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

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A B S T R A C T

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.

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The polymerization of deoxygenated hemoglobin S (HbS) causing erythrocyte deformation, rigidity and fragility has been considered to be the basic physiopathologic mechanism of sickle cell anemia (SCA), being responsible for the hemolytic anemia, vaso-occlusive phenomena and progressive functional asplenia that characterize the disease. Additional factors such as inflammation, number of polymorphonuclear (PMN) leukocytes in the circulation, endothelial activation, and abnormal adhesion of leukocytes, erythrocytes and platelets to the endothelium are considered to play a determinant role in the severity of the vaso-occlusive phenomena.[1-3]

Adhesion molecules and their ligands on the surface of erythrocytes and of endothelial cells and in plasma may actively participate in the process of erythrocyte adhesion in sickle cell anemia. Endothelial receptors involved in the adhesion of erythrocytes in SCA include the vitronectin $\alpha v\beta 3$ receptor (CD51/CD61), VCAM-1 (CD106), CD 36, and glycoprotein Ib.[4-6] Thrombospondin (TSP), von Willebrand factor, laminin, fibrinogen and fibronectin are present in plasma and in the endothelial matrix [7-9] Several receptors such as very late activation antigen 4 (VLA-4), CD 49d/CD29 and the TSP receptor (CD36) are present on the erythrocyte surface. The CD36 receptor is expressed on both reticulocytes and mature red cells, although the levels in mature cells are extremely low. The integrin $\alpha 4\beta 1$ is not expressed on mature erythocytes, only on reticulocytes. Other adhesive molecules on the erythrocyte surface include aggregated band 3 of the membrane, Lutheran blood group antigen, sulfated glycolipids, and phosphatidylserine (PS).[10-15] PS is asymmetrically distributed in the cell membrane of eukaryotes, predomi-

nating on the inner surface.[16] This asymmetry is inverted in a subpopulation of sickle cell erythrocytes that present PS on the outer surface of the cell membrane.[14,17] This exposure of PS accelerates the formation of thrombin[18] which may contribute to the hemostatic changes observed in sickle cell anemia and predispose to vasoocclusive phenomena.[19] Treatment with hydroxyurea (HU) ameliorates the clinical manifestations of sickle cell anemia, such as the frequency and seventy painful crises, and increases the level of hemoglobin in a significant number of patients. The mechanisms by which hydroxyurea produces its effects are not completely understood, although hydroxyurea-induced increases in the concentration of fetal hemoglobin and in the percentage of red cells containing hemoglobin F (HbF) (F-cells) are generally accepted to be the most important changes responsible for this drug's beneficial effects. However, the association between signs of clinical and laboratory improvement and the rise in HbF is not complete, nor are their time courses synchronous. Thus, other changes that occur in response to the use of hydroxyurea may be involved in mediating the clinical response. These could include a decrease in inflammatory responses owing to a reduction of the number of neutrophils and modification of the expression of surface molecules of erythrocytes and leukocytes that are directly involved in cell adhesion. A change in cell adhesion molecules has been demonstrated for L-selectin and $\alpha L\beta 2$ integrin on leukocytes, and CD36 and CD49d on the red cell surface.[19,20] Furthermore, HU therapy decreases the *in vivo* adhesion of sickle erythrocytes to TSP and laminin.[21] However, we suspect that the effects of hydroxyurea are far broader than so far demonstrated, and probably involve other molecules and cells. In the present study we evaluated hydroxyurea-induced changes in the expression of various cell surface molecules in red cells and platelets.

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 $S\beta^0$ 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, [22] 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 1.5×10^9L or the number of platelets fell below 100×10^9L, 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 *http://www.bd.com*), Pharmingen (San José, CA, USA (*http://lwww.pharmingen.com*) and DAKO (Vila Real, Carpintaria, CA, USA *http://www.dakousa.com*). The following monoclonal antibodies were used: CD49d-PE, CD62-P, IgG2a-PE, CD64-FITC, IgG1-FITC, glycophorinA-FITC, CD71-FITC, CD36-FITC, CD61-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).

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% NaN_3) 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 granulosity (SSC) and gate purity was determined by specific labeling with glycophorin 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) in cytometry was determined by dividing the difference between the maximum and minimum FSC presented by the erythrocytes into four equal quadrants, each corresponding to 25% of the total FSC distribution (gates 1 to 4). Gate 5 (annexin V$^+$ erythrocytes) and gate 6 (annexin V$^-$ erythrocytes) were then created and erythrocyte distribution was assessed by the intersection of gates 1 to 4 with gate 5 and later with gate 6, and the percentage corresponding to each quartile (Q1 to Q4) was recorded.

