Fibrinogen Baltimore II: Congenital hypodysfibrinogenemia with delayed release of fibrinopeptide B and decreased rate of fibrinogen synthesis

(dysfibrinogenemia/hypofibrinogenemia/catabolism)

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ABSTRACT A congenital hypofibrinogenemia, fibrinogen Baltimore II, was found in a young asymptomatic Caucasian female. Prothrombin, partial thromboplastin, and euclolubin lysis times were normal, as were platelet function and coagulation factor assays. Subnormal plasma fibrinogen levels were found using chronometric, rate-independent, and immunologic assay methods. Kinetic analysis of fibrinopeptide release revealed a delay in the thrombin-catalyzed release of fibrinopeptide B from the abnormal protein. Proteolysis of fibrinopeptide A by thrombin or Arvin, fibrin monomer polymerization, and fibrin polymer ligation occurred at normal rates. Catabolism of radiolabeled autologous and homologous fibrinogen was also normal, but the fibrinogen synthetic rate was less than half the normal value. Comparison of the coagulation characteristics of fibrinogen Baltimore II with those of other abnormal fibrinogens indicates that it represents a unique example of hypofibrinogenemia.

Congenital dysfibrinogenemia is an inherited disorder of fibrinogen synthesis in which a presumed structural abnormality results in altered coagulation characteristics of the protein. The clinical manifestations of the disease vary from hemorrhagic diathesis to thrombosis. The characteristic clinical laboratory finding in dysfibrinogenemia is an apparently low plasma fibrinogen concentration using an assay dependent on the rate of clot formation. This value usually falls within normal limits when a rate-independent method is used.

In lieu of information concerning the precise location of a molecular defect, dysfibrinogens have been classified on the basis of their functional characteristics (1-3). The etiology of dysfibrinogenemia has been related to alterations in the primary structure of the protein. Blomback et al. (4) were the first to identify such a defect, and more recently four different abnormal proteins have been similarly characterized (5-8).

Fibrinogen. Fibrinogen was purified from citrate/phosphate/dextrose plasma by three glycine precipitation steps (9) at room temperature. Fibrinogen isolated in this way from normal or abnormal plasma was >98% coagulable when assayed by the method of Laki (10) and appeared similarly homogeneous when analyzed by NaDodSO4/polyacrylamide gel electrophoresis (11) under reducing conditions. Commercial normal human fibrinogen (Kabi, grade L, Stockholm, Sweden) was further purified as above by glycine precipitation. This preparation was used as "control" fibrinogen unless otherwise noted.

Thrombin. Thrombin (bovine, topical; Parke-Davis) was stored at -20°C in a solution [1,000 units (u)/ml] of 25 mM barbital buffer (pH 7.5) containing 135 mM NaCl. Human α-thrombin, a generous gift of C. Murano (National Institutes of Health), was prepared by the method of Fenton et al. (12). All references to thrombin activity are in US (formerly NIH) units.

Coagulation Studies. The following procedures were used for clinical laboratory studies: one-stage prothrombin time (PT) by the method of Quick et al. (13) with rabbit brain thromboplastin (Pel-Freez; ref. 14); partial thromboplastin time (PTT) by the method of Nye et al. (15) with Thrombopax Reagent (Ortho Diagnostics); euclolubin lysis time according to von Kaula and Schultz (16); factors VIII, IX, XI, and XII according to Hardisty and MacPherson (17) with the appropriate factor-deficient plasma; and Factor XIII according to Tyler (18). Factors VII and X were measured as follows: citrated patient plasma was diluted 1:10 with 0.9% NaCl, and 0.15 ml of this solution was combined with an equal amount of factor-deficient plasma. A one-stage PT was then performed as described above. The results were compared with a standard curve generated in the same manner using pooled normal plasma.

