Fetal Hemoglobin (HbF) Synthesis in Baboons, *Papio cynocephalus*. Analysis of Fetal and Adult Hemoglobin Synthesis During Fetal Development

By Joseph De Simone and Anne L. Mueller

Fetal hemoglobin (HbF) and adult hemoglobin (HbA) synthesis was studied in fetal baboons, *Papio cynocephalus*, to determine the normal pattern of hemoglobin production during fetal development. Fetuses ranging from 53 to 180 days gestation (term gestation 184 days) were used. Erythroid cells were incubated with 3H-L-leucine, and the rates of globin chain synthesis and the distribution of radioactivity into hemoglobin intermediates and completed hemoglobin molecules were determined. Gamma chain synthesis accounted for approximately 97% of the total nonalpha chain synthesis up to 140 days gestation; beta chain synthesis accounted for the remainder. After 140 days gestation, approximately equal quantities of gamma and beta chain were synthesized in the bone marrow. Prior to 140 days gestation, total alpha chain synthesis was 30% greater than total non-alpha chain synthesis, while there was balanced chain synthesis after 140 days gestation. During the period of excess alpha chain synthesis, fetal erythrocytes contained a large pool of alpha-hemoglobin (alpha chain with heme attached) molecules uncoupled with beta or gamma chains. In view of the possibility that alpha chains may have a lower affinity for gamma chains than beta chains, excess alpha chain synthesis may be required to maintain low levels of free gamma chains.

STUDIES RELEVANT to the regulation of fetal hemoglobin (HbF) synthesis are not only important for increasing our knowledge of cell biology but also may be important therapeutically in sickle cell disease and beta-thalassemia. Perrine et al summarized evidence that HbF levels on the order of 20% distributed in all red blood cells are associated with a benign course in sickle cell disease. In view of the controversy over human fetal research, it is obvious that studies of the regulation of HbF synthesis cannot be done in the human, and therefore a nonhuman primate model would be advantageous. Recently we reported that the baboon, *Papio cynocephalus*, is a model experimental animal for studies of the control of HbF synthesis. The kinetics of adult hemoglobin (HbA) synthesis in this species are the same as those reported for human HbA. The kinetics of baboon HbF assembly indicated that gamma chains have less affinity than beta chains for association with alpha chains. Although this preferential association of alpha and beta chains has not been reported in synthesis studies of human HbF, it is consistent with reports showing an association of alpha hemoglobin with beta chains from hemoglobin H (beta4) in preference to gamma chains from hemoglobin Bart's (gamma4).14

The present study was undertaken to determine whether *P. cynocephalus*...
could be a useful animal model for studies relating to the switch from HbF to HbA synthesis that occurs during fetal development.

**MATERIALS AND METHODS**

**Preparation of fetal erythroid cells.** Baboon fetuses with gestational ages of 53–180 days (term gestation 184 days) were used. Since a single male was kept with a female for only 3 days at ovulation, the estimated gestational ages of the fetuses were known within 1–2 days. Fetuses with known gestational ages were obtained by cesarean section; before the umbilicus was clamped, each was heparinized and exsanguinated through the umbilical vein and the blood was placed on ice. Unless stated otherwise, all further procedures were performed at 4°C. The cells were centrifuged free of plasma and washed three times by centrifugation at 1000 g for 5 min in NKM (153 mM NaCl, 5 mM KCl, 5 mM MgCl₂).

The fetal bone marrow and fetal liver were perfused by pumping isotonic saline into the left ventricle to remove the majority of peripheral blood contamination. When the perfusate coming out of the right auricle was water clear, the perfusion was stopped. After the perfusion the fetal liver and long bones were dissected out and the liver placed in α medium (Flow Laboratories) containing 2% fetal calf serum (αFCS). The ends of the long bones were cut off, and the bone marrow cells were collected by flushing the marrow cavity with a 20-gauge hypodermic needle, and the plasma and fat were removed by centrifugation at 1000 g for 10 min. The cells were resuspended and washed twice in αFCS by centrifugation at 1000 g for 5 min. The fetal liver was minced with a scissors and passed through a 40-mesh screen. A single-cell suspension was prepared by repeated passage of the cells through a 20-gauge hypodermic needle. The liver cells were then suspended in 20 vol NKM and centrifuged at approximately 150 g for 5 min. The nonpelleted cells were discarded, the pellet was resuspended in 20 vol NKM, and the above procedure was repeated two additional times.

