**RED CELLS**

**Hereditary Spherocytosis and Elliptocytosis Erythrocytes Show a Normal Transbilayer Phospholipid Distribution**

By Kitty de Jong, Sandra K. Larkin, Stefan Eber, Paul F.H. Franck, Ben Roelofsen, and Frans A. Kuypers

Phosphatidylserine (PS) asymmetry was determined in red blood cells from patients with hereditary spherocytosis and elliptocytosis. No PS-exposing subpopulations were detected using the very sensitive method with fluorescently labeled annexin V. Treatment with N-ethylmaleimide or adenosine triphosphate (ATP) depletion to inactivate the flipase did not lead to formation of PS-exposing subpopulations in these cells, but elevated intracellular calcium levels did lead to extensive scrambling of the PS asymmetry. Although interactions of the membrane skeleton with the phospholipid bilayer have been suggested to stabilize the asymmetric distribution of PS across the bilayer, our data show that red blood cells with a severely damaged membrane skeleton are able to preserve asymmetry, even under conditions in which restoration of the asymmetric distribution is excluded. Moreover, the loss of membrane asymmetry in these cells requires active scrambling involving high levels of intracellular calcium as in normal cells. Our data show that the severe disorder of the membrane skeleton found in these cells does not affect the activity of flipase or scramblase, indicating that these proteins are not regulated by, or coupled to, the membrane skeleton assembly, and that possible thrombotic events in spherocytosis patients are not likely associated with altered PS topology of the red blood cells.

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**HEREDITARY SPHEROCYTOSIS** (HS) and hereditary elliptocytosis (HE) are heterogeneous disorders characterized by alterations in the interactions between proteins of the red blood cell (RBC) membrane skeleton, with reported molecular defects in ankyrin, spectrin, band 3, band 4.1, or band 4.2.1-3 The resulting instability of the membrane skeleton can be detected by altered morphology, osmotic fragility, or deformability.4 In normal red blood cell membranes, phosphatidylserine (PS) is exclusively located on the inner leaflet of the membrane (see Kuypers5 for review). This asymmetric distribution of PS is thought to be preserved by activity of the aminophospholipid translocase, or flipase,6 and can be disrupted by action of the scramblase,7 provided that the flipase activity is inhibited. Interactions of the membrane skeleton with the phospholipid bilayer have also been suggested to play a role in the stabilization of the asymmetric distribution of PS across the bilayer.8,9 It was reported that proteins such as spectrin and band 4.1 and 4.2 interact with PS,10-13 and it was logically assumed that the abnormal interaction between skeleton and the membrane bilayer, resulting from altered skeletal proteins, would affect phospholipid distribution. The phospholipid asymmetry in red blood cells from HS and hereditary pyropoikilocytosis patients with different levels of spectrin deficiency was studied using phospholipase degradation.14,15 No significant alterations were observed, but the presence of small subpopulations with altered phospholipid asymmetry would likely go undetected using this approach. Recently, fluorescently labeled annexin V (AV) has been used to detect subpopulations of red blood cells that expose PS in vivo.16,17 Some anemias, such as sickle cell disease17,18 and some forms of thalassemia,19,20 are characterized by the presence of a small but significant subpopulation of cells that expose PS. The presence of PS-exposing cells in these hemoglobinopathies is thought to result in an imbalanced hemostasis and to increase a risk for stroke.5 Whereas a thrombotic risk is usually not associated with HS or HE, cases of both arterial and venous thrombosis have been described.21 Interestingly, the Mg-adenosine triphosphatase (ATPase) activity, related to the flipase activity, was shown to be increased in HS red blood cell membranes, suggesting that the increased movement of PS from outer to inner monolayer compensates for the decreased interaction of PS with the membrane skeleton.22 We hypothesized that we would be able to identify (small) subpopulations of red blood cells that expose PS in the blood of HS and HE patients using fluorescently labeled AV, and that inhibition of the flipase would increase the size of this subpopulation. The decreased interaction with the skeleton could lead to an increased movement of PS to the outer monolayer, which would not be compensated by a deactivated flipase activity.

In this study, we inhibited the flipase in HS and HE red blood cells by rapid adenosine triphosphate (ATP) depletion or N-Ethylmaleimide (NEM) treatment, both well established procedures to inhibit Mg-ATPase-driven movement of PS from outer to inner monolayer. Fluorescently labeled AV in combination with flow cytometry was used to probe for the presence of PS-exposing cells, and treatment with calcium and ionophore was used as a control to scramble the glycerophospholipids across the membrane. We report that HS or HE erythrocytes do not lose PS asymmetry, even after overnight storage after the inhibition of the flipase, whereas increase in cytosolic calcium efficiently scrambles the PS asymmetry in these cells as in normal red blood cells.

