

### Hydroxyurea Enhances Fetal Hemoglobin Production in Sickle Cell Anemia

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**A**bstract. Hydroxyurea, a widely used cytotoxic/cytostatic agent that does not influence methylation of DNA bases, increases fetal hemoglobin production in anemic monkeys. To determine its effect in sickle cell anemia, we treated two patients with a total of four, 5-d courses (50 mg/kg per d, divided into three oral doses). With each course, fetal reticulocytes increased within 48–72 h, peaked in 7–11 d, and fell by 18–21 d. In patient I, fetal reticulocytes increased from  $16.0 \pm 2.0\%$  to peaks of  $37.7 \pm 1.2$ ,  $40.0 \pm 2.0$ , and  $32.0 \pm 1.4\%$  in three successive courses. In patient II the increase was from  $8.7 \pm 1.2$  to  $50.0 \pm 2.0\%$ . Fetal hemoglobin increased from 7.9 to 12.3% in patient I and from 5.3 to 7.4% in patient II. Hemoglobin of patient I increased from 9.0 to 10.5 g/dl and in patient II from 6.7 to 9.9 g/dl. Additional single-day courses of hydroxyurea every 7–20 d maintained the fetal hemoglobin of patient I at 10.8–14.4%, and the total hemoglobin at 8.7–10.8 g/dl for an additional 60 d. The lowest absolute granulocyte count was  $1,600/\text{mm}^3$ ; the lowest platelet count was  $390,000/\text{mm}^3$ . The amount of fetal hemoglobin per erythroid burst colony-forming unit (BFU-E)-derived colony cell was unchanged, but the number of cells per BFU-E-derived colony increased. Al-

though examination of DNA synthesis in erythroid marrow cells in vitro revealed no decreased methylcytidine incorporation, Eco RI + Hpa II digestion of DNA revealed that hypomethylation of gamma-genes had taken place in vivo after treatment. This observation suggests that hydroxyurea is a potentially useful agent for the treatment of sickle cell anemia and that demethylation of the gamma-globin genes accompanies increased gamma-globin gene activity.

#### Introduction

The beneficial effect of increased levels of fetal hemoglobin (HbF)<sup>1</sup> in sickle cell anemia (SS) is demonstrated by asymptomatic SS individuals with pancellular hereditary persistence of HbF (1) and by the relatively mild course of SS Saudi Arabians with high levels of HbF (2). It is the course of these patients that encourages drug-induced stimulation of HbF synthesis as a therapeutic strategy in SS. Five-azacytidine (5-azaC), an investigational cancer chemotherapeutic agent, augments HbF production in anemic baboons (3, 4) and patients with thalassemia and SS (5, 6). The mode of action of 5-azaC has been thought to relate to the capacity of the drug to inhibit the methylation of deoxycytidine 5' to the gamma gene. However, there are justifiable concerns about the safety of 5-azaC (7), and controversy exists about this proposed mechanism of action (4, 8). Five-azaC is an S-phase cytotoxic agent that interferes with dividing cells (9), a characteristic which in itself may cause increased HbF production by altering the kinetics of erythropoiesis (4), as seen in stress erythropoiesis (10, 11).

Recently, Letvin et al. showed that anemic monkeys treated with hydroxyurea (HU), an S-phase cytotoxic agent that does

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1. Abbreviations used in this paper: 5-azaC, five-azacytidine; BFU-E, erythroid burst colony-forming units; CFU-E, erythroid colony-forming units; F, fetal; Hb, hemoglobin; HbF, fetal hemoglobin; HU, hydroxyurea; kb, kilobase; retic, reticulocyte; SS, sickle cell anemia.

not cause demethylation of DNA (12), responded with increased levels of HbF, suggesting that mechanisms other than those effecting methylation may be relevant (13). Papayannopoulou and co-workers have demonstrated a similar effect of cytosine arabinoside (14). HU, itself an iron chelator, inhibits DNA synthesis by inhibition of the iron-containing enzyme, ribonucleoside diphosphate reductase (15). The drug is widely used in the longterm treatment of chronic myeloproliferative disorders in oral doses ranging from 20 to 80 mg/kg per d (16), with rapidly reversible hematologic toxicities. Though it is a powerful mutagen (17), there is no evidence to date that this drug is leukemogenic when given regularly to a susceptible population (P. Berk, personal communication). If successful, treatment with HU to increase HbF levels in SS would have the advantages of ease of administration and control of toxicities, and is likely to be less carcinogenic than azaC. Therefore, we have cautiously investigated the effects of HU in two patients with SS and report here our initial experience.

