Altered Plasma Membrane Phospholipid Organization in *Plasmodium falciparum*-Infected Human Erythrocytes

By R.S. Schwartz, J.A. Olson, C. Raventos-Suarez, M. Yee, R.H. Heath, B. Lubin, and R.L. Nagel

The intraerythrocytic development of the malaria parasite is accompanied by distinct morphological and biochemical changes in the host cell membrane, yet little is known about development-related alterations in the transbilayer organization of membrane phospholipids in parasitized cells. This question was examined in human red cells infected with *Plasmodium falciparum*. Normal red cells were infected with strain FCR3 or with clonal derivatives that either produce (K) or do not produce (K') knobby protuberances on the infected red cells. Parasitized cells were harvested at various stages of parasite development, and the bilayer orientation of red cell membrane phospholipids was determined chemically using 2,4,6-trinitrobenzene sulphonic acid (TNBS) or enzymatically using bee venom phospholipase A2 (PLA2) and sphingomyelinase C (SMC). We found that parasite development was accompanied by distinct alterations in the red cell membrane transbilayer distribution of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). Increases in the exoplasmic membrane leaflet exposure of PE and PS were larger in the late-stage parasitized cells than in the early-stage parasitized cells. Similar results were obtained for PE membrane distribution using either chemical (TNBS) or enzymatic (PLA2 plus SMC) methods, although changes in PS distribution were observed only with TNBS. Uninfected cohort cells derived from mixed populations of infected and uninfected cells exhibited normal patterns of membrane phospholipid organization. The observed alterations in *P. falciparum*-infected red cell membrane phospholipid distribution, which is independent of the presence or absence of knobby protuberances, might be associated with the drastic changes in cell membrane permeability and susceptibility to early hemolysis observed in the late stages of parasite development.

MALARIA is the world's most common form of hemolytic anemia and remains a major public health problem in many areas of the world. In 1983 the number of malaria cases reported worldwide was 5.5 million, and it is estimated that approximately 65% of the world's population lives in malarious areas.

Involvement of red cell membrane glycoproteins has been implicated in the attachment of parasites to the membrane, and parasite maturation has been shown to result in a redistribution of spectrin, the major membrane skeletal protein. Alterations in the morphology of the infected cells as well as changes in their osmotic fragility, membrane deformability, and permeability to cations, amino acids, and glucose strongly suggest that membrane lipids are also affected by parasitization.

In normal human erythrocytes, the phospholipids comprising the plasma membrane are distributed asymmetrically between the bilayer leaflets. This asymmetric distribution results in the exoplasmic leaflet being enriched in phosphatidylcholine (PC) and sphingomyelin (SM) while the cytoplasmic leaflet is enriched in phosphatidylethanolamine (PE) and contains all of the phosphatidylserine (PS). Alterations in the bilayer distribution of membrane phospholipids have been noted in several pathologic erythrocytes including sickle cells, spherocytes due to spectrin deficiency, elliptocytes due to protein 4.1 deficiency, as well as in erythrocytes undergoing drug-induced endocytosis. We have investigated the organization of membrane phospholipids in human red cells infected with knobby and knobless strains of *Plasmodium falciparum* and determined that significant changes occur in their bilayer distribution during the intracellular maturation of the parasites.

MATERIALS AND METHODS

Cultivation of *P. falciparum*. Parasites were grown at a 5% cell suspension by the method of Jensen and Trager. The FCR3 strain was maintained in synchronous development by a combination of sorbitol treatment and gelatin enrichment, as proposed by Kile-jian. Sorbitol treatment resulted in cultures highly enriched in "early ring" stage-infected cells. Gelatin enrichment resulted in cultures that were predominantly (75%) late-stage parasites (trophozoites and schizonts). Knobby (A2,K') and knobless (D4,K') clones of FCR3 were synchronized with sorbitol treatment only. The extent of parasitemia and stage of development was determined by Giemsa-stained cells.

For experiments using the FCR3 strain of *P. falciparum*, fresh, washed normal human red cells were inoculated with late schizonts obtained from gelatin supernatants (parasitemia >80%) to a final parasitemia of 5% and plated at 1% cell suspension prior to incubation. For experiments using the K' or K clones of FCR3, cultures were first grown at a 1% cell suspension to a parasitemia of 30%. This material was diluted to 5% parasitemia with fresh, washed red cells and plated as for FCR3. For controls, uninfected red cells from the same donor as the infected samples were incubated simultaneously.