Platelet aggregometry was performed on a platelet aggregation profilometer (BioData, Horsham, PA) at 37°C with platelet-rich plasma (=300,000 platelets per mm²) and the following as aggregating agents in a final volume of 0.5 ml: 2–10 μM ADP (Sigma), 1.2 mg of ristocetin (Abbott) per ml, 5.5 and 11 μM epinephrine (Elkins-Sinn, Cherry Hill, NJ), 0.26 mg of collagen ( Worthington) per ml, and 0.2–0.4 μ of bovine thrombin (Parke-Davis) per ml.

Release of Fibrinopeptides. Release of fibrinopeptides A and B was measured by a modification of the Martinelli and Scheraga (19) method. The clotting reaction was initiated at 35°C by adding 10 μl of human thrombin (1.33 u/ml) or Arvin (A-38414, Abbott; lot no. 23-126-DH; 3.3–6.7 u/ml) to 50 μl of a fibrinogen solution (4–10 mg/ml) containing 20 mM sodium phosphate (pH 7.4) with 0.36 M L-α-aminoaniprac acid and 0.15 M NaCl. At the indicated times the reaction was stopped and the clot was solubilized by adding 15 μl of 5% phosphoric acid.

METHODS

Blood. Blood was obtained by venipuncture and plasmapheresis performed with citrate/phosphate/dextrose (Fenwall Laboratories, Deerfield, IL) as anticoagulant. Patient plasma used for clinical studies was prepared by diluting 9 vol of whole blood with 1 vol of 3.8% trisodium citrate dihydrate.

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Abbreviations: u, unit(s); PT, prothrombin time; PTT, partial thromboplastin time; IEF, isoelectric focusing.

7318
A 10-μl aliquot (≈60 μg) was then analyzed by reverse-phase HPLC on a Varian model 5000 liquid chromatograph. Peptides were detected by absorbance at 205 nm and eluted from an octadecylsilane column with an isocratic mobile phase containing 22% acetonitrile in 0.1% trifluoroacetic acid. Additional details of this method, which permits rapid determination of the kinetics of fibrinopeptide release, will be published elsewhere.

Electrophoretic Studies. NaDodSO4/polyacrylamide gel electrophoresis was performed in a Pharmacia horizontal slab gel apparatus using the Laemmli method (11).

Isoelectric focusing (IEF) of reduced fibrinogen was done in 2-mm horizontal slabs of 1% agarose (IEF grade, Pharmacia) containing 12% (wt/vol) sorbitol, 6 M urea, 6% (vol/vol) Pharmalyte 4–6.5, and 2% (vol/vol) Pharmalyte 3–10. Fibrinogen was dissolved in a solution of 6 M urea (freshly deionized by passage through a 2.5 × 22 cm column of Resyn I-300, mixed-bed ion exchanger; Fisher) and 5% (vol/vol) 2-mercaptoethanol. Complete reduction of disulfide bonds occurred after incubation of this solution for 3–6 hr at 37°C. Ten microliters (∼50 μg) was applied to the gel near the anode, and focusing was carried out at 10 W constant power for 75–120 min at 13°C using 1 M NaOH as the cathode solution and 10 M glutamic acid as the anode solution. Gels were fixed and stained with Coomasie blue G-250 (Bio-Rad). All protein bands focused between pH 5.5 and 7.4.

Studies of Fibrinogen Metabolism. Fibrinogen Baltimore II was labeled with 125I by the iodine monochloride method of McFarlane (20). Free iodine was removed by gel filtration on a 2.4 × 82 cm column of Sephadex G-100 (particle size 40–120 μm) equilibrated with 10 mM sodium citrate buffer (pH 7.0) containing 0.25 M NaCl in sterile pyrogen-free water. Three-milliliter fractions were collected into sterile nonpyrogenic glass tubes, each containing 0.12 ml of 5% human serum albumin (sterile, for injection; NYBCEN, New York) to stabilize the fibrinogen (21). Protein eluting at the excluded volume (specific activity, 3.5 μCi/μg; 1 Ci = 3.7 × 1010 Bq) was 98% coagulable by thrombin and >99.5% precipitable. Immediately prior to injection the fibrinogen solution was sterilized by passage through a 0.22-μm filter (Milllex-CV, Millipore).

