Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 4842–4846, August 1983
Medical Sciences

Treatment of sickle cell anemia with 5-azacytidine results in increased fetal hemoglobin production and is associated with nonrandom hypomethylation of DNA around the γ–δ–β-globin gene complex

(DNA methylation)

SAMUEL CHARACHE*†‡, GEORGE DOVER*§, KIRBY SMITH*¶, C. CONOVER TALBOT, JR.¶, MARTHA MOYER*, AND SAMUEL BOYER*¶

Departments of *Medicine, §Pediatrics, and †Laboratory Medicine, The Johns Hopkins University School of Medicine, and ¶The Howard Hughes Medical Institute, Laboratory for Human Biochemical Genetics, Baltimore, Maryland 21205

Communicated by John W. Littlefield, April 13, 1983

ABSTRACT **Increased production of fetal hemoglobin (HbF) was observed in a patient with sickle cell anemia treated with 5-azacytidine. Each of four courses of therapy resulted in a rapid and prolonged increase in the percentage of HbF containing reticulocytes (F reticulocytes) and HbF containing erythrocytes (F cells). The percentage of HbF in peripheral blood rose from 1.8 to 8.9%. The rise in HbF production was accompanied by an increase in peripheral blood hemoglobin concentration from 8 to 12 g/dl and an increase in mean erythrocyte volume. Treatment with 5-azacytidine resulted in hypomethylation of total genomic and a Y-chromosome-specific DNA fragment isolated from both peripheral blood and bone marrow. Of 15 restriction enzyme sites around the γ–δ–β-globin gene complex, only 2 became hypomethylated: one 107 bases 5′ to the γ^G and the other 107 bases 5′ to the γ^A globin genes.**

Erythrocytes containing fetal hemoglobin (HbF), termed F cells, are resistant to sickling in most patients with sickle cell disease (1). Two observations help explain this phenomenon. (*i*) Polymerization of sickle hemoglobin (HbS) is highly dependent on its intracellular concentration (2). (*ii*) Because HbF does not easily copolymerize with HbS, substitution of HbF for HbS decreases the concentration of polymerizable hemoglobin within the erythrocyte (3). Newborns with sickle cell anemia (4), rare individuals with HbF and HbS in every cell (compound heterozygotes for HbS and pancellular hereditary persistence of HbF) (5), and individuals with sickle cell anemia from certain inbred populations, who produce high levels of F cells, tend to have relatively mild or nonexistent disease (6, 7). It seems reasonable that any therapy that substantially increased HbF levels would benefit patients with sickle cell disease.

Increase in HbF production might be achieved by altering control of globin gene expression. It has been suggested that the degree of DNA methylation in sequences in or near genes may play some role in controlling their function (8). DNA associated with several eukaryotic genes is less methylated in tissues that express the genes than in tissues that do not (9–14). Several studies show that addition of 5-azacytidine (azaC) to tissue cultures results in hypomethylation of DNA and the activation of genes (14–18). Proposed mechanisms for this effect include the inherent inability of azaC-substituted DNA to serve as a methyl acceptor (19) and the inhibition of DNA methyl transferase by azaC (20) and by azaC-substituted DNA (21).

DeSimone *et al.* (22) recently demonstrated that adult baboons rendered anemic by bleeding increased their HbF levels 2- to 6-fold after treatment with azaC. Studies of human bone marrow, obtained after administration of azaC to a patient with thalassemia (23), demonstrated hypomethylation in the γ-globin gene region and increased γ-globin gene expression, potentially explaining the finding of DeSimone *et al.*

Accepting the possibly mutagenic and carcinogenic (24, 25) effects of azaC, a man severely affected with sickle cell anemia consented to a trial of the drug. The cellular mechanisms by which HbF levels increased were documented and the changing patterns of methylation of the DNA around the γ–δ–β-globin complex were measured.

METHODS

Case Report and Administration of azaC. The individual studied is a 32-year-old nurse (patient 3 in ref. 26; "Beta" in ref. 27), who had spent 70 of the 166 days prior to treatment as an inpatient and had been transfused with 224 units of blood over the preceeding 9 yr, with some symptomatic amelioration. His last transfusion was 55 days prior to treatment with azaC. Informed consent was obtained in accordance with the guidelines of The Johns Hopkins University Medical School Joint Committee on Clinical Investigation. The patient received 4-hr infusions of azaC (supplied by the National Cancer Institute) at 8-hr intervals. A total of nine infusions with 47 mg of azaC (30 mg/m^2 per infusion) were given on three occasions. On day 73, he received subcutaneous injections of the same amount of drug, on the same schedule.

