A Unique Factor XIII Inhibitor to a Fibrin-Binding Site on Factor XIIIa

By Hidetaka Fukue, Kristen Anderson, Peter McPhedran, Lionel Clyne, and Jan McDonagh

An 81-year-old woman, who presented with sudden episodes of spontaneous bleeding, was found to have a specific inhibitor of factor XIII. Her fibrin clots had ~70% γ-γ and no α polymer formation, under conditions where normal fibrin was fully cross-linked; the patient's clots were soluble in urea or monochloroacetic acid. Factor XIII activity in her plasma was 24%, measured by the dansylcadaverine incorporation assay. When mixed with normal plasma, the patient's plasma inhibited fibrin cross-linking; however, in mixtures of patient and normal plasma, there was no inhibition of factor XIII activity when assayed by the incorporation of dansylcadaverine into casein. Thus, this inhibitor was active against fibrin cross-linking but not against ligation of small molecules to casein. Consequently, gel electrophoresis of reduced, sodium dodecyl sulfate-solubilized fibrin clots was a simple, quantitative method that was used to measure inhibitor activity. This inhibitor is unique and has been designated inhibitor New Haven. It was neutralized by anti-IgG and anti-κ. It did not inhibit the activation of factor XIII but did inhibit fibrin cross-linking. There was complex formation between the inhibitor and activated factor XIII (A', A*) but not between A0 or fibrinogen. Only A', A* and the 56-Kd fragment bound to affinity columns made with this IgG. The inhibitor significantly decreased the binding of A', A* to fibrin clots. These data indicate that the epitope for this inhibitor is in a fibrin binding site. It is hidden in the zymogen and expressed on A' and A*, indicating that the conformational change occurring with the cleavage of the activation peptide is sufficient to expose the fibrin binding site.

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THE FORMATION and dissolution of a fibrin gel in vivo is a seminal event, resulting in various physiologic and pathologic reactions. These processes are controlled by multiple feed-forward and feed-backward loops at every stage. Factor XIII plays an important role in the final stages of fibrin assembly and in regulation of fibrinolysis. Factor XIIIa* cross-links fibrin γ chains and fibrin α chains, resulting in a gel that is mechanically more stable and more resistant to fibrinolysis. It also covalently cross-links α2-plasmin inhibitor (α2-PI) to the fibrin surface, resulting in more efficient inhibition of plasmin. Fibronecitin is likewise cross-linked to fibrin in plasma, causing the gel to have different mechanical properties. Factor XIII circulates as a zymogen that must be activated in a concerted fashion by thrombin and Ca2+ with non-cross-linked fibrin I or II as a cofactor. The salient features of the final stage in fibrin assembly are the proteolytic cleavages of fibrinogen and factor XIII, which release fibrinopeptide A, the factor XIII activation peptide, and fibrinopeptide B, leading to the polymerization of fibrin and formation of a cross-linked fibrin gel. Concomitant with γ chain cross-linking of fibrin, there is cross-linking of α2-PI to fibrin.

In this report, we have used a spontaneously occurring antibody to the final stages of blood coagulation to dissect this system further. Acquired inhibitors of fibrin stabilization are quite rare; approximately 12 cases have been reported. However, they may provide significant insights into the molecular details of this system. A spontaneously occurring antibody to fibrin stabilization was purified, and its inhibitory function was characterized. These experiments provide more evidence for the mechanism and function of factor XIII.

*In plasma, factor XIII has a molecular formula of A2B2. Its activation proceeds through distinct steps, giving rise to several molecular forms:

\[ A_2B_2 \xrightarrow{\text{Thrombin}} A'_2B_1 \xrightarrow{\text{Ca}^{2+}} A'_3 + 2B. \]

The intracellular form has only A subunits; its activation is similar:

\[ A_2 \xrightarrow{\text{Thrombin}} A'_2 \xrightarrow{\text{Ca}^{2+}} A'_3. \]

Only A'3 (or factor XIIIa) is the active enzyme.

