

## "Fibrinogen Tokyo II". An abnormal fibrinogen with an impaired polymerization site on the aligned DD domain of fibrin molecules.

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### Research Article

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FIGURE 1

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# “Fibrinogen Tokyo II”

## AN ABNORMAL FIBRINOGEN WITH AN IMPAIRED POLYMERIZATION SITE ON THE ALIGNED DD DOMAIN OF FIBRIN MOLECULES

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**ABSTRACT** A hereditary dysfibrinogenemia associated with defective aggregation of fibrin monomers was found in a 39-yr-old female and in the members of her immediate family, who had all been asymptomatic.

The abnormality was probably due to an impaired polymerization site exposed in the DD domain of two adjacent fibrin molecules, because plasmic fragment DD derived from the propositus' cross-linked fibrin bound far less tightly to insolubilized normal fragment E than that from the normal one. Its complementary polymerization site in the E domain of fibrin, which was exposed by thrombin cleavage, and the polymerization site in the D domain of fibrinogen, which was available without activation by thrombin, were both found to be normal.

More anodal migration of the abnormal fragment DD than the normal one, as shown by immunoelectrophoresis, seemed to support our concept that the mutation most likely resides in the D domain of the abnormal fibrinogen molecule at or near a region closely related to the polymerization site that is exposed when two fibrin molecules are linearly aligned.

The work of others on the polymerization of normal fibrin with different techniques yielded results consistent with our conclusions.

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## INTRODUCTION

Many of the dysfibrinogenemias reported thus far fall into a category with abnormal polymerization of fibrin monomer with or without altered releases of fibrinopeptides (1-3). The functional abnormalities associated with abnormal polymerization have not been located on a molecular level, except in fibrinogen Detroit (4, 5), to the replacements of amino acid residues or to the functional domains where the polymerization sites have been identified.

In this paper, we report a congenital dysfibrinogenemia, designated as fibrinogen Tokyo II, with a substantially delayed polymerization of fibrin monomer that is probably due to an abnormality in the polymerization site on the DD domain of fibrin molecules, which are unfolded so that they can function when two fibrin molecules are linearly aligned.

## METHODS

**Blood collections.** Venous blood was collected by the two-syringe method. A 2-ml portion was placed in a plain glass tube and allowed to clot at 37°C for 2 h to harvest serum. Another 2-ml portion was transferred to a glass tube containing EDTA and used for blood cell counting and preparation of EDTA plasma for assays of plasma proteins including fibrinogen. The other portion was anticoagulated with 1/9 vol of 3.8% trisodium citrate; platelet-rich and platelet-poor plasmas were prepared for the platelet aggregation and blood coagulation studies, respectively.

**Hemostasis and coagulation tests.** Hemostatic and coagulation studies were performed by standard procedures (6, 7) or as described elsewhere (8), unless otherwise stated. Factor XIII (XIII)<sup>1</sup> was determined immunochemically by

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<sup>1</sup> Abbreviations used in this paper: XIII, factor XIII; XIIIa, activated factor XIII; IBS, imidazole-buffered saline; KIU, kallikrein inhibitor unit; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Laurell's method (9) using antisera purchased from Behring-Werke AG, Marburg/Lahn, West Germany as well as by measuring the fluorescence of incorporated monodansyl cadaverine into casein, basically according to Lorand et al. (10). Plasma fibrinogen was determined by four methods: (a) the thrombin time method essentially according to Clauss (11) using a fibrinogen determination kit (Dade, Miami, FL), (b) the method of Ratnoff and Menzie (12), (c) the method of Laurell (9), and (d) the turbidimetric method—0.1 ml of EDTA-plasma diluted with 3.0 ml of 0.5% EDTA-K<sub>2</sub> in physiological saline was heated at 56°C for 10 min and the resulting turbidity was measured at 660 nm. Concentrations of fibrinogen in the sample were obtained on a calibration curve made with dilutions of a pooled plasma containing a known amount of fibrinogen. The thrombin time was performed as follows: 0.1 ml of either imidazole-buffered saline (IBS; one part of 0.257 M imidazole buffer, pH 7.4, and nine parts of physiological saline) or 0.025 M CaCl<sub>2</sub> was added to 0.1 ml of plasma, and the mixture was warmed at 37°C for 2 min. 0.1 ml of 10 NIH-U/ml thrombin ([Thrombin Topical from Parke, Davis & Co., Detroit, MI] was dissolved in 50% glycerol to give 1,000 NIH-U/ml, stored at -20°C and diluted as previously described with IBS just prior to use) was then added, and a clotting time was obtained in duplicate with a fibrometer (Becton, Dickinson & Co., Cockeysville, MD). The Reptilase time was performed by replacing thrombin with a 50 µg/ml solution of Reptilase, venom of *Bothrops atrox* (Reptilase-R, Pentapharm, Switzerland).

