCl_2 , and incubated for 30 min at 4°C with monoclonal mouse anti-CD71, a marker for reticulocytes (24). Thereafter, the sample was washed twice with PBS, incubated with phycoerythrin-labeled goat anti-mouse antibody for 30 min at 4°C, washed twice with PBS, and incubated with FITC-AnV and 2.5 mmol/L CaCl_2 as described above. The sample was then analyzed by flow cytometer, in which phycoerythrin fluorescence was monitored at 560 to 590 nm. The flow cytometer's software was used to calculate in each sample the percentage of CD71-positive cells, annexin V-positive cells, and cells positive for both CD71 and annexin V.

Reconstitution Experiments with Isolated RBC and Plasma

Isolated RBC from healthy subjects were resuspended at an Hct of 50% in plasma from patients on HD, whereas RBC from patients on HD were resuspended at an Hct of 50% in plasma obtained from healthy subjects. RBC from each group of subjects were also resuspended at 50% Hct in autologous plasma. RBC suspensions were then incubated at room temperature. After 5 min of incubation (incubation time 0) and subsequently at designated time points (2, 4, and 8 h), an aliquot of the suspension was removed, and further processed as described for determination of PS exposure by flow cytometer. In another set of experiments, RBC from healthy subjects were resuspended at 50% Hct in plasma from HD patients and incubated for 2 h. Thereafter, an aliquot of the suspension was processed and analyzed for annexin-V binding to RBC, while another aliquot was washed twice with 0.9% NaCl, incubated with autologous plasma, and at prescribed time points further aliquots were processed and analyzed for annexin-V binding to RBC.

To assess the approximate molecular size and thermal stability of the putative uremic plasma factor(s) involved in RBC PS exposure, uremic plasma was passed over a Centriscart I filter (Sartorius, Göttingen, Germany) with a nominal molecular weight cutoff of 10 and/or 20 kD, and the ultrafiltrate was either used as such or boiled for 5 min. The ultrafiltrate or heat-treated ultrafiltrate specimens were added to isolated RBC from healthy subjects at a Hct of 50%. After 2 h of incubation at room temperature, aliquots of red cells were processed and analyzed for annexin-V binding to RBC. In all reconstitution experiments, care was taken to ensure ABO and Rh blood group compatibility.

Phosphatidylserine Transport Assay

Phosphatidylserine transport in RBC was examined using a cell morphology assay based on the established pattern of RBC shape changes occurring in response to the addition of exogenous DLPS vesicles (25). FITC-AnV-labeled RBC from HD patients were FACS-sorted (FACStar; Becton Dickinson, San Jose, CA) to obtain either a PS-positive or a PS-negative fraction. Sorted cells (either PS-expressing or PS-non-expressing red cells) were collected into tubes containing 1 ml of PBS, incubated with EGTA (2.5 mmol/L) to remove FITC-annexin, and washed twice with saline. Unilamellar DLPS vesicles were prepared by sonicating the lipid mixture in PBS at pH 7.4. RBC at 50% Hct were incubated with DLPS vesicles (250 μM) in the presence of 5 mM glucose at 37°C. At prescribed time points, aliquots of RBC suspensions (5 μl) were fixed for 15 min at room temperature in 50 μl of glutaraldehyde (1% in PBS, pH 7.4). Samples

were examined by light microscopy, and their morphology was used as a measure of transbilayer PS distribution. Echinocytes were assigned scores of +1 to +5 (increasing value denoting more severe crenation); discocytes were scored 0; and stomatocytes were given scores of -1 to -4 with increasingly severe stomatocytosis (25). The average score for a field of 100 cells was taken as the morphologic index.

