5-Azacytidine Increases HbF Production and Reduces Anemia in Sickle Cell Disease: Dose-Response Analysis of Subcutaneous and Oral Dosage Regimens

By G.J. Dover, S. Charache, S.H. Boyer, G. Vogelsang, and M. Moyer

Varying doses of 5-azacytidine (5-aza) were given to four sickle cell individuals for 500, 200, 100, and 30 days. The percentage of fetal hemoglobin (HbF) containing reticulocytes (F reticulocytes) increased two- to five-fold within five days of 5-aza therapy in all patients, with a two- to three-fold rapid response (<48 hours after initial dose) in three patients. Reticulocyte suppression was not observed prior to, during, or after therapy in those patients who responded within 48 hours. Subcutaneous 5-aza was given in 35-day courses consisting of every day, every other day, or three consecutive days a week. No marrow toxicity was observed on any of the regimens. For three patients, the highest average F reticulocyte level was observed on the three consecutive day a week regimen. Oral 5-aza, given with trihydroxuridine, produced comparable F reticulocyte response. In the two patients treated for more than 100 days, Hb levels increased to 11 to 12 and 9 g/dL, MCV and MCH increased by 25%, and lysate HbF levels peaked at 12% and 20%. Fetal erythroid characteristics (i-antigen, galactokinase activity, and GY/A7 ratios) did not correlate with maximal HbF production. The frequency of vaso-occlusive crises appeared to decrease in both patients followed for more than 100 days.

5-AZACYTIDINE (5-aza), a cell-cycle-specific DNA hypomethylating agent, has been shown to increase fetal hemoglobin (HbF) production in animals, in one individual with thalassemia, and in several individuals with sickle cell (SS) anemia. In the first phase of our study of repeated doses of 5-aza in one SS patient treated for 100 days, we observed: (1) a rapid increase (within 48 hours after each dose of 5-aza) in the percentage of reticulocytes containing HbF (F reticulocytes), (2) increase in the percentage of HbF and hemoglobin levels in the peripheral blood without evidence of marrow toxicity; and (3) no apparent decrease in the frequency of vaso-occlusive crisis. Alterations in the pattern of DNA methylation at CpG dinucleotide sequences around the β-globin gene complex were associated with increased HbF production in individuals treated with 5-aza, but the mechanism by which 5-aza increased levels of HbF production remained uncertain.

In this article we expand our observations concerning the effect of 5-aza therapy in patients with SS anemia. An optimal dose has been defined as that which produces an average F reticulocyte count of at least 20%, which leads to minimal marrow and gastrointestinal toxicity. The 20% level was chosen because one of us had shown that Saudi Arabian SS patients with clinically mild disease associated with increased production of HbF had F reticulocyte levels between 20% and 50%. We varied the dose and frequency of subcutaneous (SC) 5-aza administration in order to determine an optimal dose regimen, and then determined an optimal oral regimen of 5-aza administration, prompted by observations that 5-aza was orally effective when given to mice or baboons in conjunction with a cytidine deaminase inhibitor, trihydroxuridine (THU).

MATERIALS AND METHODS

Patients. All patients were adult homozygous SS patients who had become refractory to chronic transfusion therapy for complications of their disease. Patient A (J.P.) was described previously. Patients B (D.P.), C (W.T.), and D (M.J.) were 26, 45, and 23 years old, respectively. Patient A was treated for 500 days. Patient B was dropped from the study due to noncompliance after day 100. Patient C was treated for 200 days. Patient D discontinued therapy at day 30 because he elected to undergo bilateral hip replacement for preexisting aseptic necrosis of both femoral heads. All patients were treated according to protocols approved by The Johns Hopkins University Joint Committee on Clinical Investigation and gave consent after being informed of the potential carcinogenic and cytotoxic side effects of 5-aza. All patients were hospitalized during the first 45 days of therapy and during regimens of therapy when 5-aza was given daily or on every other day. The remainder of therapy was accomplished as outpatients with the subjects making visits to the hospital two to three times a week.