MATERIALS AND METHODS

Materials. Protein A-Sepharose CL-4B, blue-Sepharose, and CNBr-activated Sepharose were obtained from Pharmacia-LKB (Piscataway, NJ); DEAE HPLC (5 pW) columns from Waters (Milford, MA); barbital buffer from Bio-Rad (Richmond, CA); Seakem agarose from FMC Marine Colloids (Rockland, ME); immobilon from Millipore (Bedford, MA); biotinylated antibody and Vectastain avidin-biotin complex (ABC) reagent from Vector (Burlingame, CA); immunoelectrophoresis gels and Antigen Stain Kit from Bio-Rad (Arlington, CA); and SEAlab (Arlington, CA); defibrinated plasma was made from citrated plasma by heating it to 56°C for 5 minutes and centrifuging it. In some cases, plasma was used from a known factor XIII-deficient patient after he had been transfused. It had a factor XIII activity of 6% in the dansylcadaverine incorporation assay.

Bovine thrombin (Parke-Davis, Morris Plains, NJ), activity = 1,000 NIH U/mL, or more highly purified human thrombin (Enzyme Research Laboratories, South Bend, IN), activity = 3,333 U/mL.
NIH U/mL was dissolved in 25 mmol/L Tris-HCl, 25% glycerol, pH 7.5, to 500 U/mL and stored at ~70°C. Hirudin was dissolved in 50 mmol/L Tris-HCl, pH 7.5, to 1,000 U/mL and stored at 4°C; it was used to terminate activation of factor XIII by thrombin.

Isolation of IgG. IgG from the patient and from normal pooled plasma was isolated on protein A-Sepharose. Protein A-Sepharose was absorbed in 10 mL of water and washed three times with 0.1 mol/L phosphate buffer. The final suspension (~5 mL) had a binding capacity of 20 mg of IgG/mL. The gel was equilibrated with 0.1 mol/L phosphate buffer-0.15 mol/L NaCl, pH 8.0. After elution of unbound protein, the Igs were eluted with 0.1 mol/L glycine-HCl buffer, pH 3.0. IgG fractions were collected and dialyzed against 50 mmol/L Tris-HCl-0.1 mol/L NaCl, pH 7.4. The purified IgG proteins had the two bands characteristic of IgG on reduced polyacrylamide gels.

Purification and analysis of factor XIII. Intracellular factor XIII (A2) was purified from placenta concentrates (Fibrogammin, kindly provided by Behringwerke AG, Marburg, Germany) by chromatography on blue-Sepharose. After dialysis against buffer A (50 mmol/mL Tris-1 mmol/L EDTA, pH 7.4), the partially purified protein was applied to a high performance liquid chromatography (HPLC) with a diethyl aminoethyl column. Proteins were eluted with a linear gradient of 0 to 0.25 mol/L NaCl in buffer A at a flow rate of 1 mL/min. Plasma factor XIII (A,B2) was purified from normal, pooled human plasma (American Red Cross Blood Program, Northeast Region, Dedham, MA) by (NH4)2SO4 precipitation and heat treatment at 56°C.

Intracellular factor XIII (A2) preparations were concentrated with Centricell centrifugal ultrafilters (Polyscience, Warrington, PA) and stored at 4°C in buffer A. Purity of all preparations was determined by 7% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified A2 had only the band characteristic of this protein. Purified A2,B2 proteins had two bands, under nonreducing conditions, characteristic of the zymogen complex. The A2 and A2,B2 proteins were assayed for factor XIII activity by dansylcadenervaine incorporation into casein. Protein concentrations of purified materials were determined spectrophotometrically at 280 nm with E280 = 13.8.

Formation of fibrin clots and analysis by SDS-PAGE. Normal plasma, patient plasma, mixtures of the two, or purified fibrinogen (100 μL) was clotted with thrombin (2.5 μL, 0.5 U) and CaCl2 (100 mmol/L). The clot was allowed to gel for 2 minutes or 2 hours before hirudin (1 μL/μL, 5 μL) was added. The clots were washed extensively. In some experiments, normal plasma was mixed with normal or patient’s IgG (1 μg/μL, 200 μL) for 30 minutes before thrombin was added. With normal plasma, these conditions produced completely cross-linked fibrin (see Fig 1, lane 8). After syneresis, the clots were solubilized by overnight incubation at 37°C in 0.025 mol/L Tris buffer, pH 6.8, containing 3% SDS, 5% 2-mercaptoethanol, and 10% glycerol. After boiling for 5 minutes, 10% SDS-PAGE was performed at room temperature and at a constant current. The gel was scanned at 633 nm in a Laser Densitometer (Pharmacia-LKB). Areas under the curves were estimated either by planimetry or by weighing appropriate peaks of the tracing.