125I-Labeled normal human fibrinogen (for injection) was obtained from Amersham (IBRN; lot no. 0179) and when administered had a specific radioactivity of 75.3 μCi/mg. This material was 92% coagulable by thrombin and 95% of the radioactivity was precipitated by 10% trichloroacetic acid.

After providing informed, witnessed written and verbal consent, the affected father of the proposita was injected with a sterile solution containing 0.6 ml (45.2 μCi, 0.6 mg) of 125I-labeled normal fibrinogen and 2.6 ml (27.4 μCi, 7.5 mg) of 125I-labeled fibrinogen Baltimore II. Five-milliliter blood samples were collected at the indicated times over a period of 7 days, and 1–2.5 ml of plasma derived therefrom was assayed for radioactivity on a Beckman (Gamma 8000) gamma counter. The radioactivity in each of seven 24-hr urine samples collected during the same period was determined. Beginning 3 days prior to the study and for 17 days thereafter, the patient received daily 200 mg of potassium iodide orally.

Rates of fibrinogen synthesis and catabolism were calculated from plots of plasma radioactivity vs. time, assuming a two-compartment model (22, 23). Kinetic constants were obtained by fitting a biexponential equation (24) to the data with the aid of a computer program by using the Newton–Raphson method for nonlinear parameter estimation (25). A correction for non-protein-bound iodide was made (23). The fractional catabolic rate was determined from urinary excretion data by dividing the total cpm in urine collected during a 24-hr period by the total plasma radioactivity at the midpoint of the collection peiod. The average of all values obtained on days 2–7 is reported in Table 3. Plasma volume (3.04 liter; 37.2 ml/kg) was estimated by dividing the total protein-bound radioactivity injected by the radioactivity concentration in the first blood sample.

RESULTS

Clinical Laboratory Findings. This 25-year-old Caucasian female has been in excellent health all of her life. At age 18 yr, symptomatic impacted wisdom teeth necessitated dental extraction, and, because of a history of some post-tonsillectomy bleeding, routine coagulation studies were performed. PT, activated PTT, euglobulin lysis time, factor assays, and platelet function were all within normal limits (Table 1). However, as is typical of dysfibrinogenemia, plasma fibrinogen concentrations appeared to be low when estimated by two methods dependent on the rate of coagulation (Table 2). However, when a method that did not depend on the rate of fibrinogen coagulation was used, the estimate of plasma fibrinogen concentration more than doubled but remained subnormal (Fig. 1 and Table 2). These results were confirmed by serial dilution experiments using the Ouchterlony double-immunodiffusion technique (30) (data not shown).

Chromometric fibrinogen determinations (Fig. 1) on the patient’s 11 relatives were normal except for her father and only son, whose symptoms and clinical laboratory findings closely resembled those reported here. The pattern of inheritance of the disorder (Fig. 1) is consistent with an autosomal dominant mode of transmission.

Coagulation Studies. The coagulability of the abnormal plasma was studied under a variety of conditions (Fig. 2). For each of these experiments the fibrinogen concentration in the control plasma was diluted to match that of the proposita (estimated at 82 mg/dl), but, because the pH and ionic strength of the reaction mixtures differ, coagulation times between experiments are not comparable. Thrombin added in increasing amounts to normal and abnormal plasma reduces the coagulation time for both but restores the latter to near normal levels (Fig. 2A), a finding consistent with a proteolytic phase coagulation defect. Addition of calcium to normal or abnormal plasma is also effective in reducing the coagulation time (Fig. 2B). However, the net reduction in each case (normal vs. abnormal) is to ≈60% of the coagulation time in the absence of calcium, thus indicating no difference in the net effect of calcium on coagulation behavior. The relationship between coagulation time and pH over a range of 5.7–8.5 was similar for the abnormal and control plasma (Fig. 2C). The addition of abnormal plasma to normal

