Use of a Synthetic Homologue of Human Fibrinopeptide A for Production of a Monoclonal Antibody Specific for the Free Peptide

By B. Kudryk, M. Gidlund, A. Rohoza, M. Ahadi, D. Coiffe, and J.I. Weitz

It has been shown that epitopes reactive with one group of rabbit antibodies to human fibrinopeptide A (hFPA, Aα 1-16) are included in its COOH-terminal region (Aα 7-16). It was further established that Asp-7, Phe-8, and Arg-16 contribute to immunoreactivity and that intact fibrinogen and hFPA-containing fragments react poorly with such antibodies. The purpose of this investigation was to prepare a synthetic peptide corresponding to Aα 7-16 and use it for generation of FPA-specific monoclonal antibodies (MoAbs). Such probes would allow for development of assays that could measure hFPA directly in plasma. In our approach, an ovalbumin-conjugate of the hFPA homologue was further established that Asp-7, Phe-8, and Arg-16 contribute to immunoreactivity and that intact fibrinogen and hFPA-containing fragments react poorly with such antibodies. The purpose of this investigation was to prepare a synthetic peptide corresponding to Aα 7-16 and use it for generation of FPA-specific monoclonal antibodies (MoAbs). Such probes would allow for development of assays that could measure hFPA directly in plasma. In our approach, an ovalbumin-conjugate of the hFPA homologue served as immunogen. Mouse spleen cells were fused with the immunoglobulin nonsecreter myeloma (P3X63Ag8.653). A hybridoma (8C2-5) has been isolated that secretes an antibody (MoAb/8C2-5) with the following characteristics: (a) IgG1, kappa isotype; (b) equilibrium dissociation constant of $1.2 \times 10^{-11}$ mol/with the [125I]-labeled N-tyrosyl derivative of hFPA (Tyr-hFPA) as ligand; (c) reacts with hFPA and dog FPA (dFPA) but not

with the des Arg (Aα 1-15) or shorter peptides; (d) does not react with intact fibrinogen or Aα-chain of human or dog origin; (e) does not react with the elastase-generated hFPA-containing peptide Aα 1-21. Enzyme-based immunoassays (EIAs) have been developed for measuring plasma hFPA levels in the range $3 \times 10^{-14}$ to $5 \times 10^{-13}$ mol/L. Since it has already been shown by a number of investigators that hFPA levels in patients with overt defibrination fall into this range, we propose that the MoAb/8C2-5-based assays may serve as useful clinical tools in screening patients at risk of thrombosis. The 8C2-5 antibody may also be helpful in studies dealing with congenital dysfibrinogenemias, particularly in identifying heterozygous propositi with amino acid substitutions at any position within the Aα 7-16 region. Finally, due to its cross-reactivity with dFPA, assays using this antibody should also be valuable in the canine experimental thrombosis model studies.

In THE fibrinogen-to-fibrin transition, thrombin cleaves arginyl-glycine bonds to release two activation peptides called fibrinopeptide A (FPA, Aα 1-16) and B (FPB, Bβ 1-14). Since fibrinogen is a dimer, two moles of FPA and FPB are released when fibrinogen is transformed to fibrin monomer. Measurement of FPA in blood can serve as an in vivo index of intravascular thrombin action. Nossel et al were first to develop a specific radioimmunoassay (RIA) for estimation of FPA in blood. Since that time, many different investigators have attempted to improve not only on the specificity and sensitivity of assays, but also the ease with which free FPA and FPA-containing degradation products are measured in clinical material. In all the early studies, polyclonal antibodies raised against carrier protein conjugates of human FPA (hFPA) were used. Most of these antibodies failed to distinguish between free FPA and fibrinogen or FPA-containing degradation products. Nossel et al performed detailed studies on the immunochromatographic reactivity of a large number of rabbit anti-FPA sera. Results from these studies showed that such sera could be divided into at least three distinct categories. Group I antisera showed the highest level of discrimination in that a large molar excess, as high as 200-fold, of FPA-containing peptide was required to achieve the same level of inhibition as that obtained with the free peptide. More recently, Leekskma et al identified a rabbit antiserum (R 6216) that could discriminate between FPA and its phosphorylated variant (hFPAP, Aα 1-16 with Ser-3 in phosphorylated form).

Since the development of hybridoma technology, several laboratories, including our own, have attempted to prepare monoclonal antibodies (MoAbs) specific for neoantigenic markers which may be present on fibrinogen-derived fragments formed as a consequence of cleavage by thrombin, plasmin, elastase, or other enzymes. A number of MoAbs directed to neoepitopes present on degradation products derived from the central E domain of fibrinogen have already been identified (for review see reference 11). Dawes et al obtained positive, but very low titer, sera in 11 of 20 mice sensitized with hFPA. Following fusion of mouse spleen cells, with NS-1 myeloma cells, a good number of hybridomas (36 of 319) produced antibody that bound radiolabeled FPA. Despite the fact that most of the antibodies reacted equally well with FPA and fibrinogen, some were highly specific for the free peptide. One of these, designated MoAb/ESF9, has been used in RIA with labeled FPA. Results from these assays have shown that an excess of fibrinogen could not significantly inhibit binding of the ligand to MoAb/ESF9. Using a different antibody (MoAb/ESF1), Dawes et al have shown only slight cross-reactivity between FPA derived from either human or Rhesus monkey fibrinogen. Such
results are very interesting in that FPA's from both species are identical in size (16 amino acid residues) and differ in structure by only a single residue (Aα Ser-3 in human, Aα Thr-3 in monkey).