Hematologic Determinations and Single Cell Methods. Peripheral blood erythrocyte indices and the number of leukocytes and platelets were estimated with a Coulter Counter (Model S plus 2). Reticulocytes were stained with new methylene blue and counted manually. The percentage of HbF in hemolysates was measured by alkali denaturation (28). "F reticulocytes" (reticulocytes containing HbF) and "F cells" (peripheral erythrocytes containing HbF) were defined by microscopic immunoprecipitate reactions by using rabbit anti-human HbF antibody (29). Microdensitometric analysis of individual immunoprecipitate reactions was used to determine the amount of HbF in individual erythrocytes (30). Relative γ- and β^s-globin chain synthesis by bone marrow cells was estimated from the incor-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: kb, kilobase(s); NRBC, nucleated erythrocytes; azaC, 5-azacytidine; HbF, HbS, and HbA, hemoglobins F, S, and A, respectively.

‡ To whom reprint requests should be addressed at: Johns Hopkins Univ., 600 N. Wolfe St., Baltimore, MD 21205.

poration of [^{3}H]leucine into both globin fractions (31).

Analysis of DNA Methylation. The extent of overall DNA methylation was monitored by digestion with a number of restriction endonucleases whose activity is sensitive to methylation at their restriction sites. DNA was prepared from samples of peripheral blood and bone marrow as described (32). Following digestion with restriction enzymes, DNA fragments were fractionated according to molecular weight by electrophoresis in 1% agarose gels (33). The size distribution of DNA fragments was determined by visual examination of ethidium bromide-stained gels.

The state of methylation of a repeated Y-chromosome-specific DNA fragment was examined by comparing *Hpa* II and *Msp* I digests (unpublished data). DNA fragments were transferred to nitrocellulose paper (34) and hybridized with a cloned probe specific for the Y fragment. When sites surrounding this Y-chromosome-specific sequence are fully methylated, digestion with *Msp* I releases primarily 2.4-kilobase (kb) fragments, whereas digestion with *Hpa* II releases multiple higher molecular weight fragments. With decreased methylation more sites become susceptible to digestion by *Hpa* II, producing more 2.4-kb fragments (unpublished data). The extent of methylation was judged by visual examination of the relative intensity of the radiographic bands.

DNA methylation in the γ-δ-β-globin gene cluster was measured by restriction analysis as described by van der Ploeg and Flavell (15). Restriction enzymes insensitive to the state of DNA methylation (*Eco*RI, *Bam*HI, *Hin*dIII, and *Msp* I) were used to release restriction fragments containing one or more restriction sites whose cleavage is sensitive to DNA methylation (*Hpa* II, *Hha* I, *Sal* I, and *Xho* I). To ensure digestion with the methylation-sensitive restriction enzymes a 10- to 20-fold excess of enzyme to DNA was used.

Size-fractionated DNA digests were hybridized with specific globin gene probes derived from cDNA clones JW102 (β) and JW103 (γ) (35). DNA fragments for use as probes were recovered after digestion of JW102 with *Hin*dIII and *Mbo* II and digestion of JW103 with *Taq* I and were radiolabeled by nick-translation. Filter hybridization, washing procedures, and autoradiography were done as described (33), with the addition of three washes with 15 mM NaCl/1.5 mM sodium citrate at 65°C to decrease detection of cross-hybridization between each probe and other globin genes. The state of methylation was judged by visual examination of the relative intensities of bands corresponding to the fragments generated.

RESULTS

Hematologic determinations are presented in Fig. 1. Prior to treatment and without transfusion, the patient had a hemoglobin concentration of 7.5–8.5 g/dl. On day 0, his blood contained 43% hemoglobin A (HbA), from previous transfusions; no HbA was detectable after day 48. Hemoglobin concentration increased after the initial azaC therapy and remained between 10 and 12 g/dl. Mean corpuscular volume rose from 94–98 fl to 104.5 fl by day 25 and then remained between 101 and 107 fl; mean corpuscular hemoglobin did not change significantly. The total number of leukocytes varied between 1–3 × 10^4 leukocytes per mm^3 and the differential leukocyte count did not change significantly. The number of platelets rose after each course of azaC, reaching levels of 0.5–1.0 × 10^6 platelets per mm^3.

