# Influence of Cell Cycle Phase-specific Agents on Simian Fetal Hemoglobin Synthesis

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## Abstract

**To determine the influence of cell cycle-specific agents on primate hematopoiesis and fetal hemoglobin production, two juvenile cynomolgus monkeys (*Macaca fascicularis*) were repeatedly bled to maintain their hemoglobins at ~6.5 g/dl and fetal hemoglobin levels at 3–5%. Six separate 5-d courses of hydroxyurea at 100 mg/kg per d were then administered over the next 200 d while phlebotomy was continued. These courses of hydroxyurea progressively raised the fetal hemoglobin levels to 17 and 18%, respectively. The drug had very little effect on the frequency of immature erythroid progenitors (BFU-E) in the bone marrow, but caused a marked reduction in the frequency of later progenitors (CFU-E) and a transient fall in the reticulocyte count. Following the courses of hydroxyurea, the number of F cells and the fetal hemoglobin level fell to base line over a period of 4 wk. Two control animals which were not phlebotomized showed no detectable increase in F cells or fetal hemoglobin when treated with the same regimen of hydroxyurea.**

**A 5-d course of 5-azacytidine at 8 mg/kg per d was then given to each of the phlebotomized animals. This produced a more profound, albeit transient, reticulocytopenia, a fall in the CFU-E/BFU-E ratio, and a prompt increase in the fetal hemoglobin to levels even higher than were seen following a single 5-d course of hydroxyurea at 100 mg/kg/d. Subsequently, the animals were given a single dose of vinblastine at 0.4 mg/kg which reduced reticulocytes and CFU-E to the same extent as hydroxyurea; however, vinblastine at this dose had no effect on hemoglobin F (HbF) production. In contrast, when vinblastine was administered to the phlebotomized monkeys as a 5-d course at 0.2 mg/kg/d, prolonged reticulocytopenia followed by dramatic F cell and HbF responses were seen. Combinations of single dose vinblastine and a 5-d course of hydroxyurea were subsequently administered using two different schedules. When the animals received vinblastine on the first day of a 5-d course of hydroxyurea, the F cell response was double that seen following hydroxyurea treatment alone. In contrast, when vinblastine was administered on the final day of hydroxyurea treatment, the magnitude of the F cell response was the same as that which occurred following hydroxyurea treatment alone, but the onset of the rise was delayed for 4 d and HbF/F cell response was much higher.**

**These results establish several important features of the fetal hemoglobin response to cytotoxic agents in the primate model. The response requires accelerated erythropoiesis and is preceded by transient reticulocytopenia. The response is produced by S phase- and M phase-specific agents when given in sufficient doses and at appropriate schedules. Passage of erythrocyte progenitors through M phase appears to be necessary for expression of the effect produced by S phase agents. The fetal hemoglobin response induced by cytotoxic drug administration occurs during the recovery of erythropoiesis following marrow suppression.**

## Introduction

We have previously reported that a single course of hydroxyurea at 100 mg/kg per d for 5 d markedly increases fetal hemoglobin production in *Macaca fascicularis* with hemorrhagic anemia (1). In that report and elsewhere, we (2) and others (3) suggested that the increase in fetal hemoglobin induced by hydroxyurea and other cell cycle-dependent, phase-specific cytotoxic drugs might be due to preferential destruction of mature erythroid progenitor cells (CFU-E)[1] and precursors.[2] This would be followed by replacement of the marrow erythroblast pool with cells immediately derived from less mature progenitors in which the fetal hemoglobin synthesis program is retained (4). This proposed switch of the origin of erythroid precursors failed, however, to account for the very rapid increment of fetal hemoglobin and F cells and the lack of reticulocytopenia following treatment of experimental animals and patients with such agents (5–7).

Here, we examine the effects of cell cycle-dependent, phase-specific drugs on HbF production in a simian model in more detail. First, we evaluated the effects of repeated courses of hydroxyurea, an S phase-specific drug, on fetal hemoglobin synthesis in order to define the potential for its practical application in the treatment of patients with hemoglobinopathies. Second, we measured the impact of hydroxyurea on reticulocyte production and on CFU-E and immature erythroid progenitors (BFU-E) and compared these effects to those exerted by 5-azacytidine, another S phase-specific agent. Finally, we investigated the relationship of the cell cycle-phase specificity of drugs to their influence on fetal hemoglobin production by evaluating the effects of vinblastine, an inhibitor of mitosis active in the M, rather than the S phase of the cell cycle. The results of these experiments show that, in this simian model,

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1. *Abbreviations used in this paper:* BFU-E, immature erythroid progenitors; CFU-E, mature erythroid progenitors.
2. The term precursors describes recognizable nucleated erythroid cells whereas progenitors are morphologically unrecognizable blast cells committed to development into precursors.

transient reduction of CFU-E and depression of reticulocyte production by cytotoxic agents does not itself lead to stimulation of fetal hemoglobin production. Optimal response is schedule-dependent requiring a course of daily administration of S or M phase-specific drug for several days and occurs only in animals with accelerated erythropoiesis. The response is regularly associated with recovery of reticulocytosis following its suppression by drug administration.

## Methods

*Animal care.* The juvenile *M. fascicularis* used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (8). Two animals were regularly phlebotomized to maintain hemoglobins at ~6.5 g/dl and received parenteral cyanocobalamin, folic acid, and iron dextran as previously described (2). Two additional animals were maintained as above but were not bled.

Courses of hydroxyurea were administered via a nasogastric tube under ketamine anesthesia as single doses daily for up to 5 d. 5-Azacytidine was administered intramuscularly as a single dose daily for 5 d. Vinblastine was administered as an intravenous bolus dose for either 1 or 5 d.

