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A Monoclonal Antibody, Specific for Human Fibrinogen, Fibrinopeptide A-Containing Fragments and Not Reacting With Free Fibrinopeptide A

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Spleen cells of BALB/c mice, immunized with fragments Y of normal human fibrinogen, were fused with P3 × 63 Ag 8653 myeloma cells. A clone was found which produces monoclonal antibodies (Mab-Y18) of the IgMκ type. Mab-Y18 is immunoreactive with normal human fibrinogen, and its fragments X, Y, *N*-terminal disulphide knot, Aα-chain, and Aα stretch 1-51. The immunoreactivity with these same fragments disappears upon treatment with thrombin or arvin. This strongly suggests that fibrinopeptide A is an essential component of the Mab-Y18 epitope. This is supported by the finding that Mab-Y18 prolongs the thrombin and arvin clotting times of human fibrinogen by inhibition of the fibrinopeptide A release. More detailed information about the nature of the Mab-Y18 epitope was obtained from studies with genetic variants of human fibrinogen (especially fibrinogen Metz) and with fibrinogens from other mammalian species. These studies show that amino acid residue Aα16 (arginine) of fibrinopeptide A is essential for the Mab-Y18 epitope. Mab-Y18 does not react with free fibrinopeptide A.

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ONE OF THE crucial steps of blood clot formation is the conversion of fibrinogen to fibrin by the thrombin-catalyzed release of fibrinopeptide A and B from the amino-terminal ends of the two fibrinogen Aα and Bβ chains. Fibrin is the insoluble protein matrix of a clot. It is a transient structure and, after it has fulfilled its role in hemostasis and in tissue repair, it is degraded to soluble degradation products by the fibrinolytic system. Increased blood levels of fibrin degradation products (FDPs) may be indicative of an activated fibrinolysis. However, even in situations of substantially activated fibrinolysis, FDP levels remain generally relatively low, ie, in the range of micrograms per milliliter, and as a consequence they have to be assessed by immunologic means.

Problems encountered with conventional antisera (eg, cross-reactivity with fibrinogen) could be overcome with monoclonal antibodies. It is known that FDPs have specific epitopes (neoantigenic determinants), not expressed by fibrinogen and fibrin.[1,2] It is conceivable that such epitopes are not only expressed by degradation products of fibrin but also by those of fibrinogen, and we tried to develop monoclonal antibodies, among others, against one single purified plasmin-generated fibrinogen degradation product, fragment Y. This fragment was chosen since it has (in contrast with fibrinogen) an accelerating effect on the plasminogen activation, catalyzed by tissue-type plasminogen activator. We presented evidence that the effect is due to the exposure of amino acids Aα 148-197 upon degradation of fibrinogen to fragment Y.[3] This stretch Aα 148-197 may represent a neoantigenic determinant.

During this work, we observed an interesting clone, which produces an antibody specifically reactive with normal human fibrinogen, fibrinopeptide A-containing fragments, but not with free fibrinopeptide A. This article describes the development and characterization of this monoclonal antibody, which has great potential to set up an assay for fibrinogen degradation products in plasma, without interference by free fibrinopeptide A, and in the discrimination between fibrin and fibrinogen deposits in tissues.

MATERIALS AND METHODS

Fibrinogens. Fibrinogens of humans (normal and genetic variants), rat, cat, pig, and cow were purified as described.[4]

Fibrinogen Milan II is an abnormal fibrinogen with decreased thrombin-binding and a slow thrombin-catalyzed fibrinopeptide A release.[5] Fibrinogen Metz (a gift of Drs J. and C. Soria, Hôtel Dieu, Paris) is a homozygous fibrinogen variant in which arginine at position Aα 16 (the thrombin/arvin cleavage site) is replaced by cystein.[6] From fibrinogen Amsterdam (a gift of Drs H.L. Haak and F. Haverkate, Gaubius Institute TNO, Leiden, The Netherlands) only one of the two fibrinopeptides A can be released.[6]

Fibrinogen Kassel (a gift from Dr H. Janzarik, Klinikum der Justus-Liebig Universität, Giessen, FRG) has a prolonged thrombin time, but the molecular defect is not known at present.

