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Stimulation of fetal hemoglobin synthesis in baboons by hemolysis and hypoxia

(Hb F regulation/phenylhydrazine/hypobaric chamber/erythropoietin)

J. DESIMONE, S. I. BIEL, AND P. HELLER

Center for Genetics, University of Illinois College of Medicine, and the Departments of Medicine, University of Illinois and Veterans Administration West Side Hospitals, Chicago, Illinois 60612

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ABSTRACT **Fetal hemoglobin (Hb F) levels in the peripheral blood of baboons (*Papio cynocephalus*) increased from an average value of 0.78% to 18.1% during the recovery phase from phenylhydrazine-induced hemolytic anemia. A similar increase was observed in animals exposed to hypobaric hypoxia. Large individual variations in the maximal Hb F levels were observed which could not be correlated with the ages of the animals. Reinduction of hemolysis in two fully recovered animals resulted in Hb F levels that were of similar magnitude as in the preceding episode, suggesting the possibility of genetically determined individual variations in the rate of Hb F synthesis under the same conditions of erythropoietic stimulation. Reticulocytes from the animals subjected to hemolysis or hypobaric hypoxia synthesized similar absolute quantities of Hb F *in vitro*. The results of the present studies indicate that the physiological switch from the synthesis of Hb F to that of Hb A during ontogeny can be reversed in adult nonhuman primates by conditions of erythropoietic stress known to be associated with high erythropoietin levels. These findings open the possibility that Hb F synthesis in adult humans may be therapeutically modulated in individuals who might benefit from increased levels of Hb F, such as patients with sickle cell anemia.**

Fetal hemoglobin (Hb F; $\alpha_2\gamma_2$) is the major hemoglobin component during intrauterine life in man. Synthesis of adult hemoglobin (Hb A, $\alpha_2\beta_2$) starts during the 6th week of gestation (1), but its proportion remains only about 5% of total hemoglobin for the next 24–26 weeks (2). The newborn has 10–30% Hb A which gradually increases so that approximately 1 year after birth only trace amounts of Hb F remain (3). The frequency of Hb F-containing cells in adult blood ranges from 0.5 to 7.0%, and most of these cells also have Hb A and have been designated "F-cells" (4, 5). The factors regulating the switch from γ chain to β chain synthesis are unknown. Because fetal erythropoiesis with predominant synthesis of Hb F has been shown to be under control of erythropoietin (6), it is unlikely that this hormone is instrumental in this switch. Moreover, in culture, erythropoietin has been shown to stimulate the emergence of erythropoietic clones producing Hb F and the number of clones was proportional to the quantity of erythropoietin in the culture medium (7). The clinical counterpart of this experimental observation of a reverse switch is less impressive because, in acquired, nongenetically determined anemias, Hb F levels are only slightly increased, if at all (8). It is, of course, conceivable that, in these anemias, erythropoietin levels are insufficient to stimulate the differentiation and maturation of erythroid precursor cells giving rise to F-cells. Differences in erythropoietin sensitivity within populations of erythropoietic cells also may play a role in the regulation of the synthesis of the two hemoglobins.

Experimental stimulation of Hb F synthesis *in vivo* has been hampered by the lack of a suitable animal model. Because *Papio cynocephalus*, the long-limbed yellow baboon of Rhodesia, has been shown to have fetal and adult hemoglobins that are similar to the corresponding human hemoglobin components in chemical structure, physical properties, and relative amounts in the neonate and adult (9, 10), it appeared to be attractive to explore the possibility that this primate is a suitable model for the study of the control of Hb F synthesis *in vivo*. Because of the known indispensable role of erythropoietin in the stimulation of erythropoiesis in culture, Hb F synthesis *in vivo* was studied after induction of hemolysis by phenylhydrazine or by exposure of the animals to hypoxia in a hypobaric chamber.

METHODS

Induction of Hemolytic Anemia. Six juvenile (8–12 months old) and three adult (8–10 years old) baboons received daily intraperitoneal injections of phenylhydrazine (0.4 ml of a 5% solution per kg) for 5 days (11). All animals received daily injections of vitamin B_{12} (10–20 μg) and injections of folate (0.6–1.0 mg) on days 4 and 5 of treatment and for 5 days thereafter.

