Effects of hydroxyurea on the membrane of erythrocytes and platelets in sickle cell anemia

Background and Objectives. Adhesion molecules on the surface of erythrocytes, leukocytes and platelets are involved in vascular occlusion in sickle cell anemia. Hydroxyurea treatment of sickle cell anemia patients leads to clinical improvement and reduces the incidence of vaso-occlusive episodes. It has been previously demonstrated that hydroxyurea treatment also reduces the expression of adhesion molecules on the surface of erythrocytes. Phosphatidylserine (PS) exposure on the surface of erythrocytes has been considered to be the main determinant of altered erythrocyte adhesion in sickle cell anemia. In this study we examine the expression of PS on the surface of erythrocytes and platelets of sickle cell anemia patients before and during treatment with hydroxyurea.

Design and Methods. Blood samples from 15 sickle cell anemia patients were analyzed before and during treatment with hydroxyurea. The profile of PS expression was examined by flow cytometry.

Results. Hydroxyurea was effective, as determined by the patients' clinical improvement and increased hemoglobin (8.3 vs 9.1 g/dL, $p < 0.005$), F cells (15.9% vs 37.1%, $p < 0.005$) and mean corpuscular volume (82 fl vs 101 fl, $p < 0.005$). PS expression on the surface of erythrocytes and platelets decreased from 6.27% to 2.96% ($p < 0.005$) and from 13.5% to 4.7% ($p < 0.005$), respectively.

Interpretation and Conclusions. Hydroxyurea treatment reduces PS expression on the surface of erythrocytes and platelets, thus contributing to the favorable effects of this therapy.

Key words: sickle cell disease, phosphatidylserine exposure, annexin V, hydroxyurea.
nating on the inner surface. This asymmetry is inverted in a subpopulation of sickle cell erythrocytes that present PS on the outer surface of the cell membrane. This exposure of PS accelerates the formation of thrombin which may contribute to the hemostatic activity, up to a maximum of 40 mg/kg/day. Myelotoxicity was defined as a 20% decrease in hemoglobin level, less than 2.5 × 10^9 neutrophils/L or less than 150 × 10^9 platelets/L. Treatment was temporarily interrupted if the number of neutrophils fell below 1.5 × 10^9/L or the number of platelets fell below 100 × 10^9/L, and was resumed when the counts normalized. The treatment protocol was approved by the Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo. All patients or people responsible for them signed informed consent to participation in the study. Monthly blood tests were used to assess the patients.

The various parameters under study were evaluated at 3-month intervals until the end of the 12 months of treatment. Samples from 10 normal blood donors (3 females, 7 males; average age 37 years; range: 21 to 57 years) were also studied.

**Materials**

For the cytometric analyses we used phycoerythrin (PE) or fluorescein isothiocyanate (FITC)-labeled murine monoclonal antibodies directed against human antigens, isotypic control antibodies, and goat antibody against mouse immunoglobulin G (GAM) from Becton Dickinson (San José, CA, USA), Pharmingen (San José, CA, USA) and DAKO (Vila Real, Carpintaria, CA, USA). The following monoclonal antibodies were used: CD49d-PE, CD62-P, IgG2a-PE, CD64-FITC, IgG1-FITC, glycophorin A-FITC, CD71-PE, CD64-FITC, IgG1-FITC, GAM-FITC, and pure CD63. The monoclonal antibody against HbF was kindly provided by J. Elion and R. Krishnamoorthy from the Inserm 4458, H. Robert Debré, Paris. The PS on the surface of erythrocytes and platelets was labeled using the Apoptest kit (Nexins Research, Amsterdam, The Netherlands).