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<th>Table 1. Coagulation laboratory findings</th>
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<td>Test</td>
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<tr>
<td>PT</td>
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<tr>
<td>PTT</td>
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<tr>
<td>Euglobulin lysis time</td>
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<tr>
<td>Factors VII–XIII</td>
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<td>Platelet aggregation*</td>
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* By ADP, ristocetin, epinephrine, collagen, and thrombin.
plasma did not affect the coagulation time of the latter (Fig. 2D), which indicates that the abnormal plasma does not contain a circulating anticoagulant.

**Determination of the Functional Abnormality.** When the kinetics of individual fibrinopeptide release were examined, fibrinopeptide A was released at a rate and to an extent similar to that of the control (Fig. 3A). The release of fibrinopeptide B was delayed, although the total amount of peptide was ≈80% of normal at later time points (Fig. 3B). For other experiments (not shown) in which higher concentrations of thrombin were used, the total amount of fibrinopeptide B released was identical to that of the control. The delay is completely abolished at a final thrombin concentration of >3 u/ml and is substantially prolonged at enzyme concentrations of <0.1 u/ml.

The observation of a prolonged Arvin clotting time (Table 2) indicates that fibrinopeptide A is released more slowly than normal in plasma. We attempted to confirm this using HPLC to monitor fibrinopeptide A release from isolated fibrinogen but found the rate and extent of peptide generation to be normal (data not shown). As expected, no fibrinopeptide B was detected under these conditions.

No abnormalities regarding the initial rates of fibrin monomer polymerization were found (Fig. 4), and the plasma clot

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**Fig. 1.** Family studies of fibrinogen Baltimore II. Numbers indicate plasma fibrinogen concentrations (mg/dl) determined by chromometric (26) or, if in parenthesis, non-rate-dependent (28) methods. The arrow denotes the proposita. Squares, males; circles, females. Open symbols, normal individuals; cross-hatched symbol, deceased, not studied; solid symbols, affected individuals.

**Fig. 2.** Coagulation behavior of control and abnormal plasma. To determine the effect of thrombin concentration on coagulation time (A) 0.1 ml of bovine thrombin [in 25 mM sodium diethyl barbiturate (pH 7.5) containing 125 mM NaCl] was added to 0.1 ml of citrated plasma. The effect of calcium on thrombin-catalyzed clotting time (B) was studied by combining 0.2 ml of citrated plasma with 0.1 ml of a 14.5 mM NaCl solution containing 15 u of bovine thrombin per ml and 5–60 mM CaCl2. The effect of pH on coagulation time (C) was determined by mixing one part citrated plasma with two parts Michaelis buffer (pH 5.7), after which each solution was diluted to the same final volume with water to ensure equivalent fibrinogen concentrations. Coagulation was initiated by adding 0.1 ml of bovine thrombin (25 u/ml) to 0.2 ml of buffered plasma solution. , Control in A–C; ○, Baltimore II in A–C. The effect of mixing normal and abnormal plasma on thrombin-catalyzed clotting time (D) was studied by combining 0.2 ml of normal plasma (previously diluted with either patient plasma (○) or normal saline (•)) with 0.2 ml of bovine thrombin (10 u/ml). For each of the above reactions, clot formation was monitored visually at 37°C, and each point represents the mean of triplicate determinations.

**Fig. 3.** Time course of thrombin-catalyzed fibrinopeptide release, measured by reverse-phase HPLC. Time to form visible clot: 9 min. (A) Release of fibrinopeptide A; (B) release of fibrinopeptide B. , Control fibrinogen; ○, fibrinogen Baltimore II. One area count = 1 µV sec.