Our approach for preparing neoepitope-specific MoAbs to FPA has been to use a synthetic peptide homologue of hFPA as an immunogen, specifically the Aα 7-16 decapetide. We selected this segment of FPA because Wilner et al.13 had already shown that epitopes reactive with one group of rabbit antibodies to hFPA were included in the Aα 7-16 region of the peptide, that Asp-7, Phe-8, and Arg-16 contributed significantly to immunoreactivity, and that intact fibrinogen and hFPA-containing fragments of fibrinogen reacted very poorly with such antibodies. This study describes the preparation and characterization of an MoAb that is absolutely specific for FPA. We propose that immunoassays with this probe may prove to be useful for studies dealing with the following: screening patients at risk of thrombosis; in vitro fibrinogen proteolysis mediated by thrombin or enzymes of comparable specificity; genetic variants associated with the NH2-terminal part of fibrinogen Aα-chain; canine experimental thrombosis model.

MATERIALS AND METHODS

Synthetic peptides. Using the Merrifield solid-phase method,14 we manually synthesized the Aα 7-16 decapetide [Aα 7-16(m)]. In addition, we also obtained from Biosearch, Inc. (San Rafael, CA) a supply of the same peptide [Aα 7-16(a)] and from Applied Protein Technologies (Cambridge, MA) its N-tyrosyl derivative [Tyr-Aα 7-16(a)]. The peptide Tyr-Aα 1-21 was a generous gift of Dr. G.D. Wilner, Albany Medical College of Union University, Albany, NY. Finally, the peptide Tyr-hFPA (Lot No. 7034) was synthesized on a Biosearch Model 9600 peptide synthesizer by the Microchemistry Laboratory of The New York Blood Center.

Immunization and production of hybridomas. The Aα 7-16(a) peptide obtained from Biosearch, Inc., without further purification, was coupled to ovalbumin (crystallized, Grade V; Sigma, St Louis) by both the carbodiimide15 and glutaraldehyde16 methods. In each coupling procedure, the Aα 7-16(a) to ovalbumin ratio was 1:1 on a weight basis. Equal volumes (50 μL each) of the two conjugates were mixed with 100 μL complete Freund's adjuvant and injected intraperitoneally (IP) into BALB/c (Jackson Laboratory, Bar Harbor, ME) mice. The subsequent six booster injections (IP), at weekly intervals, consisted of 150 μL of the emulsion prepared with incomplete Freund's adjuvant. The animals were bled 1 week after the last booster. After a 3-week rest period, the animals were boosted intravenously (IV) with the Aα 7-16(a) peptide conjugated by the carbodiimide method to poly-D-glutamic acid [sodium salt approx molecular weight (mol wt) 24.8 Kd, Sigma]. The latter was used to get the NH2-terminal part of fibrinogen Aα-chain; canine experimental thrombosis model.

Testing of prefusion/fusion antiserum and hybridoma culture media. Antiserum were used for titer estimation by enzyme-linked immunosorbent assay (ELISA) and also for immunoblot analysis (see below). In the ELISA procedure, microtiter plates were coated with intact human fibrinogen as well as ovalbumin conjugates prepared with either Aα 7-16(a), hFPA, or hFPAP. Similar plates were also used in screening hybridoma culture media. Coating of polyvinyl microtiter plates (Costar, Cambridge, MA), washing, blocking, and antibody detection was similar to that described previously.12

Production of ascites, purification, and isotyping of antibody. Since antibody levels in ascites are known to be in the 3 to 15 mg/mL range, hybridoma cell line 8C2-5 (see below) was grown in the peritoneal cavity of BALB/c mice using the following protocol. Mice were primed (IP) with 0.5 mL Freund's incomplete adjuvant18. One day following this stimulation, approximately 105 hybrid cells were injected (IP) into animals. Ascites were collected 8 to 12 days later, filtered on a Milliplex filter unit (Millipore Corp, Bedford, MA), adjusted to 0.1% with NaNO3, and stored frozen (−70°C) until needed. Antibody titer in ascites was usually estimated by high-performance liquid chromatography (HPLC) using DEAE or Bio-Gel HPHT columns (see below). Antibody from ascites (45 mL) was purified by chromatography on Bakerbond Aβ (J.T. Baker Chemical Co, Phillipsburg, NJ). The column (3.1cm2 x 10 cm) was equilibrated with buffer Aβ-A (see below). Ascites fluid was dialyzed against several changes of equilibration buffer and later applied to the Bakerbond Aβ column at a flow rate of 20 to 25 mL/h. After all nondesorbed material was eluted, antibodies were recovered using a linear gradient constructed with equal volumes (200 mL) of buffers Aβ-A and Aβ-B. In the antibody elution step, the column flow rate was lowered to 10 to 12 mL/h. The isotype of the purified antibody was determined by ELISA. Polyvinyl microtiter plates were coated with antibody at a concentration of about 0.5 μg/mL in Na2CO3/NaHCO3, pH 9.6, and screening was accomplished using the ScreenType kit and procedure obtained from Boehringer Mannheim (Indianapolis).

