F Reticulocyte Response in Sickle Cell Anemia Treated With Recombinant Human Erythropoietin: A Double-Blind Study

By Ronald L. Nagel, Elliot Vichinsky, Maya Shah, R. Johnson, E. Spadacino, Mary E. Fabry, L. Mangahas, Robert Abel, and G. Stamatoyannopoulos

Studies on baboons and preliminary observations in three patients with sickle cell anemia (SS) suggested that high doses of pulse administered recombinant human erythropoietin (rHuEPO) stimulate F-reticulocyte production. We now report on the administration of rHuEPO in a double-blind format to ascertain frequency of response and potential precipitation of side effects. Ten patients were enrolled, but one was discontinued due to the indication of a blood transfusion. Of the other nine, five received rHuEPO in escalating doses (from 400 to 1,500 U per kg twice daily [BID] per week), alternating with a placebo, in blinded fashion. The second group, consisting of four patients, followed an identical protocol (except starting dose was 1,000 U/Kg, BID per week) and were iron supplemented during treatment. The criterion of response was a transient doubling (as a minimum) of the steady-state F-reticulocyte level. We found that none of the five patients in the first group responded to rHuEPO, and two of them became iron deficient, as judged by a significant decrease in ferritin. Of the second group, four patients responded with F-reticulocyte increases. In three patients, open label administration of rHuEPO confirmed the effect. We observed seven painful episodes during this study, two during the EPO administration and five during the placebo arm. Three patients were phlebotomized because the hemoglobin level increased 1.5 g/dL more than steady-state levels. Of the six patients followed-up by percent dense cell determinations, one exhibited increased levels during periods of the treatment, whereas the other five showed no change. No anti-rHuEPO antibodies were detected. We conclude that rHuEPO can stimulate F-reticulocyte response in some patients with sickle cell anemia, without apparent negative clinical side effects. The state of iron stores may be critical. Whether higher doses of HuEPO and/or a different regimen might induce sustained F cells and fetal hemoglobin increases remains to be determined.

© 1993 by The American Society of Hematology.

A SIGNIFICANT increase in the intraerythrocytic concentration of fetal hemoglobin (HbF) is very likely to inhibit polymerization of sickle cell Hb (HbS) and, consequently, the sickling phenomena. Several efforts have been recently made to pharmacologically induce this effect. Cytotoxic agents such as Ara-C and hydroxyurea have exhibited some promise and the use of the latter agent is being pursued actively. Nevertheless, concerns about potential long-term toxicity and the need to elicit the highest possible increase in HbF expression have led other groups to continue the search for HbF-inducing agents that can be administered alone or in combination with cytotoxic agents.

The observation that bleeding in baboons induced an F-reticulocyte response similar to cytotoxic agents (vinblasticine and Ara-C) prompted Stamatoyannopoulos et al to postulate that erythropoietin (EPO) could be useful in the induction of HbF. Further studies in baboons with pulse doses gave credence to this approach because HbF reticulocyte response was observed. Also, preliminary, uncontrolled administration of recombinant human EPO (rHuEPO) to three sickle cell anemia patients suggested that F-reticulocyte response could be elicited.

We report here, using a double-blind format, on a study of the administration of rHuEPO to ascertain frequency of response (defined as a doubling of the F reticulocytes) and potential side effects in a group of 10 sickle cell anemia patients. Of note is that this protocol did not result in the observation of, nor is it expected to lead to increased levels of HbF cells or percent HbF because neither effect was obtained with high pulse doses in the baboon.

MATERIALS AND METHODS

Patient Selection

The study population consisted of 10 sickle cell anemia patients who met the following inclusion criteria at the prestudy examination:

