Perturbations in the Erythroid Marrow Progenitor Cell Pools May Play a Role in the Augmentation of HbF by 5-Azacytidine

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In vivo observations on the kinetics of F cells and of fetal hemoglobin (HbF) synthesis and in vitro studies of erythroid progenitors, their number, and the γ-gene expression in their progeny were carried out in baboons (Papio cynocephalus) treated with 5-azacytidine. Maximum effect on the increase of HbF production in vivo was observed only when an expanded erythroid marrow population was present. In these animals, as well as in normal animals, treatment resulted in a significant reduction of the late erythroid progenitor cell pools (erythroid clusters and erythroid colony-forming units, CFU-E) in the marrow. This reduction was more pronounced among those progenitors grown in the absence of added erythropoietin, and it was followed by a rebound a few days after treatment cessation, reflecting the accumulation of regenerating progenitors. An early increase in the in vitro synthesis of HbF in erythroid clusters and CFU-E colonies was observed. This increase was further documented at the cellular level, with immunofluorescent labeling of colonies with monoclonal anti-γ-globin chain antibodies. In contrast to the findings in late progenitors, the number of erythroid burst-forming unit (BFU-E) colonies and the synthesis of HbF in these colonies was not influenced significantly by 5-azacytidine treatment. It is proposed that the toxic effects of 5-azacytidine on late progenitors, leading to faster mobilization of earlier progenitors to the next more mature compartment, play a role in the in vivo augmentation of HbF synthesis by this drug. This perturbation in the progenitor cell population kinetics and the presumed hypomethylation of the surviving differentiating cells may act synergistically to produce a maximum HbF response after 5-azacytidine treatment.

In MAN AND OTHER PRIMATES, there is continuation of fetal hemoglobin (HbF) in adult life in the form of production of a small number of HbF-containing red cells—the F cells.1,2 Previous studies have shown that F cells derive from the same progenitors as the erythrocytes that do not contain HbF3–10 and that their numbers increase when there is a sudden erythropoietic stress.11–15 The effect of erythropoietic stress on F-cell production is especially noticeable in manipulations done in the baboon (Papio cynocephalus); in this animal, significant increments in the number of F cells can be induced by acute hemolysis, phlebotomy, or hypobaric hypoxia.16–20

Recently, DeSimone et al. have shown that 5-azacytidine administered in vivo in anemic baboons brings about an exuberant stimulation of fetal hemoglobin synthesis.21,22 Presumably, this effect was exerted through induction of hypomethylation of γ-globin genes,21 in accord with other evidence correlating expression with degree of methylation of globin genes.23,24 5-Azacytidine was also administered in patients with either severe homozygous β-thalassemia or sickle cell anemia,25–27 resulting in significant, albeit transient, stimulations of fetal hemoglobin synthesis.

In this article we report studies of HbF expression in baboons treated with 5-azacytidine. Our purpose was to examine the effect of this compound on the kinetics of F-cell production, as well as on the populations of erythroid progenitors. In contrast to previous studies,21,22,25,27 we combined the in vivo observations on the kinetics of HbF synthesis and of F cells with studies of erythroid progenitors (their numbers and the γ-gene expression in their in vitro progeny). Our findings indicate that administration of 5-azacytidine, either to the normal or to the anemic baboon, at doses previously presumed to cause no significant hematologic toxicity21 results in selective elimination of a significant portion of late erythroid progenitors; erythroid burst-forming units (BFU-E), the early erythroid progenitors, are seemingly unaffected under the experimental conditions used. It is proposed that this distortion in progenitor cell pools plays a role in the in vivo augmentation of HbF. It is speculated that this role, depending on the treatment circumstances (normal or anemic marrow; low or high dose of 5-azacytidine), may be of major or lesser importance for the in vivo HbF augmentation.

MATERIALS AND METHODS

Animals

Two male juvenile baboons (2.5 yr old), housed and cared for at the Regional Primate Center, University of Washington, were used in this study. Blood and bone marrow samples were obtained before treatment and at desired intervals throughout the study. Bone marrow was aspirated by rotating bone marrow sites (anterior or posterior iliac crest, humeral head) and under general anesthesia.

