Fibrinogen Bethesda III:
A Hypodysfibrinogenemia

By Harvey R. Gralnick, Barry S. Coller, Joseph C. Fratantoni, and Jose Martinez

A qualitative and quantitative defect of fibrinogen in a white female was associated with first trimester abortions and hemorrhage. This congenital hypodysfibrinogenemia was inherited in an autosomal pattern. The functional defect was an abnormality in the fibrin monomer aggregation. Metabolic studies in the propositus revealed that homologous fibrinogen had a normal intravascular survival and fractional catabolic rate, while her fibrinogen, when injected into a normal volunteer, had a very short intravascular survival and high fractional catabolic rate. Coagulation changes were monitored through 36 wk of one pregnancy. The observed coagulation changes included elevation of factors VIII, VII-X, and plasminogen; however, the fibrinogen measured by immunologic or fibrin tyrosine content did not increase during the pregnancy, while the functional fibrinogen assay became undetectable and the thrombin and reptilase times were markedly prolonged. This suggested that this patient either was synthesizing more of her abnormal fibrinogen during pregnancy or that her normal fibrinogen was being catabolized at a different rate than her abnormal fibrinogen. Comparative studies with fibrinogen Philadelphia revealed several similarities in the two fibrinogens; however, significant differences were also noted. This new congenital hypodysfibrinogenemia is designated fibrinogen Bethesda III.

Since the first detailed description of a qualitative congenital abnormality of fibrinogen,1 over 30 families with congenital dysfibrinogenemia have been described.1-32 The most common functional defect has been an abnormality of fibrin monomer polymerization, and patients with this defect have almost always been asymptomatic.33 Hypofibrinogenemia in addition to congenital dysfibrinogenemia has been reported in only three instances.10,20,25 In two of these families, with fibrinogens Bethesda II20 and Philadelphia25 the hypofibrinogenemia has been due, at least in part, to increased catabolism.

We report a white family with a new hypodysfibrinogenemia (Bethesda III) in which the major clinical manifestation in the propositus included three first trimester abortions associated with extensive hemorrhage and two pregnancies associated with bleeding and postpartum thrombophlebitis. The prolonged thrombin and reptilase times of this patient’s plasma were related to a delay in fibrin monomer polymerization. In further studies, two fibrinogen populations could be separated from the propositus’ plasma, one of which had normal and the second, abnormal fibrin monomer polymerization.

Homologous radioactive fibrinogen had a normal fractional catabolic rate and intravascular half-disappearance time in the propositus, while her purified radiolabeled fibrinogen (Bethesda III) was catabolized at a rapid rate and had a
markedly shortened intravascular survival in a normal recipient. Investigation of the abnormal fibrinogen did not yield evidence of proteolysis or degradation. Because of the similarities between fibrinogen Bethesda III and fibrinogen Philadelphia, we compared some of the biologic functions and physicochemical properties of these two fibrinogens. These studies showed that fibrinogen Bethesda III and fibrinogen Philadelphia behave similarly in DEAE-cellulose chromatography and polyacrylamide gel electrophoresis; however, three major differences were noted: (1) the abnormal thrombin time of fibrinogen Bethesda III was shortened by increased thrombin concentrations while that of fibrinogen Philadelphia was not; (2) fibrinogen Bethesda III had a greater inhibitory effect on normal fibrinogen clotting than did fibrinogen Philadelphia; and (3) fibrin monomers from fibrinogen Bethesda III polymerized at ionic strengths of 0.21 and 0.30 while fibrin monomers from fibrinogen Philadelphia did not. The spectrum of qualitative and quantitative fibrinogen abnormalities in this family distinguishes it from other described dysfibrinogenemias.

MATERIALS AND METHODS

Unfractionated plasma. Blood from family members and normal individuals was collected in 0.1 vol 40% sodium citrate or 0.02 vol 20% potassium oxalate. Plasma was separated after centrifugation at 3000 g at 4°C for 15 min. Coagulation studies were performed immediately or the sample was frozen at −60°C and tested at a later time.

Preparation of fibrinogen. Blood from normal individuals and family members was collected in 0.1 vol 4% sodium citrate. The plasma was separated by centrifuging at 5000 g for 15 min, after which it was centrifuged a second time. Fibrinogen was precipitated from the cell-free plasma by (NH₄)₂SO₄ as previously described.¹ A portion was tested for clottable protein, and the rest was frozen at −60°C until used. The percentage of clottable protein varied between 90% and 96%. The percentage recovery of normal fibrinogen varied between 64% and 77% (n = 6). The mean recovery of the abnormal fibrinogen was 64% (range 55%–71%, n = 4). Fibrinogen was also prepared by the method of Barnhart and Forman.³⁴

Thrombin. The human thrombin was a gift of Dr. D. L. Aronson, Bureau of Biologics, Food and Drug Administration. It was kept frozen at a concentration of 40 U.S. U/ml in Tris-chloride (0.02 M) buffer pH 7.4. Bovine thrombin (Parke Davis, Detroit, Mich.) was dissolved in 50% (v/v) glycerol in normal saline. Dilutions of thrombin were made with normal saline.

