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Discrimination between fibrin and fibrinogen by a monoclonal antibody against a synthetic peptide

(blood coagulation/peptide-specific monoclonal antibodies/fibrin determination)

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ABSTRACT **Circulating soluble fibrin, observed in the blood of patients with ongoing intravascular coagulation, is generated from the plasma protein fibrinogen by the limited proteolytic action of thrombin. We report the production of a monoclonal antibody that discriminates between fibrin and fibrinogen in blood. The synthetic hexapeptide Gly-Pro-Arg-Val-Val-Glu, representing the amino terminus of the α chain of human fibrin, was used as immunogen. This hexapeptide is located within the Aα chain of fibrinogen but becomes the amino terminus of the fibrin α chain, after fibrinopeptide A is removed by the action of thrombin, and thus becomes accessible for antibody binding. The monoclonal antibody we have prepared can discriminate between fibrin and fibrinogen and thus can be used in assay systems to quantitate soluble fibrin or, potentially, to image fibrin-rich thrombi.**

Blood coagulation reflects the transformation of the soluble plasma protein fibrinogen to an insoluble and visible fibrin clot. This final step in the coagulation process is catalyzed by thrombin sequentially splitting off one pair of fibrinopeptides A (FPA, M_r 1800 each) and one pair of fibrinopeptides B (FPB, M_r 1800 each) from one fibrinogen molecule (1–3). By removal of the FPA, fibrinogen is converted to fibrin monomer (desAA-fibrin). DesAA-fibrin may polymerize to form a clot but can also form soluble intermediates with fibrinogen (4) or fibrinolytic degradation products (5). The generation of soluble fibrin in the circulating blood has been identified as an intermediate step in the activation of intravascular coagulation (4–8). Soluble fibrin injected intravenously into rabbits can polymerize to microclots in kidneys, lungs, liver, and spleen, occluding the capillaries and causing necrosis of the organ tissue (9, 10). Soluble fibrin in the circulating blood has been observed in animals (6, 11) as well as in patients (7, 12–16) with ongoing intravascular coagulation. Since fibrin monomer differs from fibrinogen only by the fibrinopeptides cleaved off, soluble fibrin in blood is difficult to distinguish from fibrinogen and previously has been measured only indirectly or qualitatively (6, 7, 12, 13, 15–18).

After FPA are split off from the Aα chains of fibrinogen, sites on the generated desAA-fibrin become available that permit polymerization of desAA-fibrin to fibrin strands (19–21). After a lag phase (and presumably after a conformational change of the desAA-fibrin molecule) the two FPB of the Bβ chains are cleaved off by thrombin, resulting in the generation of two further polymerization sites (3, 22, 23). Thus, the first step in the transformation of fibrinogen into fibrin is the generation of desAA-fibrin. Our hypothesis is that the amino-terminal amino acids of the α chain of fibrin are shielded within the fibrinogen molecule and thus are not accessible to an antibody directed against this epitope but become available after FPA is cleaved off (Fig. 1). The aim of the present study was to produce an antibody that might discriminate between fibrin and fibrinogen by recognizing the amino-terminal amino acids of the α chain of fibrin.

MATERIALS AND METHODS

Peptide Synthesis and Coupling of the Peptide to a Carrier Protein. The hexapeptide Gly-Pro-Arg-Val-Val-Glu, representing the amino terminus of the α chain of fibrin (Fig. 1), was synthesized by conventional procedures of fragment condensation (24, 25). To allow optimal exposure of the hexapeptide, 6-aminohexanoic acid was used as a spacer. The protected peptides Boc-Glu(OBz)-6Ahx-OMe, Boc-Val-Val-OMe, and Boc-Gly-Pro-Arg-OMe were produced by activation of the COOH terminus of the amino acids with dicyclohexylcarbodiimide in the presence of hydroxybenzotriazole (26). The *t*-butoxycarbonyl (Boc) and methoxy groups were removed with 1.2 M HCl/glacial acetic acid or with 2 M sodium hydroxide, respectively. The di- and tripeptides were connected to each other by use of the dicyclohexylcarbodiimide/hydroxybenzotriazole method. Finally, the peptide Boc-Gly-Pro-Arg-Val-Val-Glu(OBz)-6Ahx-OMe was treated with sodium hydroxide to generate the free carboxyl groups. Sequencing of the final hexapeptide confirmed that the desired amino acid sequence was present. The purity of the hexapeptide was evaluated by thin-layer chromatography, and the racemization was tested by gas chromatography (27). The racemization rate of each amino acid was <2%. The spacer-coupled hexapeptide was activated with dicyclohexylcarbodiimide in the presence of hydroxysuccinimide and then reacted with albumin (28).

