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Reticulocyte Parameters and Hemoglobin F Production in Sickle Cell Disease Patients Undergoing Hydroxyurea Therapy

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Hemoglobin F (HbF) is an effective inhibitor of HbS polymerization. Hydroxyurea (HU) is used to increase HbF synthesis and improve the clinical course of sickle cell disease (SCD) patients. We studied a series of laboratory parameters concerning HbF production and reticulocyte response, and compared data between two groups: 1) 13 SCD patients treated with HU, and 2) 33 untreated SCD patients. Higher values of Hb concentration, mean cell volume (MCV), mean cell hemoglobin (MCH), mean reticulocyte volume (MRV), HbF concentration, percentage of F-cells, and amount of HbF/F-cells were observed in the treated group of patients. There was no correlation between Hb and HbF elevations. The reticulocyte count, immature reticulocyte count, mean fluorescence index (MFI), and neutrophil count were significantly lower in treated patients. Taken together, these findings suggest that a decreased hemolytic process occurred in patients undergoing HU treatment. There was a significant correlation between MCV and HbF, between MRV and HbF, and between MRV and F-cell in patients taking HU. These data indicate that macroreticulocytes correspond to F-reticulocytes, and that an increase in MRV in SCD patients using HU may be an indirect signal of F-cell production. The concentration of HbF/F-cells was higher in patients treated with HU, but this increase apparently was independent of F-cell production. Reticulocyte (RTC) parameters, as assessed by hematological analyzers, may be useful for following erythropoietic changes in patients receiving HU, and can indirectly indicate HbF and F-cell production induced by HU therapy. J. Clin. Lab. Anal. 17:66–72, 2003. © 2003 Wiley-Liss, Inc.

Key words: sickle cell anemia; F-cells; hydroxyurea; reticulocyte immaturity

INTRODUCTION

High hemoglobin F (HbF) levels may improve the clinical course of sickle cell disease (SCD) patients. HbF is an effective inhibitor of deoxyhemoglobin S polymerization and, consequently, diminishes the sickling process (1). Hydroxyurea (HU), an S-phase cytotoxic agent, is widely used in the treatment of chronic myeloproliferative disorders. HU interferes in DNA synthesis by inhibition of the ribonucleoside diphosphate reductase, an enzyme that participates in the transformation of the deoxyribonucleotides in DNA synthesis (2). The increase in the synthesis of HbF is associated with hypomethylation of the γ-globin gene promoter. Although the exact mechanism by which HU increases HbF is not completely understood, it is clear that HU has therapeutic value in sickle cell anemia (3,4). Immature and larger reticulocytes are released from bone marrow during erythroid expansion in response to hemolysis. These reticulocytes coexpress adult and fetal globin (F-reticulocytes) (5,6). SCD patients receiving HU showed a decrease in the number of immature reticulocytes and in cell density, but there was no statistically significant increase in percent HbF or absolute HbF levels (7). Immature reticulocytes can be identified by automated hematological analyzers according to fluorescent RNA content, as evaluated by flow cytometry. Parameters such as the mean corpuscular volume of reticulocytes (MRV) and mean fluorescence index (MFI) have been used to monitor bone marrow transplantation and aid in the diagnosis and treatment of anemia (8–13).

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We studied the effect of HU administration on HbF production and reticulocyte parameters in SCD patients.

PATIENTS AND METHODS

Eligibility Requirements

This study included adult SCD patients who were undergoing HU treatment at the University Hospital of Campinas, and, as a control group, adult patients with SCD who were not receiving HU as part of their treatment. Transfusion-dependent individuals were excluded from the study. The diagnosis of SCD was based on clinical, familial, and laboratory data, including cellulose acetate and acid agar gel electrophoresis, and the estimation of A2 hemoglobin by elution (14,15).

Informed consent was obtained from all individuals included in the study, consistent with the guidelines of the local ethics committee.

