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Abstract

Fibrinogen was isolated from the plasma of a 25-year-old female with a history of mild bleeding and several recent moderate to severe hemorrhagic episodes. Coagulability with thrombin approached 100% and varied directly with the time of incubation with the enzyme. High-performance liquid chromatography analysis of thrombin-induced fibri-nopeptide release demonstrated retarded fibrinopeptide A (FPA) and fibrinopeptide B (FPB) release and the presence of an abnormal A peptide (FPA*) amounting to 50% of the total. The same biochemical abnormalities were found in her asymptomatic father. Amino acid analysis and carboxypeptidase digestion of FPA* demonstrated the substitution of His for Arg at Aα 16. In contrast to the thrombin- and reptilase-sensitive Arg-Gly bond in the normal Aa chain, the abnormal Aa chain (A*α) sequence is resistant to reptilase attack but is slowly cleaved by thrombin. To evaluate whether Birmingham A*α and Aα chains had been assembled nonselectively into heterodimeric (ie, 50% Aα, A*α) and homodimeric (ie, 25% Aα, Aα; 25% A*α, A*α) species, the clot and the clot liquor resulting from reptilase treatment of normal or Birmingham fibrinogen were separated, and each was then further incubated with thrombin to release remaining fibrinopeptides. Assuming that fibrin- ogen Birmingham contained heterodimeric molecules and that these and the normal molecules were completely incorporated into a reptilase clot, the expected coagulability would be 75%. In addition, subsequent thrombin treatment of the reptilase clot would release 50% of the total FPA* and 75% of the total FPB present in the original sample. On the other hand, if only homodimeric fibrinogen species (50% Aα, Aα; 50% A*α, A*α) existed, the maximum reptilase coagulability would be 50%, and after thrombin treatment, 50% of the total FPB and no FPA* would be recovered from the reptilase clot. We found the propositus's fibrinogen to be 68% coagulable, and we recovered 45% of the FPA* and 70% of the FPB from the reptilase clot. Essentially the same coagulability and distribution of fibrinopeptides was found in the reptilase clot from her father's fibrinogen. We therefore conclude that fibrinogen Birmingham contains heterodimeric species (Aα, A*α) amounting to approximately 50% of the circulating fibrinogen molecules. The existence of heterodimers is consistent with a nonselective intracellular process of constituent chain assembly of dimeric plasma fibrinogen molecules.

Introduction

Congenital Dysfibrinogenemia is a disorder of fibrinogen synthesis in which a structural abnormality results in altered functional characteristics of the protein. To date, such fibrinogen molecules exhibit either altered fibrinopeptide release or defective fibrin polymeriza-tion.[1., 2., 3.] Several amino acid exchanges in the vicinity of the Aα Arg 16 Gly 17 cleavage site are now known to affect the interaction between thrombin and fibrinogen and the rate of fibrinopeptide release.[2] The Aα 16 Arg → His substitutions are the most common and include homozygous fibrinogens Bicetre[3] and Giessen [4., 5.] and heterozygous fibrinogens New Albany,[2] Petoskey,[6., 7.] Manchester,[8., 9., 10.] Louisville,[11] Sydney I,[12] Bern II,[13] Seattle II,[14] Amiens I, Amiens II,[15] and Bergamo III.[16]

In the case of the Aα 16 Arg → His substitution, heterozygous members of reported families have had little or no clinically significant hemostatic disorders.[6., 7., 8., 9., 10., 11., 12., 13., 14., 15., 16.] Similarly, heterozygous Aα 16 Arg → Cys family members are likewise free of hemostatic disorders.[16., 17., 18., 19., 20., 21.] In contrast, homozygous Aα 16 Arg → His and Aα 16 Arg → Cys individuals have a history of significant hemorrhagic episodes.[3., 4., 5.,22., 23.] This suggests that in heterozygous individuals fibrin formation is not sufficiently compromised to result in a bleeding tendency.

