Prenatal Diagnosis of Hemoglobinopathies: Comparison of the Results Obtained by Isoelectric Focusing of Hemoglobins and by Chromatography of Radioactive Globin Chains

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Isoelectric focusing (IEF) of hemoglobin was compared to the classical chromatography of labeled globin chains for 22 antenatal diagnoses of hemoglobinopathies: 11 for β thalassemia, and 11 for sickle cell disease. In all cases, the two methods gave identical results. The diagnosis was confirmed after birth or abortion. Three fetuses homozygous for β thalassemia and one homozygous for sickle cell disease exhibited no Hb A by IEF, in contrast to normal fetuses or those heterozygous for one of the two hemoglobinopathies. In addition, blood samples obtained in other centers after abortion of 22 fetuses homozygous for β or β thalassemia exhibited no Hb A when analyzed by IEF. When Hb A was present, the respective proportions of Hb A and acetylated Hb F were determined by densitometry of the IEF gel. The Hb A/acetylated Hb F ratio obtained by IEF correlated well with the β/γ ratio of globin chain synthesis. IEF requires 0.1 mg of unlabeled hemoglobin. It is performed in 90 min and several samples can be analyzed simultaneously. If present, maternal contamination of fetal blood must be eliminated by selective lysis of maternal (RBC) using the Ørskov reaction. Improvements in this method to obtain suitable samples for IEF analysis are described.

Several hundred antenatal diagnoses of hemoglobinopathies have been performed in the last few years. In most cases, fetal blood was studied in a three-step procedure: incubation of the red blood cells with ³H-Leucine, removal of heme, and finally chromatographic separation of the globin chains.1 The diagnosis was based on the ratio of radioactivity incorporated into the globin chains.2,3

Although a fascinating new approach, using DNA mapping of amniotic fluid cells, has been developed for the antenatal diagnosis of hemoglobinopathies,5,6 it is not yet applicable to all couples at risk for sickle cell disease and for the usual types of β thalassemia.

This paper compares the classical chromatography of labeled globin chains to a nonbiosynthetic method using isoelectric focusing of hemoglobin on acrylamide gel slabs4 to analyze the hemoglobins of pure fetal RBC. IEF is a fast method (90 min) and does not require radioactivity or the sophisticated equipment used for column chromatography and radioactivity measurement. Several samples can be analyzed on one slab. Isoelectric focusing of hemoglobin was compared to chromatography of labeled globin chains for 22 antenatal diagnoses of hemoglobinopathies (11 involving β thalassemia and 11 sickle cell disease) at 18-22 wk of pregnancy. Blood samples obtained after abortion of 22 fetuses homozygous for β thalassemia were also studied by IEF.

Since the study of fetal blood hemoglobins by IEF requires an absence of maternal contamination, maternal cells when present, must be selectively lysed by the Ørskov, Jacobs and Stewart reaction.7 A modification of this method to obtain suitable samples for IEF analysis, even when the proportion of fetal RBC is below 10%, is also described.

MATERIALS AND METHODS

Samples

Thirty-three samples of fetal blood were drawn on heparin at 18-22 wk of gestation, 32 by fetoscopy, and 1 by placentocentesis, in London and in Paris. With consent of the mother, 11 control samples were obtained before abortion for Duchene muscular dystrophy or hemophilia, according to the Helsinki declaration. Twenty-two antenatal diagnoses of hemoglobinopathies were performed, 11 for β thalassemia, and 11 for sickle cell disease. Contamination of the fetal blood by maternal cells was determined by the cytochemical method of Kleihauer (Boehringer Kit, Mannheim, West Germany). In all cases but one, the contamination by maternal cells was below 0.1%. One sample referred to as case No. 6 contained only 2% of fetal red blood cells. Blood samples obtained after abortion in New Haven (Conn.), Cagliari (Italy), and Athens (Greece) of 22 fetuses homozygous for β thalassemia were also studied.

Methods

The red blood cells were washed and incubated with ³H-Leucine as described.24 At the end of the incubation, the cells were washed in NaCl (0.15 M). An aliquot was analyzed by ion exchange chromatography of labeled globin chains.12 A fraction of red blood cells packed at 10,000 g was used for isoelectric focusing. The remaining material was kept frozen at −80°C.