HU Dose

The initial dose of HU (Bristol, Regentsburg, Germany) was 10 mg/kg/day. This was increased by 5 mg/kg/day every 8 weeks, unless toxicity was present, to a maximum dose of 20 mg/kg/day. Toxicity was defined by the presence of at least one of the following characteristics: reticulocyte count $< 50.0 \times 10^9$/L, neutrophil count $< 2.0 \times 10^9$/L, platelet count $< 100.0 \times 10^9$/L, or decreased Hb concentration.

Laboratory Studies

Complete blood count (CBC)

The CBC was determined by use of the Pentra 120 Retic (ABX-Horiba, Montpellier, France).

Reticulocytes

Analyses were performed by the Pentra 120 Retic. Whole blood samples were incubated with thiazole orange fluorescent dye for 25 sec, and the following parameters the following parameters were provided: number of reticulocytes in percentage (RTC%) and absolute counts (RTC#), three different reticulocyte maturity classes (low (LFR), medium (MFR), and high (HFR)) according to the RNA content, MFI, mean reticulocyte volume (MRV), and corrected reticulocyte count according to the degree of anemia degree (CRC).

HbF and F-Cell Measurement

HbF was quantified by the alkali denaturation method (16). The F-cell count was determined by qfixing cells with glutaraldehyde 25% (Sigma Chemical Co., St. Louis, MO). The cells were permeabilized with Triton X-100 (Reagen Indústria Química SA, Rio de Janeiro, Brazil), immunostained with mouse monoclonal antibody to human glycophorin, and conjugated with R-Phycoerythrin (Caltag Laboratories, South San Francisco, CA) and mouse monoclonal antibody to human fetal hemoglobin FITC (Caltag Laboratories). The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountai, View, CA). For each sample 50,000 events were collected, and the data were analyzed by CellQuest software (Becton Dickinson).

The amount of HbF in picograms per F-cell (HbF/F-cell) was calculated according to the formula (MHCx%HbF)/%F-cell (17).

Statistical analysis

The Mann-Whitney test was used to compare differences between groups. The association between variables was tested using the Spearman correlation coefficient. A P-value ≤ 0.05 was considered significant. All of the calculations were made in the SAS System for Windows, version 8.1, from SAS Institute Inc. (Cary, NC).

RESULTS

Thirteen adult SCD patients (11 with SC anemia, one with Sβ thalassemia, and one with hemoglobin SC) undergoing HU treatment, and 33 controls (25 with SC anemia, six with Sβ thalassemia, and two with hemoglobin SC) were enrolled in the study.

HU dosage varied from 500 to 1,500 mg/day (mean ± SD: 910.0 ± 319.36) and treatment periods varied between 7 and 72 months (mean ± SD: 26.4 ± 21.7) when the blood was collected for the study. No hematological toxicity was observed in the patients receiving HU treatment.

The hematologic data from SCD patients undergoing HU treatment and the controls are shown in Table 1. The Hb, MCV, and mean cell hemoglobin (MCH) values were higher in patients receiving HU compared to those in controls. The RTC% and RTC# values were significantly lower in patients receiving HU than in controls (Table 1). Also the absolute number of immature reticulocytes, represented by MFI#, was lower in treated patients than in controls (Fig. 1). On the other hand, the MRV values, percentages of HbF and F-cells, and amount of HbF/F-cells were higher in patients undergoing HU treatment than in controls (Table 1, Figs. 2 and 3).