Erythrophagocytosis Assay

For the erythrophagocytosis assay, human monocyte-derived macrophages were used. Peripheral heparinized whole blood from healthy subjects was diluted 1:1 with 0.9% NaCl solution. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque discontinuous centrifugation, according to the method of Boyum (26). Mononuclear cells, collected at the interface, were washed twice in RPMI 1640 and then suspended at 10^6 cell/ml in a complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin). Monocytes were purified from peripheral blood mononuclear cells by adherence to the surface of plastic dishes for 1 h at 37°C, with 5% CO_2 . Nonadherent cells were removed, and adherent monocytes were washed with PBS to remove residual nonadherent cells. Monocytes were cultured in a complete medium (the viability determined by trypan blue exclusion was >99%) at a density of 1×10^6 cells/ml for 6 d before use in the phagocytosis assay. All reagents used for monocyte and lymphocyte isolation and culture were endotoxin-free as evaluated by the limulus amoebocyte lysate assay. Monocyte-derived macrophages (1×10^5) were added to each well of a 96-well flat-bottomed plate (Nunc, Intermed, Roskilde, Denmark). RBC were added to wells containing cultured human monocytes (RBC to monocyte ratio, 100:1) and incubated for 1 h at 37°C in a humidified CO_2 incubator. The cells were then washed three times with PBS to remove unbound RBC. Noninternalized RBC were lysed with 0.2% PBS for 30 s. After another rinse with PBS, cells were fixed with methanol and stained with Giemsa. RBC ingestion by macrophages was determined by light microscopy. The results are expressed as a percentage of macrophages ingesting one or more RBC.

Statistical Analyses

Differences were analyzed by parametric (*t* test and ANOVA) and nonparametric (Mann-Whitney and Kruskal-Wallis ANOVA on ranks) methods according to the normal or skewed distribution of data, respectively. Pearson correlation coefficient (*r*) was used to test the association between pairs of variables. Statistical analyses were performed using the statistical software SigmaStat version 2.0 for Windows (Jandel Scientific Software, San Rafael, CA). Results are expressed as a mean \pm SEM, and *P* < 0.05 was considered significant.

Results

Measurement of Annexin V-Positive Red Cells in Healthy Subjects and Uremic Patients

We initially compared results of experiments in which either whole blood or isolated RBC were labeled with FITC-AnV. In agreement with previous studies (13,14), virtually identical results were found in terms of RBC PS exposure in isolated cells and whole blood samples. Thus, for routine assays, the whole blood sample was used. Erythrocytes showed virtually no fluorescence in FACS analysis after incubation in binding buffer without calcium (Figure 1A), consistent with the fact