The proportion of F reticulocytes rose from pretreatment levels (3–6%) to 13% by the third day of treatment, reached a peak of 50% by day 9, and returned to baseline by day 30 (Fig. 2, top panel). Subsequent treatment produced similar responses. The percentage of F cells (Fig. 2, center panel) rose from a pretreatment range of 8–12% to a peak of 51% on day 57 after multiple doses of the drug. The number of nucleated erythrocytes (NRBC) in peripheral blood (Fig. 2, bottom panel) rose in parallel with F reticulocytes.

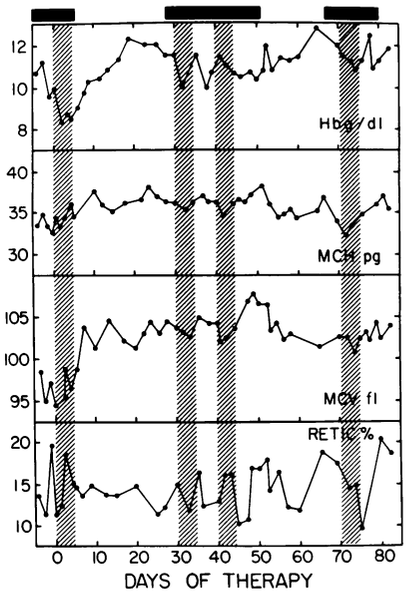

FIG. 1. Measurements of hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and percentage of reticulocytes (RETIC) during azaC therapy. Shading indicates days of azaC administration. Bars at the top indicate onset and duration of three vaso-occlusive crises suffered by the patient during therapy.

The percentage of HbF in the patient's blood rose from 1.5% to a maximum of 8.9% on day 59, and γ-globin chain synthesis, (γ:γ + β^s), rose from 0.03 to 0.10 during the first course of therapy. The average amount of HbF in individual reticulocytes and F cells also increased. Between day 0 and 9 mean HbF per cell rose from 5.7 to 8.1 pg in F reticulocytes and from 6.2 to 7.8 pg in F cells. The distribution of HbF concentrations in individual reticulocytes is shown in Fig. 3. Pretreatment, HbF/F reticulocyte ranged from 3 to 7 pg per cell (solid line). On day 3 (dotted line), 30% of the F reticulocytes contained 7 to 13 pg of HbF. By day 6 some cells contained as much as 21 pg of HbF, although the fraction of F reticulocytes containing increased HbF had not changed. By day 14 HbF/F reticulocyte had returned to the pretreatment distribution.

Erythrocyte half-life, as measured with ^{51}Cr on day 50, was 10 days. It had been 4 and 6 days when studied on two occasions pretreatment. Painful crises continued to occur during the period of study, with more-or-less their prior severity and frequency (Fig. 1).

DNA Methylation. Ethidium bromide-stained gels of peripheral blood DNA restriction digests (Fig. 4 *Upper*) showed a rapid and reversible hypomethylation of DNA after azaC treatment. The distribution of DNA fragment sizes generated by methylation-insensitive enzymes *Eco*RI and *Msp* I (also *Hin*dIII and *Bam*HI; data not shown) was unaffected by azaC treatment. After treatment there was a marked decrease in the average fragment length generated by methylation-sensitive restriction enzymes (*Hpa* II and *Hha* I) on days 5 (Fig. 4, lane 3) and 7 (Fig. 4, lane 4); by day 23 (Fig. 4, lane 5), the sizes of DNA fragments were similar to those obtained pretreatment. Subsequent azaC treatments also produced substantial hypomethylation of peripheral blood and bone marrow DNA relative to the pretreatment blood sample. Comparison of the *Hpa* II and *Msp* I digests indicates that the peak response re-