**Fig. 4.** Polymerization of fibrin monomers. Purified normal and abnormal fibrinogen (3.0 ml of a solution at 2.5 mg/ml) was combined with human thrombin (5 u in 50 µl) and the mixture was allowed to clot for 30–45 min at 25°C. The clot was removed with a glass rod, washed three times by immersion in 7 ml of distilled water for 10 min, and dissolved in 1 ml of 20 mM sodium acetate buffer (pH 4.2). The protein concentration was determined by absorbance at 280 nm (31) and the Lowry method (32). Polymerization was initiated by mixing 0.5 ml of a fibrin solution at 1 mg/ml (in sodium acetate buffer) with an equal volume of 80 mM Na2HPO4 to produce a pH 7.4 solution with an ionic strength of 0.10. The extent of polymerization was measured by turbidity at 350 nm in a Varian DMS-90 spectrophotometer against a blank containing equal volumes of 20 mM sodium acetate (pH 4.2) and 80 mM Na2HPO4. , Control fibrinogen; ○, fibrinogen Baltimore II.
was stable in 5 M urea or 2% monochloroacetic acid. These results indicate that the polymerization and stabilization phases of coagulation are normal.

Electrophoresis of Isolated Fibrinogen. A comparison of the mobility of normal and abnormal fibrinogen using NaDodSO₄/polyacrylamide gel electrophoresis revealed no detectable differences in size or relative amounts of the Aα, Bβ, or γ subunits (data not shown).

IEF of isolated fibrinogen (Fig. 5) revealed the typical pattern of 13-15 protein bands (33). No difference between the normal and abnormal fibrinogens was found. Immunoelectrophoresis (3% agar, pH 8.6) of fibrinogen Baltimore II also failed to reveal any differences from normal (data not shown).

Metabolic Studies. The plasma clearance curves for radio-
labeled autologous and homologous fibrinogen were virtually identical (Fig. 6). The corresponding half-lives, intravascular distributions, and fractional catabolic rates were similar and within normal limits (Table 3). However, the rate of fibrinogen synthesis was well below normal for the autologous fibrinogen (Table 3). Thus, the subnormal fibrinogen concentration results from a decreased rate of fibrinogen synthesis or secretion.

**DISCUSSION**

In accordance with the nomenclature suggested by Beck et al. (35), the hypodysfibrinogenemia described in the present study has been designated fibrinogen Baltimore II. Clinical laboratory studies of the proposita were unremarkable, except for the presence of subnormal amounts of fibrinogen with a prolonged clotting time. Examination of all 11 immediate blood relatives revealed that the father and only son of the proposita were similarly affected, thereby confirming the heritability of the disorder and suggesting that the dual traits of hypo- and dysfibrinogenemia are not transmitted independently.

Assessment of fibrinopeptide release kinetics reveals that fibrinogen Baltimore II is functionally abnormal in the proteolytic phase of coagulation, the specific defect being a delay in release of fibrinopeptide B. Although dysfibrinogenemia may sometimes be accompanied by severe bleeding or thrombosis, it is apparent in the present case that no serious bleeding, thrombotic episodes, or abnormal wound healing could be attributed to the abnormal fibrinogen. This is consistent with, if not predicted from, the functional defect, for it is generally accepted that cleavage of fibrinopeptide B is not essential for fibrinogen coagulation (3, 31, 36).

The results in Fig. 2A suggest that the delay in plasma coagulation can be abolished at increased thrombin concentrations. Consistent with these data is the observation that the kinetics of fibrinopeptide B release are indistinguishable from normal at thrombin concentrations >3 u/ml. Thus, the functional defect is corrected in vitro by relatively high thrombin concentrations, and this characteristic may contribute to the unremarkable case history of the index patient.

Experiments to assess the initial rates of fibrin monomer polymerization revealed no difference between the abnormal and control fibrinogen. However, the structure of the abnormal fibrin polymer may well be different from normal, as evidenced by the divergence in solution turbidity at later time points (Fig. 4), despite the fact that the initial protein concentration (measured by two methods) was identical to that of the control.