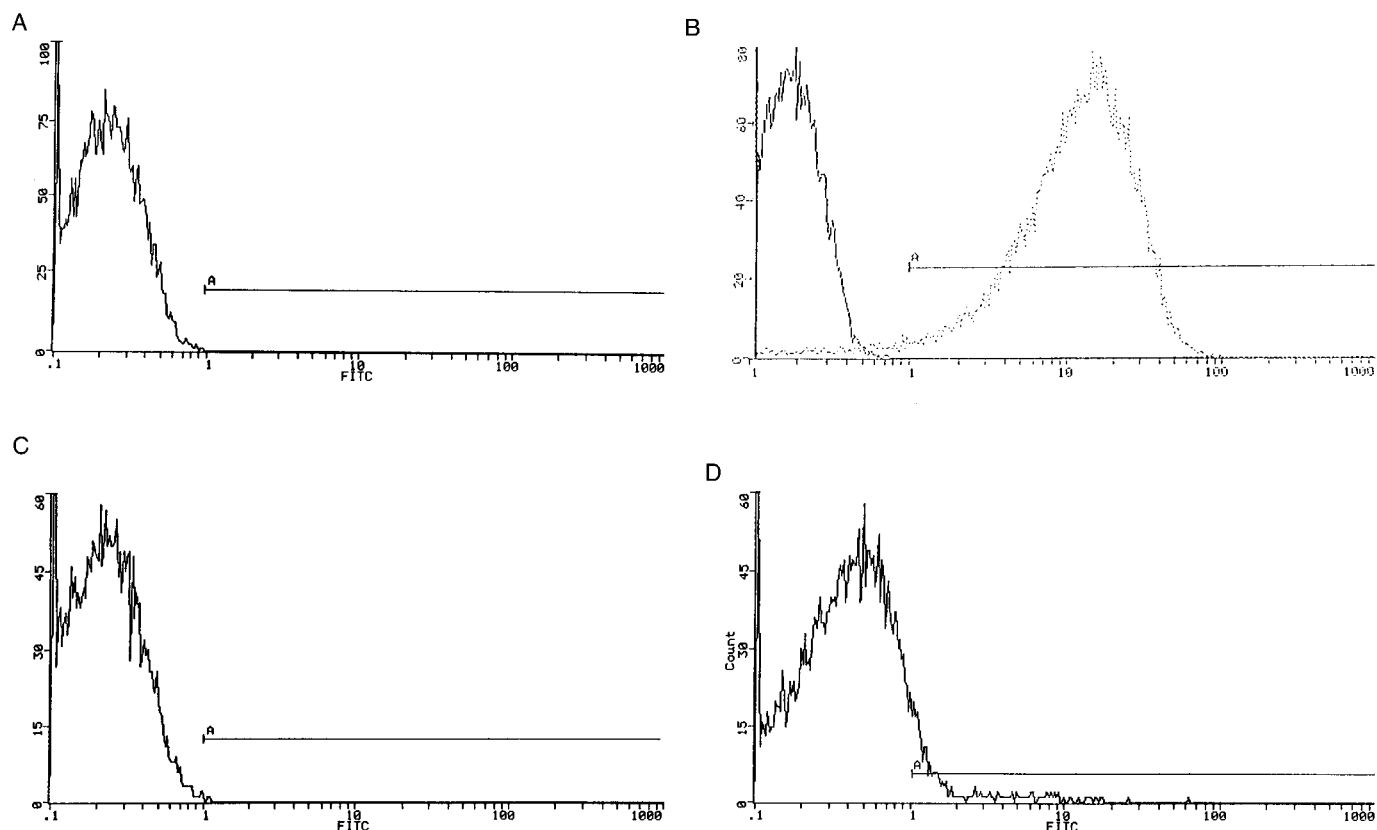


Figure 1. Red blood cell (RBC) flow cytometric analysis. (A) FITC annexin V (FITC-AnV) labeling of RBC incubated in binding buffer without calcium. (B) FITC-AnV labeling of RBC treated first with *N*-ethyl maleimide and then calcium plus A23187. FITC-AnV labeling of RBC from a healthy subject (C) and a uremic patient on hemodialysis (HD) (D). Measurement of RBC labeled with FITC-AnV was carried out by a flow cytometric assay described in Materials and Methods.

that AnV exhibits Ca^{2+} -dependent phospholipid binding properties (23). Furthermore, incubation of FITC-AnV-labeled RBC with EGTA caused loss of fluorescence in FACS analysis (data not shown). As a positive control for the assay, we measured the percentage of annexin V-positive erythrocytes in conditions known to induce an elevated expression of PS (14). Indeed, treatment of RBC with NEM, which inhibits the aminophospholipid translocase (27), and then with calcium plus ionophore A23187, leads to an acceleration of PS movement from the inner to the outer monolayer of RBC membrane. When RBC were submitted to such treatment, high levels of positive red cells (on average 90%) were found, as shown in Figure 1B. A typical flow-cytometer histogram for binding of annexin V to RBC from a healthy subject is reported in Figure 1C, whereas Figure 1D shows a flow-cytometer histogram for annexin V binding to erythrocytes from one chronic uremic patient on hemodialysis.

Figure 2 illustrates the results concerning the percentage of annexin V-positive red cells in the study population. A low but measurable percentage of annexin V-positive cells was found in whole-blood samples from healthy subjects (Figure 2), in agreement with previous studies (14,15). The mean percentage of annexin-positive red cells in chronic renal failure patients was significantly higher ($P < 0.05$) than in healthy control subjects, regardless of whether the patients were dialyzed

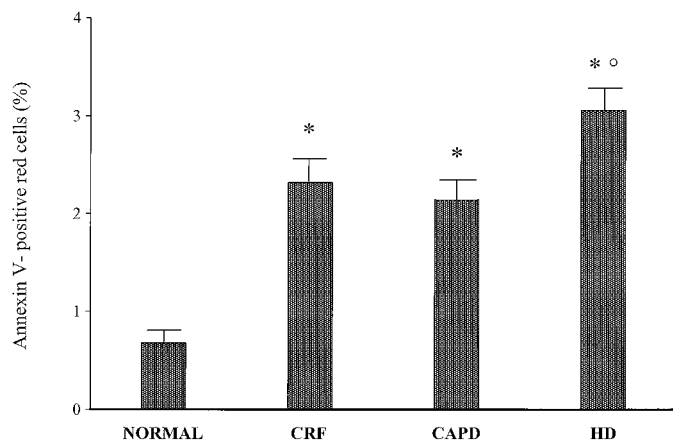


Figure 2. Binding of FITC-labeled annexin V to RBC from healthy subjects ($n = 15$), undialyzed chronic renal failure patients (CRF; $n = 30$), hemodialysis patients (HD; $n = 30$), and continuous ambulatory peritoneal dialysis patients (CAPD; $n = 24$). * $P < 0.05$ versus healthy subjects; ° $P < 0.05$ versus CAPD.