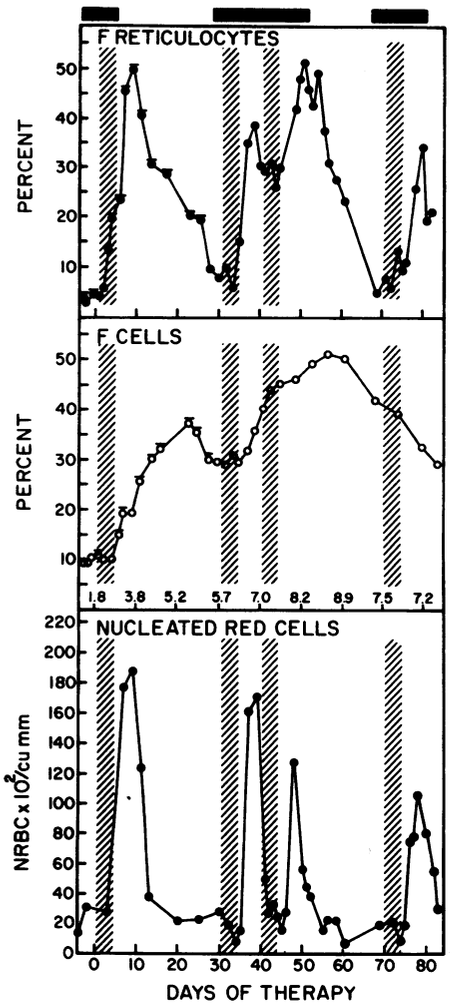

FIG. 2. Alterations in the percentage of F reticulocytes, the percentage of F cells, and the absolute number of nucleated erythrocytes (red cells) found in the peripheral blood during azaC therapy. Shading and bars are as described in the legend to Fig. 1. Numbers at the bottom of F cell section indicate % HbF in hemolysates. The standard deviation of F reticulocytes and F cell measurements are shown for first 30 days and were thereafter unchanged.

sulted in not more than 50% reduction of methylation.

The effect of azaC on DNA methylation was also assessed by the use of a repeated DNA fragment from the Y chromosome (Fig. 4 *Lower*). *Msp* I digestion of pretreatment DNA yields a

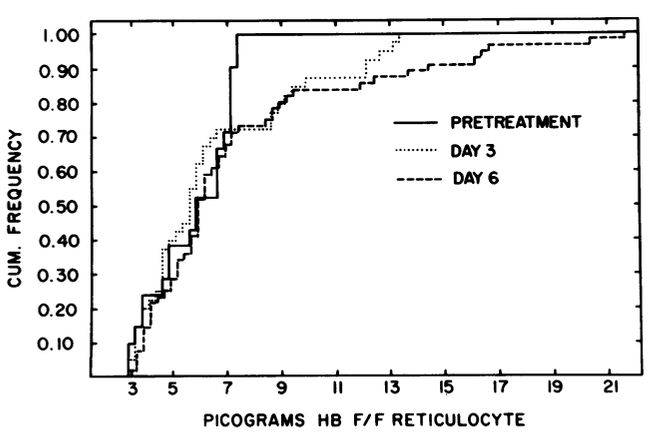

FIG. 3. Cumulative frequency plot of the amount of HbF in F reticulocytes on days 0, 3, and 6.