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Globin Biosynthesis in Uncultured Cells

Bone marrow specimens (0.2 ml) or the reticulocyte-rich (by centrifugation) fractions of peripheral blood were incubated in the presence of \( ^3H \)-leucine (specific activity 60 Ci/mmol) in leucine-free alpha medium and 10% fetal calf serum at 37°C for 12-16 hr. At the end of incubation, the samples were washed with NKM saline and either analyzed immediately or stored in liquid nitrogen. Cell lysates were subjected to globin-chain isoelectric focusing, followed by fluorography, as previously described. The relative proportion of synthesized globin chains was determined by automated densitometry of the resulting fluorograms (Gelman, ACD-18 Automatic Computer Densitometer, Ann Arbor, MI).

Results of Colonies by Immunofluorescence

For these studies, plasma clots were used (at day 3, for erythroid clusters and CFU-E colonies; at days 7-14 for BFU-E colonies); they were fixed, as previously described, and labeled with an anti-\( \gamma \)-chain monoclonal antibody plus anti-mouse F(ab')2-FITC, followed by an anti-\( \beta \)-chain monoclonal antibody directly conjugated to rhodamine.

Experimental Design

The experiments were designed (A) to quantitate the effect of 5-azacytidine alone, or in combination with mild or severe bleeding, on in vivo HbF production and (B) to assess, at the same time, any perturbations on the erythroid progenitor cell pools introduced by the treatment.

To study the effects of azacytidine in the nonanemic state, an animal (animal 1, Fig. 1) with high F-cell levels was chosen. Since it has been previously stated that, in the absence of anemia, administration of 5-azacytidine produces only a small induction of HbF synthesis, treatment of an animal with a high F-cell number (high F responder) was desirable to optimize the quantitative effects on HbF and F cells. A dose previously shown to lead to maximum response without any significant toxicity was chosen. Thus, this animal was given 10 injections of 5-azacytidine (5 mg/kg body weight per injection) in a period of 14 days; HbF and F-cell production and effects on erythroid progenitors were evaluated before, during, and after treatment.

To test effects of 5-azacytidine in the anemic state (mild or severe), an animal with low F cells was chosen (animal 2, Fig. 2). Since the combination of severe bleeding and 5-azacytidine led to a drastic stimulation of HbF synthesis, it was desirable to conduct our
observations in an animal in which baseline HbF production was low. Animal 2 was initially submitted to moderate bleeding (hematocrit decreased to approximately 30%) followed by a 5-day course of 5-azacytidine (treatment A, Fig. 2). The animal was left to rest for 30 days, and then it was subjected to intense bleeding, aimed to keep the hematocrit between 20%–25%. A 5-day course of 5-azacytidine was again given (treatment B), while the bleeding was continued; subsequently, a new 5-day course of 5-azacytidine (treatment C) was administered, again on a background of continuous bleeding. Since the animal was kept throughout the last two treatments at about the same hematocrit, possible effects of 5-azacytidine, which are additive to those of phlebotomy-induced anemia, were expected to produce discrete waves of HbF response.

**Changes in Hematologic Parameters**

Changes observed were essentially similar to those described before in baboons[21] or humans,[22-27] but certain differences were noted. In animal 1, (nonanemic baboon treated with 5-azacytidine), WBC and reticulocyte counts decreased moderately at the end of the treatment, suggesting that there were transient cytotoxic effects of 5-azacytidine at the dosage used. Subsequently, reticulocyte counts increased and exceeded the baseline levels 16 days following the end of treatment (Fig. 1). In animal 2 (bleeding plus 5-azacytidine), WBC declined mildly after each treatment. When this animal was first treated with bleeding followed by 5-azacytidine (treatment A, Fig. 2) a striking decline in reticulocyte counts was produced. The expected rise in hematocrit when the bleeding was discontinued did not occur; later, rebound reticulocytosis took place. When the animal was severely bled and received 5-azacytidine, while bleeding was continued (treatments B and C), there was a transient downward change in reticulocyte counts during each treatment period (Fig. 2). Bone marrow smears were prepared before treatment and a few times after the cessation of treatment. Specifically, the data concerning treatment C were as follows: preparations before treatment were...
highly cellular and the proportion of nucleated erythroid cells was 38%, increased from a baseline of 16% before bleeding. Preparations made after treatment appear to be less cellular than before treatment, although technical reasons are not excluded for this difference, but the proportion of erythroid cells remained more or less high (it was 49%, 25%, and 31%, 4 days, 7 days, and 15 days after treatment, respectively). Platelet count remained above \( 3 \times 10^5 / \text{cu mm} \) throughout all treatments.