The correlation coefficient data comparing hematologic parameters observed in the SCD patients are presented in Table 2. There was no correlation between Hb values and HbF levels in controls and patients

TABLE 1. Hematological data in 13 SCD patients under hydroxyurea treatment and 33 controls*

Variable	Patients under HU	Controls	*P* value
WBC ($X10^9$/l)	7.6 (3.3–12.5)	10.5 (5.4–21.4)	**0.0001**
Neut ($X10^9$/l)	3.53 (1.5–7.8)	5.45 (1.9–17.1)	**0.0097**
PLT ($X10^9$/l)	301.0 (144.0–625.0)	374.0 (100.0–605.0)	**0.09235**
Hb (g/dl)	9.1 (7.3–11.7)	8.1 (6.3–11.1)	**0.0490**
MCV (fl)	110.0 (91.0–128.0)	91.0 (64.0–109.0)	< **0.0001**
MCH (pg)	35.7 (28.7–44.1)	28.8 (19.4–36.7)	**0.0035**
RDW (%)	16.4 (15.0–24.7)	22.6 (16.3–29.6)	**0.0002**
RTC (%)	2.4 (1.1–7.8)	6.59 (0.32–11.2)	**0.0058**
RTC# ($X10^9$/l)	74.9 (26.5–216.7)	174.5 (11.3–397.8)	**0.0015**
MFI (%)	17.4 (5.77–26.60)	17.4 (9.0–31.2)	0.4207
MFI# ($X10^9$/l)	13.86 (1.89–43.97)	29.4 (1.9–97.4)	**0.0128**
MRV (fl)	125 (108–142)	110.0 (78.0–137.0)	**0.0003**
HbF (%)	14.2 (5.1–20.2)	5.6 (0.8–16.6)	**0.0003**
Fcell (%)	57.0 (27.4–94.3)	25.6 (2.6–73.0)	**0.0008**
HbF/Fcell (pg/cell)	7.9 (5.3–13.4)	5.9 (2.6–13.1)	**0.0003**
TBil (mg/dl)	2.0 (0.9–3.3)	3.1 (1.0–10.9)	0.1372

*Values in median and range.
WBC, white blood cells count; Neut, neutrophil count; PLT, platelet count; Hb, hemoglobin concentration; MCV, mean cell volume; MCH, mean cell hemoglobin; RDW, red cell distribution width; RTC#, reticulocyte absolute count; MFI, mean fluorescence index; MRV, mean reticulocyte volume; HbF, hemoglobin F; Fcell, Fcell count measured by FACSCalibur Flow Cytometer; HbF/Fcell, amount of HbF per Fcell; TBil, total bilirubin; MFI#, MFI absolute count.
bold = significant *P* value.

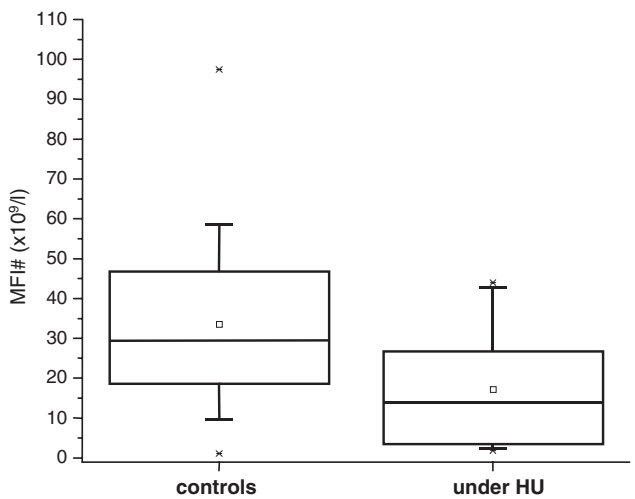

Fig. 1. Boxplots related to MFI ($\times 10^9$/L) in 33 controls and 13 SCD patients undergoing HU treatment.

receiving HU. There was a moderate correlation between MCV and HbF in patients treated with HU, and this correlation was weak but significant in controls. There was no correlation between MCV and Hb in either group. MRV showed a moderate correlation with F-cell count in both groups, and the correlation between MRV and HbF was significant only in the patients receiving HU (Table 2). No correlation was observed between Hb values and RTC# count in either group.

To ascertain whether HU dosage interfered with reticulocyte count and HbF levels, we tested the correlation between these variables. The results showed that the HU dosage was not correlated with reticulocyte count ($r = -0.1426$, $P = 0.6419$), MFI# ($r = 0.0931$, $P = 0.7621$), or HbF% ($r = 0.2911$, $P = 0.3344$).

