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Immunology

The sequence Aα-(148–160) in fibrin, but not in fibrinogen, is accessible to monoclonal antibodies

(synthetic peptide/enzyme immunoassay/neoantigen/blood coagulation)

WIM J. G. SCHIELEN*, MARIJKE VOSKUILEN†, GODEFRIDUS I. TESSER*, AND WILLEM NIEUWENHUIZEN†‡

†Gaubius Institute, Nederlandse Organisatiè voor Toegepast Natuurwetenschappelÿk Onderzoek, Leiden, The Netherlands; and *Laboratory of Organic Chemistry, University of Nijmegen, Nijmegen, The Netherlands

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ABSTRACT **Fibrin, but not fibrinogen, accelerates the activation of plasminogen catalyzed by tissue-type plasminogen activator. Previous work showed that essential information for this accelerating capacity of fibrin resides in the sequence corresponding to residues 148–160 of the Aα chain of fibrinogen [Aα-(148–160)]. Our working hypothesis, based on those findings, is that Aα-(148–160) is buried in fibrinogen and becomes accessible to proteins such as plasminogen and/or tissue-type plasminogen activator when fibrinogen is converted to fibrin. To test this hypothesis we have raised a monoclonal antibody against synthetic Aα-(148–160) and found that this antibody reacts with fibrin and not with fibrinogen. This finding shows that Aα-(148–160) becomes accessible when fibrinogen is converted to fibrin and that Aα-(148–160) is a fibrin-specific neoantigenic determinant.**

When the coagulation system is activated, thrombin is generated. Thrombin converts circulating fibrinogen to fibrin monomer by the cleavage of fibrinopeptides, A and B, from the amino-terminal ends of the two fibrinogen Aα and Bβ chains, respectively. The fibrin monomers aggregate through the resulting new amino-termini of the α and β chains, which constitute sites that bind to complementary sites in the carboxyl-terminal domains (1, 2) of other fibrin(ogen) molecules. The carboxyl-terminal sites already exist in fibrinogen, and at low concentrations fibrin is kept in solution by complexation with fibrinogen. Beyond a critical (local) concentration, fibrin aggregates to form a fibrin gel—i.e., a thrombus is formed.

With the advent of monoclonal antibody (mAb) technology it has become possible to raise antibodies against molecular features that are specific for fibrin and do not occur in fibrinogen (neoantigenic determinants). Examples are mAbs that have been raised against the new amino-termini of the fibrin α chains (3, 4) and β chains (5). Those antibodies were elicited by using conjugates of a carrier protein and synthetic peptides with an amino acid sequence identical to that occurring in the amino-terminal stretches of the fibrin α and β chains, respectively.

The activation of plasminogen by tissue-type plasminogen activator (t-PA) is greatly accelerated by fibrin, but not by fibrinogen. Previous work showed that the sequence Aα-(148–197) (6) and in particular Aα-(148–160) and Lys-157 of the Aα chain (7) play an essential role in this acceleration. Our working hypothesis based on those findings is that this part of the Aα chain is buried in fibrinogen and becomes accessible to proteins such as plasminogen and t-PA during the fibrinogen-to-fibrin conversion. If this hypothesis is correct, Aα-(148–160) would also become accessible to antibodies upon fibrin formation, and antibodies against Aα-(148–160) would be expected to react with fibrin and not with fibrinogen.

In this paper we describe the production of mAbs against this part of the Aα chain. A conjugate of bovine serum albumin (BSA) and a synthetic peptide with the sequence of Aα-(148–160) was used as immunogen. The resulting antibodies react exclusively with fibrin, and not with fibrinogen. This demonstrates that Aα-(148–160) is a fibrin-specific neoantigenic determinant, and supports our hypothesis that the sequence Aα-(148–160) is inaccessible in fibrinogen and becomes accessible in fibrin.