**Cell staining.** Cell fractions were suspended in αFCS. Slides were prepared from these cell suspensions using a cytocentrifuge (Shandon). Duplicate slides from each cell fraction were stained by the benzidine peroxide procedure using hematoxylin counter stain and also by the Wright-Giemsa technique. Cells containing (stainable levels of) hemoglobin could easily be identified using the benzidine peroxide procedure, while basophilic erythroblasts and proerythroblasts could be identified more easily using Wright-Giemsa stain.

**Incubations.** Erythroid cells were incubated with 3H-L-leucine at 37°C, and the soluble phases of the cell lysates were isolated. The packed cells were lysed in 4 vol 1.5 mM MgCl₂. Reticulocytes were stirred on ice for 2 min to affect lysis, while the bone marrow and liver erythroid cells were stirred on ice for 5 min. Supernates were stored frozen at −20°C until analyzed.

**Analysis of globin synthesis.** Total incorporation of 3H-L-leucine into α, β, and γ globin chains was determined after the globin chains had been separated using carboxymethyl cellulose (CMC) chromatography as described. The distribution of total lysate protein radioactivity was determined after electrophoresis of the soluble cell phases on cellulose acetate membranes. Analysis of the radioactive composition of completed hemoglobin molecules and hemoglobin intermediates was performed after separating these components by cellulose acetate electrophoresis.

**RESULTS**

Analysis of the stained slides of the perfused fetal bone marrow cell suspensions showed that about 20% of the cells were nonnucleated red cells. Of the nonnucleated erythroid cells, approximately 80% were reticulocytes. Since there were only 9%–10% reticulocytes in the peripheral blood, it was assumed that the large majority of the reticulocytes found in these cell preparations were bone marrow reticulocytes. Therefore there was no appreciable contamination of bone marrow erythroid cells by peripheral blood. Analysis of the stained slides of the purified liver erythroid cell suspensions showed that about 80%–
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Fig. 1. CMC column chromatography of globin prepared from hemolysates of 151-day baboon fetus. Distribution of radioactivity into (A) peripheral blood reticulocytes and (B) bone marrow erythroid cells after incubation for 5 min in presence of \(^{3}\)H-leucine.

90% of the cells were nucleated erythroid cells, 10%-15% nonnucleated red cells, and less than 5% nonerythroid cells.

Eight baboon fetuses (gestational ages 53-180 days) were used in this study. Peripheral blood reticulocytes, bone marrow erythroid cells, and liver erythroid cells were incubated with \(^{3}\)H-L-leucine as described above and the soluble phase of the cells isolated. Figure 1 shows the pattern of globin radioactivity after CMC chromatography of reticulocyte and bone marrow cell lysates from a fetus with a gestational age of 151 days following a 5-min pulse label. Un-
Table 1. Summary of Hemoglobin Synthesis in the Fetal Baboon

<table>
<thead>
<tr>
<th>Gestational Age (days)</th>
<th>HbA as Percent of Total Hb</th>
<th>Incubation Time (min)</th>
<th>α/β + γ Ratio</th>
<th>β/β + γ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>&lt;1.0</td>
<td>30</td>
<td>1.23</td>
<td>1.30</td>
</tr>
<tr>
<td>103</td>
<td></td>
<td>60</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>2.8</td>
<td>5</td>
<td>1.20</td>
<td>1.28</td>
</tr>
<tr>
<td>142</td>
<td>2.9</td>
<td>5</td>
<td>1.30</td>
<td>1.20</td>
</tr>
<tr>
<td>147</td>
<td>9.0</td>
<td>5</td>
<td>1.51</td>
<td>1.40</td>
</tr>
<tr>
<td>151</td>
<td>12.0</td>
<td>5</td>
<td>1.56</td>
<td>1.18</td>
</tr>
<tr>
<td>162</td>
<td>18.0</td>
<td>5</td>
<td>1.96</td>
<td>0.94</td>
</tr>
<tr>
<td>180</td>
<td>39.0</td>
<td>30</td>
<td>1.04</td>
<td></td>
</tr>
</tbody>
</table>

labeled autologous hemoglobin was used in preparing the labeled samples for chromatography. As can be seen, the ratio of newly synthesized β chains to newly synthesized γ chains was approximately 0.6 in the peripheral blood reticulocytes and approximately unity in the bone marrow cells. Since the peripheral blood and bone marrow cells contained 12% and 40% HbA, respectively, it is obvious that the switch from HbF to HbA synthesis has already occurred in this animal. It is interesting to note that the relative rate of β to γ chain synthesis in the bone marrow was about two times greater than that in the peripheral blood.