(Figure 2). No difference was found in annexin-positive cells between undialyzed patients with CRF and chronic uremic patients on renal replacement therapy, whether CAPD or HD. By contrast, the percentage of annexin V-positive red cells

significantly differed between patients on HD and patients on CAPD, the mean values being significantly higher ($P < 0.05$) in HD than in CAPD (Figure 2). The results of two-color flow cytometric analysis for assessment of the relative age of annexin V-positive red cells in the three groups of uremic patients (Table 1) indicate that many, but not all, annexin V-positive red cells are still young enough to be expressing a marker for reticulocytes, and that only some young erythrocytes are positive for FITC-AnV.

PS exposure was also studied by the prothrombinase assay, an established assay for the presence of PS on a membrane surface (28). Indeed, several investigators have shown that PS, when expressed on the external leaflet of cell membrane, provides the necessary lipid cofactor for the formation of the prothrombinase complex. The amount of thrombin released (nM/min) in samples from healthy control subjects ($n = 6$) and from HD patients ($n = 6$) was 4.8 ± 0.1 and 6.6 ± 0.1 , respectively ($P < 0.001$). Although these data cannot be directly compared with the FITC-AnV experiments in terms of percentage of cells expressing PS, the prothrombinase activation would support the evidence of PS exposure obtained with the annexin V procedure in HD patients.

Correlation with Degree of Renal Function

In the group of 30 patients with a wide spectrum of chronic renal insufficiency on conservative treatment (mean serum creatinine 4.4 mg/dl; range, 1.6 to 9.8), a strong correlation was observed between the percentage of annexin V-positive red cells and the serum creatinine concentration (Figure 3; $r = 0.903$). A lower inverse correlation was also observed when comparing with calculated creatinine clearance, based on age and body surface area ($r = -0.836$). A correlation between serum creatinine concentration and annexin-positive red cells was not found within any of the other study groups (data not shown).

Furthermore, undialyzed CRF patients were subdivided into three groups ($n = 10$ for each group) according to serum creatinine concentration: mild CRF (<2 mg/dl), moderate CRF (between 2.1 and 5.9 mg/dl), and advanced CRF (>5.9 mg/dl). In each CRF group, annexin V-positive erythrocytes were significantly higher ($P < 0.05$) than in healthy subjects. Significant ($P < 0.05$) differences in annexin-positive red cells were found between mild CRF ($0.97 \pm 0.07\%$) and both moderate ($2.46 \pm 0.23\%$) and advanced ($3.54 \pm 0.17\%$) CRF. Patients with advanced CRF also had significantly higher annexin-positive cells than patients with moderate CRF ($P <$

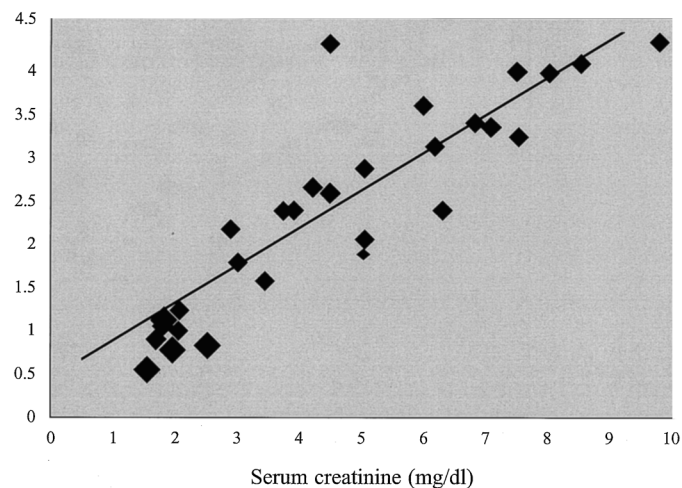


Figure 3. Correlation between annexin V-positive red cells and the degree of renal insufficiency, expressed by the serum creatinine concentration, in 30 undialyzed patients with chronic renal failure ($r = 0.903$; $P < 0.0001$).