**Design and Methods**

**Patients**

The study was conducted on 15 patients with sickle cell anemia (7 females, 8 males; average age 22 years; range: 7 to 38 years). Twelve patients were SS homozygotes and 3 Sβ^+^ heterozygotes. The criteria for the introduction of treatment with HU included a clinical history of at least three painful crises requiring hospitalization during the preceding year. Exclusion criteria were the presence of renal or hepatic dysfunction, hypersplenism, chronic hepatitis, and human immunodeficiency virus or hepatitis C virus infection. The patients were treated with hydroxyurea for 12 to 18 months according to a previously published protocol, i.e., hydroxyurea administered at the dose of 20 mg/kg/day on four consecutive days per week and with a monthly increment of 5 mg/kg/day in the absence of myelotoxicity, up to a maximum of 40 mg/kg/day. Myelotoxicity was defined as a 20% decrease in hemoglobin level, less than 2.5 × 10^9 neutrophils/L or less than 150 × 10^9 platelets/L. Treatment was temporarily interrupted if the number of neutrophils fell below 1.5 × 10^9/L or the number of platelets fell below 100 × 10^9/L, and was resumed when the counts normalized. The treatment protocol was approved by the Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo. All patients or people responsible for them signed informed consent to participation in the study. Monthly blood tests were used to assess the patients.

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**Flow cytometry**

**Erythrocytes**

Five milliliters of peripheral blood collected into EDTA was centrifuged at 500 g for 10 min and the plasma and buffy-coat were removed. The remaining red cell concentrate was washed three times with 10 mL phosphate-buffered saline (PBS, 10 mM phosphate, 150 mM NaCl, 0.01% NaN₃) with 1% bovine serum albumin (BSA) at 4°C. The final volume was diluted 1/10 with PBS. Next, 5 µL of the desired antibody (CD36, CD49d, CD71, glycophorin A, IgG1 and IgG2a) were added to the 100 mL volume of the suspension. The suspension was then incubated for 20 min at 4°C in the dark, washed twice with PBS and resuspended in 500 mL PBS. The erythrocytes were analyzed with a FACsort cytome-
ter (BD, San José, CA, USA) using CellQuest software. The erythrocytes were identified by size (forward scatter, FSC) and granularity (SSC) and gate purity was determined by specific labeling with glycoporphin A. Isotypic antibodies were used as the labeling control.

Cell counts and the determination of hemoglobin concentration were performed using an automated cell counter (ActDiff, Beckman-Coulter, Fullerton, CA, USA).

**Reticulocyte quantification**

Reticulocytes were counted by labeling 5 µL of total blood with 1 mL thiazole orange (BD) and using the Reticount protocol of a FACsort cytometer.

**Quantification of PS on the erythrocyte cell membrane**

Ten microliters of the erythrocyte suspension diluted 1/10 with PBS were diluted with 485 µL of the binding buffer that accompanies the kit and with 5 µL of annexin-V-FITC diluted 1/10 with the same buffer. The final cell suspension was incubated for 10 min on ice in the dark and analyzed with the cytometer. The distribution of annexin V-positive and –negative (annexin+ and annexin−) erythrocytes in relation to relative size (FSC) and granulosity (SSC) and gate purity was determined by specific labeling with glycophorin A. Isotypic antibodies were used as the labeling control. Next, 100 µL of this suspension were mixed with 1 mL PBS containing 0.2% SDS and allowed to stand for 1 min at RT. The SDS was removed by washing 3 times in PBS, and the cells were adjusted to a final volume of 100 µL in PBS. For immunophenotyping, 5 µL of the final suspension were incubated for 1 h at RT in 200 µL of anti-γ chain MoAb, and then for 30 min at RT in 100 µL of FITC-labeled F(ab')2 anti-mouse IgG (GAM) diluted to 10 µg/mL in PBS containing 1% BSA. Cells were washed 3 times in PBS containing 0.2% Triton X-100 after each incubation and finally the RBC were resuspended in PBS and analyzed.

**Statistical analysis**

Data for paired samples with a normal distribution were analyzed by the two-tailed paired t-test. Paired samples with a small N or with non-normal distribution were analyzed by the non-parametric Wilcoxon test. Unpaired samples with a normal distribution were analyzed by the unpaired t-test and samples with non-normal distribution were analyzed by the non-parametric Mann–Whitney test. Correlations were tested by Pearson’s test when the samples showed a normal distribution and by Spearman’s test when they had a small N or showed non-normal distribution. The level of statistical significance was set at 5% (p < 0.05) for all analyses.