Agarose gel electrophoresis and immunoblotting. Agarose gel electrophoresis of mixtures of the patient’s IgG or normal IgG and three molecular forms of the active subunit of factor XIII (A, A'B, A'2) was performed under nonreducing and nondenaturing conditions similar to those previously described. The electrophoresis was performed at 10°C and at 10 V/cm for 1.5 hours in 1.5-mm thick gels containing 0.6% agarose in 0.1 mol/L barbital buffer. These proteins were transferred to immobilon membrane by capillary action, and immunoblotting with antifactor XIII A or anti-IgG was performed.

Immuno-logic assays. Factor XIII A protein was assayed by rocket immunoelectrophoresis. Electrophoresis was performed in 1.0% agarose gel, containing 0.4% rabbit antiserum to human factor XIII A in Tris-barbital buffer, pH 8.6, at constant voltage (10 V/cm) at 10°C for 18 hours. The gel was stained with Coomassie blue. Alternatively, enzyme-linked immunosorbent assays (ELISAs) were used to quantitate A and B proteins. Factor XIII A subunit was also analyzed by crossed immunoelectrophoresis (CIE). Electrophoresis in the first dimension was performed in an 0.8% agarose gel at a constant voltage of 10 V/cm at 10°C for 1.5 hours. The second dimension was performed in the same gel, containing 0.4% rabbit antiserum to human factor XIII A subunit at a constant voltage of 2 V/cm at 10°C for 18 hours.

Fibrinogen was also analyzed by CIE, with rabbit antiserum to human fibrinogen (0.5%).

Neutralization of the patient’s IgG. Neutralization of activity from the patient’s IgG with specific antisera to Igs was determined.

Fig 1. 10% SDS-polyacrylamide gel of reduced fibrin clots obtained from normal and patient plasma in mixing assays. Lane 1, purified fibrinogen (the Aα and Bβ chains migrate slightly slower than the α and β chains). Lane 2, fibrin from a XIII-deficient (transfused) plasma, preincubated with 2% antifactor XIII antiserum. Lanes 3 through 8 represent patient and normal plasma mixed before clotting. Lane 3, patient only; lane 4, mixture of 1:1; lane 5, 1:2; lane 6, 1:4; lane 7, 1:8; lane 8, normal only. Molecular weights and the chain composition of non-cross-linked and cross-linked fibrin are indicated.
RESULTS

Case report. An 81-year-old white woman was hospitalized with an anterior wall myocardial infarction. The infarction was complicated by pulmonary edema, for which she required intubation, and by atrial and ventricular arrhythmias. She improved and was discharged on digoxin, furosemide, procainamide, and her long-standing desiccated thyroid.

A year and one-half later, she came to the emergency room with pain and swelling in her right thigh, with no antecedent injury. Her hematocrit was 25 (her hematocrits are usually 37 to 40). She was transfused and sent home; she returned a few days later with further swelling of her thigh, and she was then admitted. There was no history of trauma or fracture. Angiography showed no abnormalities, but a computerized tomographic scan of her thigh showed two large hematomas medially. There was no prior personal or family history of spontaneous hemorrhage. Routine coagulation tests were normal. She was transfused with two more units, and the bleeding stopped; she was discharged on the same medications. Two months later, she was readmitted for bleeding around her right knee and a hematocrit of 23. She was again transfused and discharged.

Two months later, she was readmitted for pain and swelling due to a spontaneous hematoma over her right pectoral muscle and axilla, with dissection down her right flank to the supraperic area. Results of laboratory tests on this admission are given in Table 1. The conclusions then were that the patient had: (1) a weak lupus inhibitor because of her prolonged partial thromboplastin time (PTT), its failure to correct in mixing studies, and a ratio of 1.36 in the thromboplastin inhibition test; and (2) a specific factor XIII inhibitor because her clots and the mixtures with normal plasma were soluble in urea. She was again transfused; there was no further bleeding. Procainamide was discontinued, and the patient was treated with prednisone. Six months later, the PTT and factor XIII screening tests were repeated and were normal. Over the ensuing 3 years, she had four more admissions for arrhythmias and cardiac failure but no further hemostatic problems.

