

Prenatal Diagnosis of Hemophilia B by an Immunoradiometric Assay of Factor IX

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An immunoradiometric assay of factor IX was developed based on homologous antibodies that arose in a hemophilic patient. With this assay, 11 of 12 patients with severe hemophilia B had factor IX antigen levels below 1 U/dl and 6 patients with mild hemophilia B had various levels. Factor IX antigen in 8 fetuses (16th–20th gestational week) aborted for therapeutic reasons ranged from 1.8 to 10.0 U/dl. Six amniotic fluids contained 0.28–1.2 U/dl factor IX antigen. Using the immunoradiometric assay, we could diagnose hemophilia B prenatally in one fetus at risk. No factor IX antigen (<0.2 U/dl) was detectable in the fetoscopic sample. After termination of the pregnancy, analysis of blood from the abortus confirmed the diagnosis of severe hemophilia B. We conclude that very sensitive immunologic assays, such as the one described here, will prove useful in prenatal diagnosis of severe hemophilia B, since determination of factor IX activity in fetoscopic samples is unreliable because of possible contamination with thromboplastic material.

MANY WOMEN who are known carriers of hemophilia have chosen to terminate their pregnancies with male fetuses to avoid the possibility of having a hemophilic son. This line of action entails a 50% risk of aborting a healthy fetus. However, the development of sensitive immunoradiometric assays for the antihemophilic factor A (factor VIII) and the improvement in the techniques for obtaining fetal blood have made prenatal diagnosis of hemophilia A possible in fetuses at risk.¹⁻⁴ Immunologic assays are preferable to coagulant assays, since they are not influenced by thromboplastic materials in the fetoscopic samples. We have developed an immunoradiometric assay for factor IX that seems to be more suitable for prenatal diagnostic purposes than hitherto available immunologic assays of factor IX. This article describes the new method and its clinical application in our first prenatal examination for factor IX in a fetus at risk for hemophilia B.

MATERIALS AND METHODS

Diagnostic Case

The patient was a woman, born in 1954, who had recurrent hemarthroses and other bleeding symptoms during childhood. In 1960, female hemophilia B was diagnosed with a factor IX activity that was only 2% of normal.⁵ It was first thought that the patient might be a homozygote for the hemophilia B gene. If so, it would mean that any son she might have would be a hemophiliac. During pregnancy in 1977, the woman declined genetic counseling. She delivered a son who turned out to be healthy. It thus became evident that she was a heterozygous carrier of hemophilia B with an unusually low level of factor IX.⁶ She became pregnant again, and when fetal sexing showed a male fetus, the woman decided to proceed with prenatal diagnosis on fetal blood obtained at fetoscopy.

Fetal blood for factor IX determination was obtained in the 19th week of gestation. Ultrasound scanning showed that the placenta covered the entire anterior uterine wall. The fetoscope (Dyonics Needlescope) was therefore inserted transvaginally.⁷ A sample of amniotic fluid was collected through the fetoscope. Two placental vessels were punctured and mixed samples (0.6–0.8 ml) of blood and amniotic fluid were aspirated into plastic syringes containing 0.2 ml of 3.8% (0.13 M) trisodium citrate solution. Evidence that the fetal

blood samples were uncontaminated by maternal blood was obtained by the Kleihauer-Betke fetal hemoglobin stain. Erythrocyte volume fraction-hematocrit (EVF) was measured gravimetrically to estimate the dilution factor of the sample, assuming a fetal EVF of 37%.^{8,9}

Immediately after fetoscopy, the woman was given factor IX concentrate intravenously.

Controls

Amniotic fluid for factor IX determination was collected from women admitted for therapeutic abortion at 16–20 wk gestation. After the pregnancy had been terminated by hysterotomy, the fetal blood was sampled either by catheterization of one of the umbilical arteries (nos. 1–3 in Table 2) or by free flow from the pendant umbilical cord. In addition, factor IX was determined in one sample obtained at fetoscopy of a fetus at risk for hemophilia A but found to be normal. The fetoscopic sample in this case had a volume of 0.9 ml, which was made up to 1.1 ml by addition of 0.2 ml citrate solution.