Drugs. Injectable 5-aza was obtained from the Division of Cancer Treatment, National Cancer Institute. Purified pyrogen-free 5-aza and THU were provided by Drs J. Posada and P. Davignon of the National Cancer Institute, and stored at −70 °C. Encapsulation of 5-aza and THU was performed by The Johns Hopkins Hospital Pharmacy, using precautions suitable for chemotherapeutic agents. Claimed exemptions for new drugs (INDs) were filed with the Food and Drug Administration for all phases of the study. For SC injection, 100 mg of injectible azacytidine was suspended in 4 mL of water for injection and injected as a slurry.

HbF levels. The percentage of F reticulocytes and the amount of HbF/F cell were determined using polyclonal rabbit anti-human HbF. The percentage of mature erythrocytes containing HbF (percentage of F cells) was assayed using a mouse monoclonal anti-human HbF antibody developed in our laboratory. Lysate HbF levels were measured by alkali denaturation and GY/A7 ratios were kindly measured by Dr Blanche Alter and Dr Walter Schroeder.

RBC indices were measured by an electronic cell counter that had been standardized with blood collected in K3EDTA. Twenty-step phthalate ester density gradients, supplied by Dr C. Noguchi, were used to measure the mean corpuscular hemoglobin concentration (MCHC) and the percentage of dense cells (MCHC > 37 g/dL).

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Vasocclusive crises. Vasocclusive crises were defined as episodes of localized pain associated with tachycardia, fever, an elevated WBC, or an increase in total bilirubin. Only one painful episode in patient A was not associated with such findings.

Miscellaneous. Activity of galactokinase was measured by Dr E. Beutler. Erythrocyte folate, serum B12, α-fetoprotein, and carcinoembryonic antigen levels were measured in The Johns Hopkins Hospital Clinical Laboratory using standard methods. RBC i-antigen titers were measured by a standard agglutination technique using antisera kindly provided by Dr M. Crookston. Cytidine deaminase levels were measured in peripheral blood mononuclear cells separated on Percoll. DNA polymorphism haplotypes were determined by restriction enzyme analysis of peripheral blood by Dr S. Antonarakis.

RESULTS

Onset of F reticulocyte response. Figure 1 indicates that the rapid increase in percentage of F reticulocytes described previously for patient A (5) occurred in three of four patients treated with 2 mg/kg/d SC 5-aza given as a single dose for three consecutive days. In the three patients exhibiting a rapid response, total reticulocyte levels remained at pretherapy levels during this period. It is unclear why patient D responded more slowly. Only he exhibited nausea and vomiting and a decrease in reticulocyte production (2.9 to 5.3 x 10^6/μL) of peripheral blood mononuclear cells separated on Percoll. DNA polymorphism haplotypes were previously described.

Effect of various 5-aza doses on F reticulocyte production. Table 1 summarizes the mean F reticulocyte levels achieved for patients A and B with daily therapy at the same dose. Alternate-day administration (regimen II) or divided daily doses (regimen IV) resulted in F reticulocyte levels comparable to those obtained with the optimal SC regimen (regimen III) for three successive days each week (regimen III). In subject A, administration of 5-aza for three consecutive days per week was less effective than regimen III.

Table 1. Average F Reticulocyte Response to Various Doses of Subcutaneous (SC) and Oral (PO) 5-Azacytidine

<table>
<thead>
<tr>
<th>Patients</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>4.4 ± 1.0* (0.2)†</td>
<td>3.8 ± 0.8 (0.2)</td>
<td>14.4 ± 1.2 (0.5)</td>
</tr>
<tr>
<td>I</td>
<td>25.0 ± 5.6 (1.2)</td>
<td>17.6 ± 2.7 (0.9)</td>
<td>26.1 ± 3.5 (1.3)</td>
</tr>
<tr>
<td>II</td>
<td>31.9 ± 8.7 (1.1)</td>
<td>31.6 ± 8.7 (1.1)</td>
<td>26.3 ± 10.0 (1.0)</td>
</tr>
</tbody>
</table>

*Mean ± 1 SD percentage of F reticulocytes.
†Mean absolute F reticulocyte level x 10^6/μL.

