Relation of Beta to Gamma Synthesis During the First Trimester: An Approach to Prenatal Diagnosis of Thalassemia

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Extract

Washed fetal erythrocytes, obtained at hysterotomy, were incubated with ^{14}C - or (^3H)leucine for 2 hr. Adequate separation of β^A and β^S from γ chains on 8 M urea columns was achieved by minor refinements of standard techniques. Contamination of the β peak with embryonic chains was excluded by simultaneous labeling with isoleucine. The β/γ synthetic ratio showed significant correlation with fetal crown-rump length and with gestational age. The mean β/γ ratio for 20 fetuses removed after 5 to 16 gestational weeks was 0.087 ± 0.014 . β^S production and decreased β^A production were demonstrated in two additional fetuses. We have also shown that in artificial mixtures of fetal and maternal blood, or in mixtures obtained by placental aspiration, the β/γ synthetic ratio is proportional either to the reciprocal value of the fraction of fetal cells in the mixture, or to $1 +$ the fraction of maternal erythrocytes in a suspension of placental or amniotic fluid cells. It was thus possible to calculate the β/γ synthetic ratio of fetal cells by extrapolation from the β/γ ratios obtained in certain placental or amniotic fluid blood samples.

Speculation

The β/γ synthetic ratio of fetal blood cells can be determined accurately by examining fetal blood or mixtures of fetal and maternal blood obtained by placental aspiration. If a reliable and safe method were developed for obtaining fetal or mixed placental blood, and if decreased β chain synthesis in thalassemia were expressed in the 1st trimester, the prenatal diagnosis of thalassemia and of sickle cell anemia should be feasible.

Introduction

The possibility of prenatal diagnosis of thalassemia and sickle cell anemia, the two major inherited disorders of hemoglobin, has been suggested by the observations of Hollenberg *et al.* [3], Kazazian and associates [6, 7] and Kan *et al.* [5] who provided evidence that

the human β chain is synthesized in the 1st trimester of fetal life. All of these groups of investigators have shown quite clearly that homozygous sickle cell anemia might be diagnosed during the 1st trimester if an adequate sample of fetal cells could be obtained. The diagnosis would be apparent from the absence of

newly synthesized hemoglobin A, or β^A chain, and their replacement with hemoglobin S or β^S chain following suitable separation.

The valuable approaches utilized by these workers have raised several important technical questions, some of which we attempt to deal with in the present paper. First, we favor separation of labeled α , β , and γ chains, with exclusion of labeled ϵ or ζ chains, by urea carboxymethylcellulose (CM-cellulose) chromatography. The diagnosis of thalassemia is much more accurately performed by this method (at least in adults). Second, hemoglobin Portland has been shown to migrate with hemoglobin A during zone electrophoresis [9] and might, if present and labeled, contaminate hemoglobin A during column chromatography of hemoglobin tetramer. On the other hand, the original techniques of Kan *et al.* [5] are not sufficient, inasmuch as adequate separation of γ and β chains are not achieved. In this paper we present a minor modification of the urea CM-cellulose method which provides excellent separation of γ and β chains and documents the freedom of α and β peaks from ζ or ϵ chains.

The diagnosis of β thalassemia in the 1st trimester depends upon two important additional assumptions. (1) The globin synthesis defect in thalassemia must be expressed in the 1st trimester, a fact which will not be established without further experience. (2) The relation of normal β chain synthesis to γ chain synthesis during the 1st trimester must be defined clearly if the rate of β chain production in a particular sample is to be considered normal or abnormal. In this paper we present the range of values for the ratio of β to γ chain synthesis in the erythrocytes of fetuses ranging in size from 1.5 cm to 14 cm or from 5 to 16 weeks of fetal life.

Finally, a practical application of the biochemical studies must begin. We have suggested previously [5] that a mixture of fetal and maternal cells aspirated from a placental vessel could, under certain circumstances, permit assessment of fetal cell globin chain synthesis. However, the influence of maternal cell contamination on the results must be determined. A new methodologic approach to the correction for this contamination is also discussed in this paper.