Late ring-infected cells were collected from culture approximately 24 hours after inoculation. Trophozoite-infected cells were collected at approximately 36 hours and schizont-infected cells at approximately 48 hours. In some experiments FCR3 trophozoite- or schizont-infected cells were enriched from uninfected red cells by gelatin treatment.

Determination of erythrocyte plasma membrane phospholipid...
Red cell membrane phospholipid organization was determined enzymatically using phospholipase A$_2$ (PLA$_2$, from bee venom) and sphingomyelinase C (SMC, from Staphylococcus aureus) or chemically using the amino-labeling reagent trinitrobenzenesulfonic acid (TNBS). In the absence of extensive cell hemolysis, these probes do not penetrate the membrane bilayer and react only with those phospholipids exposed on the red cell membrane surface. Therefore, the extent to which the various classes of phospholipids react with these probes is a measure of their exposure in the membrane exoplasmic leaflet.

At various stages of parasite development, 0.1 mL washed packed red cells were treated with 12.5 or 25 units PLA$_2$ at 37°C for 45 minutes followed by 1.5 or 3 units SMC for 30 minutes, or with 5 mmol/L TNBS for 1 hour in a total volume of 4 mL (final hematocrit of 2%). The buffer used for the PLA$_2$/SMC experiments contained 10 mmol/L sodium phosphate, pH 7.4, 10 mmol/L sodium chloride, 0.25 mmol/L magnesium chloride, 0.5 mmol/L calcium chloride, 130 mmol/L potassium chloride and 10 mmol/L glucose; while that used for the TNBS experiments contained 50 mmol/L sodium phosphate, pH 8.5, 1.2 mmol/L potassium phosphate, 120 mmol/L sodium chloride, 5 mol/L potassium chloride, 0.5% bovine serum albumin, and 5.5 mmol/L glucose. Following the incubation the cells were collected by centrifugation, and the extent at cell hemolysis was determined by the supernatant absorbance of 540 nm. Enzyme-treated cells were immediately lysed with 0.5 mL 5 mmol/L EDTA (pH 7.4), while TNBS-treated cells were washed once in the incubation buffer (pH 7.4), twice in 0.9% saline (pH 7.4), and then lysed with 0.5 mL water. The lipids were extracted, individual phospholipid classes separated by thin-layer chromatography, and quantitated by phosphorous determination. The percentage of phospholipid hydrolysis (for enzyme-treated cells) or modification (for TNBS-treated cells) was calculated as the ratio of native (hydrolysed or modified) phospholipid to the amount of total phospholipid in the extractable membrane phospholipid.

Hemoglobin electrophoresis. Aliquots of the red cell lysates prepared from the control or TNBS-treated cells were subjected to hemoglobin electrophoresis on cellulose acetate strips. Each sample contained the equivalent of from 5 to 10 μL packed cells; the electrophoresis was performed at pH 8.5 at 380 V for 20 minutes at 24°C in a Helena Electrophoresis Chamber (Helena Labs, Beaumont, TX). The electrophoresed cellulose acetate strips were stained in Ponceau S stain (0.5% wt/vol) Ponceau S, 3.5% wt/vol) sulfosalicylic acid, 3.5% wt/vol) trichloroacetic acid and then destained in 5% acetic acid.