Buffers and HPLC solvents. Buffer A-3, used for antibody stock solutions, was composed of 0.04 mol/L Tris, 0.11 mol/L NaCl, 0.1% NaNO3, pH 7.5; buffer A-5, used in RIA, was composed of Dulbecco's phosphate buffered saline (D-PBS, Gibco Laboratories, Grand Island, NY) containing 0.1% NaNO3 and 1.0 mg/mL ovalbumin (crystallized, Grade V, Sigma, St Louis). Buffer A-5B, used in ELISA, was made fresh just before use, was composed of D-PBS containing 1.0 mg/mL ovalbumin (crystallized, Grade V, Sigma). Buffer “NID”, (used for immunoblotting) was composed of 0.01 mol/L Tris, 0.15 mol/L NaCl, 0.1% NaNO3, 0.1% KIU/mL aprotinin (Trasyiol, Mobay Chemical Corp, New York), 1 μg/mL soybean trypsin inhibitor (Worthington Biochemical Corp, Freehold, NJ), 0.87 μmol/L D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (Calbiochem-Behring Corp, La Jolla, CA), pH 7.4. HPPHT-A, (solvent A used in high-performance hydroxylapatite chromatography) was composed of 0.01 mol/L Na2HPO4, 10.0 μmol/L CaCl2, 0.05% NaNO3, pH 6.8. HPPHT-B, (solvent B used in high-performance hydroxylapatite chromatography) was composed of 0.30 mol/L Na2HPO4, 10.0 μmol/L CaCl2, 0.05% NaNO3, pH 6.8. DEAE-A, (solvent A used in high-performance DEAE chromatography) was composed of 0.02 mol/L Tris, pH 8.5. DEAE-B, (solvent B used in high-performance DEAE chromatography), was composed of 0.30 mol/L Na2HPO4, 10.0 μmol/L CaCl2, 0.05% NaNO3, pH 6.8. DEAE-A, was composed of 0.02 mol/L Tris, 0.30 mol/L NaCl, pH 7.0. Aβx-B, (Bakerbond Aβx equilibration buffer) was composed of 0.01 mol/L KH2PO4, pH 6.0, additionally containing 0.1% NaN3 (Bakerbond Aβx limit buffer), was composed of 0.20 mol/L KH2PO4, pH 6.8 additionally containing 0.1% NaN3 of μ-Bond-A, (solvent A used in high-performance μ-Bondpak C18 chromatography), was composed of 0.05% trifluoroacetic acid (Pierce Chemical
Co, Rockford, IL). μ-Bond-B, (solvent B used in high-performance μ-Bondapak C$_18$ chromatography), was composed of 0.025% trifluoroacetic acid containing 50% acetonitrile.

**HPLC analysis of peptides and antibodies.** Analytical and preparative HPLC were performed on three different columns using a Model 1090 Hewlett-Packard (Palo Alto, CA) liquid chromatography. DEAE column was used for titration of antibody present in ascites and for antibody purification. For each application, the column (7.5 x 75 mm, Waters Chromatography Div, Millipore Corp, Milford, MA) was developed at room temperature using the following gradient constructed with DEAE-A and B buffers: at 5% B, three minutes; at 100% B, three minutes; 100% to 5% B, two minutes; at 5% B, 15 minutes. Before HPLC, all samples were dialyzed against DEAE-A and filtered using a 0.2 μm Millex-GS filter unit (Millipore Corp). Column flow rate was 1.0 mL/min and, in preparative runs, 1 mL fractions were collected. HPHT column (Bio-Gel hydroxylapatite column) used for titration of antibody present in ascites and antibody purification. The column (7.8 x 100 mm, Bio-Rad Laboratories, Richmond, CA) was developed at room temperature using the following gradient constructed with HPHT-A and B buffers: 10% to 40% B, 20 minutes; at 40% B, two minutes; at 70% B, two minutes; at 70% B, 15 minutes. Before chromatography, all samples were dialyzed against HPHT-A and filtered using a 0.2 μm Millex-GS filter unit (Millipore Corp). Column flow rate was 0.8 mL/min. μ-Bondapak column was used for titration of antibody present in ascites and antibody purification.