*Bone marrow samples.* Bone marrow was obtained from each animal on several occasions. Great care was taken to minimize peripheral blood contamination of these samples. Under ketamine anesthesia, a long bone in the leg was exposed and a hole drilled into the bone using an orthopedic drill. A preheparinized catheter was then fed into the marrow cavity and 1–2 ml of marrow aspirated from three separate sites. Any aspirate in which there were fewer than 20,000 total nucleated cells/$\mu$l of marrow sample was discarded.

Each sample was diluted in tissue culture medium and low density cells obtained by centrifugation over Ficoll-Hypaque (specific gravity 1.077). The low density cells were washed three times, counted, and examined under the microscope. The vast majority of nucleated cells were erythroblasts, but many reticulocytes were also present. The low density marrow cells were therefore subjected to a second centrifugation on Ficoll-Hypaque to reduce the number of erythroblasts and reticulocytes in the marrow samples. The separate samples from the same animal were then pooled to reduce potential cell population differences between different samples.

*Routine hematological investigations.* At each phlebotomy, an initial blood specimen was used for measurement of the hemoglobin and leukocyte count on an automated cell counter. Differential counts were performed on freshly made smears. Reticulocyte counts were made after vital staining with new methylene blue. Measurements of F cells and total HbF were performed as previously described (1).

*Erythroid progenitor cell assays.* To investigate the effects of drugs on mature erythroid progenitor cells, the ratio of CFU-E/BFU-E in $10^5$ cells was established. This obviated inherent variability of the recovery of marrow low density cells, a variability that would be expected to be particularly noticeable following the administration of cytotoxic drugs. CFU-E were cultured in a plasma clot assay, as previously described (9), using 1 U/ml of Step III Connaught erythropoietin. The CFU-E-derived colonies were scored on day 3. The plasma clot assay was utilized because its plating efficiency for CFU-E-derived colonies was three to five times that of the methyl cellulose assay. BFU-E were cultured in a methylcellulose assay (10), again using 1 unit/ml of erythropoietin. BFU-E-derived colonies were scored at day 10. For detection of the colonies derived from these progenitors, the methyl cellulose assay proved distinctly superior, providing more and larger colonies than those observed in the plasma clot assay. Low density bone marrow cells were plated at different cell concentrations ranging between $1.24 \times 10^4$ and $2.5 \times 10^5$/ml to ensure that a maximal cloning efficiency was obtained. In general, optimal growth was obtained between $1.5 \times 10^4$ cells and $2 \times 10^5$ cells/ml in the plasma clot assay, and $2.5 \times 10^4$ cells and $1 \times 10^5$ cells/ml in the methylcellulose assay.

## Results

*Effects of hydroxyurea in unbled animals.* To evaluate whether hydroxyurea has a direct effect on fetal hemoglobin production in the nonanemic state, the drug was administered daily at a dose of 100 mg/kg per d for 5 d to two unbled monkeys with completely normal hematopoiesis. No effect on fetal hemoglobin or F cell production was detected (data not shown).

*Effects of phlebotomy.* Repeated phlebotomy totalling ~100 ml/wk maintained the hemoglobin at ~6.5 g/dl in two monkeys throughout the nearly 300 d of this study. In the absence of drug administration, this caused a reticulocytosis of ~18% in animal I and 20% in animal II (Fig. 1). These base-line values rose to ~24 and 30%, respectively, as the study progressed (Fig. 1).

*Fetal hemoglobin production by BFU-E-derived colonies.* To determine the potential fetal hemoglobin program of primitive erythroid progenitor cells in this simian species, peripheral blood BFU-E were cultured in methylcellulose. This was done after phlebotomy had been started, but before any drug therapy commenced. BFU-E-derived colonies were plucked at 12 d when they appeared well hemoglobinized, and a lysate of the pooled colonies was subjected to electrophoresis in a Triton-urea gel. Densitometry of the stained gel revealed that the percentage of fetal hemoglobin comprised 39% of the total hemoglobin in the BFU-E-derived colonies of animal I and 56% with respect to animal II (data not shown). These values are very much higher than those found in man (11), but are similar to those described previously in the Rhesus monkey (4) and in the baboon (12).

*Effects of the initial doses of hydroxyurea on fetal hemoglobin production.* As previously reported (1), hydroxyurea administration was instituted 62 d after regular phlebotomies

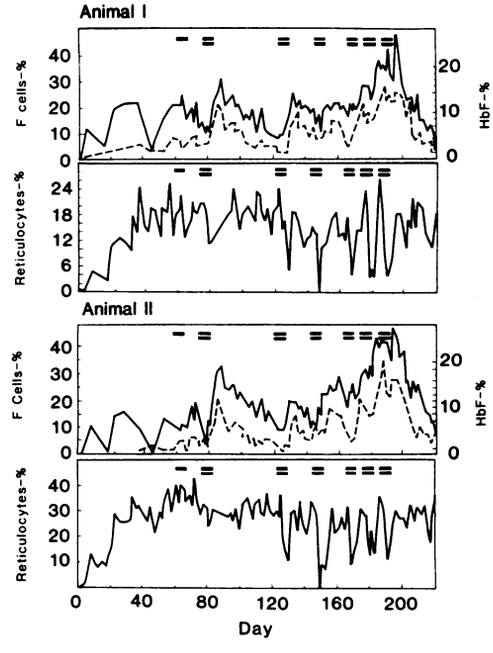


*Figure 1.* Effects on animals I and II of repeated 5-d courses of hydroxyurea at either 50 (——) or 100 (═══) mg/kg per d on the reticulocyte count, fetal hemoglobin, and F cell responses.

were begun (Fig. 1). The first course of hydroxyurea at 50 mg/kg for 5 d had no significant effect on either animal nor did a single dose of 100 mg/kg. However, the effects of hydroxyurea at 100 mg/kg per d for 5 d on both animals were remarkably similar. Within 2 d of completion of the course of drug, the F cells rose to 34% in animal I and 30% in animal II, while the fetal hemoglobin level rose to 14 and 12%, respectively. Following this response, F cells and percentage of fetal hemoglobin returned to base-line values over a period of 3 to 4 wk.