DeSimone baseline F reticulocyte levels seemed to influence the response to treatment. On all dose schedules, the mean F reticulocyte level achieved for patients A and B (pretreatment levels 4.4% ± 1.0% and 3.8% ± 0.8%, respectively) was always less than that in patient C (pretreatment level, 14.4% ± 1.2%). None of the drug regimens listed in Table 1 caused marrow toxicity (defined by decreased WBC, platelet, or reticulocyte counts compared to pretherapy values), even after administration of 5-aza for 30 consecutive days at 2 mg/kg/d (regimen I). In subject A, administration of 5-aza for three successive days each week (regimen III) resulted in F reticulocyte levels comparable to those obtained with daily therapy at the same dose. Alternate-day administration (regimen II) or divided daily doses (regimen IV) were less effective than regimen III.

Oral administration of 5-aza with THU (regimen V) in patient A and C increased F reticulocyte production, although oral doses of 5-aza (2 mg/kg/d) or THU (200 mg/d) given alone did not (data not shown). THU (1.5 to 2 mg/kg) was given one hour before 5-aza, and an equal second dose was given with 5-aza; no food was given until one hour after the second dose. When 5-aza, 2 mg/kg/d, was given with THU for three successive days, nausea and vomiting were prominent complaints and significant suppression of WBC, platelets, and reticulocytes were seen in both patients with maximum depression ten to 15 days after beginning treatment. Marrow function recovered within three weeks of the initial dose. When 0.2 mg/kg/d of 5-aza was given orally with THU (regimen V, Table 1) F reticulocyte levels comparable to those achieved with the optimal SC regimen (regimen III of 2 mg/kg/d) were observed with no evidence of cytotoxicity.

An oral THU dose of 1.5 to 2 mg/kg (divided in two doses, the first given one hour before, the second with 5-aza; fasting) resulted within 24 hours in a suppression of peripheral blood WBC cytidine deaminase levels to 31% and 33% of pretherapy levels in patients A and C, respectively. Cytidine deaminase levels had returned to 85% to 100% of pretherapy values seven days later when the drug was given for two consecutive days each week. When given three consecutive days each week (see regimen V), day 7 cytidine deaminase

Fig 1. The F reticulocyte response in four sickle cell (SS) patients given subcutaneous (SC) 5-aza (5-aza). Patient A, □; patient B, ♦; patient C, ○; patient D, O. Note that total reticulocyte counts were unchanged during this period of time in all patients except for patient D (see Results). Shaded bar, top of the graph, refers to days of administration of 5-aza. 2 mg/kg/d (SC) for three consecutive days. Data on patients A, B, and C have been previously described.
levels were 54% and 69% of pretherapy levels for both patients.

Effect of various 5-aza doses on other hematologic measurements: hemoglobin, reticulocyte count, percentage of HbF and percentage of F cells. Table 2 summarizes average hemoglobin concentrations, reticulocyte counts, percentage of HbF, and percentage of F cells obtained on patients A and C on various regiments of 5-aza. Only those data obtained when no transfused cells were present are summarized. Drug regimens were changed before hemoglobin concentrations had returned to pretreatment levels, but after F reticulocyte levels had dropped to pretreatment levels.

Hemoglobin concentrations rose in both patients A and C while they were on 5-aza (Table 2). Patient A’s levels were consistently higher than those of Patient C even though HbF levels behaved conversely. Although patient B’s hemoglobin values rose on regimen III from 8.5 g/dL (pretherapy) to 11.5 g/dL (day 84), he was too noncompliant to obtain sufficient data after transfused cells had disappeared. Patient D left the study (see Materials and Methods) shortly after a second dose of 5-aza. Except for transient suppression of reticulocytes following the inception of high-dose oral 5-aza therapy (2 mg/kg/d + 200 mg THU), the proportions of reticulocytes in patients A and C were unchanged during treatment.

Because we were unwilling to allow our patients to return to their pretreatment hemoglobin concentrations before changing dosage regimens, we cannot conclude from these data whether one drug regimen results in a higher hemoglobin concentration than another regimen. It is apparent, however, that hemoglobin concentrations remained persistently higher than pretreatment levels in patient A over a prolonged period of time (500 days posttherapy).