0.05). In both dialysis groups, the percentage of annexin V-labeled RBC was significantly increased compared with mild CRF ($P < 0.001$ for both). Patients on HD showed significantly higher annexin-positive cells than moderate CRF patients ($P < 0.05$), and no difference from advanced CRF. By contrast, those under CAPD treatment exhibited no difference from patients with moderate CRF, but a significantly lower percentage of annexin V-positive red cells than advanced CRF ($P < 0.001$).

Phosphatidylserine Transport Assay

To assess whether abnormalities in PS transport may contribute to the increased exposure of PS at the extracellular face of the RBC membrane in CRF, erythrocyte shape changes during incubation with DLPS vesicles were examined using a cell morphology assay (25,29). DLPS-treated normal RBC became echinocytic within 5 min (Figure 4), reverting rapidly to discocytic (10 to 20 min) and then stomatocytic shapes, in keeping with previous reports (25). A similar response to DLPS treatment was observed for uremic PS-negative erythrocytes, FACS-sorted (Figure 4). By contrast, uremic FACS-sorted, PS-expressing erythrocytes crenated rapidly but did not revert to discocytic or stomatocytic shapes (Figure 4). These results indicate that the ability of uremic erythrocytes to transport PS seems to be altered in the PS-positive cell fraction.

Table 1. Relative age of annexin V-positive red cells in the three groups of patients^a

Category	CRF ($n = 10$)	HD ($n = 10$)	CAPD ($n = 10$)
CD71-positive cells (%)	3.33 ± 0.20	3.70 ± 0.22	3.13 ± 0.17
Annexin V-positive cells (%)	2.53 ± 0.21	3.05 ± 0.18	2.28 ± 0.13^b
CD71 + annexin V-positive cells (%)	1.94 ± 0.17	2.33 ± 0.17	1.72 ± 0.13^b

^a CRF, chronic renal failure; HD, hemodialysis; CAPD, continuous ambulatory peritoneal dialysis.

^b $P < 0.05$ versus HD (ANOVA on ranks followed by Tukey test).

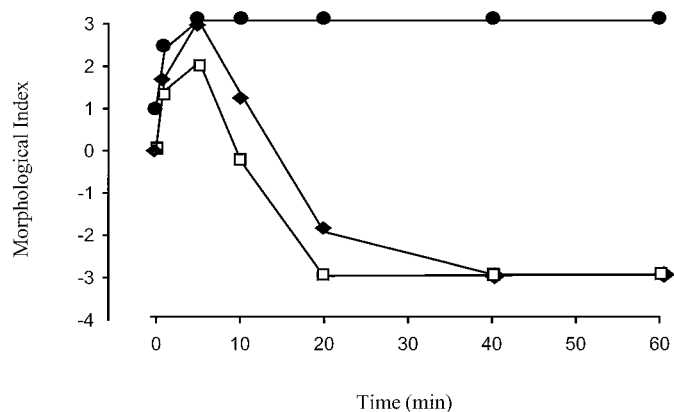


Figure 4. Time course of erythrocyte shape change upon treatment with dilauroylphosphatidylserine (DLPS) vesicles. RBC were incubated with DLPS. Aliquots of cells were removed, fixed in glutaraldehyde, and analyzed by light microscopy for cell morphology as described in Materials and Methods. ◆, shape change of erythrocytes from healthy subjects ($n = 3$); □, shape change of FACS-sorted, PS-negative erythrocytes from hemodialysis patients ($n = 3$); ●, shape change of FACS-sorted, PS-positive erythrocytes from hemodialysis patients ($n = 3$).