It is well recognized that reptilase does not cleave the abnormal Aα chains (A*α) of fibrinogens containing an Aα 16 Arg → His substitution, although fibrinopeptide A containing histidine at position 16 (FPA*) can be released from A*α chains slowly and completely by thrombin.[6., 7., 8.,11] Galanakis et al[11] studied fibrin(ogen) Louisville from which FPA had been released by reptilase treatment. They found that all FPA*-containing fibrinogen molecules were in the incoagulable reptilase clot liquor. Because reptilase cleavage of FPA from hybrid species should have resulted in significant incorporation of half-activated A*α, α fibrin molecules into the reptilase clot, they therefore concluded that fibrinogen Louisville contained only homodimeric species (ie, Aα, Aα or A*α, A*α). Hybrid molecular species (ie, Aα, A*α) would be expected to occur if intracellular fibrinogen chain assembly were a nonselective process. Thus, their observation suggests that there is selective intracellular association of constituent Aα subunit chains during hepatic assembly of Louisville fibrinogen dimers. In this study, we examined the question of homodimers v heterodimers in fibrinogen prepared from a newly discovered family with an Aα 16 Arg → His substitution (fibrinogen Birmingham). This fibrinogen is accompanied in the propositus by moderate to severe bleeding episodes.

MATERIALS AND METHODS

Tris and glycine were obtained from Aldrich Chemical Co, Milwaukee. Phenylmethylsulfonylfluoride, orthophthalaldehyde (OPA), carboxypeptidase A (CPA), carboxypeptidase B (CPB), and reptilase (Atroxin) were purchased from Sigma Chemical Co, St Louis. Trasylol (aprotinin) was from Mobay Chemical Corp, New York. Reagents for amino acid analysis were purchased from Varian Instrument Group, Sunnyvale, CA. Human thrombin was a generous gift from Dr J Fenton III (Division of Laboratories and Research, New York State Department of Health, New York). All other reagents were of the highest purity available from commercial sources.

Normal fibrinogen fraction 1–2 (from pooled normal plasma) and fibrinogen Birmingham fraction 1–2 were isolated from citrated plasma by glycine precipitation[24] and further purified as described by Mosesson and Sherry.[25] Fibrinogen concentrations were determined spectrophotometrically at 280 nm by using an absorbance coefficient ($A_{1cm}^{1\%}$) of 15.1 for fibrinogen.[26]

Clinical coagulation studies were performed by standard methods at the University of Alabama Hospitals in Birmingham. Multimeric analysis of von Willebrand factor (vWf) was performed at the Blood Center of Southeastern Wisconsin by electrophoresis of plasma in 0.65% Seakem high gelling temperature ultra pure agarose gels (HGT-(P); FMC Corp, Rockland, ME) in the presence of 0.1% sodium dodecyl sulfate (SDS) and visualized by using radiolabeled rabbit antihuman vWf and autoradiography as previously described.[27] The reference standard for vWf assays was pooled plasma from 50 normal donors (stored at -80°C).

Thrombin time measurements were made in a Fibrometer Precision Coagulation Timer (BBL, Cockeysville, MD) by adding 0.1 mL human thrombin at a concentration of 4 National Institutes of Health U/mL to 0.3 mL fibrinogen in 50 mmol/L Tris and 120 mmol/L NaCl, pH 7.4. Clottability of the purified fibrinogens was determined as described by Laki.[28]

SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli[29] on 9% slab gels.

Kinetics of fibrinopeptide release were analyzed by incubating fibrinogen (3 mg/mL) in 0.15 mol/L ammonium acetate buffer, pH 8.0, with human thrombin (0.2 U/mL) at room temperature for zero to 24 hours. Released fibrinopeptides were analyzed and quantified by using the high-performance liquid chromatography (HPLC) system described by Kehl et al and Kehl and Henschen.[30,31] Specifically, the fibrinopeptides were detected at 215 nm with a Varian Vista model 5000 system controlled by a Varian model 401 microprocessor on an Altex Cl8 reverse-phase column (0.46 x 25 cm; Rainin Instrument Co, Woburn, MA) using a linear gradient of 12% to 28% acetonitrile in 25 mmol/L ammonium acetate, pH 6.0.