Materials and Methods

General Methods

A few drops of blood obtained from the umbilical cord of fetuses removed at hysterotomy, sometimes as little as 4 μ l, were transferred into tubes containing

heparinized saline solution. Tubes were refrigerated at 4° and transferred to the laboratory as soon as possible. There the cells were washed three times in Krebs-Ringer-phosphate (KRP) buffer, pH 7.4, and incubated at 37° for 2 hr with 3 μ Ci (14 C)leucine, 0.3 Ci/mM [12], or 30 μ Ci (3 H)leucine, 30 Ci/mM [12], in a 1/1 dilution of dialyzed AB Rh-positive plasma in Krebs-Ringer-phosphate buffer, as described previously [5]. Lysate obtained from washed incubated cells was stored at -20° until needed.

Globin chains were separated on columns (0.8 $\times$ 15 cm) of CM-cellulose in 8 M urea as described previously [5]. Less than 5 mg globin were put on the columns to permit improved separation of γ and β chains. In order to improve this separation, the four chambers of the Varigrad were modified to contain 100 ml sodium phosphate buffer, pH 6.7, at respective concentrations of 3.73, 9.88, 12.96, and 34.5 mM. When the amount of radioactive globin was less than 2 mg, approximately 2 mg nonradioactive globin obtained from adult or neonatal cord blood were added as carrier. Adult globin containing leucine labeled with a different isotope from that incorporated into the fetal globin was also added as a marker in some experiments.

An aliquot of 1 to 3 ml from each fraction was diluted with Aquasol [12] and counted in a Packard liquid scintillation spectrometer [13]. When both 3 H and 14 C were present, the correction for 14 C in the 3 H spectrum was based on the "spilling" of 14 C into the 3 H spectrum in a solution of 9.88 mM sodium phosphate in 8 M urea. This is the approximate buffer composition at which β and γ chains are eluted from the columns. Synthetic ratios were calculated from the sum of radioactivities under the respective peaks.

Detection of Isoleucine-containing Globin Chains

To determine whether embryonic globin chains such as ϵ or ζ chains might be present in the non- γ peaks eluted from the column, erythrocytes of two fetuses were also incubated and chromatographed as above except that 30 μ Ci (3 H)isoleucine, 30 Ci/mM [12] were substituted for labeled leucine, and isoleucine rather than leucine was omitted from the incubation mixture. The (3 H)isoleucine-labeled globin chains were then chromatographed simultaneously with (14 C)leucine-labeled globin chains from the same fetus.

Relation of β Chain to γ Chain Synthesis

To determine the rate of β chain synthesis relative to γ chain synthesis in fetal cells, the area under the β

chain radioactivity peak was divided by the area under the γ chain peak.

The β/γ synthetic ratios determined in this manner were plotted against crown-rump (C-R) length and fetal age (the latter determined anamnesticly and from C-R length) and a regression line determined by the method of least squares.

Influence of Maternal Cell β Chain Synthesis on β/γ Synthetic Ratio in Fetal Cells

The method utilized to subtract maternal β chain synthesis from the observed fetal cell β/γ synthetic ratio was based on a previous observation that the rate of labeling of fetal and maternal globin proceeds independently in mixtures of the two cell types and that maternal γ chain synthesis relative to fetal γ chain synthesis is negligible. When these facts were established, one of us (GC) appreciated that a linear relation must exist between the β/γ ratio of a series of dilutions of fetal cells in maternal cells and the reciprocal of the fraction of fetal cells in the cell mixture. If various points on this line were determined experimentally, the β/γ synthetic ratio in pure fetal cells could be obtained by extrapolation to 0.

To test this mathematical conclusion experimentally, cells obtained by open placental aspiration during hysterotomy, or artificially prepared mixtures of fetal and maternal cells, were incubated with (^3H)leucine both directly and after further dilution with maternal cells. Globin chain separation and determination of the β/γ synthetic ratio in the various mixtures and in pure fetal cells was then determined by the methods described above. Certain computations used in these experiments are presented in the *Appendix*.