Purification of Fibrinogen. Fibrinogen was purified from citrate-treated human blood by the glycine precipitation procedure (29) as described (30).

Preparation of DesAA-Fibrin and DesAABB-Fibrin. DesAA- and desAABB-fibrin were prepared by incubating purified fibrinogen (6.5 mg/ml) with batroxobin (2 units/ml; Pentapharm, Basel, Switzerland) or bovine thrombin (1 unit/ml; Behringwerke, Marburg, F.R.G.), respectively. The clots were isolated after incubation for 2 hr at room temperature in phosphate-buffered saline (0.15 M NaCl/10 mM phosphate, pH 7.2) containing 5 mM EDTA and 3.0 M urea. The dissolved fibrin was repolymerized by removing the urea by dialysis. Clotted material was again dissolved in phosphate-buffered saline/5 mM EDTA/3.0 M urea and stored at 4°C.

Immunization and Preparation of Monoclonal Antibodies. BALB/c mice were immunized twice with 200 μg of carrier-bound synthetic hexapeptide in Freund's complete adjuvant. Mice received a final intravenous injection of 100 μg of

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Abbreviations: FPA, fibrinopeptide(s) A; FPB, fibrinopeptide(s) B.

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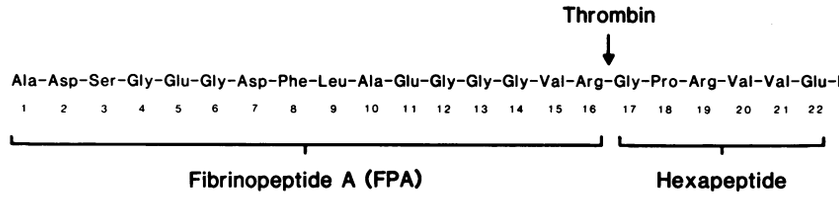

FIG. 1. Amino terminus of the Aα chain of human fibrinogen. Thrombin, as well as batroxobin, removes FPA by cleavage between amino acids 16 and 17. The hexapeptide 17–22 represents the amino terminus of the α chain of human fibrin. R, remainder of the α chain.

carrier-bound peptide in phosphate-buffered saline 3 days before they were killed. Monoclonal antibodies were prepared according to Köhler and Milstein (31). Culture supernatants were screened 2 weeks after fusion for the presence of specific antibodies. Selected cultures were cloned twice by the limiting-dilution technique (32).

Assay of Fibrin-Specific Antibodies. The screening for fibrin-specific antibodies either in plasma of mice or in culture supernatants was carried out by a solid-phase ELISA. Polystyrene microtiter plates (Nunc Immunoplate II F) were coated by overnight incubation at 4°C with antigen (10 μg/ml, 50 μl per well) in phosphate-buffered saline/5 mM EDTA. In the cases of desAA- and desAABB-fibrin, the phosphate-buffered saline contained 3.0 M urea. After five washes with washing buffer (phosphate-buffered saline/0.05% Tween 20), 50 μl of plasma (diluted 1:1000) of immunized mice or 50 μl of culture supernatant was added for 1 hr at room temperature. The wells were washed as described above and incubated for 1 hr with 50 μl of peroxidase-conjugated rabbit anti-mouse Ig antiserum (Dako, Copenhagen) diluted 1:400 in washing buffer. After washing, 150 μl of the substrate, 2 mM ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), Boehringer Mannheim] in 0.1 M sodium acetate/0.05 M NaH_2PO_4 containing 2.5 mM H_2O_2, was added, and 45 min later the optical density was measured at 414 nm in a Titertek Multiskan (Flow Laboratories, Meckenheim, F.R.G.).

Ig classes and subclasses of monoclonal antibodies were determined by adding purified antibody (10 μg/ml) or culture medium to ELISA plates coated with desAA-fibrin. After washing, bound antibody was determined by adding 50 μl of peroxidase-conjugated rabbit anti-mouse antiserum to IgG1, IgG2a, IgG2b, or IgG3 (diluted 1:500 in washing buffer) (a gift from P. Bitter-Suermann, Institute of Microbiology, University of Mainz, Mainz, F.R.G.). After incubation for 1 hr at room temperature, the ELISA procedure was completed as described above.

