5-Azacytidine Increases γ-Globin Synthesis and Reduces the Proportion of Dense Cells in Patients With Sickle Cell Anemia

By Timothy J. Ley, Joseph DeSimone, Constance Tom Noguchi, Patricia H. Turner, Alan N. Schechter, Paul Heller, and Arthur W. Nienhuis

We previously demonstrated that 5-azacytidine can selectively increase γ-globin synthesis in a patient with β-thalassemia, prompting us to treat two patients with sickle cell anemia and two additional patients with β-thalassemia. 5-Azacytidine (2 mg/kg/day) was continuously infused for 7 days with no apparent clinical toxicity. The γ/β-globin biosynthetic ratio increased fourfold to sixfold in the bone marrow cells of each patient after treatment and remained elevated for 7-14 additional days. Hypomethylation of DNA near the γ-globin genes in bone marrow cells was demonstrated 2 days after beginning the 5-azacytidine infusion. The peripheral blood fetal hemoglobin (HbF) level increased from 6.0% to 13.7% in one patient with sickle cell anemia and from 1.8% to 8.9% in the second. Stractan gradient analyses of peripheral blood from patients with sickle cell anemia revealed a marked decrease in the percentage of dense cells (cells that contain increased amounts of HbS polymer when deoxygenated) following treatment. These observations provide an impetus to investigate the effects of repeated courses of 5-azacytidine in a small group of severely ill patients to determine whether this drug may have a role in the treatment of patients with sickle cell anemia and β-thalassemia.

We recently reported that 5-azacytidine specifically increased γ-globin synthesis in a patient with β-thalassemia. Treatment of four additional patients, two with β-thalassemia and two with sickle cell anemia, have now provided data implying that this response may be consistent and reproducible in humans. This article also reports the effect of 5-azacytidine administration on the properties of red cells from sickle cell anemia patients.

Sickle cell anemia is a severe debilitating disease caused by the inheritance of two abnormal β-globin genes, each containing a single nucleotide substitution in codon 6. The product of this abnormal β-globin gene (β-globin) combines with α-globin chains to form hemoglobin S (HbS) molecules (αβ2); when the red cell is deoxygenated, these abnormal molecules aggregate to form a polymer of HbS inside the red cell. Cells containing large amounts of HbS polymer are dense and relatively rigid; such cells are thought to be particularly important in the pathogenesis of this disease.

The polymerization of deoxygenated HbS is in part dependent on the concentration of HbS within an individual red cell. If the amount of HbS per cell could be diminished by replacing HbS with fetal hemoglobin (HBF) (αγ2), then the tendency of that cell to sickle may be reduced. Furthermore, αγββ tetramers have almost no tendency to form polymer; in contrast, ααββ tetramers do have a tendency to form polymer, but to a smaller extent than ααββ tetramers. Therefore, a larger proportion of cells that contain HBF, and an increased amount of HBF per cell, might particularly improve the course of sickle cell anemia.

5-Azacytidine is a cytidine analog that has been shown to increase HBF production in baboons (animals with globin gene structure and function that is similar to humans) and in a patient with β-thalassemia. Furthermore, this drug has been shown to activate a variety of repressed genes in tissue culture cells, in some cases, gene activation has been associated with hypomethylation of DNA in the treated cells. When 5-azacytidine is incorporated into newly synthesized DNA in tissue culture cells, the activity of DNA methyltransferase in these cells is markedly reduced. DNA that is newly synthesized in these cells is therefore relatively hypomethylated.

In the previous study of a patient with β-thalassemia, we discovered that bone marrow cell DNA in the region of the duplicated fetal (γ) and embryonic (ε) globin genes became relatively hypomethylated after treatment with 5-azacytidine. Hypomethylation of DNA near the γ-globin genes was associated with a high degree of expression of these genes, but the ε-globin gene was transcribed at a very low level despite hypomethylation. Similarly, hypomethylation of the embryonic α-globin-like gene (γ) after 5-azacytidine treatment was not associated with expression of that gene (our unpublished data). These findings sug-
gest that hypomethylation of DNA near a gene may be necessary—but not sufficient—for a high degree of gene expression.11

MATERIALS AND METHODS

Patients Studied

A summary of each patient’s clinical and biochemical characteristics is presented in Table 1. Patient 1 is a 58-yr-old American black man with frequent severe pain crises that were treated with intermittent transfusions of red cells for about 25 yr prior to this study; the patient’s last transfusion was 51 days before admission. At the time of admission, Hbs concentration was 63%, HbA was 30%, HbF concentration was 5%, and HbA2 was 2%. The relatively low absolute reticulocyte count (about 100,000/cu mm) may have reflected the recent transfusions. During treatment with 5-azacytidine, the patient received transfusions of 300 ml, 140 ml, and 140 ml of packed red blood cells on days 1, 5, and 7, respectively, to maintain a hemoglobin concentration of at least 7.5 g/dl and to reduce the risk of painful crises.