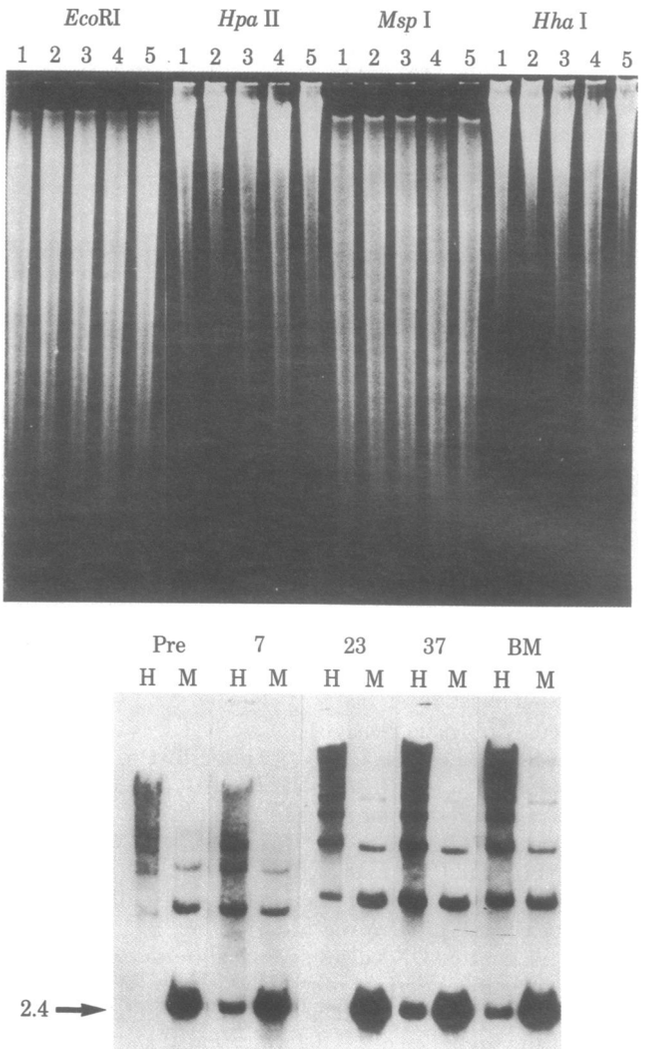

FIG. 4. Analysis of genomic DNA methylation. (*Upper*) Ethidium bromide-stained agarose gels of DNA from various stages of azaC therapy digested with methylation-insensitive (*Eco*RI and *Msp* I) and methylation-sensitive (*Hpa* II and *Hha* I) restriction enzymes. The numbers above each lane designate the following days of treatment: 1, pretreatment; 2, 2 days; 3, 5 days; 4, 7 days; and 5, 23 days. (*Lower*) Autoradiogram of *Hpa* II (H) and *Msp* I (M) digests of genomic DNA hybridized with a cloned probe specific for a repeated 2.4-kb DNA fragment from the Y chromosome. The numbers at the top refer to days of azaC therapy. Day 37 was 7 days after the second azaC treatment. Pre, pretreatment; BM, bone marrow DNA isolated 4 days after a later treatment with azaC. The position of the 2.4-kb fragment is denoted at the left of the figure. The higher molecular weight bands are multiples of the 2.4-kb band.

prominent hybridizing band at 2.4 kb, typical of the unmethylated repeats, whereas *Hpa* II digestion generates a smear of higher molecular weight fragments expected for methylated repeats. *Hpa* II digests on days 5 and 7 show a substantial increase in hybridization to fragments of 2.4 kb or multiples of 2.4 kb, indicating significant hypomethylation. By day 23, the majority of these fragments were again methylated, as judged by the decreased intensity of 2.4-kb bands from *Hpa* II digests. Samples taken after the second azaC treatment, days 37 and 41 (data not shown), and both posttreatment bone marrow preparations (one is shown in Fig. 4 *Lower*) showed levels of hypomethylation equivalent to that obtained after the earlier treatment.

A summary of methylation changes in the γ–δ–β-globin gene region in peripheral blood DNA and its methylation state in posttreatment bone marrow DNA is presented in Fig. 5. Five