The relative rates of β and γ chain synthesis in fetal hemopoietic tissues at different stages of development are given in Table 1 together with the relative proportions of HbF and HbA present in the blood at the time of sampling. Beta chain synthesis is low during early fetal development and begins to increase in the bone marrow only by 142 days gestation. By 147 days gestation β chain synthesis accounts for 50% of the total non-α chain synthesis, and it is maintained at that level for the remainder of fetal development. Therefore the switch to β chain production begins at about 140 days gestation.

Analysis of the ratios of total α chain to total β + γ chain synthesis (Table 1 and Fig. 2) showed that there is unbalanced synthesis of α and non-α chains prior to the switch to β chain synthesis. In all fetuses of 142 days gestation or less α chain synthesis was 30% greater than non-α chain synthesis. During the switch to β chain synthesis there was a concomitant decrease in the relative rate of α chain synthesis; after the switch occurs there is an approximate balance of α and non-α chain synthesis.

Detectable levels of HbA were not present in the 53-day fetus. However, HbA was found to represent about 3.0% of the total hemoglobin of the 103-day fetus, and it remained at that level until the time of switching. Although HbA was not identified in the peripheral blood or liver erythroid cells of the 53-day fetus, a hemoglobin component was visible on cellulose acetate electrophoresis
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Fig. 2. CMC column chromatography of globin prepared from hemolysates of 142-day baboon fetus. Distribution of radioactivity into (A) peripheral blood reticulocytes and (B) bone marrow erythroid cells after incubation for 5 mm in presence of 3H-leucine.

of both the peripheral blood and liver erythroid cell lysates that was neither HbA nor HbF. Figure 3A depicts the distribution of radioactivity in the total protein of the reticulocyte lysate from the 53-day fetus following electrophoresis on cellulose acetate. The compositions of the radioactive peaks labeled a globin and HbF have been described previously. In the region of the radioactive peak designated a-hemoglobin is a hemoglobin component that accounted for 8% of the total hemoglobin of this fetus. The migration distance of this component was similar to that expected for soluble a-hemoglobin. All fetuses with gestation...
tional ages below 147 days had this component after electrophoresis, although the component accounted for only 2% of the total hemoglobin protein of the cells from the 142-day fetus. This hemoglobin component, designated α-hemoglobin in Fig. 3A, was excised from an unstained cellulose acetate strip and the protein and radioactivity eluted into an unlabeled carrier solution of equal amounts of HbA and HbF. The mixture was converted to globin by acid-acetone treatment, and column chromatography was subsequently performed on CMC to separate the α, β, and γ chains. Analysis of the distribution of globin radioactivity associated with this hemoglobin component is shown in Fig. 3B. Greater than 90% of the radioactivity was associated with carrier α chains, indicating that this minor hemoglobin may be soluble α-hemoglobin. The same analysis performed on all erythropoietic tissue samples of the 103-, 138-, and 142-day fetuses showed that they also had a minor hemoglobin component whose globin composition was chromatographically identical to α chains.

DISCUSSION

The developmental changes that occur in the hemoglobins of preterm fetal baboons were studied in animals of 53–180 days gestation. Our results indicate that the switch to HbA synthesis occurs at approximately 140 days gestation. This gestational age in these baboons is equivalent to 31 wk gestation in man. Previous reports estimated the switch in human fetuses to occur at the 32nd to 34th week of gestation. Therefore it appears that the switch time in these baboons is proportional to that in man.
Fig. 4. Changes in relative rates of $\alpha$, $\beta$, and $\gamma$ chain synthesis during fetal development. Note abrupt changes in the $\beta/(\beta + \gamma)$ and $\alpha/(\beta + \gamma)$ radioactivity ratios that occurred at approximately 140 days gestation.

Synthesis of $\beta$ chains was not found in the 53-day fetus, either in the peripheral blood or in liver erythroid cells, in agreement with the observation that HbA was not found in either tissue. In human fetuses, however, there appears to be about 5% HbA present as early as 8 wk gestation, indicating that there may be species differences between man and baboon with respect to the time during gestation when HbA first appears.