RBC and Plasma Reconstitution Studies

To examine whether CRF is associated with a plasma factor(s) affecting the exposure of PS at the external face of RBC membrane, reconstitution experiments were performed with normal or uremic isolated RBC resuspended in plasma. RBC from healthy subjects were resuspended in plasma from patients on chronic HD, while erythrocytes from chronic HD patients were resuspended in plasma from healthy subjects. Erythrocytes from healthy subjects expressed a higher level of PS exposure when assayed in uremic plasma compared to normal plasma at any time point measured (Figure 5). Also, the percentage of annexin-positive normal red cells increased after 2 h of incubation in uremic plasma, when compared to the beginning of incubation, but thereafter plateaued (Figure 5). With regard to uremic erythrocytes, no difference was found at the beginning of incubation in the percentage of annexin-positive cells between RBC incubated in normal plasma compared to RBC incubated in autologous (uremic) plasma. During the incubation time, however, uremic erythrocytes showed a decrease in the percentage of annexin V-positive cells when assayed in normal plasma compared to autologous plasma (Figure 5). That chronic renal insufficiency is associated with a plasma factor or retained uremic toxin affecting the exposure of PS is confirmed by further reconstitution experiments. When normal RBC, preincubated for 2 h with uremic plasma, were resuspended in autologous plasma, a progressive decline in annexin V-positive red cells was observed (Figure 6).

Preliminary Characterization of the Uremic Plasma Factor(s)

To get some insight into the approximate molecular weight and nature of the putative uremic plasma factor(s) influencing the exposure of PS on the outer face of red cell membrane,

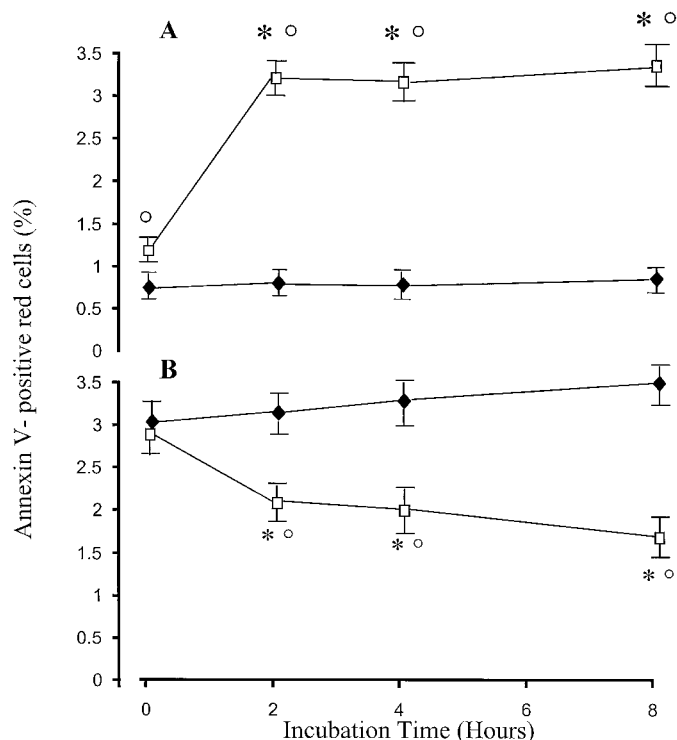


Figure 5. Erythrocyte and plasma reconstitution experiments. (A) Normal RBC incubated with autologous plasma (◆) or uremic plasma from a patient with the same blood group (□). (B) Uremic RBC incubated with autologous plasma (◆) or normal plasma from a subject with the same blood group (□). Measurements of annexin V-positive red cells at indicated time points were performed by flow cytometric assay. Data are means \pm SEM from four subjects. Each reconstitution experiment was carried out in duplicate. * $P < 0.05$ versus incubation time 0; ° $P < 0.005$ versus autologous sample at the same time point of incubation.

studies ($n = 4$) were done with plasma from chronic HD patients. Erythrocytes isolated from healthy subjects were incubated with uremic plasma (control sample) or its ultrafiltrate obtained through either a 20 or 10 kD cutoff filter. Incubation of normal RBC with the 20-kD ultrafiltrate caused a significant PS exposure ($2.78 \pm 0.1\%$ annexin V-positive red cells) as in the untreated uremic plasma sample ($2.9 \pm 0.1\%$ annexin V-positive red cells), whereas incubation with 10-kD ultrafiltrate had a poor effect ($0.5 \pm 0.07\%$ annexin V-positive red cells). In addition, heat treatment of the 20-kD ultrafiltrate strongly inhibited the ability of the uremic ultrafiltrate to cause exposure of PS ($0.9 \pm 0.04\%$ annexin V-positive red RBC).