1. Homozygous SS for sickle cell anemia, as evidenced by two methods of Hb electrophoreses; (2) monitored clinical status of at least 3 months; (3) aged between 18 and 45 years; (4) clinically stable as otherwise evidenced by medical history, complete physical examination (including vital signs and weight), and 12-lead electrocardiogram, each performed within 2 weeks of the start of the study; (5) no clinically significant, acute abnormalities on chest x-ray (within 6 months before study participation); (6) no clinically significant abnormal laboratory values for the following tests, except for those values out of the normal range due to the disease process: in addition, all medications and dosages must have been stable for 2 weeks before study entry, and were continued throughout the duration of the study; (7) all females were either postmenopausal for at least 1 year, had a hysterectomy or tubal ligation, or had taken oral contraceptives for at least 1 month before study entry, or agreed to use spermicide and barrier methods of protection (the patients continued with the same medication).
method for the duration of the study.); (8) all females of childbearing potential had a negative serum pregnancy test (human chorionic gonadotropin β radioimmunoassay [HCGβ RIA] test) and history of a normal menstrual flow within 1 month before study entry; and (9) an informed consent was signed after the nature of the study has been fully explained.

The criteria for exclusion were: (1) presence of significant hepatic, cardiovascular, pulmonary, malignant, hematologic, neurologic, infectious, or inflammatory diseases unrelated to sickle cell anemia; (2) sickle cell crisis within 3 weeks of study admission; (3) uncontrolled hypertension (ie, diastolic blood pressure > 100 mmHg) or history of seizures; (4) presence of asthma or severe atopic disease; (5) presence of current (last 3 months) ischemic heart disease; (6) presence of thrombocytopenia (<100,000 cells/µL); (7) presence of neutropenia (<2,000 cells/µL); (8) history of Coombs-positive hemolytic anemia; (9) presence of apparent iron-deficiency anemia; (10) corticosteroid therapy or immunosuppressant therapy within 1 month before study entry; (11) history of substance abuse; (12) exposure to an investigational drug within 1 month of the start of the study; (13) acute illness within 7 days of the start of the study; and (14) plasma creatinine greater than 1.5 mg/dL.

The procedures for screening were performed within 2 weeks before dosing, except where otherwise stated, and included the following: (1) medical history, including sickle cell crisis history; (2) physical examination, including weight and vital signs; (3) 12-lead electrocardiogram; (4) chest x-ray (within 6 months); (5) the following routine laboratory tests: (A) CBC plus differential, and platelet count; (B) serum chemistry and serum ferritin; and (C) urinalysis, including pH, specific gravity, and microscopic; (6) the following special laboratory measurements: (A) percent and total reticulocyte count; (B) F reticulocytes; (C) F cells; and (D) HbF, by alkali denaturation; (7) pregnancy test, RIA, if applicable; (8) rHuEPO antibody; (9) naturally occurring rHuEPO levels; and (10) signed consent form in the presence of witnesses.

Sickle cell anemia patients were defined by two methods of Hb electrophoreses (cellulose acetate at pH 8.6 and agar electrophoresis, pH 6.4) as having exclusively HbS, HbF, and HbA2 in their red blood cells (RBCs). S/β thalassemia combinations were excluded by family studies, normal mean corpuscular volume (MCV), and, in some cases, Mst II digestion of their DNA.

Table 1 depicts steady-state hematologic characteristics of the patients that completed the study.