## Methods

**Case reports and administration of HU.** Patients I and II are, respectively, 17 and 23-yr-old SS females who had each been hospitalized more than 10 times in the previous year with protracted painful crises. They were untransfused and had normal creatinine clearance and normal serum levels of hepatic enzymes. After giving informed consent, the patients were initially treated with 5-d courses of HU at 50 mg/kg per d divided into three oral doses.

Patient I received three 5-d courses of therapy starting on days 0, 30, and 86. She has since been followed for 170 d during which time she received additional single-day courses of HU (50 mg/kg per d) on days 110, 119, 125, 140, 155, and 167. Patient II was treated with one 5-d course of therapy and had bone marrow aspirated from the posterior iliac crest before therapy (day 0), and again 72 h after the completion of the 5-d course of HU (day 8). This patient has not yet received subsequent treatment, as she developed hypoxia with pneumonia and required transfusion.

**Hematologic determinations.** Blood hemoglobin (Hb), erythrocyte counts and indices, leukocyte counts, and platelet counts were done with a diagnostics counter (model ELT-8/ds; Ortho Diagnostic Systems, Inc., Westwood, MA). Reticulocyte (retic) and differential leukocyte counts were performed manually by standard techniques. The percentage HbF in hemolysates was measured by alkali denaturation (18). Samples were collected in Boston, stored at 4°C, and shipped weekly on ice to Baltimore for enumeration of fetal (F)-retics by the microscopic immunoprecipitation assay described by Dover et al. (19). F cells were stained and enumerated by the method of Betke et al. (20). Erythroid activity of marrow was estimated morphologically by examination of stained smears.

**Analysis of DNA methylation.** DNA was prepared from bone marrow as described by Bell et al. (21). 15 µg of Eco RI (Boehringer Mannheim Biochemicals, Indianapolis, IN) digested DNA was cleaved with excess Hpa II (New England Biolabs, Beverly, MA), and electrophoresed on 0.8% agarose. Fragments were transferred to nitrocellulose filters (Schleicher and Schuell, Inc., Keene, NH) by the procedure of Southern (22). Filters were hybridized with nick-translated Taq I gamma-globin insert of the plasmid JW151 (23) under conditions described by Bell (21).

After stringent washing (68°C and  $0.1 \times$  salt-sodium citrate), filters were exposed to X-ray film by use of an intensifying screen (Lightening Plus; DuPont Instruments, Wilmington, DE).

The capacity of cells to methylate newly replicated DNA was measured as described (Beardsley, G. P., and M. M. Klaus, manuscript in preparation). Briefly, washed marrow mononuclear cells were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum and [ $^{14}$ C]deoxycytidine and incubated for 16 h at 37°C in 5% CO<sub>2</sub>. After the cells were harvested and washed, the DNA was isolated and hydrolyzed enzymatically to nucleosides (24). Unlabeled nucleoside markers were added, and the nucleosides were separated by reverse-phase high-pressure liquid chromatography. Fractions were collected, and radioactivity associated with the deoxycytidine and 5-methyl deoxycytidine peaks was determined by scintillation counting. Percentage methylation was calculated as: [(counts per minute [ $^{14}$ C]5-methyl deoxycytidine)/(counts per minute [ $^{14}$ C]deoxycytidine + counts per minute [ $^{14}$ C]5-methyl deoxycytidine)]  $\times$  100.

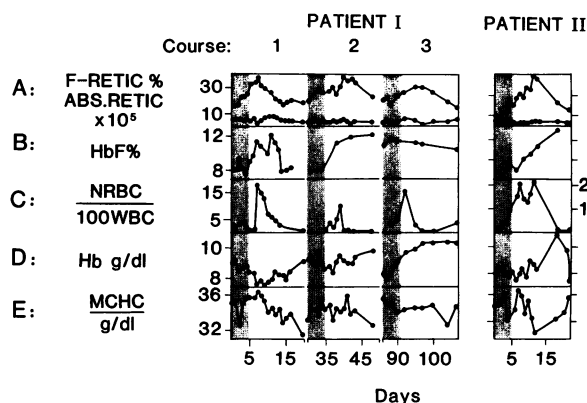
**Cultures.** Bone marrow and peripheral blood low density mononuclear cells were isolated by Ficoll-diatrizoate centrifugation and cultured in methylcellulose at 37°C in 5% CO<sub>2</sub> by the method of Iscove et al. (25). Cells were plated at  $1-2 \times 10^5$  cells/ml. Red colonies at 7 d in culture were scored as erythroid colony-forming units (CFU-E) (marrow only), and at 14 d in culture as erythroid burst colony-forming units (BFU-E) (blood and marrow). Colonies were then plucked with Pasteur pipettes and pooled. The cells were counted and assayed for HbF by modification of the Javid, Pettis, and Miller radioligand immunoassay (26).