A good correlation was observed between HbF levels and F-cell numbers in both groups of patients enrolled in the study. No correlation was observed between HbF values and RTC#, HbF values and MFI#, and HbF/F-cell and F-cell values in treated patients and controls (Table 2).

DISCUSSION

The concentration of HbF influences clinical and laboratory features of SCD. HbF reduces polymerization of deoxy HbS because hybrids of HbF and HbS do not enter the polymer. Consequently, SCD patients with HbF levels have attenuate clinical and laboratory manifestations (18).

HU is a chemotherapeutic drug that inactivates the ribonucleoside diphosphatase enzyme, thereby inhibiting cell division (19). The effects of HU on HbF production in patients with SCD have been reported in several studies (3,4,7,29,37). The exact mechanisms involved in increasing HbF production are not completely understood. It has been suggested that new stem cells are recruited during hematological stress, and that gamma globin gene expression has not yet been turned off in these progenitor cells (2). However, the clinical and laboratory benefits derived from administering HU to SCD patients are due not only to a rise in HbF

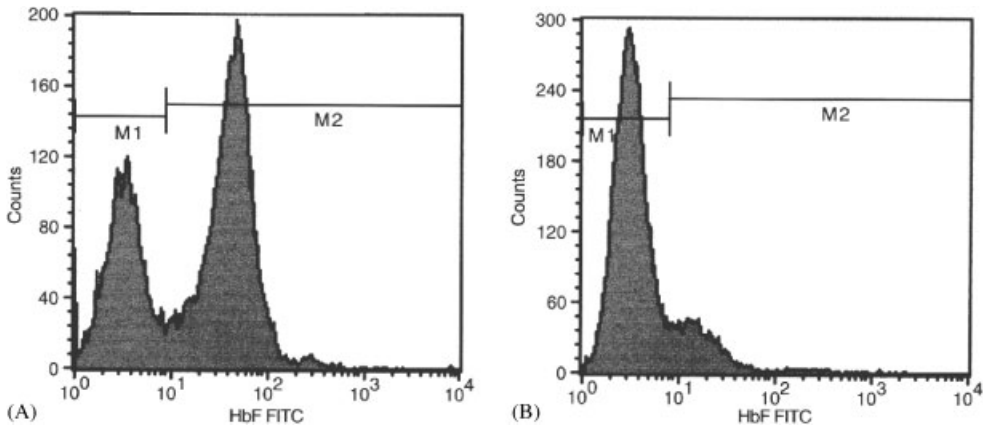

Fig. 2. Erythrocytes and F-cell counts as measured by a FACSCalibur flow cytometer using human fetal hemoglobin FITC as the fluorochrome (HbF FITC). **A:** An SCD patient receiving HU, showing erythrocytes that do not contain HbF (M1) and erythrocytes that contain 65% HbF (M2). **B:** A control, showing erythrocytes that do not contain HbF (M1) and erythrocytes that contain 16% HbF (M2).

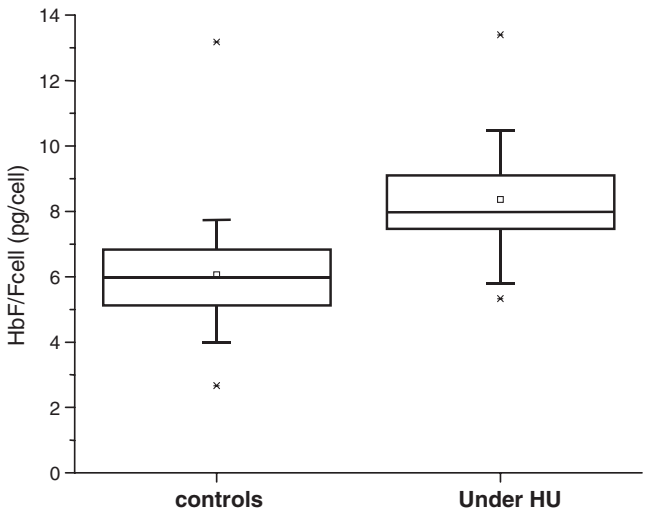

Fig. 3. Boxplots related to HbF/F-cell in 33 controls and 13 SCD patients undergoing HU treatment.

