The Ontogenesis of Hemoglobins in *Macaca nemestrina*

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*Macaca nemestrina* have two fetal hemoglobins which differ in the structures of their γ chains. Thus animals of this species, like man, appear to possess duplicate γ-chain loci. In this study, the ontogenesis of hemoglobins in *M. nemestrina* was followed in animals ranging in age from the 60th day of gestation to adulthood. Fetuses in the 60th day of gestation had the two fetal hemoglobins and a small amount of presumed embryonic hemoglobin. Fetuses between 60 and 132 days' gestation had only the two fetal hemoglobins. Adult hemoglobin appeared in the last month of gestation. The subsequent transition from γ- to β-chain formation was nearly completed by the end of the second postnatal month. The two fetal hemoglobins were present in 2:1 proportions throughout fetal development and the period spanning the shift from γ- to β-chain formation. The pattern of hemoglobin development in these macaques differs from that in man in the following ways: (1) The relative activities of the two γ-chain loci do not change. (2) α-Chain synthesis is adequate throughout gestation (no γ-chain tetramers were found). (3) The transition from γ- to β-chain synthesis is more abrupt.

Each of the normal major human hemoglobins is composed of one or two of four different polypeptide chains that are synthesized in various proportions at different times during the life cycle. Epsilon and alpha chains are found in the embryonic hemoglobins, Hb Gower 1 (ε4) and Hb Gower 2 (α2ε2). These hemoglobins predominate in embryos during the second month of gestation and are completely supplanted by fetal hemoglobin (α2γ2) by the end of the first trimester.1 Fetal hemoglobin accounts for over 95% of the total hemoglobin in the fetal erythrocytes during the sixth month of gestation, declining to levels of approximately 80% at birth. The decline in the Hb F continues after parturition until levels of less than 2% are attained between the ages of 5 and 6 mo. Adult hemoglobin (Hb A or α2β2) is found in small amounts after the fourth month of gestation, and comprises 5-10% of the total hemoglobin in the fetus during the sixth month of gestation, about 20% in the neonate, and approximately 97% of the total from the age of 6 mo until death.2

The mechanisms involved in determining the levels of activity of different hemoglobin chain loci during human development are unknown. An under-
standing of these mechanisms would be of theoretical and practical significance. Genetically determined developmental anomalies involving the continuation of synthesis of fetal hemoglobin in adult life have been found in man, but their interpretation remains a matter of speculation. Similarly, non-hereditary factors are associated with atypical production of human fetal hemoglobin. Given an understanding of these phenomena it may be possible, with the proper manipulation, to arrest the synthesis of adult hemoglobin in the thalassemic fetus or neonate and thus cause continued synthesis of fetal hemoglobin throughout the life of an affected individual. The existence of detailed information on hemoglobin development in various nonhuman primates would aid in investigations aimed ultimately at influencing the course of hemoglobin development in man.

In this report, we describe the ontogenesis of hemoglobin in *Macaca nemestrina* (pig-tailed macaques). Animals of this species are of particular interest because, like man, they possess duplicate \( \gamma \)-chain loci, which direct the synthesis of structurally different \( \gamma \) chains. In *M. nemestrina*, the two \( \gamma \)-chain loci are involved in the production of two fetal hemoglobins, designated Hb \( F_{saw} \) and Hb \( F_{fast} \) according to their relative electrophoretic mobilities in starch gels of alkaline pH.

**Materials and Methods**

Blood samples were drawn from 15 fetal and 40 infant macaques ranging in age from the 60th day of gestation to 334 days postpartum. The duration of gestation in *Macaca nemestrina* is approximately 170 (162–186) days. Fetuses were taken by Caesarean section and bled by direct cardiac puncture. Twelve infants were examined at intervals of approximately 10 days during the first 2 to 3 mo of life. Blood samples were also drawn from 36 of the 53 different mothers and from 14 of the 23 different fathers of these animals.

