Prenatal Diagnosis of Classic Hemophilia (Hemophilia A) by Immunoradiometric Assays

By Leon W. Hoyer, Carl A. Carta, Mitchell S. Golbus, John C. Hobbins, and Maurice J. Mahoney

During the period from 1978 to 1983, 92 pregnancies have been evaluated by fetoscopy for the prenatal diagnosis of hemophilia A. Satisfactory fetal plasma samples were obtained in 80 instances and the diagnosis—or exclusion—of hemophilia was made by immunoradiometric assay of the factor VIII coagulant protein (VIII:C). The accuracy of the diagnosis established by fetoscopy has been verified after delivery or termination, and there have been no misdiagnoses resulting from laboratory error. Additional evidence for the accuracy of the analysis was the observation that the frequency of hemophilia in pregnancies of obligate carriers of the hemophilia gene, and of women whose plasma assays were indicative of the carrier state, was 29 of 59 fetuses at risk. In one case of cross-reacting antibodies, whose plasma assays were indicative of the carrier state.

The development of reliable and reasonably safe methods for obtaining fetal plasma samples has permitted the prenatal diagnosis of a number of severe hereditary diseases. One of them, classic hemophilia (hemophilia A), is an X-linked deficiency of coagulation factor VIII (antihemophilic factor, VIII:C) that affects approximately one in every 10,000 men. The severity of the factor VIII deficiency is generally similar within families, and the plasma has less than 1% of the normal factor VIII level in half of these patients. These boys have frequent spontaneous or traumatic hemorrhages, and they require plasma derivatives to treat bleeding episodes. In addition, there is often progressive joint damage. Although the development of factor VIII concentrates, home transfusion programs, and comprehensive care centers have markedly reduced the frequency of hospital visits and have modified to some extent the impact of the disease, hemophilia continues to represent an emotional, financial, and physical burden for the patient and his family.

Because the genetic transmission of hemophilia is well recognized, families of patients with hemophilia are concerned about the possible transmission of this severe bleeding disorder. The development of fetoscopy with fetal blood sampling and immunologic assays for factor VIII have made it possible to evaluate those pregnancies that are at risk for transmission of hemophilia, and we and others have demonstrated the feasibility of prenatal diagnosis for this condition.

This report summarizes six years' experience, in which prenatal diagnosis of classic hemophilia was carried out using the techniques first described in 1979. We can now identify the frequency with which such evaluation is sought and document the safety and accuracy of the procedure.

MATERIALS AND METHODS

Patients

Women were referred for prenatal diagnosis of hemophilia by genetic counselors, obstetricians, and hematologists throughout the United States and Canada. Before fetoscopy was considered, the family history was carefully reviewed, and the diagnosis was verified. The likelihood of the hemophilia carrier state was evaluated by family history and by coagulation and immunologic assays of the woman's plasma. Because these techniques do not identify all obligate carriers, normal plasma values do not exclude the hemophilia carrier state and they did not preclude fetoscopy in women who were potential carriers by family history. Whenever possible, a plasma sample was obtained from an affected male relative to determine if that family's hemophilia was caused by the synthesis of nonfunctional cross-reacting material (CRM)-positive protein. Amniotic fluid was obtained at 16 weeks of gestation to determine the sex of the fetus. Two women presented so late in pregnancy that this step was not feasible, and fetal sex was determined by ultrasound evaluation and by cytologic staining for X or Y chromatin in uncultured amniotic fluid cells.

A total of 92 pregnancies have been evaluated during the period from 1978 through 1983. All but four of the procedures were performed at Yale-New Haven Hospital or at the University of California, San Francisco. Two fetoscopies were done for prenatal evaluation of hemophilia by Tracy Perry at the Royal Victoria Hospital, Montreal, and two were done by Robert Carpenter at...
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Baylor University Hospital, Houston. In all cases, the plasma and fetoscopy samples were evaluated at the University of Connecticut Health Center.