**Immunoblotting.** Reduced samples of human and dog fibrinogen, crosslinked fibrin, as well as other proteins were subjected to electrophoresis on acrylamide gels (7.5% to 15% gradient gels) in sodium dodecyl sulfate (SDS-PAGE). Electrophoresis (18 to 20 h/room temperature/9mA constant current) was on gel slabs (36 cm(w) x 26 cm(l) x 0.75 mm(thick)) in a buffer containing 0.025 mol/L Tris, 0.19 mol/L glycine, 0.1% SDS, 5 mg antibody to mouse immunoglobulin (rabbit anti-mouse immunoglobulin (rabbit anti-mouse in the case of MoAb/8C2-5 and goat anti-rabbit in the case of R-33) conjugated to agarose was added to each assay tube. The second incubation (with the antibody immunoglobulins) involved end-over-end mixing (10 rotations/min) for two hours at 4°C using a homemade apparatus. Following this, all second antibody-containing tubes were centrifuged (2,000 g, ten minutes, 4°C), washed three times with cold saline, and the pellet counted for radioactivity. In some instances, antibody-bound and free tracers were separated by the addition of charcoal (Norit A, decolorizing carbon; Pfanzteich Laboratories, Inc, Waukegan, IL) suspensions made in Dulbecco’s phosphate buffered saline containing 0.1% Na$_2$NO$_3$ and 4.0 mg/mL ovalbumin. Due to different charcoal dose-response curves obtained with the tracers, the coal concentrations in assays with R-33 and MoAb/8C2-5 were 6 mg/mL and 1.2 mg/mL, respectively.

**Affinity measurements.** $^{[125]}$Tyr-hFPA, at constant concentration (21 and 42 pmol/L), was incubated with various concentrations of pure MoAb/8C2-5 (14.7 to 137 pmol/L) for 60 hours at 4°C. Antibody-bound tracer was isolated using rabbit anti-mouse immunoglobulin conjugated to agarose (RAM) as described above. In these experiments, nonspecific binding (without antibody) was ≤5%. Calculations of bound antigen and free antibody at equilibrium were obtained from radioactivity measurements as follows. The concentration Ag(b) of bound antigen is related to the total concentration Ag(t) of antigen by:

$$\frac{Ag(b)}{Ag(t)} = \frac{R - R(nb)}{R(t)}$$

where R is conjugate-bound radioactivity when initial concentration of antibody was $Ab_R$, R(nb) was bound radioactivity in absence of antibody, and R(t) is total radioactivity in each assay tube. The concentration $Ab$ of free antibody binding sites at equilibrium is then obtained by the difference: $\Delta Ab = Ab_R - Ag(b)$.

**Preparation of enzyme conjugates.** Horseradish peroxidase (HRPO, Boehringer Mannheim, Indianapolis) was conjugated to both hFPA and MoAb/8C2-5 by a method involving oxidation of the sugar moieties of the enzyme by periodate ions. In the case of HFP, 1.0 mg peptide was linked to 5.0 mg enzyme. In the preparation of MoAb/8C2-5-HRPO, 5 mg antibody was coupled to 10 mg enzyme. The protocol used for the preparation of both conjugates was as previously described with only slight modification.31

**Competitive ELISA procedure using hFPA-HRPO.** Polystyrene microtitre plates were coated with 100 μL/well of MoAb/8C2-5 at a concentration of about 1.0 μg/mL in Na$_2$CO$_3$/NaHCO$_3$, pH 9.6. Generally, plates were coated overnight at 4°C. However, antibody-coated plates can be prepared in as little as two to three hours. Coated plates are subsequently "blocked" with buffer A-5B (15 minutes at 20°C). After this time, plates are washed (100 μL, 3 ×) with D-PBS containing 0.05% Tween 20 (TPBS) and later incubated (30 minutes at 20°C) with a fixed concentration of hFPA-HRPO (dilution made in buffer A-5B) mixed with an equal volume of either buffer A-5B or a known but variable concentration of hFPA standard or an unknown test sample. Standard hFPA or unknown test samples are diluted in buffer A-5B. Following three wash cycles (100 μL each) with TPBS, 50 μL of a commercially available substrate-dye (TMB, 3,3',5,5'-tetramethylbenzidine) solution (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, MD) is added to each well and plates are incubated 15 minutes at 20°C with gentle shaking. Color development is quenched by adding 50 μL N HCl/ and color intensity is measured (at 450 nm) using a microelisa autoreader.

**Competitive ELISA procedure using MoAb/8C2-HRPO.** In this assay, microtitre plates were coated with ovalbumin conjugates made with either B. coli-7-16(a), hFPA, or hFPAP. It has been difficult
to estimate the number of “active” (antibody-binding) peptides present on such conjugates. Plates are coated with 100 μL/well of conjugate solution (about 1.0 μg/mL with respect to ovalbumin, dilutions made in Na₂CO₃/NaHCO₃, pH 9.6). Generally, plates are coated overnight at 4°C but, if need be, can be used within several hours. In this assay, the binding of the MoAb/8C2-5-HRPO to the peptide-ovalbumin plates is competitively inhibited by an added standard or test peptide solution. Incubation, washing, substrate-dye solutions and all other procedures are identical to those described above for the competitive ELISA using hFPA-HRPO.