Patient 2 was a 42-yr-old American black man with homozygous sickle cell anemia and frequent severe pain crises. This patient had previously received red cell transfusions for severe pain crises between 1977 and 1980, but had received none during the 2 yr prior to treatment. No transfusions were given during or following 5-azacytidine treatment. From 1980 until the time of admission, the patient had approximately 1 crisis per month, but he infrequently required hospitalization.

Patients 3 and 4 had severe beta-thalassemia and transfusional hemochromatosis; these two young men of Italian descent were not transfused for 30–40 days prior to the 5-azacytidine infusion in order to allow expansion of the erythroid cell mass in the bone marrow. Patient 3 received 300 ml of packed RBC on days 2 and 3 of the protocol, but received no further transfusions until day 83. Patient 4 remained untransfused during and for 2 mo following 5-azacytidine.

The protocol for drug administration was approved by the National Heart, Lung, and Blood Institute review board and informed consent was obtained. All patients received 5-azacytidine as a continuous infusion for 7 consecutive days. The drug was mixed in 100 ml of lactated Ringer’s every 4 hr, and was administered as previously described.1 Compazine, 5 mg p.o., t.i.d., was administered during the 5-azacytidine infusion. The patients suffered no ill effects during or after drug administration. Peripheral blood was obtained daily for complete blood counts, reticulocyte counts, differential counts, and SMA-18 analysis. Blood was drawn biweekly for determination of the concentration of HbF and Hbs, and for carcinoma- bryonic antigen and alpha fetoprotein levels. Bone marrow aspirations were obtained before, during, and after the infusion for morphological study, for determination of globin biosynthetic ratios, and for RNA and DNA analysis.

Protein Synthesis

A quantity of $2 \times 10^7$ nucleated cells obtained from the bone marrow was incubated in leucine-free Iscove’s modification of Eagle’s medium with 50 μCi of $^3$H-leucine (Amersham, Arlington Heights, IL) at 37°C in 5% CO2 for 2 hr.15 Cell lysates were analyzed on Triton-X/urea polyacrylamide gels, as previously described,16 or with carboxymethylcellulose (CMC) chromatography; $\gamma/\beta$ ratios (plotted in Fig. 2) were all determined by CMC chromatography.15

RNA Analysis

RNA and DNA were recovered from bone marrow cells after lysis in guanidinium HCl/Sarcosyl, as previously described.18 Two hundred and fifty nanograms of total bone marrow RNA were annealed with uniformly radiolabeled DNA probes prepared on single-stranded templates of M13 MP7-globin gene recombinants, as previously described.17 Probe DNA was annealed to bone marrow RNA in 50% formamide for 18 hr at 65°C. Samples were treated with S, nuclease (Miles, Elkhart, IN), ethanol precipitated, heat denatured, and analyzed on 8% polyacrylamide sequencing gels with subsequent autoradiography.18

DNA Analysis

The only methylated base in eukaryotic DNA is cytosine (C); approximately 5% of cytosine residues in human DNA are methylated.19 The frequency of cytosine methylation near the $\gamma$-globin genes was ascertained by digesting total bone marrow DNA to completion with a methylation-insensitive enzyme (Eco RI), and then further digesting with a fivefold excess of the enzyme Hpa II (these restriction endonucleases were obtained from Bethesda Research Laboratories and were used according to the manufacturer’s specifications). Hpa II recognizes the nucleotide sequence CCGG, but cuts the sequence only when the internal C residue or both C residues are unmethylated. Completeness of digestion with Hpa II was determined by standard techniques.20 After each double digestion was performed, the samples were electrophoresed on 1% agarose gels.