Hemophiliacs

The immunoradiometric assay of factor IX was tested on 12 patients with severe hemophilia B from 11 different kindreds and on 6 patients with moderate or mild hemophilia B.

Immunoradiometric Assay of Factor IX

Factor IX antigen was determined with a new immunoradiometric assay. Serum from a patient with hemophilia B, who had developed an inhibitor to factor IX, was used as a source of antibody. The serum had no detectable antibodies to other plasma

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proteins, but a low titer (1:1) against HB_sAg (RIA test). The titer against factor IX was 5000 U/dl (1 U being the amount that neutralizes 1 U of factor IX during incubation for 2 hr at 37°C).

IgG from the serum was isolated with protein-A-Sepharose (Pharmacia Fine Chemicals) chromatography. Five milliliters of serum was applied to a 5-ml column. The column was washed with 0.5 M NaCl and the IgG eluted with 0.1 M glycine-HCl buffer, pH 2.5, and dialyzed against 0.04 M phosphate buffer, 0.1 M NaCl, pH 7.4. IgG (0.25 mg) containing 1 inhibitor unit was labeled with 37 MBq (megabecquerel) ¹²⁵I with the lactoperoxidase method.

The specific labeled antibody to factor IX was purified by complexing it with insolubilized factor IX and elution at acid pH according to the following procedure. Sixty milligrams of factor IX concentrate powder (Preconativ, Kabi) was dissolved in 4 ml 0.1 M bicarbonate buffer, 0.5 M NaCl, pH 8.0, and added to 6 ml CH-activated Sepharose 4B (Pharmacia Fine Chemicals). The coupling was allowed to proceed for 2 hr at room temperature. The supernatant was removed, and unreacted groups in the gel were blocked by addition of 0.1 M Tris-HCl buffer, pH 8.0. The gel was then washed until A₂₈₀ in the wash approached zero. The labeled IgG was added to the gel and incubated over night at 4°C. The gel was then washed first with phosphate buffer, pH 7.4, and then with 0.1 M Tris-HCl, pH 9.0, followed by 0.1 M acetate, 0.5 M NaCl, pH 4.75. Finally, the specific labeled antibody was eluted with 0.1 M glycine-HCl buffer, 1% bovine serum albumin, pH 3.0.

The test was performed in the following way: 55 × 11 mm polystyrene tubes (Ellerman tubes, Medart, Sweden) were coated with IgG from the serum by incubation with 0.6 ml of a dilution 1:1000 in 0.1 M carbonate buffer, pH 9.6, for 12 hr at 4°C. The tubes were washed 3 times with 1 ml of phosphate-buffered saline, 0.1% bovine serum albumin. The samples to be tested in various dilutions with 0.01 M phosphate buffer, 0.1 M NaCl, 6% BSA, pH 7.4, and in a total volume of 0.4 ml were then incubated in the tubes for 12 hr at 23°C. The tubes were again washed 3 times, and 0.4 ml labeled antibody solution was added. The labeled antibody was diluted 20–40 times with 0.01 M phosphate buffer, 0.1 M NaCl, containing 0.1% BSA, 0.05% normal IgG (Kabi) to about 10,000 cpm/0.4 ml. After reincubation for 12 hr at 23°C and washing 3 times with 1 ml of water, the tubes were counted in a gamma scintillator.