Transabdominal Placental Aspiration

Before the standard procedure for NaCl solution-induced abortion, ultrasonic placental localization was performed. When it appeared that the placental integrity would be necessarily violated by the abortion procedure, preparations were made to collect either bloody amniotic fluid or gross blood obtained during aspiration. When it was certain that the needle was in the amniotic space, the saline solution infusion was begun. In three of nine cases, blood was obtained which contained fetal cells. These cells were pelleted, resuspended in AB Rh-positive plasma, and stained for fetal hemoglobin [8]. If any fetal cells were detected, the sample was washed and incubated with

(^3H)leucine both directly and after further dilutions with maternal cells as described previously.

Results

Figures 1, 2, and 3 demonstrate the chromatographic separation of newly synthesized globin chains labeled with radioactive leucine in three fetuses, one of which has sickle trait. Adequate separation of β^A and β^S from γ is achieved and the identity of the fetal β and α chains is established by cochromatography with labeled chains from a normal adult and an adult with sickle trait. Figure 2 also demonstrates that the β peak of a 4.7-cm fetus contains less than 1.5% of the isoleucine newly incorporated into the γ peak, thus excluding significant contamination of the β peak with any of the known embryonic chains which are known to have a very high isoleucine content [2, 10]. Similar results were obtained in an older 7.8-cm (C-R length) fetus. Other than γ chains, only one isolated protein, which was rapidly eluted from the column, appeared to have a high isoleucine content. This conceivably could represent one of the embryonic globin chains, but proof is lacking because nonglobin proteins may be rich in isoleucine. Synthesis of β chain was detected in all of the fetuses and the β/γ synthetic ratio showed significant correlation ($r = 0.725$, $P < 0.01$) with fetal C-R length (Fig. 4). A poorer correlation was expectedly

Fig. 1. CM-cellulose radiochromatography of globin from a 2-cm (crown-rump length) fetus, and from a normal adult. ●—●: ^3H radioactivity of the globin chains of fetal blood incubated previously with (^3H)leucine; ○---○: ^{14}C radioactivity of the globin chains of adult blood incubated previously with (^{14}C)leucine.

Fig. 2. CM-cellulose radiochromatography of globin from a 4.7-cm (crown-rump length) fetus, labeled with both (^{14}C)leucine and (^3H)isoleucine. ●—●: (^{14}C)leucine radioactivity; ○---○: (^3H)isoleucine radioactivity.

Fig. 3. CM-cellulose radiochromatography of globin from an 8-cm (crown-rump length) fetus with hemoglobin S production and of globin from the mother with sickle cell trait. ●—●: ^{14}C radioactivity of the globin chains of fetal blood after incubation with (^{14}C)leucine; ○---○: ^3H radioactivity of the globin chains of maternal blood after incubation with (^3H)leucine.

found with anamnestic gestational age, probably because of inaccuracy in determination of the latter. The slopes of the plots for β/γ and β/α synthetic ratios during the 2nd to 4th months of pregnancy, obtained by linear regression, were, however, relatively small. The mean values for 20 Black and Caucasian fetuses

removed after 5 to 16 gestational weeks were $0.087 \text{ sd} \pm 0.014$ for β/γ and $0.096 \text{ sd} \pm 0.023$ for β/α . Figure 3 demonstrates β^S production in a 12-gestational week, 8-cm (C-R length) fetus. Both in this and in another 9-cm (C-R length) fetus, β^A synthesis exceeded β^S synthesis (Fig. 4), as is the case in adults heterozygous for the sickle trait. The relatively small sd of the mean β/γ synthetic ratio permitted clear definition of low β^S and β^A production in these fetuses. The sum of the two was normal.

The results of comparison of extrapolated determinations of $(\beta/\gamma)_t$ with actual measurements of $(\beta/\gamma)_t$ in pure fetal cells are shown in Figure 5. Here, the various β/γ synthetic ratios in artificial mixtures of fetal and adult cells $(\beta/\gamma)_m$ are plotted against $1/q$, the reciprocal value of the fraction of fetal cells, as described under *Materials and Methods*. Note that in both cases the extrapolated value of $(\beta/\gamma)_m$ at $1/q = 0$ is very close to the actual $(\beta/\gamma)_t$ measured independently in pure fetal blood. Furthermore, the ratio of synthetic activities in fetal *versus* adult cells calculated from the slopes of the graph as described in the *Appendix* were 6250/1 in a 10-week, 6-cm (C-R length) fetus, and 588/1 in a 17-week, 12.5-cm (C-R length) fetus. These ratios of synthetic activities are much higher than those reported by Kazazian and coworkers [6], and are even higher than those obtained by more direct measure-

