Maintenance of Fetal Hemoglobin (HbF) Elevations in the Baboon by Prolonged Erythropoietic Stress

By J. DeSimone, M. Biel, and P. Heller

We have previously shown that acute erythropoietic (Ep) stress by hemolysis or hypobaric hypoxia causes elevations of HbF in the baboon. The magnitude of these elevations is genetically determined, ranging from 3% to 60% (low, intermediate, and high responders). These genetic differences in HbF levels among animals are mainly due to differences in the number of HbF-containing cells ("F-cells"). The present study was undertaken to study the influence of prolongation and of the severity of Ep stress on HbF levels and the number of F-cells. The packed cell volume (PCV) of the blood of 4 animals, approximately 3 yr old, was maintained at 20% by daily phlebotomies, and the animals were exposed to varying degrees of hypobaric hypoxia for up to 40 days. In these experiments, the number of F-cells increased rapidly and reached individually constant levels ranging from 60% to 80%, when the PCV reached 20%, and no further increase was observed regardless of the subsequent degree of hypoxia. On the other hand, HbF levels, and with it the values for HbF per F-cell, increased proportionally to the severity of the Ep stress and could be maintained at a constant level dependent on the degree of the hypoxia, e.g., at 19,000 feet HbF levels of one animal remained 20%–25% throughout the duration of the exposure of 14 days. These data are indicative of separate control of F-cell numbers and of the levels of HbF per F-cell. It appears that with the increase of Ep stress, those Ep stem cells that have retained the HbF program are mobilized into maturation. A model, attempting to explain this phenomenon is presented.

PREVIOUS STUDIES1-3 in the baboon (Papio cynocephalus) revealed that under conditions of erythropoietic stress, such as hemolytic anemia or hypobaric hypoxia,1 the production and peripheral blood levels of HbF are increased. The maximal HbF levels attained in stressed juvenile baboons were either high (28%–60%), intermediate (10%–25%), or low (3%–10%). In older animals these values were somewhat lower and there was a negative correlation with age.1 The magnitude of these responses was shown by half-sibship analysis to be genetically determined.2 Also, normal ("resting") HbF levels, as determined by radioimmunoassay, were found to be clearly correlated with maximal peripheral blood HbF levels attained during the recovery phase from acute hemolytic anemia. Furthermore, the correlation of resting HbF values in parents and offspring was significant (p<0.001), demonstrating that resting HbF levels are genetically determined.3

A correlation was also found between the HbF levels in hemolysates and the number of HbF-containing erythrocytes (F-cells).3 This was the case in the absence and presence of erythropoietic stress, suggesting that genetic differences in blood levels of HbF among animals are mainly due to differences in the number of F-cells.

Because the termination of the acute erythropoietic stress led to prompt diminution of HbF levels and of F-cells,1,3 the question arose whether prolongation of the stress period could maintain or further increase the levels of HbF and the number of F-cells. Such experiments, designed to produce prolonged erythropoietic stress, might also help to clarify the mechanism of gene activation leading to HbF elevation in adults.

MATERIALS AND METHODS

Two juvenile baboons (3 yr old) were subjected to prolonged erythropoietic stress by acute hemolysis, phlebotomy, and hypobaric hypoxia. Hemolysis was produced by daily intraperitoneal injections of phenylhydrazine (PHZ), 0.4 ml of a 5% solution/kg, for 3 days. Afterward, daily phlebotomies (25%–30% of blood volume) were performed to maintain the PCV at 20%. In addition, hypobaric hypoxia was produced by exposure to simulated altitudes in a hypobaric chamber. Two other animals of similar age were only bled and exposed to hypobaric hypoxia. All animals received injections of vitamin B12 (10–20 μg), folate (300 μg), and iron dextran (100 mg) every other day throughout the course of the experiment.

HbF levels in hemolysates, F-cell numbers, reticulocyte counts, and PCV values were determined before, during, and for varying periods after the start of the regimen producing erythropoietic stress (see Results). The reticulocyte percentage achieved by bleeding to a PCV of 20% was used as reference for the calculation of the reticulocyte index (R1) at other PCVs (R1 = Percent reticulocytes × PCV/20).