Fibrin monomer polymerization studies were performed by the method of Belitser et al,[32] as modified by Gralnick et al.[33] Fibrin monomer was prepared by treating fibrinogen (1 mg/mL) with thrombin (0.5 U/mL) in 0.06 mol/L KPO_4 buffer, pH 6.8, containing Trasylol (5 KIU/mL), for 18 hours at room temperature, winding the clot on a glass rod, and dissolving the clot in acetic acid (0.02 mol/L). Reaggregation was initiated by diluting an aliquot of the fibrin solution into at least a tenfold excess of 0.06 mol/L KPO_4 or 0.15 mol/L KPO_4 buffer, pH 7.0 (final). The final fibrin concentration was 0.5 mg/mL; polymerization was monitored at 350 nm in a Gilford model 240 spectrophotometer.

Normal FPA and Birmingham FPA and FPA* were isolated by HPLC as described earlier. Approximately 50 Mg of each was hydrolyzed under vacuum in 6 N HC1 for 20 hours at 110°C. Released amino acids were identified and quantified by HPLC on a Varian MicroPak Hydrolysate column (0.4 x 15 cm) as recommended by Varian. Amino acids were reacted after fractionation with OPA[34] and detected with a Varian Fluorochrom Fluorescence Detector with an excitation filter of 340 to 380 nm and an emission filter of 390 nm.

CPA digestion was performed on the isolated fibrinopeptides by mixing the peptide (50 µg) in 0.2 mol/L N-ethylmorpholine acetate, pH 8.5, with CPA (15 µg) and incubating the mixture at 37°C for four hours. Digestion was terminated by lowering the pH to 2.5 to 3 by the addition of 5 mol/L acetic acid. After lyophilization, the released amino acids were detected and quantified as described earlier. CPB digestion was done under identical conditions using 0.072 enzyme commission units of CPB.

Fibrinogen Birmingham was examined for the presence of het-erodimers as follows. Normal or Birmingham fibrinogen (3 mg/mL, 2 mL) in 0.15 mol/L ammonium acetate buffer, pH 8.5, was incubated with reptilase (1 U/mL) for two hours at room temperature. The resulting clots were wound on a glass rod, washed, and dissolved in 300 µl of 0.02 mol/L acetic acid. After determining the protein concentration, the fibrin solution was diluted into 1.5 mL of the ammonium acetate buffer containing thrombin (10 U/mL) and incubated at room temperature for two hours. An aliquot (200 to 400 µl) of the reptilase clot supernatant was saved for HPLC analysis. Solid ammonium sulfate (30% saturation wt/vol) was added to the remaining clot supernatant solution, the solution mixed at 4°C for 30 minutes, and the precipitate harvested by centrifugation. The pre- cipitate was dissolved in l mL of the ammonium acetate buffer and treated with thrombin (10 U/mL) for two hours at room temperature. Released fibrinopeptides were detected in these samples by HPLC and quantified by comparison with recovery of FPA and fibrinopeptide B (FPB) from a thrombin-treated normal fibrinogen sample.

RESULTS

Case report. The propositus (C.D.) is a 25-year-old female from Birmingham, AL, who had experienced several relatively mild bleeding episodes since her childhood including protracted nosebleeds and

occasional hematomas. In the spring of 1984 she presented at the University of Alabama Hospitals with a hemarthrosis of her knee. Results of laboratory investigations (Table 1) were compatible with congenital dysfibrinogenemia. Occasional prolonged bleeding times were also noted. Subsequent evaluation of her parents (Table 1) revealed that she had inherited the fibrinogen abnormality from her asymptomatic father. Since 1984 the propositus has had several hospitalizations, usually for hematoma formation, frequently causing neurological compression. Platelet function evaluations made during a quiescent period demonstrated normal platelet aggregation in response to adenosine diphosphate (1 x 10^{-6} mol/L to 2 x 10^{-5} mol/L), collagen, arachidonic acid, epinephrine, and ristocetin. Analysis of plasma vWf multimers on several occasions showed an intermittent reduction of the highest-molecular weight forms. Administration of desmopressin acetate (DDAVP) did not consistently correct the bleeding time prolongation (when present) even though an increase in high-molecular weight vWf multimers was usually observed.