TABLE 2. Correlation coefficient data comparing hematological parameters observed in 13 patients under hydroxyurea treatment and 33 controls

	With HU		Controls	
Variables	R	*P* value	R	*P* value
Hb × HbF	0.0247	0.9359	0.2277	0.2099
MCV × HbF	**0.6437**	**0.0176**	**0.4053**	**0.0214**
MCV × Hb	−0.0013	0.6936	−0.1812	0.3127
MRV × HbF	**0.5777**	**0.0387**	0.2739	0.1292
MRV × MFI#	0.1210	0.6936	0.0620	0.7316
MRV × Fcell	**0.5667**	**0.0434**	**0.4046**	**0.0216**
HbF × Fcell	**0.7582**	**0.0027**	**0.9342**	<0.0001
HbF × RTC#	−0.5109	0.0743	0.2742	0.1287
HbF × MFI#	−0.3296	0.2713	−0.0170	0.9262
Hb × RTC#	0.2644	0.3826	0.2021	0.2592
HbF/Fcell × Fcell	0.0494	0.8725	0.2895	0.1079

Hb, hemoglobin concentration; HbF, hemoglobin F concentration; MCV, mean cell volume; MRV, mean reticulocyte volume; MFI, mean fluorescence index; RTC #, reticulocyte absolute count; Fcell, Fcell count; HbF/Fcell, amount of HbF per Fcell.

concentration, but also to a variety of other effects, such as an increase in red cell size, improvements in deformability, and cellular hydration (20). Other hematological changes observed in patients undergoing treatment were an increase in total hemoglobin level and a decrease in reticulocyte count, which suggest a reduction of hemolysis or a suppressive effect on effective erythropoiesis, as demonstrated by erythrocyte survival and ferrokinetics studies (19).

Patients treated with HU for SC anemia showed a marked decrease in Hb content of reticulocytes, an increase in MRV, and a significant increase in the RBCHb/RTCHb ratio, which indicate improved red blood cell survival (21).

We have introduced a new parameter for investigations of hemoglobinopathy: the MFI. This is a precise parameter that quantifies the global fluorescence intensity of the immature reticulocyte population, and has been shown to be useful as a predictor of engraftment in bone marrow transplantation (22). In a study of the clinical significance of the immature reticulocyte fraction (IRF), Chang and Kass (23) suggested that the integration of the IRF and the reticulocyte enumeration may indicate the erythroid response to anemia. A decrease of the IRF in patients with anemia reflects a nonresponsive or underresponsive marrow.

In the present study, all patients treated with HU showed MFI% within the reference range. Sixty-three percent of the patients presented normal RTC# values and 23% of patients showed RTC# values slightly above normal (according to the reference range for normal populations), as evaluated by the Pentra 120 Retic (24). The majority of controls (78%) presented RTC# values higher than normal. Although the percentage of MFI was similar in both groups, MFI# indicated that an elevated number of immature reticulocytes were being released from bone marrow to the circulation in controls.

We found higher Hb levels and lower mature and immature reticulocyte numbers in patients receiving HU treatment than in controls; therefore, we supposed that hemolysis was reduced in the treated patients, in agreement with suggestions made by other authors (3,25,26). However, since we found no difference in total bilirubin levels between both groups of patients in this study, and we did not perform erythrocyte survival or ferrokinetics studies, we could not exclude the possibility of a myelossuppressive effect due to HU in our cases.