Human fibrinogen Aα, Bβ, and γ chains. Human fibrinogen Aα, Bβ, and γ chains were separated by reduction and carboxymethylation of fibrinogen and purified as described by Doolittle and co-workers.[7]

Plasmin-generated fibrin(ogen) degradation products. Fragment X was prepared and purified as described[8]; Y was prepared as described by Nieuwenhuizen et al,[9] and D_{cate}, D_{EGTA}, and E were prepared as described by Van Ruijven et al.[10]

Cyanogen bromide (CNBr) fragments of fibrin(ogen). The amino-terminal disulphide knots (NDSK) of fibrinogen and fibrin were prepared and purified as described by Blombäck et al[11] and Olexa et al,[12] respectively.

Fragment FCB-2[13,14] (also known as HO1-DSK[15]), its Aα chain remnant (Aα 148-207), and fragment FCB-3[14] were purified as described before.[13]

Fragment Aα 1-51 was prepared either by reduction and carboxymethylation of the NDSK of fibrinogen (NDSK-fbg) or by CNBr treatment of purified Aα chain.

Purification (partial) was achieved in both cases by gel filtration on Sephadex G50 in 50% acetic acid. Aα 1-51–containing fractions were identified by end-group analysis[16] (alanine and some aspartic acid) and mol wt determinations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Table 1 summarizes the chain (remnant) composition of the fibrin(ogen)-derived fragments used in this study.

Immunization scheme. Female BALB/c mice (Centraal Proefdierenbedrijf TNO, Zeist, the Netherlands) were injected intraperitoneally with 25 μg of purified fibrinogen fragment Y in Freund complete adjuvant (Difco, Amsterdam) and then at three-week intervals with 25 μg of fragment Y in incomplete Freund adjuvant. An intravenous (IV) injection of 25 μg of fragment Y in phosphate-

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Table 1. Summary of the Chain (Remnant) Composition of Fibrin(ogen)-derived Fragments

Product	Amino Acid Stretches Derived From			
	Aα Chain	Bβ Chain	γ Chain	References
Fibrinogen	$(1-610)_2$	$(1-461)_2$	$(1-410)_2$	14
Fibrin monomer	$(17-610)_2$	$(15-461)_2$	$(1-410)_2$	14
X	$(1-208)_2$	$(1-461)_2$	$(1-410)_2$	17, 18
Y	1-208/1-78	1-461/54-122	1-410/1-58	17, 18
D_{cate}	111-197	134-461	86-410	19
D_{EGTA}	111-197	134-461	86-303	19
E	$(1-78)_2$	$(54-122)_2$	$(1-58)_2$	20
NDSK (fbg)	$(1-51)_2$	$(1-118)_2$	$(1-78)_2$	11
NDSK (fb)	$(17-51)_2$	$(15-118)_2$	$(1-78)_2$	11
FCB-2	148-207	191-224 225-242 243-305	95-265	13-15
FCB-3	241-476	—	—	14
Fibrinopeptide A	1-16	—	—	21
Fibrinopeptide B	—	1-14	—	21

Values indicate the numbers of amino acids.

buffered saline (PBS) was given three days before fusion (see below).

Fusion. The immunized mice were killed in ether vapor, and spleen cells were harvested. Spleen cells (8×10^7) were fused with 2×10^7 nonproducing myeloma P3 × 63 Ag 8653 cells in the presence of 40% polyethyleneglycol 4000 (Baker, Deventer, The Netherlands), essentially as described by Köhler and Milstein.[22]

The cell suspension was diluted and divided over 96-well microtiter plates (Costar, Cambridge, Mass). 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 cells per well) as described by McKearn.[23]

Screening procedure enzyme-linked immunosorbent assay (ELISA). Screening was performed by a procedure analogous to that originally described by Engvall and Perlman[24] on plates, coated with fibrinogen, fibrin monomers or fragment Y, and using a rabbit anti-mouse immunoglobulin coupled with urease (Sanbio, Mistelrode, The Netherlands) for visualization. In some cases such as the cross-reactivity assay (see below) a horse radish peroxidase conjugate was used in combination with 5-aminosalicylic acid (0.8 mg/mL) and hydrogen peroxide (0.005%).

Cross-reactivity assay according to Soria et al.[25] In this assay, a dilution of a solution of monoclonal antibody is chosen, giving 80% of the maximum response in the assay described under the heading "Screening Procedure" on fragment Y-coated plates. The antibody solution at this dilution is preincubated (16 hours at 4 °C) with different concentrations of fibrin(ogen) fragments (0 to 200 μg/mL) to be tested for cross-reactivity with fragment Y and then applied to a fragment Y-coated plate. A concentration-dependent decrease of response from 80% downward is indicative for cross-reactivity.