On further investigation of her factor XIII problem, surprising values were observed (Table 2). Factor XIII activity was measured with a dansylcadaverine incorporation assay. In this assay, dansylcadaverine is a small analogue of lysine, which is incorporated into the glutamine residues of the casein substrate. The A and B proteins of factor XIII were measured with ELISAs. Normally, patients who are congenitally deficient in factor XIII have less than 1% activity in the dansylcadaverine assay and ~1% A protein and ~50% B protein. It has been reported that...

Table 1. Laboratory Results for the Patient, Obtained at Her Fourth Admission

<table>
<thead>
<tr>
<th>Tests</th>
<th>Patient</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit</td>
<td>26%</td>
<td></td>
</tr>
<tr>
<td>ANA* titer</td>
<td>1:2,000</td>
<td>&lt;1:40</td>
</tr>
<tr>
<td>PT</td>
<td>11.5 s</td>
<td>11.7 s</td>
</tr>
<tr>
<td>PTT</td>
<td>45.0 s</td>
<td>29.0 s</td>
</tr>
<tr>
<td>Mix</td>
<td>40.6 s</td>
<td>37.8 s</td>
</tr>
<tr>
<td>Thromboplastin inhibition test</td>
<td>39.2 s</td>
<td>29.7 s</td>
</tr>
<tr>
<td>Thrombin time</td>
<td>21.3 s</td>
<td>21.4 s</td>
</tr>
<tr>
<td>Fibrinogen (turbidometric)</td>
<td>450 mg/dL</td>
<td>250-450 mg/dL</td>
</tr>
<tr>
<td>Factor VIII:C</td>
<td>75%</td>
<td>50-200%</td>
</tr>
<tr>
<td>Factor VIII:Ag</td>
<td>192%</td>
<td>50-200%</td>
</tr>
<tr>
<td>Ristocetin cofactor</td>
<td>216%</td>
<td>50-200%</td>
</tr>
<tr>
<td>Factor IX</td>
<td>113%</td>
<td>50-150%</td>
</tr>
<tr>
<td>Factor XI</td>
<td>90%</td>
<td>50-150%</td>
</tr>
<tr>
<td>Factor XIII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mix:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:2 (patient: normal)</td>
<td>Urea-soluble</td>
<td></td>
</tr>
<tr>
<td>1:4</td>
<td>Urea-soluble</td>
<td></td>
</tr>
<tr>
<td>1:8</td>
<td>Urea-insoluble</td>
<td></td>
</tr>
<tr>
<td>Bleeding time</td>
<td>11 min</td>
<td>&lt;9 min</td>
</tr>
<tr>
<td>Platelet aggregation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal to collagen, ADP, epinephrine, ristocetin, arachidonic acid</td>
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Table 2. Factor XIII Values for the Patient

<table>
<thead>
<tr>
<th>Date</th>
<th>Activity</th>
<th>A Protein</th>
<th>B Protein</th>
<th>Solubility in Monochloroacetic Acid</th>
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</thead>
<tbody>
<tr>
<td>Pretransfusion</td>
<td>24%</td>
<td>14%</td>
<td>39%</td>
<td>Soluble</td>
</tr>
<tr>
<td>Posttransfusion:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>38%</td>
<td>23%</td>
<td>44%</td>
<td>Soluble</td>
</tr>
<tr>
<td>3 d</td>
<td>38%</td>
<td>33%</td>
<td>49%</td>
<td></td>
</tr>
<tr>
<td>6 d</td>
<td>42%</td>
<td>24%</td>
<td>67%</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Date</th>
<th>Mix</th>
<th>Activity Observed</th>
<th>Calculated Activity*</th>
<th>Solubility in Monochloroacetic Acid</th>
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</thead>
<tbody>
<tr>
<td>Pretransfusion</td>
<td>1:1</td>
<td>69%</td>
<td>62%</td>
<td>Soluble</td>
</tr>
<tr>
<td>Posttransfusion:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>1:1</td>
<td>60%</td>
<td>69%</td>
<td>Soluble</td>
</tr>
</tbody>
</table>

Values reported as percent of normal plasma.