Purification of Monoclonal Anti-Fibrin Antibodies from Ascites. Hybridoma cells (10^7) were injected intraperitoneally into Pristane-primed BALB/c mice. The Ig-fraction was isolated by two precipitation steps with sodium sulfate and was further purified by affinity chromatography on an anti-mouse Ig column (anti-IgA, anti-IgM, anti-IgG). Antibodies were eluted from the column with 0.2 M glycine/HCl (pH 2.4). Eluted fractions were neutralized by the addition of 2 M Tris/HCl (pH 8.4).

Generation of Fibrin in Plasma Samples. Human blood was collected into a syringe containing 0.1 vol of the following anticoagulant: 0.15 M NaCl/0.05 M EDTA/0.02 M 6-aminohexanoic acid/0.02% sodium azide/aprotinin (2000 units/ml; Bayer, Leverkusen, F.R.G.) at pH 7.2. Cellular particles were removed by two consecutive centrifugations at 1500 × *g*. Plasma samples were stored at −80°C. To generate soluble fibrin in plasma, thrombin or batroxobin was added to plasma at a final concentration of 0.4 unit/ml or 0.1 unit/ml, respectively; enzyme treatment was for 2 min or 4 min, respectively, at 20°C. After incubation, the plasma samples did not show any turbidity or clot formation. Coagulation was interrupted by addition of dilution buffer (phosphate-buffered saline containing 5 mM EDTA, 2 mM 6-aminohexanoic acid, and 200 units of aprotinin/ml) to give a final dilution of 1:20. The same results were obtained when the coagulation in thrombin-treated plasma was interrupted by the addition of hirudin (10 units/ml; Pentapharm, Basel, Switzerland).

Detection of Fibrin in Plasma Samples by ELISA. The wells of polystyrene microtiter plates were coated with monoclonal antibody (10 μg/ml in phosphate-buffered saline, 50 μl per well) overnight at 4°C, washed extensively with phosphate-buffered saline/0.05% Tween 20, and incubated with 50 μl of plasma diluted in diluting buffer for 1 hr. To measure the amount of fibrin bound, a second antibody (50 μl of a peroxidase-conjugated rabbit polyclonal anti-fibrinogen antibody) was added. After an incubation of 1 hr and removal of the second antibody by washing, the activated substrate ABTS was added and the optical density was measured at 414 nm.

Quantitative Determination of FPA. FPA concentration in plasma samples was determined by a radioimmunoassay (Mallinckrodt, St. Louis, MO).

RESULTS

Preparation of Monoclonal Antibodies. BALB/c mice were immunized with the carrier-bound synthetic hexapeptide representing the amino terminus of the fibrin α chain. Plasma samples from the majority of immunized mice showed a positive immune response against the unconjugated peptide, desAA-fibrin, and desAABB-fibrin but not against fibrinogen. Spleens of mice whose plasma showed the strongest immune response against fibrin and no response against fibrinogen were chosen to generate hybridomas. From one fusion experiment, 31 hybridomas out of 100 wells were obtained. Of these 31 hybridomas, 18 clones produced antibody that bound to desAA-fibrin but not to fibrinogen. Fourteen subcloned hybridoma populations whose supernatants showed the strongest anti-fibrin reaction were chosen for the production of monoclonal antibodies in ascitic fluid. Monoclonal antibodies were purified by affinity chromatography on an anti-mouse Ig column. Purified antibodies showed only two bands after $NaDodSO_4$/PAGE under reducing conditions (33).

Selection of a Fibrin-Specific Monoclonal Antibody. Different purified antibodies obtained were tested by treating human plasma with thrombin or batroxobin, respectively. Batroxobin is a snake enzyme that cleaves off FPA only. These enzymes were used at low concentrations so that the plasma did not clot during incubation. All the monoclonal antibodies tested showed a positive reaction with thrombin- or batroxobin-treated plasma samples, whereas only a very weak reaction was observed with control plasma not treated with these enzymes. The antibody with the highest anti-fibrin response was chosen for all further experiments and is designated anti-fbn 17. The class of this antibody was IgM.

