Fetal Hemoglobin and F-Cell Responses to Long-Term Hydroxyurea Treatment in Young Sickle Cell Patients

By Micheline Maier-Redelsperger, Mariane de Montalembert, Antoine Flahault, Maria Grazia Neonato, Rolande Ducrocq, Marie-Pierre Masson, Robert Girod, and Jacques Elion for the French Study Group on Sickle Cell Disease

We have studied the cellular and molecular responses to long-term hydroxyurea (HU) treatment in 29 severely affected young patients with sickle cell disease (mean age, 10.9 ± 4.1 years). Patients received HU at 20 mg/kg/d on 4 consecutive days per week initially, with a monthly escalated dose avoiding marrow-toxicity (mean steady-state dose, 34.2 ± 4.6 mg/kg/d) for 12 to 36 months (mean duration, 22 months). The studied parameters were hemoglobin F (HbF), F reticulocytes (F retics), F cells, the amount of Hbf per F cell (F/F cell), polymer tendency at 40% and 70% deoxygenation, and hemolysis. Initial HbF (Fi) was dispersed (from 0.85% to 13.9%). HbF increased in all patients but 1. HbF at maximal response (Fmax) reached a sustained level varying from a 1.5-fold to a 16-fold Fi after a variable delay (6 to 24 months). Fmax was not related to HU dosage, but ∆F (Fmax – Fi) was strongly correlated to ∆MCV (MCVmax – MCVi). HbF increase resulted from the increase of both F cells and F/F cell. In this rather short series, Fi and Fmax were not significantly associated with age, gender, or β-globin haplotype. Neither Fmax nor ∆F was related to bone marrow reserve, as measured by baseline reticulocyte or neutrophil counts. However, Fmax was highly dependent on Fi. When patients are individualized into three groups according to Fmax (group 1, Fmax > 20% [12 patients]; group 2, 10% < Fmax < 20% [11 patients]; group 3, Fmax < 10% [5 patients]), Fi is significantly different between groups, being the highest in group 1. In addition, the best responders (group 1) were significantly different from patients in the two other groups with higher levels of total hemoglobin, decreased bilirubin, and decreased polymer tendency. © 1998 by The American Society of Hematology.

SICKLING OF RED BLOOD cells in sickle cell disease (SCD) is the consequence of a single amino acid substitution in the β-globin chain (βGlu→Val) that is responsible for the polymerization of the abnormal hemoglobin S (HbS) upon deoxygenation. The extent of polymer formation at any oxygen saturation is primarily dependent on the total intracellular hemoglobin concentration and on the respective percentages of S and non-S hemoglobins within the cell. Non-S hemoglobins, such as hemoglobin A (HbA), A2 (HbA2), or F (HbF), influence the polymerization process, because they reduce the intracellular HbS concentration and because mixed hybrids with HbS and HbF (or A2) do not enter the polymer. This last property makes HbF the most potent inhibitor of deoxyHbS polymerization. Accordingly, many approaches to develop therapies for SCD have focused on preventing polymerization by the use of pharmacological agents that increase the production of HbF. Among the various drugs proposed within the last years to improve the clinical course of SCD, hydroxyurea (HU) seems to be the most effective and has now been tried in large multicentric series of adult patients. After a double-blind trial enrolling 299 adults patients, it appeared that HU significantly reduced the frequency of painful crises, acute chest syndrome episodes, and blood transfusion requirements. These promising results in adult patients, associated with the absence of toxic effects or malignancies observed during long-term HU administration in a series of young patients with cyanotic congenital heart disease, encouraged us to investigate this treatment in young SCD patients. We present here data concerning a phase II therapeutic assay performed over 3 years in a cohort of 29 young SCD patients to appreciate if HU could stimulate HbF production without inducing clinical or hematological toxicity. This cohort included a large majority of children and teenagers belonging to a first generation of African immigrants, usually of homogeneous ethnic background.