*Assuming that the patient had 24% factor XIII.
in such patients, as little as 2% to 3% activity is sufficient to control hemostasis in vivo and to produce clots that are insoluble in 5 mol/L urea or 1% monochloroacetic acid in vitro. Thus, although this patient had both low activity (24%) and low factor XIII protein concentrations (14% A and 39% B), there should have been sufficient activity for hemostasis and for insolubility of her clots in monochloroacetic acid or urea. Furthermore, mixing studies showed that there was no inhibitor of factor XIII activity with the dansylcadaverine incorporation assay. Nevertheless, her fibrin clots were soluble, as were mixtures of normal and patient plasma. This anomaly was solved by assessing the cross-linking of her fibrin clots with polyacrylamide gels (Fig 1). None of the patient’s fibrin clots or the equal mixtures with normal plasma had any α chain cross-linking, and γ chain cross-linking was modest. There was considerable γ chain monomer in all of the clots. The concentration of γ dimer was proportional to the amount of normal plasma added. Thus, it became clear that, although the inhibitor did not inhibit factor XIII activity in the dansylcadaverine incorporation assay, it did inhibit γ chain and α chain cross-linking in fibrin. This appeared to be a unique factor XIII inhibitor, and studies were undertaken to determine its specificity.

Demonstration and characterization of the inhibitor. The cross-linking of both α and γ chains in fibrin from the patient’s plasma was inhibited. In mixing studies of patient’s and normal plasmas, inhibition was demonstrated up to a ratio of 1:8. SDS-PAGE of reduced fibrin clearly showed inhibition of fibrin cross-linking (Fig 1). Furthermore, when the IgG fraction from the patient’s plasma was prepared with protein A-Sepharose, the purified IgG fraction immediately inhibited the cross-linking of fibrin in normal plasma. In contrast, the addition of normal IgG had no effect on the cross-linking process (Fig 2).

To measure this inhibition, SDS gels of reduced normal fibrin with varying amounts of the patient’s IgG were analyzed by densitometry. These data showed that γ cross-linking in 100 μL of normal plasma had been inhibited by ~30% with the addition of 100 μg of the patient’s IgG, while α cross-linking had been almost completely inhibited.

Pretreatment of the patient’s IgG preparation or plasma with goat antibodies to human Ig heavy chain (γ) or light chain (κ) eliminated its inhibitory effect. After these treatments, plasma could be cross-linked normally (Fig 3, lanes 4 and 6). However, pretreatment with goat antibody to human light chain (λ) or to IgM heavy chain (μ) had no effect (Fig 3, lanes 5 and 7).

Specificity of the inhibitor for A’ and A*. Because fibrin cross-linking was specifically inhibited, it could be reasoned that the inhibitor was directed at a fibrin cross-linking site. However, CIE of fibrinogen showed no difference between normal and patient fibrinogen. The patient’s plasma was applied to a fibrinogen-Sepharose column, and the inhibitor did not bind. Furthermore, the inhibitor activity in the patient’s plasma could be neutralized with purified factor XIII but not with purified fibrinogen. Also, the fibrin formed from the patient’s plasma, after removal of the inhibitor by affinity chromatography on protein A-Sepharose, could be cross-linked normally.

When various amounts of factor XIII A subunit (Fibrogammin) were added to the patient’s plasma, the inhibitor was neutralized, and the patient’s plasma formed normal cross-linked fibrin. Likewise, when various amounts of Fibrogammin were added to a mixture of normal plasma and the patient’s IgG, cross-linking occurred at high doses of A subunit (data not shown). The neutralization of the inhibitor in 1 mL of the patient’s plasma required ~12 U of Fibrogammin, and neutralization of 1 mg of the patient’s IgG required ~0.9 U of Fibrogammin. Thus, the concentra-
FACTOR XIII INHIBITOR INHIBITS FIBRIN BINDING

Fig 3. Neutralization of the patient's inhibitor activity by anti-lg. Normal or patient IgG (10 μg/μL, 10 μL) was incubated with specific anti-lg (2.5 μg/μL, 200 μL) overnight at 4°C and 1 hour at 37°C. The supernatants were mixed with normal plasma, clotted and assayed as in Fig 1. Lane 1, control fibrinogen. Lane 2, fibrin clots from normal plasma + normal IgG, no anti-lg. Lane 3, normal plasma + patient's IgG, no anti-lg. In lanes 4 through 7, normal plasma + patient's IgG was incubated with anti-lg: lane 4, anti-β; lane 5, anti-μ; lane 6, anti-κ; lane 7, anti-λ.