### Table 1. Hematologic Parameters of the Sample

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Hb (g/dL)</th>
<th>Hct (%)</th>
<th>MCV (fl)</th>
<th>Total Retic (%)</th>
<th>F Retic (%)</th>
<th>F Cells (%)</th>
<th>HbF (%)</th>
<th>Bilirubin (µg/dL)</th>
<th>Iron Suppl (mU/µL)</th>
<th>Creat (mg/dL)</th>
<th>LDH (U/L)</th>
<th>Ferritin Before EPO</th>
<th>Ferritin After EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KM</td>
<td>35/M</td>
<td>9.8</td>
<td>28.1</td>
<td>106.9</td>
<td>29.9</td>
<td>12.0</td>
<td>48.2</td>
<td>10.2</td>
<td>2.9</td>
<td>No</td>
<td>237</td>
<td>1.0</td>
<td>390</td>
<td>671</td>
</tr>
<tr>
<td>AL</td>
<td>26/M</td>
<td>10.2</td>
<td>30.7</td>
<td>85.2</td>
<td>13.7</td>
<td>11.0</td>
<td>40.8</td>
<td>6.3</td>
<td>1.7</td>
<td>No</td>
<td>80</td>
<td>1.0</td>
<td>316</td>
<td>700</td>
</tr>
<tr>
<td>AJ</td>
<td>39/M</td>
<td>9.1</td>
<td>27.2</td>
<td>106.9</td>
<td>13.5</td>
<td>11.8</td>
<td>34.3</td>
<td>5.5</td>
<td>1.7</td>
<td>No</td>
<td>157</td>
<td>0.8</td>
<td>352</td>
<td>1,080</td>
</tr>
<tr>
<td>HL</td>
<td>27/M</td>
<td>8.3</td>
<td>22.3</td>
<td>86.3</td>
<td>22.7</td>
<td>7.0</td>
<td>16.3</td>
<td>2.07</td>
<td>2.4</td>
<td>No</td>
<td>300</td>
<td>0.9</td>
<td>363</td>
<td>42.4</td>
</tr>
<tr>
<td>KW</td>
<td>25/M</td>
<td>9.8</td>
<td>27.9</td>
<td>98.4</td>
<td>14.1</td>
<td>11.8</td>
<td>27.3</td>
<td>4.9</td>
<td>3.0</td>
<td>No</td>
<td>79</td>
<td>0.8</td>
<td>331</td>
<td>147</td>
</tr>
</tbody>
</table>

**Group B**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Hb (g/dL)</th>
<th>Hct (%)</th>
<th>MCV (fl)</th>
<th>Total Retic (%)</th>
<th>F Retic (%)</th>
<th>F Cells (%)</th>
<th>HbF (%)</th>
<th>Bilirubin (µg/dL)</th>
<th>Iron Suppl (mU/µL)</th>
<th>Creat (mg/dL)</th>
<th>LDH (U/L)</th>
<th>Ferritin Before EPO</th>
<th>Ferritin After EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW</td>
<td>25/M</td>
<td>8.7</td>
<td>26.2</td>
<td>75.4</td>
<td>12.8</td>
<td>0.9</td>
<td>3.5</td>
<td>2.5</td>
<td>6.0</td>
<td>Yes</td>
<td>185</td>
<td>0.9</td>
<td>493</td>
<td>73</td>
</tr>
<tr>
<td>IP</td>
<td>25/M</td>
<td>9.6</td>
<td>28.1</td>
<td>101.2</td>
<td>25.9</td>
<td>10.8</td>
<td>38.9</td>
<td>7.0</td>
<td>2.3</td>
<td>IO</td>
<td>92</td>
<td>0.8</td>
<td>385</td>
<td>13,480</td>
</tr>
<tr>
<td>EP</td>
<td>24/F</td>
<td>8.7</td>
<td>28.3</td>
<td>93.2</td>
<td>8.1</td>
<td>5.5</td>
<td>38.5</td>
<td>6.3</td>
<td>1.5</td>
<td>Yes</td>
<td>63</td>
<td>0.4</td>
<td>232</td>
<td>503</td>
</tr>
<tr>
<td>JP</td>
<td>20/F</td>
<td>8.7</td>
<td>28.0</td>
<td>92.7</td>
<td>10.8</td>
<td>10.6</td>
<td>16.3</td>
<td>2.4</td>
<td>1.1</td>
<td>IO</td>
<td>54</td>
<td>0.5</td>
<td>237</td>
<td>21,240</td>
</tr>
</tbody>
</table>

Abbreviations: Hct, hematocrit; Retic, reticulocytes; Bilirubin, total bilirubin; Creat, creatinine; LDH, lactic dehydrogenase levels; IO, iron overload.

* Pretrial plasma EPO levels.

### Study Protocol

**Group A patients.** Five patients not supplemented with iron received rHuEPO or placebo once a week for 12 weeks after a blinded randomization schedule in which rHuEPO alternated with a placebo. The weekly administration was divided in two doses separated by 12 hours. Doses were escalated weekly from 400 to 1,500 U/kg weight, except for one patient who was initially treated with 1,000 U/kg twice daily (BID) per week and escalated to 1,500 U/kg BID per week.