Platelet activation

Five milliliters of whole blood were centrifuged at 200 g for 10 min to obtain platelet-rich plasma. A platelet-rich plasma (PRP) aliquot (5 μL) was diluted with 1 mL PBS/1% formalin and incubated at 4°C for 2 h. The suspension was then washed 3 times with 2 mL Tyrode solution. Five microliters of FITC γ1 and PE γ2a antibodies were added to the platelet pellet in the control tube, and 5 then μL of each of the monoclonal antibodies CD61-FITC and CD62-PE were added to the test tube. The material was incubated at 4°C for 20 min in the dark. Next, the preparation was washed twice with Tyrode solution and a final suspension of the pellet in 500 μL was prepared and submitted to the cytometric analysis.

Determination of the percentage of F cells

The percentage of red blood cells containing HbF (F cells) was determined as previously described.[23] Briefly, red-blood cells (RBC) were washed 3 times in FCS. A total of 50 mL of packed RBC were resuspended in 1 mL of PBS containing 50 mg/mL sodium dodecyl sulfate (SDS) and 1 mg/mL FCS. After standing for 1 min at room temperature (RT), 10 mL of PBS containing 1% formaldehyde and 10 mg/mL SDS were added and then incubated for 2h under constant shaking. Fixation was completed by the addition of 800 μL of 35% formaldehyde in aqueous solution and further overnight incubation at RT under shaking. The fixed RBC were washed 3 times in PBS and resuspended to a final volume of 500 μL. 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, CD 49d and annexin V. The mean hemoglobin level was lower in sickle cell anemia patients than in controls (8.03 g/dL v 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

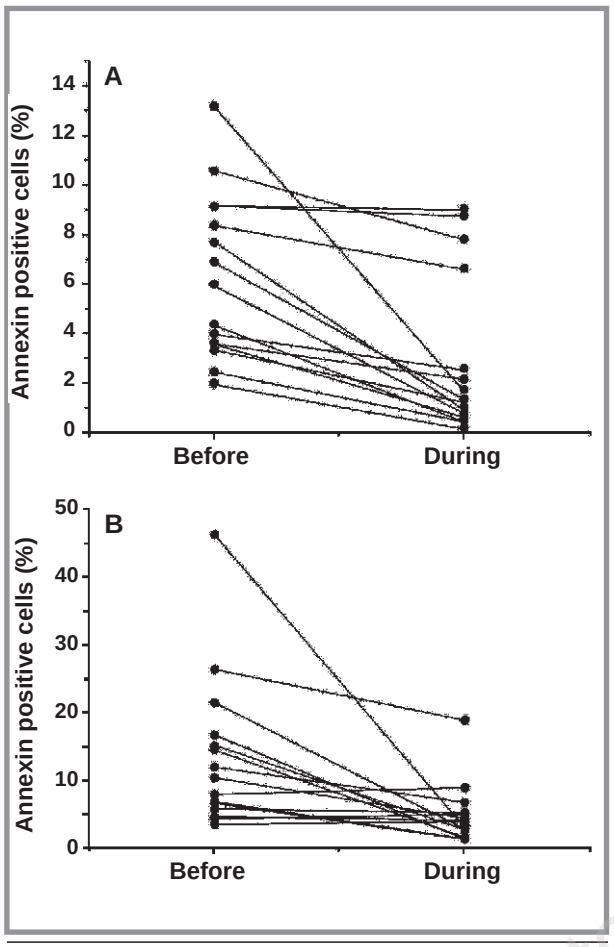

Figure 1. Percent distribution of annexin V-positive red blood cells (A) and platelets (B) for each patient before and during treatment with hydroxyurea.