<table>
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<th>Patient</th>
<th>Disease</th>
<th>Age</th>
<th>Hb When Untransfused*</th>
<th>Transfusion Burden (Units of Packed RBC)</th>
<th>Hb† Day 0</th>
<th>Max (Day)</th>
<th>Day 0</th>
<th>Day 7</th>
<th>$\gamma + \beta$ (1 and 2) or $\alpha/\alpha$ (3 and 4)</th>
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<td>0.6 3.3</td>
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* "Hb (g/dl) when untransfused" represents the mean value for at least 10 determinations over a period of several years.
† "Day 0" and "Max" Hb levels represent the mean of 3 determinations: the Hb level on the indicated day, and the level on the days before and after this day.
‡ Patient 1 received 580/ml of packed RBC between days 0 and 7 of the treatment course.
§ Patient 3 received 600/ml of packed RBC between days 2 and 7 of the treatment course.
¶ The patient began to be transfused at the age of 5; records prior to that time had been destroyed.
SS, sickle cell anemia; ND, no data.
agaroose vertical slab gels, and the DNA was transferred to nitrocellulose filters by the technique of Southern. The filters were then baked, prehybridized, hybridized, and washed as previously described. The filters were autoradiographed for 5–7 days at −70°C.

**Cellular Analysis**

Red cells obtained from peripheral blood were separated into fractions of various densities by centrifugation on discontinuous Stractan gradients. Stractan was purified as previously described, and then Stractan solutions of 19, 21, 22, 23, 24, 26, 28, and 33 g/dl at 290 mosmole/liter were prepared by diluting the stock solution with phosphate-buffered saline containing glucose. Solutions of decreasing density were successively layered into 1.5 × 9 cm cylindrical centrifuge tubes. One to six milliliters of a washed suspension of red cells was layered on top, and the tubes were centrifuged for 45 min at 20,000 rpm at 5°C. The red cells formed discrete layers between adjacent Stractan solutions. The cells were harvested with nearly complete recovery by slicing the tubes between each layer of cells. The total hemoglobin in each layer was determined from the volume of the harvested cell suspension and its hemoglobin concentration. The HbS concentrations in each layer of the gradients obtained from patient I were assessed by separately determining the HbS concentration in each layer of the gradient by isoelectric focusing of hemoglobin.

**RESULTS**

**Clinical Courses**

A summary of the clinical and biosynthetic data obtained from the patients is shown in Table 1. No patient had noticeable side effects during or after 5-azacytidine treatment.

Irreversibly sickled cells were present in fingerstick blood smears obtained before treatment in patients 1 and 2, but were markedly reduced in number in smears made from both patients on days 14 and 21. The hemoglobin concentration in patient 1 was essentially unchanged after therapy (Fig. 1). The hemoglobin concentration of patient 2 had increased from 8.5 g/dl to 10.2 g/dl by day 21 (Fig. 1). The absolute reticulocyte count of patient 1 was relatively low before treatment, presumably because of recent transfusions; about 35 days after the infusion was completed, the reticulocyte count had risen to a normal level for this patient (Fig. 1, middle panel). The absolute reticulocyte count of patient 2 declined markedly during the 14 days after treatment, but had increased to pretreatment levels by day 55. The indirect bilirubin level of both patients with sickle cell anemia decreased after 5-azacytidine (Fig. 1, lower panels). The lactate dehydrogenase declined markedly in patient 1 (800 U/liter to 280 U/liter), but changed little in patient 2 (320 U/liter to 220 U/liter). The white blood cell counts, granulocyte counts, and platelet counts did not significantly change (data not shown). Serial bone marrow examinations revealed erythroid hyperplasia in all samples obtained from both patients.

Both thalassemic patients had elevated absolute reticulocyte counts before treatment, indicating moderately effective erythropoiesis and active hemolysis. After the 5-azacytidine infusion, the Hb concentration gradually increased over a period of 2 wk (Table 1) and the reticulocyte counts declined slightly. The white blood cell counts, platelet counts, and bone marrow examinations did not change significantly after therapy.

Serial SMA-18 analysis and urinalysis revealed no significant hepatotoxicity or nephrotoxicity associated with any treatment course. Carcinoembryonic antigen levels and alpha fetoprotein levels did not change significantly in any patient after treatment.