**From Children’s Hospital Oakland Research Institute, Oakland, CA; Kinderklinik, Georg-August University Göttingen, Göttingen, Germany; Centraal Klinisch Chemisch Laboratorium, Ziekenhuis Leyenburg, Den Haag, the Netherlands; and Centre for Biomembranes and Lipid Enzymology, University of Utrecht, the Netherlands.**

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Address reprint requests to Frans A. Kuypers, PhD, Children’s Hospital Oakland Research Institute, 747 52nd St, Oakland, CA 94609.

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Fig 1. Osmotic deformability patterns of red blood cells are shown for: (A) Two patients with defined molecular disorders leading to spherocytosis (see Results); (B) Seven patients who present typical characteristics of hereditary spherocytosis, but for whom the molecular disorder is unknown; (C) Two patients with defined molecular disorders leading to elliptocytosis (see Results); (D) Five patients of the same family with defined molecular disorders leading to elliptocytosis or poikilocytosis (see Results); and (E) Three patients who present typical characteristics of hereditary elliptocytosis, but for whom the molecular disorder is unknown. In all graphs, the normal range of osmotic deformability curves is shown (±1 standard deviation [SD]), as well as one typical normal curve (Control).
**MATERIALS AND METHODS**

**Erythrocytes**

Blood in EDTA or acid citrate dextrose (ACD) anticoagulant was obtained after informed consent from healthy volunteers for control incubations, and from HS and HE patients of the Children’s Hospital Oakland (Oakland, CA) and San Francisco General Hospital (San Francisco, CA). Alternatively, cells from HS and HE patients were shipped from Göttingen, Germany, or Den Haag, the Netherlands, accompanied by shipment controls. Diagnosis of HS or HE was confirmed for each individual patient by ektacytometry using a Technicon ektacytometer (Technicon, Tarrytown, NY), as described earlier. Blood was stored at 4 to 6°C until used for the AV labeling or flipase inhibition experiments. Red blood cells were isolated by washing in Hank’s buffered saline solution (HBSS; Sigma, St Louis, MO), HEPES buffered saline (HBS: 10 mmol/L HEPES, 145 mmol/L NaCl, pH 7.4), or phosphate buffered saline (PBS: 10 mmol/L Na+/K+ phosphate, 145 mmol/L NaCl, pH 7.4) before further treatment.

**Flipase Activity and Inhibition**

NEM inhibits the flipase by reacting with a sulfhydryl group necessary for its activity. NEM (Aldrich, Milwaukee, WI) was added to a 30% hematocrit cell suspension to a final concentration of 10 mmol/L. After 30 minutes incubation at 37°C, the cells were pelleted, and the supernatant was used to determine hemolysis. The cells were then washed three times with at least 5 volumes of PBS or HBS to remove NEM. Flipase activity was measured as described before, using spin-labeled PS.

The movement of PS from outer to inner monolayer is driven by ATP consumption. To inhibit this movement, RBCs were resuspended to 10% hematocrit in ATP depletion buffer containing 10 mmol/L HEPES, 130 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L EGTA, 10 mmol/L inosine, 6 mmol/L iodoacetamide, and 5 mmol/L sodium tetraionate. After 2 hours incubation at 37°C, the cells were pelleted, and the supernatant was used to determine hemolysis. The cells were then washed three times with PBS or HBS. The treated cells were sampled.
for ATP determination directly after the incubations or after overnight storage at room temperature. Cytosolic ATP was determined as described earlier, using the bioluminescence method with luciferin/luciferase (ATP assay kit; Sigma) after precipitation of the cells with 6% trichloroacetic acid and expressed per cellular hemoglobin.

**Scrambling of Membrane Phospholipid Organization**

Membrane glycerophospholipid organization was disrupted by the use of calcium and ionophore as described before. RBCs at a 16% hematocrit were equilibrated in HBSS 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. The suspension was incubated for 1 hour at 37°C, washed with 5 mmol/L EDTA, washed with buffer containing 1% bovine serum albumin (BSA) to remove ionophore, and finally resuspended in buffer.