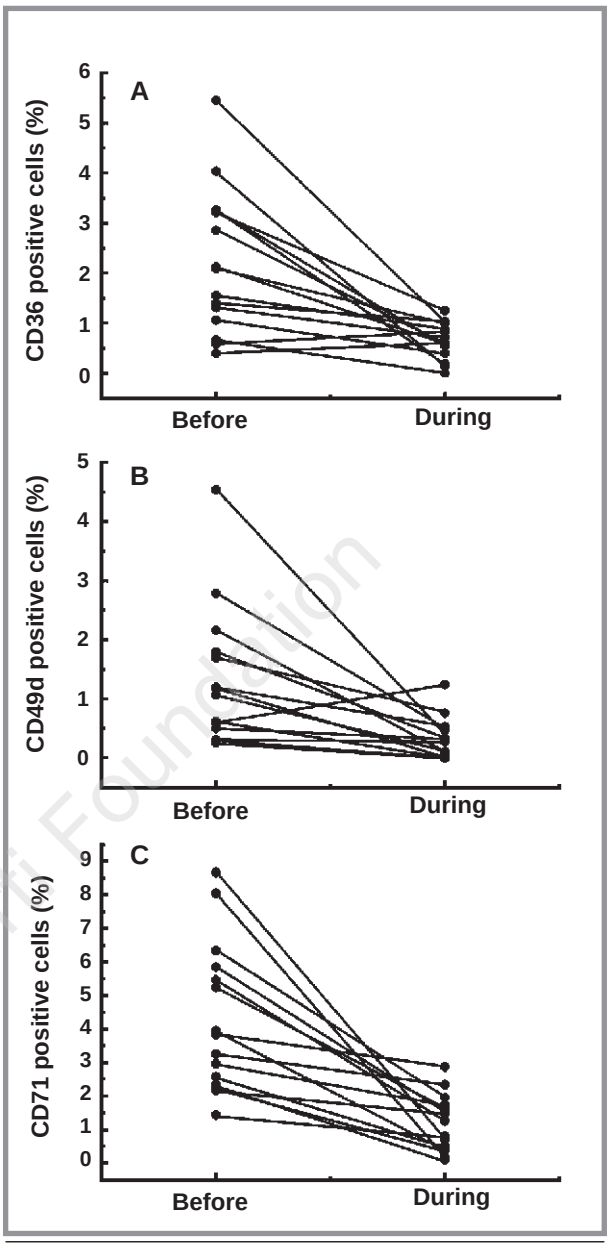

Figure 2. Percent distribution of CD36 (A), CD49d (B) and CD71 (C) positive red blood cells for each patient before and during treatment with hydroxyurea.

reticulocytes (8.44% vs 4.46%) and of erythrocytes positive for CD36, CD71, CD49d and annnexin 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, which concentrated the smaller erythrocytes, 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).

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).

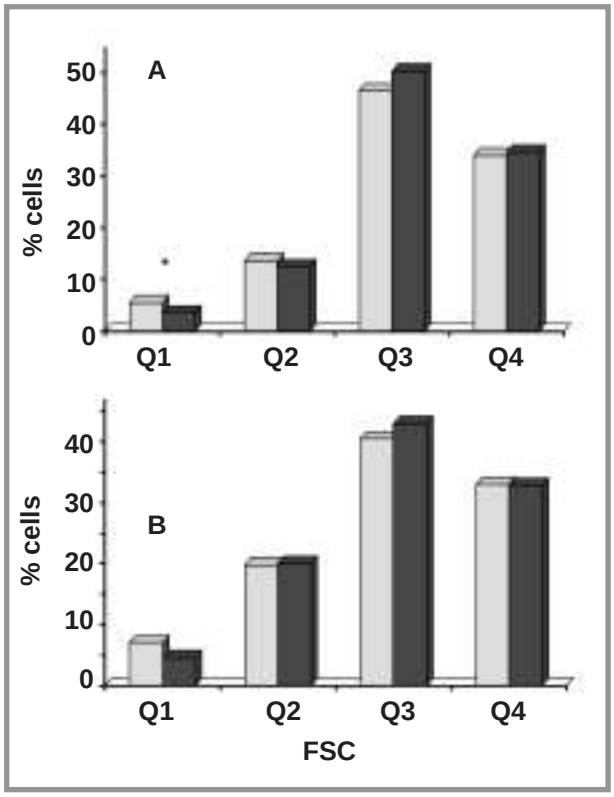

Figure 3. Percent distribution of annexin V-positive (light column) and negative (dark column) erythrocytes in relation to the forward scatter (FSC) quartiles (Q1 to Q4). A, before treatment with hydroxyurea; B, during treatment with hydroxyurea. *: significantly different ($p<0.05$).

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.

Table 1. Leukocyte and erythrocyte variables.