*Effects of repeated 5-d courses of hydroxyurea at 100 mg/kg per d.* 48 d after the first 5-d course of 100 mg/kg per d of hydroxyurea, a further similar course was given and a rapid fetal hemoglobin response was again observed (Fig. 1). The maximal values obtained were a little less than with the previous course but the kinetics of the response were similar.

Four additional 5-d courses of hydroxyurea at 100 mg/kg per d separated by 22, 20, 11, and 10 d were then administered. The last three courses were given as soon as the neutrophil count recovered to above 700 per cubic millimeter. With each of the courses of hydroxyurea, there was a prompt fetal hemoglobin response, and the rapidly repeated courses of therapy led to an incremental rise in the maximum F cell and percentage fetal hemoglobin values obtained. Following the final course of hydroxyurea, peak F cell values of 44% in animal I and 49% in animal II were obtained with percentage fetal hemoglobin values of 18 and 16%, respectively. The percentage of F reticulocytes (kindly measured by Dr. George Dover, Johns Hopkins University and Hospital, Baltimore, MD) had risen to ~70% in both animals at the time the peak F cell response was seen. A gradual fall of F cells to the base-line levels induced by bleeding occurred after the final course was completed.

*Effect of hydroxyurea on other hematological parameters.* The 5-d hydroxyurea courses at 100 mg/kg per d led to an immediate fall in the neutrophil count, often apparent by the third day of therapy. Nadirs of 300 granulocytes/$\mu$l were occasionally observed, but it must be noted that the neutrophil count in this species is lower than that of humans and varies greatly from day to day. Equally low neutrophil values were observed sporadically during the control phlebotomy period. Neutrophil recovery to the desired 700 per microliter was also variable, occurring between 7 and 30 d following cessation of treatment. There were no infectious complications throughout the period of this study and both animals maintained their body weights.

The hydroxyurea therapy also caused a significant fall in the reticulocyte count. This fall was immediate and of very brief duration (Fig. 1), and was associated with a decreased phlebotomy requirement to maintain a stable hematocrit. The recovery of the reticulocyte count was always accompanied by a rise in F cells and HbF.

*Establishing dose equivalence.* It is important to understand that comparisons between drugs in a model such as this are always complicated by the necessity to establish some measure of dose equivalence. We have utilized the degree of reticulocyte suppression for this purpose since it is a direct index of toxicity to the erythroid system. In our experience, myelosuppression was an unreliable indicator of drug effects since the neutrophil count was highly variable from day to day and neutropenia did not occur with any consistent pattern in relation to drug administration. No animal became severely neutropenic during these studies. When necessary, this dose equivalence was established by carrying out preliminary experiments in these animals, escalating doses until reticulocyte suppression equivalent or greater than that produced by the standard 5-d course of hydroxyurea was attained.

*Effect of 5-azacytidine on fetal hemoglobin production.* When both animals were at base-line F cell and percent fetal hemoglobin values 38 d after the final dose of hydroxyurea, a 5-d course of 5-azacytidine at 8 mg/kg per d was administered. The results are shown in Fig. 2 *A*. The responses induced by this schedule of 5-azacytidine administration were similar to those seen following a 5-d course of hydroxyurea, though the peak values were even higher and reticulocyte suppression more profound. A maximum F cell response of 37% and fetal hemoglobin level of 18% were observed and the trough of reticulocyte suppression lasted about twice as long as that induced by hydroxyurea. The granulocyte count and bleeding requirement also transiently declined (data not shown).

*Perturbations of the progenitor cell pools following treatment with hydroxyurea and 5-azacytidine.* The demonstration that, in this species of monkey, erythroid colonies derived from early progenitors contain large amounts of fetal hemoglobin is in accord with the hypothesis that the marked fetal hemoglobin response induced by S phase-specific cytotoxic drugs could be due to toxicity of these drugs directed toward rapidly replicating late progenitors and proerythroblasts and subsequent derivation of erythroblasts from more slowly replicating immature erythroid progenitors. To ascertain whether such differential cytoreduction did occur as a result of administration of hydroxyurea and 5-azacytidine, bone marrow was cultured to determine the frequencies of CFU-E- and BFU-E-derived colonies before and after each of two separate courses of hydroxyurea treatment and the single course of 5-azacytidine administration. The changes in ratios of CFU-E/BFU-E are shown in Fig. 3. Similar progenitor cell perturbations were produced by both drugs, a marked depression of CFU-E relative to BFU-E. The changes in CFU-E/BFU-E ratios were very similar, though more pronounced, than those detected by Torrealba-de Ron and coworkers (3) in this setting.