Methods

In utero blood samples were obtained by fetoscopy at 18 to 21 weeks of gestation, and amniotic fluid samples were taken before umbilical vessel puncture. Direct puncture of a fetal vessel was regularly attempted, and pure fetal blood or mixtures of amniotic fluid and fetal blood were aspirated under direct vision through 26-gauge needles into plastic syringes containing 3.8% sodium citrate. The estimated dilution of fetal blood in amniotic fluid was calculated assuming a fetal hematocrit of 37% and a fetal RBC count of $2.5 \times 10^9$ per microliter. Evidence that the fetal blood samples were uncontaminated by maternal blood was obtained by Kleihauer-Betke fetal hemoglobin stain and by Coulter Channelyser (Hialeah, Fla) cell-size distribution. After removal of erythrocytes, these plasma samples were frozen at $-70^\circ$C until assays were performed. When pregnancies were terminated, abortus blood (0.5 to 1.0 mL) was collected into 0.1 mL of 3.8% citrate.

Factor VIII coagulant antigen (VIII:CaG) and factor VIII-related antigen (VIIIIR:Ag) were measured by the fluid-phase immunoradiometric assays that have been previously described. Values for VIII:CaG and VIIIIR:Ag are expressed as units per deciliter, with pooled normal plasma, prepared as previously described, the standard (100 U/dL). Plasma obtained by fetoscopy from 49 normal male fetuses at 18 to 20 weeks' gestation had a mean VIII:CaG of $33.1 \pm 2.4$ (SE) with a range from 7 to 91 U/dL, a mean VIIIIR:Ag of 101.5 ± 6.1 with a range of 28 to 227 U/dL, and a mean ratio of the two assays of 0.33 ± 0.02 with a range of 0.18 to 0.60. The lower limit of VIII:CaG detection by our assay is 0.8 U/dL and this value must be multiplied by the dilution of fetal plasma with amniotic fluid to calculate the sensitivity of the assay for evaluating a fetus at risk for hemophilia.

RESULTS

The evaluation of fetuses at risk for classic hemophilia requires fetoscopy to obtain fetal plasma for analysis. Fetoscopies were performed initially in New Haven, and patients traveled there from all sections of the United States and from two foreign countries. Since 1981, fetoscopic prenatal diagnosis of hemophilia has also been carried out regularly in San Francisco, and a small number of diagnostic procedures have been done in Montreal and Houston. Frozen fetal plasma was sent to Farmington, Conn, for analysis in every case, and assay results have been available within 72 hours of the procedure. The rapid evaluation is important because these pregnancies are at 18 to 20 weeks, and the decision about continuation or termination must be made without delay.

Our total experience over the past six years is summarized in Table 1 and Fig 1. All of these procedures were carried out for women who were obligate or potential carriers of severe hemophilia A. The initial fetoscopy did not yield a satisfactory sample of fetal blood in 15 instances, most of them during the first three years of the program, but repeat fetoscopies were successful in three of the four pregnancies in which they were done. Thus, a satisfactory fetal plasma sample was obtained in 80 of the 92 pregnancies. Fetal VIII:CaG and VIIIIR:Ag values were compared with those previously determined for normal fetuses at 18 to 20 weeks' gestation, and the ratio of the two values was especially important because the magnitude of plasma dilution with amniotic fluid was estimated from the fetal RBC count.

Fifty-one of the eighty samples had normal VIII:CaG levels and were presumed to be from normal fetuses. This was subsequently verified by clinical evaluation and/or laboratory testing in 46 of the infants, but could not be verified in three cases because of fetal loss. In one instance, in evaluation of a pregnancy for a hemophilia carrier who had no living hemophilic relatives available who could provide a plasma sample for analysis, both the fetoscopy sample and the infant's plasma had normal levels of VIII:CaG and VIIIIR:Ag but the infant had mild (VIII:C, 17

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Table 1. Prenatal Diagnosis of Hemophilia From 1978 to 1983

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>New Haven</th>
<th>San Francisco</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancies evaluated</td>
<td>68</td>
<td>20</td>
<td>4</td>
<td>92</td>
</tr>
<tr>
<td>Outcome of fetoscopy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIII:CaG within normal range</td>
<td>40</td>
<td>8</td>
<td>3</td>
<td>51</td>
</tr>
<tr>
<td>Verified after delivery by VIII:C or VIII:CaG assays</td>
<td>26</td>
<td>1</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Verified clinically</td>
<td>12</td>
<td>6</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>No follow-up data because of fetal loss</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>No detectable VIII:CaG</td>
<td>23</td>
<td>5</td>
<td>1</td>
<td>29</td>
</tr>
<tr>
<td>Hemophilia verified by assay of a blood sample from the abortus</td>
<td>21</td>
<td>3</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>No sample obtained</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Unsatisfactory sample</td>
<td>5</td>
<td>7</td>
<td>0</td>
<td>12</td>
</tr>
</tbody>
</table>