Exposure to Hypobaric Hypoxia. Three juvenile baboons were exposed to gradually increasing simulated altitudes by decompression in a hypobaric chamber as follows: day 1, 14,000 feet; day 2, 17,000 feet; day 3, 20,000 feet; and days 4–11, 23,000 feet [300 torr (40 kPa)]. The animals were returned to ground level pressure for about 30 min daily for feeding, watering, cleaning, and blood sampling. Blood sampling consisted of the removal of 2 ml of blood each day. Between days 7 and 11 the values for packed erythrocyte volumes (PCV) were prevented from rising beyond 50% by increasing the volume of the blood sample to 3–8 ml.

Hb F levels, reticulocyte counts, PCV values, and relative rates of synthesis of α, β, and γ polypeptide chains were determined before, during, and for varying periods after the induction of hemolysis or hypoxia.

Hb F Levels. The method of Singer *et al.* (12) was found to be applicable to the determination of baboon Hb F by comparing the results of this method with the percentages of baboon Hb F in artificial mixtures of chromatographically purified Hb F and Hb A (13). Hb F was purified from fetal erythrocytes containing approximately 95% Hb F; Hb A was purified from adult erythrocytes containing <1% Hb F. The regression line

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Abbreviations: Hb F, fetal hemoglobin; Hb A, adult hemoglobin; PCV, packed erythrocyte volume.

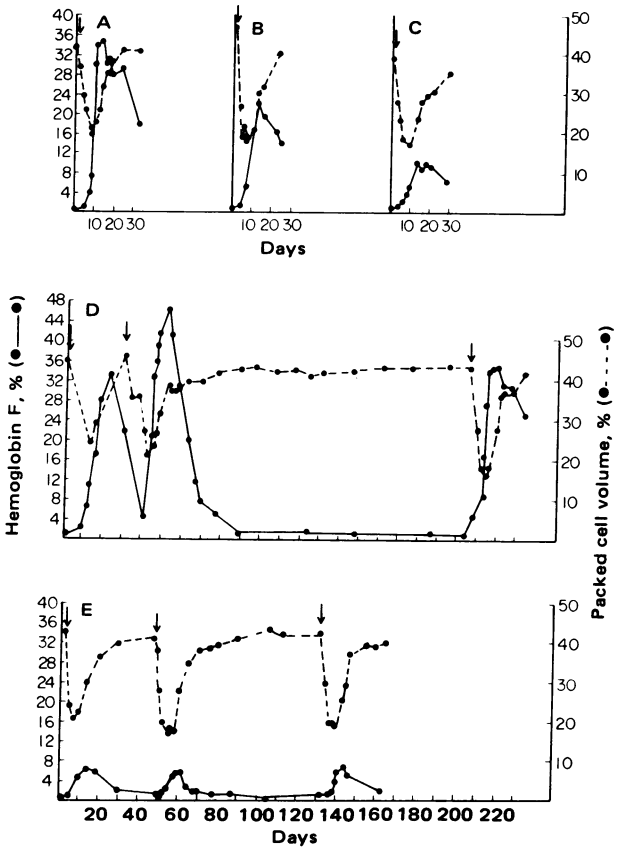

FIG. 1. Changes in peripheral blood Hb F levels and PCV after phenylhydrazine-induced hemolytic anemia in juvenile and adult baboons. (*A*) In a 1-year-old animal. (*B*) In a 9-year-old animal. (*C*) In an 8-year-old animal. (*D*) In a 0.9-year-old animal after three successive episodes of phenylhydrazine-induced hemolysis; the last two episodes were initiated after full hematologic recovery. (*E*) In a 10.0-year-old animal after three successive episodes of phenylhydrazine-induced hemolysis; the last two episodes were initiated after full hematologic recovery.

relating the results of alkali denaturation (ordinate) and actual percentage of Hb F had a correlation coefficient of 0.81 and an intercept of 1.06. The values of Hb F were calculated from this regression line.

The methods of Kleihauer *et al.* (14) for the estimation of the proportion of F-cells in man also proved to be applicable to baboon erythrocytes. This method is based on the fact that Hb A can be eluted from ethanol-fixed cells by phosphate/citrate buffer, pH 3.3, whereas Hb F is precipitated within the cells and can be visualized after staining with 0.1% eosin.

Globin Chain Synthesis in Reticulocytes. Venous blood samples were collected in heparin and placed in ice. The cells were centrifuged and the plasma was saved. Packed cells (0.5 ml) were added to 0.3 ml of plasma followed by the addition of 0.1 mCi of L-[^{3}H]leucine in 0.2 ml of α medium (Flow Laboratories). The cells were incubated at 37° for 2 hr, washed, and lysed as described (15). Incorporation of the radioactive leucine into α, β and γ chains was determined after their separation by CM-cellulose chromatography (16).