**Annexin V Labeling and Flow Cytometric Analysis**

The treated cells were labeled with AV essentially as described before, either directly after the incubations or after overnight storage at room temperature. Fluorescein isothiocyanate (FITC) conjugated human recombinant AV (FITC-AV) or green fluorescent protein AV (GFP-AV; a kind gift from Joel Ernst, University of California at San Francisco, San Francisco, CA) were used alternatively with similar results. Briefly, cells were resuspended at 0.05% hematocrit in HBSS containing 2 mmol/L CaCl2 and AV (75 to 150 ng/mL). After 30 minutes incubation at room temperature, the cells were pelleted at 10,000g for 10 seconds, and the supernatant was removed. The cells were then resuspended to approximately 106 cells/250 µL of HBSS containing 2 mmol/L CaCl2. Flow cytometry was carried out exactly as described earlier. The percentage of AV-positive cells was determined from the fluorescence signal in excess of that obtained with a negative (unlabeled) control for each sample.

**RESULTS**

**Characterization of Red Blood Cells by Ektacytometry**

All samples tested for phospholipid asymmetry were analyzed by ektacytometry. The osmotic deformability curves of all patients showed typical patterns for HS or HE (Fig 1).

For a number of patients, the molecular defect was known. Patient BB (Fig 1A) was characterized as having dominant spherocytosis with a deletion of methionin 663 or 664 in exon 16 of band 3. Patient LF (Fig 1A) had spherocytosis with a deletion of methionin 663 or 664 in exon 16 of band 3. Patient CH (Fig 1C) had spherocytosis with a frameshift in exon 11 (1369 del C) of band 3. Patient PZ and RG (Fig 1D) were of one family in which the HE defect was characterized as a C to A point mutation in exon 2 of α-spectrin (codon 28 ARG to CYS, also referred to as spectrin αLely polymorphism in the second allele, causing a poikilocytic appearance in the blood smear. In Fig 1E, the osmotic deformability patterns are observed between different unrelated patients or between patients from the same family, regardless of the presence of the α Lely polymorphism as an aggravating factor (Fig 1). All of the patients tested for phospholipid asymmetry for whom the molecular defect was unknown fell within the range indicated in Figs 1B and 1E.

**PS Exposure In Vivo**

To determine the number of cells in the circulation that expose PS, we used fluorescently labeled AV. GFP-AV has been shown to bind similarly to apoptotic cells as FITC-labeled human recombinant AV. Both probes were used in our experiments to label red blood cells that expose PS, with identical results.

RBCs from patients with HS and HE showed a similarly small fraction of labeled cells as normal control cells after labeling with fluorescently labeled AV (Table 1). The fraction of red blood cells in the blood of HS and HE patients was lower than 0.7% and not significantly increased, as compared with red blood cells in the blood of normal individuals. These results indicate that the subpopulation of PS-exposing, circulating cells in vivo is very small indeed in normal individuals and not increased in the HS and HE patients.

**Inhibition of the Flipase**

NEM treatment. Normal RBCs treated with 10 mmol/L NEM for 30 minutes showed a slight increase in the subpopulation that exposed PS from 0.3% to 0.8% (Table 2), indicating no significant increase as compared with normal levels (Table 1). The slight decrease after overnight (±20 hours) incubation of these cells coincided with a low level of hemolysis, suggesting that some of these PS-exposing cells had lysed during the incubation. The flipase activity in the NEM treated cells, as measured by the movement of spin-labeled PS from outer to inner monolayer, was inhibited as reported before (not shown). Taken together, these data indicate that NEM treatment and resulting deactivation of the flipase do not lead to the exposure of PS in a subpopulation of normal red blood cells.

Similarly, NEM treatment did not lead to PS exposure in RBCs from HS and HE patients (Table 2). No significant increase was found in the PS-exposing fraction of red blood cells as compared with normal control cells. After additional overnight storage of these treated cells at room temperature, this

<table>
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<th>Table 1. Labeling of Red Blood Cells With Fluorescent Annexin V</th>
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<td><strong>Percentage of Cells Labeled</strong></td>
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<tr>
<td>Normal</td>
</tr>
<tr>
<td>HS patients</td>
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<td>HE patients</td>
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fraction did not increase. In summary, these data indicate that flipase inhibition by NEM treatment does not lead to the exposure of PS in subpopulations of normal, HS, or HE red cells.

Rapid ATP depletion. Because NEM treatment, although effective in the inhibition of the flipase, can also lead to other effects as the result of its reaction with protein thiol groups, we inhibited flipase activity by rapidly decreasing ATP levels in a buffer containing 6 mmol/L iodoacetamide and 5 mmol/L tetrathionate. The ATP levels at the start of the experiments were 3.4 ± 0.6 µmol/g hemoglobin for normal cells and 3.9 ± 0.7 µmol/g hemoglobin for HS and HE cells and were not significantly different. Less than 2% of the original ATP level was found in the normal control cells after 2 hours ATP depletion, and less than 1% was found in the treated HS or HE cells. The flipase activity was severely inhibited as expected and reported before (Seigneuret and Devaux, not shown).