## Results

**Hematologic response.** All four of the 5-d courses of therapy were accompanied by a rapid increase in the number of circulating F-retics. The increase was evident within 48–72 h of the start of therapy, peaked within 7–11 d, and fell to baseline by 18–21 d (Fig. 1). Patient I had a baseline percentage F-retic of  $16.0 \pm 2.0$ , which increased to  $37.7 \pm 1.2$ ,  $40.0 \pm 2.0$ , and  $32.0 \pm 1.4\%$  during the first, second, and third courses of therapy. Patient II had a baseline percentage F-retic of  $8.7 \pm 1.2$ , which increased to  $50.0 \pm 2.0\%$ . The absolute reticulocyte count remained relatively stable. As observed in the patients treated with 5-azaC by Charache et al. (6), a burst of nucleated erythrocytes emerged as the F-retics rose. During the first course of treatment of patient I, peripheral blood HbF increased transiently from a baseline of 7.9% to a peak of 12.0% on day 11. F cells rose from 35 to 50.6%. During the second course, the HbF increased from 7.9 to 12.3%, with an F cell response that increased from 30.5% to 45.4%. The HbF remained elevated throughout the third course, ranging from 10.6 to 11.7%, whereas the F cells ranged between 54 and 23%. In patient II HbF rose from a baseline of 5.3% to a peak of 7.4%, and the F cells rose from 24 to 35.6%.

Hb concentration increased in both patients: in patient I, from a baseline of 9.0 to 10.5 g/dl by the end of the third course, and in patient II from 6.7 to 9.9 g/dl.

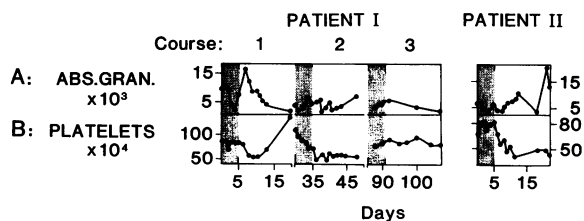
Although there was no clinically significant marrow toxicity associated with the 5-d courses of therapy, bone marrow suppression was evident with most courses of therapy (Fig. 2)



**Figure 1.** Effect of HU on peripheral blood of two patients with SS. HU (50 mg/kg per d given orally for 5 d) was administered in three courses to patient I and in one course to patient II, as indicated by the shaded areas. Percentage F-retics are shown in the upper graph of A, with the absolute (ABS.) retic count in the lower graph of A. B shows the percentage fetal hemoglobin; C shows the number of nucleated erythrocytes (NRBC)/100 white blood cells (WBC) per cubic millimeter; D shows the total hemoglobin; and E shows the mean corpuscular hemoglobin concentration (MCHC) of erythrocytes.

and in some cases was accompanied by a recovery phase overshoot of platelet count. The lowest platelet count observed was  $390,000/\text{mm}^3$ , with rebound platelet counts over a million—a pattern similar to that noted in the 5-azaC-treated patients (6). The pretreatment absolute neutrophil counts were  $>5,000/\text{mm}^3$ , and the lowest absolute neutrophil count after therapy was  $1,600/\text{mm}^3$ .