RESULTS AND DISCUSSION

Synthetic peptides. On analytical HPLC, the manually prepared decapeptide [Aα 7-16(m)] showed a single major peak with retention time (R,) ≈ 22 min. The Aα 7-16(a) peptide was less pure; however, the major peak eluted with same R,. Results from amino acid analysis and sequence analysis (Applied Biosystems Model 477A Protein Sequencer and Applied Biosystems Model 120 PTH-Analyzer) indicated that both peptides corresponded in structure to human Aα 7-16. The N-tyrosyl derivative of the decapeptide supplied by Applied Protein Technologies (Cambridge, MA) was further purified by preparative HPLC using a μ-Bondapak C₁₈ column and the purified peptide gave a single peak with an Rₜ ≈ 28 min. The Tyr-hFPA peptide also gave a single peak (Rₜ ≈ 25 min) on HPLC when monitored at either 210 or 280 nm.

Analysis of prefusion serum. Antisera collected from animals immunized with the Aα 7-16(a)-ovalbumin conjugate were initially screened on ELISA plates coated with fibrinogen or fibrin. When such sera were appropriately diluted, significant color was generally observed only on plates coated with fibrinogen (not shown). These results suggested that the animals had been sensitized and were producing antibodies to the NH₂-terminal portion of the Aα-chain (i.e., the Aα 7-16 region) of human fibrinogen. Since this region is missing in fibrin, little or no reactivity could be expected on fibrin-coated ELISA plates. The extent of reactivity would be related to the amount of fibrinogen present in the fibrin used in preparation of plates. Immunoblot analysis (not shown) was also made using a dilution of prefusion serum from an animal (mouse no. 2) showing the highest response on ELISA. As control, a preimmunization serum diluted in the same manner was also used. As expected, only background staining was obtained with the control serum. In contrast, a band coincident with the Aα-chain as well as a number of other bands were observed in lane 1 (right panel) due to the fact that prestained (blue chromophore) markers were used.

Fig 1. Immunoblotting of various reduced and nonreduced proteins. Before blotting, proteins were separated by SDS-PAGE (7.5% to 15% gradient). The following samples were electrophoresed: (lane 1) prestained marker proteins (Bethesda Research Laboratories, Gaithersburg, MD) consisting of myosin H-chain (200 Kd), phosphorylase B (97.4 Kd), BSA (68 Kd), ovalbumin (43 Kd), α-chymotrypsinogen (25.7 Kd), β-lactoglobulin (18.4 Kd) and lysozyme (14.3 Kd); (lane 3) DTT-reduced human fibrinogen; (lane 4) reduced human crosslinked fibrin; (lane 5) reduced dog fibrinogen; (lane 6) reduced dog crosslinked fibrin; (lane 9) ovalbumin; (lane 10) human FPA and FPB conjugated to ovalbumin with carbodiimide; (lane 11) fragment E from human fibrinogen; (lane 12) hFPAP conjugated to fragment E with carbodiimide. No samples were applied in lanes 2, 7, and 8. After transfer to nitrocellulose, membranes were either stained for protein (left panel) or treated with MoAb/8C2-5 at a final concentration of about 2 μg/mL (right panel). Antibody-bound protein bands were detected using peroxidase-conjugated rabbit antibody to mouse immunoglobulin, H₂O₂ and 4-chloro-1-naphthol. Other details dealing with the immunoblotting procedure are given in Materials and Methods. Antibody 8C2-5 does not react with any protein in the standard mixture, bands in lane 1 (right panel) are due to the fact that prestained (blue chromophore) markers were used.
with reduced human fibrinogen and the positive antiserum. The latter results were expected since it is well known that the Aα-chain is quite heterogeneous and that considerable proteolysis of the COOH-terminal portion of the chain occurs during purification of human fibrinogen from plasma. Of interest, the positive serum did not react with the Aα-chain of dog fibrinogen. Dog Aα-chain is much less degraded during purification and, by comparison with humans, the dog segment Aα 7-16 differs only slightly in structure (Aα Asp-7 is replaced by Glu and Aα Leu-9 is replaced by Ile).22

Screening antibodies in hybridoma culture media. The spleen of mouse no. 2 (see above) was used for production of hybridomas. Good cell growth was observed in a large number of wells (503 of 1,152) of microtiter plates ten to 14 days following fusion. Initially, culture media were screened for antibody on plates coated with either intact fibrinogen or conjugates prepared with the Aα 7-16(a) peptide and poly-D-glutamic acid. Of all the media obtained from the different clones tested by ELISA, only six contained antibody that bound to wells coated with the peptide conjugate but not with intact fibrinogen and only antibody secreted by two cell lines (8C2 and 8C3) could bind [125I]Tyr-hFPA ligand. One sample showed slight binding to plates coated with both the peptide conjugate and fibrinogen. In competition ELISA on plates coated with Aα 7-16(a)-poly-D-glutamic acid and using the rabbit anti-mouse immunoglobulin-HRPO conjugate, only antibodies secreted by hybridomas 8C2 and 8C3 were neutralized by appropriate dilutions of Aα 7-16(a), hFPA, or hFPAP. Antibody secreted by hybridoma 9A9 could be neutralized by Aα 7-16(a), but only at a very high concentration.