HbF/F cell. During the first 300 days of SC therapy in patient A, negligible differences in the amount of HbF/F cell were seen (Table 3) even though F reticulocyte production and F cell levels increased dramatically (Table 2). In contrast, during oral 5-aza therapy, the amount of HbF/F cell increased 37% in patient A (from 7.6 to 10.4 pg) and 103% in patient C (from 5.5 to 11.2 pg) (Table 3). No changes in HbF/F cell were seen in the short time that patients B and D were followed.

RBC indices and “dense cells.” After 100 days of therapy both patients A and C demonstrated increases in their MCV and MCH (Table 3). In patient A, the mean MCV and MCH were increased the most during regimen III (114 ± 2 Fl and 40 ± 1 pg) and regimen V (116 ± 4 Fl and 40 ± 1 pg). Both patient A and C had normal serum B12 and red cell folate levels throughout therapy. The MCHC as measured by an electronic cell counter (patients A and C) or by the phthalate ester technique (patient C only) did not change (Table 3). The proportion of erythrocytes with a MCHC > 37 g/dL was followed weekly in patient C (Fig 2). Dense cells disappeared between day 40 and 90, coincident with a decrease in reticulocytes which occurred during the period of marrow depression and gastrointestinal toxicity following a toxic high oral dose of 5-aza (2 mg/kg/d) given with THU. Patient A (data not shown) showed a similar suppression of dense cells and reticulocyte counts at this dosage schedule. After adjustment of dosage (Table 1, oral regimen V) when F reticulocyte levels remained above 20% and F cell levels ranged from 65% to 83%, the percentage of dense cells varied between 4% and 8%. Pretreatment levels were similar (4% to 8%). Pretreatment levels were similar (4% to 6%). However, the patient had been transfused prior to drug therapy and had 46% normal red cells in his blood at the beginning of observation. No correlation between the proportion of dense cells and vasoocclusive crisis (as noted by Fabry et al[20]) was observed in patient C. However, only two assays were performed on patient C during that period of therapy when crises occurred (day 0 through 20).

Markers of “fetal erythropoiesis.” Because all patients

| Table 2. Hematologic Response to Various Doses of 5-Azacytidine for Patients A and C |
|-----------------------------|---------------------|---------------------|---------------------|---------------------|
| Patient | Dose* | Days of Therapy | Hb(g/dL) | Reticulocytes (%) | HbF (%) | F Cells (%) |
| A | Pre | 8.5 ± 1.0† | 12.0 ± 4.0 (4.3) | 1.5 ± 0.7 | 9.3 ± 1.7 |
| I | 125-160 | 11.3 ± 0.8 | 15.3 ± 9.1 (4.5) | 7.6 ± 1.5 | 41.4 ± 5.7 |
| II | 165-200 | 11.9 ± 0.6 | 16.8 ± 6.4 (5.0) | 7.1 ± 0.2 | 35.2 ± 3.5 |
| III | 203-240 | 12.0 ± 0.7 | 17.1 ± 5.2 (5.1) | 8.0 ± 0.8 | 32.6 ± 2.8 |
| IV | 245-280 | 11.7 ± 0.3 | 17.1 ± 7.6 (5.6) | 8.6 ± 1.0 | 36.2 ± 5.8 |
| V | 415-464 | 11.3 ± 0.7 | 12.5 ± 4.1 (3.5) | 9.6 ± 0.7 | 43.0 ± 2.7 |
| C | Pre | 8.0 ± 0.5† | 13.9 ± 2.8 (3.9) | 2.9 ± 0.1 | 14.4 ± 1.2 |
| | 140-180 | 9.2 ± 0.3 | 15.3 ± 5.5 (3.4) | 17.5 ± 0.6 | 63.4 ± 3.9 |

Parentheses indicate the mean absolute reticulocyte level x 10⁴/L. *See Table 1 for doses of 5-aza. †Patient A had 46% HbA, and patient C had 56% HbA due to previous transfusions.