MATERIALS AND METHODS

Synthesis of [Ata-DL-Nle^{147}]Fibrinogen-Aα-(147–160)-tetradecapeptide. Fibrinogen-Aα-(148–160)-tridecapeptide (Lys-Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile-Arg-Ser) was extended with acetylthioacetyl-DL-norleucine (Ata-DL-Nle) at the amino terminus. Thus, methionine present in fibrinogen at Aα 147 is replaced by DL-Nle. This procedure allows the coupling to a carrier protein and (via the norleucine content) the subsequent determination of the number of coupled peptides per carrier protein molecule. The [Ata-DL-Nle^{147}]fibrinogen-Aα-(147–160)-tetradecapeptide (Ata-P_{14}) was synthesized by solid-phase peptide synthesis using the *p*-alkoxybenzyl alcohol resin (8) and N^{α}-(9-fluorenyl)-methyloxycarbonyl (Fmoc) amino acids (9). During solid-phase peptide synthesis the amino acid side chains were protected with acid-labile protecting groups: the ε-amino group of lysine with *tert*-butoxycarbonyl (Boc), the δ-guanidino group of arginine with pentamethylphenylsulfonyl (Pms), the γ-carboxyl group of glutamic acid and the β-carboxyl group of aspartic acid with *tert*-butyl ester (OBu^t), and the β-hydroxyl group of serine with *tert*-butyl (Bu^t). Fmoc-Ser(Bu^t)-OH [2 equivalents (eq.)] was coupled to the resin (1.07 mmol/g) by *in situ* activation with dicyclohexylcarbodiimide (2 eq.) and 4-(dimethylamino)pyridine (2 eq.). To suppress racemization, 1-hydroxy-1-benzotriazole (4 eq.) was added (10). All reactants were dissolved in dimethylformamide. After 16 hr at 5°C the remaining alcohol groups of the resin were capped with benzoyl chloride and pyridine in dimethylformamide for 1 hr. The subsequent cleavage of the Fmoc groups was carried out with 20% (vol/vol) piperidine in dimethylformamide. Coupling of Fmoc-Arg(Pms)-OH (3 eq.) was performed by *in situ* activation with dicyclohexyl-

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Abbreviations: BSA, bovine serum albumin; mAb, monoclonal antibody; t-PA, tissue-type plasminogen activator; Ata, acetylthioacetyl; Nle, norleucine (2-aminohexanoic acid); Pms, pentamethylphenylsulfonyl.

‡To whom reprint requests should be addressed at: Gaubius Institute, Nederlandse Organisatiè voor Toegapast Natuurwetenschappelÿk Onderzoek, P.O. Box 612, 2300 AP Leiden, The Netherlands.

carbodiimide (3.3 eq.) and 1-hydroxy-1-benzotriazole (3.6 eq.) in dimethylformamide. The Fmoc group was cleaved off with 20% piperidine in dimethylformamide. Finally, norleucine was introduced as Fmoc-DL-Nle-OH and the Ata group was added as described above.

The fully protected Ata-P_{14} was cleaved from the resin during a 2-hr reaction with 55% trifluoroacetic acid in dichloromethane; all side-chain protecting groups (except Pms) are cleaved in this mixture simultaneously. After evaporation under reduced pressure, the residue was treated with trifluoroacetic acid methanesulfonic acid/thioanisole (10:1:1, vol/vol) for 2 hr at ambient temperature to remove the Pms group.

The crude Ata-P_{14} was purified by countercurrent distribution using the extraction system 1-butanol/acetic acid/water (5:1:4) over 200 transfers. A chromatographically pure fraction was obtained. Except for serine, this fraction had the expected amino acid composition (mol/mol; Nle defined as 1; theoretical composition in parentheses): Arg, 2.15 (2.00); Asp, 2.14 (2.00); Glu, 1.03 (1.00); Ile, 3.04 (3.00); Leu, 0.98 (1.00); Lys, 2.14 (2.00); Nle, 1.00 (1.00); Ser, 0.56 (1.00); Val, 1.01 (1.00). The low amount of serine was due to deterioration in the very strongly acidic conditions during hydrolysis before amino acid analysis.