Isoelectric focusing on thin layer has been described earlier.8 Two microliters of packed red blood cells were lysed with 20 μl of water. An aliquot was used to determine the hemoglobin concentration in Drabkin’s solution at 540 nM in a 0.3 ml cuvette. The hemolysate was adjusted to 10 mg Hb/ml with water. Ten microliters were loaded onto a piece of filter paper (Whatman 3) (5 mm × 6 mm). The pieces of paper were placed in a straight line on the surface of the gel, 1.5 cm from the cathode. The gel (250 x 115 x 0.5 mm) was prepared with Ampholines (pH 6.0–8.0 or pH 6.0–9.0) (LKB) and 12 mg potassium cyanide (KCN) on a plastic sheet. Migration was performed on a Multiphor apparatus (LKB) at 10°C using...
constant power (15 W; i.e., 1.0 W/ml of gel). When focusing was complete (75–90 min), the gel was placed in 20% trichloroacetic acid for 5 min, rinsed with water and scanned at 420 nm with a Super Cellomatic (Sebia, Issy les Moulineaux, France). For storage, the gel was transferred to a sheet of filter paper (Whatman 3 mm) and dried overnight at room temperature.

In order to evaluate the amounts of Hb A detectable by IEF, Hb A and Hb F purified by chromatography on Biozex 70 (Biozad) were mixed in various proportions. The final concentration of Hb was 10 mg/ml. The proportion of Hb A increased from 1% to 6%. The proportion of acetylated Hb F present in RBCs of 20-wk-old fetuses was determined by chromatography on Biozex 70 performed as described previously.10

To test the selective lysis of maternal RBC by the Ørskov reaction, based on differences in carbonic anhydrase levels in fetal and adult RBC, fetal blood samples contaminated by maternal cells or artificial mixtures of fetal and adult RBC were lysed according to the method of Boyer7 as modified by Alter.11 The procedure for a cell suspension in 0.15 M NaCl (1.106 cells/ml determined with a Coulter F) was as follows: 0.5 ml of the cell suspension was mixed with 1 ml of acetazolamide (92 μM) and 9 ml of NH4Cl (0.183 mM); 0.5 ml of NH4HCO3 (11.04 mM) was added and mixed after a 2-min delay; lysis was performed at room temperature (22°C–25°C) and the optical density at 600 nm was recorded in order to determine the completion of maternal cells lysis. An aliquot (0.063 ml) of NaCl 1.5 M was then added to stop the lysis of red blood cells, usually after 15 min of lysis. The mixture was subsequently centrifuged at 1100 g and at 4°C for 5 min. The pellet was suspended in 2 ml of decomplemented AB Rh+ serum and layered onto 2 ml of 10% Ficoll 400 (Pharmacia, Uppsala, Sweden), dissolved in NaCl (0.15 M), and centrifuged at 1500 g at 4°C for 10 min in order to separate the membrane ghosts from the intact cells. The pellet of fetal red cells was washed three times with AB Rh+ serum and part of the sample was smeared in order to determine the proportion of intact maternal cells that could be present after the Ørskov reaction. The smear was dried with a fan for 10 min, fixed in 80% ethanol for 5 min, dried again for 10 min, and finally stained with the Betke Kleihauer method using the Boehringer Kit (Mannheim, Germany). Half of the smear was immersed in the Hb A eluting solution for 20 sec. Then the whole slide was dipped in the eosin solution for staining. The other part of the sample was washed with sodium chloride (0.15 M) and submitted to isoelectric focusing as described.

RESULTS

Initially, our goal was to compare the results obtained by IEF to those obtained by classical chro-
matography of globin chains for the prenatal diagnosis of sickle cell disease and $\beta^0$ thalassemia. In such cases, fetuses homozygous for the disease could not exhibit Hb A, whereas heterozygous or normal fetuses at 18–20 wk of gestation would. However, during the course of our study, we observed that two fetuses homozygous for $\beta^+\beta^0$ thalassemia (or $\beta^+\beta^0$ thalassemia) did not exhibit Hb A by IEF in spite of a $\beta^+$ chain synthesis that was greater than 1%. Consequently, we determined the lowest proportion of Hb A detectable by IEF under the conditions described in the method section. We shall next consider the qualitative and the quantitative results obtained by IEF.

The blood samples of 11 fetuses aborted for nonhematologic reasons exhibited Hb A that was easily detectable by IEF since it migrated between Hb F and acetylated Hb F (Hb F Ac) (Fig. 1A). Artificial mixtures of Hb A and of Hb F were used to determine the lowest proportion of Hb A detectable by scanning of a fresh gel at 415 nm or by direct vision of the dried gel. In both conditions, the detection limit of Hb A was 0.9%–1.0% in five experiments. Above this proportion, Hb A was clearly visible.

