Hydroxyurea Induction of Hemoglobin F Production in Sickle Cell Disease: Relationship Between Cytotoxicity and F Cell Production

By G.J. Dover, R.K. Humphries, J.G. Moore, T.J. Ley, N.S. Young, S. Charache, and A.W. Nienhuis

Initial alterations in fetal hemoglobin (HbF) production among eight sickle cell anemia subjects treated with hydroxyurea (Hu) are summarized. Four of these subjects had been previously treated with 5-azacytidine (5-aza). All subjects treated with Hu (50 mg/kg/d for three to five days) had suppression of their total reticulocyte counts by seven days, whereas the four subjects previously treated with 5-aza (2 mg/kg/d for three to five days) had increased reticulocyte counts at day 7. The effect of Hu on increasing the number of HbF-containing reticulocytes (F reticulocytes) is extremely variable, ranging from ten- to less than onefold differences in maximal posttherapy recovery from marrow suppression did not result in greater than twofold increases in F reticulocyte counts. Mean day 7 F reticulocyte levels in the four subjects treated with both Hu and S-aza were 4.1 x 10^7/μL and 15.4 x 10^7/μL, respectively.

Among Hu-treated subjects, increased F reticulocyte production was correlated with low serum creatinine levels and rapid removal of Hu from the plasma. Furthermore, suppression of CFU-E colony formation on day 2 of therapy with Hu was inversely correlated with maximal F reticulocyte response. We conclude that where Hu treatment results in marrow toxicity (decreased reticulocyte counts, decreased CFU-E colony formation) HbF production is less likely to increase. Those sickle cell anemia subjects with minimal renal dysfunction (serum creatinine level, >1.0 mg/dL) exhibit the most cytotoxicity and least F reticulocyte response to Hu.

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RESULTS

F reticulocyte response. Figure 1 details the F reticulocyte response of eight SS subjects given Hu. A greater than twofold increase in the F reticulocyte occurred in only four subjects (10, 12, A, E). Figure 2A illustrates that all eight subjects showed a decrease in total reticulocytes after seven days of Hu therapy. In contrast, the four subjects previously treated in a Coulter Counter (Coulter, Hialeah, Fla), and the percentage of total reticulocytes was estimated after staining with new methylene blue.

Hu levels. Plasma Hu levels were analyzed by the method of Fabricius and Rajewsky. The assay is a colorimetric procedure based on the oxidation of Hu, with iodine forming nitrite that diazotizes sulfanilic acid. The diazotized sulfanilic acid is then coupled with N-(1-naphthyl)-ethyleneamine dihydrochloride to produce a red color. Samples were separated from whole blood, frozen at -20 °C and assayed within two days of collection. Serum creatinine levels were determined at least five times prior to and during experimental therapy by routine clinical methods.

In vitro analysis. Assays of early and late erythroid progenitors (BFU-E and CFU-E) were performed as previously described in cultures that contained 0.8% methylcellulose (Dow Chemical Co, Midland Mich), 30% fetal calf serum (Flow Laboratories, McLean, Va), 2.5 μg/mL of erythropoietin (Connaught Laboratories, Willowdale, Canada), and 10% phytohemagglutinin-stimulated leukocyte-conditioned media.

MATERIALS AND METHODS

Subject selection. All subjects studied were adults with SS disease followed at Johns Hopkins Hospital (subjects A, C, and E) or the National Institutes of Health (NIH) (subjects 6, 7, 10, 11, and 12). The effects of an optimal dose of 5-aza (1.5 to 2 mg/kg/d) on subjects A, C, and 7 have been previously reported. Subjects E, 10, 11, and 12 were given only Hu. The dose of Hu, 50 mg/kg/d for three or five days, was chosen on the basis of previous reports in animals and man. Subjects 6 and 7 were given an additional single dose of Hu (25 mg/kg) and followed for seven days. All subjects gave informed consent for these experiments under the guidelines outlined by the Joint Committee on Clinical Investigation at Johns Hopkins Hospital or the National Heart, Lung, and Blood Institute Review Board.

HbF production. Previously described radial immunoprecipitation assays were used to estimate the percentage of HbF-containing reticulocytes (F reticulocytes). The red cell number was enumerated by routine clinical methods.

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was treated with 5-aza (A, C, 6, 7) showed no evidence of reticulocyte suppression at seven days following 5-aza therapy. Reticulocyte levels returned to pretreatment levels within seven days of their nadir in all Hu-treated subjects except subject 6. Subject 6 required a transfusion on day 7 after the hemoglobin level dropped from 9.8 to 5.0 g/dL following Hu administration. F reticulocyte levels increased with recovery from marrow suppression in subject 7, but the maximal F reticulocyte response (day 13) was not greater than twofold the pretreatment levels.