RESULTS

Plasma membranes from normal uninfected human erythrocytes maintained in cell culture for periods of time equivalent to those of the P. falciparum-infected cells (24 to 48 hours) contained approximately 32% PC, 28% PE, 14% PS, and 25% SM. The total amount of extractable membrane phospholipid in the late-stage parasitized cell populations was about 20% greater than in the uninfected control or early-stage parasitized cells. This increase is likely due to presence of a parasitophorous membrane that encapsulates the parasites and develops during parasite maturation. Membranes from infected cells contained similar proportions of membrane phospholipids, suggesting that parasitization and development of the parasitophorous membrane was not accompanied by conversion of one phospholipid species to another (Table 1). Subjecting normal uninfected erythrocytes to phospholipid hydrolysis using PLA$_2$ and SMC yielded the pattern given in Table 2. Combined PLA$_2$ and SMC treatments yielded hydrolysis of 75% ± 3% of the PC, 27% ± 2% of the PE, none of the PS, and 59% ± 1% of the SM. In contrast to uninfected cells, P. falciparum-infected cells containing early-stage (ring-form) parasites showed a moderate increase in PE hydrolysis (33% ± 6%, P < 0.001) with a concomitant decrease in PC hydrolysis (57% ± 5%, P < 0.001). Hydrolysis of SM (54% ± 6%) was not significantly different from that of the uninfected cells (59% ± 1%), nor was there any hydrolysis of PS. Intracellular maturation of the parasites to trophozoites and schizonts resulted in further increases in PE hydrolysis (46% ± 6%, P < 0.001). Infected cells containing clonal derivatives producing knobs (K') also showed marked elevations in PE hydrolysis that progressively increased as the parasites matured from early-stage forms to late-stage forms (34% ± 8% and 44% ± 7%, respectively, P < 0.001). Similarly, infected cells containing clonal derivatives lacking knobs (K) exhibited increased PE hydrolysis (42% ± 2% in the late-stage forms, P < 0.001, not measured in the early-stage forms). Decreases in the extent of PC hydrolysis were also observed in both the K' omitted and K-infected cells, similar to those seen in the uncloned FCR3-infected cells. Membrane organization of SM was examined only in the early-stage parasitized cells where no differences were observed, nor was there any evidence for PS hydrolysis in any of the infected cells. As noted in Table 1, maturation of the parasites resulted in an approximate 20% increase in total extractable membrane phospholipid.

Although our data indicate that parasitized cell membranes demonstrate a loss of phospholipid asymmetry, we can only speculate on whether such changes represent a complete loss of asymmetry in subpopulations of the infected cells or a partial loss of asymmetry in all of the infected cells. To address this issue we performed calculations using the data given in Table 2 and compared the calculated values to the measured values for phospholipid hydrolysis. For example, considering PE hydrolysis in the synchronous cell culture (Syn)-infected cells, calculations assuming 100% PE hydrolysis (ie, complete loss of PE asymmetry) in all of the parasitized cells yielded an average value of 40% PE hydrolysis. This calculated value compares favorably with the measured value of 46% ± 6% PE hydrolysis by PLA$_2$ and SMC in the Syn-late-stage infected cells. Calculations assuming only 50% PE hydrolysis in all of the parasitized cells yielded an average of 31% PE hydrolysis. This calculated value agrees closely with the measured value of 33% ± 6% PE hydrolysis in the Syn-early-stage infected cells. Similarly, calculations for PE hydrolysis in the K' and K-infected cells yielded an average of 36% PE hydrolysis if all of the PE had translocated to the exoplasmic leaflet and 30% if only 50% of the PE had translocated. These calculated values agree favorably with the measured values of 44% ± 7% and 34% ± 8% for K'-late- and K'-early-stage infected cells, respectively. Thus, our calculations suggest but do not prove, that in early-stage parasitemia only a portion (~50%) of cytoplasmic leaflet PE becomes accessible to the exoplasmic leaflet, whereas in late-stage parasitemia essentially all of...
the cytoplasmic leaflet PE becomes accessible to the exoplasmic leaflet.  

An alternative method for detecting phospholipids exposed on the surface of cells involves chemically labeling the aminophospholipids using 2,4,6-trinitrobenzene sulfonic acid (TNBS). This reagent only slowly penetrates living cells and has been used by many laboratories to covalently label aminophospholipids exposed on the surface of both normal and pathologic erythrocytes. We obtained similar results using TNBS as we did using phospholipases. As shown in Table 3, uninfected cells maintained in cell culture exhibited labeling of both PE (9 ± 3%) and PS (2% ± 5%). The extent of PE labeling increased dramatically in the parasitized cells; the uncloned FCR3 ring-form parasitized cells showed a threefold increase in PE labeling (P < 0.001) that continued to increase as the parasites matured to trophozoites (fivefold increase, P < 0.001). Similar increases in PE labeling were also seen in the K⁺ and K⁻ clonal derivatives; however, the extent of increase (two- to threefold, P < 0.001) was about 30% less than that seen in the FCR3-infected cells. This may be due to the lower levels of parasitemia achieved in the clonal derivatives or may represent a selective effect of cloning. The extent of PS labeling showed a slightly different pattern from that of PE labeling; none of the early stage parasitized cells showed labeling of PS above uninfected cell values regardless of whether the parasites were of the FCR3, K⁺, or K⁻ variants. However, cells infected with late (trophozoite)-stage parasites showed small but statistically significant increases in PS labeling (P < 0.05 for K⁺-trophozoites, P < 0.003 for K⁻-trophozoites, and 0.001 for K⁻-schizonts).