Red cells from each animal were washed three times with cold saline and lysed by freezing with 2–3 volumes of distilled, deionized water. Stromata were removed by centrifugation at 12,000 \( \times \) g for 30 min. Hemolysates containing 4–6 g of hemoglobin per 100 ml were prepared from the red cells of each animal and examined by horizontal starch-gel electrophoresis in Tris-horate-EDTA buffer, pH 8.6. After electrophoresis, gels were sliced; one half was stained with amido black, the other with benzidine. For estimation of the relative proportions of the various hemoglobin fractions, electrophoretic preparations stained with amido black were used.

Hemoglobins from fetuses and infants were tested for resistance to denaturation by 0.06 N NaOH, pH 12.2. Alkali-resistance tests were performed on fresh solutions of oxyhemoglobin whenever possible (within 2 days after collection); in a few instances, hemoglobins were converted to their cyanmethemoglobin derivatives by adding small amounts of \( K_3Fe(CN)_6 \) and KCN, stored at \(-20^\circ C\), and tested at a later date. There were no significant differences in the results obtained from these two types of preparations. Hemoglobin solutions prepared from the erythrocytes of four adult animals were used as controls.

The hemoglobins from fetuses taken at 132, 150, 156, 159, and 167 days of gestation were fractionated on 2.5- × 43-cm columns of DEAE-Sephadex A-50. The hemoglobins were eluted by the application of a gradient formed by mixing 900 ml of 0.03 M Tris-HCl, pH 7.45, with an equal amount of 0.10 M Tris-HCl, pH 6.00. All buffers were 0.001 M in KCN.

Separation of adult hemoglobin from the fetal hemoglobins was more readily accomplished on CM-Sephadex C-50. Hemolysates from animals sampled 2, 17, 39, 73, 94, 156, and 334 days postpartum were applied to 2.5- × 43-cm columns of CM-Sephadex and
eluted by mixing 900 ml of 0.05 M phosphate starting buffer, pH 6.5, with 900 ml of 0.05 M phosphate limiting buffer, pH 6.9. All buffers were 0.001 M in KCN and were prepared by mixing 0.05 M solutions of Na₂HPO₄, 0.001 M in KCN, and NaH₂PO₄, 0.001 M in KCN, to the desired pH.

The fractions comprising each peak in an effluent profile were pooled and the hemoglobins converted to cyanmethemoglobins by the addition of K₃Fe(CN)₆ to an approximate concentration of 0.001 M. The concentration of hemoglobin in each pool was determined spectrophotometrically after conversion to cyanmethemoglobin ($\lambda = 540 \text{ m}_{\mu}, \varepsilon = 4.6 \times 10^4$, mol wt Hb = 64,450). The relative amount of hemoglobin in each pool was expressed as a percentage of the total hemoglobin recovered from the column. The hemoglobin in each pool was concentrated by vacuum dialysis at 4°C and identified by starch-gel electrophoresis as described above.

**RESULTS**

Typical patterns produced by electrophoresis of hemoglobins from adult, newborn, and fetal *Macaca nemestrina* appear in Fig. 1. The stages of development and the proportions in which the various hemoglobins were found are given in the following paragraphs.

**Embryonic Stage:** The three youngest animals, examined in the 60th day of gestation, had small amounts of a very slowly moving hemoglobin that was
similar in electrophoretic mobility to the human embryonic hemoglobins. Although the possibility that this minor hemoglobin might be a variant of one of the normal fetal hemoglobins cannot be excluded, the fact that it appeared only in animals of this age suggests that this is an embryonic hemoglobin. The blood samples obtained from these 60-day embryos were too small to permit further studies of this minor component. The major hemoglobins from these embryos were electrophoretically identical to the Hb Fslow and Hb Ffast from fetuses. In addition, visual estimation of the proportions of these hemoglobins indicated that they are present in the approximate ratio of 2 Hb Fslow : 1 Hb Ffast.

