A Monoclonal Antibody That Recognizes a Neo-Antigen Exposed in the E Domain of Fibrin Monomer Complexed With Fibrinogen or Its Derivatives: Its Application to the Measurement of Soluble Fibrin in Plasma

By G. Soe, I. Kohno, K. Inuzuka, Y. Itoh, and M. Matsuda

Using urea-solubilized human fibrin monomer as an immunogen, we raised in mice a battery of monoclonal antibodies that reacted with the immunogen but not with urea-treated or native fibrinogen. Although they all failed to react with acid-solubilized fibrin monomer (acid-FM) alone, an antibody designated as IF-43 was found to recognize acid-FM, which was bound with fibrinogen or its derivatives to form a 1:2 complex of soluble fibrin. The epitope for this antibody, thus, appears to be exposed most probably by conformation changes induced in the acid-FM molecule upon formation of the complex. Because IF-43 was able to recognize fibrin-derivated plasmatic fragment E treated with urea but not the thrombin- and urea-treated amino-terminal disulfide knot of fibrinogen, the presence of the Aa (52-78) residue segment is expected to yield (ie, the desA- and des-AA fragments) lacking one or both of the A peptides desAABB-FM lacking both of two peptides, A and B. When they are produced in the presence of an excess of fibrinogen, they form complexes with fibrinogen and exist as soluble protein complexes called soluble fibrin (SF) Under pathologic conditions in which blood coagulation is activated and thrombin is eventually generated, such soluble fibrin complexes are known to be present in the circulating blood. Therefore, their detection and quantification in plasma derived from patients with thrombotic diseases has been expected to provide useful information on the state and degree of intravascular coagulation.

In the present communication, we describe a monoclonal antibody (MoAb) that specifically reacts with SF and discuss its epitope with special reference to possible conformation changes induced in the FM molecule complexed with fibrinogen and its derivatives. We also describe application of this antibody to the measurement of soluble FM in plasma samples derived from patients with thrombotic diseases and other disease conditions.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals and reagents were purchased from commercial sources without purification unless specified.

Preparation of urea-solubilized FM (urea-FM) and acid-solubilized FM (acid-FM). Human fibrinogen (grade I; Kabi Diagnostica, Mölndal, Sweden) was dissolved in 50 mmol/L Tris-HCl, pH 8.0, to bring 2% (wt/vol) and applied onto a lysine-Sepharose 4B column to remove plasminogen and plasmin, if any. The pass-through fraction was collected and treated with 2 mom urea to denature factor XIII, a trace contaminant present in the fibrinogen preparation. Fibrinogen thus prepared was dialyzed against 20 mmol/L disodium ethylenediaminetetraacetate (EDTA) and was brought to 10 mmol/L. Two milliliters of the fibrinogen solution was clotted for 2 hours at 37°C with 1.25 NIH U of human thrombin (Sigma, St Louis, MO). After the addition of 1 mmol/L diisopropylfluorophosphat (DFP), the clot was squeezed with a plastic rod and centrifuged for 20 minutes at 20,000g and 37°C. The pellet was solubilized with either 6 mol/L urea (urea-FM) or 20 mmol/L acetic acid (acid-FM), brought to 10 mg/mL, and stored at −40°C. These FM preparations were found to be homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 6% gels. Upon use, they were further diluted to appropriate concentrations with either 6 mol/L urea or 20 mmol/L acetic acid in accordance with the solution used for solubilization of fibrin clots.

Preparation of MoAbs that recognize urea-FM but not urea-treated fibrinogen. Using 100 μg of urea-FM in complete Freund's adjuvant followed by 100 μg of urea-FM without the adjuvant as an immunogen, MoAbs were raised in BalblC mice essentially by a hybridoma technique of Kohler and Milstein, with minor modifications as described elsewhere. We have obtained 12 antibodies that were able to react with urea-FM but not with acid-FM or urea-treated fibrinogen. The epitope for this antibody thus appeared to be exposed on treatment of FM with urea. Among them, there was an antibody that was able to react with a mixture of acid-FM and native fibrinogen (acid-FM + native fibrinogen), indicating that a certain conformational change(s) may have been induced in acid-FM on contact with fibrinogen. The Ig subclass of this antibody was determined to be IgG2a with κ type light chains. This antibody was designated as IF-43.