Hemoglobin F was determined in hemolysates by alkali denaturation according to the method of Singer et al.1 Erythropoietin levels were assayed in the mouse, rendered polycythemic by hypobaric hypoxia.1 Peripheral blood red cells containing HbF were visualized by indirect immunofluorescence6 using antisera to HbF, prepared in rabbits, and fluorescein-labeled anti-rabbit γ-globulin, prepared in goats.3

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RESULTS

Effects of Acute Hemolysis, Phlebotomies, and Hypobaric Hypoxia on HbF Levels

Two animals received PHZ for 3 days followed by phlebotomies to maintain their PCVs at 20%. In one animal (Fig. 1), the reticulocyte index reached a maximum of 48% on day 10 of treatment. On day 12, the HbF level reached a maximum of 26.3% with the F-cell number being 89%. From day 12 to 22, the F-cell number remained at approximately the same level, but HbF levels progressively decreased to 13.1%, indicating that the proportion of HbF per F-cell diminished after the discontinuation of the hemolytic phase of stress. From day 23 through 36, the animals were exposed to gradually increasing simulated altitudes by decompression in a hypobaric chamber as follows: days 24–27: 12,000 ft; days 28–29: 14,000 ft; days 30–31: 16,000 ft; day 32: 16,500 ft; days 33–36: 18,000 ft. While the animals were in the hypobaric chamber, they were bled daily until day 33 to maintain the PCV at approximately 20%. Bleeding was stopped when the altitude reached 18,000 ft on day 33. The reticulocyte index increased from 23%, when the animal was placed into the hypobaric chamber, to 62.9% on day 36. The HbF level was lowest, 11.0%, on day 29 and increased to 18.5% on day 38. This maximum occurred 4 days after a simulated altitude of 18,000 ft had been reached and bleeding had been discontinued. F-cell numbers, however, remained at approximately 87% throughout the treatment period, resulting in increased levels of HbF per F-cell during the final days of increased Ep stress. The results obtained from the second animal were comparable (Table 1). In both animals, MCV and MCH did not change during the final period of the experiment when the additional increase in stress led to an increase in HbF per F-cell.

Effect of Severe Prolonged Erythropoietic Stress Consisting of Phlebotomies and Hypobaric Hypoxia

Two juvenile baboons were bled daily so that after 4 days the PCV was approximately 20%. This PCV was

<table>
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<th>Percent HbF in Hemolysate</th>
<th>F-Cell No. (%)</th>
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*Reticulocyte index = % reticulocytes x PCV/20.
maintained throughout the experiment by daily phlebotomies. From days 7 to 23, the animals were also placed into the hypobaric chamber and the simulated altitude gradually increased as indicated in Tables 2 and 3. Again, the most noteworthy feature of the tabulated values was the rapid initial increase in the number of F-cells to a maximal number on reduction of the PCV to approximately half the original value. This maximal number changed very little with the altitude gradually increased as indicated in Tables 2 and 3. The experiments in which animals were exposed to a simulated altitude of 14,000–18,000 ft while their PCV was maintained at 20%, there is a further increase in Epo production followed by increased reticulocytosis and HbF synthesis, while F-cell numbers remain constant at a maximum. These changes in peripheral blood HbF levels must therefore result from changes in the amount of HbF per F-cell.

**DISCUSSION**

The experiments in which animals were exposed to phenylhydrazine-induced hemolysis followed by phlebotomies to maintain a PCV of 20% demonstrated that immediately following the acute severe hypoxic phase there is increased production of F-cells to a genetically determined maximum that distinguished high and low HbF responders. These F-cells contain greater amounts of HbF per F-cell than those found in resting F-cells. As the erythropoietic stress diminishes during hematologic recovery, as evidenced by a decrease in reticulocytosis, there is a decrease in peripheral blood levels of HbF, even though maximal F-cell numbers are maintained. When animals are then exposed to hypobaric hypoxia in addition to the stress maintained by phlebotomies (PCV:20%), there is a further increase in Epo production followed by increased reticulocytosis and HbF synthesis, while F-cell numbers remain constant at a maximum. These changes in peripheral blood HbF levels must therefore result from changes in the amount of HbF per F-cell.

As has been suggested and confirmed here, there are two mechanisms responsible for increases in HbF levels following erythropoietic stress: (1) increases in F-cell number and (2) increases in HbF per F-cell.