The immunoradiometric dose-response curve proved to be nearly linear for normal plasma in dilutions 1/128–1/2048 with an amount of bound radioactivity ranging from 25% to 3% (Fig. 1). The sensitivity limit of the method was thus 0.05 U/dl or even lower. To test the accuracy at very low levels, pooled normal plasma was diluted 1:1000 and 1:2000 with 0.01 M phosphate buffer, 0.1 M NaCl, 6% BSA, pH 7.4, and each dilution was tested 20 times. For plasma dilution 1:1000, the mean value was 0.090 ± 0.008 (SD) U/dl, and for dilution 1:2000, it was 0.039 ± 0.005 (SD) U/dl. To test the reproducibility at various levels, pooled normal plasma was diluted 1:10, 1:100, and 1:1000 with the same buffer, frozen, and tested on 10 different days. The means and standard deviations were 8.9 ± 0.57, 0.88 ± 0.04, and 0.095 ± 0.009 U/dl and the coefficients of variation 6.4%, 4.5%, and 9.5%, respectively.

Factor IX Coagulant Activity

Factor IX coagulant activity was determined with a one-stage recalcification assay described by Nilsson.¹²

RESULTS

Factor IX antigen was measured with the immunoradiometric assay in 24 normals. The mean and standard deviation were 108 ± 27 U/dl. Factor IX activity

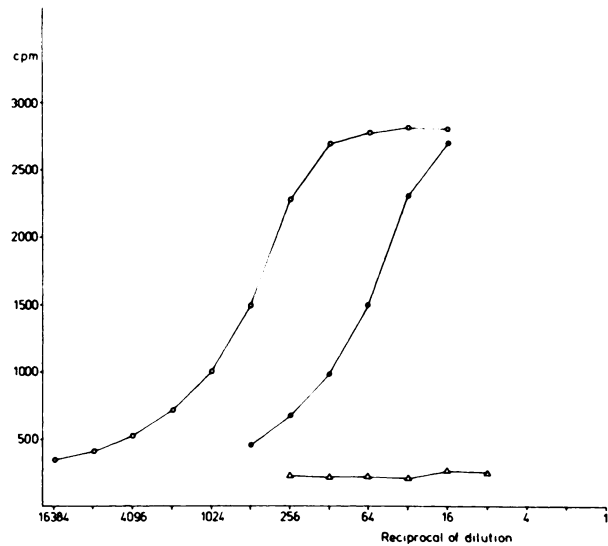


Fig. 1. Immunoradiometric assay of factor IX. The dilution curves for pooled normal plasma (○—○), a patient with severe hemophilia B (△—△), and a patient with mild hemophilia B (●—●) are shown. Counts per minute (cpm) are plotted against the reciprocal of plasma dilution.

in the same samples was 95 ± 24 U/dl. The coefficient of correlation between antigen and activity was 0.74 ($p < 0.001$). The results in 18 patients with hemophilia B tested are shown in Table 1, and dose-response curves in one case of severe and one case of mild hemophilia B in Fig. 1. Of 12 patients with severe hemophilia B (factor IX activity < 1 U/dl), 11 had a factor IX antigen below 1 U/dl. The twelfth patient had received an infusion of factor IX concentrate 5 days before being tested and had a level of 2.5 U/dl.

Table 1. Factor IX in Plasma From 18 Patients With Hemophilia B

Patient No.	Factor IX Antigen (U/dl)	Factor IX Coagulant Activity (U/dl)
1	<0.1	<1
2	<0.1	<1
3	<0.1	<1
4	<0.1	<1
5	<0.1	<1
6	0.04	<1
7	0.1	<1
8	0.2	<1
9	0.5	<1
10	0.5	<1
11	0.9	5*
12	2.4	6*
13	35	2
14	1.7	3
15	79	4
16	115	6
17	16	12
18	12	15

*The patient had received a factor IX infusion 5 days before being tested.

Table 2. Factor IX in the Controls

Sample	Gestational Age in Weeks	Fetal Plasma		Amniotic Fluid Factor IX Antigen (U/dl)
		Factor IX Antigen (U/dl)	Factor IX Coagulant Activity (U/dl)	
Surgical (hysterotomy) abortuses				
1	18	2.2	4	—*
2	17	5.1	8	—*
3	17	4.6	—*	—*
4	16	1.8	—†	0.42
5	20	7.4	7	1.14
6	18	3.9	—†	1.2
7	19	10.0	9	0.78
8	16	2.4	—†	0.96
Fetoscopy control				
9	18	4.8‡	—*	1.2

*Not determined.