Coagulation and fibrinolytic studies on samples from the propositus and her family were performed by the following techniques: the method of Ivy et al.¹³ for bleeding time, the Lee and White method for clotting,¹³ the prothrombin time by the method of Quick³⁷ utilizing rabbit brain thromboplastin (Ortho Diagnostics, Raritan, N.J.), and the partial thromboplastin time by the methods of Langdell et al.³⁸ using Celite (Warner Lambert, Morris Plains, N.J.) as the activator. The thrombin time was measured with concentrations of bovine or human thrombin of 1–100 U/ml with and without 0.025 M CaCl₂. Reptilase venom of Bothrops atrox was obtained in powder form from Abbott Laboratories, North Chicago, Ill. It was dissolved in distilled water and used at a concentration of 20 μg venom/ml. Factors V, VII-X, VIII, IX, and XI were assayed as previously described.³⁸ Factor XIII was measured by the method of Losowsky et al.⁴⁴ using 5 M urea, 2% acetic acid, and 1% monochloroacetic acid as dissolving solution. Fibrinogen was determined by the following three methods: (1) tyrosine determination on the fibrin clot,⁴¹ (2) radial immunodiffusion by the method of Feinberg,⁴² and (3) chromometric by the method of Claus.⁴³

The euglobulin clot lysis time was performed by the Milstone method.⁴⁶ The plasminogen assay was modified from the method of Alkjaersig et al.⁴⁷ alpha casein was used as the substrate. Fibrin(ogen) degradation products were measured by tanned sheep red cell hemagglutination inhibition⁴⁸ and by immunodiffusion.

Unless otherwise stated, clottability of purified fibrinogen preparations was determined as previously described.¹³ Protein concentration was estimated by the technique of Lowry.⁵⁰
Fibrinopeptide release with thrombin and ancor was investigated by a method patterned after the original procedure of Lorand as previously described.13

Fibrin monomer aggregation was studied by the technique of Belitser et al. as previously described.13 and by the method employed in the study of fibrinogen Cleveland.1

Crosslinking of normal and abnormal fibrinogen was assessed on SDS polyacrylamide gel electrophoresis (PAGE).32 Aliquots of fibrinogen in a Tris-saline-citrate buffer (0.025 M Tris, 0.1 M NaCl, 0.025 M sodium citrate, pH 7.3) were set up in six test tubes to be tested at time 0, 5 min and 1, 2, 20, and 24 hr. The incubation mixture consisted of 0.5 ml (2 mg/ml) of fibrinogen to which was added 50 µl CaCl2 (0.025 M), 50 µl (0.03 M) cysteine (pH 7.0), and 50 µl 30 U/ml human thrombin. For the time zero sample, the CaCl2 was omitted and 50 µl 0.75% EDTA was added. At the end of each incubation time, the fibrin clot was washed in 10 ml 0.9% NaCl three times, and the clot was spun at 2000 g for 10 min. The clot was then removed, blotted on filter paper, and transferred to a tube containing 4 M urea solution with 1% SDS and 5 mM dithioerythritol. The clots were left overnight at room temperature and then incubated for 3 hr at 37°C in the urea-SDS-dithioerythritol solution and analyzed on 7.5% PAGE. Approximately 40 µg protein was placed on each gel. Gels were run for 3 hr (until the tracking dye reached the bottom of the gel) and then fixed with 20% sulfosalicylic acid overnight, stained with 0.25% Coomassie blue for 3 hr, and then destained in 7.5% acetic acid. The gels were compared visually and by densitometry.

Fibrinogen digestion with plasmin. Normal and abnormal fibrinogen (1 ml; 3 mg/ml) in 0.3 M NaCl was placed in each of eight test tubes. To each of the test tubes, 0.07 U plasmin (Kabi, Stockholm, Sweden) was added and the mixture incubated for 2, 5, 10, 20, 30, 60, 120, and 240 min. The digestion was stopped by the addition of aprotinin 500 U/ml (FBA Pharmaceuticals, New York, N.Y.). An aliquot of the incubation mixture was placed in 8 M urea and 2% SDS at room temperature overnight, then at 37°C for 3 hr; the 20 µg of protein was electrophoresed in 1% SDS on 5% polyacrylamide gels. Approximately 40 µg protein was placed on each gel. Proteins used as standards included normal fibrinogen, transferrin, beta galactosidase, gamma globulin, and bovine serum albumin as previously described.33

Aliquots from each of the digestion periods (prior to incubation with SDS) were tested in a thrombin time assay. The incubation mixture consisted of 0.1 ml normal plasma, 0.1 ml fibrinogen digestion mixture, and 0.1 ml human thrombin, final concentration 2.5 U/ml. Since the abnormal fibrinogen clotted slowly at 2.5 U/ml, the abnormal fibrinogen digest-normal plasma mixture was tested at thrombin concentrations of 2.5, 5, and 10 U/ml (at 5–10 U/ml the abnormal fibrinogen clotting time was similar to that for normal fibrinogen at 2.5 U/ml). After the addition of thrombin, the time for clot formation to appear was recorded.

Platelet tests. Platelet retention in a glass bead column was measured as previously described.44 Platelet aggregation was performed in a Chronolog aggregometer on citrated platelet-rich plasma (PRP, adjusted platelet count 300,000/mm3) from the propositus and a normal control. Aggregation of PRP was tested with human thrombin, collagen, epinephrine, and ADP. ADP was obtained from Sigma Chemical, St. Louis, Mo., epinephrine from Parke Davis, and collagen from Worthington Biochemical, Freehold, N.J.

Chemical and physical studies. Sialic acid was measured by the thiobarbituric acid method of Warren and neutral sugars by the method of Hewitt.56 Gradient elution chromatography was carried out at 5°C on a column (2.2 X 33 cm) of DEAE-cellulose prepared by the method of Peterson and Sober.57 The procedure and the concave gradient were those described previously.58

Plasma (10 ml) and purified fibrinogen (15 mg) from normals and the propositus and her brother were chromatographed at 23°C on a 40 X 2.5 cm column packed with Sepharose 4B (Pharmacia, Piscataway, N.J.). The eluting buffer was Tris-NaCl (0.05 Tris, 0.10 NaCl), pH 7.3. Fibrinogen was localized in the effluent by thrombin time measurements and radial immunodiffusion against anti-human fibrinogen antibody.