**Purification of fibrinogen.** About 130 ml of acid-citrate-dextrose-plasma derived from the propositus or healthy adults was passed through lysine-agarose (13) and gelatin-agarose (14) columns connected in tandem at 22°C. The agarose was Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Fibrinogen was precipitated from this plasma by either 25% ammonium sulfate saturation (repeated three times) or the method of Blombäck and Blombäck (15). The fibrinogen fractions were dissolved in appropriate volumes of 0.3 M NaCl which was extensively dialyzed against the same solution and stored frozen in small aliquots at -80°C until used. In general, the purity of fibrinogen in various samples prepared by these two methods fell between 90 and 95% as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16).

**Studies on purified fibrinogen and its derivatives.** The thrombin and Reptilase times were measured similarly as described above on plasma samples using 0.05 M Tris-HCl, pH 7.6, containing 0.1 M NaCl, instead of IBS. Thrombin clottability was estimated by the ratio of thrombin clottable protein to total protein, as determined by the method of Ratnoff and Menzie (12). Immunoelectrophoresis was performed at a constant current of 3 mA/cm width for 90 min using 0.8% agarose (Nakarai Chemicals, Ltd., Kyoto, Japan) in barbital buffer, ionic strength 0.05, pH 8.6. Gels were stained with Coomassie Brilliant Blue R-250 (Nakarai Chemicals). DEAE-cellulose chromatography was carried out as described by Finlayson and Mosesson (17). Cross-linking of fibrin mediated by activated factor XIII (XIIIa) were examined by SDS-PAGE using 5.0 or 7.5% gels as well as by the clot solubility test in 1.0% monochloroacetic acid (18). Total release of fibrinopeptides by either thrombin or Reptilase and aggregation of fibrin monomer were studied essentially according to Gralnick et al. (19).

**Preparation of plasmic digests of cross-linked fibrin.** The patient's (30 mg) and normal (200 mg) fibrinogens, 2% in 0.3 M NaCl, were enriched with 50 U of purified human XIII (25 U/ml prepared by the method of Curtis and Lorand

[20]) per gram of fibrinogen and diluted 100-fold with 0.05 M Tris-HCl, pH 7.6, containing 0.1 M NaCl, 0.01 M CaCl<sub>2</sub>, and 25 kallikrein inhibitor unit (KIU)/ml aprotinin (Ono Pharmaceutical Co., Ltd., Osaka, Japan). Cross-linked fibrin clots were formed by adding 2,000 NIH-U of purified bovine thrombin (21) per gram of fibrinogen under gentle stirring for more than 30 s. The clots were then incubated for the initial 2 h at 37°C and for another 12 h at 4°C to induce a maximal conversion of fibrinogen to fibrin. They were transferred onto filter paper placed in funnels and the clot liquid was squeezed out. The fibrin clots were then repeatedly washed with the buffer without aprotinin and cut into small pieces. To harvest fragments DD and E<sub>1</sub> + E<sub>2</sub>, the subfragments of fragment E that retain polymerization capacity (22-24), the minced fibrin clots derived from 200 mg of normal or 30 mg of abnormal fibrinogen were suspended in 4.0 and 0.6 ml, respectively, of 0.05 M Tris-HCl, pH 7.6, containing 0.1 M NaCl, 0.01 M CaCl<sub>2</sub>, 0.04% NaN<sub>3</sub> and 12.5 U/ml streptokinase-activated human plasmin (22.5 Committee on Thrombolytic Agents U/mg). After 8 h of gentle stirring at 25°C, the remaining clots were separated by centrifugation and again digested similarly with one-half of the initial volume of the plasmin-containing buffer for another 8 h. The digestion was repeated once again so that the fibrin clots were almost completely digested. The lysates, which had been separated by centrifugation and treated each time with 500 KIU of aprotinin per unit of plasmin, were combined and subjected to two-step chromatography on Sepharose 6B (Pharmacia Fine Chemicals), essentially as described by Olexa and Budzynski (22). The fractions that retained the molecular weight for either fragment DD or fragments E<sub>1</sub> and E<sub>2</sub>, as verified by SDS-PAGE, were combined, concentrated, and used for the binding study or stored frozen until used.

**Binding study using Sepharose conjugated with fibrin monomer or with plasmic digests of cross-linked fibrin.** Fibrinmonomer-Sepharose was prepared from normal fibrinogen essentially according to Heene and Matthias (25), packed into a small column (gel vol = 4 ml), and equilibrated with 0.05 M Tris-H<sub>3</sub>PO<sub>4</sub>, pH 7.6, containing 0.1 M NaCl, 0.005 M EDTA, and 5 KIU/ml aprotinin (buffer A). A gradient employing pH (7.6 → 4.1) and NaCl (0.1 → 2.0 M) was applied for elution using 0.05 M Tris-H<sub>3</sub>PO<sub>4</sub>, pH 4.1, containing 2.0 M NaCl, 0.005 M EDTA, and 5 KIU/ml aprotinin (buffer B<sub>1</sub>). 1-ml fractions were collected.