Other antigens. The Aa-chain of fibrinogen was separated by reduction and carboxymethylation as described by Doolittle et al. Plasmatic degradation products of fibrinogen were prepared essentially as described by Nieuwenhuizen et al for fragments X and Y and by van Ruijven-Vermeer et al for fragment D, the largest plasmatic fragment D species retaining the carboxy-terminal segment of the y-chain, and fragment E. The amino-terminal disulfide knots (NDKs) of fibrinogen and fibrin, ie, CNBr-derivatives containing the...
fibrinogen-derived fragments were analyzed also by immunoblotting. Briefly, after 10 μg/mL IF-43 to 20 hours at 22°C to Immobilon polyvinylidene difluoride (PVDF) Wells of polystyrene microtitration plates were coated overnight at 4°C. The antigens include desAAABB-FM; desAA-FM; fibrinogen and its plasmic fragments X, Y, D, and E; thrombin-treated plasmic fragment X; thrombin-treated or nontreated N-DSK; XDP; the D-dimer; and fragment E(E, + E2). In the other set of experiments using an antifibrinogen polyclonal antibody, we confirmed that all antigens either in urea or acetic acid had been adequately immobi- lized onto the plates. The antigen-coated wells were washed three times with 0.15 mol/L NaCl containing 0.05% Tween 20 and incubated with 10 μg/mL IF-43 in 20 mol/L Tris-HCl, pH 7.6, containing 0.15 mol/L NaCl and 0.05% Tween 20 (TBS-Tween) for 1 hour. The bound antibody was captured by antismung Ig conjugated with horseradish peroxidase (HRP; Dako, Glostrup, Denmark) and visualized by incubation with phenol-2,4 aminoantipyrine (AAP)/H2O2 as substrate.

Reactivity of IF-43 to FM in mixtures with fibrinogen or various fibrinogen-derived fragments as determined by a sandwich ELISA. Wells of polystyrene microtitration plates were coated overnight at 4°C with 20 μg/mL IF-43 in 50 mol/L sodium carbonate, pH 9.5. To the IF-43-coated wells were added serial dilutions of mixtures of 1 vol of acid-FM in 20 mol/L acetic acid and 9 vol of fibrinogen or its plasmic fragment X, Y, D, or E or bovine serum albumin (BSA) in 50 mol/L Tris-HCl, pH 8.0, containing 0.15 mol/L NaCl at a molar ratio of 1:10. The wells were then incubated for 30 minutes at 37°C. As a control, a mixture of acid-FM and a synthetic Aα (17-26) residue peptide, GPRVRIVERHQ, as an antipolymerant in the same buffer at a molar ratio of 1:1,000 was used. The captured antigens were tagged with an antifibrinogen rabbit antibody conjugated with HRP and visualized by incubation with phenol-AAP/H2O2 as substrate.

Immunoblotting. Reactivity of IF-43 to fibrinogen and its derivatives was analyzed also by immunoblotting. Briefly, after 10 μg each of samples was subjected to SDS-PAGE using 6% to 12% gradient gels, proteins in the gels were electrophoretically transferred to 2 μA/cm² for 2 hours at 22°C to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore Co, Bedford, MA) using an electrophoret apparatus (Atto Co, Tokyo, Japan). The transfer buffer consisted of 0.1 mol/L Tris-HCl, pH 8.8, 0.192 mol/L glycine, 20% (vol/vol) methanol and 0.02% (wt/vol) SDS. After the transfer of proteins, the membranes were blocked by soaking for 1 hour in TBS-Tween containing 5% (wt/vol) skim milk and incubated for 16 hours at 4°C with 10 μg/mL IF-43 in TBS-Tween containing 1% (wt/vol) skim milk. After washing four times with TBS-Tween, the membranes were incubated for 1 hour at 25°C with an alkaline phosphatase-conjugated antismung IgG goat antibody (Dako), washed four times with TBS-Tween, and subjected to development of color using 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium as substrates, as described previously.