The IEF pattern of 22 samples of fetal blood obtained for prenatal diagnosis of hemoglobinopathies are shown in Fig. 1. Among the 11 blood samples from fetuses at risk for sickle cell disease, 5 samples (Fig. 1B, cases 1–5) had a normal pattern of Hb A and Hb F with no Hb S. Another 5 samples (cases 6–10) exhibited both Hb A and Hb S in addition to Hbs F, as expected for the heterozygous state. The last one (case 11) showed only Hbs F and S, as expected for the homozygous state of sickle cell disease. In all cases, the result obtained by IEF, a few hours after the fetoscopy, was confirmed 1 day later by the results of the chromatography of the globin chains. Fig. 1C shows the isoelectric focusing pattern of 11 samples of fetal red blood cells obtained for antenatal diagnoses of $\beta$ thalassemia. Samples of 3 fetuses, 2 homozygous for $\beta^+$ thalassemia (or $\beta^0/\beta^+$ thalassemia) (cases 20 and 21) and one homozygous for $\beta^0$ thalassemia (case 22), as identified by chromatography of the labeled globin chains and family studies, did not contain a visible amount of Hb A. In contrast, the normal fetuses or those heterozygous for $\beta$ thalassemia (cases 12–19) exhibited visible amounts of Hb A. Fig. 2 illustrates these observations showing the optical density profile of a normal sample (case 2), of a sample heterozygous

Fig. 2. Optical density recording of isoelectric focusing of 4 fetal blood samples: normal (case 2) heterozygous $\beta$ thalassemia (case 19), homozygous $\beta^+\beta^0$ thalassemia (or $\beta^+\beta^0$ thal) (cases 20 and 21).
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Figure 3. Chromatography of labeled globin chains from blood obtained after abortion of a fetus homozygous for β-thalassemia (N' 21).

for β-thalassemia (case 19) and of two samples from fetuses homozygous for β+ thalassemia (or β+/ββ thalassemia) cases 20 and 21.

Figure 3 shows the radioactivity pattern of the globin chains of a fetus (case 21) after abortion. The β/γ ratio of radioactivity (0.016) confirmed the homozygous state of β-thalassemia. The presence of a very low βA chain synthesis led to the diagnosis of β+-thalassemia Study of the family indicated that three children having Cooley's anemia exhibited βA chain synthesis (results not shown). A pattern of radioactivity similar to that of case 21 was obtained for case 20, both before and after abortion. Therefore, the two fetuses could be diagnosed as having homozygous β+-thalassemia. However, the double heterozygous state β+/ββ thalassemia is also possible and cannot be ruled out in the absence of hemoglobin variants in the two families. The 22 blood samples obtained by several centers of prenatal diagnosis after abortion of fetuses homozygous for β-thalassemia did not contain a detectable amount of Hb A when analyzed by IEF, although 5 of them exhibited from 1% to 1.6% βA chain synthesis.

In addition to its simple detection on IEF, Hb A was also evaluated quantitatively. An initial study failed to obtain the correct proportion of Hb A when Hb F was used as reference, because Hb F was overloaded and was underestimated by scanning. Consequently, Hb A was overestimated and variations were observed from one IEF to another. Lower quantities of artificial mixtures of Hb A and Hb F (3.5 mg/ml) gave better results, but Hb A could not be detected and quantified in a proportion below 2.5% of Hb F.

Recently we have been able to overcome this difficulty. We have observed that the proportion Hb A/Hb F estimated by IEF scanning was in good correlation with the βA/γ ratio of radioactivity of fetal blood samples obtained for prenatal diagnosis, as shown in Fig. 4. The two methods exhibited a high correlation for normal fetuses or those heterozygous for sickle cell disease or β-thalassemia (p < 0.001). The acetylated Hb F corresponds to a constant proportion of Hb F (7.5% ± 0.84%) at 20 wk of gestation as determined by chromatography on Biozex 70 in 12 cases. Consequently, Hb F Ac can be used as a reference to measure the proportion of Hb A by IEF.

The accuracy of the measurement of the O.D. ratio of Hb A/Hb F Ac by IEF scanning is shown in Fig. 5 for the artificial mixture of Hb A and of total Hb F (Hb F + Hb F Ac). The proportion of Hb A was from 1% to 6%.

The absence of maternal contamination in the fetal blood sample is absolutely essential for prenatal study of hemoglobinopathies by IEF. Only one sample of fetal blood contained maternal RBC; the others were thus suitable for direct analysis by IEF. However, in
Fig. 5. Correlation between the known proportion of Hb A in artificial mixtures of Hb A and Hb F and the Hb A/Hb F ratio determined by densitometry at 415 nm of the IEF gel.

spite of consistent improvements of fetal blood sampling by fetoscopy, some samples obtained by this method and many of those obtained by placentocentesis may contain various proportions of maternal cells. Therefore, conditions which induce the complete lysis of maternal cells by the Ørskov reaction were studied in various mixtures of maternal and fetal cells.