Fetal Stage: All hemolysates from fetuses produced two hemoglobin bands, which, in samples from fetuses taken during the last month of gestation, were preceded by a single faint band with the same electrophoretic mobility as the sole hemoglobin of adult animals. The two fetal hemoglobins were present in the approximate proportions of 2 Hb Fslow to 1 Hb Ffast in all fetal samples. These proportions, estimated by visual inspection of starch gel (stained with amido black), were confirmed by DEAE-Sephadex chromatography of hemoglobins from five fetuses (Fig. 2).

Transitional Stage: Small amounts of adult hemoglobin were found in all newborn animals; the amount of hemoglobin in each of the two major bands characteristic of fetal phenotypes decreased steadily with postnatal age, while the level of adult hemoglobin showed a corresponding increase. Hemoglobin Ffast was not detectable by electrophoresis of hemolysates from infants more than 60 days old, and neither fetal hemoglobin could be detected in electrophoretic patterns produced by the hemoglobins from animals more than 80 days old.

The electrophoretic patterns produced by the hemoglobins of animals sampled between birth and 60 days of age indicated that the ratio of 2 Hb
F\text{slow} to \text{Hb F}_{fast} persists throughout the period during which the shift from fetal to adult hemoglobin takes place. Chromatography of hemolysates from 2-, 17-, and 39-day-old infants on CM-Sephadex demonstrated conclusively that this ratio is maintained (Fig. 3). The amounts of fetal hemoglobin recovered from hemolysates from older animals were too low (less than 2%) to permit accurate quantitation. However, \text{Hb F}_{slow} was clearly present at higher levels than \text{Hb F}_{fast} in the 73- and 94-day-old animals.

Hemolysates from infants were subjected to alkali-denaturation tests. The alkali-denaturation rates of isolated \text{Hb F}_{slow} and \text{Hb F}_{fast} were identical; thus the alkali-resistant fractions in whole hemolysates include both fetal hemoglobins. The results of alkali-resistance tests closely approximate those obtained by starch-gel electrophoresis, and clearly illustrate the decline in levels of fetal hemoglobin from an average of 80% over the first 10 days following birth to a mean value of only 8.3% between 50 and 60 days of age (Fig. 4). Levels of fetal hemoglobin in hemolysates from older animals were too low for accurate quantitation by the methods used.

\textit{Adult Stage}: Hemolysates from all adult animals produced a single, rapidly moving band on electrophoresis; no hemoglobin was detected in the zones
normally occupied by Hb F_{slow} or Hb F_{fast}. In addition, no fetal hemoglobins were detected in fractions isolated by CM-Sephadex chromatography of 200–300 mg of hemoglobin from animals sampled at 156 and 334 days of age.

At no time during the period of development covered by these studies was there any indication of the presence of hemoglobins similar to Hb Bart's (γ₄), a homotetramer normally found in human fetuses and neonates.

**DISCUSSION**

An outline of the ontogenesis of hemoglobin chains in *Macaca nemestrina* appears in Fig. 5. The duration of gestation (about 170 days) in *M. nemestrina* is approximately three fifths as long as that in man (about 280 days). If we compare the ontogenesis of hemoglobins in *M. nemestrina* with that in man, we find evidence that this relative temporal relationship is maintained. For example, embryonic hemoglobins are no longer detectable in human fetuses by the end of the first trimester of gestation; a small amount (less than 3%, as estimated by visual inspection of starch gels) of a presumed embryonic hemoglobin was found in the red cells of three macaques in the 60th day of gestation (the end of the first third of gestation).

To date, there have been no reports of embryonic hemoglobins in primates other than man, although they have been described in other mammals. The discovery of a presumed embryonic hemoglobin in hemolysates from fetal cercopithecoids suggests that these components are also present in animals whose lineage diverged from that of man over 30 million years ago. The γ chains of the two fetal hemoglobins of *M. nemestrina* are the only non-α chains which were detected during the middle one-third of gestation. It is clear that β chains (and, of course, adult hemoglobin) appear in significant amounts only during the last few days of gestation, in contrast to their much earlier appearance in human fetuses. Similarly, the adult pattern of hemoglobin formation is attained at a much earlier postnatal age in *M. nemestrina* than in man.