<table>
<thead>
<tr>
<th>Table 2. Plasma Membrane Phospholipid Organization of Human Erythrocytes Infected with P Falciparum</th>
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<tbody>
<tr>
<td>Erythrocyte</td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>Normal erythrocytes maintained in cell culture</td>
</tr>
<tr>
<td>Syn-early stage (rings)</td>
</tr>
<tr>
<td>Syn-late stage (trophozoites + schizonts)</td>
</tr>
<tr>
<td>K⁺-early stage</td>
</tr>
<tr>
<td>K⁺-late stage</td>
</tr>
<tr>
<td>K⁻-early stage</td>
</tr>
<tr>
<td>K⁻-late stage</td>
</tr>
</tbody>
</table>

Erythrocytes (0.1 mL packed cells, 2% final hematocrit) were treated with 25 units bee venom phospholipase A₂ for 45 minutes at 37 °C followed by three units Staphylococcus aureus sphingomyelinase C for 30 minutes at 37 °C. Erythrocytes infected with P falciparum strain FCR3 were maintained in synchronous cell culture (Syn) as were the cells infected with clonal derivatives that produce (K⁺) or do not produce (K⁻) knobby protuberances. Membrane phospholipids were separated and quantitated as in Table 1. Treatment-induced cell hemolysis was < 0.5%.

Values represent the mean (± 1 SD) of from three to eight determinations. Significantly different from normal erythrocytes at P < 0.001,* or P < 0.05.†

ND = Not determined.
phospholipids were separated and quantitated as in Table 1. Staphylococcus aureus by 1.5 units (Syn-trophozoites); this increase was most marked in the schizont-infected with late-stage forms of strain FCR3 maintained in synchronous cell culture (Syn) were separated by sorbitol treatment. Membrane phospholipids were separated and quantitated as in Table 1. Treatment-induced cell hemolysis was <5%.

Table 3. Plasma Membrane Phospholipid Organization of Human Erythrocytes Infected with P Falciparum

<table>
<thead>
<tr>
<th>Erythrocyte</th>
<th>Parasitemia</th>
<th>% Phospholipid Labeled by TNBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal erythrocytes maintained in cell culture</td>
<td>0</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>Syn-rings</td>
<td>18</td>
<td>24 ± 3*</td>
</tr>
<tr>
<td>Syn-trophozoites</td>
<td>17</td>
<td>41 ± 8*</td>
</tr>
<tr>
<td>Syn-schizonts</td>
<td>20</td>
<td>43 ± 6*</td>
</tr>
<tr>
<td>K⁺-trophozoites</td>
<td>14</td>
<td>16 ± 1*</td>
</tr>
<tr>
<td>K⁺-rings</td>
<td>13</td>
<td>30 ± 7*</td>
</tr>
<tr>
<td>K⁺-trophozoites</td>
<td>12</td>
<td>29 ± 4*</td>
</tr>
</tbody>
</table>

Erythrocytes (0.1 mL packed cells, 2% final hematocrit) were treated with 5 mmol/L TNBS for 1 hour at 37 °C. FCR3-parasitized cells were maintained in synchronous cell culture (Syn) as were the cells infected with clonal derivatives that produce (K⁺) or do not produce (K⁻) knobby protuberances. Membrane phospholipids were separated and quantitated as in Table 1. Treatment-induced cell hemolysis was < 5%.