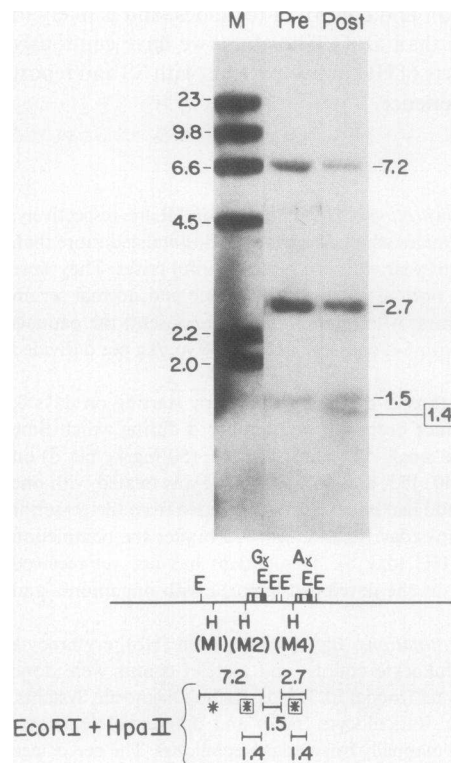
Between days 110 and 170, when patient I was treated with 6 single-day courses of HU, the percentage F-retics varied between  $16.7 \pm 1.2$  and  $34.6 \pm 4.2$ . The percentage HbF remained elevated between 10.8 and 14.4, an increase on the average of  $\sim 50\%$  over baseline. The F cells fluctuated between 36.6 and 44.8%. The Hb remained elevated, ranging between 8.7 and 10.8 g/dl. The absolute granulocyte nadir was  $5,000/\text{mm}^3$ , and platelet nadir was  $444,000/\text{mm}^3$ .



**Figure 2.** Effect of HU on peripheral blood of two patients with SS. The courses of therapy are the same as described in Fig. 1. A shows the effect on the absolute granulocyte (ABS. GRAN.) count per cubic millimeter. B shows the effect on the platelet count per cubic millimeter.

During this period of observation, there was no objective change in the clinical course of either patient.

**DNA methylation status.** Pre- and post-treatment marrows were  $\sim 85\%$  erythroid. Blot hybridization of Eco RI + Hpa II-digested DNAs using a gamma globin complementary DNA probe is shown in Fig. 3. Pretreatment DNA fragments of 7.2, 2.7, and 1.5 kilobases (kb) hybridized, consistent with complete methylation of the M2 and M4 Hpa II restriction sites located 5' to the G gamma- and A gamma-globin genes (27). Marrow DNA obtained after HU treatment contained an additional 1.4-kb fragment, indicative of reduced methylation of the M2 and/or M4 sites (27). Consistent with this, reduced hybridization of



**Figure 3.** Southern blot analysis of gamma-globin gene region of marrow DNA from patient II digested with Eco RI and Hpa II before and after HU therapy. Size markers are shown in lane M, day 0 sample in Pre lane, day 8 sample in Post lane. A diagram of the region is shown below. G-gamma- and A-gamma-coding regions are shown as black boxes, intervening sequences as open boxes. E, Eco RI recognition sites; H, potential Hpa II sites which depend upon the methylation status of the methylation sites, M1, M2, and M4. Sizes of fragments resulting from Eco RI and Hpa II digestion that would hybridize with the gamma-complementary DNA probe are shown at the bottom. When the M2 and M4 sites are fully methylated (\*), only fragments of 7.2, 2.7, and 1.5 kb are seen, as in the Pre sample. When unmethylated (□) 1.4-kb fragments derived from either or both G-gamma- and A-gamma-genes appear as seen in the post-treatment sample.

the 7.2- and 2.7-kb bands relative to the invariant 1.5-kb band was also apparent. Visual inspection suggests ~25% demethylation of the M2 and/or M4 sites. Measurement of percentage methylation of newly synthesized DNA did not reveal any difference between pre- ( $5.36 \pm 0.5\%$ ) and post- ( $5.28 \pm 0.5\%$ ) treatment samples.

**Erythroid progenitor studies.** There was no change in the peripheral BFU-E HbF program of either patient as defined by the HbF content of peripheral BFU-E-derived cells. HbF content (picograms per BFU-E-derived cell) of patient I was  $2.5 \pm 0.28$ ,  $2.34 \pm 0.24$ , and  $2.54 \pm 0.12$  on days 0, 7, and 14 respectively. HbF content (picograms per BFU-E-derived cell) of patient II was  $5.44 \pm 0.12$ , and 4.82 on days 0 and 8. Both patients did, however, show an increase in the number of cells per colony after treatment. The number of cells per peripheral BFU-E-derived colony of patient I was  $3.4 \times 10^3 \pm 0.5$ ,  $4.8 \times 10^3 \pm 1.9$ , and  $6.96 \times 10^3 \pm 3.51$  on days 0, 7, and 14. Patient II had an increase in the number of cells per peripheral BFU-E derived colony, from  $4.33 \times 10^3$  to  $12.3 \times 10^3$ ; cells per marrow BFU-E derived colony rose from  $4.87 \times 10^3$  to  $7.95 \times 10^3$ ; cells per CFU-E derived colony rose from  $2.85 \times 10^2$  to  $4.18 \times 10^2$  between days 0 and 8.