*Two fetoscopies were done in Montreal and two in Houston.
†No increased bleeding or bruising was noted at 1 to 5 years of age.
‡Two separate fetoscopies were done during one of these pregnancies, and a single procedure was done in the other 11 instances. Eleven of these pregnancies were terminated; one was continued with the delivery of a normal male. A satisfactory fetal plasma sample was obtained when a second fetoscopy was done in three other pregnancies.

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Fig 1. The outcome of 92 pregnancies in which fetoscopy was done for prenatal diagnosis of hemophilia.
U/dL) CRM-positive hemophilia A. In this family, the assay values could not differentiate a normal fetus from one affected with CRM-positive hemophilia because there was no proband sample to exclude the CRM-positive condition.

In the other 29 fetoscopies, no VIII:CAg was detected in the plasma–amniotic fluid mixture, and the dilution factor was sufficiently low that a VIII:CAg/VIIIR:Ag ratio greater than 0.15 was excluded. One of these 29 pregnancies was continued to term with delivery of a hemophilic infant. The remaining pregnancies were terminated, and the absence of detectable VIII:CAg was verified in undiluted postmortem blood samples in 23 of the 28 instances.

The outcome of the fetoscopic analysis was also compared to the predicted likelihood of hemophilia in these pregnancies (Table 2). When all of the pregnancies at 50% risk for a hemophilic offspring were analyzed—the obligate carrier women and those with plasma assays indicating that they were hemophilia carriers—there were 29 hemophilic fetuses and 30 fetuses with normal values.

Although fetoscopy for prenatal diagnosis of hemophilia is not without risk to the fetus, the complication rate was similar to that observed for other conditions.1,3 There were five fetal deaths within seven days of the fetoscopy in the 55 pregnancies that were not intentionally terminated because the fetal plasma had no detectable VIII:CAg or because it was not possible to obtain a fetal plasma sample that was satisfactory for analysis (Fig 1). Three of these fetuses had normal VIII:CAg values in the fetoscopy sample. In addition, two fetal deaths occurred in utero before the diagnosis of hemophilia was established by plasma assays. This risk, approximately 9% overall and 6% (3/52) in normal fetuses, must be recognized when counseling women whose likelihood of being a hemophilia carrier is small when family history and laboratory studies are analyzed. In addition, there were five premature births—at 26, 28, 30, 32, and 34 weeks—in the 50 pregnancies that continued past the immediate postfetoscopy period.

A longitudinal examination of our data provides some indication of the level of interest in hemophilia prenatal diagnosis in the United States and Canada (Table 3). Table 3 also demonstrates the marked improvement over time in the percentage of fetoscopies that were successful and in the concentration of the fetal plasma in the samples that were obtained. It is apparent that relatively few women seek hemophilia prenatal diagnosis, for to the best of our knowledge, our experience encompasses all studies done in North America.

Our experience with a single instance of CRM-positive hemophilia demonstrates the importance of VIII:CAg analysis of a proband sample. Although this has been possible in most cases (Table 4), there have been instances in which there was no living relative with hemophilia or in which the family situation made it impossible to obtain a plasma sample from an affected relative.

During the course of the six years, eight women have had fetoscopic evaluation of two successive pregnancies (Table 5). In five cases, the first pregnancy had been terminated because the fetus was hemophilic, and two other pregnancies had been terminated because it was not possible to obtain a satisfactory sample that

| Table 2. Relationship of Fetoscopy Outcome to Likelihood of Hemophilia |
|---------------------------|-------------------|-------------------|-------------------|
| Likelihood That Woman Is Hemophilia Carrier | Normal VIII:CAg | No VIII:CAg | Sample |
| Obligate carrier | 13 | 18 | 5 |
| Woman with >0.8 likelihood of being a hemophilia carrier | 17 | 11 | 1 |
| Woman with <0.2 likelihood of being a hemophilia carrier | 21 | 0 | 4 |