**Protein Biosynthesis and F-Cell Quantitation**

γ-Globin synthesis increased fourfold to sixfold in each patient after treatment (Figs. 2 and 3 and Table 1). In patient 1, the $\gamma$-$\gamma + \beta$ biosynthetic ratio in bone marrow cells increased from 0.04 (day 0) to 0.17 (day 7) and subsequently declined to baseline over the next 14 days. In patient 2, the $\gamma$-$\gamma + \beta$ synthetic ratio increased from 0.02 (day 0) to 0.11 (day 7), similarly declining during the next 2 wk. Analysis of radiolabeled bone marrow lysates obtained on day 7 revealed that the increase in $\gamma$-globin synthesis was due to an equal increase in $\gamma$ and $\gamma$-globin biosynthesis (Fig. 3, panels I and II). Increased $\gamma$-globin synthesis was not detected by day 2 in either patient.

Data obtained in the study of one patient with $\beta^+$-thalassemia (patient 3) suggested that there was a reciprocal change in $\gamma$- and $\beta$-chain synthesis. This patient had an elevated basal level of $\gamma$-globin production (Fig. 3, panel III), enhancing our ability to detect small changes in $\gamma$-globin synthesis. Before treatment with 5-azacytidine, the $\gamma$-$\beta$ biosynthetic ratio was approximately 0.8:1 and the $\alpha$:non-$\alpha$ globin synthetic ratio was 2:1. On day 7, the increased $\gamma$-globin synthesis was clearly accompanied by a decline in $\beta$-globin synthesis (Fig. 3, panel III), although the $\alpha$:non-$\alpha$ globin synthetic ratio had decreased to 1.5:1 (Table 1). The absolute increase in $\gamma$-globin synthesis in sickle cell anemia patients was relatively small, and thus we could not accurately determine whether it was accompanied by a reciprocal decrease in $\beta$-globin synthesis.

Increased $\gamma$-globin synthesis led to increased peripheral blood HbF levels in all patients. In patient 1, HbF increased from 6% to 13.7%, and in patient 2 the level increased from 1.6% to 8.9% (Fig. 2, lower panels). When untransfused, patient 3 had approximately 65% HbF in peripheral blood. Immediately prior to treatment, the HbF level was 17%–20% because of prior transfusion. The HbF level had increased to 30% on
Fig. 1. Clinical courses of patients 1 and 2 (sickle cell anemia). In each panel, the period of 5-azacytidine administration is shaded. The hemoglobin levels, absolute reticulocyte counts, and indirect bilirubin measurements are plotted for each patient in the indicated panels. For patient 1, the circled numbers 1, 2, and 3 in the upper panel indicate the times of transfusions of 300, 140, and 140 ml of packed RBCs, respectively. Patient 2 received no transfusions before, during, or after treatment.

day 15, 40% on day 21, and 64% on day 45. In patient 4, the HbF increased from 6% before treatment to a maximum of 29% on day 18, and then slowly declined.

Kleihauer-Betke stains of peripheral blood cells revealed <5% F cells before therapy in patient 1, increasing to 30% F cells by day 21. In patient 2, F-cell number increased from <5% to approximately 16% on day 21. Patient 3 had about 40% F cells before treatment and 90% F cells on day 21. F cells (determined by immunofluorescence) in patient 4 increased from 33% before treatment to 70% by day 14.

Patient 1 was retreated by two of us (P.H. and J.D.) 10 and 18 wk after the first course of therapy. 5-Azacytidine, at 2 mg/kg/day, was given by subcutaneous injection in divided doses every 12 hr for 4 days during week 10, and for 5 days during week 18. HbF increased from 9.6% to 21% after the second course,

Fig. 2. Globin chain biosynthetic ratios (bone marrow) and hemoglobin F levels (peripheral blood) from patients with sickle cell anemia. Globin chain biosynthetic ratios were determined by carboxymethylcellulose chromatography, as described in Materials and Methods. The biosynthetic ratios represent newly synthesized globin chains in bone marrow cells. Hemoglobin F levels in peripheral blood lysates were determined by resistance to alkali denaturation, as previously described. The peak percent HbF level in patient 1 was 13.7%.
and from 13.4% to 22% after the third. F cells (Kleihauer-Betke) increased from 20.5% to 61% after course 2, and from 46% to 80% after course 3.