The results summarized in Table 1 and Fig. 4 illustrate that the switch is marked by two developmental changes. First, there is a rapid increase in the rate of $\beta$ chain synthesis. The results obtained from the 103- and the 138-day fetuses showed that they each were synthesizing $\beta$ chains at baseline levels of 3% of the total non-$\alpha$ chain synthesis in both the peripheral blood and bone marrow cells. However, by 142 days gestation $\beta$ chain synthesis accounted for 5% of the total non-$\alpha$ chain synthesis in the peripheral blood and 8% in the bone marrow, indicating that the switch had just begun. At 147 days gestation $\beta$ chain synthesis accounted for 34% and 52% of non-$\alpha$ chain synthesis in the peripheral blood and bone marrow cells, respectively. At this time HbA in the peripheral blood accounted for 9% of the total cellular hemoglobin mass. At 151 days gestation $\beta$ chain synthesis accounted for 36% and 49% of non-$\alpha$ chain synthesis in the peripheral blood and bone marrow cells, respectively, and HbA in the peripheral blood was 12% of the hemoglobin mass. Second, there is a rapid decrease in the percentage of $\alpha$ chain synthesis. At both 103 and 138 days gestation there is a 30% excess of $\alpha$ chain synthesis. By 151 days gestation $\alpha$ and non-$\alpha$ chain synthesis was relatively balanced.

The results also show that the switch is precisely timed. If it is assumed that the proportion of HbA in the peripheral blood changes approximately 0.5%–1.0%/day after initiation of this switch, it can be estimated that the switch occurred at about 142 days gestation for the four postswitch animals listed in Table 1. Knowing the precise gestational age at which the switch begins will be important in future experiments designed to determine the physiologic component responsible for the switch. Finally, since the ratio of $\beta$ to $\beta + \gamma$ chain synthesis in the bone marrow cells of fetuses aged 147–180 days gestation was approximately 0.5, it appears that postswitch fetuses synthesize...
approximately equal amounts of HbA and HbF until some later time, at which a switch to adult levels of HbA synthesis begins.

An unexpected observation in relation to the coordinate control of α and non-α chain synthesis during fetal development was the 30% excess α chain synthesis found in all preswitch fetuses. As discussed above, the lysates of all preswitch fetuses also contained a hemoglobin component identical to α hemoglobin on the basis of electrophoretic migration on cellulose acetate and on the basis of coelution with α globin during CMC chromatography. Peptide analysis of the putative α-hemoglobin and α chains on a 95-day fetus verified their homology with authentic α globin (De Simone J, Adams JG: Unpublished results). Therefore the excess α chain in preswitch fetuses is present not only as free α chain but also as what appears to be α-hemoglobin.

The demonstration of a large excess of α chain synthesis is interesting in view of what is considered to be the fate and pathologic consequence of excess α chains in β-thalassemia homozygotes. In the β-thalassemia homozygote, excess α chains precipitate in the red cells, and these precipitates, called Fessas bodies, play an important role in the chronic hemolysis of the disease. The imbalance of α chain synthesis, however, is much greater in β-thalassemia homozygotes than that observed in the baboon fetuses. For example, the α to non-α chain synthesis ratio in β-thalassemia is consistently greater than 2, while in the preswitch baboon fetuses it averaged only 1.3. In individuals homozygous for δβ-thalassemia the α to non-α chain synthesis ratio is only 1.5. The imbalance of synthesis in these individuals only moderately affects red cell survival, and Fessas bodies have not been found in their peripheral blood. Individuals homozygous for the hereditary persistence of fetal hemoglobin gene also produce a 50% excess of α chains, and it has been well established that these individuals, aside from a slight microcytosis, are apparently healthy. It therefore appears that an excess of α chain synthesis does not have significant pathologic consequences at the levels found in preswitch fetuses. Thus α to non-α chain synthesis ratios of 1.3–1.5 appear to be consistent with relatively normal red cell function.

Data suggest that excess α chain synthesis may be normal when γ chains are the predominant non-α chains synthesized. In a recent report, data were presented supporting the concept that γ chains do not combine readily with α chains to form HbF tetramers. It appears that excess α chain mass is required for the rapid assembly of HbF. This rapid assembly of HbF maintains low levels of free γ chains, which otherwise would combine to form Hb Bart’s (γ4). Hb Bart, like HbH (δ4), is unstable, has a high affinity for oxygen showing no Bohr effect, and rapidly oxidizes to methemoglobin. Therefore free γ chains may be more detrimental to the cell than free α chains or α-hemoglobin at the levels found in preswitch fetuses. In the course of the evolutionary process the possibility of the formation of a large pool of free γ chains has been minimized.

REFERENCES

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