| Table 3. HbF Levels and Erythrocyte Indices on Patients A and C |
|-----------------------------|---------------------|---------------------|
| Patient | Day of Therapy | HbF (%) | F Cells (%) | HbF/F Cell (pg) | MCV (fl) | MCH (pg) | MCHC* (g/dL) |
| A | 0 | 1.8 | 8 | 7.6 | 95 | 34 | 35.8 |
| | 101 | 6.5 | 33 | 7.2 | 105 | 37 | 35.2 |
| | 200 | 7.3 | 40 | 6.9 | 108 | 38 | 35.2 |
| | 301 | 6.8 | 33 | 8.0 | 110 | 38 | 34.5 |
| | 410 | 8.6 | 42 | 8.1 | 121 | 40 | 33.7 |
| | 500 | 9.5 | 40 | 10.4 | 125 | 44 | 35.2 |
| C | 0 | 2.9 | 18 | 5.5 | 98 | 34 | 34.7 |
| | 52 | 10.9 | 50 | 7.4 | 101 | 34 | 33.7 |
| | 100 | 15.1 | 64 | 9.9 | 124 | 42 | 33.9 |
| | 146 | 16.8 | 62 | 11.3 | 126 | 42 | 33.3 |
| | 203 | 16.8 | 62 | 11.2 | 123 | 42 | 34.2 |

*Values on patient A were determined by Coulter Counter (Hialeah, Fla) and on patient C by phthalate ester technique.
During oral therapy the number of crisis days was also decreased (15 of 200 days). Patient C responded much more dramatically. After day 30 of treatment, he had no clearcut further painful crisis. It is important to note that these observations were uncontrolled, and that the patients knew when they were receiving treatment and when the dosages were altered. Furthermore, each patient received intense emotional and psychiatric support from medical and nursing personnel. For these reasons, no conclusions can be drawn concerning amelioration of the clinical features of the disease during 5-aza therapy.

**DISCUSSION**

**Mechanism of action.** All four SS patients treated with 5-aza demonstrated significant increases in HbF production. The mechanism of the increase is unknown, but two major hypotheses have been advanced. In one, late erythroid precursors are “reprogrammed” through some action of 5-aza27; in the other, early erythroid precursors, with an inherent program for increased production of HbF, are “recruited” concomitant with the cytotoxic destruction of later precursors.3 Some light is shed on the question by examining the timing of the in vivo F reticulocyte response to the drug. Three of our four patients exhibited a rapid response (Fig 1) and no decline in total reticulocyte production. Only patient D failed to show a significant immediate rise in F reticulocytes. Variation in the rapidity of the F reticulocyte response was also observed in six SS patients treated with a single course of 5-aza at the National Institutes of Health by Ley and Nienhuis and assayed in our laboratory: five of them responded rapidly, but one did not.27 The rapid increase in F reticulocytes within 24 to 48 hours of treatment without suppression of total reticulocytes suggests that, in most patients, 5-aza reprograms late erythroid precursors to produce HbF. Patient D exhibited gastrointestinal and bone marrow toxicity after the single course of treatment which behaved in similar fashion. It is conceivable that the late response in these two patients reflects the cytotoxic “recruitment” phenomenon, but that responses to doses of 5-aza not accompanied by toxicity reflect the alternate “reprogramming” effect.

It is intriguing to note that absolute reticulocyte levels in our patients were not significantly different from pretherapy levels (P > .10) on any of the drug regimens described (see Figure 2).
transient declines in total reticulocyte production preceded the smaller percentage of F reticulocyte counts lower during regimens that produced higher F reticulocyte responses. These observations suggest that increased F cell production was not associated with or preceded by a decline in erythropoietic activity and further makes recruitment of early precursors, as a result of marrow suppression, an unlikely cause of increased HbF production.

**Improvement in anemia.** With sustained increased F reticulocyte production, hemoglobin concentration rose, as did the MCV, MCH, and the amount of HbF/F cell. The MCHC, however, remained constant. There was also a suggestion that clinical symptoms (painful crises) decreased, raising questions of the relationships between these effects.