†Activity >20 U/dl, apparently owing to interference in the test by unspecific thromboplastic materials.

‡Level measured in fetoscopic sample with EVF (hematocrit) 26% was 3.4 U/dl. Amniotic fluid could contribute at most with 0.5 U/dl to this level (see Results) and at least 2.9 U/dl thus was contributed by the fetus. If the level in undiluted fetal plasma is calculated from this value, assuming a fetal EVF of 37%, it will be 4.8 U/dl.

Among the patients with moderately severe and mild hemophilia B, the levels of immunologically detectable factor IX were variable (Table 1).

Control Fetuses

The concentration of factor IX antigen in plasma from eight surgical (hysterotomy) nonhemophilic abortuses was 1.8–10.0 U/dl. Though in small amounts, factor IX antigen was found to be present in amniotic fluid (Table 2).

The control fetoscopic sample of the normal fetus had an EVF (hematocrit) of 26% (no. 9 in Table 2). Factor IX antigen in this sample was 3.4 U/dl. In pure amniotic fluid, factor IX was 1.2 U/dl. Assuming that fetal EVF is 37%, the dilution of the fetal plasma with amniotic fluid and citrate solution was 3:5. The amniotic fluid could therefore contribute at most 0.5 U/dl (2/5 · 1.2 U/dl), and in fact, even less (since the citrate alone could account for a dilution of the fetal plasma 3:4, see Materials and Methods) to the level of 3.4 U/dl observed in the fetoscopic sample. From these data, the fetal plasma concentration was calculated to be at least 4.8 U/dl (Table 2).

Diagnostic Case

The woman herself had a low level (1.9 U/dl) of factor IX antigen in plasma, and no detectable factor IX antigen in amniotic fluid (Table 3). She was negative for HB_sAg.

The sample obtained at fetoscopy contained >95% fetal red blood cells. Even in the sample with the highest EVF (19%), no factor IX antigen was detected (Table 3). It was not possible to assess factor IX coagulant activity in the fetoscopic samples (see Discussion).

The woman decided to have her pregnancy terminated, which was done by hysterotomy under cover of factor IX concentrate. Plasma of the male abortus was collected by catheterization of one of the umbilical arteries. No factor IX antigen and no factor IX coagulant activity could be detected in the fetal plasma.

DISCUSSION

Prenatal diagnosis of hemophilia offers several difficulties. Fetoscopic samples are often mixed with amniotic fluid. Such samples are not suitable for analysis by conventional coagulant assays, since amniotic fluid contains thromboplastic materials,¹³⁻¹⁵ which may interfere with these assays. The difficulty can be overcome by the use of immunologic methods for factor VIII and factor IX. Sensitive immunoradiometric assays of factor VIII have been developed,^{10,11,16} and can be applied to fetoscopic samples obtained for prenatal diagnosis of hemophilia A.^{1,2,17} A

Table 3. Factor IX in the Diagnostic Case

Sample	Factor IX Antigen (U/dl)	Factor IX Coagulant Activity (U/dl)
Maternal plasma	1.9	5
Amniotic fluid	<0.1	—*
Fetal plasma		
Fetoscopy sample	<0.2†	—*
Postabortal sample‡	<0.1	<2

*Activity >20 U/dl apparently owing to interference by unspecific thromboplastic materials in amniotic fluid.

†No demonstrable factor IX antigen in fetal plasma diluted about 1:2 with amniotic fluid and citrate solution. Assay sensitivity was 0.1 U/dl in undiluted samples.