*Effects of vinblastine.* Since both hydroxyurea and 5-azacytidine exerted similar influences on erythropoiesis and HbF production, we chose to examine whether such effects were characteristic of other cell cycle-dependent agents. We therefore decided to study the effects of vinblastine, a drug which is active in the M phase rather than the S phase of the cell cycle (13). More than 4 wk after any previous treatment, and with the F cells and HbF at base line, the animals received 0.4 mg/kg of vinblastine as a single dose. Though all of the erythrokinetic effects of vinblastine were identical to those exerted by hydroxyurea, including the fall in reticulocyte production and marrow CFU-E to BFU-E ratio (Fig. 3 and Table I), there was no response of either F cell production or fetal hemoglobin in either animal (Fig. 2 *B*). We then repeated a 5-d course of hydroxyurea in both animals to be certain that they remained capable of a fetal hemoglobin response. The results (Fig. 2 *C*) demonstrated that the capacity for a fetal hemoglobin response to hydroxyurea administration remained intact. This series of experiments was subsequently repeated in its entirety and gave identical results (data not shown).

To approximate the dose schedule employed with 5-azacytidine and hydroxyurea more closely, vinblastine was given at a dose of 0.2 mg/kg per d for 5 d. This treatment produced a reticulocytopenia in both animals which persisted for 8 d. 9

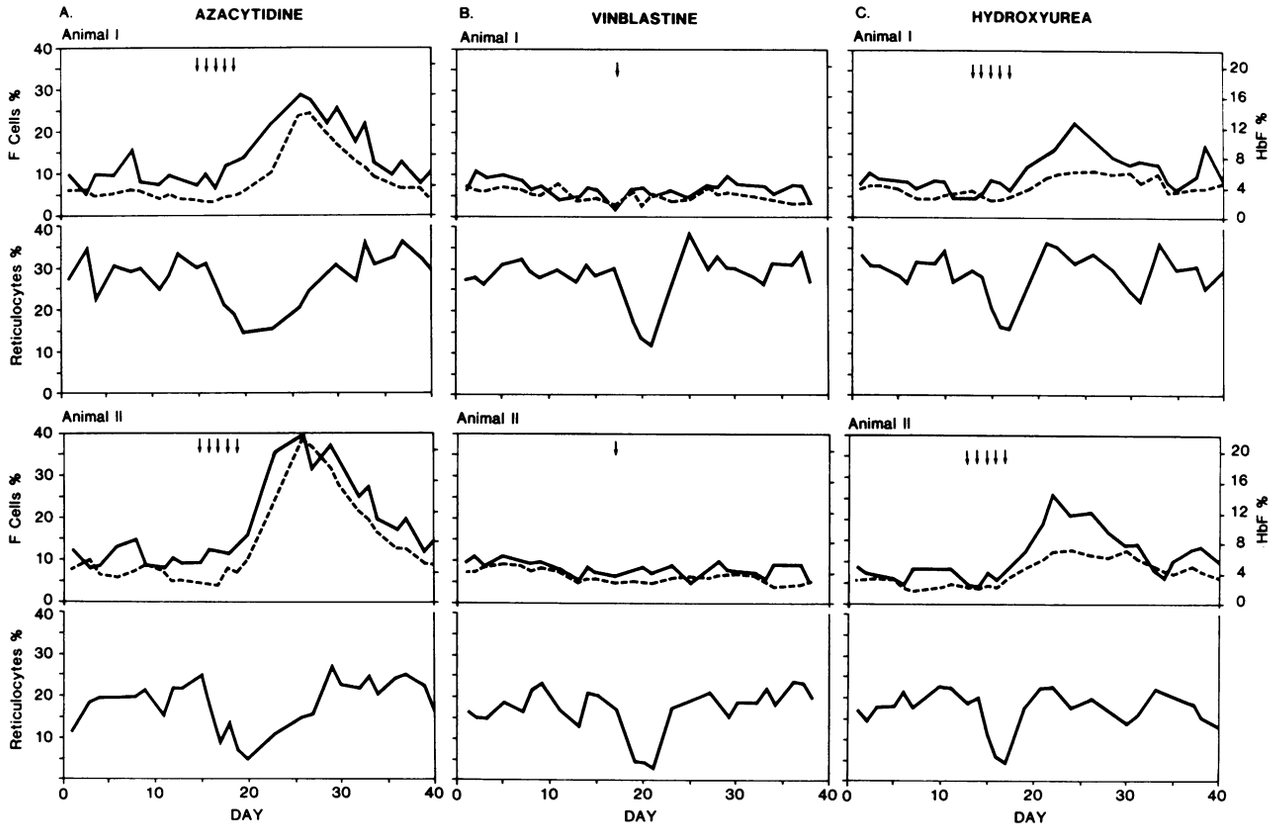


*Figure 2.* (*A*) Effects in animals I and II of a 5-d course of 5-azacytidine at 8 mg/kg per d intramuscularly (↓) on the reticulocyte counts, fetal hemoglobin (– – –), and F cell (——) responses. The course was given 42 d after the last course of hydroxyurea that is shown in Fig. 1. (*B*) Effects in animals I and II of a single dose of vinblastine at 0.4 mg/kg intravenously (↓). This dose was given after several preliminary trials with lower dosages to establish an equivalent reticulocyte suppression dose. At least 21 d elapsed between each successive trial. (*C*) Effects in animals I and II of a 5-d course of hydroxyurea at 100 mg/kg per d (↓). This course was given 36 d after the dose of vinblastine that is shown in (*B*). Note that the scale for percent fetal hemoglobin for all three panels is shown at the extreme right of the figure and that for the percent F cells is at the extreme left.

d after completion of the last dose, F cells rose from 3 to 21% and HbF from 2 to 12% in animal I (Fig. 4). The increase was less impressive in animal II in which inadvertent extravasation of vinblastine into subcutaneous tissues occurred. The increase in F cell response persisted for days in both experimental animals.