Sepharose conjugated with the plasmic digests of normal cross-linked fibrin was prepared as follows: 57.5 mg of fragment DD and 19.8 mg of fragments E<sub>1</sub> + E<sub>2</sub> (fragment E<sub>1+2</sub>), as estimated on the basis of an extinction coefficient of E<sub>280</sub><sup>1%</sup> = 20.8 for fragment DD and 10.2 for fragment E (26), were mixed with 5 ml each of CNBr-Sepharose 4B gel (27). Fragment DD was confirmed to be fairly homogeneous, but fragment E<sub>1+2</sub> was an approximately 1:1 mixture of E<sub>1</sub> and E<sub>2</sub> which contained a trace amount of E<sub>3</sub>, if any, as depicted in Fig. 1. Approximately 86.5% of fragment DD and 93.6% of fragment E<sub>1+2</sub> were conjugated to the Sepharose gels. After treatment with 1 M monoethanolamine, pH 8.0, the gels were extensively washed with chilled 0.1 M NaHCO<sub>3</sub>, pH 8.9, containing 0.5 M NaCl and packed into small columns. The packed DD- and E<sub>1+2</sub>-conjugated Sepharose gels (3.5 ml) were successively washed with 100 vol each of 6 M urea in 0.05 M Tris-H<sub>3</sub>PO<sub>4</sub>, pH 4.1, containing 1 M NaCl, 0.005 M EDTA, and 5 KIU/ml aprotinin (buffer B<sub>2</sub>); 0.1 M CH<sub>3</sub>COOH; and buffer A. After the columns were equilibrated with buffer A, samples of ~0.5 mg were applied to the columns. The adsorbed materials were eluted after the A<sub>280</sub> became <0.005 by employing pH (7.6 → 4.1), NaCl

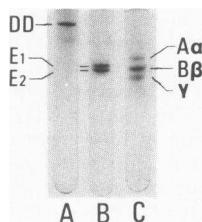


FIGURE 1 SDS-PAGE of fragments DD (gel A) and  $E_{1+2}$  (gel B) derived from normal cross-linked fibrin that was conjugated to the CNBr-activated Sepharose 4B. The three-subunit polypeptides of normal fibrinogen (mol wt:  $A\alpha = 65,000$ ,  $B\beta = 55,000$ , and  $\gamma = 47,000$ ) are shown as reference in gel C. A constant current of 7 mA/gel was applied to 7.5% gels. Gels were stained with Coomassie Brilliant Blue R-250.

(0.1 → 1.0 M), and urea (0 → 6 M) gradients, using buffer  $B_2$ . 1-ml fractions were collected.

The affinity chromatography was performed by using the same column for normal and abnormal samples in order to compare chromatograms as precisely as possible. For each run, these columns were regenerated by washing with sufficient amounts of buffer  $B_1$  for fibrinmonomer-Sepharose and buffer  $B_2$  for DD- or  $E_{1+2}$ -Sepharose as well as with 0.1 M  $CH_3COOH$ . The gels were then equilibrated with buffer A for repeated use. The binding capacity of the gels was confirmed to be unaltered since it was observed that the chromatograms were almost similar as long as identical samples were applied.

## RESULTS

### Case history and family study

A 39-yr-old female inpatient at the gynecological ward had been examined for hemostasis and blood coagulation before she underwent hysterectomy for a uterine myoma. She had experienced neither excessive bleeding nor thrombotic tendency. Since the preoperative coagulation study revealed a markedly prolonged thrombin time and variable levels of plasma fibrinogen, we were prompted to further investigate her plasma. Some of her immediate family members investigated were also found to have a similar abnormality of fibrinogen, although they are all asymptomatic. Thus, the abnormality appears to be compatible with an autosomal dominant trait; no consanguinity was known to the family (Fig. 2).