Plasma and fibrinogen of the members of the propositus' family were subjected to immunoelectrophoresis in agarose (1%) at pH 8.6 with 0.05 M barbital buffer by the procedure of Grabar and Williams.59 Double immunodiffusion was performed in 1% agarose. The antisera used in these experiments were prepared in rabbit and goat. Plasma from normals, the propositus, and her affected family members were tested in crossed antigen-antibody electrophoresis.60

Reduced and nonreduced purified fibrinogen from normals and members of the propositus' family were electrophoresed in 0.1% SDS-5% acrylamide gels.13 Gels were stained with Coomassie blue or periodic acid-Schiff reagent.
Fibrinogen survival. A homologous fibrinogen survival test was performed in the propositus with fibrinogen obtained from a pedigreed donor who has not transmitted hepatitis (despite over 35 blood donations) and had neither hepatitis-associated antigen(s) nor antibodies in his serum. A fibrinogen survival test was performed in a normal volunteer with the propositus’ fibrinogen (obtained before her exposure to the homologous fibrinogen). Both the patient and normal volunteer were informed of the risk of hepatitis (neither HBs:Ag nor HBs:Ab were detected in the plasma or fibrinogen for injection), and both signed informed consent forms.

Fibrinogen was iodinated with 125I by the method of MacFarlane as previously described to a ratio of 0.5 mole iodine/mole fibrinogen.

Comparative studies of fibrinogens Bethesda III and Philadelphia. Samples of Bethesda III fibrinogen and plasma from the propositus and her brother were analyzed simultaneously with the plasma and/or fibrinogen from the propositus with fibrinogen Philadelphia. The comparative studies included thrombin clotting times, the effect on normal fibrinogen clotting, and the effect of increasing thrombin dosage on the thrombin clotting time (all at similar fibrinogen concentrations). Elution chromatography on Whatman DE-52 DEAE-cellulose of 125I-labeled fibrinogen Bethesda III was compared to 125I-labeled fibrinogen Philadelphia or 131I-labeled normal fibrinogen. The migrations of intact and reduced radiolabeled fibrinogen Bethesda III, fibrinogen Philadelphia, and normal fibrinogen were analyzed in SDS–5% PAGE and in Tris-glycine pH 8.5–5% PAGE.

CASE REPORT

The patient, a 27-yr-old white female, was admitted to a local hospital at the 24th week of her third pregnancy because of atraumatic oozing from the gingival area and the eruption of spontaneous atraumatic hematomas over the lower extremities. She stated that 6 yr prior to her admission she had had a normal, full-term pregnancy associated with excessive postpartum bleeding. She had had a second pregnancy that terminated in the 9th week due to a spontaneous abortion with extensive uterine bleeding resulting in a decrease in her hematocrit.

On physical examination she had multiple lower extremity ecchymotic areas and some intermittent vaginal bleeding. The initial clotting studies were interpreted as being diagnostic of diffuse intravascular coagulation. Her prothrombin, partial thromboplastin, and thrombin times were prolonged, and her fibrinogen level was less than 55 mg/dl. The patient was placed on 5000 U heparin intravenously every 4 hr; because of persistent laboratory abnormalities she continued to receive heparin for 18 days. During this time some of her coagulation tests improved; however, her fibrinogen level remained low. On the 19th hospital day a cesarean section was performed with the delivery of a 1122-g male infant who died 18 hr later; autopsy showed immaturity, hyaline membrane disease of the lung, pulmonary petechiae, hemorrhages of the gastric mucosa, a subcapsular hematoma of the right lobe of the liver, retroperitoneal hemorrhage, intraventricular hemorrhage of the brain, and multifocal subarachnoid hemorrhage. The amniotic fluid was dark stained and contained small clots. The placenta had a blood clot adherent to the maternal surface that was consistent with the diagnosis of focal abruptio placentae. After the cesarean section the patient developed thrombophlebitis of the left leg that was treated with conservative measures, with relief of the signs and symptoms in 3 days. Post partum her prothrombin and thrombin times remained prolonged and her fibrinogen level reduced.

The patient had two subsequent pregnancies that were terminated by spontaneous abortion and excessive bleeding during the first trimesters. In December 1974 the patient conceived again and during the 24th week of pregnancy developed uterine bleeding with a drop in her hematocrit from 36% to 30%. Treatment with 26 units (410 ml) of cryoprecipitate resulted in rapid cessation of her bleeding. During the 32nd week of pregnancy the patient again experienced brisk uterine bleeding. Since arm veins were inadequate for infusion, a lower extremity cutdown was performed. She was treated with packed red blood cells and cryoprecipitate with immediate cessation of bleeding. Two days later the cutdown site became tender, warm, and erythematous and the leg became swollen. The catheter was removed and conservative measures were applied to the lower extremity. Five days after the initial cryoprecipitate transfusion the patient again experienced massive uterine bleeding. Packed red blood cell transfusions and treatment with cryoprecipitate infusion abruptly halted her bleeding. It was decided during the 34th week of gestation to terminate the pregnancy by cesarean section. The patient had received no cryoprecipitate for 7 days, and her Clauss-fibrinogen level was undetectable (<15 mg/dl). It was decided not to infuse cryoprecipitate unless the patient started to bleed. A 2043-g female infant was delivered by classical cesarean section. Examination of the placenta showed no pathology.
Fig. 1. Family studies. Seven members (four females, three males) of the family of fibrinogen Bethesda III had long thrombin times and long reptilase times with reduction of total fibrinogen concentration (below the slashed line; measured by fibrin tyrosine content) and severe reduction of functional fibrinogen as measured by the Clauss assay (above the slashed line). Those symbols in which there are no figures indicate that the individual was not studied.