In all eight subjects given Hu, day 7 F reticulocyte levels were lower than those attained in the four subjects given 5-aza (Fig 2B). The mean day 7 F reticulocyte level for the four subjects given 5-aza and Hu were $15.4 \times 10^4 / \mu L$ and $4.1 \times 10^4 / \mu L$, respectively.

The two subjects who exhibited the most toxicity (6 and 7) were given a lower dose of Hu (25 mg/kg) and followed for seven days. In subject 6 the total reticulocyte count decreased slightly ($41 \times 10^4 / \mu L$), and the F reticulocyte count did not change ($1.5 \times 10^4 / \mu L$). Subject 7's total reticulocyte count also decreased ($32 \times 10^4 / \mu L$), but his F reticulocyte count increased ($3.8 \times 7.0 \times 10^4 / \mu L$).

**Hu levels.** Normally more than 80% of Hu is excreted within six hours in the urine.$^{13,14}$ Plasma Hu levels were measured following a standard oral dose of 25 mg/kg of Hu in all eight subjects. Plasma levels of Hu are shown for four subjects in Fig 3. Subjects 6 and 7 who showed little if any F reticulocyte response and a marked reduction in total reticulocytes had delayed plasma Hu clearance. Subjects A and 12 had normal plasma clearances of Hu, less severe suppression in total reticulocytes (Fig 2), and ten- and fourfold increases in the F reticulocyte level.

Minimal renal dysfunction might prolong clearance of Hu from the plasma and thereby lead to increased renal toxicity. Mean serum creatinine levels during therapy for each subject (range, 0.5 to 1.1) correlated significantly with six-hour plasma Hu levels, $r = 0.85, P = 0.01$. Moreover, the six-hour level of Hu and the mean serum creatinine levels were negatively correlated with maximal F reticulocyte responses ($r = 0.80, P = 0.03$; and $r = 0.70, P = 0.05$).

**In vitro analysis.** Bone marrow–derived CFU-E colonies were assayed in all five subjects studied at the NIH (Table 1). The F reticulocyte response as measured by the ratio of maximal F reticulocyte levels to pretreatment levels was inversely correlated with the suppression of CFU-E colony growth at two days of therapy. Subject 6, who had the most profound reticulocyte suppression (Fig 2), showed the greatest reduction in CFU-E on day 2. An analysis of data regarding BFU-E colony growth suggests that suppression may also be related to the F reticulocyte response. Subject 10, who showed the highest F reticulocyte response, had no suppression of BFU-E colonies at day 2. Subject 12, who showed an intermediate response, had an early suppression of BFU-E (19%, day 2), but some later recovery (80%, day 7). Subjects 11 and 7, who showed relatively low F reticulocyte responses, showed profound suppression of BFU-E colonies on day 2 and day 7.

**Fig 1.** F reticulocyte response to Hu therapy. Each subject was treated with 50 mg/kg/d (shaded boxes). The number in parentheses refers to the serum creatinine level of each subject.

**Fig 2.** Comparison of the effect of Hu and 5-aza on (A) the total reticulocyte count and (B) the F reticulocyte count. Pretreatment (PRE) and day 7 (7) values are given. Open circles indicate subjects treated with both drugs. Closed circles are subjects treated only with Hu. Numbers and letters are the same as listed in Fig 1.

**Fig 3.** Plasma Hu levels measured following an oral dose of 25 mg/kg Hu. Open circles represent subjects A (peak level, 20 $\mu g / mL$) and 12 (peak level, 12 $\mu g / mL$), and closed circles represent subjects 6 (peak at four hours, 22 $\mu g / mL$) and 7 (peak at two hours, 23 $\mu g / mL$).
### Table 1. Comparison of F Reticulocyte Increments and CFU-E Colony Formation During Hu Therapy

<table>
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*Post = maximum F reticulocytes \( \times 10^6/\mu L \) (See Fig 1). Pre = pre-F reticulocytes \( \times 10^6/\mu L \).

†Number of CFU-E colonies expressed as a percentage of day 0 (pretherapy) CFU-E colonies. The pretherapy number of CFU-E colonies/\( 10^6 \) nucleated cells were 107, 78, 314, 60, and 85 for subject 10, 12, 11, 7, and 6, respectively.

‡Number of BFU-E colonies expressed as a percentage of day 0 (pretherapy) BFU-E colonies. The pretherapy number of BFU-E colonies/\( 10^6 \) nucleated cells were 38, 57, 152, and 50 for subjects 10, 12, 11, and 7, respectively.