**Table 2. Effect of Bleeding* and Hypobaric Stress on Level of HbF and F-Cells in a 3-yr-old Baboon (no. 3956)**

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<th>Day</th>
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<th>Percent HbF in Hemolysate</th>
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*Almost daily phlebotomies to maintain PCV at 20%.
†Last day in hypobaric chamber.

**Table 3. Effect of Bleeding* and Hypobaric Stress on Levels of HbF and F-Cells (Baboon no. 4005)**

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*Almost daily phlebotomies to maintain PCV at 20%.
†Bleeding was discontinued.
‡Last day in hypobaric chamber.
Experiments were also performed to determine whether an increase in the amount of HbF per F-cell could be made to persist for an extended period. By maintaining a PCV of approximately 20% at a simulated altitude of 19,000–20,000 ft for 12 days, the level of HbF in the hemolysates remained above 20% for a period of 14 days, thereby demonstrating that levels of HbF can persist under constant stimulation of erythropoiesis that is likely to be associated with recruitment of erythroid precursors directly from the primitive BFU-E pool.

In clonal cultures, a glycoprotein hormone (burst-promoting activity, BPA)8,10 and erythropoietin (Ep)11 are required for the growth of erythroid stem cells (BFU-E). Eaves et al. have suggested that BPA is required for cell cycling and Ep for survival of these cycling erythroid precursors.11 The more primitive the stem cell, the greater the amount of BPA required for these cells to cycle and the greater the amount of Ep necessary for these cycling cells to survive. With maturation of erythropoietic precursors, there is an increase in their sensitivity to both BPA and Ep, i.e., less BPA is required for these cells to cycle and less Ep for these cycling cells to survive. Because of the severe hypoxic stimulus in our baboons, Ep and probably also BPA12 were increased, resulting in the cycling of an even greater number of more primitive BFU-E that survive and mature. It has been suggested that with normal maturation of erythropoietic stem cells there is a change in the hemoglobin program from HbF to HbA production.13 Therefore, the more primitive the BFU-E that is stimulated to cycle by Ep stress, the greater the HbF produced by its descendant erythroblasts.

Several investigators have demonstrated13,15 that Ep has no direct effect on the production of HbF in culture. We have recently injected 1000 U of Ep every 8 hr intramuscularly for 5 days into a normal baboon of 5 kg body weight, leading to reticulocytosis of approximately 10% and to an increase in the PCV from 38% to 41%, but synthesis and blood levels of HbF were not influenced, confirming the findings in culture. It is also unlikely that BPA exerts a direct effect on stimulating HbF synthesis, but rather its stimulating effect on HbF synthesis is due to increased cycling and maturation of primitive BFU-E.

The increase in HbF production in response to erythropoietic stress and the results of in vitro studies of erythropoiesis9 are compatible with the following hypothesis, which is an extension of that proposed by Papayannopoulou et al.16 (Fig. 2): The normal maturation, which results in a progressive loss of proliferative potential and the acquisition of the capacity for hemoglobin production; (2) biochemical differentiation of the HbF program, which progresses through several stages in which the capacity for HbF production is gradually lost concomitant with an increase in the potential for HbA synthesis. It may be postulated that both BPA and Ep increase the probability that a primitive erythropoietic stem cell (BFU-E) will survive but decrease the probability that it will undergo biochemical differentiation as it matures. Neither hormone alters the rate at which a cell matures. In the presence of high levels of both BPA and Ep, greater numbers of primitive BFU-E cycle and go on to maturity maintaining their capacity for HbF production. The more primitive the erythroid stem cell the greater the probability that in the presence of high levels of BPA and Ep it will retain the potential for greater HbF synthesis, resulting in greater content of HbF per F-cell. Therefore, the greater the erythropoietic stress, the greater the amount of HbF per F-cell.

Whether this model can also be used to explain the genetic differences between animals in F-cell numbers observed following erythropoietic stress2,3 remains to be seen. In this respect it deserves emphasis that no differences in Ep levels were observed between stressed “high” or “low” HbF responders (unpublished observations), and therefore, the genetic variability is independent of Ep, which, however, is necessary for the expression of this genetic variability. It is conceivable that this variability may be due to differences in cellular receptors for BPA or differences in the levels of BPA.

ACKNOWLEDGMENT

We are grateful for the most skillful technical assistance of Lemuel Hall, Kevin Draves, and David Zwiers.

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Maintenance of fetal hemoglobin (HbF) elevations in the baboon by prolonged erythropoietic stress

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