Purity and isotyping of antibodies recovered from ascites. Antibody present in ascites induced by hybridoma 8C2-5 (subclone no. 5 of hybridoma 8C2) was purified by chromatography on a Bakerbond ABx column (see Materials and Methods). Identical patterns were obtained when 8C3-induced ascites was fractionated on a similar size Bakerbond ABx column. In contrast, however, when 9A9-induced ascites were fractionated on such columns, the second peak eluted earlier than that observed for the other two ascites. On SDS-PAGE, the gradient eluted protein peak from 8C2-5-induced ascites showed it to be better than 95% pure antibody. Similar results were obtained with the gradient eluted protein from both 8C3- and 9A9-induced ascites. Ross et al24 have shown that many different murine and rat MoAb, of both IgG1 and IgG2 isotype, can be purified to 90% to 95% homogeneity on fast protein liquid chromatography (FPLC) using ABx columns. By contrast to the just cited method, in this report we demonstrate a high degree of antibody purification from very large volumes of ascites.
using a conventional column packed with Bakerbond ABx. Immunoglobulin isotyping of the ABx-purified antibodies showed that both MoAb/8C2-5 and MoAb/8C3 were IgG,, kappa. The isotype of MoAb/8C5 was not determined.

**Immunoblot analysis using MoAb/8C2-5.** As expected, no protein bands from factor XIIIa-crosslinked human and dog fibrin (Fig 1, lanes 4 and 6, respectively) were able to bind MoAb/8C2-5. Since fibrin formation involves the loss of FPA and FPB, little, if any, reactivity of putative anti FPA MoAb should be obtained with such samples. Clearly, the extent of reaction with such antibodies would be inversely related to the degree of fibrinopeptide removal during fibrin formation. As shown in Fig 1, MoAb/8C2-5 also failed to react with reduced human or dog fibrinogen (lanes 3 and 5, respectively). Furthermore, no reactivity was observed with the carrier proteins before (lanes 9 and 11) coupling with human fibrinopeptides. In fact, the only bands that bound MoAb/8C2-5 were those from samples containing carrier protein-conjugates of hFPA/hFPB (lane 10) and hFPAP (lane 12). Since carbodiimide-induced coupling of proteins or peptides gives rise to products that are quite heterogeneous in size and composition, the streaking effect obtained in this immunoblot analysis is to be expected. Most importantly, however, the failure to observe a reaction between MoAb/8C2-5 and the fibrinogen Aα-chain indicates that, for immunoreactivity with this antibody, FPA must have a free Aα Arg-16 at its COOH-terminal end. Since FPA coupled to any carrier protein with carbodiimide is thought to be covalently linked by either the free NH₂ – or COOH groups, such conjugates may contain some peptides with free COOH-termini. Immunoblot analysis was not performed with the other two antibodies (MoAb/8C3 and MoAb/99A).

**Specificity of MoAb/8C2-5 determined by RIA.** It has been known for some time that leukocyte extracts contain enzymes that degrade fibrinogen. Recently, Weitz et al5 conclusively demonstrated that neutrophil elastase present in such extracts was the enzyme responsible for fibrinogenolytic activity and that one of the cleavage products was the FPA-containing fragment Aα 1-21. Since this peptide is probably the smallest naturally occurring FPA-containing fragment and since it, as well as its N-tyrosyl derivative (Tyr-Aα 1-21), can be readily cleaved with thrombin, we decided to study the specificity of MoAb/8C2-5 by determining its ability to bind intact and thrombin-cleaved [125I]-Tyr-Aα 1-21. As shown in Fig 2, thrombin-digestion of Tyr-Aα 1-21 at the Aα Arg 16-Gly 17 bond results in two peptides that can be well resolved by HPLC using μ-Bondapak C18 columns. The inset to Fig 2 shows that very different radioactivity profiles were obtained when intact and thrombin-digested mixtures of “cold” Tyr-Aα 1-21 and [125I]-Tyr-Aα 1-21 were fractionated by HPLC. As expected, the digested sample gave a single major radioactive peak that eluted earlier (relative to the intact ligand) and very close to the fractions that contained “cold” Tyr-Aα 1-16. The relative elution times for both “hot” and “cold” Tyr-Aα 1-21 and Tyr-Aα 1-16 were nearly identical. This HPLC analysis was performed in order to verify that the digested ligand, which was later to be used for binding studies with MoAb/8C2-5, was not degraded to more than the predicted number of peptide fragments. Figure 3 (upper panel) shows the binding of intact and thrombin-digested [125I]-Tyr-Aα 1-21 to MoAb/8C2-5. In all binding experiments, identical dilutions of both radioactive ligands—characterized on HPLC by the method just described—were used. Binding RIA data clearly shows that MoAb/8C2-5 only reacts with the thrombin-digested [125I]-Tyr-Aα 1-21 peptide. For comparison, the binding of these same two ligands to a polyclonal anti-FPA antibody (R-33) is also shown (Fig 3, lower panel). The latter antibody was prepared by Nossel et al and it was previously shown that it can cross-react extensively with fibrinogen and FPA-containing fragments. In other RIA, MoAb/8C2-5 exhibited good binding not only with thrombin-digested [125I]-Tyr-Aα 1-21 but also with [125I]-Tyr-Aα 7-16(a) and [125I]-Tyr-hFPA (data not shown). MoAb/8C3 gave similar binding properties whereas MoAb/99A failed to bind any radioactive ligand just described. Finally, short-term carboxypeptidase B diges-