### DISCUSSION

In a previous report by Platt et al., two SS subjects given Hu, 50 mg/kg/d divided into three doses for five days, increased F reticulocyte levels without significant suppression of reticulocytes. In this report, eight subjects treated with comparable doses for three to five days showed suppression of total reticulocyte counts, with marked suppression seen in two of our subjects. The number of F reticulocytes after seven days of therapy with Hu increased slightly in six out of eight subjects even though all subjects experienced suppression in total reticulocytes. Even after recovery of the marrow, F reticulocyte levels were not significantly increased. Furthermore, the rapid increase in F reticulocytes (two to three days after starting therapy) previously reported following 5-aza therapy is not seen in our subjects treated with Hu.

Our subjects showed considerable differences in their F reticulocyte responses to Hu. A clue to the origin of that variability is apparent when serum creatinine and plasma Hu levels are analyzed. Minimal renal dysfunction leads to delayed clearance of Hu from the plasma. Previous investigators have shown that peak plasma levels of Hu \( >10^{-4} \) mol/L (7.6 \( \mu g/mL \)) will cause marrow cytotoxicity in man. The peak levels obtained in all subjects given a 25-mg/kg test dose of Hu are in the cytotoxic range. In subjects with higher serum creatinine levels (Fig 3), plasma Hu levels remain elevated for a longer period of time, leading to significant marrow suppression and decreased F reticulocyte response.

The differences in the Hu-induced F response of the subjects reported herein and those of Platt et al. are unclear. Presumably, the peak drug level after a single 50 mg/kg dose given to our subjects would be much higher and possibly more toxic than the divided doses given by Platt et al. However, when the two subjects (6 and 7) with the greatest toxicity to a single dose of 50 mg/kg/d were given lower doses, both showed suppression of total reticulocyte count levels, and only one showed a marginal increase in F reticulocytes. The two subjects treated by Platt et al were younger than our eight subjects, ages 17 and 23, with ages ranging from 27 to 44 in our subjects. Both of Platt's subjects had normal serum creatinine levels, 0.3 and 0.5 (personal communication, Platt). Our data would suggest that these low serum creatinine values would favor a higher F reticulocyte response. Finally, preliminary information on two subjects (A and C) with serum creatinine values of 0.6 and 0.9 subsequently treated at Hopkins suggests that less cytotoxicity and a more sustained F response may be achieved if the amount of Hu given three days per week is reduced to 20 to 40 mg/kg/d.

This report also indicates that drugs given at doses that caused detectable marrow suppression are unlikely to be of benefit in SS subjects since the result of such suppression is a decrease in F cell production. These observations are important since others have suggested that the mechanism of action of 5-aza and Hu is the selective cytotoxic effect on late erythroid progenitors (CFU-E) and subsequent recruitment of high HbF progenitors (BFU-E). In a previous report, we have shown that CFU-E suppression is not associated with increased F reticulocyte response with 5-aza. In this report we show that patients who had the least measured toxicity to their CFU-E on day 2 of treatment (subjects 10 and 12) had the highest F reticulocyte response. Conversely, the subjects with the most profound decrease in CFU-E at two days of therapy were the ones with the least F reticulocyte response. These data along with previous information that shows no cytotoxicity of CFU-Es following 5-aza therapy suggest that selective CFU-E cytotoxicity is not necessary for the early increases in F reticulocytes following 5-aza or Hu.

It is unclear whether 5-aza and Hu increase F cell production in the same way. In those subjects treated with both drugs, the F reticulocyte response is more rapid and consistently higher with 5-aza than with Hu. 5-aza increased F cell production without suppression of CFU-E on day 2 of therapy. In this report the highest response among the patients studied at the NIH was the individual with no detectable suppression of BFU-E or CFU-E. Clearly, cytotoxicity of erythroid precursors is not necessary for a rapid increase in F cell production. Some increase in F cell production, however, may result from drug therapies that selectively inhibit CFU-E and not BFU-E. Unfortunately, our data suggest that Hu treatment is more likely to suppress both CFU-E and BFU-E. Careful adjustment of Hu dosage based on measured plasma levels after a test dose may permit a more selective effect.

Since selective cytoreduction of late erythroid precursors (CFU-E and pronormoblasts) is not associated with increased HbF productions in clinical trials using 5-aza and Hu, alternative mechanisms must now be considered. Other agents (vincristine, cytosine arabinoside) that perturb cell-cycle kinetics have been associated with increased HbF production. We now favor the hypothesis that perturbation of the cell cycle without cell death may in some way change the regulatory patterns that control fetal and adult hemoglobin production. No information is available as yet that indicates how perturbation of the cell cycle might alter gene expression. Perhaps reversible delays in cell division lead to the accumulation of unstable proteins. These proteins...
may act as trans-acting substances that modulate differential globin gene expression. Further tests are now underway to test several aspects of this general hypothesis.

Neither this study nor previous reports of 5-aza24'9 and Hu3 indicate that these drugs have therapeutic benefit in SS disease. Further studies are necessary to determine whether lower or less frequent doses of Hu will result in greater F production.

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REFERENCES

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