The recovery of fetal cells was on the order of 30%. The results, summarized in Table 1, indicated that most frequently, a complete disappearance of maternal cells occurred. However, in a few instances a very small proportion of cells unstained by the Kleihauer procedure was present. These cells could be either intact maternal cells or membrane ghosts induced by the Ørskov reaction and not eliminated during the centrifugation. We used a very simple procedure to distinguish the intact maternal cells from the membrane ghosts. The Kleihauer staining involves two steps: first, the elution of hemoglobin A present in the RBC, and second, the staining by eosin of the hemoglobin that remains in the cells. Consequently, half of the smear was dipped in the Hb A eluting solution and then the whole slide was immersed in the eosin solution. Eosin alone stains all intact maternal and fetal cells, but not the membrane ghosts. Finally, by difference of the proportion of unstained cells in the two parts of the smear, it was possible to evaluate the exact proportion of intact maternal cells remaining after the Ørskov reaction. An example is shown in Fig. 6. In three instances (samples H, I, and J of Table 1), the percentage of the intact maternal cells remaining after the Ørskov reaction was evaluated after the subtraction of membrane ghosts detected on the smear stained by eosin alone. In such cases, the intact maternal cells were in a much lower proportion than that indicated by the Kleihauer staining. For sample J, an artificial mixture of 94% normal adult RBC and 6% fetal cells from an aborted fetus homozygous for sickle cell thalassemia.

Table 1. Enrichment of Fetal RBC by Selective Lysis of Maternal Cells in Blood Samples Obtained for Antenatal Diagnosis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Risk of Pregnancy</th>
<th>Genotype of the Fetus</th>
<th>Initial Proportion of Fetal Cells in %</th>
<th>Final Proportion of Intact Maternal Cells + Membrane Ghosts in %</th>
<th>Final Proportion of Intact Maternal Cells in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>β Thalassemia</td>
<td>A/B Thalassemia</td>
<td>3</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>B</td>
<td>S†</td>
<td>A/S</td>
<td>2</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>β Thalassemia</td>
<td>A/A or A/β Thalassemia</td>
<td>30</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>D</td>
<td>S</td>
<td>A/A</td>
<td>85</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>E</td>
<td>S</td>
<td>A/S</td>
<td>90</td>
<td>0.3</td>
<td>ND</td>
</tr>
<tr>
<td>F‡</td>
<td>Hemophilia</td>
<td>A/A</td>
<td>10</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>G</td>
<td>S</td>
<td>A/S</td>
<td>40</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>H</td>
<td>S</td>
<td>A/S</td>
<td>10</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>I</td>
<td>Hemophilia</td>
<td>A/A</td>
<td>5</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>J**‡</td>
<td>S/S</td>
<td></td>
<td>5</td>
<td>1.5</td>
<td>0.2</td>
</tr>
<tr>
<td>K*</td>
<td>β* Thalassemia (homozygote)</td>
<td></td>
<td>6</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mixture of RBC from a normal adult and RBC from a fetus homozygous for "J" sickle cell disease "K" β* thalassemia obtained after abortion in both cases.
†Sickle cell disease.
‡Artificial mixture.
§Sample 6 in Fig. 1 and in text p 4 and p 11.
ND, not determined.
disease, isoelectric focusing did not allow the detection of Hb A after the Ørskov reaction. A similar absence of Hb A was observed after the Ørskov reaction for sample K, an artificial mixture of normal adult RBC and of cells of a fetus homozygous for βthalassemia.

DISCUSSION

Isoelectric focusing of hemoglobin and chromatography of labeled globin chains provided identical results for 11 fetuses at risk for sickle cell disease. The diagnosis was confirmed after birth in 10 cases and after abortion for the fetus found to be homozygous for sickle cell disease.

IEF and chromatography provided concordant results for 11 pregnancies at risk for βthalassemia. Both methods made it possible to distinguish 3 fetuses homozygous for βthalassemia from normal fetuses or heterozygotes (8 cases). In addition, IEF did not detect Hb A in blood samples obtained after abortion of 22 fetuses homozygous for βthalassemia. Eight were ascertained to be homozygous for βthalassemia by family study, five exhibited βchain synthesis by chromatography. This group of five fetuses may have the genotype βthal/βthal or βthal/βthal. The last nine fetuses did not exhibit detectable amounts of β chains synthesis; the family studies of globin chain synthesis were not performed. Consequently, the genotype of these fetuses could be (βthal/βthal, βthal/βthal or βthal/βthal). In addition, these cases derive from various populations in which the βthalassemic defects are probably heterogeneous. In spite of the absence of Hb A detectable by IEF in the samples of these 22 fetuses, more experience is needed to determine the proportion of Hb A by IEF and the limits of this method in fetuses exhibiting various combinations of βthalassemic defects.