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**Fig 3.** Binding RIA using different dilutions of MoAb/8C2-5 mixed with standard amount of [125I]-Tyr-Aα 1-21 ligand before and after digestion with thrombin (upper panel). After a suitable incubation period (18 to 20 hours), antibody-bound ligand was isolated using agarose conjugates of rabbit anti-mouse immunoglobulins (RAM) as described in Materials and Methods. For comparison, binding RIA results obtained with a polyclonal rabbit anti-FPA sera (R-33) and [125I]-Tyr-Aα 1-21 before and after thrombin digestion are also shown (lower panel). In the latter case, ligand-bound antibody was separated using agarose-insolubilized goat anti-rabbit immunoglobulins. Antiserum R-33 was prepared by Nossel et al and has been shown to cross-react extensively with intact fibrinogen and FPA-containing fibrinogen fragments.
tion of all three antibody-reactive ligands resulted in near complete loss of immunoreactivity with MoAb/8C2-5 (data not shown). Taken together, these results clearly indicate that, for immunoreactivity with MoAb/8C2-5, the NH₂-terminal part of the Aα-chain of human fibrinogen must include a segment comprising Aα 7-16 and that Aα Arg-16 cannot be linked in peptide bond at its COOH-terminal end. Furthermore, the data obtained with the carboxypeptidase B-digested ligands suggest that Aα Arg-16 is crucial for epitope expression. As shown in Fig 4, the RIA inhibition curve with MoAb/8C2-5 at 98.7 nmol/L was about 70 times less sensitive than that obtained with antiserum R-33. In this assay (100 μL of different dilution of native hFPA tested), the 50% displacement of binding was obtained with 2.5 pmol of hFPA using R-33. In order to achieve the same level of inhibition with MoAb/8C2-5, about 170 pmol of hFPA was required. A slight increase in sensitivity (50% displacement with about 42 pmol of hFPA) was obtained in an assay using a lower concentration of MoAb/8C2-5 (35.2 nmol/L) and, for recovering the immune complex, a conjugated form of second antibody in place of charcoal. In this type RIA, a five-hour preincubation with “cold” hFPA had no effect on assay sensitivity (not shown). Inhibition curves with MoAb/8C2-5 and either “cold” Tyr-hFPA or dFPA (not shown) were identical to those obtained with hFPA. The des Arg homologue of hFPA (Aα 1-15) did not show inhibition of binding in the MoAb/8C2-5 assay, even at a concentration of 100 nmol/mL (not shown). The latter results substantiate the binding data obtained with the carboxypeptidase B-digested ligands (see above) and further support the idea that Aα Arg-16 is crucial for immunoreactivity with MoAb/8C2-5. Predictably, synthetic homologues smaller (Aα 1-12, Aα 1-13, and Aα 1-14) and larger (Aα 1-18, Aα 1-19, and Aα 1-20) than hFPA also failed to react with MoAb/8C2-5 (not shown). It should be emphasized that the inhibition curve obtained with hFPA and MoAb/8C2-5 is identical when buffer or normal human plasma (containing heparin and Trasylol as described (25)) is used as diluent for the peptide.

Equilibrium dissociation constant of MoAb/8C2-5. The ligand used in these studies was [125I] Tyr-hFPA. Binding was calculated from the amount of radioactivity, corrected for nonspecific binding, associated with the agarose-conjugated RAM. Experiments were conducted with ligand concentrations of 21 and 42 nmol/L, respectively. Different concentrations (14.7 to 137 nmol/L) of MoAb/8C2-5 were used. In these experiments, the ABx-recovered antibody (see above) was further purified by HPLC using Bio-Gel HPHT as described in Materials and Methods. Results analyzed according to the Scatchard equation are shown in Fig 5. Data in Fig 5 are from an experiment where 21 nmol/L ligand and antibody-ligand mixtures were incubated for 60 hours at 4°C. Similar results (not shown) were obtained with 42 nmol/L ligand or with either ligand concentration and much shorter incubation times (18 to 20 hours). By linear regression analysis, the equilibrium dissociation constant of the MoAb/8C2-5-Tyr-hFPA interaction is: 1.5 ± 0.2 × 10⁻⁷ mol/L. By comparison with a different Aα-chain antibody (MoAb/Y18) isolated by Koppert et al. (26) MoAb/8C2-5 shows a rather low affinity. Antibody Y18 (IgM, kappa)
reacts with human fibrinogen as well as hFPA-containing peptides but not with free hFPA. The two antibodies (MoAb/8C2-5 and MoAb/Y18) are similar in that Aa Arg 16 is crucial for reactivity. In the case of MoAb/Y18, Aa Arg 16 must be present in peptide-bond linkage with at least part of the Aa-chain beginning with Aa Gly 17. As mentioned above, the Aa Arg 16 - Gly 17 bond must be cleaved in order to obtain reactivity with MoAb/8C2-5.