Coupling of latex beads with IF-43. One milliliter of 10% latex suspension in 0.1 mol/L Tris-HCl, pH 8.0 (Seradyn, Indianapolis, IN; particle size, 0.212 μm), was added to 10 mL of the F(ab')2 fragment of IF-43 (0.8 mg/mL in 0.1 mol/L Tris-HCl, pH 8.0), stirred for 1 hour at 4°C, and centrifuged for 20 minutes at 20,000g and 4°C. The F(ab')2 fragment was prepared as follows. The antibody was digested with pepsin at an enzyme:substrate ratio of 1:8 (wt/wt) for 2 hours at 37°C in 0.1 mol/L sodium citrate, pH 3.8, and the F(ab')2 fragment was isolated by gel filtration on Sephacryl S-300 HR column (Pharmacia Biotech, Tokyo, Japan). By calculating the residual proteins in the supernatant based on the difference of A280 of the F(ab')2 solution before and after coupling, we found that about 50% to 60% of proteins were bound to the latex beads. The pellet was suspended in 0.1 mol/L Tris-HCl, pH 8.0, containing 0.3% (wt/vol) BSA and gently stirred for 1 hour at 4°C. The antibody-coated latex beads were precipitated by centrifugation for 20 minutes at 20,000g and resuspended in 10 mL of distilled water containing 0.09% (wt/vol) NaNO2 to make 1% (wt/vol) suspension under gentle stirring for 1 hour at 4°C. This latex preparation was used to measure soluble FM in plasma derived from healthy volunteers and patients with a variety of thrombotic diseases.

RESULTS

Preparation of antibodies recognizing a specific conformation(s) of fibrin, but not fibrinogen. By immunizing mice with urea-FM, we have obtained a battery of MoAbs (altogether 12) reactive to urea-FM, but not to acid-FM alone or to native or urea-treated fibrinogen in a direct binding ELISA (data not shown). Among these antibodies, an antibody designated as IF-43 was found to react with acid-FM when it was complexed with native fibrinogen or its plasmic derivatives containing the D domain, as will be discussed.

Purification of IF-43 and assessment of its IgG subclass. The antibody was purified from ascites of mice injected with the hybridoma cells by affinity chromatography using protein A-Sepharose CL 4B (Pharmacia Biotech) according to the standard method. The IgG subclass was determined to be IgG2a with κ type light chains using an isotyping kit (Amer- sham, Tokyo, Japan).

Immunoblot analysis against fibrinogen and fibrin and their derivatives. Reactivities of IF-43 to fibrinogen and fibrin and their derivatives were analyzed by immunoblotting. The antigens include fibrinogen; fibrinogen-derived fragments X, Y, D, and E (Fg-X, Fg-Y, Fg-D, and Fg-E, respectively); desAAABB-FM; desAA-FM; thrombin-treated Fg-X (Fn-X); fragment E(E, + E2) derived from plasmic digests of cross-linked fibrin (Fn-E); the phase 3-digests of cross-linked fibrin (XDP); and thrombin-treated or nontreated N-DSK (Fn-N-DSK and Fg-N-DSK, respectively).

When analysis was conducted against nonreduced anti- gens, neither fibrinogen (Fg) nor its plasmic fragments, ie, Fg-X, Fg-Y, Fg-D, and Fg-E, were stained with the antibody, whereas two fibrin monomers, desAA-FM and desAAABB-FM, and fibrin-derived plasmic fragments, ie, Fn-X, Fn-Y, and XDP, were stained positively (Fig 1A). The results seemed to indicate that the epitope had been exposed in the central E domain of fibrinogen on removal of the A peptides by thrombin or aneced. Nevertheless, Fn-N-DSK closely resembled Fn-E was not stained, although the A peptide had been removed therefrom (Fig 1A, the extreme right lane).