### Hemostasis and coagulation studies

The bleeding time, whole blood clotting time, platelet counts, and platelet aggregation were normal but the one-stage prothrombin time, 15.8 s (control, 11.3 s), and activated partial thromboplastin time, 37.9 s (control, 21.3 s), were moderately prolonged. In thrombelastography, there was a moderately prolonged clot formation time, 18.0 min (normal,  $5.4 \pm 1.0$  min), and reduced maximum amplitude, 35.0 (normal,

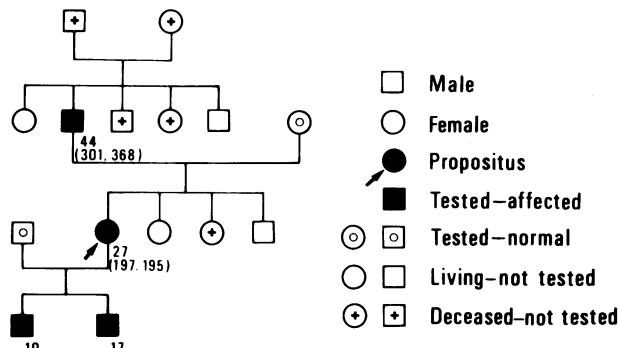


FIGURE 2 Pedigree of fibrinogen Tokyo II. Figures represent plasma fibrinogen concentrations measured by the thrombin time method and in parentheses, those determined by the method of Ratnoff and Menzie and the turbidimetric method, respectively.

$53.4 \pm 4.5$ ). The means  $\pm$  SD ( $n = 50$ ) are given. Blood coagulation factors including XIII were found to be within normal ranges. Levels of plasminogen and antithrombin III were normal both in activity and antigen. Concentrations of  $\alpha_2$ -plasmin inhibitor, 6.9 mg/dl, and plasma fibronectin, 31.3 mg/dl, were also normal; the incorporation of both, which were recently demonstrated to be cross-linked to the  $\alpha$ -chain of fibrin by XIIIa (28, 29), into fibrin was normal as evidenced by  $\sim 33$  and 23% decreases in the amounts found in serum, respectively. As has been shown in many of the disfibrinogenemia reported thus far, the thrombin and Reptilase times were markedly prolonged, but could be partially corrected by the addition of calcium ions. There was also a great discrepancy between the markedly reduced level of plasma fibrinogen determined by the thrombin clotting time method and normal levels obtained by other techniques, as shown in Table I.

TABLE I  
Thrombin and Reptilase Times and Fibrinogen Concentrations Determined on Propositus' Plasma

Studies	Patient	Normal or control
Thrombin time (s)		
Without calcium ions	154.8	11.8
With calcium ions	14.0	5.6
Reptilase time (s)		
Without calcium ions	171.7	18.7
With calcium ions	21.7	9.8
Fibrinogen concentrations (mg/100 ml)		
Thrombin time method (Clauss)	27	200-400
Ratnoff and Menzie's method	197	200-350
Turbidimetric method	195	200-400
Laurell's method	378	200-400

## Studies on purified fibrinogen and its plasmic fragments

**Gross molecular structure.** Molecular weights of fibrinogen Tokyo II and its three subunit polypeptides and the cross-linking pattern made by XIIIa were identical with those of normal fibrinogen when examined by SDS-PAGE (pattern not shown). Fibrinogen Tokyo II was also indistinguishable from normal fibrinogen by DEAE-cellulose chromatography (profile not shown) and immunoelectrophoresis (Fig. 7, wells 1 and 2).

**Thrombin and Reptilase times.** As shown in Table II, the thrombin and Reptilase times were markedly prolonged; virtually no solid fibrin clots were formed in more than 40 min. The addition of  $\text{CaCl}_2$  corrected the clotting times remarkably, but still only partially.

**Release of total fibrinopeptides.** When the release of total fibrinopeptides was studied at timed intervals after the addition of thrombin or Reptilase, no substantial differences were noted between the patient's and normal fibrinogens (pictures not shown).

**Aggregation of fibrin monomer.** Markedly altered aggregation was observed in the patient's fibrin monomer when compared with normal ones. The alteration depends on the ionic strength and thus virtually no aggregation was observed when the concentration of  $\text{NaCl}$  was increased to 0.05 M or more in 0.06 M potassium phosphate, pH 6.8. When normal fibrin monomer was mixed with 1/3 vol of either 0.02 M acetic acid or the patient's fibrin monomer, the aggregation profiles were almost similar; this shows that the patient's fibrin monomer per se would not inhibit the polymerization of normal fibrin monomer (pictures not shown).

## Binding studies by means of affinity chromatography

**Fibrinogen on the fibrinmonomer-Sepharose.** To see if the proposed binding site on the D domain of

TABLE II  
Thrombin and Reptilase Times and Thrombin-clottability on Purified Fibrinogen

Studies	Patient's	Normal
Thrombin time (s)		
Without calcium ions	>2,400	12.9
With calcium ions	18.8	11.6
Reptilase time (s)		
Without calcium ions	>2,400	25.3
With calcium ions	34.5	16.8
Thrombin clottability (%)		
Without calcium ions	72	92
With calcium ions	90	96

fibrinogen (5, 30, 31) is malfunctional in fibrinogen Tokyo II, 5.5 mg each of highly purified normal and abnormal fibrinogen fractions (fractions recovered in the study by DEAE-cellulose chromatography were combined and repeatedly used here) were subjected to affinity chromatography on fibrinmonomer-Sepharose. Gradient elutions with 50 ml each of buffers A and B<sub>1</sub> were performed. As shown in Fig. 3, adsorbed fractions of normal and abnormal fibrinogens were eluted nearly at the same positions, which suggests that the binding site on the D domain in fibrinogen Tokyo II was indistinguishable from that in normal fibrinogen.