The carrier status of 90 women undergoing fetoscopy was evaluated by family history and by measuring plasma VIII:CAg and VIIIR:Ag at the time of fetoscopy.11 In two women in whom fetal plasma was not obtained by fetoscopy, no maternal sample was obtained for carrier analysis.

| Table 3. Dilution of Fetal Plasma With Amniotic Fluid in Fetoscopy Samples |
|---------------------------|-------------------|-------------------|-------------------|
| Undiluted | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 4 | 0 |
| 1.2–2.5 | 0 | 2 | 0 | 3 | 0 | 1 | 2 | 2 | 1 | 1 |
| 2.6–10 | 2 | 7 | 0 | 14 | 1 | 7 | 1 | 7 | 0 | 4(1) |
| Total No. of fetoscopies | 9 | 16 | 20 | 16 | 15 | 16 |

The parentheses indicate the number of samples that were unsatisfactory for diagnostic purposes. One of the 1978 pregnancies was evaluated twice by fetoscopy, but neither sample was satisfactory. NH, Yale-New Haven Hospital; SF, University of California, San Francisco.

| Table 4. Evaluation of CRM Status |
|---------------------------|-------------------|-------------------|-------------------|
| CRM Status | No. of Pregnancies |
| Proband available and tested (CRM-negative) | 55 |
| No proband available | 25 |
| Maternal plasma immunoassays indicate that she is a CRM-negative hemophilia carrier | 19 |
| No detectable VIII:CAg in fetal plasma | 12 |
| Normal VIII:CAg in fetal plasma | 7 |
| Normal maternal plasma immunoassays | 6 |
| Normal VIII:CAg in fetal plasma | 6* |

This table includes only the 80 pregnancies for which an evaluable fetal sample was obtained by fetoscopy.

*One of the six fetuses had CRM-positive hemophilia.
could exclude hemophilia. Normal fetal plasma was identified in five of the subsequent pregnancies, and in a sixth instance the pregnancy was continued to term even though the fetoscopy did not yield a satisfactory plasma sample.

One additional pregnancy (not included in Table I) was evaluated by fetoscopy for factor VIII analysis during this period. In that case, both parents had mild von Willebrand’s disease, and they had had one child with the severe (homozygous) form of that disorder. To avoid a second instance of severe von Willebrand’s disease, fetoscopic evaluation was carried out at 19 weeks’ gestation. Fetal plasma VIIIIR:Ag was 142 U/dL, and the absence of severe von Willebrand’s disease was verified after delivery as it had been in one previous instance.17

DISCUSSION

Prenatal diagnosis of hemophilia requires safe collection of fetal plasma samples and accurate measurements that can establish that the fetus does or does not have hemophilia. Since our initial report of prenatal diagnosis of hemophilia in 1979,8 we have continued to offer this diagnostic procedure to families in which pregnant women are obligate or potential carriers of the hemophilia gene. Although abortion of affected fetuses is not acceptable to many women, antenatal diagnosis and selective termination of affected pregnancies is, at the present time, the only practical way of preventing hemophilia if a family wishes to have unaffected children.

The need for fetal plasma to identify hemophilia is a complicating factor, for, at present, fetal cells from amniotic fluid cannot provide the necessary information. Moreover, the sensitivity of available immunoassays requires that the fetal blood sample be at least 10% of the amniotic fluid–fetal blood mixture. As a result, several pregnancies were not evaluated because it was not possible to obtain a satisfactory fetal blood sample. Although 86% of pregnancies could be evaluated at the two centers during the six-year period, including three instances in which the fetal sample was obtained by a second fetoscopy, there has been some increase in the success rate with experience (Table 3). During the past four years, 92% of fetoscopies have obtained a satisfactory sample in New Haven (45/49) and 72% (13/18) have yielded evaluable samples in San Francisco.

Although undiluted fetal blood samples have been obtained in some instances, especially during 1983, the frequency with which there was amniotic fluid contamination made it impossible to evaluate fetal plasma routinely by coagulation assays. The confounding effect of even small amounts of amniotic fluid makes VIII:C assays suspect, and we have depended on VIII:C measurements in all instances. Although the evaluation of dilute samples by immunoassay has been criticized,19 our experience demonstrates the accuracy of immunologic diagnosis of severe (CRM-negative) hemophilia when fetoscopy samples are diluted as much as tenfold with amniotic fluid (Table 1).