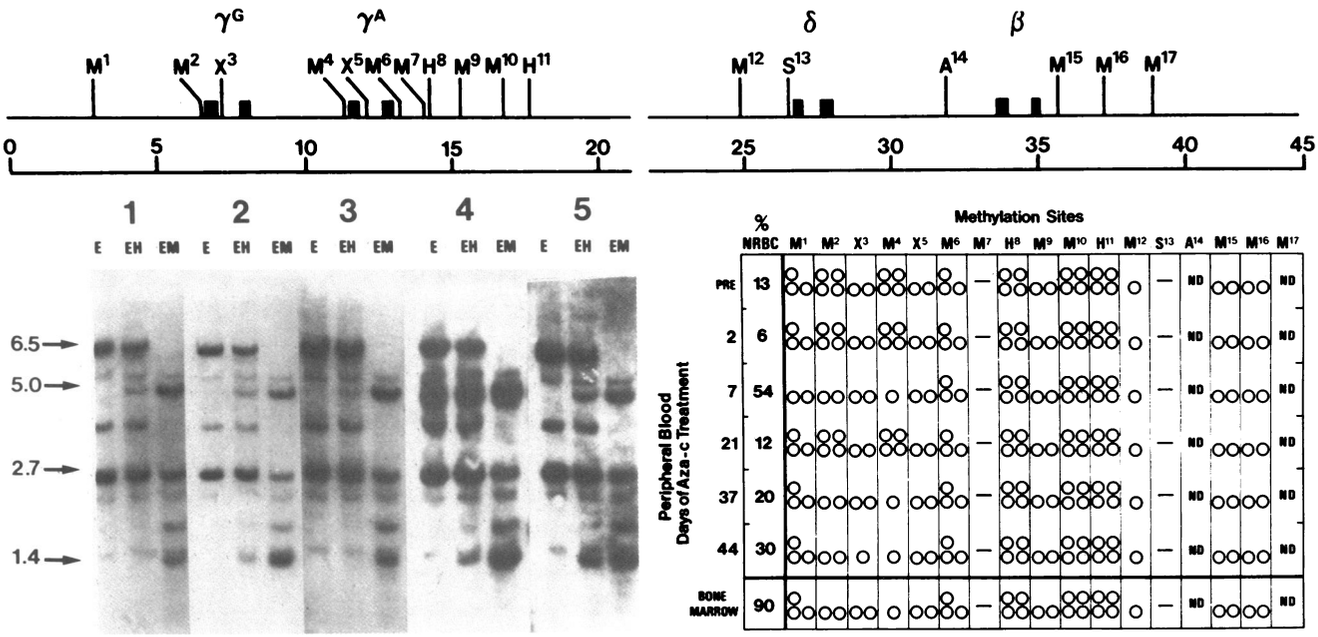

FIG. 5. Analysis of DNA methylation around the γ–δ–β-globin gene region. (*Upper*) The map of methylation-sensitive restriction sites is redrawn from van der Ploeg and Flavell (15). (*Lower Left*) The autoradiogram displays analysis of methylation at the M_1 and M_2 sites as determined by *Eco*RI and *Hpa* II (EH) or *Eco*RI and *Msp* I (EM) double-digests with a γ-gene hybridization probe. Digestion with *Eco*RI (E) gives a 6.5-kb band containing the γ^G gene and a 2.7-kb band containing the γ^A gene. Cleavage of the 6.5-kb *Eco*RI γ^G fragment at the M_1 site yields a 5.0-kb double-digest fragment, whereas cleavage at M_2 gives a 1.4-kb double-digest fragment. The extent of methylation at the M_2 site was determined by visual comparison of the intensity of hybridization of the 1.4-kb band with the sum of the 6.5-, 5.0-, and 1.4-kb bands. The minor bands seen are the result of heterologous hybrids, such as crossreacting δ and β genes. The numbers on the left give the positions of γ-gene containing fragments in kilobases. The numbers along the top of the gels denote the days of azaC therapy: 1, pretreatment; 2, 7 days; 3, 21 days; 4, 44 days; and 5, DNA from bone marrow taken 4 days after later treatment. (*Lower Right*) The approximate level of methylation at each of the methylation-sensitive restriction sites. One-hundred percent methylation is indicated by ⊗ and 0% by —. ND, no determination was made. The methylation levels at the sites closest to the gene sequence can be measured accurately. Additional sites farther from the gene can only be measured if the closest site is methylated. For example, modification at M_1 can be measured accurately if the M_2 site is fully methylated. If methylation at M_2 is <100%, the measurement at M_1 represents only those fragments whose M_2 site is methylated.

restriction sites associated with the δ- and β-globin genes were significantly unmethylated throughout the study and no changes in methylation were detected. With one exception (M_7), 11 sites surrounding the γ-globin genes were heavily methylated in pretreatment DNA. Nine of these sites showed no change in methylation after azaC treatment. The sites found 107 bases 5′ to the γ^G (M_2) and γ^A (M_4) genes became less methylated after treatment. To illustrate the method for assessing methylation levels, analysis of methylation at the M_2 (*Msp* I/*Hpa* II) site is also presented in Fig. 5.

DISCUSSION

Ley *et al.* (23) have reported that continuous infusion of 140 mg of azaC per day for 7 days in a β^+-thalassemia homozygote results in hypomethylation of bone marrow DNA near the γ-globin locus, increased γ mRNA, increased γ-globin synthesis, and increased HbF levels in peripheral blood. After a transient increase in reticulocyte count, reticulocytopenia, leukopenia, and neutropenia were observed. Our results indicate that lower doses of azaC given for only 3 days had similar effects on HbF production but did not produce bone marrow suppression. Increased HbF production was characterized by a rapid increase in F reticulocytes, which lasted ≈30 days and was associated with a transient increase of HbF/F reticulocyte. In addition, the mean corpuscular volume of erythrocytes increased. The mean corpuscular hemoglobin did not change significantly, suggesting that HbS reciprocally decreased in those cells producing HbF.

The mechanism by which azaC induces synthesis of HbF is unclear. Heretofore, drug-related increased HbF production in sickle cell disease had only been documented at the cellular level in an individual recovering from bone marrow suppression (36) produced during cancer chemotherapy. It is unlikely that such "expanded" erythropoiesis accounted for the repeated increases in F reticulocyte levels seen here because no marrow toxicity was noted and alterations in reticulocyte counts did not correlate with the percent of F reticulocytes.

The dose of azaC used to treat this patient resulted in significant, but not complete, hypomethylation of total genomic DNA and a repeated DNA fragment from the Y chromosome (Fig. 4). The surprising finding is the apparent specificity of the azaC effect on methylation within the γ–δ–β-globin gene complex. Only two of the sites examined, M_2 and M_4, showed a rapid and reversible hypomethylation (Fig. 5).