Table 1. Coagulation Studies in the Propositus With Fibrinogen Bethesda III

<table>
<thead>
<tr>
<th></th>
<th>Patient</th>
<th>Control</th>
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<tbody>
<tr>
<td>Bleeding time (min)</td>
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<td>Clotting time (glass) (min)</td>
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<td>6–14</td>
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<td>Partial thromboplastin time (sec)</td>
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<td>Thrombin time (sec)</td>
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<td>Reptilase time (sec)</td>
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<td>19–24</td>
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<tr>
<td>Euglobulin clot lysis (min)</td>
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<td>&gt;180</td>
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<tr>
<td>Plasminogen (casein units/ml)</td>
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<td>Platelet count (platelets x 10^3/mm^3)</td>
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<tr>
<td>Factors V, VII, VIII, IX, XI, XIII</td>
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<td>Normal</td>
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<tr>
<td>Fibrinogen degradation products (μg/ml)</td>
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<td>&lt;8</td>
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<tr>
<td>Fibrinogen (mg/dl)</td>
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<tr>
<td>Fibrin tyrosine content</td>
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<td>186–360</td>
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<tr>
<td>Immunologic</td>
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<td>200–390</td>
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<tr>
<td>Thrombin clotting time (Clauss)</td>
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<td>190–380</td>
</tr>
<tr>
<td>Platelet function tests</td>
<td>Normal</td>
<td>Normal</td>
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</table>

* Range of six measurements when the patient was not pregnant.

examine placental blood were unsuccessful at the time of delivery. The patient did well post partum except for a severe exacerbation of the lower extremity thrombophlebitis. Wound healing was normal. Conservative therapy without anticoagulation over a period of 10 days resulted in diminution of the thrombophlebitis, and the patient and her infant child were discharged.

One sister of the propositus had bled extensively after a vaginal delivery followed by a tubal ligation and required transfusion of blood and cryoprecipitate.

RESULTS

Coagulation studies in the propositus showed prolonged prothrombin, thrombin, and reptilase times with only slight prolongation of the partial thromboplastin time. The fibrinogen level was reduced when measured by immunologic or fibrin tyrosine content to approximately 100 mg/dl, while the chronometric (Clauss) value was always significantly lower (30–41 mg/dl) (Table 1). All coagulation factors were normal, fibrinogen degradation products were not detectable, and the plasminogen concentration was normal. The euglobulin clot lysis was performed on eight occasions, and on five it was shortened to 90–170 min (normal >180).
Table 2. Inhibition of Normal Plasma Thrombin Time

<table>
<thead>
<tr>
<th>Percentage of Normal Plasma</th>
<th>NP and Fibrinogen Bethesda Ill Plasma</th>
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<tr>
<td>100</td>
<td>25.0</td>
<td>24.8</td>
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<td>90</td>
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<tr>
<td>10</td>
<td>49.2</td>
<td>—</td>
</tr>
<tr>
<td>0</td>
<td>67.2</td>
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</tr>
</tbody>
</table>

NP designates a pool of normal plasma.

*The fibrinogen value of the normal plasma was 2.65 mg/ml and 1.04 mg/ml for the abnormal plasma.

Family studies showed that the propositus' daughter, two sisters, one brother, and two nephews had coagulation abnormalities similar to those of the propositus (Fig. 1, Table 1), while her mother, one other sister, and two nephews of affected siblings had normal coagulation studies.

Thrombin times performed on normal and propositus plasma with increasing concentrations of thrombin showed that the abnormal thrombin time in the patient was shortened with the increased thrombin concentration; however, even at 100 U/ml the thrombin clotting time was still twice that of the normal plasma at the same thrombin concentration. When the thrombin and reptilase times were performed in the presence of CaCl₂, the abnormal coagulation times in the propositus' plasma were shortened; however, again this value was at least twice that found with normal plasma. Studies of the propositus' plasma and fibrinogen showed an inhibitory effect of the coagulation of normal plasma and/or fibrinogen (Table 2).

Fibrinopeptide release determinations were performed on normal and patient fibrinogen with both thrombin and Arvin. The time course and the amount of non–TCA precipitable protein released from the abnormal fibrinogen were indistinguishable from normal (Fig. 2). Fibrin monomers prepared with either thrombin or reptilase from the patient's fibrinogen polymerized with a longer lag time and a decreased slope compared to normal fibrin monomer aggregation (Fig. 3); however, the final amplitude was similar to that seen with normal fibrin.
monomers. The abnormalities in fibrin monomer aggregation were accentuated by increasing the ionic strength from 0.12 to 0.30 and were more pronounced with fibrin monomers prepared with reptilase than with thrombin.

When fibrin monomers were prepared from 8% ethanol-precipitable fraction of fibrinogen and from the ethanol-soluble fraction, two separate populations of fibrin monomers were found in the patient's fibrinogen. The patient's fibrin monomers from the ethanol-precipitable fraction had a prolonged lag phase before the onset of aggregation and had a reduced slope compared to normal; however, the patient's fibrin monomers prepared from the 8% ethanol supernatant were similar to the normal ethanol supernatant fibrin monomers in onset, slope, and total amplitude of aggregation (Fig. 4).