**Results**

**Erythrocytes**

The results of the variables analyzed in the erythrocytes and leukocytes are listed in Table 1. Before hydroxyurea treatment, sickle cell anemia patients had higher mean values of F cells and reticulocytes than did controls and more erythrocyte positivity for CD36, CD71, CD49d and annexin V. The mean hemoglobin level was lower in sickle cell anemia patients than in controls (8.03 g/dL vs. 15.18 g/dL), as was the mean corpuscular volume (MCV) (87.21 fL vs. 92.08 fL). Hydroxyurea treatment caused an increase in mean hemoglobin concentration (8.03 to 9.51 g/dL), F cell percentage (15.96% to 37.16%) and MCV (87.21 to 101.83 fL). On the other hand, hydroxyurea treatment reduced the percentage of

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reticulocytes (8.44% vs 4.46%) and of erythrocytes positive for CD36, CD71, CD49d and annexin V (Figures 1 and 2). Despite this reduction, mean CD36⁺, CD71⁺ and annexin V⁺ erythrocyte levels continued to be significantly higher than in controls. Hydroxyurea treatment reduced the percentage of annexin V⁺ erythrocytes in all patients, but one (Figure 1A).

During hydroxyurea treatment, the percentage of CD49d⁺ erythrocytes fell to a level not differing from that in controls. Before hydroxyurea treatment, the percent distribution of annexin V⁺ erythrocytes with respect to FSC, which reflects cell size, was the same as that of negative erythrocytes in the 2nd, 3rd and 4th quartiles (13.7% vs 12.7%, 47.4% vs 49.7%, 33.4% vs 33.9%). However, 5.9% of annexin V⁺ and 3.1% of annexin V⁻ erythrocytes were found in the 1st quartile, with the difference being significant (p = 0.006) (Figure 3A).

During hydroxyurea treatment, the percent distribution of annexin V⁻ and annexin V⁺ erythrocytes was the same with respect to FSC (Figure 3B).

Figure 1. Percent distribution of annexin V-positive red blood cells (A) and platelets (B) for each patient before and during treatment with hydroxyurea.

Figure 2. Percent distribution of CD36 (A), CD49d (B) and CD71 (C) positive red blood cells for each patient before and during treatment with hydroxyurea.

Leukocytes

Hydroxyurea treatment significantly reduced total leukocyte counts from pretreatment values. However, the leukocyte counts of treated patients continued to be higher than those of controls (Table 1).

Platelets

Platelet counts and percent platelet activation, determined by CD62 labeling, did not differ between treated and untreated sickle cell anemia patients and controls. The percentage of annexin V-labeled platelets was reduced during hydroxyurea treatment (Table 2) in all but two patients (Figure 1B).
Correlations

In sickle cell anemia patients, before and during hydroxyurea treatment, MCV was positively correlated with percent F cells (r = 0.5589, p = 0.0020) (Figure 4A) and with hemoglobin concentration (r = 0.60, p = 0.0004). MCV was negatively correlated with reticulocyte count (r = -0.42, p = 0.018) (Figure 4B), with percent annexin V+ erythrocytes (r = -0.379, p = 0.0386) (Figure 4C), with percent CD71+ erythrocytes (r = -0.42, p = 0.019) (Figure 3D), with percent CD36+ erythrocytes (r = -0.44, p = 0.015) (Figure 4E), and with percent CD49d+ erythrocytes (r = -0.45, p = 0.01) (Figure 4F).

Hemoglobin concentration was positively correlated with percent F cells (r = 0.48, p = 0.007) and MCV, and negatively correlated with percent CD36+ (r = -0.52, p = 0.003), CD71+ (r = -0.64, p = 0.0001), CD49d+ (r = -0.42, p = 0.02), and annexin V+ (r = -0.56, p = 0.001) erythrocytes and with reticulocyte count (r = -0.59, p = 0.0006).

Reticulocyte counts were positively correlated with percent CD36+ (r = 0.51, p = 0.004), CD71+ (r = 0.72, p < 0.0001), CD49d+ (r = 0.47, p = 0.009) and annexin V+ (r = 0.59, p = 0.0005) erythrocytes and negatively correlated with percent F cells (r = -0.39, p = 0.03). The percentage of F cells was negatively correlated with the percentage of CD49d+ erythrocytes (r = -0.49, p = 0.005) and did not show a significant correlation with the percentage of CD36+, CD71+ or annexin V+ erythrocytes.