In a purified system, anti-fbn 17 showed the following characteristics. It did not react with fibrinogen and bovine serum albumin, the carrier protein to which the peptide was coupled, but it reacted with desAA-fibrin, with desAABB-fibrin, and with the unconjugated fibrin-specific hexapeptide

Table 1. Specificity of the monoclonal anti-fibrin antibody 17 in ELISA of purified antigens

Antigen	OD_{414}
Fibrinogen	0.061
DesAA-fibrin	1.298
DesAABB-fibrin	0.873
Bovine serum albumin	0.05
Hexapeptide*	1.096

*Gly-Pro-Arg-Val-Val-Glu.

(Table 1). The ability of anti-fbn 17 to recognize fibrin in plasma that had been treated with thrombin or batroxobin, respectively, is demonstrated in Fig. 2. Fibrin could be measured in the presence of fibrinogen and in the presence of other plasma components. Control plasma samples showed only a negligible reaction with anti-fbn 17. This minor reaction might indicate the presence of small amounts of fibrin in plasma under normal conditions. Fibrinogenolytic and fibrinolytic degradation products generated by incubation of purified fibrinogen or clotted fibrin, respectively, with plasmin for different intervals (15 min to 6 hr) did not react with anti-fbn 17. This was true also for samples containing mainly X, Y, or D and E fragments (37) as demonstrated by $NaDodSO_4$/PAGE. Anti-fbn 17 recognized fibrinogen in plasma samples to which $NaDodSO_4$ had been added (0.05% final concentration).

Specificity and Sensitivity in Plasma. To calculate the concentration of fibrin present in plasma samples, the FPA concentration of thrombin-treated plasma was measured and related to the total amount of FPA that could be released from the plasma fibrinogen (1 mg/ml). From these values the fibrin concentrations were calculated. Fig. 3 shows that anti-fbn 17

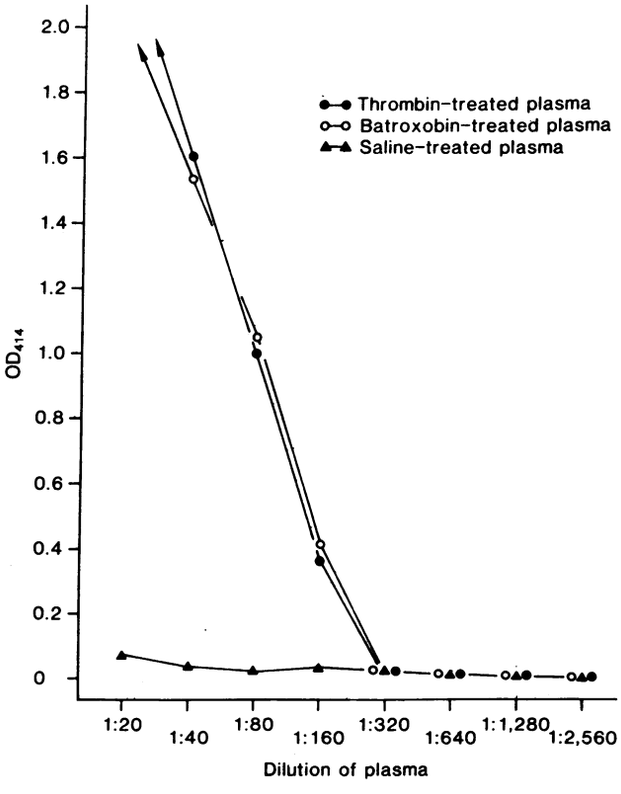

FIG. 2. ELISA of fibrin with the monoclonal anti-fibrin antibody (anti-fbn 17) in plasma treated with thrombin (●) or batroxobin (○), respectively. Control plasma (▲) was treated with isotonic saline.