In human embryos and fetuses, α-chain synthesis does not appear to match that of non-α chains. Thus Hb ε₄ is found in embryos, and Hb γ₄ is normally present in the red cells of fetuses and newborns. The absence of detectable amounts of γ₄ homotetramers in the red cells of fetal and newborn macaques indicates that there is little or no discrepancy in the levels of α- and γ-chain synthesis in these animals.
The two human \(\gamma\) chains differ in the exchange of glycine and alanine at position 136.\(^{11}\) The hemoglobin tetramers containing either of these two chains have identical charges and are inseparable by electrophoretic and chromatographic techniques. Detection and quantitation of the two types of human fetal hemoglobin relies on isolation of the C-terminal cyanogen bromide fragments and determination of the Gly/Ala ratios within these fragments.\(^{11,12}\)

Although developmental studies involving the two fetal hemoglobins are difficult and time-consuming, data concerning the relative proportions of the chains synthesized during development have been collected. For example, the ratio of \(\gamma^{136\text{Gly}}\) to \(\gamma^{136\text{Ala}}\) chains at birth is 3:1.\(^{11,12}\) This ratio was originally thought to reflect the presence of four different \(\gamma\)-chain loci, three of which produced \(\gamma^{136\text{Gly}}\) chains. However, subsequent studies\(^{12}\) indicate that the relative activities of the two types of loci change as the infant grows older, until the ratio is nearly reversed. Thus, it is impractical to assign numbers of loci which fit observed ratios of chain types.

It is evident that postnatal repression of the human \(\gamma\)-chain loci produces dissimilar effects on the two types of loci. The shift from fetal to adult hemoglobin formation is characterized not only by synthesis of fewer \(\gamma\) chains, but of different proportions of the two types of \(\gamma\) chains as well. An altered ratio of \(\gamma^{136\text{Gly}}\) to \(\gamma^{136\text{Ala}}\) chains has also been noted in cases of heterozygous beta thalassemia.\(^{13}\) In \textit{M. nemestrina}, no evidence of differential repression of the two \(\gamma\)-chain loci was observed. The two fetal hemoglobins were found in 2:1 proportions in 60-day embryos, fetuses, neonates, and infants up to 2 mo of age. This ratio may change later in life; our attempts to determine accurately the amount of each fetal hemoglobin in blood from older animals were unsuccessful. However, during the period of rapid decline in fetal hemoglobins (Fig. 5), the ratio of Hb F\(_{\text{slow}}\) to Hb F\(_{\text{fast}}\) remained at 2:1. These findings indicate that the regulation of the shift from synthesis of fetal to adult hemoglobins is, to some extent, species-specific. The steady decline in levels of human fetal hemoglobin from over 95% to about 80% during the last trimester of gestation is not paralleled in \textit{M. nemestrina}. The fetal hemoglobins in this species remain at consistently high levels until the last few days of gestation, dropping to about 90% at birth. These data indicate that the shift from synthesis of \(\gamma\) to \(\beta\) chains occurs nearer the end of gestation in \textit{M. nemestrina} than in man. Moreover, the rate of decline of levels of fetal hemoglobin following parturition is much higher in \textit{M. nemestrina} than it is in human infants. Fetal hemoglobins comprise about 10% of the total hemoglobin in hemolysates from human infants at the age of 3 mo; this level is attained in \textit{M. nemestrina} within approximately 50 days. The rapid reduction in levels of fetal hemoglobin in infant \textit{M. nemestrina} might result from any one or more of the following conditions: (1) a shorter life span of fetal macaque erythrocytes; (2) selective destruction of macaque erythrocytes which contain fetal hemoglobin; and (3) a more abrupt shift from the synthesis of \(\gamma\) to \(\beta\) chains in \textit{M. nemestrina}. Further studies will enable us to decide among these possibilities.

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