The percentage of annexin V+ erythrocytes was positively correlated with the percentage of CD36+ (r = 0.52, p = 0.003), CD71+ (r = 0.42, p = 0.0005), and CD49d+ (r = 0.53, p = 0.004) erythrocytes.

The percentage of CD36+ erythrocytes was positively correlated with the percentage of CD49d+ (r = 0.84, p < 0.0001) and CD71+ (r = 0.83, p < 0.0001) erythrocytes.

Discussion

The adherence of erythrocytes, leukocytes and platelets to the cell endothelium is considered to be an important event in the pathophysiology of the vaso-occlusive phenomena that characterize sickle cell anemia. In the present study we assessed the expression of 5 adhesion molecules located on the surfaces of erythrocytes and platelets in normal individuals and in sickle cell anemia patients before and during hydroxyurea treatment. The results show, for the first time, that hydroxyurea treatment reduces the percentage of erythrocytes and platelets that express phosphatidylserine on their surface. Additionally, we confirmed previous reports demonstrating reduced expression of CD36, CD49d and CD71 molecules on erythrocytes during hydroxyurea treatment.

Hydroxyurea has multiple effects on the erythrocyte lineage, including increases in HbF, F cells, F reticulocyte levels and MCV. These phenomena may contribute to the reduction of reticulocyte counts and to the increase in HbF levels. Hydroxyurea also has an effect on non-erythroid cells, reducing the number of neutrophils and the expression of adhesion molecules on

Table 1. Leukocyte and erythrocyte variables.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls mean ± SD</th>
<th>Sickle-cell anemia Pre treatment mean ± SD</th>
<th>Under treatment mean ± SD</th>
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<tbody>
<tr>
<td>N. of leukocytes (× 10^6/µL)</td>
<td>6.87±1.52</td>
<td>13.70±3.60</td>
<td>8.04±2.14</td>
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<td>Hemoglobin (g/dL)</td>
<td>15.18±1.25</td>
<td>8.03±0.95</td>
<td>9.51±1.59</td>
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<td>F cells (%)</td>
<td>3.4±0.34</td>
<td>15.96±12.21</td>
<td>37.16±23.83</td>
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<td>Reticulocytes (%)</td>
<td>0.96±0.34</td>
<td>8.44±3.58</td>
<td>4.46±3.61</td>
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<td>MCV (fL)</td>
<td>92.08±7.14</td>
<td>87.21±8.37</td>
<td>101.83±17.81</td>
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<tr>
<td>CD 36 (%)</td>
<td>0.35±0.37</td>
<td>2.21±1.43</td>
<td>0.65±0.36</td>
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<tr>
<td>CD 71 (%)</td>
<td>0.12±0.09</td>
<td>4.29±2.22</td>
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<tr>
<td>Annexin V (%)</td>
<td>0.08±0.07</td>
<td>6.27±3.33</td>
<td>2.96±3.27</td>
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<td>CD 49 d (%)</td>
<td>0.14±0.33</td>
<td>1.36±1.20</td>
<td>0.32±0.35</td>
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</table>

*: values from the samples collected at the time of peak HbF. All control values were significantly different from pre-treatment values (p < 0.005), and all values under treatment were significantly different from pre-treatment values (p < 0.005).
Table 2. Platelet variables.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls mean±SD</th>
<th>Pre-treatment mean±SD</th>
<th>Under treatment mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N. of platelets</td>
<td>Platelet activation</td>
<td>Platelet annexin V (%)</td>
</tr>
<tr>
<td></td>
<td>(x1000/μL)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>N. of platelets</td>
<td>240.45±51.93*</td>
<td>34.47±25.71</td>
<td>12.96±11.62</td>
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<tr>
<td>Platelet activation</td>
<td>376.50±92.90</td>
<td>38.51±22.14</td>
<td>14.66±11.27</td>
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<tr>
<td>Platelet annexin V</td>
<td>312.13±121.13</td>
<td>30.81±26.97</td>
<td>4.67±4.47°</td>
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<td></td>
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</table>

Data not shown

Values from samples collected at the time of peak Hb F; *: control versus pre-treatment significantly different (p < 0.005); °: pre-treatment versus under treatment significantly different (p < 0.005).