When these antigens were stained with IF-43 after reduc- tion, the fibrin α-chain and its remnants in plasmic fragments were stained positively (Fig 2A, middle 5 lanes), as referred to the polypeptides stained with a polyclonal antibody (Fig 2B). As anticipated, none of the subunit polypeptides of fibrinogen-derived fragments were stained when IF-43 was used.
SOLUBLE FIBRIN-SPECIFIC ANTIBODY

Fig 1. Immunoblot analysis of fibrinogen and its derivatives under nonreducing conditions using IF-43 (A) and an antifibrinogen polyclonal antibody (B). Fg, fibrinogen; Fg-X, Fg-Y, Fg-D, and Fg-E, plasmic fragments X, Y, D, and E of fibrinogen, respectively; desAA-FM, fibrin monomer depleted of both A peptides; desAABB-FM, fibrin monomer depleted of both of the two A and B peptides; Fn-X, plasmic fragment X of fibrin; Fn-E, plasmic fragment E of fibrin; XDP, plasmic phase 3-digests of cross-linked fibrin; Fg-N-DSK, the amino-terminal disulfide knot of fibrinogen; Fn-N-DSK, the thrombin-treated Fg-N-DSK. The proteins were resolved by SDS-PAGE under nonreducing conditions and subjected to immunoblotting. Note that all proteins reactive to IF-43 contain fragment E depleted of the A peptides (desAA). Despite lacking both A peptides, Fn-N-DSK was not recognized by IF-43, indicating that the Aα (52-78) residue segment was critical for the epitope expression.

Fibrinogen and its plasmic fragments or two N-DSKs were stained (Fig 2A, 5 lanes from left and 2 lanes from right, respectively). Removal of the A peptide from the N-terminus of the Aα-chain thus appeared to be mandatory for epitope expression, but the newly exposed N-terminal segment of the fibrin α-chain itself might not be directly involved in eliciting the epitope. Indeed, when the Aα (17-26) residue peptide was tested for inhibition of the binding of IF-43 to the isolated fibrin α-chain in a direct binding EIA, no substantial inhibition was observed in the range of 10- to 3,000-fold molar excesses of the peptide over the fibrin α-chain (data not shown). Because the epitope was found to reside in the α-remnant of Fn-E but not that of Fn-N-DSK, the C-terminal 27 residue peptide of Fn-E, corresponding to the Aα (52-78) residues and lacking in Fn-N-DSK, was suspected to be responsible for eliciting the epitope. We, therefore, synthesized a 27 residue peptide with a sequence for the Aα (52-78) residue segment and analyzed the reactivity of IF-43 to this synthetic peptide by a direct binding EIA and immunoblotting. Although not shown here, IF-43 was unable to react with this synthetic peptide either. Thus, the epitope was located to the Aα (17-78) residue segment, but it was lost on removal of its C-terminal 27 residue peptides.

To see whether the disulfide-linked structures of fibrinogen and its derivatives are required for thrombin-mediated expression of the epitope for IF-43 or whether the α-chain polypeptide alone is sufficient for it, we have isolated the fibrinogen Aα-chain and examined the reactivity to IF-43 by immunoblot analysis before and after treatment with thrombin. IF-43 was unable to recognize the isolated Aα-
chain itself (Fig 3A, lane 2), but was able to do so when the Aα-chain had been treated with thrombin (Fig 3A, lanes 3 and 4). Thus, removal by thrombin of the A peptide from the isolated Aα-chain also seems to be essential for expression of the epitope. Positions for the Aα- and α-chains are shown by immunoblotting using a polyclonal antifibrinogen antibody as reference (Fig 3B). Therefore, the epitope for IF-43 was found to be buried in the isolated Aα-chain (Fig 3A, lane 2) and exposed in the polypeptide chain on removal of the A peptide from its amino-terminus (Fig 3A, lanes 3 and 4).