**RNA Analysis**

Bone marrow RNA obtained from each patient contained an increased concentration of correctly initiated and processed γ-globin mRNA after 5-azacytidine treatment (Fig. 4). Uniformly 32P-labeled DNA fragments complementary to globin mRNAs were used as the probes for these analyses. The γ-globin RNA-specific probe was derived from a fragment spanning the 5’ end of the γ-globin gene, and the β-globin gene-specific probe was derived from intron I and part of exon II of the β-globin gene (see line drawing at bottom of Fig. 4). Correctly initiated and processed γ-globin mRNA protects probe fragments of 145 nucleotides (nt) (derived from exon I) and 206 nt (most of exon II) from degradation with 5′ nuclease. Correctly processed β-globin mRNA protects a 134-nt fragment of the β-globin gene probe from degradation by S1 nuclease.

Using hybridization conditions of 65°C and 50% formamide, each probe was entirely specific for transcripts derived from its own gene. RNA derived from K562 cells contains ε, γ, and δ mRNA (Ley TJ and Dean A, manuscript in preparation) but no β-globin RNA. On analysis of K562 cell RNA with a mixture of the two probes, the two γ-specific bands were detected, but no β-specific band was observed. An RNA sample that contained β but no γ mRNA generated only the β-specific band, proving the specificity of this probe for processed β-globin mRNA.

Two hundred fifty nanograms of each RNA sample from patient 2 were annealed to a mixture of the γ- and β-globin gene probes (Fig. 4). Samples from days −2, 0, and +2 contained only trace amounts of correctly initiated and processed γ mRNA; large quantities of β-globin mRNA were present. Increased intensity of the γ-specific bands was noted in samples obtained on days 7, 14, and 21. However, increased intensity of the β-specific bands (and decreased intensity of the band at the position of the reannealed β probe) indicated that samples obtained on days 7, 14, and 21 contained a higher level of total globin mRNA per measured microgram of total bone marrow RNA. Despite the variation in total mRNA content among the samples, the relative amounts of γ and β mRNA in each sample could be determined because both probes were included in each hybridization reaction. The γ:β mRNA ratios correlated closely with the γ:β globin protein biosynthetic ratios in each bone marrow sample (Table 1). Identical analyses performed on bone marrow RNAs from patients 1 and 3 yielded similar results.

A similar analysis was performed with a probe derived from the ε-globin gene (data not shown). Before treatment with 5-azacytidine, no ε mRNA was detected.
5-AZACYTIDINE AND SICKLE CELL ANEMIA

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5-AZACYTIDINE AND SICKLE CELL ANEMIA

DNA Methylation

The frequency of cytosine methylation at the 5' ends of both the ε-γ and δ-γ genes decreased after treatment with 5-azacytidine (Fig. 5). Total bone marrow DNA was digested to completion with Eco RI, an enzyme that releases a fragment of 7.2 kb (kb = kilobase = 1,000 basepairs) containing the 5' end of the ε-γ gene and a fragment of 2.7 kb containing the 5' end of the δ-γ gene. Each of these fragments hybridizes with the nick-translated probe, a 0.9-kb Bam-Eco RI intron II fragment obtained from the ε-γ gene. Both the 7.2- and the 2.7-kb fragments contain a single Hpa II recognition site (CCGG) located near the 5' end of each gene. Cutting of these sites by Hpa II releases a 1.5-kb Hpa II–Eco RI fragment from each larger fragment that will hybridize with the γ intron II probe. This probe crosshybridizes with the 5.2-kb Eco RI fragment containing the 5' end of the human β-globin gene, a fragment that contains no Hpa II sites.

In patient 1 (Fig. 5, panel I), a faint band of 1.5 kb on day 0 indicates a relatively high frequency of cytosine methylation at the 5' end of both γ genes before treatment. By day 7, markedly increased intensity of the 1.5-kb band indicates that the frequency of cytosine methylation has decreased. The intensity of the 1.5-kb band declines steadily on days 14 and 21, indicating a return to the baseline methylation frequency. Similarly, in patient 2 (Fig. 5, panel II), the 1.5-kb band is barely identified before treatment and is of strikingly increased intensity by day 2. Hypomethylation of DNA near the γ genes was also observed on days 2 and 14 in patient 4 (Fig. 5, panel III). A similar pattern was noted in patient 3 (data not shown).