Coupling of the Tetradecapeptide to BSA. 6-(1-Maleimido)hexanoic acid 1-succinimide ester (4.5 mg) was added (11) to 50 mg of BSA (Carl Roth, Karlsruhe, F.R.G.; 99% pure) dissolved in 1 ml of 0.15 M phosphate buffer (pH 8.0). After 5 min the reaction mixture was subjected to gel filtration on a Sephadex G-25 column (Pharmacia), run in 0.15 M phosphate buffer (pH 6.0).

The peptide derivative was activated by cleaving the acetyl group from the acetylthioacetyl moiety by dissolution in 4 M NaOH/methanol/dioxane (1:5:14) for exactly 15 sec and subsequent neutralization with acetic acid. The solution containing the neutralized thioacetyl-tetradecapeptide (Tha-P_{14}) was added to the functionalized BSA and allowed to react for 2 hr at ambient temperature (12). The reaction mixture was dialyzed against distilled water for 24 hr and lyophilized. The amount of incorporated norleucine, as determined by amino acid analysis, showed that on average four to five Tha-P_{14} molecules were linked per BSA molecule.

Antigens. Human fibrinogen was purified as described (13). Fibrin monomers were prepared according to Haverkate and Timan (14) and stored as a 13.4 mg/ml solution in 20 mM acetic acid at −20°C. Prior to use they were diluted to 0.45 mg/ml with 20 mM acetic acid (fibrin monomer solution, see below). Fibrinogen Aα chain was obtained by reduction and carboxymethylation of fibrinogen and purified according to Doolittle *et al.* (15). Cyanogen bromide digestion of fibrinogen was performed according to Blombäck *et al.* (16). CNBr fragment FCB-2 [comprising the sequence Aα-(148–207)] was isolated from the digest as described before (17), by a method adapted partly from the procedure of Olexa and Budzynski (18).

Soluble fibrin was generated in plasma by addition of thrombin (Leo, Ballerup, Denmark; 10 μl, 2 NIH units/ml) to 0.1-ml portions of normal pooled citrated plasma. At timed intervals (between 0 and 75 sec) 40-μl aliquots were taken in which the thrombin reaction was quenched by 20-fold dilution in 0.10 M NaCl/0.05 M phosphate, pH 7.4, containing 0.05% Tween 20 and hirudin (0.2 unit/ml). In some experiments *Agkistrodon rhodostoma* venom protease (Defibrase, Pentapharm, Basel, Switzerland) was used instead of thrombin under the same conditions. In these experiments, however, the reaction was quenched by the addition of diisopropyl fluorophosphate to a final concentration of 1 mM.

Immunization. Female BALB/c mice were injected intraperitoneally with 125 μg of immunogen (corresponding to $\approx$10 μg of BSA-linked peptide) in Freund's complete adjuvant (Difco) and then twice at 2-week intervals with 125 μg of immunogen in Freund's incomplete adjuvant. An intraperitoneal injection of 250 μg of immunogen in 0.15 M NaCl was given 3 days before fusion.

Fusion. The immunized mice were killed in ether vapor and spleen cells were harvested. Spleen cells (7.8×10^7) were fused with 1.4×10^7 nonproducing myeloma cells (Sp2/0-Ag14) in the presence of 40% (wt/vol) poly(ethylene glycol) 4000 (Baker) essentially as described by Köhler and Milstein (19). The cell suspension was diluted and divided over 96-well microtitration plates (Costar). Media of growing cells were screened for antibody production as described below. Cell lines producing reactive antibodies were subcloned twice by limiting dilution (0.5 cell per well) as described by Oi and Herzenberg (20).

Hybridoma Selection Procedure. Hybridomas were selected by using an enzyme-linked immunosorbent assay (ELISA). The ELISA was performed essentially as described (21, 22) with purified Aα chains or fragment FCB-2 as immobilized antigen. Immunoreactive mouse immunoglobulin was visualized with horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin (Nordic, Tilburg, The Netherlands), using 3,3′,5,5′-tetramethylbenzidine (Aldrich), and H_2O_2 as the substrate mixture (23).