The fetoscopy diagnosis was verified after delivery for 48 fetuses whose plasma VIII:C was within the normal range. The only instances in which follow-up data were not available were the three pregnancies in which a miscarriage occurred shortly after the procedure. In addition, the absence of VIII:C in fetoscopy samples was verified in undiluted fetal blood samples in 24 of 29 instances. The other five pregnancies were terminated after the women had returned to the care of the referring obstetrician and a fetal blood sample was not obtained.

The frequency of hemophilia in those pregnancies with a 50% likelihood of a hemophilic fetus (Table 2) also verified the accuracy of hemophilia identification in utero. The number of hemophilic fetuses (29) was exactly what one would expect for 59 fetuses with a 50% likelihood of hemophilia.

The only diagnostic problem in this series was CRM-positive hemophilia in the child of a woman whose evaluation was incomplete in that there was no proband sample for analysis. This underscores the need to test a proband sample for any woman considering fetoscopy for prenatal diagnosis of hemophilia. The
only exception would be for those women whose plasma VIII:C:Ag and VIIIR:Ag values are sufficiently characteristic of a CRM-negative hemophilia carrier that the likelihood of a CRM-positive hemophilic son is remote. As is apparent in Table 4, only six of 80 pregnancies did not meet these criteria. In one of these six cases, CRM-positive hemophilia was diagnosed after delivery. Although this family had been informed of the inherent limitations of prenatal diagnosis in the absence of a proband sample, they accepted the 10% risk that the fetus would have CRM-positive hemophilia in preference to abortion. If undiluted fetal plasma could have been obtained in this situation, accurate evaluation by coagulation assays would have permitted antenatal evaluation. Unfortunately, amniotic fluid contamination prevented accurate evaluation of this pregnancy by coagulation assays. Although all published information about severe (VIII:C < 1 U/dL) hemophilia indicates that the CRM-positive state is extremely uncommon—it has been identified only once in several large series—the characterization as “severe” may be inaccurate because of the difficulty in measuring low levels of VIII:C with precision. For this reason, we emphasize to families that there is a 10% possibility of CRM-positive hemophilia, the incidence in mild and moderate disease, unless it has been excluded by analysis of a proband sample or of the maternal plasma.

Although fetoscopy is sought infrequently (Table 1), it is of interest to note the frequency with which women have had two fetoscopies for prenatal diagnosis (Table 5). In five of the seven instances in which the initial procedure identified hemophilia or was inadequate so that the pregnancy was terminated, the subsequent fetoscopy identified a normal fetus and that pregnancy was continued because hemophilia had been excluded. This pattern indicates the importance of prenatal diagnosis in some families and it provides a strong rationale for the continuing availability of this procedure. Although fetoscopy is sought by only a small fraction of the nearly 500 hemophilia carrier women who are pregnant each year in the United States, the low utilization (approximately 3%) may be related to uncertainty about the safety and accuracy of the procedure. Our experience over the past six years addresses these questions and offers better information than that previously available. Although there have been no maternal complications from the fetoscopies, there is a significant risk to normal fetuses from the procedure, approximately 6% (3/52). The frequency of premature deliveries in the other 49 pregnancies (10%) was similar to that in normal pregnancies not undergoing fetoscopy.

The isolation and cloning of the factor VIII gene should make prenatal diagnosis possible by gene analysis and thereby reduce the requirement for fetal blood sampling. Molecular diagnosis will use DNA extracted either from amniocytes obtained during the second trimester or chorionic tissue obtained by biopsy during the first trimester. It will depend on characterization of mutant factor VIII genes or on linked polymorphisms within or adjacent to the factor VIII gene. An example, a clinically useful X chromosome restriction fragment length polymorphism, has recently been described, and this approach will be applicable for those families in which the linkage relationship can be established and in which the pregnant woman is heterozygous. Because the molecular defect in hemophilia will undoubtedly be heterogeneous—and affected family members will not always be available for proband studies (Table 4)—there will be some families for whom prenatal diagnosis will not be possible by DNA analysis. This is the situation for the thalassemias today. For these families, fetoscopy will continue to provide a method for accurate diagnosis. Its availability is an option that should be recognized by all who are responsible for genetic counseling of families with hemophilia.

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