Stractican Gradient Analyses

Peripheral red blood cells obtained from both sickle cell anemia patients revealed a decrease in the proportion of dense cells after treatment. Patient 1 had been transfused prior to initiation of therapy and had 63% HbS in peripheral blood when the first Stractican gradient was performed (Fig. 6, panel I). Determination of the HbS concentration in each band revealed that most HbS was found in cells of high density, as expected for a patient with sickle cell anemia. On day 14, the HbS was predominantly located in cells of reduced density; at this time, the HbS concentration in

Fig. 4. Analysis of globin RNAs by simultaneous S, nuclease mapping with β-globin-specific and γ-globin-specific probes. At the bottom of the figure, a prototypical globin gene is represented. Flanking sequence DNA is represented by a line, coding sequences (exons) are represented by black boxes, and introns or intervening sequences (IVS) are represented by open boxes. The 3' end of the gene is not shown. The origin of the probes used in these analyses are shown below the prototypical gene. The β-specific probe (β probe, reference 17) is derived from 272 nucleotide (nt) fragment of the human β-globin gene that includes all of IVS 1 and part of exon 2. The γ-specific probe is derived from the 2.7-kb Bam HI fragment that includes the 5' end of the γ gene. The lengths of probe fragments (in nt) that are protected from 5', nuclease digestion by normal globin gene. The lengths of probe fragments (in nt) that are protected

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peripheral blood (52%) was only slightly lower than on day 0. However, newly transfused red cells may have contributed to the alteration in the Stractan gradient profile of this patient’s blood.

Stractan gradient analysis revealed similar results in patient 2, who had not been transfused for several years prior to therapy and who received no transfusions during his treatment course. This patient had approximately 97% HbS in peripheral blood on day 0 and exhibited a characteristic Stractan profile for a patient with sickle cell anemia. Cells with widely varying densities were present (Fig. 6, panel II, part A); a large percentage of these were distinctly abnormal dense cells (fractions 6–8). However, by day 14 (part B) and day 21 (part C) the dense cells were markedly reduced in number. On day 21, the cells were predominantly lighter than normal, although the absolute reticulocyte count at that time was only 5,000/cu mm (Fig. 1, right middle panel). Red cell density determined by Stractan gradients varies linearly with mean corpuscular hemoglobin concentration (MCHC),26 gradients of this patient’s blood were not specifically calibrated for MCHC. However, red cells obtained from normal individuals are never found predominantly in fractions 1 and 2 of identically prepared gradients,27 suggesting that the MCHC of red cells obtained on day 21 from

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**Fig. 5.** Southern blot analysis of DNA methylation in the region of the human γ-globin genes. Each panel represents an analysis of a different patient (I. patient 1; II. patient 2; III. patient 4), and each lane represents an analysis of total bone marrow DNA obtained on the indicated day. Samples were digested to completion with Eco RI and Hpa II, as described in Materials and Methods. The diagram at the bottom of the figure schematizes the region of the γ-globin genes. Flanking sequence DNA is indicated by a line, coding sequences are indicated by black boxes, and intervening sequences are indicated by open boxes. The locations of the αγ- and γγ-globin genes are indicated. Pertinent Eco RI (E) and Hpa II (H) recognition sites are shown, although the map is not complete. The sizes of fragments that would be expected to hybridize with the nick-translated γ probe (derived from the large intervening sequence of the γ gene) are indicated. In each panel, the 7.2-kb and 2.7-kb fragments representing the 5’ ends of the αγ- and γγ-globin genes are indicated. Additionally, a 5.2-kb band is seen in each panel, representing cross hybridization of the 5.2-kb Eco RI fragment that contains the 5’ end of the human β-globin gene. This fragment contains no Hpa II sites. The position of the 1.5-kb fragment caused by Hpa II digestion of unmethylated recognition sites near the 5’ ends of the αγ- and γγ-globin genes is also indicated in each panel.
patient 2 was in fact less than normal. Coulter-counter-determined MCHC values did not significantly change after treatment, but these automated measurements are inaccurate when measuring dehydrated cells.\(^7\) By day 55 (part D), the Stractan profile revealed a return to a population of more dense cells.

In order to assess whether the shift in cellular density seen in patients 1 and 2 was specific for sickle cell anemia, we performed serial Stractan gradients on blood samples from patient 4 (Fig. 6, panel III). This patient received 600 ml of packed RBC 2 mo prior to therapy, but received no transfusions during treatment; the Stractan profile obtained on day 0 is shown in part A of panel III. This profile, abnormal because the mean MCHC of thalassemic red cells is low, changed only slightly after treatment (Fig. 6, panel III, parts B and C).