Erythrophagocytosis Assay

Erythrophagocytosis by human macrophages was examined in conditions characterized by a different RBC PS exposure. RBC were obtained from a healthy subject (0.6% FITC-AnV-positive red cells) and a chronic uremic patient on HD (3.1% FITC-AnV-positive red cells); in addition, normal RBC were resuspended at an Hct of 50% in uremic plasma and incubated for 2 h (3.2% FITC-AnV-positive red cells after incubation time). Erythrophagocytosis, expressed as the percentage of macrophages ingesting

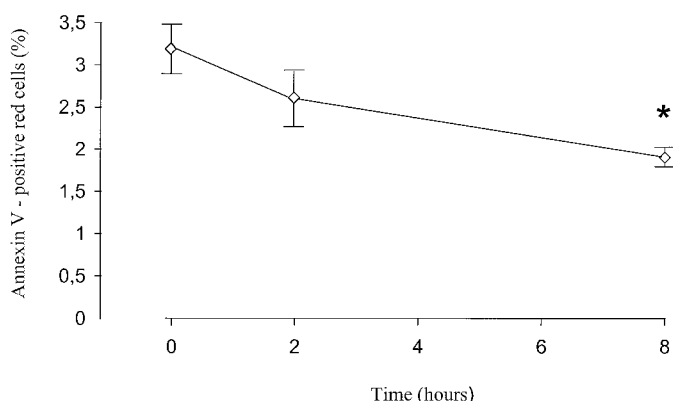


Figure 6. Disappearance of PS-positive normal red cells formerly exposed to uremic plasma. Normal RBC ($n = 4$) were preincubated with uremic plasma for 2 h and then resuspended in autologous plasma. Time 0 indicates the beginning of incubation in autologous plasma. For further experimental details, see Materials and Methods. Measurement of RBC labeled with FITC-AnV was carried out by a flow cytometry assay. * $P < 0.05$ versus time 0.

one or more Giemsa-stained RBC, was 14% for normal RBC. However, when normal RBC were preincubated in uremic plasma, erythrophagocytosis rose to 49%, a value comparable to that found for uremic RBC (51%).

Discussion

The appearance of PS at the extracellular face of cell plasma membrane is associated with several physiologic and pathologic phenomena (7,8), with particular regard to the processes of cell-cell interaction, hemostasis, and cell activation. The data reported here indicate that there is an increased PS exposure in erythrocytes from chronic uremic patients, regardless of whether the patients are on dialysis. The abnormality predominantly affects red cells that are still expressing a marker for reticulocytes. Our results show that in chronic uremia, the percentage of annexin V-positive RBC increases with the progressive decline in renal function, and persists elevated on renal replacement therapy. The increased PS exposure in RBC from hemodialyzed patients was further supported by an independent assay for phospholipid asymmetry, the prothrombinase activation. Compared to healthy subjects, we indeed found a significantly higher rate of prothrombin to thrombin conversion when RBC from HD patients were used.

Our data indicate that the form of dialytic treatment does seem to influence the abnormal exposure of PS in uremic erythrocytes. Patients on CAPD showed in fact a lower percentage of annexin-positive RBC than patients on HD. In addition, PS-expressing RBC were significantly lower in CAPD patients than in undialyzed patients with advanced CRF, whereas there was no difference between the latter patients and HD patients. These findings, coupled with the better degree of anemia found in peritoneal dialysis patients, are consistent with the notion of a better effect of CAPD over HD in terms of anemia (30,31).

Some of the several derangements in the metabolic function and membrane stability of uremic RBC (16–21), such as the

defective activity of the Na^+/K^+ -ATPase (16) and Ca^{2+} pump (20), can be acquired by normal erythrocytes upon exposure to uremic plasma. Our data indicate that uremic plasma strongly influences the exposure of PS in RBC as well. Indeed, normal RBC incubated in uremic plasma show an increase in annexin V-positive cells reaching values similar to those found in chronic HD patients. The increased exposure of PS in RBC exposed to uremic plasma, however, does not seem an irreversible abnormality. When RBC are taken out of the uremic environment and reincubated in normal plasma, outer-exposed PS disappears in a large subpopulation of erythrocytes (Figures 5 and 6).