The beneficial effect of hydroxyurea treatment was first attributed to the rise in hemoglobin F which may cause a reduction in HbS polymerization. However, several studies have indicated that the rise in HbF concentration is not the only or indeed the most relevant result of treatment with hydroxyurea. It has been demonstrated that many patients have a clinical improvement before a significant increase in HbF occurs. A multicenter clinical study of adult patients showed that HbF level was correlated with a reduction of painful crises only during the first 3 months of treatment, whereas the correlation between the reduction of painful crises and neutrophil counts persisted for more than 2 years. In the present study we observed that the percentage of F cells was positively correlated with hemoglobin concentration and with MCV, and negatively correlated with CD49d and annexin V (Figure 4). This observation suggests that the increase in MCV may have a direct effect on the adhesive phenotype of sickle cells and that monitoring the increase of MCV may be used as indirect evidence of the increase in the expression of adhesive molecules on sickle cells.

Unlike normal individuals, patients with sickle cell anemia have erythrocytes that express PS on their surface. The population of erythrocytes ranges from 0.4% to 12% according to previous studies. In the present study this population ranged from 1.6% to 10.5%. PS expression seems to be one of the major determinants of the increased adhesion of sickle cells to the vascular endothelium. Recent studies have demonstrated that sickle cells with marked exposure of PS on their surface present ex vivo a three-fold higher adhesiveness to the vascular endothelium than red cells with low PS expression. This was not the case for the expression of CD36 and VLA-4, whose higher or lower expression did not modify the low adhesiveness. The intensity of PS expression seems to be directly correlated with the risk of cerebrovascular accidents as assessed by transcranial Doppler imaging.

The study of erythrocyte fractionation on density gradients showed that about 45% of the erythrocytes expressing PS were concentrated in the fractions of lower density (1.07 to 1.08 g/mL) and about 10% in the higher density fraction (>1.13 g/mL). Reticulocytes and megaloblasts are concentrated in the lower density fraction, whereas irreversibly sickled cells are found in the higher density fraction. More than 50% of the erythrocytes that express PS are reticulocytes identified by the presence of RNA in the cytoplasm or by CD71 labeling. In the present study we observed that the greatest PS exposure occurred on the largest erythrocytes, as demonstrated by flow cytometry (larger FSC) (Figure 3). This population also showed a concentration of PS-expressing CD71, CD36 and CD49d and of reticulocytes (data not shown). Reticulocytes are the cells with the greatest adhesiveness to vascular endothelium.
Hydroxyurea reduces phosphatidylserine in sickle cell anemia

Phosphatidylserine (PS) exposure on the surface of sickle cells has been attributed to the adhesion molecules CD36 and CD49d and, more recently, to PS exposure on the cell membrane. The stress reticulocyte CD71+ has a higher concentration of PS molecules than of CD36, with its adhesive potential deriving exclusively from PS. CD36 does not, in itself, seem to be responsible for the altered adhesion of sickle cells since there is no correlation between adhesion and the stress reticulocytes CD36+. Thus, the reduction of annexin V expression on erythrocytes during hydroxyurea treatment could be one of the main factors involved in the reduction of the adhesive phenotypes of red blood cells observed in patients treated with hydroxyurea.

PS exposure on the surface of sickle cells, in addition to affecting erythrocyte adhesion to the vascular endothelium, exacerbates anemia by enhancing phagocyte recognition and removal of these cells and favors the development of a thrombophilic state. There is a direct correlation between PS exposure on sickle erythrocytes and the generation of thrombin. PS expression on the surface of platelets is also significantly reduced during treatment with hydroxyurea (Table 2). This fact, taken together with the reductions in platelet number and platelet activation observed in about 30%
of the treated patients, may attenuate the pro-thrombotic state that characterizes sickle cell anemia.

In summary, we demonstrate here, for the first time, that treatment of sickle cell anemia with hydroxyurea causes a significant reduction of PS exposure on the surface of erythrocytes and platelets, a fact that may explain part of the beneficial effects of this type of treatment.

References