Fig 4. Effect of patient's IgG on thrombin-dependent conversion of A to A'. Purified A (0.1 μg/μL, 5 μL) was incubated with normal or patient IgG (6 μg/μL, 5 μL) or saline for 10 minutes. Then thrombin (5 μL, 0.1 U) or thrombin-CaCl₂ (0.1 U, 20 mmol/L) was added. After 20 minutes, hirudin (1 U/μL, 5 μL) was added, and the gel was blotted with anti-A. Samples were analyzed on 10% SDS-polyacrylamide gels. Positions of A and A'/A* are indicated. Lanes 1 through 3, saline incubated with zymogen (lane 1), A' (lane 2), A* (lane 3). Lanes 4 through 6, normal IgG incubated with A (lane 4), A' (lane 5), A* (lane 6). Lanes 7 through 9, patient's IgG incubated with A (lane 7), A' (lane 8), A* (lane 9).
Fig 5. Formation of a complex between patient’s IgG and A’ or A*. Samples were treated as in Fig 4, then electrophoresed in 0.6% agarose under non-denaturing, nonreduced conditions and analyzed by Western blotting. In blot A: lane 1 represents factor XIII A protein + normal IgG; lane 2 represents A’ + n. IgG; lane 3 represents A* + n. IgG; lane 4 represents A + patient IgG; lane 5 represents A’ + p. IgG; and lane 6 represents A* + p. IgG. Blot A: blot with anti-A. Blot B: same samples as in blot A, but blot with anti-IgG.

Between binding of this IgG to A’ and A*. However, purified A protein migrated normally in the presence of both normal and patient IgG.

The affinity of the patient’s IgG toward A’ and A* was shown definitively by binding the patient’s IgG to protein A-Sepharose and using this affinity absorbent to remove A’ or A* from defibrinated normal plasma. After incubation of A, A’, and A* with normal IgG, the zymogen and its activated forms were constant in the supernatant. In contrast to normal IgG, with the patient’s IgG, A’ and A* were decreased significantly, while the concentration of A protein was not affected (Table 3). The adsorbed proteins on the solid-phase adsorbent were eluted with Tris buffer containing 3% SDS, 5% 2-mercaptoethanol, and 10% glycerol. These eluted proteins were analyzed by SDS-PAGE and immunoblotting with anti-A (Fig 6). Only A’ and A* (lanes 5 and 6) were eluted from the patient IgG affinity matrix. No factor XIII proteins were eluted when normal IgG was used as the affinity matrix. This experiment will differentiate A’ and A* and show that the IgG bound to both molecules.

Inhibition of fibrin binding to the A’/A* protein of factor XIII by the patient’s inhibitor. When factor XIII functions in the clotting of normal plasma, there is only a small amount of A* protein left in the serum. This suggests binding of A* to fibrin during the clotting process. The presence of A* protein in the clot with the patient’s inhibitor was tested directly. After three saline washings of the clotted fibrin, the reduced fibrin clot was analyzed by SDS-PAGE and immunoblotting with anti-A. Only trace amounts of A* protein could be faintly detected in the patient’s clot, while A protein was easily detected in normal fibrin clots (Fig 7). When normal plasma was preincubated with the patient’s inhibitor, the same results were observed. In this case, there was inhibition of complex formation between normal fibrin and normal factor XIII A. Therefore, these results suggested inhibition of fibrin binding to A’/A*.

Having previously established that this IgG specifically bound to A’ and A*, and thinking that the inhibitor functioned primarily to prevent fibrin binding, experiments were performed to define the antigenic epitope more precisely. Purified A protein was digested with higher

<table>
<thead>
<tr>
<th>Affinity Matrix</th>
<th>Species of A</th>
<th>Concentration of A in Supernatant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal IgG*</td>
<td>A</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>A’</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>A*</td>
<td>96</td>
</tr>
<tr>
<td>Patient IgG</td>
<td>A</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>A’</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>A*</td>
<td>34</td>
</tr>
</tbody>
</table>