## Discussion

The studies of Ley et al. (5) and Charache et al. (6) demonstrated that the administration of 5-azaC can induce DNA hypomethylation and enhance HbF production in SS. Letvin et al. showed that HU, an S-phase cytotoxic agent without known effects on the methylation of DNA bases also increased HbF production in anemic monkeys (13). Therefore, we studied the effect of hydroxyurea in two SS patients to see whether this commonly used chemotherapeutic agent would stimulate HbF production in these patients.

The effects of HU can be summarized as follows: (a) there was a rapid, dramatic increase in percentage F-retics, generally equivalent to what has been reported with 5-azaC treatment; (b) there was no clinically significant marrow toxicity; (c) there was a measurable loss of DNA methylation in the region 5' to the gamma-globin genes; (d) the peripheral blood BFU-E HbF program was unchanged; and (e) the size of peripheral and marrow BFU-E- and CFU-E-derived colonies was increased. Taken together, these observations suggest that HU can increase HbF production, but fail to illuminate an obvious mechanism of action.

The F-retic number begins to increase 24–72 h after the start of treatment, a rate that is much faster than one would expect if the effect merely represented recovery from marrow toxicity. In fact, the speed of the response implies that the enhancement of HbF production is induced in very mature erythroid progenitors or even in erythroid precursors. This recalls the effect of HU in hypertransfused mice in which increased erythroid precursors were observed in the marrow 48 h after administration of the drug (28), as though very late CFU-E or erythroblasts matured more rapidly as a result of treatment.

That drugs such as HU can stimulate erythroid differentiation has been demonstrated in cultures of Friend erythroleukemia cells by Ebert and co-workers (29). It is formally possible that HU treatment caused amplification of gamma-globin genes as seen in vitro in selected Chinese hamster ovary cells with respect to dihydrofolate reductase genes (30). The strategy we used to examine gamma-globin gene methylation does not rule out a two- to threefold gene amplification in our patient.

Of interest is the fact that though HU has no direct effect on the methylation of deoxycytidine, HU treatment in vivo resulted in reduced methylation of the M2 and/or M4 Hpa II sites 5' to the gamma-globin genes, critical areas for expression of gene activity (31). It should not be inferred from these data that the change in methylation pattern causes increased gamma-globin gene expression. More likely, it reflects increased gamma-globin gene expression in a selected cell population or is a consequence of increased gamma-globin gene expression per se (32, 33).

The HbF content of BFU-E derived colonies was unchanged during treatment, suggesting that HU did not cause hemoglobin reprogramming or reverse switching at this early stage of erythroid development. The changes in the growth patterns of the colonies, however, may indicate that cell cycle time is altered by the drug. This kinetic effect could influence gamma-gene expression by a mechanism as yet undetermined. It is important to emphasize that the observed change in HbF production is representative of the focus of this inquiry but that cellular characteristics, such as oxygen affinity and membrane properties, may also be altered in this process. Indeed, these changes may contribute to potential clinical benefit even more than the alterations in HbF.

In the two patients reported here, HU had a measurable and sustainable effect on HbF production with a minimum of toxicity. The levels of HbF achieved exceed the threshold levels calculated by Powars and colleagues (34) necessary to ameliorate major organ failure and approach the levels calculated to ameliorate recurrent painful crises. However, the cellular distribution of HbF in these patients is broadened as indicated by the increased F-retic and F cell percentages, an effect which may lead to slow clinical improvement even below the thresholds calculated on the basis of total HbF percentage.

Although these preliminary results are encouraging, many unresolved issues remain: the precise mechanism of action, the optimal drug dosage and schedule, the long- and short-term benefits and risks, and the fraction of patients likely to respond. In fact, the toxicities of HU therapy may ultimately preclude its use in this setting. These issues need to be resolved in small controlled studies before HU can be recommended for clinical use.

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