RESULTS

Effects of Hemolysis. The relationship of the values for PCV and Hb F levels is illustrated in Fig. 1 and Table 1. After discontinuation of phenylhydrazine, the proportion of Hb F increased almost parallel to the increasing PCV. Maximal Hb F

Table 1. Maximal Hb F levels during recovery from phenylhydrazine-induced hemolytic anemia

Animal age, years	Lowest PCV after treatment	Hb F, % of total Hb: Before treatment	Hb F, % of total Hb: Peak value during recovery
10.0	21	0.9	6.6*
9.0	18	0.7	21.1
8.0	17	0.8	10.7
1.2	20	0.7	6.4
1.0	20	0.5	34.8
0.9	21	1.0	33.2†
0.8	18	0.9	16.8
0.7	22	1.1	17.7
0.7	21	0.4	14.5
$\bar{X}$	—	0.78	18.10
SEM	—	±0.08	±3.50

* After two additional treatments, the maximal Hb F levels were 5.9 and 6.8%.

† After two additional treatments, the maximal Hb F levels were 46.1 and 34.6%.

levels were obtained approximately 12–14 days after the first injection, when the PCV values were approaching normal values. Afterward, the porportion of Hb F decreased as the PCV continued to increase. In the six juvenile animals, the Hb F levels ranged from 6.4 to 34.8%; in the adult animals they ranged from 6.6 to 22.1%. Because of the marked individual variations of these values, two animals were subjected to phenylhydrazine treatment two additional times to determine whether the observed Hb F levels were reproducible in individual animals. After the first treatment, one animal had 6.6% Hb F and after subsequent episodes of hemolysis these levels were 5.9 and 6.8%. The other animal that responded to the first treatment with 33.2% Hb F had 46.1 and 34.6% HbF during subsequent episodes of phenylhydrazine-induced hemolysis.

The F-cell distribution was determined on blood smears from selected animals with peripheral blood Hb F levels ranging from 5.1 to 46.1%. Fig. 2 demonstrates the F-cell distribution obtained from an animal with 28% Hb F. There were approximately 40–50% F-cells, with varying intensity of the stain. Because the proportion of F-cells was found to be always greater than the proportion of Hb F in the peripheral blood, it can be assumed that most of the cells contained both Hb A and Hb F.

Globin chain synthesis was studied in intact reticulocytes of two phenylhydrazine-treated juvenile baboons. Fig. 3 demonstrates the pattern of α, β, and γ chain synthesis in the reticulocytes of one animal 5 and 10 days after the first injection. On the 5th day, γ chain synthesis accounted for 5.5% of total non-α-chain synthesis, and on the 10th day, for 45.4%. On days 14 and 17, γ chain synthesis was 15.2 and 4.3% of total non-α-chain synthesis. The maximal peripheral blood level of Hb F in this animal was 34.8% on the 14th day of treatment. In the other animal, the values on days 5, 12, and 17 were 4.3, 57.5, and 21.7%, respectively. This animal reached the maximal level of Hb F on the 16th day, with 46.1%. In both animals, maximal γ chain synthesis occurred shortly before maximal Hb F levels were obtained.

Effects of Hypoxia. Upon exposure to simulated high altitudes, all three animals exhibited some degree of hypophagia and weight loss. Two animals also vomited during the first 2 days of decompression.