**Binding of fragment  $E_{1+2}$  to fragment DD-Sepharose.** The binding of fragment  $E_{1+2}$  to fragment DD-conjugated Sepharose was tested. A sample of 0.46 mg (0.5 ml) of fragment  $E_{1+2}$  that was derived from fibrinogen Tokyo II or one from normal fibrinogen was applied to a fragment DD-conjugated Sepharose column, and 1-ml fractions were collected. Approximately 30% of each fragment  $E_{1+2}$  was not adsorbed; this was probably because an excess amount of protein had been applied as judged from the SDS-PAGE of the eluted fractions. The adsorbed materials were eluted in a similar fashion at 3.4 M or higher urea concentrations (Fig. 4). The eluted fractions were combined as indicated, concentrated, and subjected to SDS-PAGE to confirm the molecular size and structure. As shown in Fig. 5, left panel, they were found to be composed solely of fragments E<sub>1</sub> and E<sub>2</sub> that had been applied to the column. Thus, it appears that the

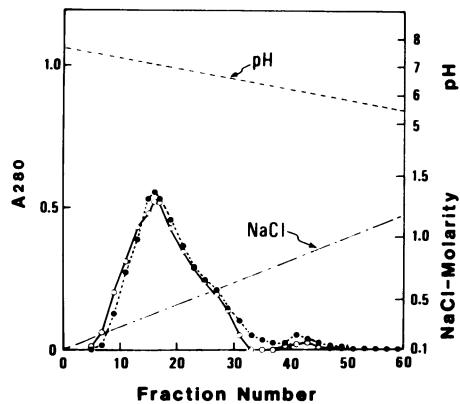


FIGURE 3 Affinity chromatography of fibrinogen Tokyo II (○) and normal fibrinogen (●) on fibrinmonomer-Sepharose. A 5.5-mg sample of highly purified fibrinogen (fractions recovered in the study of DEAE-cellulose chromatography were combined and used in this study) was applied to a fibrinmonomer-Sepharose 4-ml column at 25°C, and adsorbed protein was eluted with 50 ml each of buffers A and B<sub>1</sub> by employing gradients of NaCl (0.1 → 2.0 M) and pH (7.6 → 4.1). 1-ml fractions were collected.

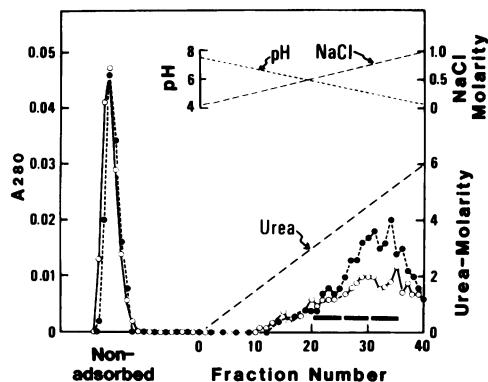


FIGURE 4 Affinity chromatography of plasmic fragment  $E_{1+2}$  ( $E_1 + E_2$ ) derived from fibrinogen Tokyo II (○) and normal fibrinogen (●) on fragment DD-conjugated Sepharose. Samples of 0.46 mg of fragment  $E_{1+2}$  were individually applied to a 3.5-ml column at 25°C, and adsorbed proteins were eluted by employing gradients of NaCl (0.1 → 1.0), pH (7.6 → 4.1), and urea (0 → 6 M), using 20 ml each of buffers A and B<sub>2</sub>. 1-ml fractions were collected. Fractions containing proteins were combined as indicated by horizontal bars, concentrated, and subjected to SDS-PAGE to ascertain the molecular weight of eluted protein fractions (see Fig. 6).

polymerization site postulated in the NH<sub>2</sub>-terminal region of fragment E (which is present in  $E_1$  and  $E_2$ , but not in  $E_3$ ) (31) may function normally and bind to a unique complementary site on the DD domain formed by two adjacent fibrin molecules.