Subclass Assessment and Purification of the mAb. Subclass assessment was carried out by immunoelectrophoresis (24). Antisera against mouse immunoglobulin α, μ, δ, ε, γ, κ, and λ chains were gifts from J. Radl (Institute for Experimental Gerontology, Nederlandse Organisatiè voor Toegepast Natuurwetenschappelÿk Onderzoek, Rijswijk, The Netherlands). Since the mAb appeared to precipitate at low ionic strength, purification was carried out by simply dialyzing the ascites fluids against 5 mM sodium phosphate (pH 6.0).

RESULTS

Preparation of the mAb. Two weeks after fusion and selection, cell growth was observed in 260 of the 380 seeded wells. The media of 4 wells reacted with Aα chain in the ELISA system. The cells from 2 of the positive wells were cloned and recloned. After cloning and recloning, four clones were chosen for further study. One is described in this paper. This line has been in culture for 2 months and has been injected into the peritoneal cavity of BALB/c mice for *in vivo* production of ascites fluid. The clone appeared to be stable *in vivo* (>2 months, three passages).

We will refer to the mAb produced by this clone as anti-Fb-1/2. Subclass assessment showed that anti-Fb-1/2 is of the IgM(κ) subclass. Therefore it could not be purified by protein A-Sepharose chromatography. It precipitates, however, during dialysis against a low ionic strength buffer (5 mM sodium phosphate, pH 6). It can easily be redissolved by addition of a high ionic strength buffer (1.7 M NaCl/0.05 M sodium phosphate, pH 6) and remains in solution upon dilution to physiological ionic strengths.

Specificity of the mAb. Purified anti-Fb-1/2 showed the following characteristics in the ELISA system. It did not react with BSA, which was used as the carrier protein for immunization, or with fragment E and reacted only weakly with pure fibrinogen. It reacted strongly, however, with fibrinogen Aα chain and FCB-2. Apparently, Aα-(148–160) in FCB-2 and in Aα chain, but not in fibrinogen, can interact with the mAb. Anti-Fb-1/2 is able to interact with fibrin (in the μg/ml range) in a plasma milieu—i.e., in the presence of excess fibrinogen—as shown in Figs. 1 and 2. (The fibrinogen concentration of the plasma was 2.4 mg/ml.) Fig. 1 shows results of an experiment in which microtitration plates coated with anti-Fb-1/2 were used. The coated wells were incubated with serial dilutions of a pooled normal plasma that was spiked with fibrin monomer solution. Captured antigen was tagged with horseradish peroxidase-conjugated rabbit poly-

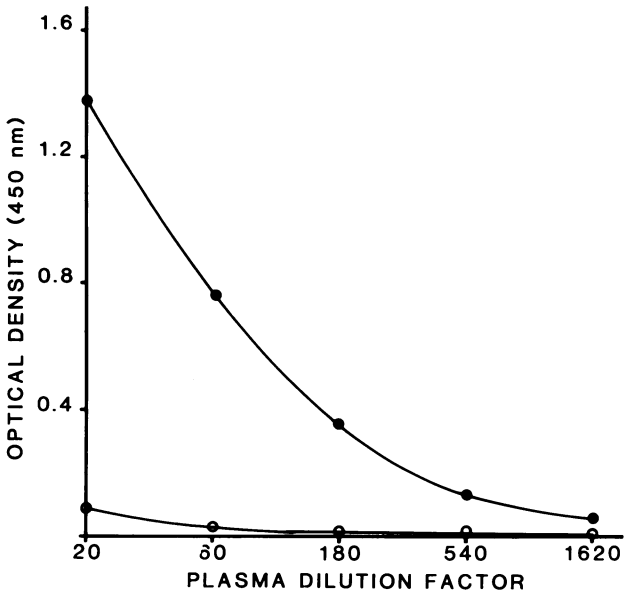

FIG. 1. Dose–response curves of 20-fold diluted normal plasma with a fibrinogen concentration of 2.4 mg/ml (○) and of the same 20-fold diluted normal plasma but with fibrin monomer added to a final concentration of 15 μg/ml (●).