*Two minutes incubation at 25°C of affinity matrix with defibrinated plasma containing the different species of A.
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Fig 6. Complex formation between patient’s IgG and A⁺ or A*. Normal or patient Ig G (10 μg/μL, 10 μL) was incubated with protein A-Sepharose (50 μL) for 10 minutes at 25°C. After centrifugation, the pellet, containing normal IgG (lanes 1 through 3) or patient IgG (lanes 4 through 6), was incubated with A⁺ (3 μg/μL, 5 μL) + saline (lanes 1 and 4), A⁻ + thrombin (lanes 2 and 5), or A⁻ + thrombin-CaCl₂ (lanes 3 and 6). Hirudin was added, and the pellet was washed extensively. Proteins adsorbed to the pellet were solubilized with SDS-PAGE reduced sample buffer, electrophoresed, and blotted with anti-A.

Fig 7. Binding of factor XIII A in plasma to fibrin clots. Normal plasma (lanes 1 through 3), XIII-deficient plasma (lanes 4 through 6), or patient’s plasma (lanes 7 through 9) was clotted and extensively washed. The clots were analyzed by SDS-gel electrophoresis and immunoblotting with anti-A antiserum. Plasma was also analyzed. Lane 1, normal plasma. Lane 2, normal 2-minute clot. Lane 3, normal 1-hour clot. Lane 4, XIII-deficient plasma. Lane 5, XIII-deficient 2-minute clot. Lane 6, XIII-deficient 1-hour clot. Lane 7, patient plasma. Lane 8, patient 2-minute clot. Lane 9, patient 1-hour clot.

concentrations of thrombin and analyzed as above. The patient’s IgG, but not normal IgG, bound to A⁻ and also to the 51,000- to 54,000-dalton fragment that arises from thrombin digestion under these conditions. The fibrin binding region has previously been located in this fragment. There was no interaction between the 19,000- to 21,000-dalton fragment and this IgG (Fig 8).

DISCUSSION

Plasma factor XIII is important for both hemostasis and fibrinolysis. In its active form, factor XIIIa (A⁺) is a transglutaminase and acts to catalyze the formation of covalent bonds between fibrin and other molecules by forming ε-(γ-glutamyl)-lysyl cross-links. In fibrin, A⁺ forms cross-linked bonds between γ chains to form γ-γ dimers and α chains to form α polymers. It also cross-links other molecules to fibrin, notably fibronectin, α₂-PI, and von Willebrand protein, and it cross-links certain other matrix proteins.

Patients who have acquired inhibitors to fibrin stabilization have a tendency to bleed severely. In this case, a general inhibitor to fibrin stabilization was suspected from
the urea solubility of clots, which were obtained from recalcified mixtures of normal plasma in different proportions with the patient’s plasma. In accordance with established convention, this inhibitor to factor XI will be denoted as IgG New Haven. This inhibitor appears to be unique. The reported cases of factor XI inhibitors are primarily Igs, and all have the property of inhibiting fibrin stabilization; except for two, they all inhibit the incorporation of amine into casein. The two inhibitors that did not inhibit the incorporation of lysine analogues into casein were in the patients from Oslo, Norway and Boston, MA. The inhibitor in the patient from Boston was directed toward fibrin and probably inhibited fibrin cross-linking by binding to the cross-linking site on fibrin. The mechanism of inhibition in the case from Oslo may be the same. In those two patients, there was normal factor XIII activity when measured with amine incorporation assays. This was clearly not the case with this patient. The factor XIII activity in the New Haven patient was low, but there was no evidence of inhibition in the dansylcadaverine incorporation assay (Tables 1 and 2). As a consequence, the most applicable and convenient method for monitoring inhibition was that of reduced gel electrophoresis, where the formation of cross-links in fibrin could be quantitated (Figs 1 and 2). The inhibitor was of high titer. After clotting, there were ~30% free γ chains and ~100% free α chains, and the clot was completely soluble in monochloroacetic acid or urea (Figs 1 and 2, and Tables 1 and 2). It had been previously found that fibrin with 74% or more of monomeric γ chains was partially soluble. Totally insoluble fibrin clots had 100% of γ chains converted to γ dimers, and there was ~8% to 10% of fibrin composed of polymers with a molecular weight (Mr) > 100,000. In this patient, it is apparent that solubility was correlated with the absence of α polymer formation.