Crosslinking of fibrin as judged by PAGE was normal in the time of onset and the amount of gamma dimers and alpha polymers formed as compared to normal fibrinogen.

Immunodiffusion of the propositus' plasma and fibrinogen showed precipitin arcs of complete identity with normal plasma and purified fibrinogen when reacted with goat or rabbit antifibrinogen antibody.

The migration and shape of the propositus' plasma fibrinogen precipitin arc were identical to normal in immunoelectrophoresis; however, immunologic determination of fibrinogen by radial immunodiffusion showed hypofibrinogenemia in the propositus and her affected family members (Fig. 1). Crossed antigen-antibody electrophoresis against an anti-human fibrinogen antibody showed that plasma precipitin arcs of fibrinogen Bethesda III and fibrinogen Philadelphia were similar to normal.
Fibrin monomer aggregation. Fibrinogen was prepared from normal and fibrinogen Bethesda III plasma by precipitation with 8% ethanol, and the supernatant was then precipitated with 25% saturated ammonium sulfate. Fibrinogens from both 8% ethanol precipitation and supernatant were then washed several times and treated with thrombin. Fibrin monomer aggregation in the 8% ethanol fraction was 1.0 mg/ml and in the supernatant fraction 0.68 mg/ml. The 8% ethanol precipitable fibrinogen Bethesda III had a markedly abnormal fibrin monomer aggregation, while the fibrinogen found in the supernatant had an aggregation curve similar to normal. It should be noted that the normal supernatant aggregation curve was more prolonged than the normal 8% ethanol fraction.

PAGE in the presence of SDS of the normal and the propositus’ purified fibrinogen showed identical migration of the protein bands. After reduction of the fibrinogen with dithioerythritol, the subunit structure of the abnormal fibrinogen could not be distinguished from normal fibrinogen in the relative amount or position of the chains. No additional chains or protein bands were identified. The β and γ chains of Bethesda III and normal fibrinogen reacted with the periodic acid Schiff stain.

Plasmin proteolysis of purified fibrinogen from the propositus and her brother was abnormal when analyzed by PAGE (Fig. 5). With normal fibrinogen, plasmin proteolysis results in the early appearance of the high molecular weight fragments X and Y with the subsequent appearance of the smaller fragments D and E. After 120 min no appreciable amounts of fragments X and Y remain (Fig. 5A densitometric scan: X, 10%; Y, 17%; D, 66%; E, 7%). In contrast, after 120 min of plasmin proteolysis of the propositus’ fibrinogen, significant amounts of fragments X and Y persisted (Fig. 5B densitometric scan: X, 40%; Y, 24%; D, 34%; E, 2%). The digests were also tested for their inhibitory effects on the thrombin clotting time of normal plasma. With the normal fibrinogen-plasmin digest, the peak of inhibition occurred at 20 min, and by 120 min little inhibitory effect remained. In contrast to this, the peak anticoagulant effect of the digestion mixture of the abnormal fibrinogen occurred at 20 min and then persisted through the 120-min digestion process (Fig. 6; not shown is that the inhibition persisted for at least 240 min).

Elution chromatography of purified fibrinogen on DEAE cellulose of the abnormal and normal fibrinogens showed identical elution of peaks 1 and 2. There was no difference in clottability of fibrinogen in these peaks.
Fig. 5. (A) Polyacrylamide gel electrophoresis (PAGE) analysis of plasmin degradation of normal and fibrinogen Bethesda III fibrinogen. Fibrinogens were incubated with purified plasmin, and the proteolysis was followed by SDS-PAGE. Note that with normal fibrinogen after the 60-min sample, very small amounts of fragments X (32%) and Y (22%) were present with fragments D (32%) and E (4%). (B) PAGE of plasmin degradation of fibrinogen Bethesda III. Note that in contrast to the normal (Fig. 5A) fibrinogen proteolysis pattern there was a persistence of the large molecular weight fibrinogen fragments in fibrinogen Bethesda III (at 60 min X = 68%, Y = 19%, D = 13%). Scan data at 120 min given in the text. Not shown, these fragments persisted for at least 240 min of proteolysis.
FIBRINOGEN CONGENITAL ABNORMALITY

Fig. 6. Inhibitory effect of fibrinogen proteolysis on the normal plasma thrombin clotting time. Samples of normal and Bethesda III fibrinogen proteolysis were tested for the effect on thrombin clotting time of normal plasma. At the intervals indicated 0.1 ml of the fibrinogen degradation mixture was added to 0.1 ml of normal plasma at 37°C and incubated for 10 sec; then 0.1 ml of human thrombin, final concentration 2.5 U/ml, was added. Note that the normal (closed circles) fibrinogen proteolysis mixture peak of inhibition of normal plasma clotting time occurred at 20 min. Peak of inhibition of the fibrinogen Bethesda III proteolysis mixture (open circles) occurred also at 20 min; however, the inhibition from 20 min persisted to 120 min with fibrinogen Bethesda III. Not shown, this inhibition persisted for over 240 min. Thrombin time mixtures with fibrinogen Bethesda III fibrinogen were treated with either 5 U or 10 U thrombin with almost identical results.