A complicating feature of these data is the possible effect of a changing cell population in peripheral blood after azaC treatment. Because changes in methylation correlate with the percentage of peripheral blood NRBC (Fig. 5), NRBC might always be hypomethylated at the M_2 and M_4 sites and the apparent methylation change a reflection of their increase. We think this unlikely for several reasons. (*i*) Although we were unable to obtain a pretreatment marrow from this patient, normal adult bone marrow is fully methylated at the M_2 and M_4 sites (15). The hypomethylation observed at these sites in two posttreatment marrows is likely to be the result of azaC therapy. (*ii*) In a thalassemic patient one or both of the M_2 and M_4 sites, fully methylated in pretreatment bone marrow, became hypomethylated after treatment with azaC (23). (*iii*) The M_2 and M_4 sites are specifically hypomethylated in erythroid tissues producing HbF and in a human leukemia cell line (K562) that can be induced

Table 1. Cellular distribution of HbF in clinically mild forms of sickle cell disease, American individuals with sickle cell disease, and the patient treated with azaC

Condition	F reticulocytes, %	F cells, %	HbF/F reticulocytes, mean pg	Total HbF, %*	*n*
S/HPFH†	100	100	6–8	27–35	3
SS (Saudi Arabian)‡	34 ± 9	62 ± 13	5–8	18.7 ± 0.1	15
SS (American)	11 ± 8	24 ± 20	5–8	8.6 ± 7.1	40
azaC patient§	52	51	8.1	8.9¶	1

All values were obtained by using methodology outlined in refs. 29 and 30.

* Determined by alkali denaturation (28); expressed as range of values or mean ± SD.

† S/HPFH, double heterozygote for HbS and pancellular hereditary persistence of hemoglobin.

‡ Sickle cell disease patients from Eastern Saudi Arabia (6).

§ Peak values.

¶ Note that peak % HbF of 8.9% was found at a time when 51% of the cells contained HbF and the mean corpuscular hemoglobin was 37 pg. Thus, F cells at that time contained 6.5 pg of HbF per F cell. This value is not different than pretreatment levels of 6.2 and 6.8 pg of HbF per F cell. Therefore, transient increases in the pg of HbF/F reticulocyte were not prolonged enough to increase significantly the HbF per F cells.

to produce HbF (15). (*iv*) Hypomethylation of similar sites 5′ to expressed globin genes has been described in other species (13). (*v*) A similar selective hypomethylation around azaC-activated retrovirus genes has been reported in chicken leukocytes under conditions that did not produce detectable methylation changes around the globin genes (37). Thus, the azaC effect on methylation in the γ-globin gene region appears to be specific and associated with increased HbF production, although these studies do not establish a causative relationship between the two observations. For example, we have not addressed the issue of an azaC effect on other loci, which may, in turn, stimulate HbF production or the observation that the principal azaC effect appears to be on F cell number and not on the amount of HbF per cell.

Do our preliminary trials suggest that azaC therapy is potentially useful in sickle cell disease? Some tentative conclusions can be drawn by comparing the cellular distribution of HbF obtained in this study (Figs. 2 and 3) to previously reported distributions in black American sickle cell patients (1) and individuals with milder forms of sickle cell anemia, S/HPFH heterozygotes (38) and Saudi Arabian sickle cell patients (7) (Table 1). If the dose of azaC used in this report could be given at more frequent intervals without toxicity, the number of F cells and the amount of HbF/F cell might be altered to the pattern seen in mildly affected Saudi Arabian patients with sickle cell anemia. If the drug could be administered in such a manner that all erythrocytes were affected, levels of F cells and HbF similar to those of asymptomatic S/HPFH individuals might be achieved. Because this drug has been classified as a potential carcinogen (25), studies to evaluate possible therapeutic benefit should only be performed on severely affected sickle cell patients.

We thank R. Burk for his helpful suggestions during the course of this study and K. E. Young for providing the Y chromosome 2.4-kb probe. This work was supported by National Institutes of Health Grants HL027996 (S.C.), HL28028 (G.D.), HL15026 (K.S.), and RR-35 (General Clinical Research Center). G.D. is the recipient of National Institutes of Health Research Career Award AM00689 and M.M. is supported in part by a grant from the Central Maryland Committee for Sickle Cell Anemia.