MATERIALS AND METHODS

Patients

Twenty-nine young homozygous SCD patients (21 males and 8 females) were selected for this study from the clinics of centers belonging to the French Study Group on Sickle Cell Disease. The age range was 4 to 19 years (mean age, 10.9 ± 4.1 years). One child was less than 5 years of age, 10 were between 5 and 10 years of age, 10 were between 10 and 15 years of age, and 8 were more than 15 years of age. Diagnosis of SCD was established for each individual on the basis of hemoglobin electrophoresis and family studies. To be eligible, patients had to have reported at least three painful crises necessitating an hospitalization in the precedent year. Exclusion criteria were renal insufficiency (creatinine clearance <120 mL/min/1.73 m²), hepatic...
HYDROXYUREA IN SCD CHILDREN

prescribed if reticulocytes decreased below 50

Statistical Analysis

RESULTS

Patients

Follow-up varied from 12 to 36 months (mean duration, 22

Hematological Data

Table 1 shows the mean hematological response of the cohort

Table 1. Laboratory Values During Treatment With HU

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pretreatment Value (n = 29)</th>
<th>HbF Maximal Response (n = 28)</th>
<th>Year 1 (n = 28)</th>
<th>Year 2 (n = 20)</th>
<th>Year 3 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total hemoglobin (g/dL)</td>
<td>8.4 ± 1.2</td>
<td>8.9 ± 1.1*</td>
<td>9.0 ± 1.2</td>
<td>9.1 ± 0.9</td>
<td>9.2 ± 0.6</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>84.5 ± 10.1</td>
<td>101.4 ± 13.4*</td>
<td>101.6 ± 13.6</td>
<td>101.8 ± 15.9</td>
<td>102.4 ± 14.8</td>
</tr>
<tr>
<td>Neutrophils (×10^9/L)</td>
<td>7.2 ± 2.9</td>
<td>4.2 ± 2.6*</td>
<td>4.3 ± 2.9</td>
<td>5.2 ± 2.3</td>
<td>4.7 ± 2.9</td>
</tr>
<tr>
<td>Platelets (×10^9/L)</td>
<td>393 ± 170</td>
<td>340 ± 114</td>
<td>338 ± 117</td>
<td>299 ± 175</td>
<td>340 ± 109</td>
</tr>
<tr>
<td>Reticulocytes (×10^9/L)</td>
<td>417 ± 214</td>
<td>229 ± 129*</td>
<td>225 ± 109</td>
<td>266 ± 140</td>
<td>252 ± 73</td>
</tr>
<tr>
<td>HbF (%)</td>
<td>40.0 ± 3.4</td>
<td>17.3 ± 8.5*</td>
<td>13.9 ± 7.4</td>
<td>13.0 ± 9.4</td>
<td>22.5 ± 2.8</td>
</tr>
<tr>
<td>F reticulocytes (%)</td>
<td>7.1 ± 3.9</td>
<td>21.7 ± 10.7*</td>
<td>24.6 ± 15.0</td>
<td>26.0 ± 16.9</td>
<td>32.7 ± 35.1</td>
</tr>
<tr>
<td>F cells (%)</td>
<td>24.4 ± 14.3</td>
<td>59.9 ± 19.4*</td>
<td>57.0 ± 23.2</td>
<td>54.2 ± 22.1</td>
<td>55.5 ± 27.3</td>
</tr>
<tr>
<td>F/F cell (pg)</td>
<td>4.1 ± 1.3</td>
<td>8.7 ± 2.7*</td>
<td>8.4 ± 2.3</td>
<td>6.9 ± 3.3</td>
<td>11.8 ± 1.7</td>
</tr>
</tbody>
</table>

* P < .05 from Wilcoxon signed test, comparing the pretreatment values with values at HbF maximal response.
.0005). The platelet count also decreased from 393 ± 170 to 340 ± 14 × 10^9/L, but this decrease was not significant.

**HbF Production**

**Variations in HbF.** Table 1 summarizes the mean HbF response of the cohort before HU, yearly during the treatment, and at the time of HbF maximal response.

Initial HbF levels (Fi) before treatment were dispersed (from 0.85% to 13.9%). Fi values were highly correlated to MCV (r = .57, P = .002). Female patients (n = 8) had a higher mean Fi than males (n = 21: 6.1% ± 5.1% v 3.5% ± 2.1%), but this difference was not significant. Mean Fi values for Senegalese, Benin, and CAR homozygous patients were 5.5% ± 0.8%, 3.3% ± 1.6%, and 4.3% ± 4.1%, respectively, but these differences were not significant. In this series, Fi was not correlated to age.

An increase in HbF was consistently observed in all the patients except 1 (treatment was stopped for this patient). The variations of HbF percentages are presented in Fig 1. Individual data for all the patients are represented in Fig 1A, which shows the great variability of response from patient to patient. To better illustrate the various types of response described below, eight of these curves were selected and are shown in Fig 1B.