Gel filtration on Sepharose 4B of plasma and/or fibrinogen from the propositus and her brother showed a fibrinogen elution pattern identical to normal when tested by thrombin clotting time or radial immunodiffusion. The only differences noted were prolonged thrombin clotting times and reduced amounts of immunologic fibrinogen with the abnormal plasma and fibrinogen. When identical quantities of purified fibrinogen were chromatographed, the immunologic fibrinogen levels were similar in the normal and abnormal column fractions. There were no "shoulders" or other peaks detected by either immunologic or thrombin clotting assays.

Sialic acid content of the propositus' and her brother's fibrinogen were normal. Normal fibrinogen contained 16.3 ± 1.2 nmol sialic acid/mg protein or 0.51% ± 0.04% (mean ± SD, n = 8 different preparations of fibrinogen). The propositus' fibrinogen contained 15 nmol sialic acid/mg protein or 0.47%, and her brother's fibrinogen contained 16 nmol sialic acid/mg protein or 0.50%. Neutral sugar content of fibrinogen Bethesda III was similar to normal.

Comparative studies of fibrinogen Philadelphia and fibrinogen Bethesda III. At a thrombin concentration of 1 U/ml the plasmas of fibrinogen Bethesda III (67 sec) and fibrinogen Philadelphia (62 sec) had prolonged thrombin times; however, the thrombin time of fibrinogen Bethesda III was shortened, while that of
fibrinogen Philadelphia increased at a thrombin concentration of 2 U/ml. Both fibrinogens Bethesda III and Philadelphia inhibited the coagulation of normal plasma; however, fibrinogen Bethesda III caused greater inhibition than fibrinogen Philadelphia (Table 3).

Simultaneous gradient elution chromatography on DEAE cellulose of \(^{125}\text{I}\)-fibrinogen Bethesda III, \(^{131}\text{I}\)-fibrinogen Philadelphia, and unlabeled normal fibrinogen showed delayed elution of the first peak of both abnormal fibrinogens (Fig. 7A). When \(^{125}\text{I}\)-fibrinogen Bethesda III, \(^{131}\text{I}\)-normal fibrinogen, and normal unlabeled fibrinogen were chromatographed simultaneously, the first peak of fibrinogen Bethesda III still eluted four tubes after the peak of radioactivity and absorbance of the normal fibrinogen (Fig. 7B).

Reduced and nonreduced radiolabeled fibrinogens Bethesda III and Philadelphia were indistinguishable from normal by SDS-PAGE. In Tris-glycine (pH 8.5) 5% polyacrylamide gels, radiolabeled fibrinogens Bethesda III and Philadelphia behaved similarly in that the peak of radioactivity migrated 3–4 mm more toward the anode than normal.

**Metabolic studies.** Analysis of radiolabeled normal (homologous) and Bethesda III fibrinogen by SDS-PAGE showed no differences in the position of the individual chains or the intact protein. When homologous normal iodinated fibrinogen was infused into the propositus, the \(t\frac{1}{2}\) was 3.25 days (normal 3.69 ± 0.45, mean ± SD, \(n = 21\)) and the fractional catabolic rate was normal (27%). When the patient’s abnormal fibrinogen was iodinated and infused into a normal volunteer, there was a rapid plasma disappearance of radioactivity, with a \(t\frac{1}{2}\) of approximately 1.42 days (Fig. 8). The fractional catabolic rate of this fibrinogen was 85% (normal 22% ± 2.7%, \(n = 23\)). When the same normal volunteer received the same homologous normal iodinated fibrinogen as the propositus, the survival was 3.33 days and the fractional catabolic rate was 24%. The absolute fibrinogen synthesis rate of normal fibrinogen in the normal donor was 21.7 mg/kg/day, while with the abnormal fibrinogen the absolute synthetic rate was 69.3 mg/kg/day (normal 16.9–40.1). The percentage of plasma radioactivity excreted in the urine was always lower in the first 24 hr than in any other 24-hr period.

**Platelet function tests.** A standardized template bleeding time was normal (3.5 min), as was platelet aggregation. At a final epinephrine concentration of 9.1 \(\mu M\) the patient’s PRP gave the typical double wave response. A final ADP concentration of 9.1 \(\mu M\) produced a large single wave of aggregation. Thrombin at a final concentration of 0.45 U/ml produced a large single wave, while at 0.23 U/ml a

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**Table 3. Thrombin Time**

<table>
<thead>
<tr>
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<th>Thrombin Time (sec)</th>
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<tbody>
<tr>
<td>Normal Plasma (fibrinogen concentration 2.44 mg/ml)</td>
<td>19.3</td>
</tr>
<tr>
<td>Normal Plasma diluted 1:1 with buffer (final fibrinogen concentration 1.22 mg/ml)</td>
<td>24.7</td>
</tr>
<tr>
<td>Fibrinogen Philadelphia plasma mixed with normal plasma 3:1 (final fibrinogen concentration 1.20 mg/ml)</td>
<td>33.5</td>
</tr>
<tr>
<td>Fibrinogen Bethesda plasma mixed with normal plasma 3:1 (final fibrinogen concentration 1.23 mg/ml)</td>
<td>48.3</td>
</tr>
</tbody>
</table>