Expression of the epitope for IF-43 in FM on formation of complexes with fibrinogen or its plasmic derivatives. Because acid FM, which by itself was not recognized by IF-43, was able to expose the epitope after treatment with denaturing agents such as urea, we speculated that certain conformation changes might be involved in expression of the epitope. FM molecules have been shown to form complexes with fibrinogen and its derivatives and, during these interactions, FM molecules are anticipated to undergo certain conformation changes. To explore this possibility, we examined whether the epitope for IF-43 would also be exposed in FM on formation of complexes with fibrinogen or its derivatives (Fig 4). Indeed, the epitope was found to be expressed in the mixture of acid-FM and fibrinogen and its plasmic fragments X, Y, and D, all apparently possessing a structure corresponding to the D domain endowed with a binding site with the E domain of fibrinogen or its derivatives. However, the epitope was not expressed in acid-FM mixed with fibrinogen-derived fragment E or BSA as well as in the control with the Aα (17-26) residue peptide (Fig 4). The results appeared to be in good agreement with our presumption that the epitope for IF-43 was buried in FM alone, but was expressed in the α-chain polypeptide of its E domain, which has been shown to bind with the D domain of fibrinogen or its plasmic derivatives via the set of "A"-"a" polymerization sites. To show the complex formation between FM and its plasmic fragments containing the structure of D domain, we selected a mixture of acid-FM and fragment D, and analyzed the mixture by gel filtration. Namely, 2 mL of a mixture of 0.3 mmol/L acid-FM and 9 mmol/L fragment D, (the molar ratio of acid-FM:fragment D = 1:30) was incubated for 30 minutes at 37°C and applied onto a Sephacryl S-300
HR column (1.5 x 80 cm). Eluted fractions were monitored by A_{280} for proteins and by the sandwich EIA for soluble FM. Soluble FM was all eluted together with fragment D, in the first small protein peak corresponding to a molecular size of approximately 5 x 10^{5} (Fig 5). This protein fraction was found to be a complex of one molecule of FM and two molecules of fragment D, based on the molecular size of about 3.3 x 10^{5} for desAABB-FM and 1.0 x 10^{5} for fragment D, and densitometric measurement of their silver-stained bands on SDS-PAGE run under nonreducing conditions (Fig 5, lane 2 in inset). Calibration curves were constructed for respective proteins by measuring the band intensity for known amounts of the proteins on SDS-PAGE, and the molar ratios of desAABB-FM:fragment D, were estimated to be 1:2.12, 1:2.16, 1:2.18, and 1:2.15 for fractions 82, 84, and 86 and their pooled fraction, respectively. They were theoretically assigned to be 1:2. The major, second protein peak was found to be fragment D, alone (lane 3 in inset).

The status of soluble FM in plasma. To examine the status of soluble FM in plasma, normal plasma was spiked with 200 ng/mL desAABB-FM and subjected to gel filtration on Sephacryl S-300 HR. The amount of soluble FM in each fraction was measured by the sandwich EIA. The spiked desAABB-FM was recovered nearly totally in a sharp peak immediately after the void volume position (V_{0} in Fig 6), indicating that the FM molecules had been complexed with fibrinogen, most probably with two molecules on the basis of a relative molecular mass of the protein complex of about 10^{6}. This presumption was confirmed by the presence of the Aα- and Bβ-chains representing fibrinogen and the α- and β-chains representing desAABB-FM as visualized by immunoblotting using an antifibrinogen polyclonal antibody and by measurement of intensities of respective polypeptide bands (Fig 6, lane 2 in inset).