**Group B patients.** Five patients were enrolled in the study, but one patient did not complete the study because he required exchange transfusion therapy because of acute chest syndrome in the initial part of the protocol. Patients in this group received a supplement of 325 mg of ferrous sulphate three times a day throughout the study, except for the two patients who had very high steady-state ferritin levels (>10,000 U). The escalating doses of rHuEPO began at 1,000 and escalated to 1,500 U/kg week.

Because the increase of total Hb is considered an unwelcome event in the absence of antisickling therapy, the increase in 1.5 g/dL over steady-state during the protocol was an indication for phlebotomy.

If the patient doubled the F-reticulocyte percentage, the patient was eligible to an open label phase at the doses at which the effect was seen. In this follow-up protocol, rHuEPO was administered weekly and was not alternated with placebo.

### Laboratory Procedures

Patients were monitored three times a week in group A and four times a week in group B. Complete blood counts with differential white blood cell counts were determined with a Coulter Counter (Coulter, Hialeah, FL). Reticulocyte counts were performed in 2,000 cells using the brilliant cresyl blue method. For the determination of red RBCs that contained HbF (F cells), the cells were washed and resuspended in fetal calf serum. Smears made with these cells were dried and fixed in a mixture of acetone, methanol, and ethanol and reacted with murine anti-γ-globin and a goat antimouse γG(αβ), fragment conjugated to fluorescein isothiocyanate. F reticulocytes were counted as previously described by Papayannopoulou et al. HbF levels were determined by alkaline denaturation and RBC densities as previously described. Density gradients were performed as described by Fabry et al. Liver and renal function test and ferritins were performed weekly. Baseline serum EPO was determined by ra-
dioimmune assay (SmithKline Bioscience Laboratories, Evansville, IN) and serum anti-EPO antibodies were determined by Ortho Pharmaceutical Corp (Raritan, NJ).

Criteria of Response

The doubling of the F-reticulocyte level after the administration of rHuEPO (particularly when appropriately delayed by 1 or 2 days) was used as a criterion of response. This protocol was not designed to result in an increase in HbF, nor were significant increases in HbF levels expected to be seen. Its objective was limited to qualitatively reproduce the baboon response to EPO in humans, that is, the increase in F-reticulocytes secondary to EPO administration.

RESULTS

Group A Patients

None of the five patients in this group had a frank response to rHuEPO administration, according to our established criteria, except that one patient reached the exact doubling of F reticulocytes on one occasion. In most of the patients, there were smaller but consistent increases in the F reticulocytes in association with the administration of rHuEPO, but not with placebo. In this group of patients that were not supplemented with iron before or during treatment, interesting changes in ferritin levels were observed. Serum ferritins decreased in all patients and two patients developed frank iron deficiency; coincidentally, with hypoferritinemia, they exhibited microcytosis and hypochromia (Table 1).

Group B Patients

Complete results were obtained in four of the five patients recruited. Three were supplemented with 325 mg of ferrous sulphate three times daily (TID) and the other two had iron overload (due to previous chronic transfusion protocols) with ferritin levels greater than 10,000. No significant decrease of ferritin was observed in these four patients (Table 1). The fifth patient was discontinued early in the protocol due to medical indication for transfusion (acute chest syndrome).

All four patients had an F-reticulocyte response according to our criteria. In three patients (examples are presented in Figs 1 through 3), the increase in F reticulocytes was consistent and was confirmed in an open label format in which the administration of rHuEPO was made weekly, and not alternated with placebo. The fourth patient's (JP) F reticulocytes also reached doubling (Fig 4), and an open label study was attempted, but due to a cluster of painful crises, no reliable data were collected. The F-reticulocyte response, as in baboons, was transient and occurred 1 to 3 days after rHuEPO administration.

All four patients in group B had increased total reticulocytes after rHuEPO administration, which slightly preceded the maximal F-reticulocyte response.

Of the three patients that responded with a confirmed F-reticulocyte increase, one (CW) had a significant increase in F cells during the period of treatment (from 3.5% to 7.6%). The others showed no change.

Dense cells were determined for five SS patients, one from group A and four from group B. Only one patient (CW) exhibited an increase of dense cells, not accompanied with any clinical symptoms. A decrease to steady-state levels was observed before the open label phase was finished and before the last two doses of rHuEPO. The increase in dense cells preceded painful crises, particularly on day 132 (see Discussion).