Although reconstitution studies were performed with uremic plasma from HD patients only, one may well expect similar results concerning plasma from either CAPD or undialyzed CRF patients, given the similarities (although to a possibly different degree) in the uremic product retention pattern. This assumption would be supported by the strong correlation found between PS exposure in erythrocytes and the decrease in renal function. Such correlation also suggests a pathogenetic role, in the increased exposure of outer leaflet PS in RBC, played by humoral factor(s) retained in proportion to the degree of nephron loss during progression of renal disease. Our *in vitro* experiments indicate that the ability of uremic plasma to increase the exposure of PS in RBC is associated with a molecular weight range between 10 and 20 kD. This could explain the lower percentage of PS-expressing RBC that we found in CAPD patients compared to HD patients, since CAPD more efficiently removes high molecular weight solutes (32,33). In addition, we observed that the ability of uremic plasma is strongly inhibited by boiling. These observations suggest that the putative uremic factor(s) is a large heat-labile molecule, and possibly a protein or peptide. However, it may also be a low molecular weight substance that behaves like middle molecules (molecular weight between 300 and 12,000) due to high protein binding. In addition, a synergism between several accumulated solutes cannot be ruled out at present.

Various distinct activities are involved in the regulation and maintenance of membrane lipid sidedness. Several studies have shown that an active transport mechanism, aminophospholipid translocase, flips PS from the outer to the inner leaflet of cell plasma membrane (34,35). This PS transport seems to be affected in chronic uremia, as indicated by an RBC morphologic assay. However, such defect is most likely present only in the RBC fraction FACS-sorted as PS-positive (Figure 4). Elevated intracellular Ca^{2+} levels, which can be found in uremic RBC (20,36), might play a role in such abnormality (37). However, the increase in intracellular Ca^{2+} of uremic RBC has been attributed to either PTH-induced Ca^{2+} entry into the cell (38) or to a uremic circulating inhibitor of the membrane Ca^{2+} pump (20). According to the results of our reconstitution studies, the molecular weight of both substances (9424 and <3000 Daltons, respectively) would exclude their being involved in the increased appearance of PS in RBC exposed to uremic plasma. In addition, while Ca^{2+} -dependent loss of membrane phospholipid asymmetry is not corrected (8), we observed a significant decrease in PS-expressing RBC after

removal of cells from the uremic milieu. Thus, although a role for cytoplasmic Ca^{2+} cannot be unequivocally ruled out, these findings suggest the presence of another mechanism causing the loss of PS asymmetry in CRF.

The exposure of PS on the surface of RBC represents a signal for their recognition by phagocytes and their subsequent elimination from the circulation (13–17). Our data for erythrophagocytosis by human monocyte-derived macrophages, a cell population that binds to RBC (13), show more than a threefold increase for uremic RBC compared to normal RBC in macrophage RBC ingestion. Interestingly, normal RBC preincubated in uremic plasma displayed a percentage of erythrophagocytosis, as well as of annexin-positive cells, comparable to that of uremic red cells. Surface-exposed PS has been suggested as a pathogenic mechanism in the reduced RBC survival that characterizes sickle cell disease (16,21). Although the limited number of observations via erythrophagocytosis assay do not allow one to draw definitive conclusions, we propose that increased exposure of PS in uremic RBC may contribute to shortening their life span. In addition, the appearance of PS at the erythrocyte's outer leaflet may have other pathophysiologic implications relevant to CRF such as coagulation abnormalities (39–41).

In summary, the data reported here indicate that increased exposure of erythrocyte PS is present in both undialyzed patients with CRF and patients on CAPD or HD. Since surface-exposed PS may trigger several reactions, such abnormality might contribute to the red cell pathology commonly encountered in chronic uremia. In addition, since membrane phospholipid asymmetry is thought to be ubiquitous, our findings demonstrating an increased PS exposure in RBC may not be limited to RBC alone. It has been shown that the acquired erythrocyte-defective sodium transport in CRF can also be acquired by other cells (42). If surface-exposed PS was increased in other cells as well, this could at least partly explain some of the various cell derangements present in uremia.

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