**Effects on HbF**

In animal 1, an increment in F-cell number took place during the time 5-azacytidine was administered. F cells increased from an 18% pretreatment level to a peak of 39% 22 days after cessation of treatment (Fig. 1). High F-cell levels were retained until day 65 when the observations discontinued. Four months later, the F-cell number was 16%. Concomitantly with the increase in F cells, there was an increment in chemically determined HbF (from 1.8% pretreatment to 6.9% 22 days after treatment). HbF per F cell (estimated from the number of F cells and the amount of chemically determined HbF in hemolysates) increased from 2.06 pg/F cell pretreatment to a maximum of 5.7 pg/F cell at day 26 posttreatment. The rate of \( \gamma \)-globin synthesis was determined in reticulocytes and bone marrow cells before treatment and 1 day, 7 days, and 5 wk after completion of treatment. One day after treatment, \( \gamma/\gamma + \beta \) in bone marrow was 0.24, and 7 days after treatment, 30% of the synthesized non-\( \alpha \)-chains were \( \gamma \), while 5 wk later \( \gamma \)-chain synthesis in both bone marrow and peripheral blood was at the pretreatment levels (~5%) (Fig. 3). Overall, this animal, which normally had a high number of in vivo F cells, responded to 5-azacytidine treatment with a moderate increase in numbers of F cells and in HbF/F cell (twofold and 2.5-3-fold, respectively).

Moderate degree of bleeding in animal 2 elicited a threefold increment in the numbers of F cells (Fig. 2). Administration of 5-azacytidine following this bleeding slightly increased the number of F cells (2%-3% F cells prebleeding; 8%-9% in response to bleeding; 10%-11% following 5-azacytidine treatment). The amount of HbF/F cell increased 2-2.5-fold (Fig. 2). The subsequent two treatments with 5-azacytidine, with the animal's hematocrit maintained, by bleeding, in the low 20s, generated two distinct peaks of increments of F cells and HbF (Fig. 2). That the two waves of increased F cells are the result of action of 5-azacytidine alone is suggested by the descending curves of F-cell numbers while the animal was continuously being bled in the period between treatments B and C (Fig. 2). It is of interest that the effect of 5-azacytidine on F-cell numbers following treatment C exactly duplicated the pattern produced after treatment B. Of interest also is that the amount of HbF/F cell did not increase above the level seen following the administration of 5-azacytidine to the mildly anemic animal (compare, in Fig. 2, the peaks of HbF/F cell after treatments A, B, and C) and therefore was not dependent on the magnitude of F-cell response.

Globin biosynthesis was performed on circulating reticulocytes and bone marrow cells of animal 2 at various intervals (Fig. 4). Before treatment, the \( \gamma/\gamma + \beta \) ratio in bone marrow cells and in peripheral blood reticulocytes was 0.01. Following treatment A, the \( \gamma/\gamma + \beta \) ratio in bone marrow cells was 0.05 and 0.02 at 4 and 7 days posttreatment, respectively; it was 0.04 in peripheral blood reticulocytes 7 days posttreatment. Five days following treatment B, the \( \gamma/\gamma + \beta \) ratios were 0.49 and 0.55 in bone marrow and peripheral blood, respectively. These ratios were 0.27 and 0.38, 4 days following treatment C. Since globin biosynthesis was measured only at limited times following treatment, these findings may not reflect the maximum levels reached. The transient nature of the increments in \( \gamma/\gamma + \beta \) ratios strictly parallel changes in F-reticulocyte kinetics, as is apparent from the data presented in Fig. 4.

**F-Reticulocyte Kinetics**

The kinetics of F-cell response were further evaluated with counts of F reticulocytes in animal 2 (Figs. 2

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![Diagram](https://via.placeholder.com/150)
5-aza: Effects on F Cells and E-Progenitors

Fig. 4. Measurements of globin-chain biosynthesis (γ/γ + β) in peripheral blood reticulocytes (upper panel) and in bone marrow cells (middle panel) of animal 2, before and at different intervals following treatments A, B, and C. For comparison, in the lower panel, concurrent measurements of F reticulocytes are shown.

and 4). 5-Azacytidine administration on the mildly anemic animal (treatment A) increased F reticulocytes from 4% to 25%. F-reticulocytes reached a peak value 4 days after the end of treatment A, and they remained at about the same level until posttreatment day 10 (while the animal was at rest). The administration of 5-azacytidine on the severely bled animal (treatment B) evoked an F-reticulocyte response up to 70%. This value was observed 4 days after discontinuation of treatment B. F-reticulocytes decreased, thereafter, to a level of about 10%, while the animal was being bled. A new wave of F reticulocytes, similar in magnitude to that following treatment B, was observed again 4 days after the third treatment (treatment C) was completed.