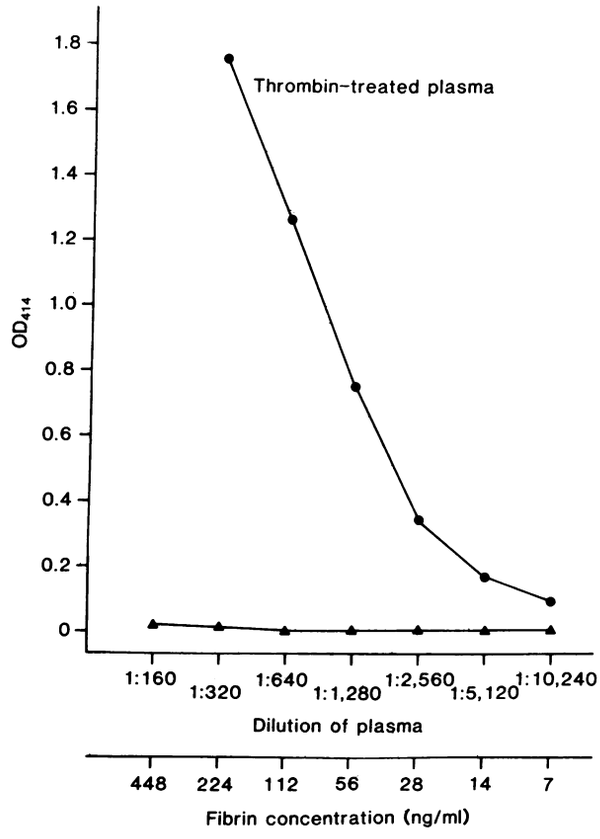

FIG. 3. Sensitivity of anti-fbn 17 towards different concentrations of fibrin in plasma treated with thrombin (●). Control plasma was treated with isotonic saline (▲). Plasma was treated with thrombin (0.4 unit/ml) for 2 min. Coagulation was interrupted by the addition of hirudin (10 units/ml), and FPA was measured according to Nossel *et al.* (17). From the FPA content in the plasma samples, the concentration of fibrin present at different dilutions was calculated.

can be used to detect fibrin at concentrations as low as 14 ng/ml.

In a separate experiment, known concentrations of desAA-fibrin in phosphate-buffered saline containing 3 M urea were added to plasma, and this mixture was diluted for testing (Fig. 4). Fibrin concentrations as low as 78 ng/ml could be detected, in the presence of fibrinogen concentrations that were >20 times higher. Anti-fbn 17 shows a lower sensitivity toward desAA-fibrin that has been kept in 3 M urea before being added to plasma (compare Fig. 4 to Fig. 3). This might be due to a urea-induced conformational change in the fibrin molecule.

Determination of Soluble Fibrin in Blood. Blood was drawn from volunteers and kept in polystyrene test tubes at 37°C, without addition of any anticoagulants. At different times, aliquots were removed, and ongoing coagulation was interrupted by the addition of an anticoagulant solution. After blood cells were removed by centrifugation, the plasma was diluted (1:10) and the presence of fibrin was measured by ELISA (Fig. 5). Fibrin could not be detected by anti-fbn 17 up to 20 min after the blood was drawn. At 30 min and 40 min, a 12-fold and a 30-fold, respectively, increase in fibrin concentration was measured. This increase in fibrin concentration goes in parallel with the increase in FPA concentration that indicates thrombin activity. Visible fibrin strands could be detected only in the 40-min sample. This experiment indicates that soluble fibrin generated in blood in the absence of an anticoagulant can be recognized by the monoclonal antibody anti-fbn 17.

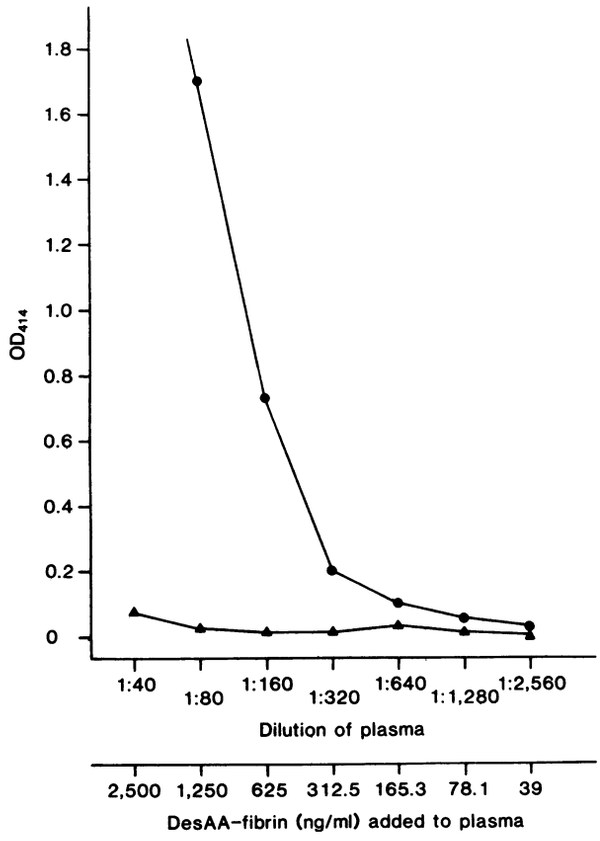

FIG. 4. Detection of purified desAA-fibrin added to plasma. Purified desAA-fibrin in 20 μl of phosphate-buffered saline/2.6 M urea was added to 180 μl of anticoagulated plasma to give a final concentration of 100 μg of desAA-fibrin/ml. Dilutions of this mixture (●) were subjected to ELISA with anti-fbn 17. In the control samples (▲), desAA-fibrin was omitted.