It has long been known that FM is present as soluble FM complexes in plasmas of patients with thrombotic diseases, including the disseminated intravascular coagulation syndrome (DIC). To explore the status of such complexes in plasma, we chose plasma samples derived from patients with DIC in which concentrations of soluble FM were determined to be greater than 100 μg/mL by a latex aggregation assay that will be described later. We performed gel filtration of these plasma samples on Sephacryl S-300 HR and found that the elution profiles were essentially identical among themselves and also with that for the FM-spiked normal plasma (Fig 7). We attempted to analyze the FM-enriched peak fraction, fraction 76, by immunoblotting using an antifibrinogen polyclonal antibody and found that the proteins in the fraction were predominantly a complex with fibrinogen of desAA-FM but not desAABB-FM based on the profile of subunit polypeptides lacking the β-chain. This finding indicates that thrombin preferentially attacks the Aα-chains of fibrinogen in blood circulation and that the resultant desAA-FM yields a soluble complex with two molecules of fibrinogen.

Application of IF-43 to the measurement of soluble FM in plasma by a latex aggregation assay. The FM-spiked plasma was examined by an aggregation assay using latex beads coated with the F(ab')_{2} fragment of IF-43 (Fig 8). Namely, 10-μL aliquots of test samples were mixed with 40 μL of 0.4% (wt/vol) latex beads suspension, and the mixture was brought to 400 μL by adding 350 μL of 50 mmol/L Tris-HCl, pH 8.0, containing 70 mmol/L NaCl. The rate of aggregate formation was assessed by reading an absorbance at 950 nm (A_{950}) at 15-second intervals for 10 minutes on an autoanalyzer (LPIA-200; Mitsubishi Chemical Corp, Tokyo, Japan) based on a latex photometric immunoassay (LPIA) with near infrared turbidity and expressed in terms of ΔA_{950} as a function of the concentration of FM or other antigens.
tested. This LPIA-system was found to be able to measure FM spiked to normal plasma up to 200 µg/mL. Using this assay, the concentration of soluble FM in normal plasma was determined to be 0 to 3.97 µg/mL (mean ± SD, 3.82 ± 1.31 µg/mL; n = 98).

Using this latex aggregation assay, we measured soluble FM in plasma derived from 71 patients with a variety of thromboembolic diseases, including DIC, and compared the data with those for the D-dimer measured by a latex agglutination test using an MoAb JIF-23 that recognizes the amino-terminal conformation of plasmic fragment D species (fragments D1, D2, and D3) (Table 1). The patients were classified into four groups according to the presence or absence of ischemic diseases in one or more organs and bleeding tendencies, ie, group I, 11 patients with both ischemic diseases and bleeding; group II, 15 patients with ischemic...
diseases only; group III, 15 patients with bleeding only; and group IV, 30 patients manifesting neither ischemic diseases nor bleeding. Under this criterion, most of the patients with a clinical diagnosis of DIC are classified into either group I or III, and patients with a cerebral infarction, thrombophlebitis, or liver cirrhosis are classified into group II. Patients in group IV were associated with one or more of miscellaneous diseases, including systemic lupus erythematosus, malignant lymphoma, multiple myeloma, and solid cancers in the gall bladder, thymus, esophagus, and ovary. In group I, soluble FM and the D-dimer were both markedly elevated, but in group II, only soluble FM was increased. On the other hand, soluble FM was moderately increased despite of distinctly elevated D-dimer in group III. When ischemic diseases were not present, the level of soluble FM in plasma remained in the normal limit. As such, there was no significant correlation between the two parameters in these patients (Fig 9).

**DISCUSSION**

The epitope for IF-43 was located in the α-chain remnant of thrombin-treated plasmic fragment E of human fibrinogen, i.e., the Aα (17-78) residue peptide segment (Figs 1A and 2A). Although its exact location has not been pinpointed, the epitope is apparently buried in fibrinogen and exposed when the A peptides are removed by thrombin or thrombin-like enzymes (Fig 3A), and certain conformation changes are subsequently induced in the α-chain polypeptide of the E domain. This presumption is based on the finding that IF-43 failed to recognize acid-FM alone, but was able to recognize acid-FM complexed with fibrinogen or its plasmic fragments X, Y, and D1, all containing a structure corresponding to the D domain (Fig 4). Thus, the contact between the thrombin-activated E domain of FM and the D domain of another molecule via the set of "A"-"a" polymerization sites seems to be prerequisite for the epitope expression. As shown by gel filtration chromatography conducted on a mixture of acid-FM and fragment D1 at a molar ratio of 1:30, the first protein peak eluted at positions corresponding to a relative molecular mass of about 5 × 10^5 was immunoreactive to IF-43 (Fig 5). This protein species was found most likely to be a complex of one molecule of FM with two molecules of fragment D1 held together by a pair of "A"-"a" sets of polymerization sites.