Of special note are the F-reticulocyte values during the course of treatments B and C, i.e., when the animal was given 5-azacytidine while it was kept severely anemic with continuous bleeding (Figs. 2 and 4). Definite increases were observed at day 5 of treatment (last day of treatment) in both animals, although in treatment B, F reticulocytes might have increased even before that time. The apparent decrease in F reticulocytes in treatment C from days 1 through 4 of treatment was atypical, and it could not be reproduced in another animal treated in an identical manner (data not shown).

Effects on Progenitor Cell Pools

The number of aggregates of 4–8 nucleated erythroid cells (E-clusters), of CFU-E colonies (8–64 cells) and of BFU-E colonies (multilobed colonies or colonies with over 64 cells each at culture days 7–14) were assessed in bone marrow plasma clot and methylcellulose cultures from the two baboons under study. Since we have observed that a significant number of colonies can be obtained in baboon erythroid cell cultures in the absence of exogenous erythropoietin (unpublished observations), cultures done without added erythropoietin were also evaluated.

Figure 5 summarizes results from animal 1. The main findings are: (A) Immediately following 5-azacytidine (1 day after), there is a significant reduction in the number of erythroid clusters and CFU-E colonies; erythroid clusters and CFU-E grown without added erythropoietin (Ep) are most severely affected. (B) Following this reduction stage, a rebound in the number of CFU-E and of E-clusters takes place; this rebound, at least at the time the culture was done, is more pronounced in CFU-E and E-clusters grown under optimal concentrations of erythropoietin. (C) The number of BFU-E colonies is essentially unaf-
fected by treatment with 5-azacytidine. One day after the end of treatment, the number of bone marrow BFU-E is at the pretreatment range; a small rebound in BFU-E colonies was observed in cultures done (in the presence of erythropoietin) 7 days after the completion of treatment.

The results obtained with animal 2 are summarized in Fig. 6. The findings show: (A) Acute and sustained anemia produces a striking increment in the number of E-cluster-forming cells and of BFU-E colonies recovered from cultures with no added erythropoietin. (B) The net effect of 5-azacytidine treatment is a striking reduction in this pool of E-clusters and CFU-E. For example, the total number of E-clusters and CFU-E grown in no added erythropoietin was 1,934/10^5 after bleeding, but they were reduced to 340/10^5 1 day after treatment and to 167/10^5 4 days after treatment (See Fig. 6). The number of erythropoietin-dependent colonies (E-clusters and CFU-E) was 330 and 655/10^5 1 and 4 days after treatment, respectively. (C) 5-Azacytidine treatment did not affect the number of burst-forming units at times that it affected the CFU-E and E-clusters.

**HbF Biosynthesis in Erythroid Cultures**

Table 1 summarizes results of globin biosynthesis in CFU-E-derived colonies and in E-clusters. Pre-5-azacytidine treatment, E-clusters and CFU-E grown on no added erythropoietin produce basal levels of HbF, which were 6% in animal 1 and 5% in animal 2 (post bleeding). With increasing levels of erythropoietin in culture, those levels increase; this finding is typical of normal baboon CFU-E cultures (unpublished observations). A further increase in HbF synthesis, at all doses of erythropoietin used, was characteristic of E-clusters and CFU-E colonies in cultures of bone marrow samples obtained 1 and 7 days post-5-azacytidine treatment (Table 1).

The synthesis of fetal hemoglobin in BFU-E-derived colonies was strikingly high in the unmanipulated animals, and it remained at high levels following bleeding (Table 2). The effect of bleeding plus 5-azacytidine was assessed with measurements of globin biosynthesis in BFU-E colonies produced by plating bone marrow samples obtained 1, 3, and 7 days after the end of 5-azacytidine treatment. There was no difference in HbF synthesis between pretreatment BFU-E and 1 day posttreatment BFU-E colonies (Table 2); a decline in γ/γ + β ratios was observed 3 days after treatment C. The significance of this finding is not clear.