**DISCUSSION**

All four patients described in this study exhibited a fourfold to sixfold increase in \(\gamma\)-globin synthesis after 5-azacytidine administration. Two additional patients (one treated by us\(^1\) and another treated by Charache, Dover, and colleagues\(^29\)) exhibited a similar response. All six patients treated to date have therefore experienced a significant increase in HbF production when given 5-azacytidine. These results encourage us to believe that most patients who receive adequate amounts of this drug will exhibit a significant increase in \(\gamma\)-globin synthesis.

Our data also imply that 5-azacytidine may alter the physiologic manifestations of sickle cell anemia. Hemolysis decreased, and irreversibly sickled cells were markedly reduced in number after treatment; Stractan gradient analyses revealed a marked reduction in the proportion of dense cells and also suggested that a cohort of cells even lighter than normal had been produced. The striking shift in the Stractan profiles probably reflected two concurrent processes: first, red cells newly formed under the influence of 5-azacytidine presumably had a reduced tendency to accumulate polymer and become dense. Secondly, irreversibly sickled cells and dense cells containing large amounts of HbS polymer were probably removed rapidly from peripheral blood. Since new dense cells were not being formed after 5-azacytidine administration—and since the dense cells already present were being rapidly removed—the proportion of dense cells declined precipitously.

The reduction in the proportion of dense cells may cause clinical benefit for patients with sickle cell anemia. Dense cells probably have a disproportionate effect on the abnormal rheologic behavior of blood from sickle cell anemia patients, since they contain HbS polymer even at very high oxygen saturation values.\(^26\) The reduction in the size of the dense cell
fraction (which includes the irreversibly sickled cells) should therefore have therapeutic benefit.

5-Azacytidine caused a profound alteration in the properties of newly formed red cells in sickle cell anemia, even though the absolute level of HbF in peripheral blood (9%–14%) was relatively small. Increased levels of HbF are frequently encountered in patients with sickle cell anemia, and very high levels are associated with a mild clinical course. However, the levels of HbF reached after 5-azacytidine in our patients have not previously been associated with reduced clinical severity. Why did such a “small” change in the absolute production of HbF result in such striking amplification at the cellular level?

First, HbF was distributed in a large percentage of newly synthesized cells. Using a relatively insensitive assay (Kleihauer-Betke stains), we found that a substantial proportion of cells (16%–30%) had detectable amounts of HbF after treatment. This finding suggests that an even larger percentage of cells contained increased amounts of HbF. Charache, Dover, and colleagues have also treated a sickle cell patient with multiple courses of 5-azacytidine. Their elegant and sensitive assays for intracellular HbF showed that as many as 50% of the reticulocytes contained HbF immediately after treatment; 50% of all circulating red cells were also found to contain HbF after three treatment cycles. Furthermore, our patient 1 had 80% F cells after his third course of 5-azacytidine. These data show that a large proportion of the red cells formed under the influence of 5-azacytidine contained increased amounts of HbF.

Secondly, the newly formed “F cells” made under the influence of 5-azacytidine presumably have a reduced tendency to accumulate deoxy-HbS polymer. The major determinants of the tendency of deoxy-HbS to polymerize are the intracellular concentration of deoxy-HbS and the total intracellular hemoglobin concentration. Both of these determinants may have been affected by 5-azacytidine, as discussed below.

5-Azacytidine may directly reduce the concentration of HbS in newly formed red cells, since increased γ-globin synthesis may be associated with an equivalent reduction in β-globin synthesis. A complete “reverse switch” from β- to γ-globin synthesis has been documented in baboons treated with 5-azacytidine. A similar reciprocal change is implied by our data obtained in the study of one patient (patient 3) with β+ thalassemia. Slightly reduced amounts of β-globin synthesis in our patients with sickle cell anemia were not readily detected, but the animal data and data from patient 3 suggest that the changes in globin synthesis in patients with sickle cell anemia may also be reciprocal. Furthermore, increased production of γ-globin means increased formation of αγβ8 tetramers, which do not participate in polymer formation. The αγβ8 tetramers therefore effectively reduce the number of β chains that may participate in polymer formation.

5-Azacytidine may also reduce the tendency of deoxy-HbS to polymerize by reducing the mean cell hemoglobin concentration (MCHC), a change that lowers the chemical “activity” of the intracellular hemoglobin. A striking decrease in cellular density (and presumably MCHC) to values below that observed in normal individuals was noted for patient 2 on day 21 (Fig. 6, panel III, part C). This decrease in cell density occurred despite the low number of reticulocytes (5,000/cu mm) noted on this day (Fig. 1). The reason why 5-azacytidine might cause the production of cells with reduced MCHC is unknown, but such a change would certainly be of benefit. Further studies to document the magnitude, consistency, and mechanism of this possible effect are indicated.