Table 1. Results of Laboratory Tests

Test	**Propositus**	**Father**	**Mother**	**Normal**
Plasma				
Thrombin time (s)	52.1	59.6	21.4	22.6
Reptilase time (s)	25.8	—	—	9.7
Fibrinogen, ACA (mg/dL)	50	—	—	200–400
Fibrinogen (M partigen RID) (mg/dL)	342	240	294	200–400
FDP (ng/mL)	>360	—	—	<10
Factor Vlll:vW antigen (%)	110	—	—	50–150
Purified fibrinogen				
Thrombin time (s)	164	185	23	22
Coagulability with thrombin (%)	97	95	98	>95
Coagulability with reptilase (%)	68	66	—	>95
Other				
Bleeding time (min)	5–15	7.5	5	2.5-9

Abbreviations: ACA, automated chemical analysis; FDP, fibrinogen degradation products; RID, radial immuno diffusion.

Characterization of purified fibrinogen and fibrin. The thrombin time of purified propositus fibrinogen was markedly prolonged when compared with normal fibrinogen (Ta-ble 1). Thrombin clottability increased with the time of incubation and approached 100%. In the presence of repti-lase, the coagulability was 68%. SDS-PAGE of Birmingham fibrinogen, fibrin, and factor XIIIa-cross-linked fibrin demonstrated that this abnormal fibrinogen contained subunit chains of normal size and XIIIa reactivity (results not shown). Similar results were obtained with her father's fibrinogen. Fibrin monomer, prepared from proband fibrinogen at physiological or at high ionic strength, polymerized at the same rate and to the same extent as did normal fibrin (results not shown).

Fibrinopeptide analysis and characterization. Total FPA (FPA plus FPA*) was released from Birmingham fibrinogen at a slower rate than that observed for normal fibrinogen (FPA $t_{1/2}$, 30 to 40 minutes v 10 minutes; Fig 1). FPB release from the abnormal fibrinogen also was moderately retarded compared with normal (FPB $t_{1/2}$, 25 and 15 minutes, respectively). Delayed FPB release accompanying slow FPA release has previously been observed in the case of fibrinogen Sydney I by Southan et al[12]who suggested that

this abnormality was due to coupled release of FPA and FPB. FPA* was first detected at later time points of the kinetic analysis (Fig 2) and was eluted with a retention time of 18.4 minutes, whereas normal FPA eluted at 19.5 minutes. This elution pattern is characteristic of that previously observed for FPA* released from fibrinogens containing an A α 16 Arg → His substitution.[6., 7., 8., 9., 10., 11., 12., 13., 14., 15., 16.] An identical early-eluting FPA* peak was also observed with thrombin-treated fibrinogen isolated from the father's plasma.