**HbF in Colonies as Assessed by Immunofluorescence**

Several plasma clots, doubly labeled with anti-γ and anti-β-chain antibodies, were evaluated for the

![Fig. 6. Assessment of erythroid clusters (E-clusters), CFU-E, and BFU-E-derived colonies in bone marrow samples from animal 2. Findings in (A) untreated animal; (B) after bleeding but before treatment with 5-azacytidine, and (C) 4 days after cessation of treatment C (bleeding plus 5-azacytidine). (Upper panel) 0.0 IU of erythropoietin/ml of culture; (Lower panel) 0.5 IU of erythropoietin added per ml of culture. Note the increase in both E-clusters and CFU-E after bleeding and their decline following treatment with 5-azacytidine. Changes are more pronounced in colonies grown without added erythropoietin.](image-url)
presence of HbF and HbA using the criteria previously described.\textsuperscript{3,9,10} Colonies were classified as F+ A+ if all their cells contained both HbF and HbA; as A+ F− if they contained only HbA-expressing cells; as F+ A− if they contained cells expressing HbF but not HbA.

**E-clusters and CFU-E.** Evaluation of HbA and HbF in CFU-E colonies and E-clusters was done on culture day 3. At this day, over 95% of the colonies were mature and expressed HbA in their cells. In approximately 5% of colonies, only HbF, but not HbA, was detectable (F+ A− colonies). Such colonies were usually composed of larger cells and were excluded from our analysis because we felt that they represented immature colonies. Since the number of excluded colonies is very small, there is no impact to the conclusions drawn from these evaluations.

In cultures done in the absence of exogenous erythropoietin, from 50 to 200 colonies and clusters were evaluated for immunofluorescence. In the normal, untreated animal (animal 2), 85% of the E-clusters and 54% of the CFU-E colonies contained only HbA-expressing cells (Fig. 7). Following bleeding, the number of HbF-positive E-clusters increased from 15% to 40%, but the number of F-positive CFU-E remained virtually unchanged (Fig. 7). Following 5-azacytidine treatment, the HbF-positive E-clusters increased further to 90%, and HbF-positive CFU-E colonies, which did not change after bleeding, increased to 75%.

In cultures done at optimal concentrations of erythropoietin, 37% of the E-clusters and 70% of CFU-E colonies were positive for HbF (Fig. 7). Apparently, the presence of erythropoietin in culture increases the frequency of HbF-positive E-clusters and CFU-E colonies, in agreement with previous observations by Macklis et al. in rhesus monkeys.\textsuperscript{35} After bleeding, the frequency of HbF-positive E-clusters and CFU-E was identical to that of nonbled animals, i.e., 38% for E-clusters and 72% for HbF-positive CFU-E colonies. After 5-azacytidine, 84% of the clusters and 91% of CFU-E colonies were HbF-positive in cultures with optimal levels of erythropoietin.

**BFU-E Colonies.** Studies of BFU-E colonies labeled with fluorescent antibodies showed that, in the normal baboon, 100% of the erythroid bursts are uniformly positive for HbF. This is in sharp contrast to the findings in the normal human BFU-E cultures, where mixed bursts (i.e., bursts containing subcolonies or sectors with only HbA-expressing cells) predominate.\textsuperscript{39,10} This uniform cellular expression of HbF remained unchanged in the animal made moderately or severely anemic by bleeding or after treatment with 5-azacytidine. These findings are in agreement with the results of globin-chain biosynthesis described in the previous section.

**DISCUSSION**

The results of the present study, as well as those of others,\textsuperscript{39} clearly show that 5-azacytidine requires an expanded erythroid marrow population to bring about its maximum effect on the increase of hemoglobin F synthesis in vivo. Since, in our experiments, repeated courses of 5-azacytidine were administered in the same animal, direct quantitative comparisons of the magnitude of 5-azacytidine action on mildly or severely anemic marrow were possible. In the mildly anemic animal, 5-azacytidine had a minimal effect; for maximum effect not only severe anemia was required, but also, continued bleeding during drug administration was necessary.

Previous studies on 5-azacytidine treatment had placed major emphasis on the induction of DNA hypomethylation by the 5-azacytidine as the major underlying event,\textsuperscript{21,22,25-27} and the kinetics of F-cell production in the 5-azacytidine treated baboons were not well defined. The notion was advanced that 5-azacytidine, at least in some animals, did not change the F-cell numbers, but only the hemoglobin F production per F cell.\textsuperscript{21} In the present study, we focused our attention on cellular events superimposed by 5-azacytidine treatment on a marrow with either basal or stimulated erythropoiesis.