The concentration of HbS within the red cell is a major determinant of HbS polymerization. A previous report suggested that improvement in anemia seen in 5-aza treatment is associated with decreased MCHC and the disappearance from the blood of very dense cells (ie, those with the higher MCHC). However, in those patients, the disappearance of dense cells and the change in MCHC were also associated with transient reticulocyte suppression. In our patients A and C, dense cells decreased only when reticulocytes were suppressed after the patients were given ten times the optimal oral dose of 5-aza/THU. Recent data by Noguchi et al suggest that these dense cells are relatively young cells and therefore might be expected to disappear if reticulocyte levels fall. However, during optimal therapy (regimens III and V), when reticulocyte percentages were not decreased, Hb levels remained elevated, the MCHC did not change, and dense cells persisted (ie, new ones were formed as fast as old ones were removed). It appears, therefore, that the partial compensation for the anemia in these patients is not attributable to either a decreased MCHC or the disappearance of dense cells.

A second possible explanation for the decreased anemia in our patients is their increased HbF production. Initially, improvement in anemia was associated with increased F cell production without an increase in HbF/F cell. This indicates that a decrease in anemia in SS patients may be accomplished by increasing F cell production without increasing HbF/F cell. In contrast, HbF/F cell did increase on oral regimens. Surprisingly, in both patients A and C the increase in the MCH (10 pg and 8 pg, respectively) was greater than the increase in HbF/F cell (3.8 pg in A and 4.7 pg in C) indicating that Hbs/cell also had increased. Assuming that the MCH of F cells and non-F cells are equal, the percentage of total hemoglobin per cell attributed to HbF did not increase substantially in patient A (22% to 24%, day 0 to day 500), but did rise in patient C (16% to 27%, day 0 to day 200). Despite these differences in HbF production, both patients had less anemia; the lower HbF level in patient A being associated with higher average hemoglobin levels. It appears that increased HbF production alone cannot account for the changes in anemia seen in our patients.

**Fetal characteristics of RBCs.** Additional fetal erythrocyte characteristics were observed during the course of therapy. Elevated MCV was seen in both patients. Erythrocyte i-antigen titers were increased in patient A and not in patient C even though patient C had the higher F cell level at the time of assay. Only patient A had higher Gr/AY ratios than predicted from their restriction enzyme polymorphism haplotypes. Fetal levels of erythrocyte enzymes were not observed in either patient. This mixed pattern of fetal and adult erythrocyte markers is more consistent with the changes seen with stress erythropoiesis rather than a true pattern of fetal erythropoiesis.

**Variability of response.** We have observed that the rapidity of response to 5-aza varies between patients. Although the reasons for this variability are unknown, cytotoxic effects clearly are associated with the delayed F reticulocyte response. Because baseline levels of F cell production in SS disease are genetically determined and since pertherapy levels of F reticulocytes were predictive of subsequent F reticulocyte responses (Table 1), it might be expected that SS patients with high baseline F reticulocyte levels may need less intensive therapy than patients with low (<5%) F reticulocyte levels. However, it is unclear what minimal percentage of F reticulocyte, if any, can reduce the clinical severity of sickle cell disease. Each person brings to therapy his own genetic constraints on F cell production. Such constraints may influence the amount of drug and the magnitude of response needed to reduce clinical symptoms. Furthermore, it is not clear whether improvements in any one hematologic parameter (percentage of HbF, MCH of F cell, Hb level, and percentage of dense cells) predicts clinical improvement as defined by frequency of severity of vasoocclusive crisis. The absence of such a definable marker makes assessment of therapeutic maneuvers more difficult.

**Overall role of 5-aza in SS disease.** We have increased HbF in a limited number of SS patients. Little toxicity was observed, but the long-term effects of this form of therapy are unknown. Although probably carcinogenic in animals, little information is available regarding the carcinogenic potential of 5-aza in humans. In the absence of controlled clinical trials, we cannot determine whether elevations in HbF levels, produced as described here, will significantly alter the clinical course of patients with sickle cell disease. The question of carcinogenicity impedes such trials, but other cell-cycle-specific agents (which may be less carcinogenic) have been shown to increase HbF in animals and in humans. These agents should be evaluated in a manner similar to that outlined in this report before controlled clinical trials are begun.

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