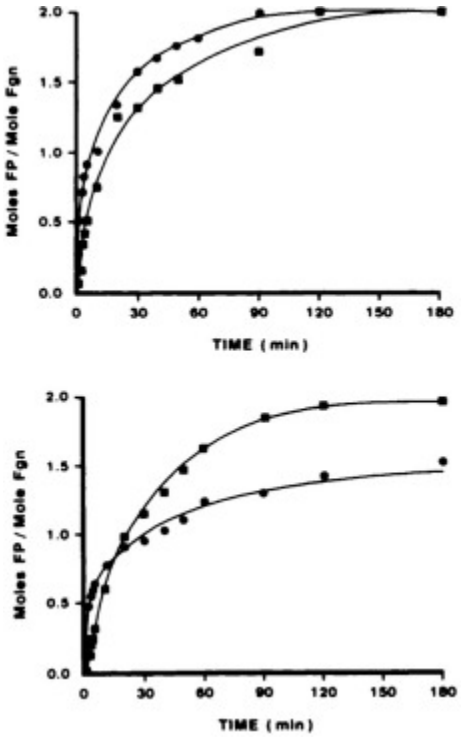

Fig 1. Time course of fibrinopeptide release from normal and Birmingham fibrinogen. Thrombin (0.2 U/mL, final) was added to fibrinogen solutions (3 mg/mL) in 0.15 mol/L ammonium acetate buffer, pH 8.5. Top panel, normal fibrinogen; bottom panel, Birmingham fibrinogen, •, FPA; ■, FPB; FP, fibrinopeptide; Fgn, Fibrinogen.

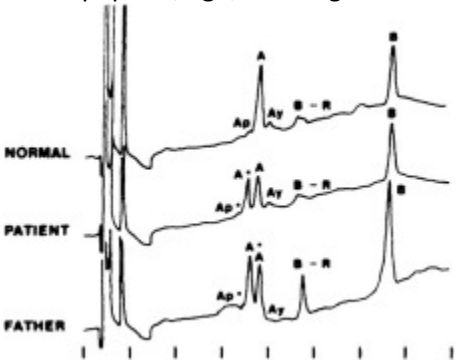

Fig 2. HPLC analysis of the fibrinopeptides released by thrombin from normal and Birmingham fibrinogen. Fibrinogen (3 mg/mL) was incubated with thrombin (0.2 U/mL, final) for 24 hours. Ap, phosphorylated FPA; A, FPA; Ap*, phosphorylated FPA*; A*, FPA*; Ay, FPA lacking N-terminal Ala; B-R, FPB lacking the C-terminal Arg; B, FPB.

Amino acid analysis of FPA* indicated the absence of the arginine residue found in FPA and the presence of 0.8 mol histidine/mol peptide. All other amino acids normally present in FPA were present in FPA* in the expected amounts. The carboxy-terminal sequence of FPA and FPA* was obtained from carboxypeptidase digestion. Normal FPA from the subject or the control released arginine upon CPB digestion and no amino acids with CPA. In contrast, no amino acids were released from FPA* by CPB digestion, whereas incubation with CPA resulted in the release of histidine and the penultimate valine from this peptide (results not shown).

Identification of heterodimeric Birmingham fibrinogen molecules. As expected, reptilase cleaved only FPA from normal fibrinogen. Subsequent incubation of the reptilase clot with thrombin resulted in FPB release, which accounted for all available FPB (Table 2, Fig 3A). Fibrinogen Birmingham incubated with reptilase released FPA amounting to 49% of the total FPA obtained from normal (Table 2, Fig 3B). This sample was 68% coagulable with reptilase, whereas normal was 96% coagulable. When the Birmingham reptilase clot was subsequently treated with thrombin, 45% of the FPA* and 70% of the total FPB were recovered. The remaining FPA* and FPB was recovered from the reptilase-incoagulable supernatant. As assessed by SDS-PAGE under the conditions of this experiment, fibrin cross-linking did not occur. Nearly identical results were obtained with fibrinogen isolated from the subject's father (Table 2, Fig 3C).

Table 2. Fibrinopeptide Recovery (Moles Fibrinopeptide per Mole Fibrinogen)

Sample	**FPA (%)**	**FPA• (%)**	**FPB (%)**
Normal fibrinogen			
Reptilase supernatant	1.92	0	0
Reptilase supernatant + lla	0.17	0	0
Reptilase Clot + lla	0	0	1.93
Total	2.09 (105)	0	1.93 (97)
Propositus's fibrinogen			
Reptilase supernatant	0.98 (49)	0	0
Reptilase supernatant + lla	0.04 (2)	0.54 (27/55)	0.58 (29)
Reptilase clot + lla	0	0.44 (22/45)	1.40 (70)
Total	1.02 (51)	0.98 (49/100)	1.98 (99)
Father's fibrinogen			
Reptilase supernatant	0.97 (49)	0	0
Reptilase supernatant + lla	0	0.58 (29/57)	0.60 (30)
Reptilase clot + lla	0	0.43 (22/43)	1.38 (69)
Total	0.97 (49)	1.01 (51/100)	1.98 (99)