Steinberg et al. (7) hypothesized that myelossuppression may be a prerequisite for HU to increase HbF. According to Ohene-Frempong and Smith-Whitley (27), two toxicity parameters are absolute neutrophil counts $<2{,}000/\mu L$ and absolute reticulocyte counts $<80{,}000/\mu L$. Our results showed that patients taking HU had lower neutrophil and reticulocyte counts than the controls, but no patients reached values indicative of marrow toxicity, according to the criteria used in this study.

The patients receiving HU showed MCV values higher than controls, as reported previously (3,26). Because the percentage of patients with other hemoglobinopathies that course with microcytosis was higher in the group not receiving HU, we analyzed MCV only in sickle cell anemia patients. We observed that 78% of controls presented MCV below 100 fl, and 81.8% of patients receiving HU showed MCV above 100 fl. Thus, the difference in MCV between groups probably was determined by the effect of HU in patients undergoing treatment. The correlation between MCV and HbF supports this concept, as noted previously (3). Changes in cellular hydration may be indicated by the high MHC, and can explain the high median value observed in groups receiving HU. The expanding volume of cells appears to be a result of the high Hb content, rather than an effect of chemotherapy (3,28).

In a study (29) of the laboratory and clinical responses to HU therapy in pediatric patients with severe SCD, a significant increase in HbF and F-cell percentages, without changes in hemoglobin concentrations, reticulocyte counts, and total bilirubin measurements, was observed. All of the patients improved clinically, and the increase in HbF parameters was much higher than in the adult pilot trial–probably because adults with Hb are subject to a long-term genetic silencing of the γ globin genes (29).

The HbF levels in SCD depend on the degree of contribution of three mutually independent processes: F-cell production, HbF biosynthesis with F-cells, and preferential F-cell survival (30). F-cell quantification is an important laboratory parameter for evaluating HbF production in SCD (31). Wood et al. (32), using anti-HbF antibodies conjugated with FITC in fixed smears, determined that the distribution of HbF-containing cells in normal persons was $2.7\% \pm 1.4\%$, and ranged from 30% to 59% in five patients with SC anemia. More recently, the F-cell percentage was quantified in a large group of children with SCD by use of a flow cytometer. The results were similar to those obtained in our group of adult patients receiving HU ($x = 55.9 \pm 19.9$, and $x = 58.75 \pm 19.46$, respectively) (33). Although the amount of HbF/F-cells was higher in patients taking HU than in controls, none of the patients reached the threshold value of 15 pg of HbF/F-cell, which has been suggested to be adequate for interfering in the sickling process (17). Our median value of HbF/F-cell was lower than those reported in other studies (1,30), but it was close to that observed in two patients treated with HU

for more than 7 years (34). There was no correlation between the amount of HbF/F-cells and the F-cell count. These data support the idea that both variations of HbF/F-cell and F-cell count are independent and may be regulated by different genetic factors (7,35).

The significant correlation between MRV and HbF levels, and between MRV and F-cell levels observed in patients receiving HU suggests that macroreticulocytes contain higher HbF concentration than the oldest erythroid cells and correspond to the so-called F-reticulocytes. These reticulocytes coexpress adult and fetal globin, and are produced with acute erythroid stimulation (5). The F-cell number is the result of F-cell production and selective peripheral lysis of non-F-cells. On the other hand, F-reticulocytes directly reflect changes in F-cell production (31).

RTCs are particularly active in the adhesion of sickle cells to endothelial cells. A reduction in the adhesive process would facilitate movement of erythrocytes through the capillary bed before sickling occurs. Therefore, the reduction in RTC numbers following HU therapy could account for an improved clinical course in SCD patients, independently of HbF synthesis induction (36). The decreased neutrophil counts resulting from HU administration probably provide a clinical benefit to SCD patients. Lower neutrophil numbers may limit the extent of tissue destruction and the severity of pain caused by vaso-occlusion and infarction (37). Our results support these previous findings. Evaluations of RTC immaturity and changes in RTC volume associated with HbF synthesis may provide interesting insights into the effects of HU on HbF production and erythroid kinetics in SCD patients.

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