In contrast, Hb A was detectable in blood samples of normal fetuses or of those heterozygous for βthalassemia. The correlation between the Hb A/Hb F Ac ratio obtained by IEF and the β/γ ratio of globin chain synthesis indicates that Hb F Ac is present as a constant proportion of Hb F. This observation was confirmed by chromatography on Biozex 70.

Artificial mixtures of Hb F and Hb A were analyzed by isoelectric focusing on five occasions. Hb A in a proportion below 0.9% was not detectable by IEF.
These results led us to conclude that the red blood cells of the two fetuses homozygous for \( \beta^+ \) thalassemia (or \( \beta^0/\beta^+ \) thalassemia) (cases 20 and 21) exhibited a proportion of Hb A below 0.9%, slightly lower than expected according to the synthesis of the \( \beta^0 \) chain in the reticulocytes of the same blood sample (case 21: \( \beta/\gamma = 0.016 \), case 20: \( \beta/\gamma = 0.012 \)).

This small discrepancy could be the consequence of differences in the sensitivity of the methods used to evaluate the \( \beta^0 \) chain synthesis in reticulocytes and the proportion of Hb A in the total RBC population. In some circumstances, the \( \beta \) chain synthesis may be slightly overestimated by chromatography when present in very low proportion. Another explanation could be a difference in the proportion of Hb A synthesis in reticulocytes and the mean proportion of Hb A present in the total RBC population at a given time of fetal development.\(^1\)

When Hb A was detectable by IEF, the Hb A/Hb F Ac ratio obtained by IEF and the \( \beta^0/\gamma \) ratio of globin chain synthesis were well correlated. However, the distinction between normal fetuses and those heterozygous for \( \beta \) thalassemia is sometimes difficult because the ratios of the two populations overlap each other with both methods.

One of the main problems in the antenatal diagnosis of hemoglobinopathies is the contamination of fetal blood by maternal red cells. Several means are used to overcome this difficulty, such as dilution and extrapolation to zero contamination,\(^1\) subtraction\(^1\) repression of maternal erythropoiesis by transfusion before sampling,\(^2\) differential agglutination of the fetal red cells using the i antigen,\(^3\) and selective hemolysis by the \( \text{\AA}rskov \) reaction.\(^7\,\,11\)

It must be emphasized that isoelectric focusing of fetal blood samples necessitates a specific approach in order to solve the problem of maternal blood contamination. The maternal cells cannot be present in a proportion as high as 0.5%. Consequently, evaluation of the proportion of maternal red cells has to be performed by a careful Kleihauer staining on all samples. The use of the \( \text{\AA}rskov \) reaction as described by Boyer\(^7\) and by Alter\(^1\) and modified as described in this paper, proved to be efficient for sample 6, which was obtained by placentocentesis and contained only 2% of fetal red cells.

As shown in Table 1, a complete disappearance of maternal cells and of cell membranes after the \( \text{\AA}rskov \) reaction could be obtained 8 out of 14 times. Three other times (cases H–J), most of the contaminating cells were not intact maternal cells but membrane ghosts, and the samples were suitable for IEF analysis. Another three times, the distinction between intact maternal cells and membrane ghosts was not made and the proportion of intact maternal cells could be overestimated.

Cells from women at 20 wk of pregnancy (A/A, A/S, or heterozygous for \( \beta \) thalassemia) are similarly susceptible to the \( \text{\AA}rskov \) lysis. However, the observed variation of the half-time of lysis of maternal cells indicates that the kinetics of maternal cell lysis has to be determined in all cases before the study of the fetal blood samples contaminated by maternal cells.

In conclusion, IEF was compared to classical chromatography of globin chains for the prenatal diagnosis of hemoglobinopathies. In all cases, identical results were obtained by both methods and the diagnosis was confirmed at birth when possible. Isoelectric focusing was reliable for the antenatal diagnosis of sickle cell disease and \( \beta^0 \) thalassemia. Pure fetal red blood cells must be obtained directly by fetoscopy or after the selective lysis of all maternal cells. Further studies are needed in the field of \( \beta^+ \) thalassemia in order to evaluate the limits of this method, in particular with regard to the various combinations of \( \beta \) thalassemias that may occur in different populations.

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