The most significant finding in the present studies was that 5-azacytidine, whether it acted on normal or anemic marrow, affected the size of the erythroid progenitor cell pools. In particular, this effect, at least at the doses and time administered, concerned almost exclusively the late progenitor cell pools (E-clusters and CFU-E). Since late progenitors, either in the normal or the anemic state, remain continuously in cycle,\textsuperscript{36,37} and since 5-azacytidine requires cells with

![Fig. 7. Proportion of HbF-positive colonies in day-3 cultures (plasma clots) after labeling with an anti-γ-globin chain monoclonal antibody. \(\textcircled{C}\) Findings before bleeding; \(\textcircled{C}\) after bleeding: and \(\textcircled{C}\) after 5-azacytidine plus bleeding (4 days after cessation of treatment C). (A) Cultures in the absence of exogenous erythropoietin. (B) Cultures with 0.5 IU erythropoietin/ml.](image-url)
active DNA synthesis for its action, it is not surprising that this population of late progenitors (and perhaps actively proliferating differentiated cells as well) were the target cells for its action. Late progenitors could have been killed because of 5-azacytidine toxicity; alternatively, they could have decreased in numbers because they were drawn to downstream differentiation. If the latter possibility was correct, we would expect to see an increase in reticulocytes during the days of administration of 5-azacytidine; instead, a decrease was observed both in the normal and in the anemic animal. Thus, the most likely interpretation for the reduction in late progenitor cell numbers is their killing by 5-azacytidine. Although this reduction in late progenitor pool size was documented 1 and 4 days after the end of treatment, we presume that it is an ongoing process during the treatment course. Seven days after the cessation of treatment, we demonstrated a “rebound” of late progenitors, reflecting the accumulation of regenerating progenitors once the toxic insult was no longer present.

The reduction in the size of the late pools in the face of continuing erythropoietic demands is expected to create a faster mobilization or recruitment of earlier progenitors to the next more mature compartment. What would be the consequences of this shift in progenitor cell pools? We have previously postulated that such kinetic perturbations in vivo are responsible for the transient elevations of hemoglobin F observed after bleeding or in bone marrow recovery in patients with transient erythroblastopenia of childhood.12 The effect is transient until the late progenitor pools are reconstituted and expanded, at which time there is no (or there is minimal) further hemoglobin F production, because the expanded late pools can meet the demands of greater red cell production. This concept would explain why there is no continuing increase in F cells after repeated phlebotomies. The F-cell kinetic data in our two baboons treated with 5-azacytidine are compatible with this sequence of events. First, we have clearly shown, in contrast to previous studies,21 that 5-azacytidine in the normal and the anemic animal elicited a wave of F cells and of F reticulocytes at a time when their late progenitor pools were insulted, as shown by culture studies and by the reduction in reticulocytes during the treatment period. This wave of F-reticulocyte production was virtually over when the late progenitor pools were reconstituted despite the fact that continuing regeneration was taking place because of the demands imposed by bleeding. However, it is impossible, on the basis of the present studies alone, to decide to what extent, this wave of F-cell response is due to precursor pool kinetic changes or to a direct effect of 5-azacytidine (presumably by hypomethyl-
dependent not only on the dose but also on the mode of drug administration (i.e., continuous versus intermittent, fusion, i.v. versus s.c. route, etc.).

There are several other aspects of this study that require comment. Early erythroid progenitors of the BFU-E type appear to remain unaffected by the doses of 5-azacytidine used in our experiments. Since these progenitors, by and large, are not cycling normally (or they are only partially or transiently cycling after marrow regeneration), it is not surprising that these early progenitors would be the least affected. In the study reported by Ley et al., it was observed that the methylation pattern returned to normal very soon after 5-azacytidine discontinuation; this observation is compatible with the notion that early progenitors were not affected by 5-azacytidine. Nevertheless, it is anticipated that if the drug is given continuously these precursors will eventually be affected.

It has been previously reported that, after bleeding and hypobaric hypoxia or bleeding and 5-azacytidine treatment in baboons, the numbers of F cells increase first, and they reach a maximum before any change is observed in the amount of HbF/F cell, implying that the mechanism responsible for the increase in HbF/F cell operates only after maximum F-cell levels are achieved. In contrast, the results of the present study indicate that the increment in HbF/F cell is independent of the magnitude of F-cell response; both parameters (i.e., F cells and HbF/F cell) increase at the same time. This finding is interpreted to indicate that the mechanism that increases F-cell numbers and HbF/F cell may be common, but the abundance of the two parameters may vary independently and be related to the type of manipulations used (i.e., 5-azacytidine or bleeding), the magnitude of stress, or the ability of bone marrow precursor pools to meet the erythropoietic demand imposed.

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Perturbations in the erythroid marrow progenitor cell pools may play a role in the augmentation of HbF by 5-azacytidine

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