1. Dover, G. J., Boyer, S. H., Charache, S. & Heintzelman, K. (1978) *N. Engl. J. Med.* **299**, 1428–1435.
2. Noguchi, C. T. & Schechter, A. N. (1981) *Blood* **58**, 1057–1068.
3. Bertles, J. F., Rabinowitz, R. & Dobler, J. (1970) *Science* **169**, 375–377.
4. Watson, R. J., Stalman, A. W. & Bitello, F. W. (1948) *Am. J. Med. Sci.* **215**, 419.
5. Herman, E. C. & Conley, C. L. (1960) *Am. J. Med.* **29**, 9–17.
6. Pembrey, M. E., Wood, W. G., Weatherall, D. J. & Perrine, R. P. (1978) *Br. J. Haematol.* **40**, 415–429.
7. Dover, G. J., Boyer, S. H. & Pembrey, M. E. (1981) *Science* **211**, 1441–1444.
8. Riggs, A. D. (1975) *Cytogenet. Cell Genet.* **14**, 9–25.
9. McGhee, J. D. & Ginder, G. D. (1979) *Nature (London)* **280**, 419–420.
10. Mandel, J. L. & Chambon, P. (1979) *Nucleic Acids Res.* **7**, 2081–2103.
11. Compere, S. J. & Palmiter, R. D. (1981) *Cell* **25**, 233–240.
12. Bird, A., Taggert, M. & Macleod, D. (1981) *Cell* **26**, 381–390.
13. Felsenfeld, G. & McGhee, J. (1982) *Nature (London)* **296**, 602–603.
14. McKeon, C., Ohkubo, H., Pastan, I. & deCrombrugghe, B. (1982) *Cell* **29**, 203–210.
15. van der Ploeg, L. H. T. & Flavell, R. A. (1980) *Cell* **19**, 947–958.
16. Mohandas, T., Sparkes, R. S. & Shapiro, L. J. (1981) *Science* **211**, 393–396.
17. Constantinides, P. G., Jones, P. A. & Gevers, W. (1977) *Nature (London)* **267**, 364–366.
18. Christy, B. & Scangos, G. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6299–6303.
19. Jones, P. A. & Taylor, S. M. (1980) *Cell* **20**, 85–93.
20. Friedman, S. (1979) *Biochem. Biophys. Res. Commun.* **89**, 1328–1333.
21. Creusot, F., Acs, G. & Christman, J. K. (1982) *J. Biol. Chem.* **257**, 2041–2048.
22. DeSimone, J., Heller, P., Hall, L. & Zwiers, D. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4428–4431.
23. Ley, T. J., DeSimone, J., Anagnou, N. P., Keller, G. H., Humphries, R. K., Turner, P. H., Young, N. S., Heller, P. & Neinhuis, A. W. (1982) *N. Engl. J. Med.* **307**, 1469–1475.
24. Benedict, W. F., Banerjee, A., Gardner, A. & Jones, P. A. (1977) *Cancer Res.* **37**, 2202–2208.
25. International Agency for Research on Cancer (1981) *IARC Monogr. Eval. Carcinog. Risk Chem. Man* **26**, 37–44.
26. Charache, S., Duffy, T. P., Jander, N., Scott, J. C., Bedine, M. & Morrell, R. (1975) *Arch. Intern Med.* **135**, 1043–1047.
27. Charache, S. & Walker, W. G. (1981) *Blood* **58**, 892–896.
28. Singer, K., Chernoff, A. I. & Singer, L. (1951) *Blood* **6**, 428–438.
29. Dover, G. J., Boyer, S. H. & Bell, W. R. (1978) *Blood* **52**, 646–672.
30. Dover, G. J. & Boyer, S. H. (1980) *Blood* **56**, 1082–1091.
31. Charache, S., Fox, J., McCurdy, P., Kazazian, H. H. & Winslow, R. (1977) *J. Clin. Invest.* **59**, 652–658.
32. Kunkel, L. M., Smith, K. D., Boyer, S. H., Borgoankar, D. S., Wachtel, S. S., Miller, O. J., Bug, W. R., Jones, W. W. & Rusy, J. M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1245–1249.
33. Schmeckpeper, B. J., Willard, H. F. & Smith, K. D. (1981) *Nucleic Acids Res.* **9**, 1853–1872.
34. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
35. Wilson, J. T., Wilson, L. B., de Riel, J. K., Villa-Komaroff, L., Efstratiadis, A., Forget, B. G. & Weissman, S. M. (1978) *Nucleic Acids Res.* **5**, 563–581.
36. Dover, G. J., Boyer, S. H. & Zinkham, W. H. (1979) *J. Clin. Invest.* **63**, 173–176.
37. Groudine, M., Eiseman, R. & Weintraub, H. (1981) *Nature (London)* **292**, 311–317.
38. Dover, G. & Boyer, S. H. (1980) *Tex. Rep. Biol. Med.* **40**, 43–54.