Expression of the epitope in FM on formation of such a members? 

**Fig 8.** Aggregation assay on plasma spiked with various concentrations of deaAABB-FM by using latex beads coated with the F(ab')2 fragment of IF-43. The rate of aggregate formation was assessed by reading A450 at 15-second intervals for 10 minutes on an autoanalyzer based on the latex photometric immunoassay (LPIA) with near infrared turbidity and expressed as ΔA450 as a function of the concentration of soluble FM.

**Fig 9.** Comparison of levels of soluble FM and the D-dimer in plasmas derived from patients with thrombosis. Plasma samples were obtained from patients with a variety of thrombotic diseases with or without accompanying bleeding. The soluble FM was measured by the latex aggregation assay as described in Fig 8 and the D-dimer was measured by a latex aggregation assay using an antibody that recognizes the amino-terminal conformation of the plasmic fragment D species (D1, D2, and D3).

**Table 1. Concentrations of Soluble FM and the D-Dimer in Patients With Thrombotic Diseases and/or Bleeding Tendencies**

<table>
<thead>
<tr>
<th>Group*</th>
<th>No. of Patients</th>
<th>Soluble FM (µg/mL)</th>
<th>D-Dimer (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>11</td>
<td>67.60 ± 28.38</td>
<td>40.01 ± 17.04</td>
</tr>
<tr>
<td>II</td>
<td>15</td>
<td>67.30 ± 59.10</td>
<td>10.90 ± 5.00</td>
</tr>
<tr>
<td>III</td>
<td>15</td>
<td>9.67 ± 8.71</td>
<td>34.54 ± 24.37</td>
</tr>
<tr>
<td>IV</td>
<td>30</td>
<td>2.41 ± 3.49</td>
<td>4.51 ± 3.31</td>
</tr>
</tbody>
</table>

* Patients were classified according to the presence or the absence of ischemic diseases and bleeding. Group I, both ischemic diseases and bleeding were present; group II, only ischemic diseases were present; group III, only bleeding was present; group IV, neither ischemic diseases nor bleeding was present. 

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protein complex was also observed when acid-FM had been added to normal plasma and the FM-fibrinogen complex formed (Fig 6). The high molecular weight proteins eluted at positions corresponding to a relative molecular mass of 10^6 in gel filtration were found to be immunoreactive to IF-43 and most likely to be a complex consisting of one molecule of FM and two molecules of fibrinogen based on the band intensity of the Bβ- and β-chains (Fig 6, lane 2 in inset). Thus, triads composed of one molecule of FM and two molecules of fibrinogen appear to be the predominant species of SF in plasmas spiked with acid-FM.

The epitope was also expressed by treatment of the antigens with denaturing agents such as urea and SDS, but, here again, removal of the A peptides was found to be inevitable. Interestingly, this holds true with the isolated Aα-chain of fibrinogen, as shown by immunoblot analysis of the isolated Aα chain with or without prior treatment with thrombin [Fig 3A]. This may sound queer because denaturation with urea or SDS of the isolated Aα-chain is expected to expose the putative epitope possibly hidden in or near the Aα (52-78) residue segment. Based on nuclear magnetic resonance studies, Zheng et al. suggested the presence of a long-range interaction of an Asp at position Aα-7 with a remote residue of fibrinogen not included in the Aα (1-20) residue segment. On the other hand, Brown and Prevec observed an increased electrophoretic mobility of the N-protein of the tsD1 mutant of vesicular stomatitis virus on SDS-PAGE, which was totally corrected by enzymatic removal of residues near the carboxyl-terminus identical with those from the wild-type protein. Thus, they also proposed a long-range interaction of the side chain of the amino acid substituting the side chain of one or more amino acids in the carboxy-terminal residues of the protein complexed with SDS. Such a long-range interaction may also exist in the isolated SDS-denatured Aα-chain, involving a certain amino acid in the A peptide and one or more amino acids in or near the putative epitope likely to be related to the Aα (52-78) segment. If this is the case, the epitope may still be buried in the Aα-chain SDS complex, and prior removal of the A peptide is necessary for exposure of the epitope.