Fig. 4. Nomogram showing correlation between β/γ synthetic ratio and fetal size or gestational age. ●: synthetic ratio in each of 20 normal fetuses; ---: boundaries of the normal range at a distance of 2 sd from the regression line calculated for these ratios; ○: β/γ synthetic ratios in two fetuses synthesizing β^S as well as β^A chains; □: β^S/γ synthetic ratios in these fetuses. C-R: crown-rump.

Fig. 5. The β/γ synthetic ratios in mixtures of fetal and adult blood cells. $\bullet$: $(\beta/\gamma)_m$ measured in mixtures with different concentrations of fetal cells; $\circ$: $(\beta/\gamma)_f$ measured directly in pure fetal cells. C-R: crown-rump.

ments of labeled leucine incorporation into trichloroacetic acid precipitates of erythrocyte lysate (data not shown).

Because of the much higher synthetic activity of fetal cells, $(\beta/\gamma)_m$ (the β/γ synthetic ratio in a mixture of fetal and adult cells) in blood obtained by open placental aspiration and containing more than 5% fetal cells (as estimated by the acid elution technique [8]) was not measurably different than the $(\beta/\gamma)_f$ in the corresponding fetal cells. The $(\beta/\gamma)_f$ of placental aspirates with less than 5% fetal cells could be estimated quite accurately from the ratios in mixtures of placental and maternal blood as explained under *Materials and Methods*, even when the percentage of fetal cells in the placental blood sample was much lower. In fact, Figure 6A shows that in two cases extrapolation of $(\beta/\gamma)_m$ to a value of 0 for $(1+b)$ provides a good approximation of the independently determined $(\beta/\gamma)_f$. In three of nine aminocenteses performed before saline-induced abortions, fetal cells were detected by the Betke-Kleihauer technique [8] in the amniotic fluid. In one of these cases, fetal cells were observed in what was thought to be a placental aspirate as well as in the amniotic fluid. Results of studies performed in both the amniotic fluid and placental blood samples from the second case are represented in Figure 6B. It should be appreciated that, although the slopes of the curves differ (because of a different percentage of fetal cells in each sample), they both give an extrapolated β/γ syn-

Fig. 6. The computation of $(\beta/\gamma)_f$ from β/γ synthetic ratios in placental blood and in mixtures of placental and maternal blood. A: regression line was drawn through the closed symbols ($\bullet$) representing $(\beta/\gamma)_m$ in two experiments. When the regression line is extrapolated to 0, the intercept is fairly close to $(\beta/\gamma)_f$ measured directly in pure fetal cells. The latter is represented by $\circ$. $(\beta/\gamma)_m$ at $(1+b) = 1$ represents the β/γ synthetic ratio in the undiluted, mixed placental blood. B: results obtained on dilutions of two separate samples of placental blood obtained by transabdominal amniocentesis.

thetic ratio for pure fetal cells of about 0.115, which is close to that found in cord blood cells obtained from fetuses of approximately the same gestational age. The cells observed in the first case were also studied, but by a different technique involving concentration with anti-i [4]. These results were also satisfactory but are not relevant to the approach described in this report.

Discussion

The results presented in this paper add support to the belief that adequate techniques for prenatal diagnosis of sickle cell anemia and thalassemia can be developed. Actually, the present data and those of Hollenberg *et al.* [3], Kazazian and associates [6, 7], and Kan and coworkers [5] suggest that the biochemical, if not the obstetrical, aspects of the problem are very nearly solved.

Adaptation of the urea CM-cellulose column for small samples provides excellent separation of γ and β chains without comigration of the β and α chains with any isoleucine-rich chains. Therefore, the concern over comigration of hemoglobin A with hemoglobin Portland expressed by Pataryas and Stamatoyannopoulos [9] has been relieved, at least for fetuses over 9 gestational weeks.