‡The sample was collected by catheterization of one of the umbilical arteries.

similar method for factor IX is described in this article. It is far more sensitive than hitherto available immunologic methods for factor IX.¹⁸⁻²⁰ The accuracy and reproducibility of the method were good even at very low levels of factor IX, which is essential in prenatal examinations, since the factor IX level is normally very low in 16-20-wk fetuses. In normal adults, there was a significant and strong, although not complete, correlation between factor IX antigen and coagulant activity. The lack of complete correlation is probably due mainly to an unavoidable variation in factor IX activity determinations. The immunoradiometric method gave somewhat lower values for factor IX antigen in our control fetuses (Table 2) compared with previously published values for coagulant factor IX activity in fetal plasma,^{3,9} probably because it is not influenced by thromboplastic materials.

However, immunologic assays also offer difficulties when used for prenatal diagnosis. Hemophilia B exists in several genetic variants. In one type, so-called CRM⁻ (cross-reacting material negative), factor IX antigen is not detectable. In a second type, CRM⁺, the amount of immunologically detectable protein is normal, while factor IX activity may still be very low. In other types, factor IX antigen is lowered either to the same level as, or less than that of, factor IX activity.¹⁸⁻²¹ The situation in severe hemophilia B may thus be more complicated than in hemophilia A, where the severe form is usually associated with no detectable factor VIII:C antigen.^{10,16,17} Nevertheless, the majority of patients with severe hemophilia B (factor IX activity <1 U/dl) should be diagnosable immunologically, since at least 60% of them have very little or no demonstrable factor IX antigen.¹⁹⁻²¹ With our immunoradiometric assay for factor IX, we have found only 1 patient among 12 with severe hemophilia B to have factor IX antigen above 1.0 U/dl and this patient had been given a factor IX infusion 5 days before sampling. In patients with moderate and mild hemophilia B, we found the different types described by others. Our method is based on a homologous antibody with a presumably narrow specificity and

thus perhaps with a lower capacity than rabbit antibodies²⁰ to detect abnormal variants of factor IX protein.²² Still, prenatal diagnosis should not be attempted in an individual case before a known affected member of the family has been evaluated.

Another difficulty in the prenatal diagnosis of hemophilia B is that amniotic fluid contains small amounts of factor IX. This was detected with our immunoradiometric assay. Factor IX occurs in amniotic fluid in a concentration of about 0.5%-1% of that in the maternal plasma, and its origin is probably almost entirely maternal.^{23,24} This has to be taken into account in the calculation of the fetal plasma level of factor IX from the values of the samples obtained at fetoscopy and contaminated with amniotic fluid. In this respect, factor IX differs from factor VIII, which does not occur in amniotic fluid.¹¹

In our diagnostic case, the woman had a low level of factor IX antigen in plasma and was obviously a carrier of a CRM⁻ variant of hemophilia B. She had no demonstrable factor IX antigen in the amniotic fluid, which facilitated the evaluation of the fetoscopic samples consisting of mixtures of fetal blood and amniotic fluid. Assay of these samples for factor IX coagulant activity gave false high values (>20 U/dl), apparently owing to the presence of unspecific thromboplastic materials in amniotic fluid. Coagulant assays of such samples therefore seem unsuitable for prenatal diagnosis of hemophilia B.

In our patient, the conditions for prenatal diagnosis were thus unusually favorable. But in our fetoscopic control case, factor IX was present in amniotic fluid. Nevertheless, the fetal plasma concentration of factor IX could be calculated. We therefore feel that our method is applicable also to common carriers of hemophilia B provided that the following prerequisites are met: the hemophilia is of the CRM⁻ type, the fetoscopic sample is not too diluted, and factor IX antigen in pure amniotic fluid is determined simultaneously.