The recovery of FPA, FPA*, and FPB was quantified by comparison of the peak area obtained from the chromatograms depicted in Fig 3 to the peak areas obtained from normal fibrinogen (3 mg/mL) treated with thrombin (10 U/mL) for two hours. HPLC analysis of samples incubated for longer periods of time indicated that complete release of all fibrinopeptides had occurred under these conditions. The proportions of FPA or FPB recovered in any given fraction is indicated, where appropriate, in parentheses as the percentage of normal; for FPA* the first number in parentheses indicates the percent recovery in relation to total fibrinopeptide A (FPA plus FPA*), whereas the second number indicates the recovery in terms of the total FPA*.

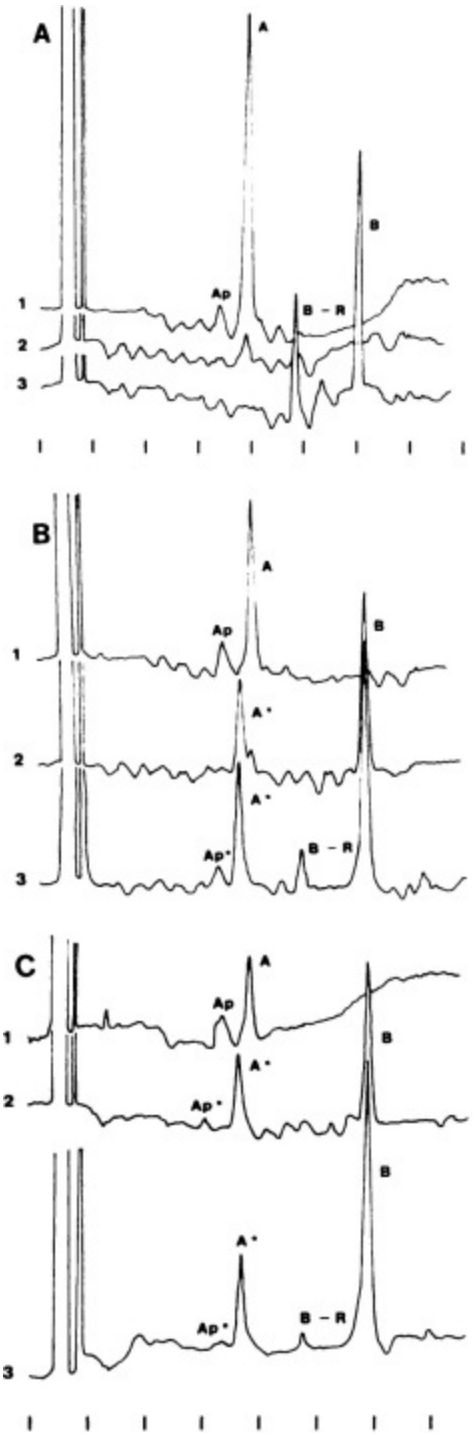

Fig 3. Comparison of fibrinopeptides released from normal and Birmingham fibrinogen. (A) Normal fibrinogen. (B) Birmingham fibrinogen (proband). (C) Birmingham fibrinogen (father). Curve 1. reptilase supernatant; curve 2. reptilase supernatant plus thrombin; curve 3, reptilase clot plus thrombin. A*, His containing A peptide; B-R, FPB lacking C-terminal Arg.