*Binding of fragment DD to fragment  $E_{1+2}$ -Sepharose.* The reverse of the situation discussed above was investigated. 0.5 mg (1.0 ml) each of fragment DD derived from fibrinogen Tokyo II and from normal fibrinogen were applied to fragment  $E_{1+2}$ -Sepharose, and the chromatograms were compared. As shown in Fig. 6, fragment DD of fibrinogen Tokyo II was eluted much earlier between fractions 6 and 13, which corresponded to 1.0–2.2 M urea. On the other hand, fragment DD derived from the normal cross-linked fibrin

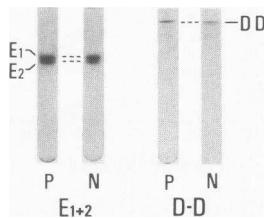


FIGURE 5 SDS-PAGE of the eluted fractions  $E_{1+2}$  from DD-Sepharose and DD from  $E_{1+2}$ -Sepharose. Representative electrophoretograms are those on the combined fractions 26 to 30 for both patient's (P) and normal (N)  $E_{1+2}$  fractions, and the combined peak fractions 8 and 9 for the patient's (P) and 26 and 27 for normal (N) fragment DD. 7.5% gels were stained with Coomassie Brilliant Blue R-250.

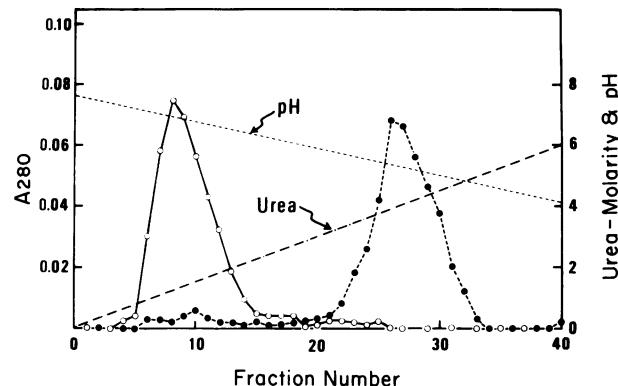


FIGURE 6 Affinity chromatography of plasmic fragment DD derived from fibrinogen Tokyo II and normal fibrinogen on fragment  $E_{1+2}$ -conjugated Sepharose. 0.5 mg each of fragment DD derived from fibrinogen Tokyo II (○) and normal fibrinogen (●) were applied to a 3.5-ml column, and adsorbed proteins were eluted as mentioned previously (see legend for Fig. 4).

was eluted between fractions 23 and 32, which corresponded to much higher concentrations of 3.4–4.8 M urea. The eluted proteins of fragment DD derived from abnormal as well as normal fibrinogens both exhibited the DD-structure as shown by SDS-PAGE (Fig. 5, right panel). The discrete elution profiles clearly show that fragment DD derived from fibrinogen Tokyo II has a significantly lower affinity than that from normal fibrinogen to fragment  $E_{1+2}$ .

*Immunoelectrophoresis of fragment DD.* When fragment DD derived from fibrinogen Tokyo II was compared with that from normal fibrinogen, more anodal migration was observed (Fig. 7, wells 3 and 4).

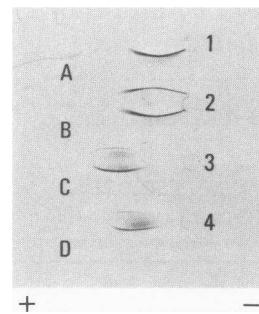


FIGURE 7 Immunoelectrophoretic comparison of fibrinogen and fragment DD derived from fibrinogen Tokyo II with those from a normal individual. Electrophoresis was performed at a constant current of 3 mA/cm width for 90 min using 0.8% agarose in barbital buffer, ionic strength 0.05, pH 8.6. Gels were stained with Coomassie Brilliant Blue R-250. Well 1, normal plasma; well 2, propositus' plasma; well 3, fragment DD derived from fibrinogen Tokyo II; well 4, fragment DD derived from normal fibrinogen; troughs A, B, and D, anti-human fibrinogen; trough C, anti-fragment D.

Such an abnormal electrophoretic mobility was not as noticeable in fragment  $E_{1+2}$  (picture not shown) and fibrinogen (Fig. 7, wells 1 and 2).