At the time of the patients’ HbF maximal response, HbF reached an absolute level (Fmax) varying from 3.3% to 36.2% (patient no. 1 in Fig 1B reached an Fmax of 34.7%). From patient to patient, this represented a variable increase of Fi, from 1.5-fold (patient no. 3 in Fig 1B) to 16-fold (patient no. 5 in Fig 1B). Eighteen patients achieved at least a twofold increase of Fi at 3 months and 8 patients at 6 months. Two patients achieved a twofold increase after 6 months, with their HbF increase being 1.5- and 1.8-fold, respectively, at 6 months (patient no. 3 in Fig 1B). The delay to reach Fmax was variable, from 6 months (patients no. 7 and 4 in Fig 1B) to 18 months (patient no. 2 in Fig 1B) and even 24 months (1 case) of treatment. For that matter, patients no. 7 and 8 are interesting to compare, because they reached the same sustained Fmax value, but at very different times (6 and 15 months, respectively). In some cases, HbF values reached a plateau after 6 months (patient no. 7 in Fig 1B) or were still increasing after 12 months (patient no. 2 in Fig 1B). The slope of HbF increase was not predictive of Fmax (see patients no. 6 and 7) and was not correlated to age. Once the peak of maximal HbF value was reached, most of the time, HbF stabilized at a lower level, except for 5 patients for whom the maximal HbF value was sustained (patients no. 8, 7, 4, 3, and 2 in Fig 1B).

Fmax was not correlated to the maximum HU dosage, but it was correlated to MCVmax (r = .56, P = .002). Similarly, ΔF (Fmax – Fi) was strongly correlated to ΔMCV (MCVmax – MCVi) (r = .62, P = .0007). Fmax was not related to reticulocyte, neutrophil, or platelet initial values, but it was highly correlated to initial F retics (r = .63, P = .003). It was not correlated to age, gender, or β-globin haplotypes. Because the vast majority of the patients belonged to the L (boys) or LL (girls) phenotype concerning the FCP locus, no conclusion can be drawn from our series as to the potential influence of this locus on HbF response. Similarly, the number of patients for whom the α-globin gene status was determined was too small to conclude on its eventual influence.

We chose arbitrarily 10% and 20% Fmax levels to individualize three groups of patients: group 1, whose Fmax were greater than 20%; group 2, whose Fmax were greater than 10% but lower than 20%; and group 3, whose Fmax remained less than 10%. HbF variation from Fi to Fmax for each individual in these three groups is shown in Fig 2. Noticeably, the 20% level (group 1) was reached by 12 patients in a delay varying from 6 to 15 months. Nine of them had a pretreatment HbF value greater than 4%. The 10% level (group 2) was reached by 11 patients. Five patients did not reach the 10% level (group 3) and 4 of them had an initial HbF value less than 2%. Comparisons between the three groups were performed for all the parameters that were found to be significantly different before HU and at the time of Fmax on the whole cohort analysis (Table 2). Pretreatment hemoglobin levels, MCV values, and reticulocyte counts were not significantly different from one group to another. However, Fi values were significantly different between the three groups (P = .005). At the time of HbF maximal response, significant differences were observed between the three groups for hemoglobin levels (P = .004) and MCV values (P = .03). From pretreatment to the time of HbF maximal response, the reticulocyte count decreased by 55%, 37%, and 25% for groups 1, 2, and 3, respectively, but these differences are not significant. Within each group, significant variations were observed from pretreatment to the time of HbF maximal response for total hemoglobin, MCV, reticulocytes, and HbF for group 1; MCV, reticulocytes, and HbF for group 2; and MCV and HbF for group 3 (see Table 2 for details). Considering the whole series, the average bilirubin level decreased from a pretreatment value of 40.4 ± 24.8 mg/L to 33.3 ± 20.2 mg/L at the time of HbF maximal response (P = .07). But when this parameter was analyzed within each of the three groups, a significant decrease was only observed for group 1 (Table 2).