**Competition ELISA using MoAb/8C2-5.** Two different inhibition EIA have been developed with MoAb/8C2-5. From a practical point of view, both these methods require no radiolabeling and, therefore, no possibility of structural alteration to any of the N-tyrosyl homologues of FPA. Furthermore, the assays save time and effort and make use of very small amounts of reagents most of which are quite stable at 4°C for at least 6 months. In our experience to date, the only reagent that has had a rather limited shelf life has been the hFPA-HRPO conjugate. A typical dose-response curve of reactivity between hFPA and MoAb/8C2-5-HRPO is shown in Fig 6. Regarding precision, the mean of the standard errors in this assay was ±1.3%. The 50% inhibition of binding was obtained with about 6.5 pmol hFPA (in the assay, 50 μL antibody-enzyme is mixed with an equal volume of diluted competitor). The reason for the higher degree of sensitivity in this method as compared with the RIA (see above) is due to the fact that the MoAb/8C2-5-HRPO can be diluted to such a high degree (about 50,000-fold or more). It is difficult to estimate the level of “active” antibody in such conjugates, but, the protein concentration is about 0.2 nmol/L. As with the RIA, it should be emphasized that the ELISA inhibition curve shown in Fig 6 is identical when buffer or normal human plasma (containing heparin and Trasylol as described) is used as diluent for the hFPA standard. In terms of time, the ELISA, using precoated peptide plates, can be performed in about 60 minutes. An inhibition curve of a similar degree of sensitivity as that shown in Fig 6 has also been obtained using a plate coated with pure MoAb/8C2-5 and the freshly prepared hFPA-HRPO conjugate. The reason for the above-mentioned time-dependent “decay” of the latter conjugate is presently under investigation.

MoAbs reactive with FPA and/or FPA-containing fragments have been identified by Koppert et al.19 as well as Shainoff et al.20 The antibody described in this study is unlike any reported to date in that it is absolutely specific for free FPA. As mentioned above, Dawes et al identified several antibodies that had preferential but not absolute specificity for free FPA.12 In quantitative terms, it is impossible to compare MoAb/8C2-5 with the latter antibodies since details on their preparation and specificity have been reported only in abstract form12 and in a brief review article dealing with monoclonal antibodies and their potential use in the Hemostasis Laboratory.28

In summary then, assays, particularly the EIAs, developed with the antibody described in this report may prove to be useful in selected clinical applications. Although the sensitivity of all the assays described herein is less than that observed with R-33 (and other polyclonal or monoclonal antibodies), the unique specificity of MoAb/8C2-5 should make it a highly useful reagent. For example, since this antibody shows no reactivity with fibrinogen, incomplete removal of fibrinogen from clinical material should not result in the overestimation of FPA levels in such samples. The question of sensitivity of the MoAb/8C2-5 assays can be addressed by simply using larger sample volumes and/or more concentrated ethanol-extracted clinical material. The availability of MoAb/8C2-5 also offers the opportunity for development of a “capture-tag” type EIA with a less specific probe. In such an assay, MoAb/8C2-5 would serve as the “capture” probe and an HRPO-labeled second antibody, directed to a different epitope on FPA, could be the “tag” reagent. As already mentioned, the latter antibody could be panspecific, that is, one that would react with fibrinogen as well as FPA-containing fragments. The only requirements for such an antibody is that it would have a similar affinity constant and that it be directed to an epitope other than the one with which MoAb/8C2-5 reacts (Aa 7-16). Binding of two different antibodies to a peptide the size of FPA would not be unique, since Jackson et al29 very recently reported that two antibodies recognizing distinct epitopes, the outer boundaries of which are separated by only three amino acids, bind simultaneously to a 24 residue peptide containing both epitopes. In addition to its use as a clinical tool for screening patients at risk of thrombosis, MoAb/8C2-5 may also be helpful in identifying genetic variants of FPA, particularly affected heterozygous probands as found in fibrinogen Louisville,30 Petoskey,31 or Stony Brook32 to name but a few. Finally, due to its complete cross-reactivity with dFPA, MoAb/8C2-5-based EIA should also be very useful in monitoring the kinetics of coil-induced clot formation in the canine experimental thrombosis model studies as reported by Rosebrough et al.33-35 One of the questions such studies have persistently raised is whether or not the aggressive fibrino-
lytic system in the dog results in a steady formation of coil-induced thrombi.\textsuperscript{15} Using the MoAb/8C2-5-based assays, this question can now be addressed.

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