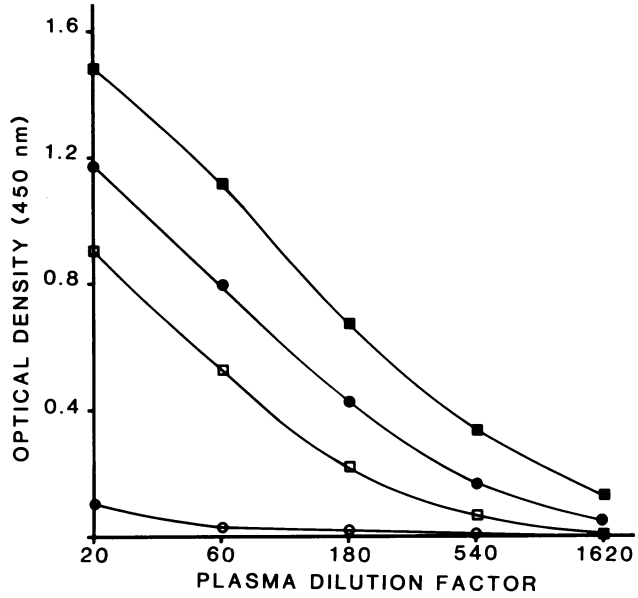

FIG. 2. Dose–response curves of normal plasma with a fibrinogen concentration of 2.4 mg/ml (○) and of the same normal plasma treated with thrombin for 25 sec (□), 50 sec (●), or 75 sec (■).

clonal antibodies to fibrin(ogen)-derived material (21, 22) and visualized by incubation with tetramethylbenzidine/H_2O_2 substrate. Fibrin concentrations down to 1 μg/ml were readily detectable.

Virtually identical dose–response curves were obtained when the fibrin monomer concentration was varied and the plasma dilution was kept constant (data not shown). Fig. 2 shows a similar experiment. Soluble fibrin was generated in plasma by treating the plasma for various periods of time with thrombin. A progressive increase in response with time of thrombin treatment is seen. Treatment with *A. rhodostoma* venom protease, which releases only fibrinopeptide A, yielded comparable results. Normal plasma gave virtually no response.

DISCUSSION

Other investigators have shown that the new amino-terminal ends resulting from cleavage of the fibrinopeptides A and B from the amino-terminal ends of the fibrinogen Aα and Bβ chains, respectively, constitute fibrin-specific epitopes (3–5). Several fibrin-specific antibodies of this type are currently available (for review see ref. 25).

Over the last years our laboratory has studied in detail the accelerating effect exerted by fibrin on the t-PA-catalyzed plasminogen activation. Not only fibrin but also nonphysiological fragments of fibrinogen (but *not* intact fibrinogen) such as the plasmin fragment D_{EGTA} (26) and the CNBr fragment FCB-2 (17) accelerate plasminogen activation by t-PA (27, 28). The activity of FCB-2 and D_{EGTA} resides in their Aα-chain remnants. Since the Aα-chain remnants of D_{EGTA} and FCB-2 are composed of the sequences Aα-(111–197) and Aα-(148–207), respectively, it was concluded that essential information must reside within Aα-(148–197) (6). In a more recent study (7), the essential structure was delineated even further, to Aα-(148–160), and it was shown that Lys-157 of the Aα chain plays a crucial role.

On these results we based our working hypothesis, that the sequence Aα-(148–160) is buried in fibrinogen and is exposed upon transformation of fibrinogen to fibrin or by digestion of fibrinogen with plasmin or with CNBr. This concept is supported by our present findings that a mAb directed against Aα-(148–160) reacts with fibrin and not with fibrinogen. The weak reaction that is observed with purified fibrinogen and with 20-fold diluted plasma (Figs. 1 and 2) is probably caused by trace contamination with soluble fibrin. Normal plasma levels of soluble fibrin are between 0.5 and 13.5 μg/ml (29). It is very unlikely, therefore, that fibrinogen preparations can be obtained with lower fibrin concentrations.

In summary, we have shown that Aα-(148–160) is a fibrin-specific epitope.

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