Of the three responders, one of them (IP) had six episodes in which total Hb exceeded 1.5 g/dL. In each of these episodes, 1 U (500 mL) was removed by phlebotomy. This patient had a significant iron overload with a ferritin level greater than 10,000 (Table 1).

Fig 1. Double-blind phase of patient CW. In this graph, F reticulocytes, total reticulocytes, HbF, F cells, and dense cell determinations are depicted. In the lower part of the diagram, the period of a painful crises is depicted by a horizontal line, as well as the days the patient received placebo (Pla) or EPO.
All patients in group A responded maximally with the lower doses administered (1,000 U/kg BID per week). The size of the response was comparable with each dose when measured from the nadir of the placebo week.

Serial physical and laboratory examinations, and, in some cases, patient diaries, gave no indication of clinical complications, except for the instances of iron deficiency in group A. Seven painful crises were observed during the treatment of the nine patients, two after rHuEPO administration and five after placebo. Anti-rHuEPO antibodies were not detected in any of the patients.

FIG 2. Open label phase of patient CW. Same parameters as in Fig 1.

DISCUSSION

There are two important reasons to pursue alternatives to hydroxyurea treatment aimed at enhancing HbF synthesis in sickle cell anemia: (1) the concerns that exist that this cytotoxic agent could represent long-term health hazards\(^\text{15}\); and (2) the possibility of finding other agents that, in conjunction with a lower dose of hydroxyurea, could induce a higher HbF response.

The results presented here show that the administration of rHuEPO in high pulse doses can induce a transient increase
in F reticulocytes in sickle cell anemia patients, similar to, although less intense than, those previously found using a similar protocol in the baboon. This is significant, because experiments in baboons have a good record in predicting human responses (if not quantitatively, at least qualitatively). The F-reticulocyte response was commonly preceded by a burst of all reticulocytes, as expected. These results also confirm the preliminary study of Al-Khatti et al.

The results are at variance, nevertheless, with the report of Goldberg et al. The lack of iron supplementation and the infrequent monitoring of F reticulocytes might be responsible for their apparent negative findings.

The fact that the F-reticulocyte response was observed almost exclusively among patients supplemented with iron (or in iron overload) raises the possibility that iron for erythropoiesis might be a limiting factor in rHuEPO response. While iron deficiency is more common in sickle cell anemia disease than previously thought, the diagnosis is difficult because ferritin levels are not a good predictor. In addition, the experience of Peterson et al. during the cyanate trials shows that iron can be a limiting factor in erythropoietic response in sickle cell disease.

No clinical complications were observed with the administration of rHuEPO in sickle cell anemia, and the double-blind design of the study helped to put to rest the notion that administration of high doses of rHuEPO could induce painful crises, because more were observed associated with the use of a placebo than the use of rHuEPO.

The only laboratory abnormality was the significant increase in dense cells observed in one patient (unaccompanied by clinical symptoms), which disappeared before rHuEPO was discontinued. The interpretation of this phenomena is not clear, but it could be related to impending painful crises. Ballas and Smithe have detected an increase in dense cells preceding the appearance of crises, and have confirmed the decrease in dense cells observed during painful crises, as reported by Fabry and Nagel. Nevertheless, this observation should be kept in mind and all patients be monitored for dense cells in future studies.

We conclude that the administration of rHuEPO at high doses and in a pulse regimen is capable of producing a transient F-reticulocyte response in sickle cell anemia, apparently without any negative effects. The effect observed here in sickle cell anemia is qualitatively similar to the response to pulse doses of EPO in baboons, including the lack of HbF response. The challenge is to find the dosage and protocol to obtain an F-cell- and HbF-sustained response by rHuEPO alone or in combination with other drugs, such as hydroxyurea.

REFERENCES

8. Stamatoyannopoulos G, Veith R, Al-Khatti A, Papayanno-


F reticulocyte response in sickle cell anemia treated with recombinant human erythropoietin: a double-blind study

RL Nagel, E Vichinsky, M Shah, R Johnson, E Spadacino, ME Fabry, L Mangahas, R Abel and G Stamatoyannopoulos