As was found with NEM treatment, neither normal RBC nor HS and HE cells showed exposure of PS (Table 3), indicating that flipase inhibition by ATP depletion did not lead to a scrambling of the bilayer. Even overnight incubation did not lead to the exposure of PS.

Scrambling In Vitro

The data above indicate that PS moves very slowly from inner to outer monolayer in normal, HS, or HE cells. Even in the absence of flipase activity, PS exposure is only observed in a very small subpopulation. To determine if scrambling of the phospholipids could be invoked in HS and HE cells as in normal cells, RBCs were treated with calcium and the ionophore A23187 to increase the intracellular calcium level, and subsequently labeled with fluorescent AV. Additionally, the cells were treated with calcium and ionophore after pretreatment with NEM or after ATP depletion to observe the additional effect of flipase inhibition on the potential of the cells to scramble their membrane bilayers.

Approximately half of the normal RBCs lost PS asymmetry on treatment with 1 mmol/L calcium in the presence of 4 µmol/L ionophore for 1 hour. It seemed likely that, in the other half of the cells, the flipase is able to restore asymmetry rapidly when calcium is removed after the incubation. This was confirmed by the fact that this restoration process is inhibited in normal cells pretreated with NEM to inhibit the flipase, leading to scrambling of the bilayer in virtually all RBCs after calcium ionophore treatment. Although the flipase is inhibited in ATP-depleted cells, the fraction of cells able to scramble does not increase in these cells and is usually slightly lower than in cells treated with calcium and ionophore only, indicating that ATP is needed for scrambling of the bilayer. Again, no difference was observed between normal and HS, or HE cells.

DISCUSSION

In the early 1980s, the membrane skeleton was suggested to be of major importance in sustaining the distribution of the main phospholipids across the red blood cell membrane, and many publications followed, addressing the interaction between PS and components of the membrane skeleton. After discovery of the ATP-consuming flipase, the role of skeleton became redefined as an “energy preserving” system. Studies implied that membrane asymmetry would be lost on disconnection of the bilayer from the underlying skeleton and that the flipase was the assigned “repair” mechanism for these occasions. Recently, a publication by Vermeulen et al. confirmed this concept by showing an increased Mg-ATPase activity in HS red blood cell membranes, suggesting that the increased outward translocation (flop) of PS, expected to result from the disorder in the membrane skeleton, would be compensated by an increase in flipase activity.

On the other hand, in the past few years, evidence has cumulated that membrane phospholipid asymmetry is maintained unless a process is activated to scramble the bilayer. In studies of cell structures without an intact membrane skeleton, membrane asymmetry was shown to be preserved even in absence of flipase activity, reducing the role of the membrane skeleton to a minimum. Recently, a calcium-induced scramblase was cloned, indicating protein involvement in activating the scrambling process. Although the current assumption seems to be that membrane phospholipid asymmetry is regulated entirely by contributions of flipase and scramblase, a small role for the membrane skeleton in maintaining PS at the inner face of the bilayer would be adequate based on the findings by Vermeulen et al.

We hypothesized that inhibition of the flipase would increase the number of scrambled, PS-exposing cells in a population of HS or HE red blood cells. The decreased interaction with the skeleton in these cells would lead to an increased movement of PS to the outer monolayer, which would not be compensated by the deactivated flipase.

We chose HS and HE patients identified by ektacytometry, which indicates the severity of the membrane disorder. Previous reports have shown that the spectrin decrement correlates well with the degree of spherocytosis, which is a function and measure of the ratio of membrane surface area to volume and is the determining factor for the reduction of deformability that can be shown by ektacytometry. Our data in Fig 1 show that the red blood cells of our patients were as severely affected as the ones that were characterized at a molecular level.

We applied both sulfhydryl modification of the flipase and ATP depletion on the selected cells to account for side effects...
that each of these methods might have. To allow for slow translocation of the phospholipids, we subsequently incubated the cells overnight at room temperature. Using AV in combination with flow-cytometric analysis, we are able to identify subpopulations of PS-exposing cells as low as 0.5% of the total population. Our data (Tables 1 to 3) show that under no condition did the subpopulation of PS-exposing HS or HE cells increase compared with control cells. We can conclude that asymmetry is maintained in HS or HE cells, despite inhibition of the flipase. This indicates that intact HS or HE red blood cells do not need to compensate for loss of phospholipid asymmetry by increased flipase activity and implies that the membrane skeleton does not seem to play a significant role in sustaining membrane asymmetry in the RBCs. On the other hand, the study by Vermeulen et al.\textsuperscript{22} was done with isolated erythrocyte membrane asymmetry in the RBCs. On the other hand, the skeleton does not seem to play a significant role in sustaining membrane asymmetry by increased flipase activity and implies that the membrane skeleton does not seem to play a significant role in sustaining membrane asymmetry in the RBCs. On the other hand, the study by Vermeulen et al.\textsuperscript{22} was done with isolated erythrocyte membranes (ghosts), and spectrin may have been detached from the membranes of HS ghosts during preparation. Although our experiments contraindicate involvement of the membrane skeleton in the regulation of membrane phospholipid asymmetry, some remaining interaction of spectrin with phospholipids cannot be excluded.