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REFERENCES

1. Firshein SI, Hoyer LW, Lazarchick J, Forget BG, Hobbins JC, Clyne LP, Pitlick FA, Muir AW, Merkatz IR, Mahoney MJ: Prenatal diagnosis of classic hemophilia. *N Engl J Med* 300:937-941, 1979
2. Gustavii B, Cordesius E, Löfberg L, Strömberg P: Fetoscopy. *Acta Obstet Gynecol Scand* 58:409-410, 1979
3. Mibashan RS, Rodeck CH, Thumpston JK, Edwards RJ, Singer JD, White JM: Plasma assay of fetal factors VIII:C and IX for prenatal diagnosis of haemophilia. *Lancet* 1:1309-1311, 1979
4. Rodeck CH, Campbell S: Sampling pure fetal blood by fetoscopy in second trimester of pregnancy. *Br Med J* 2:728-730, 1978
5. Niléhn JE, Nilsson IM: Haemophilia B in a girl. *Thromb Diath Haemorrh* 7:552-557, 1962
6. Holmberg L, Nilsson IM, Henriksson P, Ørstavik KH: Homozygous expression of haemophilia B in a heterozygote. *Acta Med Scand* 204:231-234, 1978
7. Gustavii B, Cordesius E: Transvaginal approach for sampling of fetal blood. *Lancet* 2:375, 1976
8. Oski FA, Naiman JL: *Hematologic Problems in the Newborns* (ed. 2) Philadelphia, W.B. Saunders, 1972, p. 5

9. Holmberg L, Henriksson P, Ekelund H, Åstedt B: Coagulation in the human fetus. *J Pediatr* 85:860–864, 1974
10. Holmberg L, Borge L, Ljung R, Nilsson IM: Measurement of antihaemophilic factor A antigen (VIII:CAg) with a solid phase immunoradiometric method based on homologous nonhaemophilic antibodies. *Scand J Haematol* 23:17–24, 1979
11. Peake IR, Bloom AL: Immunoradiometric assay of procoagulant factor VIII antigen in plasma and serum and its reduction in haemophilia. *Lancet* 1:473–475, 1978
12. Nilsson IM: *Haemorrhagic and Thrombotic Diseases*. London, John Wiley & Sons, 1974, pp 220–223
13. Courtney LD, Allington M: Effect of amniotic fluid on blood coagulation. *Br J Haematol* 22:353–355, 1972
14. Phillips LL, Davidson EC: Procoagulant properties of amniotic fluid. *Am J Obstet Gynecol* 113:911–919, 1972
15. Hastwell GB: Accelerated clotting time: An amniotic fluid thromboplastic activity index of fetal maturity. *Am J Obstet Gynecol* 131:650–654, 1978
16. Lazarchick J, Hoyer LW: Immunoradiometric measurement of the factor VIII procoagulant antigen. *J Clin Invest* 62:1048–1052, 1978
17. Peake IR, Bloom AL, Giddings JC, Ludlam CA: An immunoradiometric assay for procoagulant factor VIII antigen: Results in haemophilia, von Willebrand's disease and fetal plasma and serum. *Br J Haematol* 42:269–281, 1979
18. Roberts HR, Grizzle JE, McLester WD, Penick GD: Genetic variants of hemophilia B: Detection by means of a specific PTC inhibitor. *J Clin Invest* 47:360–365, 1968
19. Ørstavik KH, Østerud B, Prydz H, Berg K: Electroimmunoassay of factor IX in hemophilia B. *Thromb Res* 7:373–382, 1975
20. Thompson AR: Factor IX antigen by radioimmunoassay. *J Clin Invest* 59:900–910, 1977
21. Kasper CK, Østerud B, Minami JY, Shonick W, Rapaport SI: Hemophilia B: Characterization of genetic variants and detection of carriers. *Blood* 50:351–366, 1977
22. Denson KWE: Molecular variants of haemophilia B. *Thromb Diath Haemorrh* 29:217–219, 1973
23. Johnson AM, Umansky O, Alper CA, Everett C, Greenspan G: Amniotic fluid proteins: Maternal and fetal contributions. *J Pediatr* 84:588–593, 1974
24. Sutcliffe RG: The nature and origin of the soluble protein in human amniotic fluid. *Biol Rev* 50:1–33, 1975