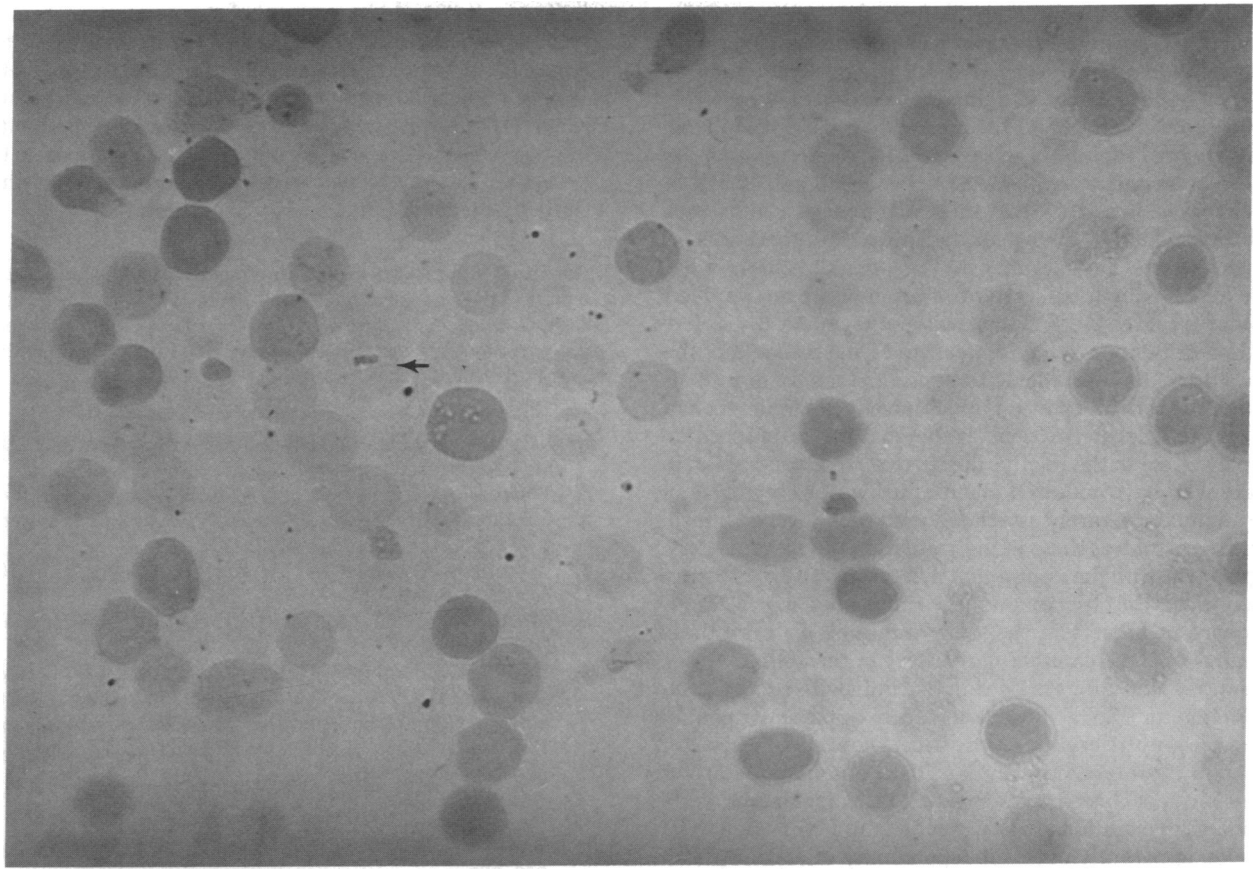

FIG. 2. F-cell distribution in the peripheral blood of an animal with 28% Hb F. There are approximately 50% F-cells in the preparation. Arrow, a cell apparently free of demonstrable Hb F. Note the marked variation in the intensity of staining among F-cells.

Initial PCV values in the three animals were 42, 45, and 47%. By day 6 they increased to 51, 52, and 53%, respectively. After that day until day 11, when the animals were removed from the hypobaric chambers, only one animal had a PCV as high as 56%. Maximal reticulocyte counts of the three animals were 10.4, 18.1, and 8.0%, respectively, between days 4 and 7. Initial Hb F levels were 1.6, 0.6, and 0.8%. They gradually increased from the 4th day onward and on the last day (day 11) of decompression they were 9.2, 8.0, and 16.5%. The maximal levels in these animals were 10.8% (day 14), 8.0% (day 11), and 21.4% (day 16), respectively.

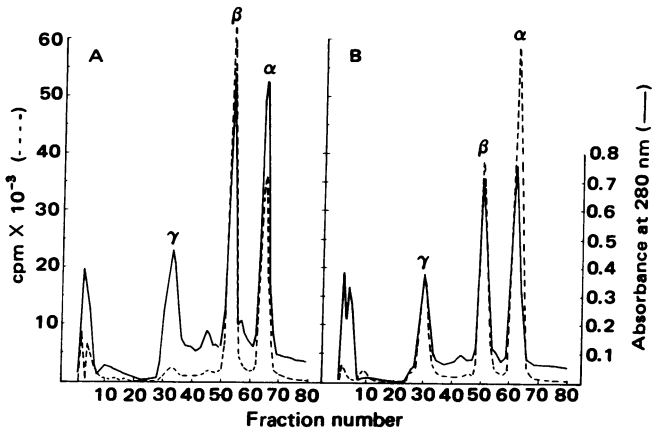

FIG. 3. CM-Cellulose column chromatography of globin prepared from hemolysates of a phenylhydrazine-treated juvenile baboon to show distribution of leucine radioactivity into α, β, and γ chains. (*A*) At 5 days after the initiation of hemolysis. (*B*) At 10 days after the initiation of hemolysis. Unlabeled Hb A and Hb F were added as carriers.