Only 5 of the 110 congenital dysfibrinogenemias have been found to exhibit delayed release of fibrinopeptide B. Fibrinogens Bethesda I (37), New York (38), and New Orleans (39) were reported to have slow release of both fibrinopeptides A and B and are thus different from the present dysfibrinogen. A review of the procedure used to measure fibrinopeptide re-

**Table 3. Metabolic studies of normal and abnormal fibrinogen**

<table>
<thead>
<tr>
<th>Fibrinogen</th>
<th>Half-life, days*</th>
<th>Mean residence time, days*</th>
<th>Intravascular distribution, % of total pool</th>
<th>Fractional catabolic rate†</th>
<th>Synthesis rate, mg/kg per day</th>
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<tbody>
<tr>
<td>Autologous</td>
<td>3.48</td>
<td>3.45</td>
<td>70</td>
<td>29</td>
<td>31.8</td>
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<tr>
<td>Homologous</td>
<td>3.93</td>
<td>2.86</td>
<td>74</td>
<td>35</td>
<td>25.8</td>
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<tr>
<td>Normal mean (34)</td>
<td>4.14</td>
<td>4.17</td>
<td>71</td>
<td>24</td>
<td>23.0</td>
</tr>
<tr>
<td>Normal range (34)</td>
<td>3.1-5.6</td>
<td>2.9-5.9</td>
<td>45-87</td>
<td>17-35</td>
<td>NR</td>
</tr>
</tbody>
</table>

*Calculated from slope of line determined by least-squares regression on last five points in Fig. 6.
†The reciprocal of the plasma-derived fractional catabolic rate.
‡Data were derived from plasma and urine and are expressed as % of plasma pool per day.
lease from fibrinogen Detroit (40) indicates that under similar conditions of low thrombin concentration (0.05 NIH unit/ml), fibrinogen Baltimore II would also have not released fibrinopeptide B to any appreciable extent. However, a comparison of cofactors, such as the plasma fibrinogen concentration, PT, PTT, and the patient’s clinical presentation (41), serves to distinguish these two abnormal proteins. Unlike the present dysfibrinogen, fibrinogen Seattle (42, 43) was present in plasma at normal concentrations, repolymerized abnormally, and released only 54% of fibrinopeptide B. Thus, we are able to conclude that fibrinogen Baltimore II is functionally unique among those dysfibrinogens with delayed release of fibrinopeptide B.

Congenital hypofibrinogenemia has been described in six families in addition to that reported here (44–49). Fibrinogen Bethesda II (44), which manifests a slight delay in total fibrinopeptide release, also exhibits a major delay in fibrin monomer polymerization and is therefore dissimilar to fibrinogen Baltimore II. The remaining proteins (45–49) are delayed only in the polymerization phase of coagulation. Thus, fibrinogen Baltimore II is readily distinguished from the other hypofibrinogenons on the basis of its functional defect, a delay in fibrinopeptide B release.

The steady-state plasma fibrinogen concentration is essentially a function of its synthetic rate and rate of plasma clearance. We found the catabolic rate for fibrinogen Baltimore II to be within normal limits and conclude that the hypofibrinogenemia results from a synthetic rate about one-third of normal (Table 3).

Plasma fibrinogen survival has been studied in four of the six previously described hypofibrinogenemias. Two of these, fibrinogens Bethesda III (45) and Philadelphia (46), were markedly hypercatabolized. A third, fibrinogen Bethesda II (44), was reported to have a slightly increased fractional catabolic rate, a somewhat shortened plasma half-life (70–72 hr), and a normal synthetic rate. Thus, the etiology of the subnormal fibrinogen levels in these three cases differs from that of fibrinogen Baltimore II. In the fourth instance (49), subnormal plasma fibrinogen concentrations were not due to hypercatabolism and thus resulted from a decreased synthetic rate. However, the functional and solubility characteristics of this protein readily distinguish it from the present one.

On the basis of the foregoing we conclude that fibrinogen Baltimore II is unique among the dysfibrinogens and suggest that a similarly unique structural defect is present in this protein.

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[References]