The unique specificity of this inhibitor was demonstrated by several experimental methods. The inhibitor was not directed against fibrin cross-linking sites, as in the previous cases. There was no demonstrated interaction between the inhibitor and fibrin or fibrinogen, and the inhibitor could be overcome by adding purified factor XIIIa to the patient’s plasma. The conversion of the zymogen to A’ by thrombin proceeded normally in the presence of the inhibitor (Fig 4), and a complex between the inhibitor and the factor XIII zymogen could not be demonstrated (Figs 4 and 5). However, there was a complex formed between the inhibitor and A’ or A* (Figs 5 and 6, Table 3); 100 μg of the purified inhibitor bound ~65% of the A’ or A* in 0.08 mL of defibrinated plasma in an affinity system. Normal IgG did not bind to any molecular forms of factor XIII, nor did the patient’s IgG bind to the factor XIII zymogen (Figs 5 and 6, Table 3). Furthermore, when the patient’s IgG was used as an affinity absorbent for factor XIII, only A’ and A* bound to this matrix. When normal IgG was used, no form of factor XIII bound to the matrix (Fig 6). Thus, there are several experiments that point to a complex being formed between A’ and A* with the patient’s IgG but not with the A zymogen.

High concentrations of thrombin will fragment A protein into three peptides: the 4,000-dalton activation peptide from the NH2-terminal, a 56,000-dalton central peptide containing the active center, and a 24,000-dalton carboxy-terminal fragment. Purified A protein was fragmented with thrombin, and the binding of the patient’s inhibitor was studied. The inhibitor bound specifically to the 54,000-dalton fragment (Fig 8). This fragment is composed of residues 38 to 515, and this portion of the A chain of factor...
XI11 has been previously found to be the fibrin binding site,\(^\text{32}\) The most reasonable explanation for the mechanism by which this IgG inhibits fibrin cross-linking is that the IgG is directed toward the fibrin binding site on the A'/A\(^*\) protein. Furthermore, we have also directly observed the activity of inhibitor to the fibrin binding site of the A'/A\(^*\) proteins (Fig 7). From all of these experiments, we would conclude, therefore, that this inhibitor is directed toward a fibrin binding site on the A protein and that this site is relatively hidden in the zymogen and is revealed on the thrombin-catalyzed intermediate (A') and the active enzyme (A\(^*\)). This would also explain the low factor XIII activity in the dansylcadaverine assay, because fibrin has been found to be a cofactor that stimulates factor XIII activity by forming a complex between non-cross-linked fibrin polymers and factor XIII. This provides in vivo validation for experimental findings in vitro.\(^\text{3}\)

This binding site for fibrin on the A molecule appears to be relatively hidden in the zymogen and is revealed by conformational change(s) that occur(s) with thrombin cleavage of the activation peptide. Ca\(^{2+}\) is not necessary for exposure of this site. It has been shown that thrombin cleavage plus a conformational change induced by Ca\(^{2+}\) are necessary for the cysteine active center to become exposed.\(^\text{7}\) In contrast, the results presented here indicate that only cleavage of the activation peptide is necessary for the fibrin binding site to be revealed. Factor XIII A protein is a globular molecule.\(^\text{37}\) This fibrin binding site is not near the thrombin cleavage site for the activation peptide in the tertiary structure of the molecule.

This inhibitor was found to be an IgG; it was completely neutralized by antibodies to \(\gamma\) and \(\kappa\) chains. There was very little neutralization by either antibodies to \(\mu\) or \(\lambda\) chains (Fig 3). In reality, one does not know how immune tolerance can be broken. The majority of the factor XIII inhibitors arose after a relatively prolonged treatment with isoniazide.\(^\text{39}\) Isoniazide can be incorporated into fibrin by factor XIIIa, and this abnormal metabolite could serve as a foreign molecule.\(^\text{39}\) In this case, the patient had been treated for \(~8\) months with procainamide. Procainamide is known to be associated with lupus-like inhibitors,\(^\text{40}\) and the patient did have a lupus-like inhibitor in addition to her factor XIII inhibitor. The patient is also elderly and had a highly positive antinuclear antibody titer, which was temporarily associated with her other immune phenomena. There was clearly some disorder in her immune tolerance mechanism, and this developed into an antibody that was specifically directed toward the factor XIII binding function to fibrin.

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A unique factor XIII inhibitor to a fibrin-binding site on factor XIII-A

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