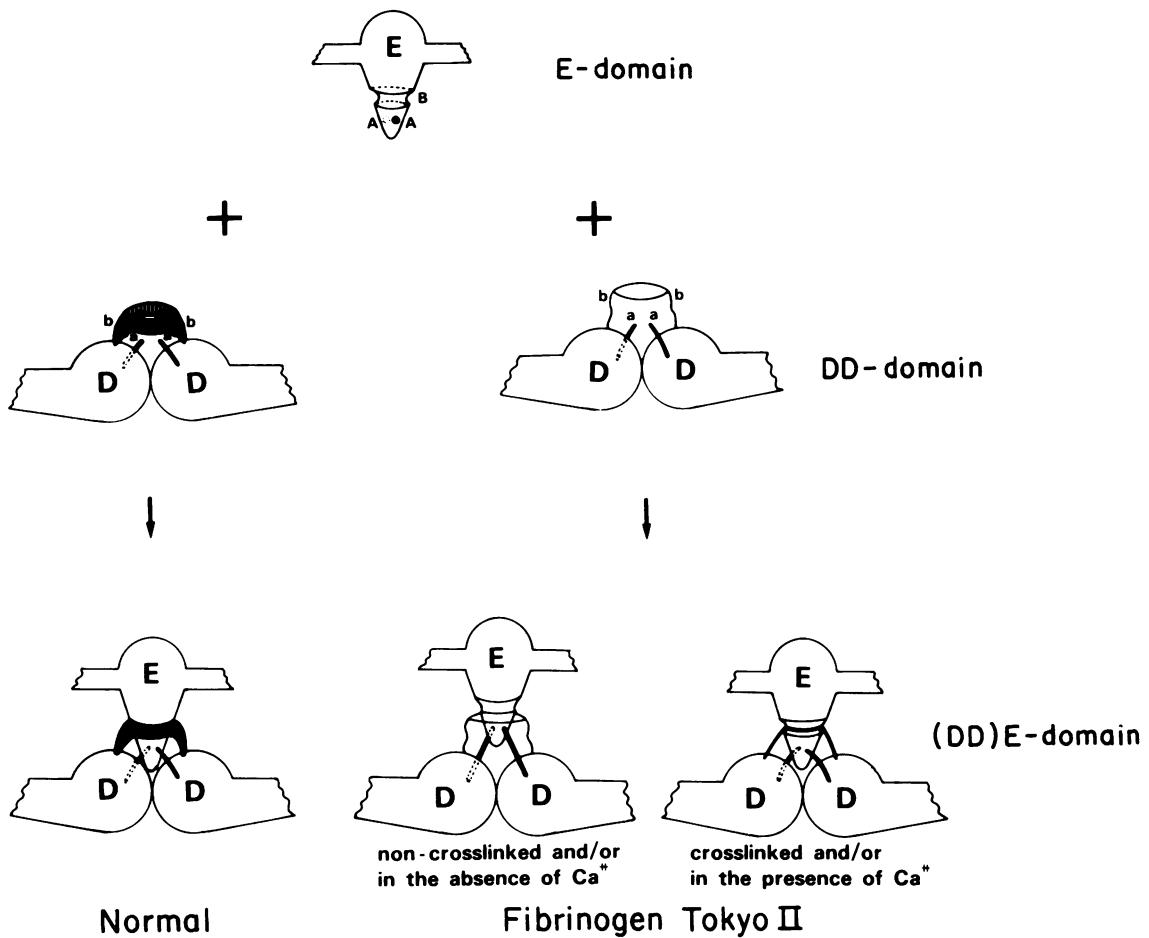
## DISCUSSION

Although impaired polymerization of fibrin monomer is the most common among the dysfibrinogenemias reported thus far (1-3), the affected polymerization sites are not necessarily characterized in relation to the functional domains of fibrinogen molecules. Kudryk et al. demonstrated two functional sites of polymerization—one on the amino-terminal disulfide knot domain that is unfolded by thrombin and the other on the D domain which is already available in the molecule of fibrinogen—by using functional domains of the well-characterized abnormal fibrinogen, fibrinogen Detroit (5, 30). Recently, however, Olexa and Budzynski postulated that four functional sites are involved in the polymerization step and depicted them by their flexible banana model of a bivalent fibrinogen molecule (31). According to their model, there are two sets of binding sites on the  $\text{NH}_2$ -terminal domain of the fibrinogen molecule that are designated as "A" and "B", their respective complementary sites are designated as "a" and "b" on the D domain. The "A" and "B" sites become available upon cleavage of fibrinopeptides A and B, respectively. While the "a" site does not need activation and is thus functional in the fibrinogen molecule, the "b" site becomes functional only when two D domains of different molecules have been linearly aligned to form a bivalent "bb." By using Sepharose conjugated with either normal noncross-linked fibrin, plasmic fragment  $E_{1+2}$  (a mixture of fragments  $E_1$  and  $E_2$ ), or fragment DD, we showed that fragment DD derived from the patient's cross-linked fibrin was eluted at distinctly lower concentrations of urea (1.0-2.2 M) and around neutral pH (7.1-6.4) while that derived from cross-linked normal fibrin was recovered at much higher concentrations of 3.4-4.8 M urea and lower pH (5.5-4.8). In the reverse situation, i.e., the elution of the patient's and the normal fragments  $E_{1+2}$  from the DD-conjugated Sepharose, the elution profiles were nearly identical. Thus, the set "B"- "bb" would not function properly in fibrinogen Tokyo II owing to the impaired "bb" site on the aligned DD domain. It has been suggested that the set of polymerization sites "B"- "bb" induces side-to-side polymerization to form a fibrin sheet (31). The defective binding between the E and DD domains found in fibrinogen Tokyo II could be also explained by the recently proposed model of Fowler et al. (32). This model is based on an electron micrographic study in which they clearly showed the presence of trimer and pentamer complexes. In these complexes, the E domain of a fibrin monomer is attached to the linearly