Assay: Plasma 0.1 ml, imidazole-buffered saline 0.2 ml, thrombin 0.1 ml of 10 U/ml solution in imidazole-buffered saline.
Fig. 7. (A) Elution chromatography of fibrinogen. Relative elution patterns of normal fibrinogen Philadelphia, and fibrinogen Bethesda III were compared on DEAE cellulose. $^{131}$-fibrinogen Bethesda III (closed circles), $^{131}$-Philadelphia (closed squares), and nonlabeled normal fibrinogen (open circles) were chromatographed together. Elution of fibrinogens Bethesda III and Philadelphia was monitored by radioactivity and normal fibrinogen by absorbance at 280 nm. First peak of fibrinogen Philadelphia and Bethesda III eluted approximately four tubes later than normal fibrinogen. (B) Closed circles, $^{131}$-labeled fibrinogen Bethesda III; closed squares, $^{131}$-normal fibrinogen; open circles, normal unlabeled fibrinogen. These samples were chromatographed simultaneously. Elution of labeled normal fibrinogen or fibrinogen Bethesda III was monitored by radioactivity and normal unlabeled fibrinogen by absorbance at 280 nm. Labeled and unlabeled normal fibrinogen eluted three or four tubes before fibrinogen Bethesda III. Thus the altered elution pattern of fibrinogen Bethesda III was not related to the radiolabeling process.
small single wave occurred followed by a disaggregation pattern. A normal sample tested at the same time gave identical results. The patient's platelet retention was 94% (mean of 20 normals: 93%).

**Coagulation studies during pregnancy.** Serial coagulation studies were performed from December 1974 to June 1975 (at the termination of the pregnancy) (Fig. 9). Six studies were performed between 1972 and 1974, and the means of these values are depicted in Fig. 9. From the onset through the termination of pregnancy most of the coagulation findings of pregnancy were seen, i.e., the factor VIII level increased from 100% to 265%, the plasminogen level increased from 2.7 to 5.13 casein units, and the factor VII-X increased from 70% to 140%. However, the fibrinogen level, as measured by either immunologic or fibrin tyrosine content, did not change during pregnancy. The chronometric (Clauss) fibrinogen dropped from a prepregnancy level of 35–40 mg/dl to completely undetectable levels during the second and third trimesters of the pregnancy (lower limit of sensitivity, ~15 mg/dl). Another reflection of the persistently decreased fibrinogen level was the low erythrocyte sedimentation rates (2–4 mm/hr) obtained over her entire
Fig. 9. Coagulation studies during pregnancy. Serial coagulation studies were performed on the propositus with fibrinogen Bethesda III during her pregnancy in 1974–1975. Ordinates: white boxes represent the mean values of six studies performed between 1972 and 1974. During the pregnancy most of the usual coagulation findings associated with pregnancy were seen: elevation of the factor VIII level, plasminogen level, and factor VII–X. However, the fibrinogen level, as measured by immunologic or fibrin tyrosine content, did not change and the Clauss fibrinogen dropped from a prepregnancy level of approximately 40 mg/dl to undetectable during the second and third trimesters of pregnancy. During this time the thrombin time rose from 100 to over 400 sec and the reptilase time rose from 42 to 65 sec. Associated with these coagulation findings related to fibrinogen was the fact that the erythrocyte sedimentation rate did not change at all during pregnancy.

pregnancy. During the pregnancy the platelet count remained between 280,000/mm$^3$ and 330,000/mm$^3$, and several assays of factor V activity were always in the normal range.

In concert with the drop in the chronometric fibrinogen value, the thrombin time rose to greater than 400 sec by the third month of pregnancy (prepregnancy value
100 sec). From March through June the thrombin clotting time was essentially infinite. Plasma to which thrombin (2.5–3.0 U/ml) had been added did not clot after 2 hr; then one aliquot was placed in the refrigerator overnight and another stored at 37°C. Inspection the next morning showed that no clot had formed in either one. Thus the thrombin clotting time was at least 19 hr. The reptilase time, 37 sec initially, rose during the course of the pregnancy to a peak level of 64 sec. Since the total fibrinogen content measured by both immunologic and fibrin tyrosine content methods remained constant but the level of functional fibrinogen dropped to undetectable limits, it appears as if there was a preferential synthesis of the abnormal fibrinogen. These findings did not reflect a general inability to synthesize coagulation proteins, since serial studies of factors VII-X, plasminogen, and factor VIII levels all showed a rise of at least 100% during pregnancy compared to the baseline studies.

DISCUSSION

Three of the reported families with dysfibrinogenemia had associated hypofibrinogenemia, but none of the fibrinogens appear to be identical to fibrinogen Bethesda III. Fibrinogen Bethesda III can be distinguished from fibrinogen Leuven because the affected family members other than the propositus with fibrinogen Leuven were not hypofibrinogenemic. Fibrinogen Bethesda II has abnormal fibrinopeptide release and an abnormal immunoelectrophoretic pattern, while fibrinogen Bethesda III does not. The differentiation of fibrinogen Bethesda III and fibrinogen Philadelphia is more difficult. The fibrinogens are similar in having a severe quantitative deficiency, functional abnormalities, and normal immunoelectrophoresis and in inhibiting the coagulation of normal fibrinogen. When normal fibrinogen was injected into both propositi, it survived normally, but both abnormal fibrinogens had shortened survivals and increased catabolic rate when injected into the propositus (fibrinogen Philadelphia) or a normal individual (fibrinogen Bethesda III). Elution chromatography on DEAE cellulose of 125I-labeled purified fibrinogen Bethesda III and 131I-labeled fibrinogen Philadelphia showed similar elution pattern(s). In both instances these were different than a normal radiolabeled fibrinogen, with the peak of radioactivity of both of the abnormal fibrinogens eluting four tubes later than the normal. Both fibrinogen Philadelphia and fibrinogen Bethesda III had normal migration on PAGE in the presence of SDS and a normal subunit pattern, and both the abnormal fibrinogens had increased anodal migration compared to normal in PAGE in a Tris-glycine pH 8.5 buffer.