**Variations in cellular parameters.** Table 1 shows the mean values of F cells, F/F cell, and F retics before HU, yearly during treatment, and at the time of HbF maximal response. An increase of these parameters was constant, but it was very variable from patient to patient both in terms of kinetics and of the maximal levels achieved. F cells exhibited a 1.2- to 5.1-fold increase of the pretreatment value and F retics a 1.5- to 7.4-fold increase of these parameters was constant, but it was very different between the three groups at the time of HbF maximal response. But a significant difference was observed between the three groups at the time of maximal HbF response. We conclude on its eventual influence.
groups before treatment, in agreement with the difference in Fi. Within each group, significant variations were observed from pretreatment to the time of HbF maximal response for F retics, F cells, and F/F cell for groups 1 and 2, but not for group 3 (see Table 2 for details).

**Polymer Formation at Different Oxygen Saturations**

In the F-cell population, the rate of polymer tendency decreased significantly during the treatment in all cases (except 1; Table 2). At 40% oxygen saturation, calculated values were...
0.28 ± 0.07 (0.16 to 0.42) and 0.20 ± 0.04 (0.13 to 0.30) before HU and at the time of HbF maximal response respectively (P < .0003). At 70% oxygen saturation, calculated values were 0.05 ± 0.05 (0.00 to 0.17) and 0.01 ± 0.02 (0.00 to 0.07) before HU and at the time of HbF maximal response, respectively (P < .003). Considering groups 1, 2, and 3, neither pretreatment values nor values at the time of HbF maximal response were significantly different from one group to another (Table 2). A decrease in polymer tendency at 40% and 70% oxygen saturation during treatment occurred for all three groups of patients, but the decrease was significant only for group 1 (P = .01 and P = .02). In the non–F-cell population, no variation of polymer tendency was observed as predicted from the lack of changes in HbF levels.

## DISCUSSION

Contrasting with the large body of data concerning the use of HU in adult patients, including the multicentric series, only a few pediatric trials have been reported to date in the United States, Belgium, and France respectively. These reports deal mostly with global HbF response, clinical benefit, and tolerance. We report data here concerning the cellular and molecular parameters of the response to HU, including variations of F retics, F cells, F/F cell, and polymer tendency in the F-cell population, in 29 patients of the French cohort.

Two parameters can be used to describe HbF response: (1) the rate of HbF increase and (2) the maximal value achieved.

### Table 2. Laboratory Values in Groups of Patients No. 1, 2, and 3

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pretreatment Values</th>
<th>Values at HbF Maximal Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 (n = 12)</td>
<td>Group 2 (n = 11)</td>
</tr>
<tr>
<td>Hemoglobin g/dL</td>
<td>8.4 ± 1.3</td>
<td>7.9 ± 1.0</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>88.3 ± 12.3</td>
<td>82.9 ± 7.3</td>
</tr>
<tr>
<td>Neutrophils (× 10⁹/L)</td>
<td>7.6 ± 2.4</td>
<td>7.0 ± 3.6</td>
</tr>
<tr>
<td>Reticulocytes (× 10⁹/L)</td>
<td>443 ± 213</td>
<td>450 ± 247</td>
</tr>
<tr>
<td>HbF (%)</td>
<td>6.3 ± 6.7</td>
<td>3.3 ± 2.1</td>
</tr>
<tr>
<td>F reticulocytes (%)</td>
<td>10.8 ± 6.8</td>
<td>5.3 ± 2.4</td>
</tr>
<tr>
<td>F cells (%)</td>
<td>33.6 ± 14.5</td>
<td>20.1 ± 7.8</td>
</tr>
<tr>
<td>F/F cell (pg)</td>
<td>5.6 ± 1.6</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td>P40</td>
<td>0.26 ± 0.06</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>P70</td>
<td>0.04 ± 0.04</td>
<td>0.07 ± 0.07</td>
</tr>
<tr>
<td>Bilirubin (mg/L)</td>
<td>47.5 ± 28.1</td>
<td>36.9 ± 24.6</td>
</tr>
</tbody>
</table>

Abbreviations: NS, not significant. P > .05; P40 and P70, polymer tendency at 40% and 70% oxygen saturation in the F-cell population.

*P values from Kruskal-Wallis test comparing the values of the three groups either before treatment or at the time of HbF maximal response.

†P < .05 from Wilcoxon signed test within group categories, comparing the pretreatment values with values at HbF maximal response.
We find that both parameters are highly variable; thus, their accurate evaluation depends both on the size of the series and on the duration of follow-up. Here, 29 young patients have been observed for periods varying from 12 to 36 months (average, 22 months), as compared with 6 months of follow-up of 25 patients in the Belgian series23 and a 6 to 39 months (average, 24 months) of follow-up of 13 patients in the American series.24 We find that the maximal level of HbF is not reached at 6 months for 68% of the children and only after 12 months for 14% of them. In addition, we find that the HbF value at 6 months is not predictive of the maximal achieved value.