aligned DD domain of fibrinogen molecules, which is covalently cross-linked by XIIIa. In fibrinogen Tokyo II, the formation of a fibrin sheet by side-to-side aggregation or even protofibril formation by the "DE-stag contact" designated by Fowler et al. (32) may thus be impaired. The polymerization site "b" on the D domain may be arranged at or near the XIIIa-mediated cross-link bonds, or these covalent bonds may align and stabilize the sites on the two fragment D moieties, as suggested by Olexa and Budzynski (31). The XIIIa-mediated stabilization or facilitation of an aligned "bb" site may, at least in part, account for an accelerated polymerization and gelation of fibrin in the presence of  $\text{Ca}^{++}$ , which is observed in most abnormal fibrinogens (1-3) as well as the normal one (33, 34). The XIIIa-mediated stabilization of the "bb" site may thus contribute to the formation of hemostatic thrombi in vivo where XIII and  $\text{Ca}^{++}$  are sufficiently available. This may also explain why little or no hemorrhagic tendency has been observed in many of the dysfibrinogemias in spite of a greatly prolonged thrombin clotting time in vitro. In our case, the propositus underwent surgery without any pathologic bleeding, though the "B"- "bb" set of Olexa and Budzynski (31) and the "DE-stag contact" of Fowler et al. (32) have been shown to function abnormally. The reason for this is not completely clear, but we presumed that the "bb" site without stabilization by cross-link bonds may not properly react with its complementary "B" site. However, the "bb" site when stabilized, may bind to the "B" site tightly enough to form the (DD)E moiety and promote polymerization of fibrin, although the binding capacity is significantly reduced compared with that of normal molecules. We did not conclusively study another set of polymerization sites, "A"- "a". However, we presumed that at least the "a" site on the D domain of fibrinogen Tokyo II functioned normally because the binding of the highly purified fibrinogen fraction to fibrinmonomer-Sepharose was virtually indistinguishable from that of the normal.

Based on these results and presumptions, we constructed a model which may illustrate the abnormal polymerization site on the DD domain in fibrinogen Tokyo II (Fig. 8).

There is a strong possibility that the abnormality resided in the DD domain of linearly aligned fibrin molecules; this was also supported by the abnormal electrophoretic mobility of fragment DD derived from the patient's fibrin. Since electrophoretic mobilities of fibrinogen in the patient's and normal plasma are indistinguishable from each other, possible explanations include the presence of conformational changes in the DD domain or a different mode of cleavage by plasmin. Because of a probable conformational change(s) due to mutation at or near the plasmic cleavage site(s),



**FIGURE 8** Schematic presentation of the malfunctioning "bb" site on the DD domain of fibrinogen Tokyo II. The defective "bb" site is represented by a loose thin ring, compared with a thick tight ring in normal fibrinogen. In the absence of  $\text{Ca}^{++}$  and/or when the reciprocally aligned two D domains are not cross-linked, this loose ring would not adequately fit the furrow on the E domain representing the complementary "B" site (bottom, center). The ring may be tightened, however, in the presence of  $\text{Ca}^{++}$  and/or upon cross-link formation between the two D domains, so as to fit the furrow and to form a more tightly bound (DD)E domain (bottom, right). Thus, the "B"- "bb" set should function somewhat properly to promote polymerization in fibrinogen Tokyo II. The two "A" sites are symmetrically arranged on a single E domain, to each of which is bound an arm that represents the "a" site on a monomeric D domain.

the patient's fragment DD may contain a negatively charged polypeptide segment(s) which should be cleaved off by plasmin, or it may have lost a positively charged one(s) which should be retained in normal molecules. Further studies are currently in progress, including an amino acid sequence analysis which is intended to clarify the abnormal structure of fibrinogen Tokyo II in relation to its functional abnormalities.

The other two functions ascribed to fibrinogen were apparently normal in fibrinogen Tokyo II, i.e., the release of total fibrinopeptides and the cross-linking by XIIIa to itself and other plasma proteins (28, 29, 35).

Other features of fibrinogen molecules, including the gross molecular weight and subunit compositions as examined by SDS-PAGE, the chromatographic pattern on DEAE-cellulose, the circular dichroism spectra, and the immunologic reactivity, were all found to be normal.

We demonstrated that the impaired polymerization of fibrin monomer found in the dysfibrinogenemia, designated as fibrinogen Tokyo II, is partly, if not completely, attributable to a dysfunction of the polymerization site on the DD domain. Such an abnormality has not been observed in congenital dysfibrinogenemias reported thus far.

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