Furthermore, there must be an additional much weaker interaction between the amino-terminal Gly-Pro-Arg segment of the fibrin α-chain and a certain region related to the putative epitope. Indeed, binding of the Gly-Pro-Arg segment as the polymerization "A" site to its complementary "a" site appears to liberate the epitope, as observed in the formation of a complex with fibrinogen or its derivatives containing the D domain (Figs 4 through 6). Moreover, the presence of the Aα (52-78) residue segment may also be required for the epitope expression, because IF-43 was able to recognize the thrombin-treated fragment E but not the thrombin-treated N-DSK lacking the Aα (52-78) residue segment. Nevertheless, IF-43 failed to react with a synthetic peptide with this sequence when examined by a direct binding EIA and immuno blotting (data not shown). Regardless of whether this peptide segment may constitute an essential part of the epitope, the epitope appears to be buried in fibrinogen or acid-FM alone and to be expressed by certain conformational changes induced in the Aα (17-78) residue segment.

Because the FM molecule consists of two identical molecular halves, two epitopes are expected to emerge on formation of a complex with two molecules of fibrinogen. Therefore, aggregation using IF-43-coated latex beads would be applicable to the detection of soluble FM in plasma. Indeed, we were able to measure up to 200 mg of acid-FM spiked to 1 mL of normal plasma. As has been extensively studied, fibrin clot formation is generally accompanied by tissue-type plasminogen activator-catalyzed activation of plasminogen to plasmin. In fact, under pathologic conditions in which massive microthrombi are rapidly formed, overwhelming secondary fibrinolysis is induced and the blood coagulation and fibrinolysis systems are profoundly perturbed. Under such conditions, including DIC, molecular markers for both blood coagulation and fibrinolysis, such as the thrombin-antithrombin III complex, the plasmin-α2-plasmin inhibitor complex, and the D-dimer, may serve as powerful tools for the diagnosis and treatment of the patients.

On the other hand, under conditions in which blood coagulation is preferentially activated and thrombosis is a predominant feature of the disease condition, molecular markers representing intravascular thrombin generation and enzymatic modification of its physiologic substrates appear to be of great use for an early diagnosis of the disease. Using a calibration curve constructed with known amounts of acid-FM spiked to normal plasma, we found that the level of soluble FM in plasma was widespread in patients with thrombotic diseases, including DIC, and that the concentration of soluble FM in plasmas was not significantly correlated with that of the D-dimer, suggesting that these two parameters reflected different aspects and stages of intravascular coagulation associated with or without fibrinolysis. Interestingly, the SF species, in plasmas derived from patients was found to be predominantly a complex of one molecule of desAA-FM, but not desAABB-FM, with two molecules of fibrinogen, as evidenced by the absence of the β-chain representing desAABB-FM in the soluble FM species on immunoblotting run after reduction (Fig 7, lane 2 in inset). On the basis of clinical manifestations of ischemic diseases in one or more organs and overt bleeding, a high level of soluble FM accompanied by a low level of D-dimer seems to be predominantly associated with thrombosis, and a low level of soluble FM accompanied by a high level of D-dimer seems to be predominantly associated with severe DIC manifesting bleeding.

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SOLUBLE FIBRIN-SPECIFIC ANTIBODY


A monoclonal antibody that recognizes a neo-antigen exposed in the E domain of fibrin monomer complexed with fibrinogen or its derivatives: its application to the measurement of soluble fibrin in plasma

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