Once the peak of maximal HbF value is reached, HbF stabilized at a slightly lower level, except for 5 patients for whom the maximal HbF value was sustained. The plateauing of HbF at 2 or 3 years of HU contrasts with Steinberg’s observation in adults,25 who shows that only half of patients have a sustained increase of HbF after 2 years of HU treatment. Whether HbF response under HU is better in children than in adults is an hypothesis that may be suggested on another issue: a fourfold increase of HbF level is observed in the two series that include the youngest children (Ferster’s series [children 2 to 22 years of age]25 and our series [children 4 to 19 years of age]), whereas a twofold increase is only observed in Scott’s series, which includes older children (children 10 to 17 years of age).24 Furthermore, a negative relationship was found between the age and the slope of HbF increase in de Montalembert’s series,15 which includes the patients of the series studied here and also 6 additional children, most of them very young. However, comparison between series is difficult, because we used a different drug dosing than Scott and Ferster. It results in the bone marrow reserve, ie, the capacity of the marrow to withstand moderate doses of HU with acceptable myelotoxicity.27 The number of myelotoxic episodes in our series was clearly lower than that reported in Steinberg’s series.25 It was actually limited to three episodes defined according to our criteria: one was a neutropenic episode (neutrophils, 1.3 × 10^9/L), one a thrombopenia (platelets, 90 × 10^9/L), and the third one a reticulopenia (reticulocytes, 80 × 10^9/L). This may be related either to the modified schedule of administration that we used (4 days/wk instead of daily) or to a better hematological tolerance of the drug in children. In this short pediatric series, there was no correlation between myelotoxic events and HbF increase, suggesting that perhaps marrow reserve does not influence HbF response in children.

Indeed, we found a clear relation between Fmax and Fi. However, when we divide the patients in three groups according to the Fmax levels, based on the assumption that this parameter is the most relevant to the expected clinical benefit,28-32 then all the Fi-related parameters in the three groups are significantly different. In contrast, these three groups are indistinguishable on the bases of initial hematological and hemolysis parameters. This finding also contrasts with Steinberg’s observation in adults,27 who found no association between HbF response and baseline HbF levels. At the time of HbF maximal response, group 1 (that with the maximal response, HbF >20%) is clearly singled out. All the HbF-related parameters are at the highest, including F retics. As a result, polymer tendency is reduced and so is total bilirubin. In accordance, total Hb is increased and reticulocyte count is low. This decrease in polymer tendency agrees with Bridges’s results that HU increases Hbs polymerization delay time.33

We find that Fi were correlated to the two parameters F cells and F/F cell. In agreement with the data of Charache et al.,24 we find that HbF increase during HU treatment resulted both from the increase of F cells and F/F cell and thus might proceed from cellular as well as molecular mechanisms. When the factor of increase of F cells from the initial value is determined at the time of the patients’ HbF maximal response, we find that the highest values are achieved for the patients exhibiting the lowest pretreatment values of F cells. This observation contrasts with our result that the best responders (group 1) are those with the highest Fi. However, this finding may be an indirect effect of the sensitivity of the method of detection. Indeed, when scoring F cells, cells containing amounts of HbF just below the threshold of detection are naturally scored with non-F cells and minor variations in HbF production during treatment may be sufficient to shift the distribution of these cells across the threshold.35,36 In conclusion, our data tend to support the fact that response to HU treatment in young patients is better than in adults, because we observed only one nonresponder. It results in a HbF level that is sustained at a level slightly lower than the HbF maximal value. We find that the HbF response in children is dependent on the initial HbF value but not, as observed in the adults, on the bone marrow reserve. The best responders form a group that distinguishes clearly from the others, with higher Hb levels, decreased bilirubin, and decreased polymer tendency. Our study was focused on the parameters of HbF response to
HU treatment. Given the fact that HU clearly has pleiotropic effects, other parameters will have to be studied before a clear correlation could be established between the clinical and biological response to treatment. These additional factors will be likely to provide further insights into the polygenic modulation of HU response and to generate more definitive predictive markers of the robustness of the response and of the ultimate clinical utility.

APPENDIX

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