Although urea chromatography is somewhat cumbersome, the resolution and estimation of individual globin chains provided by the technique is probably preferable for the diagnosis of thalassemia. Isoelectric focusing is much simpler, but acetylated hemoglobin F migrates so close to hemoglobin A [1] that careful adaptation of the focusing procedure will be necessary to apply it in prenatal diagnosis. Present data confirms the validity of the prenatal diagnosis approach with respect to sickle cell disease. Sickle cell trait had been detected previously in fetuses by Kan *et al.* [5], Kazazian and associates [6, 7], and Pataryas and Stamatoyannopoulos [9], and we present two more cases here. The close correlation of the β/γ synthetic ratio with fetal age throughout the 1st trimester, a relatively small SD of the data, and a very gradual slope renders possible the detection of low synthetic rates of β^A and β^S in sickle trait fetuses. Therefore, it seems very likely that low or absent β^A production could be detected in thalassemia trait or homozygous thalassemia if the disease is expressed during the 1st trimester. More experience is needed to confirm the latter fact.

The obstetric aspects of the procedure are, of course, only beginning to be unraveled. Valenti [11] has shown that it is possible to obtain apparently pure

samples of fetal cells from an umbilical cord puncture through an aminoscope. However, placental aspiration is probably less traumatic to both mother and fetus. We have obtained stainable fetal cells with this technique applied in a routine fashion in only three of nine attempts in this series of studies and in one attempt in late pregnancy described previously [5]. Presumably, fetal cells could be acquired with a higher frequency in actual diagnostic settings when multiple aspirations and even radiologic placental contrast studies for placental localization would be justified. Because globin synthesis by fetal cells is so much greater than globin synthesis by maternal cells (even with respect to β chain synthesis), more than 10% fetal cells in placental blood would provide a fairly accurate estimate of fetal β chain synthesis (Fig. 5). In older fetuses, or when the percentage of fetal cells in the placental sample is very low, enrichment with fetal cells is possible by treatment with anti-i serum [4]. The anti-i technique may not, however, be ideal for very small samples of placental blood. This is because of the relatively low recovery of fetal cells after agglutination and centrifugation. Therefore, we have adopted in this paper a second technique described in Figures 5 and 6. This mathematical approach appears to be sufficiently precise to provide an accurate estimate of $(\beta/\gamma)_f$ from a series of determinations of $(\beta/\gamma)_m$. With the use of high specific activity tritiated amino acids, "bloody" amniotic fluid might provide enough fetal cells to permit determination of $(\beta/\gamma)_f$ with these methods.

Finally, the ethical considerations inherent in this research require comment now while the technology is being developed. Antenatal diagnosis of the clinically severe abnormalities of hemoglobin structure or function must be considered in the light of present attitudes toward interruption of pregnancy. The justifiable climate of fear surrounding the birth of a child with homozygous thalassemia or sickle cell disease has prompted many prospective mothers at risk to seek interruption of all pregnancies. Such interruptions would, of course, destroy many healthy fetuses, an action to be deplored. A reliable and safe method for the detection of the 25% of fetuses of such mothers with a homozygous defect would greatly reduce both the climate of fear and the number of interrupted pregnancies.

The obstetric aspects of these investigations are fraught with difficulties which may not be easily resolvable by studies in human pregnancy. The development of a

suitable primate model for refinement of these procedures will certainly be both useful and important.

Appendix

Computations

The β/γ synthetic ratio in a mixture of fetal and adult cells $(\beta/\gamma)_m$ is equal to the rate of β chain synthesis in the fetal cells times the fraction (q) of fetal cells in the mixture plus the rate of β chain synthesis in the adult cells multiplied by the fraction $(1 - q)$ of adult cells in the mixture. This sum is divided by the rates of γ chain synthesis in the fetal and adult cells each multiplied by the appropriate fractions which represent their presence in the mixture. This concept is represented by

$$(\beta/\gamma)_m = \frac{K_f(\beta/G)_f \cdot q + K_a(\beta/G)_a \cdot (1 - q)}{K_f(\gamma/G)_f \cdot q + K_a(\gamma/G)_a \cdot (1 - q)} \quad (1)$$

where K_f and K_a are the rate coefficients for total globin synthesis in fetal and adult cells, respectively, and β/G and γ/G are the fractions of total globin synthesis devoted to β chain and γ chain synthesis, respectively, in fetal (f) and adult (a) cells.