These findings prompted an examination of vinblastine in combination with hydroxyurea. Two different schedules of drug administration were employed (Fig. 5). In the first experiment, vinblastine was given concurrently with the first dose of a 5-d course of hydroxyurea (Fig. 5 *A*). The magnitude of the F cell response was markedly augmented in comparison to that observed with hydroxyurea alone (Fig. 2 *C*). There was also augmentation of the fetal hemoglobin response although this was less pronounced. In a second separate experiment, performed after full recovery from the first, each animal was given vinblastine on the final day of the 5-d course of hydroxyurea (Fig. 5 *B*). Reticulocytopenia was severe and prolonged. The F cell response was no greater than that produced by hydroxyurea alone but the onset and peak of the response were delayed markedly. They occurred 4–5 d later than following administration of either hydroxyurea or 5-azacytidine alone. Furthermore, the HbF per F cell was considerably higher when vinblastine was given at the end rather than at the beginning of the hydroxyurea course.

Finally, single doses of hydroxyurea and vinblastine were administered concurrently. Whereas a single dose of hydroxyurea had no effect, the combination did. A peak F cell response occurred 8 d later in both animals, rising from a base line of ~2 to 12% in animal I and to 8.2% in animal II. These data (not shown) give some sense of the time required for development of the F cell response and also suggest that the two agents may act synergistically.

## Discussion

The experiments reported here define a number of important characteristics of the induction of the F cell/HbF response with cytotoxic drugs in this primate model. The results provide several important clues as to the possible mechanism(s) by which the effect is produced and suggest important avenues of further investigation.

First, the effect appears to require an expanded and active erythroid compartment. No detectable rise in F cell number or fetal hemoglobin was seen in nonanemic animals using the same dose and schedule of hydroxyurea which produced a strong response in the phlebotomized animals with accelerated erythropoiesis. In addition, the effect is produced by agents which are active during the S or M phases of the cell cycle as exemplified in these studies by hydroxyurea, 5-azacytidine, and vinblastine (Beardsley, G. P., M. M. Klaus, and N. L. Letvin, manuscript in preparation). Two additional S phase agents, cytosine arabinoside (14) and 5-azadeoxycytidine (15),

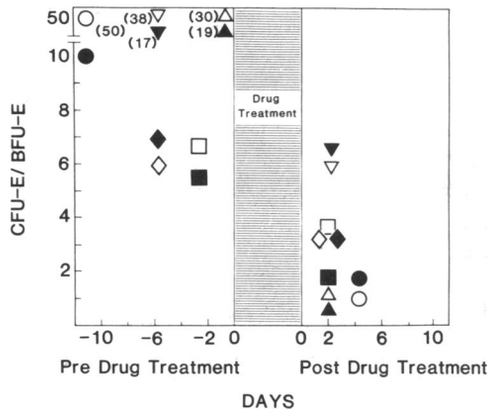


*Figure 3.* Effects of two separate 5-d courses of hydroxyurea, one 5-d course of 5-azacytidine, and a single dose of vinblastine on the ratios of CFU-E to BFU-E in the marrow cells of animals I and II. The abscissa represents the days before and after the single dose or the 5-d course during which the progenitor measurements were made. The symbols represent the following: ●, ○, third hydroxyurea course, 100 mg/kg per d *per os* for 5 d to animals I and II, respectively; ■, □, fourth hydroxyurea course, 100 mg/kg per d *per os* for 5 d to animals I and II, respectively; ♦, ◇, 5-azacytidine 8 mg/kg per d subcutaneously for 5 d to animals I and II, respectively; ▲, △, vinblastine, 0.4 mg/kg i.v. single dose to animals I and II, respectively; ▼, ▽, repeat of vinblastine course.

have each been reported to produce increases in F cells and total fetal hemoglobin very similar to those reported here. Dose and schedule of administration of drugs are obviously critical. Neither a single dose of the hydroxyurea at 100 mg/kg nor a 5-d course at 50 mg/kg were effective. Our studies of vinblastine not only emphasize the critical influence of dose and schedule but also direct our attention to the mode of action of these drugs on the HbF response. Vinblastine given as a single intravenous bolus at a dose that is at least twice the highest dose usually recommended for humans had an effect on erythropoiesis that was identical to that observed after 5 d of hydroxyurea administration at 100 mg/kg per d. There was a prompt decrease in reticulocyte production and in the frequency of CFU-E in the marrow, but, in contrast to the effects of hydroxyurea, there was no discernible influence on F cell production during the recovery phase.