DISCUSSION

Abnormal fibrinogens that contain amino acid replacements in the amino terminal region of the Aα chain make up the majority of the defects that have been determined at the molecular level.[1., 2., 3., 4., 5., 6., 7., 8., 9., 10., 11., 12., 13., 14., 15., 16.] Fibrinogen Birmingham is an example of the commonly reported A α 16 Arg → His substitution. Assuming that the normal and abnormal A α chain genes are transcribed and translated to the same extent and that the component chains are nonselectively assembled intracellularly to form dimeric fibrinogen molecules, one would expect hybrid molecules to amount to 50% of the circulating fibrinogen in a heterozygous individual. Homodimeric species (ie, Aα, Aα; A*α, A*α) would account equally for the remainder. Our results are the first to support such an assumption. The reptilase coagulability and the distribution of FPA* and FPB in the reptilase-clotted specimens imply that hetero-dimeric species (ie, Aα, A*α) comprise the theoretical 50% of circulating Birmingham fibrinogen molecules, that is, the release of nearly half of the total FPA* from the reptilase clot of both the proband's and father's fibrinogen indicates that reptilase-activated heterodimeric Birmingham fibrinogen molecules had been incorporated into the fibrin clot, evidently as the result of activation of one of its two available "A" polymerization domains. In addition, finding 69% to 70% of the total FPB in the Birmingham reptilase clot approaches the theoretical 75% that would be expected if all heterodimeric species in the Birmingham fibrinogen sample as well as all the normal molecules had been incorporated into the clot. Presumably, the reptilase clot supernatant contains the "unactivated" homodimeric (A*α, A*α) fibrinogen species plus a small proportion of "activated" hetero-dimeric molecules complexing with the soluble fibrinogen.

If, on the other hand, only homodimeric species existed in heterozygous Aα 16 Arg → His fibrinogen preparations, one would anticipate 50% coagulability with reptilase, 50% recovery of FPB from the reptilase clot, and complete recovery of available FPA* in the incoagulable supernatant of the reptilase clot. Indeed, Galanakis et al[11] reported 40% reptilase coagulability and no FPA* in the reptilase clot of fibrinogen Louisville, which is characterized by an Aα 16 Arg — His substitution. On that basis, they concluded that only homodimeric fibrinogen molecules were present in their patient. We do not understand the biologic mechanism underlying these different findings; however, one possibility is that the hepatic mechanism of intracellular fibrinogen assembly is different in the Louisville subject as compared with ours.

Data reported on fibrinogen Kawaguchi and Osaka (Aα 16 Arg → Cys)[21] are consistent with the existence of homodimers in these fibrinogens. Similarly, hybrid mole- cules were not detected in fibrinogen Seattle I (Bβ 14 Arg — Cys).[35] It seems plausible that substitution of Cys for Arg confers selectivity on the intracellular assembly process by virtue of disulfide bridging at sites that are in register. Support for the formation of disulfide bonds at the Aα Cys replacement sites comes from reports on homozygous fibrinogen Metz molecules (Aα 16 Arg → Cys),[22., 23.] which have no free sulfhydryl groups and whose amino termini are evidently close enough to undergo disulfide bridging at this position.[36] Thus, these observations on Cys-substituted molecules do not necessarily contravene our present results suggesting that fibrinogen Aα chain assembly in forming dimeric molecules is normally a nonselective process.

The bleeding episodes experienced by our subject are evidently not attributable to the Aα 16 Arg → His substitution, especially because her father is clinically normal in this regard and because other heterozygous individuals with this replacement have minimal or no bleeding tendencies.[6., 7., 8., 9., 10., 11., 12., 13., 14., 15., 16.] As assessed by analysis of her father's fibrinogen, the existence of

hybrid molecules per se appears to have little if any deleterious effect on normal hemostasis. Furthermore, it seems that a second mutation affecting the formation of fibrin is unlikely as a cause for the bleeding because fibrin monomers prepared from the proband's fibrinogen polymerized normally.

A more likely cause for the bleeding episodes is the coexistence of a second hemostatic abnormality involving either platelets or vWf. We observed an intermittent loss or absence of the highest-molecular weight multimers of vWf and occasional prolongation of the bleeding time. Platelet aggregation in the presence of various levels of ristocetin was normal, which indicated that the multimer abnormality could not be classified as a form of type IIB von Willebrand disease.[37., 38.] Even though platelet aggregation was normal, additional studies on her platelets seem warranted to investigate the possibility that her bleeding tendency could be due to storage pool deficiency.[39]

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