Variables	*Controls mean ± SD*	*Sickle-cell anemia Pre treatment mean ± SD*	*Sickle-cell anemia Under treatment§ mean ± SD*
N. of leukocytes (× 1000/μL)	6.87±1.52	13.70±3.60	8.04±2.14
Hemoglobin (g/dL)	15.18±1.25	8.03±0.95	9.51±1.59
F cells (%)	3.40±3.45	15.96±12.21	37.16±23.83
Reticulocytes (%)	0.96±0.34	8.44±3.58	4.46±3.61
MCV (fL)	92.08±7.14	87.21±8.37	101.83±17.81
CD 36 (%)	0.35±0.37	2.21±1.43	0.65±0.36
CD 71 (%)	0.12±0.09	4.29±2.22	1.19±0.84
Annexin V (%)	0.08±0.07	6.27±3.33	2.96±3.27
CD 49 d (%)	0.14±0.33	1.36±1.20	0.32±0.35

§: 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 ($p <0.005$).

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.[22,24-28] 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 2. Platelet variables.

Variables	Controls mean±SD	Sickle-cell anemia Pre treatment mean±SD	Sickle-cell anemia Under treatment§ mean±SD
N. of platelets (×1000/μL)	240.45±51.93*	376.50±92.90	312.13±121.13
Platelet activation (%)	34.47±25.71	38.51±22.14	30.81±26.97
Platelet annexin V (%)	12.96±11.62	13.46±11.27	4.67±4.47°

*§: 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 surface of lymphocytes and monocytes.[19,29] In the present study we observed that hydroxyurea treatment caused increases in hemoglobin concentration, percentage of F cells and MCV, and a decrease in reticulocytes, in agreement with previous reports (Table 1).

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.[30] 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.[29] 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, but was not correlated with the expression of CD36, CD71 or annexin V, indicating that the increase in the number of F cells does not have a significant effect on the adhesive properties of erythrocytes.

The reduction of leukocyte, platelet and reticulocyte counts and the changes in the expression of adhesive molecules on the surface of these cells may contribute to a reduction of vaso-occlusive crises. Patients with sickle cell anemia treated with hydroxyurea show a reduced percentage of reticulocytes expressing the adhesion molecules $\alpha4\beta1$ and CD36[31] and reduced erythrocyte adhesion to TSP, to laminin, and to endothelial cells in culture.[21,26] Treatment with hydroxyurea causes an early increase in MCV, suggesting that MCV may have an impact on the adhesive phenotype.[31] The increase in MCV may result from the increase in cell hydration caused by hydroxyurea, perhaps caused by modulation of K/Cl transport.[26] The level of erythrocyte hydration is known to affect red cell adhesion to the endothelium.[21,32,33] In the present study, MCV increased early, before the number of F cells had increased significantly (*data not shown*). The increase in MCV was positively correlated with the increase in F cells in the circulation and in hemoglobin concentration and negatively correlated with the percentage of reticulocytes and of cells expressing the adhesive molecules CD36, 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 decrease of the adhesive phenotype of erythrocytes in patients with sickle cell anemia treated with hydroxyurea.

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.[14,16] In the present study this population ranged from 1.6% to 10.5%. PS exposure seems to be one of the major determinants of the increased adhesion of sickle cells to the vascular endothelium.[34] 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 importance of PS in erythrocyte adhesion in sickle cell anemia was also confirmed by the study of stress reticulocytes ($CD71^+$) which are cells known to have greater adhesiveness to the endothelium. In a group of patients with sickle cell anemia, about 21% of $CD71^+$ reticulocytes expressed surface PS, whereas only 9.3% of these cells expressed CD36.[34] The intensity of PS expression seems to be directly correlated with the risk of cerebrovascular accidents as assessed by transcranial Doppler imaging.[31] 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).[19] 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 erythrocytes expressing CD71, CD36 and CD49d and of reticulocytes (*data not shown*). Reticulocytes are the cells with the greatest adhesiveness to vascular endothe-