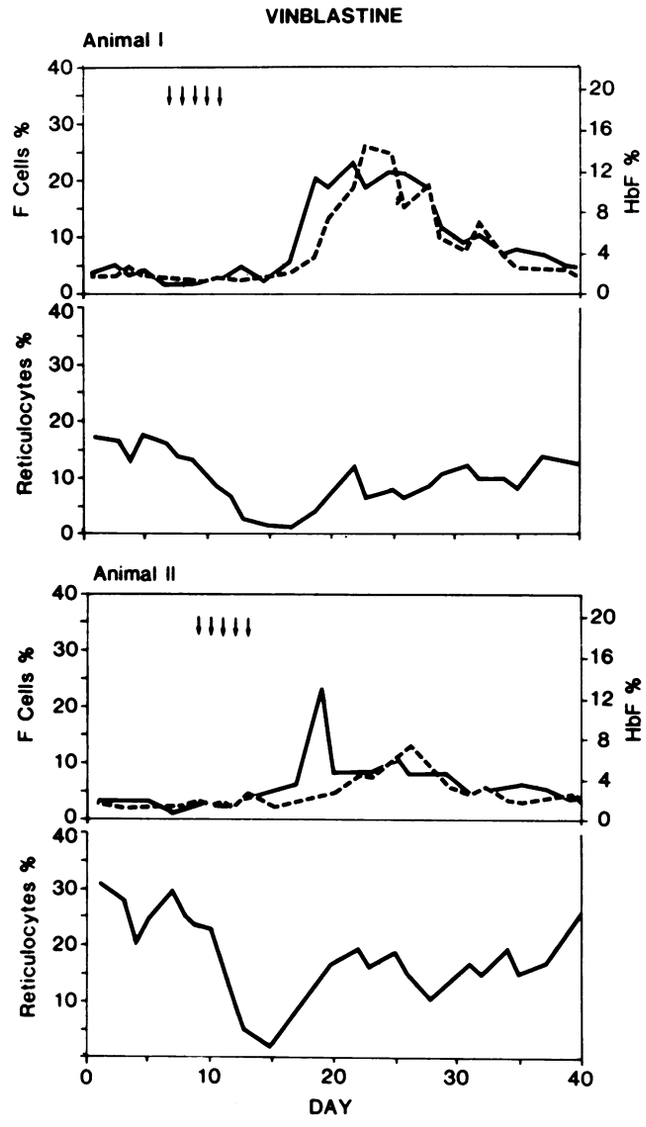


*Figure 4.* Effects on the reticulocytes, fetal hemoglobin (– – –), and F cell responses (——) of animals I and II given a 5-d course of vinblastine at 0.2 mg/kg per d i.v. as a single bolus. Extravasation damage at the site of intravenous injection was noted at day 5 in animal II.

*Table I. CFU-E- and BFU-E-derived Colonies Pre- and Posttreatment with Hydroxyurea, 5-Azacytidine, and Vinblastine*

| | Erythroid progenitor-derived colonies/$10^5$ nucleated cells | | | | | |
|---|---|---|---|---|---|---|
| | Animal A | | | Animal B | | |
| Treatment | Day 3 CFU-E (PC) | Day 10 BFU-E (MC) | CFU-E/BFU-E | Day 3 CFU-E (PC) | Day 10 BFU-E (MC) | CFU-E/BFU-E |
| 2 d before hydroxyurea | 1150 | 168 | 6.8 | 1050 | 180 | 5.8 |
| 2 d after hydroxyurea | 603 | 168 | 3.6 | 562 | 304 | 1.8 |
| 6 d before 5-azacytidine | 1362 | 229 | 5.9 | 1324 | 195 | 6.8 |
| 2 d after 5-azacytidine | 376 | 102 | 3.7 | 458 | 143 | 3.2 |
| 1 d before vinblastine | 1954 | 65 | 30.1 | 2382 | 127 | 18.8 |
| 2 d after vinblastine | 295 | 212 | 1.4 | 457 | 320 | 1.4 |

CFU-E were cultured in plasma clot (PC) at $1.0 \times 10^5$ marrow low density cells/ml and BFU-E were cultured in methylcellulose (MC) at $2.5 \times 10^4$ marrow low density cells/ml. The standard deviations of the colony frequencies are 1–10% for CFU-E and 10–15% for BFU-E. Each treatment is separated from the previous one by at least 3 wk.

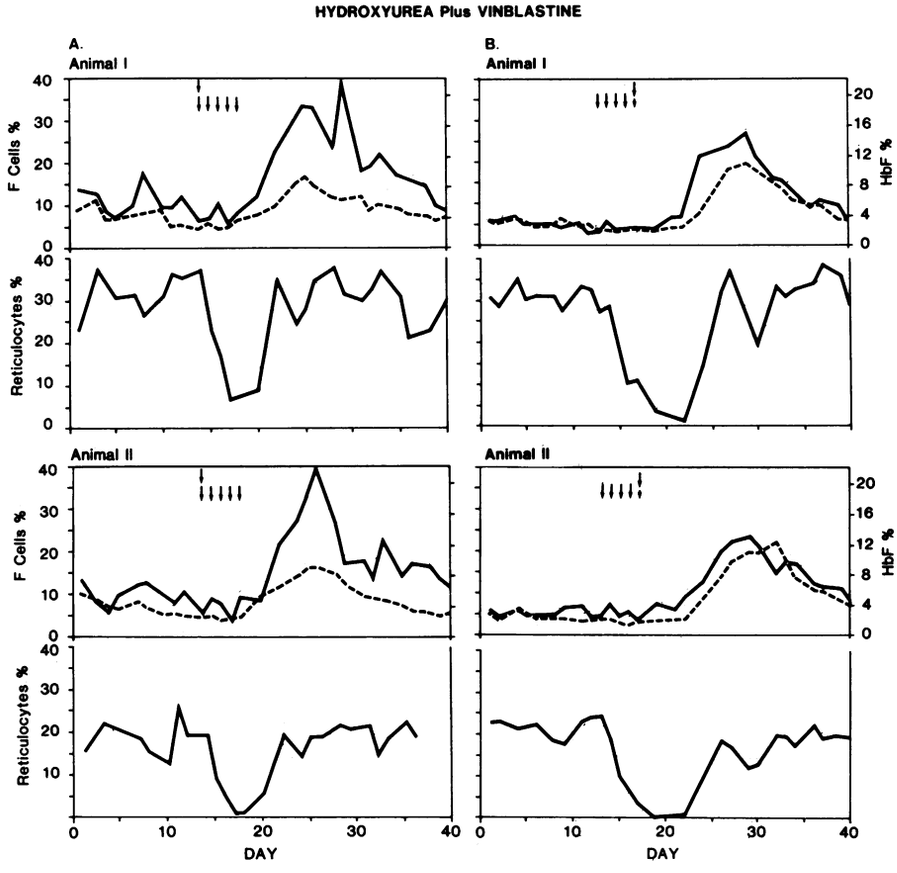


*Figure 5.* Effects on the reticulocytes, fetal hemoglobin (- - -), and F cell responses (——) of animals I and II given a 5-d course of hydroxyurea at 100 mg/kg per d together with a single dose of vinblastine at 0.4 mg/kg i.v. given on the first day of hydroxyurea (*A*) and on the final day of hydroxyurea (*B*). The courses of treatment were separated by 4 wk.