Increased γ-globin chain synthesis is accompanied by a decrease in the frequency of methylation of cytosine residues near the γ genes. This change occurs rapidly, since decreased methylation of the γ genes can be demonstrated in total bone marrow DNA 2 days after the infusion begins; 5-azacytidine may therefore directly reduce the methylation of DNA in dividing erythroid cells. However, the change in DNA methylation that was noted in the day-2 DNA samples was not accompanied by detectable increases in the concentration of γ mRNA or of newly synthesized γ-globin protein in bone marrow cells in this patient. However, in two other patients subsequently treated by us (see note added in proof), hypomethylation of the γ genes and an increased concentration of γ-globin mRNA was demonstrated in bone marrow cells two days after the 5-azacytidine infusion was started. These data, along with the observation of Charache et al. that F-reticulocytes increased 3 days after 5-azacytidine was started, indicate that this drug stimulates γ gene expression in late stage erythroid cells.

Although DNA near the γ-, ε-, and ζ-globin genes became hypomethylated in bone marrow cells after treatment with 5-azacytidine, only γ gene transcription increased significantly in these patients (and in the previously reported patient). Therefore, we are not certain that hypomethylation alone was responsible for causing increased expression of the γ genes. Since 5-azacytidine is cell cycle specific, it may be most toxic to the rapidly dividing CFU-E (colony-forming units–erythroid). If these cells are selected against, the erythroid “pool” could be repopulated by BFU-E (burst-forming units–erythroid) that tend to produce progeny making increased amounts of HbF.
continued experiments with cultured bone marrow cells and cell lines that contain repressed human γ genes may help to determine the mechanism of action of this drug.

This study has demonstrated that 5-azacytidine is capable of causing a predictable increase in HbF synthesis in patients with sickle cell anemia or β-thalassemia at a dose that produces little or no short-term toxicity. Since the effect on HbF synthesis is transient, the use of 5-azacytidine as a therapy for these diseases would either require repeated courses of the drug or a method to prolong the effect. However, 5-azacytidine is capable of transforming tissue culture cells, and limited animal studies indicate that the drug should be considered carcinogenic. The primary risk to patients participating in continuing trials is that of developing cancer; the relative risk for each patient is currently unknown. Many patients with acute and chronic leukemia have been treated with multiple courses of 5-azacytidine, but none have developed secondary malignancies (NCI, Investigational Drug Branch); however, in such patients, the carcinogenic potential of this drug cannot be accurately assessed.

These considerations have led us to limit the use of 5-azacytidine to a group of patients who have either a shortened life expectancy or severe disability. Recent studies suggest that if iron chelation with desferrioxamine is started after the age of 10, the premature death of patients with transfusion-dependent β-thalassemia may not be prevented. If 5-azacytidine administration could eliminate the need for transfusions in patients with severe iron overload, chelation and/or phlebotomy might allow more effective iron removal and thereby prolong life. The prognosis of individuals with sickle cell anemia is more uncertain, but selected patients with severe debilitating disease may choose to accept 5-azacytidine because of a poor quality of life and/or a lack of other treatment options.

The number of patients who receive this drug should be limited to the minimum required to answer specific, well-formulated experimental questions. Experimental studies should be performed with patients who might directly benefit if 5-azacytidine should prove to be clinically efficacious. All patients should be carefully educated about the potential risk of cancer. Children should not participate in studies of 5-azacytidine under any circumstances. Definite therapeutic efficacy of 5-azacytidine has not yet been established; therefore, this drug should not be used to treat patients with β-thalassemia or sickle cell anemia except for the purposes of careful controlled investigation.

NOTE ADDED IN PROOF

Since submission of this manuscript, we have treated four additional patients having sickle cell anemia with 1.0–1.5 mg/kg/day of 5-azacytidine administered over 8 hr for five consecutive days. All four patients responded with increased production of HbF. All four patients experienced mild to moderate nausea and vomiting, perhaps because of the shortened infusion time. These four patients had fewer dense cells and less baseline hemolysis than patients 1 and 2 described in this paper; the reductions in the dense cell fractions were therefore smaller than those reported here.

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