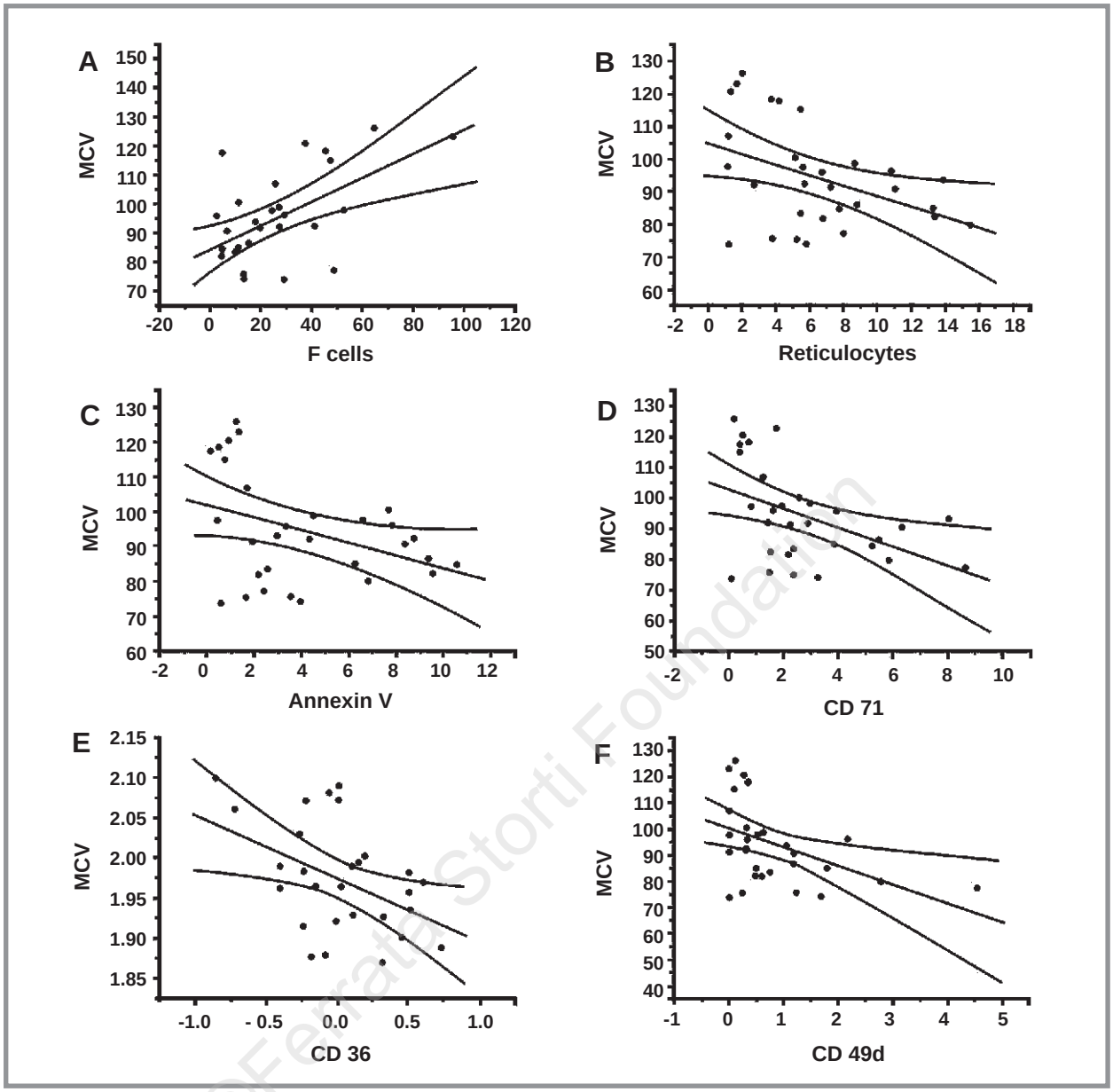

Figure 4. Correlations observed between MCV and percent F cells (A), reticulocytes (B), annexin V (C), CD71 (D), CD36 (E), and CD49d (F).

lium in sickle cell anemia.[35-37] This increased adhesiveness has been attributed to the adhesion molecules CD36 and CD49d and, more recently, to PS exposure on the cell membrane.[13,34]

The stress reticulocyte $CD71^+$ has a higher concentration of PS molecules than of CD36, with its adhesive potential deriving exclusively from PS.[34] 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 $CD36^+$.[34,38]

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[17] and favors the development of a thrombophilic state. There is a direct correlation between PS exposure on sickle erythrocytes and the generation of thrombin.[39] 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.

DTC: conception, design, interpreatation of the results, and draft of the article. ILA: patient selection and follow-up, data collection and interpretation. PVBP: flow cytometry experiments, and interpretations of results. MAZ: conception and design, interpretation of results. All authors reviewed the article critically and approved it for publication. The authors reported no potential conflicts of interest.

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