Table 4. Plasma Membrane Phospholipid Organization of Uninfected Cohort Populations from P Falciparum-Infected Human Erythrocytes

<table>
<thead>
<tr>
<th>Erythrocyte</th>
<th>Parasitemia</th>
<th>Phospholipid Hydrolysed, % of Total Phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal erythrocytes maintained in cell culture</td>
<td>0</td>
<td>PC 64 ± 1 PE 14 ± 2 PS 0 SM 26 ± 2 Total PL 1.7 ± 0.2</td>
</tr>
<tr>
<td>Uninfected cohort cells from Syn-late-stage infected mixed cell populations</td>
<td>&lt;1</td>
<td>PC 72 ± 8 PE 15 ± 1 PS 0 SM 31 ± 7 Total PL 1.8 ± 0.2</td>
</tr>
</tbody>
</table>

Erythrocytes (0.1 mL packed cells, 2% final hematocrit) were treated with 12.5 units bee venom phospholipase A₂ for 45 minutes at 37 °C followed by 1.6 units Staphylococcus aureus sphingomyelinase C for 30 minutes at 37 °C. Uninfected erythrocytes from mixed populations of erythrocytes infected with late-stage forms of strain FCR3 maintained in synchronous cell culture (Syn) were separated by sorbitol treatment. Membrane phospholipids were separated and quantitated as in Table 1. Treatment-induced cell hemolysis was < 3%.

Results for total extractable membrane phospholipid (Total PL) are given as µg phosphate/0.1 mL packed cells. Values represent the mean (±1 SD) of from eight to 12 determinations. Significantly different from normal erythrocytes at P < 0.001, * P < 0.05, † or P < 0.003‡.

It should be noted that our measurements given in Tables 1 to 3 were made using mixtures of both infected and uninfected (cohort) cells. Since <20% of the cells contained parasites, it is likely that the relatively small changes in phospholipid organization we found for PS in these cell mixtures actually represented much larger changes in the parasitized cells, assuming that cohort cells were unaffected. To address this issue directly we separated cohort cells from parasitized cells by sorbitol treatment²⁴ and examined their membrane phospholipid organization using phospholipases. As shown in Table 4, we found no evidence to indicate that membrane phospholipid organization was abnormal in cohort cells. To minimize hemolysis during cell treatment we used smaller amounts of enzymes in the cohort cell experiments than in the experiments reported in Table 2; therefore, the values obtained for cohort cell membrane phospholipid hydrolysis were somewhat lower.

It is obvious that the validity of using either enzymatic hydrolysis or chemical modification of phospholipids to probe for the exoplasmic leaflet content of phospholipids depends on the reagents being restricted to exoplasmic leaflet phospholipids. Although the extent of cell hemolysis is the most often used indicator of an intact (and thereby impermeable to the probe) cell membrane, it is certainly not the most accurate method, since even small amounts of penetration by the probes could result in the hydrolysis or modification of cytoplasmic leaflet phospholipids. To more accurately determine the extent of probe penetration in our assay system, we made use of the fact that TNBS covalently labels both phospholipids and proteins containing primary amines. Penetration of intact cells by TNBS would, therefore, result in the labeling of hemoglobin. TNBS-labeled hemoglobin is more negatively charged than is native hemoglobin and can be separated and quantitated by standard hemoglobin electrophoresis techniques. When we analyzed hemolysates prepared from the TNBS-treated cells, we did not find evidence for hemoglobin modification unless the cells were first lysed prior to the addition of TNBS (Fig 1). It can be argued that a TNBS-hemoglobin adduct might not be visible electrophoretically if only a small percentage of the added TNBS actually penetrated the cell membrane. Therefore, to evaluate the sensitivity of our system for detecting TNBS-hemoglobin, we incubated normal uninfected erythrocyte hemolysates (made from a 2% hematocrit) with varying concentrations of TNBS (0 to 5 mmol/L) and examined the electrophoretically separated hemoglobin for the presence of TNBS-hemoglobin. We found that TNBS concentrations of >0.25 mmol/L complete modification of hemoglo-
The evidence presented here suggests that infection and intraerythrocytic maturation of the malaria parasite *Plasmodium falciparum* is associated with profound alterations in the bilayer distribution of phospholipids in the infected host cell membrane. Using two different probes (enzymatic and chemical) to study red cell membrane bilayer phospholipid organization, we found that parasitization resulted in a partial loss of the normal asymmetric organization of membrane phospholipids as evidenced by the appearance of increased amounts of phosphatidylethanolamine (PE) and phosphatidylserine (PS) and a decreased amount of phosphatidylcholine (PC) in the exoplasmic leaflet of infected cells. The extent of these alterations was dependent upon the stage of parasite development; cells infected with early (ring-form) stages showed a partial redistribution of PE and PC but not PS, whereas cells infected with late (trophozoite- and schizont-form) stages also showed the above plus a partial redistribution of PS. Uninfected cohort cells that were cultured along with the infected cells exhibited normal patterns of membrane phospholipid organization. Other workers have similarly reported that cohort cells do not display membrane abnormalities. For example, Sherman and Greenan, who examined membrane fluidity in *P. falciparum*-infected human erythrocytes, found no evidence for abnormalities in the uninfected cohort cells.