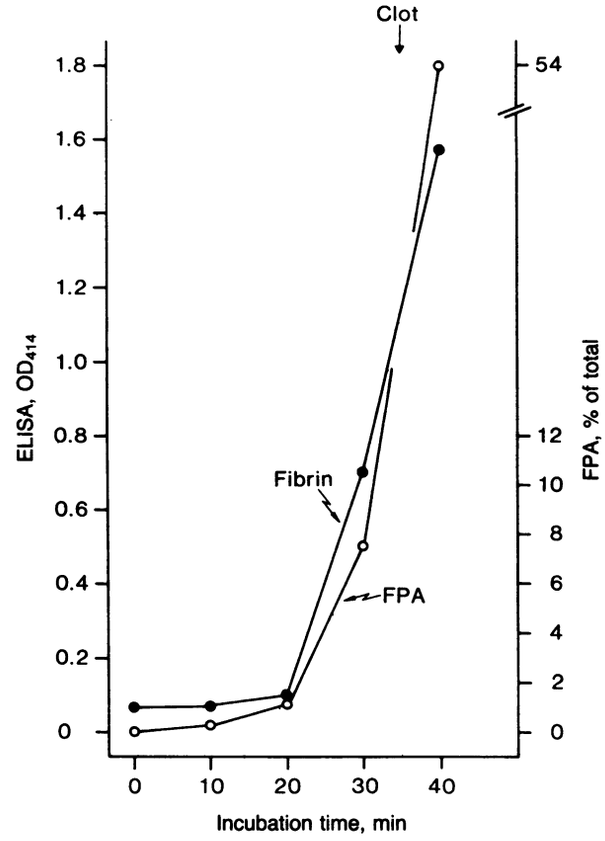

FIG. 5. Increase in fibrin (●) and FPA (○) concentrations in blood undergoing coagulation. Fibrin generated in blood was determined in diluted plasma samples by the ELISA, using microtiter plates coated with anti-fbn 17. FPA was measured by radioimmunoassay.

DISCUSSION

We have obtained a monoclonal antibody capable of distinguishing between fibrinogen and fibrin. This antibody is directed against a synthetic hexapeptide whose amino acid sequence is located within the fibrinogen molecule but which is present at the amino terminus of the α chain of fibrin after the FPA are cleaved from the fibrinogen molecule. This approach may be a useful general strategy to differentiate an activated protein from its unactivated precursor form by immunological methods.

The defined epitope of the α chain of fibrin was selected, as mainly desAA-fibrin and not desAABB-fibrin is found in the circulating blood of patients with ongoing intravascular coagulation (34, 35). FPB is believed to be cleaved only from desAA-fibrin that has polymerized to form a fibrin clot (3). For these reasons an antibody directed against the amino terminus of the α chain of desAA-fibrin seems to be the ideal antibody for detecting soluble fibrin. The antibody to the β chain of desAABB-fibrin (36) presumably will not recognize soluble fibrin.

Anti-fbn 17 recognizes an epitope of the fibrin α chain that apparently is not accessible to the antibody in native fibrinogen. However, when fibrinogen was treated with a detergent, the antibody reacted with the treated fibrinogen, probably because conformational changes of the molecule make the epitope accessible.

The ability of anti-fbn 17 to interact with fibrin may be of considerable importance in clinical assays. The antibody reacts with purified desAA-fibrin or desAABB-fibrin, detects purified desAA-fibrin added to plasma, and recognizes soluble fibrin generated in plasma and in blood during coagulation. The amount of fibrin generated in blood in the course of a coagulation process increased simultaneously with the increase in FPA concentration. Most important is that anti-fbn 17 seems to react specifically with soluble fibrin in the presence of fibrinogen. Refinements in the method may increase its simplicity and its ability to detect small amounts of soluble fibrin in the plasma of patients with arterial or venous thrombosis or disseminated intravascular coagulation. The detection and localization of thrombi, even of coronary-artery thrombi, may be possible with this antibody.

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