However, comparative studies of the two fibrinogens showed several features distinguishing between fibrinogen Bethesda III and fibrinogen Philadelphia. Both fibrinogen Bethesda III and fibrinogen Philadelphia plasmas had an inhibitory effect on the coagulation of normal fibrinogen. However, in direct comparison at similar fibrinogen levels, fibrinogen Bethesda III was more inhibitory than fibrinogen Philadelphia. In addition, increased thrombin concentrations resulted in prolongation of the thrombin clotting time of fibrinogen Philadelphia, while with fibrinogen Bethesda III it decreased the thrombin clotting time. Fibrinogen Philadelphia fibrin monomers did not polymerize when formed in plasma or when aggregated at an ionic strength of 0.2. The plasma fibrin monomers of fibrinogen Bethesda III did aggregate (despite a prolonged lag phase), as did the purified...
fibrin monomers at an ionic strength of 0.21 (Fig. 3). Aggregation of fibrinogen Bethesda III fibrin monomers was also observed at an ionic strength of 0.30. These studies suggest that although fibrinogen Bethesda III and fibrinogen Philadelphia resemble one another, they do not appear to be identical dysfibrinogenemias. The clinical expression of these two disorders was also similar but not identical. The propositus with fibrinogen Philadelphia experienced postpartum hemorrhage and had a lifelong history of excessive bleeding after minor trauma, tooth extraction, and tonsillectomy. The propositus with fibrinogen Bethesda III was symptomatic only during her pregnancies. In this she was similar to the patients with fibrinogens Vancouver, Metz, and Alba/Geneva, in whom there was a high incidence of spontaneous abortion. In our patient the abortions were associated with hemorrhagic manifestations. It is not absolutely certain that these spontaneous abortions were related to abnormal fibrinogen. However, the use of cryoprecipitate as a source of fibrinogen did stop the vaginal bleeding during her second and third trimester. It is interesting that after two of her three complete pregnancies she developed thrombophlebitis, on one occasion after cesarean section and on a second after the insertion of an intravenous catheter in the lower extremity.

During her last pregnancy, the propositus' fibrinogen level became undetectable by the chronometric technique, although the total amount of fibrinogen measured by immunologic or fibrin tyrosine content remained similar to the values obtained before her pregnancy. This suggested that there was an increase in the ratio of abnormal to normal fibrinogen. This could result from decreased synthesis of the normal fibrinogen and/or increased synthesis of the abnormal fibrinogen. Alternatively, a change in the relative rates of catabolism may have been responsible for these findings.

Several aspects of fibrinogen Bethesda III are unique. A fraction rich in the abnormal fibrinogen (as determined by fibrin monomer aggregation) could be separated from normal fibrinogen by ethanol fractionation. This suggests the presence of two populations of fibrinogen, which is consistent with an autosomal inheritance. Dual populations of fibrinogen were also found in fibrinogen Zurich I, in which a normal and abnormal population of fibrin monomers could be obtained using reptilase. The elution patterns of fibrinogen Baltimore on DEAE cellulose chromatography suggested two fibrinogen populations, as did the immunoelectrophoresis patterns of fibrinogens Cleveland I and Detroit. Recent studies have indicated that fibrinogen Paris I has two populations of fibrinogen molecules, one with a normal γ chain and one with an abnormal γ chain.

Proteolysis of fibrinogen Bethesda III with plasmin was abnormal as judged by the PAGE and thrombin time determinations. Persistence of the larger degradation products (X and Y) was reflected in marked prolongation of the thrombin time throughout the entire experimental period. Similar findings have been described in fibrinogen Geissen. Recent studies of plasmin degradation of fibrinogen Geissen and fibrinogen Paris I indicate a defect in the β chain and the carboxy-terminal part of the γ chain, respectively.

The propositus with fibrinogen Bethesda III and one sister are the only members of the family who have been symptomatic. Both have had problems with hemorrhage at the time of pregnancy or abortion. One affected sister, however, has
had two children without any bleeding. It is interesting to speculate that this abnormal fibrinogen may not provide for a firm attachment of the placenta to the uterine wall. Thus small placental tears could occur that then result in hemorrhage and abortion. Moreover, as discussed above, pregnancy itself seems to result in a deterioration in the functional activity of the fibrinogen, and this may be why the hemostatic defect was more pronounced at this time. The fibrinogen turnover studies in this patient showed that the patient herself did not have a generalized hypercatabolic state, since normal fibrinogen had a normal half-life, 3.25 days (78 hr), and a normal fractional catabolic rate, 27%. However, her fibrinogen had a 1.42-day half-life (34 hr) in a normal individual and an 85% fractional catabolic rate. It was postulated that a partial or total asialofibrinogen could result in a dysfibrinogenemia with a shortened half-life; however, the sialic acid content of fibrinogen Bethesda III was normal, and recent studies by Martinez et al. indicated that sialic acid does not play a major role in the intravascular survival of fibrinogen. Thus the mechanism of the reduced survival of this fibrinogen is unclear at the present time.

Fibrinogen Bethesda III appears to be a new congenital dysfibrinogenemia characterized by hypofibrinogenemia with delayed fibrin monomer polymerization and a shortened fibrinogen half-life. Affected individuals with this dysfibrinogenemia are asymptomatic except during pregnancy and the immediate postpartum period. There appears to be an association of spontaneous abortions with severe hemorrhage and hemorrhage during pregnancy and post partum. The site of the molecular defect of fibrinogen Bethesda III is not known, but it seems to be a defect that results in the slow conversion of fibrinogen to fibrin and delayed proteolysis of fibrinogen to its late degradation products.

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