Redistribution of membrane phospholipids was found to occur in the FCR3 as well as in the knobby (K+) and knobless (K-) variants, suggesting that the appearance of knobby protuberances in the infected cells is not associated with specific alterations in the bilayer distribution of membrane phospholipids. Since only the K+ strain is capable of venous sequestration, we conclude that phospholipid redistribution is not involved in the interaction of parasitized cells with endothelium. This conclusion is supported by other workers who have reported that alterations in the phospholipid organization of human erythrocyte membranes does not enhance their adherence to endothelium. However, increased exoplasmic leaflet content of aminophospholipids, in particular PS, has been shown to stimulate the adherence of affected red cells to mononuclear phagocytes. Whether or not alterations in membrane phospholipid organization in parasitized red cells affect this aspect of the pathophysiology of the disease remains speculative; however, parasitized cells are known to be sequestered in large numbers by the spleen and to undergo increased erythropagocytosis and adherence to autologous macrophages.

Whereas we were able to detect increases in the exoplasmic leaflet content of PE using both the enzymatic (PLA2 and SMC) and chemical (TNBS) probes, increases in the exoplasmic leaflet content of PS were only apparent using TNBS. Differences in the sensitivity of the probes for phospholipids could be due to the different region of the phospholipid molecule to which each probe reacts; the phospholipases react with the fatty acid-glycerol ester bond that is presumably buried within the hydrophobic core of the bilayer, whereas TNBS labels the aminophospholipid head group that is presumably exposed on the bilayer surface. Similarly, differences in probe sensitivity may be responsible...
for the different extents of exoplasmic leaflet PE measured using TNBS and phospholipases in the cultured uninfected erythrocytes.

Other investigators have similarly reported that the plasma membrane of *P. falciparum*-infected red cells exhibits abnormal properties, including increases in membrane disordering (fluidity) and a rearrangement of intramembranous particles. In this regard, Gupta and Mishra reported a redistribution of membrane phospholipids in rhesus monkey red cells infected with *Plasmodium knowlesi*. These authors observed large increases in the exoplasmic leaflet content of PE along with large decreases in the content of PC in that bilayer leaflet. In contrast to our results for *P. falciparum*-infected human red cells, *P. knowlesi*-infected monkey red cells did not exhibit any alteration in the bilayer distribution of PS. Such differences could be due to differences between monkey and human red cells, species differences between *P. knowlesi* and *P. falciparum*, or the different intraerythrocytic stages of infection examined (Gupta and Mishra examined only ring-stage-infected cells where we similarly did not find a redistribution of PS). However, the fact that infection of human and monkey red cells by different species of *Plasmodium* both resulted in the bilayer redistribution of membrane phospholipids strongly suggests that phospholipid reorganization may be a common event during the course of the intraerythrocytic maturation of malaria parasites. Alterations in the organization of phospholipids in the parasitized cell membranes could result in many of the observed membrane abnormalities, including enhanced permeability to cations and nutrients, and may represent a membrane whose integrity is compromised as a result of modifications in membrane lipid-lipid or lipid-protein interactions. This loss of red cell membrane integrity could be a significant component in the early hemolysis of infected cells, a necessary requisite for the continuation of the intraerythrocytic asexual portion of *P. falciparum*’s life cycle.

Although we did not specifically examine the mechanism by which phospholipid redistribution occurs in parasitized cells, it is likely to involve alterations in the rate of phospholipid translocation between bilayer leaflets. In this regard the bilayer translocation of PC in normal human erythrocytes is a relatively slow process, whereas the rate is dramatically increased in sickled erythrocytes. It is also noteworthy that sickle erythrocytes exhibit increased exoplasmic leaflet amounts of PE and PS. Thus an increased interaction of the phospholipid probes with PE and PS may not represent a static increase in the exoplasmic leaflet content of those phospholipids but rather an increased rate at which they are translocated between bilayer leaflets and, hence, become available to interact with the probes.

**ACKNOWLEDGMENT**

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