In other hemolytic anemias, such as sickle cell disease and thalassemia,\textsuperscript{5,17-19} a subpopulation of PS-exposing RBCs can be found. These hemoglobinopathies are characterized by mutations in hemoglobin that lead to damage to the membrane, which could contribute to exposure of PS on the cell surface. Our data show that impairment of the membrane skeleton by itself does not lead to this defect. Although the presence of PS-exposing cells is thought to contribute to an imbalance in hemostasis,\textsuperscript{6} reported thrombotic events\textsuperscript{21} in HS or HE can likely not be attributed to the exposure of PS on red blood cells. Moreover, our data indicate that hemolytic anemia or stress erythropoiesis does not necessarily lead to a loss of phospholipid asymmetry.

We also investigated the ability of HS and HE cells to scramble their phospholipids. Complete disruption of red blood cell PS asymmetry is only found after loading of the cells with calcium (using a calcium ionophore) after flipase inhibition by NEM (see Table 4 and De Jong et al\textsuperscript{31}). If the NEM treatment is not applied, approximately half of the RBCs will restore asymmetry on removal of calcium because of sufficient activity of the flipase. Although ATP depletion also inhibits the flipase activity, incomplete scrambling (and often reduced scrambling compared with normal RBCs treated with calcium and ionophore) of PS is observed when ATP-depleted cells are loaded with calcium (Table 4, Sulpi ce et al\textsuperscript{31} and Martin et al\textsuperscript{32}). This discrepancy suggests that ATP is involved in the calcium-induced exposure of PS. An ATP-dependent flipase was reported to play a role in the loss of phospholipid asymmetry.\textsuperscript{33} The recently characterized scramblase\textsuperscript{7} is activated by intracellular calcium by an as yet incompletely understood mechanism, but cellular levels of ATP may play a role. The ATP requirement may point at the involvement of protein phosphorylation, but it seems that tyrosine phosphorylation is not required for scrambling of membrane lipids.\textsuperscript{34} We and others have shown that scrambling may need the presence of phosphatidylinositol 4,5-biphosphate (PIP\textsubscript{2}) in the membrane.\textsuperscript{31,35} On the other hand, another ATP-dependent regulation of the scramblase might be involved.

In summary, we have shown that HS and HE red blood cells do not expose PS on their membrane surface, even after prolonged incubation in absence of flipase activity, indicating that reported thrombotic events in these diseases are not related to the appearance of PS on a red blood cell subpopulation. However, these cells are capable of scrambling their membranes and exposing PS after loading with calcium caused by activation of the scramblase, just as normal red blood cells. Our data indicate that cells with an impaired flipase and disrupted membrane skeleton can preserve their membrane asymmetry and that an active process that requires a phosphorylated state of the cell is needed to induce exposure of PS on the membrane surface. We conclude that there is no functional change in the regulation of membrane asymmetry in HS or HE cells compared with normal RBCs, regardless of a potential binding of PS to components of the membrane skeleton. Moreover, the severe disorder of the membrane skeleton found in the HS and HE cells does not affect the activity of flipase or scramblase, indicating that these proteins are not regulated by nor coupled to the membrane skeleton assembly.

**ACKNOWLEDGMENT**

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**Table 4. Percentage of Red Blood Cells Labeled With Fluorescent Annexin V After Scrambling of the Bilayer With Calcium and Ionophore**

<table>
<thead>
<tr>
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<th>Not Pretreated</th>
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<th>ATP Depleted</th>
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<tr>
<td>Normal</td>
<td>52 ± 10 (n = 11)</td>
<td>96 ± 2 (n = 9)</td>
<td>47 ± 9 (n = 6)</td>
</tr>
<tr>
<td>HS/HE patients</td>
<td>47 ± 11 (n = 9)</td>
<td>90 ± 7 (n = 9)</td>
<td>41 ± 13 (n = 9)</td>
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