These data demonstrate that limited doses of cytotoxic drugs that transiently arrest reticulocyte production and renewal of late erythroid progenitors do not necessarily produce an F cell response. More severe and prolonged inhibition of reticulocyte production and late progenitors seems to be required. This conclusion is supported by the fact that whereas a single large dose of vinblastine was without effect on the F cell response, a 5-d course of vinblastine at a standard daily dose did induce an F cell response during the recovery phase. Furthermore, a single dose of vinblastine in combination with a single dose of hydroxyurea also produced measurable stimulation of F cell production though neither were effective alone. Further evidence that the length and severity of drug-induced depression of erythroblast (and hence, reticulocyte) production from mature progenitors predicts the extent to which F cells will emerge during recovery in this model was gathered from studies of combinations of a single bolus of vinblastine with a 5-d course of hydroxyurea. The combination was more effective than either drug alone. The synergistic effect was particularly interesting when vinblastine was given at the end of a 5-d course of hydroxyurea. Though the onset of recovery of reticulocytes and F cells was retarded, the vinblastine-induced delay of entry into the M phase led to substantially higher accumulations of HbF in the F cells that were ultimately produced. Perhaps the enforced delay permitted the accumulation of larger amounts of gamma messenger RNA in the recovering erythroid cells.

Though a single unifying theory of the mode of action of these cytotoxic drugs on HbF and F cell production does not emerge from these studies, certain tentative conclusions can be drawn. First, it is certain that direct or indirect cytotoxicity must induce the F cell and HbF responses observed here. The S phase-specific agents may cause temporary arrest of reticulocyte production from proerythroblasts and indirectly lower the frequency of late erythroid progenitors by inducing their rapid differentiation into the proerythroblast pool. This "death by differentiation" (16) was observed in erythroid cell lines by Ebert and co-workers (17) and in hydroxyurea-treated mice by Rencricca and co-workers (18). Pretreatment with vinblastine just before a course of hydroxyurea as performed in one experiment described here would be expected to be particularly effective because of the known capacity of vinca alkaloids to induce a state of S phase synchronization in vitro (19) and in vivo (20). On the other hand, recovery from direct cytotoxicity toward CFU-E and precursors is equally effective as we (2) and others (3) have initially proposed. This conclusion is supported by the results of a 5-d if not a 1-d course of vinblastine, a drug with no known capacity to induce differentiation. This direct toxicity of S and M phase-specific cytotoxic agents would be expected to diminish selectively the number of CFU-E and erythroblasts because the rates of cycling in these compartments, particularly during exposure to elevated levels of erythropoietin, is higher than those in the immature progenitor compartments (21, 22). The result of this direct or indirect toxicity toward the mature compartment would be transient reticulocytopenia followed by erythroblast and reticulocyte production derived from immature progenitors initially spared from the effects of the drugs. Since the fetal hemoglobin program expressed in the colonies derived from immature progenitors is relatively high, particularly in primates (4), increased fetal hemoglobin would follow as a consequence of the compensatory wave of erythroblasts derived from them.

Though transient reticulocytopenia regularly observed in this animal model is often not seen in patients with hemoglobinopathies who have been treated with these drugs, other differences such as effective dose and splenectomy effects on reticulocyte life span may be operative. Finally, it remains possible that S phase-specific drugs might cause gamma globin gene amplification. Indeed, exposure of certain cell lines to hydroxyurea can lead to gene amplification in a small subset of cells (23). Dr. Stuart Orkin, Harvard Medical School, Boston, MA, has kindly examined the gamma globin region of the erythroblast DNA of these treated animals by restriction enzyme analysis and did not observe such amplification, but a small increase would be below the limits of detection in that system.

The stimulation of fetal hemoglobin production by cytotoxic drugs administered in vivo is the object of much interest because of obvious potential therapeutic implications, but it is important to recognize that it is not an isolated response. Hemoglobin A production decreases in concert with the rise in fetal hemoglobin. In short, a reversal of the fetal switch is seen. It is highly likely that other fetal erythroid characteristics will be observed as well, as is the case in "stress erythropoiesis" (24). If such treatment is clinically successful, any of the effects on erythropoiesis may be responsible. The fetal hemoglobin response may simply be a marker and not the clinically effective result.

As these agents are carefully studied, it is very likely that their mechanisms of action, appropriate doses and scheduling, and the contributions of important interacting drugs will be determined. Evaluations of these treatments in simian models, as reported here and elsewhere (3, 4, 25), are likely to provide important new concepts that will enhance their useful applications in the management of certain hemoglobinopathies, but determination of appropriate methods of administration will require cautious human trials.

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## References

1. Letvin, N. L., D. C. Linch, G. P. Beardsley, K. W. McIntyre, and D. G. Nathan. 1984. Augmentation of fetal-hemoglobin production in anemic monkeys by hydroxyurea. *N. Engl. J. Med.* 310:869–873.

2. Nathan, D. G. 1983. Regulation of erythropoiesis and hemoglobin F production. *In* Globin Gene Expression and Hematopoietic Differentiation. G. Stamatoyannopoulos, and A. W. Nienhuis, editors. Alan R. Liss, Inc., New York. 1:365–378.