Synthesis of γ chains by reticulocytes was undetectable (less than 1% of non-α-chain synthesis) in all three animals prior to decompression. It began to increase on the 4th day of decompression and rose to 33.7, 17.0, and 63.4%, respectively, on days 6–8. These maximal relative rates of γ chain synthesis correlate well with the respective maximal levels of Hb F in the peripheral blood for each animal. In the animal in which the relative rate of γ chain synthesis was highest (63.4% on day 7), it decreased rapidly after termination of decompression. On the 4th day after removal of the animal from the hypobaric chamber, it was 35.5% and on the 7th day it was 11.4%.

DISCUSSION

The described experiments in primates show that in the species *Papio cynocephalus* the synthesis of Hb F can be stimulated by exposing animals to hemolytic or hypoxic stress. Hb F levels as high as 46% of total hemoglobin were found in the peripheral blood of animals exposed to phenylhydrazine and 21% in animals exposed to hypobaric hypoxia. The absolute quantities of newly synthesized Hb F in these two groups are probably similar as suggested by the studies of γ globin chain synthesis.

In previous studies of the regulation of Hb F synthesis, erythropoietic cells in culture were used because no experimental animal appeared to be suitable for such studies *in vivo*. Comparison of the *in vitro* and *in vivo* conditions leading to Hb F synthesis justifies the assumption that in both experimental systems an important factor in Hb F production is erythro-

poietin. Thus, the ontogenic switch from the synthesis of Hb F to that of Hb A can be reversed by conditions of erythropoietic stress known to be associated with high erythropoietin levels. The response of the 12 animals exposed to erythropoietic stress were not uniform. In the phenylhydrazine-stimulated animals the final Hb F level achieved during the recovery phase from the hemolytic anemia varied between 5.9 and 46.1%. It was reasonable to suspect that these differences in responses may be a function of the age of the animal, but there was no such correlation. The fact that, in two animals tested, re-exposure to phenylhydrazine after full hematologic recovery led to similar levels of Hb F is suggestive of a genetically determined predisposition, which appears to be independent of the magnitude of the reticulocyte response. In fact, some animals in which the reticulocyte count was lower than in others had a higher Hb F level. It is conceivable that this predisposition may play a role in the relative absence of Hb F production in humans who have acquired anemias for various reasons, but these anemias are rarely associated with such severe erythropoietic stress such as those in the animals in the present study. In agreement with the assumption that the severity of the stress is important is the observation that, within a few days after the subsidence of hemolysis, the relative rate of Hb F synthesis decreased with the continuing recovery of the animal.

The presented data are informative only with regard to the reinduction of Hb F synthesis which apparently can be achieved by severe erythropoietic stress known to be associated with high erythropoietin levels. These observations do not permit any conclusion with regard to the regulation of the physiological cessation of Hb F synthesis and the switch to Hb A, which apparently occurs independently of erythropoietin levels. Papayannopoulou *et al.* (7) recently postulated from *in vitro* data that ontogeny of erythropoietic cells is associated with a gradual increase of highly erythropoietin-sensitive erythroid precursor cells that predominantly synthesize Hb A. The earlier erythroid precursor cells (burst-forming units), which are less erythropoietin-sensitive, can make use of their capacity of transcribing γ genes and may do so when erythropoietin levels are high. Whether or not this model will turn out to be correct, our studies *in vivo* confirm that Hb F is synthesized *in vivo* under conditions of severe erythropoietic stress. The results of the present studies also suggest the possibility of genetically determined differences among individuals of the *Papio cynocephalus* species in regard to the quantities of Hb F synthesized under the influence of equal erythropoietic stresses.

The possibility of stimulating Hb F synthesis in the adult may have therapeutic implications, especially for patients with sickle cell disease. If it could be proven in future experiments in baboons that injection of erythropoietin reproduces the results of the present studies and preferentially stimulates the production of F-cells, such a finding may be applicable to clinical situations because Hb F levels on the order of 20% are associated with a benign course of sickle cell anemia (17). It is conceivable that agents other than erythropoietin, yet to be discovered, may have a similar enhancing effect on Hb F synthesis.

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