To simplify this equation it is assumed that the term $K_a(\gamma/G)_a \cdot (1 - q)$ may be removed from the equation because it is very small relative to $K_f(\gamma/G)_f \cdot q$ whenever $(\beta/\gamma)_m$ is not higher than 0.5 and $(\beta/\gamma)_a$ is higher than 5 [14].

With the removal of the above term, the equation is simplified by division of the numerator by the denominator to

$$(\beta/\gamma)_m = (\beta/\gamma)_f + \frac{K_a(\beta/G)_a \cdot 1 - q}{K_f(\gamma/G)_f \cdot q} \quad (2)$$

$$(\beta/\gamma)_m = (\beta/\gamma)_f + K \cdot \frac{1 - q}{q} \quad (3)$$

where K is a constant for any mixture of a given sample of fetal cells with a given sample of adult cells and, in fact, when multiplied by $(\gamma/G)_f/(\beta/G)_a$, represents the ratio of the rate coefficients for total globin synthesis in adult cells *versus* fetal cells.

From this equation it is clear that when $(\beta/\gamma)_m$ is plotted against $1 - q/q$, a straight line emerges with a slope of K and a zero intercept of $(\beta/\gamma)_f$. Hence a measurement of $(\beta/\gamma)_m$ in a series of mixtures of fetal and adult cells will provide an estimate of $(\beta/\gamma)_f$ when the determinations of $(\beta/\gamma)_m$ are plotted against $1 - q/q$. The latter term can be simplified to $1/q$ when q is small. Therefore, Equation 3 becomes

$$(\beta/\gamma)_m = (\beta/\gamma)_f + K \cdot \frac{1}{q} \quad (4)$$

If it is assumed that placental blood contains both fetal and maternal blood cells, where q_0 represents the original fraction of fetal cells, then the fraction of maternal cells equals $(1 - q_0)$. If these placental cells are now diluted b times with maternal blood cells [15], the resultant fraction of fetal cells in the new mixture (q_m) is given by

$$q_m = \frac{q_0}{1 + b} \quad (5)$$

Therefore

$$1 - q_m = \frac{1 + b}{q_0} - 1 \quad (6)$$

When q_0 is small, the -1 may be eliminated and the equation is simplified to

$$\frac{1 - q_m}{q_m} = \frac{1 + b}{q_0} \quad (7)$$

Substituting the right-hand term in Equation 3 we have

$$(\beta/\gamma)_m = (\beta/\gamma)_f + K \cdot \frac{1 + b}{q_0} \quad (8)$$

Because q_0 is a constant for a given initial sample, this is further simplified to

$$(\beta/\gamma)_m = (\beta/\gamma)_f + K'(1 + b) \quad (9)$$

Therefore, when $(\beta/\gamma)_m$ is plotted against $(1 + b)$, a straight line emerges with a slope of K' and an intercept at $(1 + b) = 0$ which represents $(\beta/\gamma)_f$, the desired value.

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 12. New England Nuclear Corporation, Boston, Mass.
 13. Packard Instrument Co., Downers Grove, Ill.
 14. The mathematical proof of this statement will be supplied to interested investigators.
 15. Example: if 4 million maternal cells were added to 1 million placental cells, $b = 4$.
 16. Dr. Cividalli is a recipient of an International Postdoctoral Research Fellowship (1 F05 TW-1781-02) from the United States Public Health Service, on temporary leave from the Department of Pediatrics, Hadassah University Hospital, Jerusalem Israel.
 17. This research was supported by United States Public Health Service Grants nos. AM-05322, AM-15913, and AM-05581, and by grants from the Medical Foundation of Boston, Inc., the National Foundation-March of Dimes, and the John A. Hartford Foundation.
 18. Requests for reprints should be addressed to: D. G. NATHAN, M.D., Division of Hematology, The Children's Hospital Medical Center, 300 Longwood Ave., Boston, Mass. 02115 (USA).
 19. Accepted for publication January 3, 1974.