3. Torrealba-de Ron, A. T., T. H. Papayannopoulou, M. S. Knapp, M. F. Fu, G. Knitter, and G. Stamatoyannopoulos. 1984. Perturbations in the erythroid marrow progenitor cell pools may play a role in the augmentation of HbF by azacytidine. *Blood.* 63:201–211.

4. Macklis, R. M., J. Javid, J. M. Lipton, M. Kudisch, P. K. Pettis, and D. G. Nathan. 1982. Synthesis of hemoglobin F in adult simian erythroid progenitor-derived colonies. *J. Clin. Invest.* 70:752–761.

5. Ley, T. J., J. DeSimone, N. P. Anagnou, G. H. Keller, R. K. Humphries, P. H. Turner, N. S. Young, P. Heller, and A. W. Nienhuis. 1982. 5-Azacytidine selectively increases γ-globin synthesis in a patient with $B^+$ thalassemia. *N. Engl. J. Med.* 307:1469–1475.

6. Charache, S., G. Dover, K. Smith, C. C. Talbot, Jr., M. Moyer, and S. Boyer. 1983. 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. *Proc. Natl. Acad. Sci. USA.* 80:4842–4846.

7. Platt, O. S., S. H. Orkin, G. Dover, G. P. Beardsley, B. Miller, and D. G. Nathan. 1984. Hydroxyurea enhances fetal hemoglobin production in sickle cell anemia. *J. Clin. Invest.* 74:652–656.

8. Institute of Laboratory Animal Resources, National Research Council. 1978. Guide for the Care and Use of Laboratory Animals. National Institutes of Health (NIH). DHEW publication no. 78-23.

9. Nathan, D. G., B. J. Clarke, D. G. Hillman, B. P. Alter, and D. E. Houseman. 1978. Erythroid precursors in congenital hypoplastic (Diamond-Blackfan) anemia. *J. Clin. Invest.* 61:489–498.

10. Iscove, N. N., F. Sieber, and K. H. Winterhalter. 1974. Erythroid colony formation in cultures of mouse and human bone marrow: analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose conconvalin A. *J. Cell Physiol.* 83:309–320.

11. Friedman, A. D., D. C. Linch, B. J. Miller, M. Lipton, J. Javid, and D. G. Nathan. 1985. Determination of the hemoglobin F program in human progenitor-derived erythroid cells. *J. Clin. Invest.* In press.

12. DeSimone, J., P. Heller, and J. G. Adams. 1979. Hemopoietic stress and fetal hemoglobin synthesis: comparative studies *in vivo* and *in vitro. Blood.* 54:1176–1181.

13. Krishan, A. 1968. Time lapse and ultrastructural studies on the reversal of mitotic arrest induced by vinblastine sulfate in Earle's L cells. *J. Nat. Cancer Inst.* 41:581–595.

14. Papayannopoulou, T., A. Torrealba de Ron, R. Veith, G. Knitter, and G. Stamatoyannopoulos. 1984. Arabinosylcytosine induces fetal hemoglobin in baboons by perturbing erythroid cell differentiation kinetics. *Science (Wash. DC).* 224:617–619.

15. DeSimone, J., P. Heller, J. C. Schimenti, and C. H. Duncan. 1983. Fetal hemoglobin production in adult baboons by 5-Azacytidine or by phenylhydrazine-induced hemolysis is associated with hypomethylation of globin gene DNA. *In* Globin Gene Expression and Hematopoietic Differentiation. G. Stamatoyannopoulos, and A. W. Nienhuis, editors. Alan R. Liss, Inc., New York. 489–500.

16. Till, J. E. 1982. Stem cells in differentiation and neoplasia. *J. Cell. Physiol.* 1(Suppl.):3–11.

17. Ebert, P. S., I. Wars, and D. N. Buell. 1976. Erythroid differentiation in cultured Friend leukemia cells treated with metabolic inhibitors. *Cancer Res.* 36:1809–1813.

18. Rencricca, J. J., B. S. Morse, F. C. Monette, D. Howard, and F. Stohlman, Jr. 1975. Hydroxyurea-induced erythroid differentiation. *Proc. Soc. Exp. Biol. Med.* 149:1052–1054.

19. Pfeiffer, S. E., and L. J. Tolmach. 1967. Selecting synchronous populations of mammalian cells. *Nature (Lond.).* 213:139–142.

20. Vadlamudi, S., and A. Goldin. 1971. Influence of mitotic cycle inhibitors on the antileukemic activity of cytosine arabinoside (NSC-63878) in mice bearing leukemia L1210. *Cancer Chemother. Rep.* 55: 547–555.

21. Iscove, N. N. 1977. The role of erythropoietin in regulation of population size and cell cycling of early and late erythroid precursors in mouse bone marrow. *Cell Tissue Kinet.* 10:323–334.

22. Udupa, K. B., and K. R. Reissman. 1979. *In vivo* erythropoietin requirement of regenerating erythroid progenitors (BFU-E, CFU-E) in bone marrow of mice. *Blood.* 53:1164–1171.

23. Marian, B. D., and R. T. Schimke. 1984. Gene amplification in a single cell cycle in chinese hamster ovary cells. *J. Biol. Chem.* 259:1901–1910.

24. Nathan, D. G., and B. P. Alter. 1980. F-cell regulation. *Ann. NY Acad. Sci.* 344:219–232.

25. DeSimone, J., P. Heller, L. Hall, and D. Swiers. 1982. 5-azacytidine stimulates fetal hemoglobin synthesis (HbF) in anemic baboons. *Proc. Natl. Acad. Sci. USA.* 79:4428–4431.