Purification of the monoclonal antibody and subclass assessment. After dialysis for 16 hours at 4 °C against 0.015 mol/L of Tris-HCl, pH 7.0, the antibody-containing solutions were applied to a DE-52 cellulose column (Whatman, Maidstone, England) equilibrated with the same buffer. Elution of immunoreactive material was performed with 0.15 mol/L of Tris-HCl, pH 7.0. This material was dialyzed against 0.1 mol/L of ammonium bicarbonate, freeze-dried, dissolved in PBS/Tween, and applied to a Sephacryl S-200 column (Pharmacia, Woerden, The Netherlands) in 0.1 mol/L of $NaHCO_3$, pH 7.5. Material eluting near the void volume of this column was immunoreactive. Purity was checked by immunoelectrophoresis as described by Radl.[26] Antisera against mouse α, μ, δ, ε, γ, κ, and λ chains were gifts from Dr Radl (Institute for Experimental Gerontology TNO, Rijswijk, the Netherlands).

Influence on thrombin and arvin clotting times. The assay for influence on thrombin and arvin clotting times was performed, essentially as described by Haverkate and co-workers.[27] Increasing amounts of monoclonal antibody (pretreated with 10^{-4} mol/L of diisopropylphosphofluoridate [DFP]) were added to a constant amount of fibrinogen (0.1 mL, 2 mg/mL in PBS pH 7.4) and incubated for 2½ hours in the presence of 4 KIU of aprotinin. A monoclonal antibody raised against tissue-type plasminogen activator and not reactive with fibrinogen was used as a control. In another control experiment, human fibrinogen was replaced by rat fibrinogen.

Fibrinopeptide release. The thrombin-catalyzed fibrinopeptide release with time before and after treatment with DFP-treated monoclonal antibody was performed according to Kehl et al[28] with an LKB 2152 HPLC apparatus (Bromma, Sweden) equipped with a CP-Spher C18 reversed phase column (Chrompack, Middelburg, The Netherlands).

RESULTS AND DISCUSSION

All of the 172 wells initially filled with the fusion mixture showed cell growth upon visual inspection after three weeks. The media of the cells in only two wells were immunoreactive in ELISA with fibrinogen and fragment Y, and not with fibrin monomers. The cells in these two wells were cloned and gave nine stable clones. When one of these clones was recloned by limiting dilution (0.5 cells per well), 30% of the wells showed cell growth. The media in all wells with hybridoma growth immunoreacted with fibrinogen and with fragment Y, but not with fibrin monomers. This clone is, until now, stable in culture for over 18 months. It has also been injected into the peritoneal cavity of pristane-primed BALB/c mice for the production of ascites, where it has been productive for more than nine months (15 passages).

The monoclonal antibody described in this article will be designated as Mab-Y18. Both the results of the subclass assessment and the elution position of Sephacryl S-200 during purification (see Materials and Methods) showed that Mab-Y18 is of the IgM, κ type.

Table 2 shows the immunoreactivity of Mab-Y18 with fibrinogen and 15 fibrinogen-derived proteins and peptides, as observed in the ELISA system.

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Table 2. Immunoreactivity of Mab-Y18 With Fibrinogen and Fibrin(ogen)-derived Fragments

Antigen	Response in ELISA	Antigen	Response in ELISA
FCB-2	−	fpA	−
FCB-3	−	fpB	−
Aα 148-207	−	X	+ + +
NDSK (fbg)	+ + +	Y	+ + +
NDSK (fb)	−	D_{cate}, D_{EGTA}	−
Aα chain	+ + +	E_3 (fb)	−
Bβ chain	+	Fibrinogen	+ + +
γ chain	−	Fibrin monomers	−

The blank response is generally ~0.15 in this assay; ELISA, enzyme-linked immunosorbent assay.

−, No reaction; +, less than three times the blank response (OD); + + +, more than ten times the blank response (OD).

Only fibrinogen and its fibrinopeptide-containing fragments react with Mab-Y18. It is remarkable that both the fibrinogen Aα- and Bβ-chain give a positive result in the ELISA. This would indicate that these chains have similar or even identical epitopes, which are recognized by Mab-Y18. However, as will be demonstrated below, the explanation is more trivial, ie, our Bβ-chain preparation is contaminated with Aα-chain–related material. Free fibrinopeptides A (fpA) and B (fpB) are not reactive.

The affinity of Mab-Y18 for fragment Y is difficult to assess, since fragment Y is a plasmin-generated product, the structure of which varies from preparation to preparation. Fibrinogen and its Aα-chain are much better defined; therefore, we have estimated the affinity of Mab-Y18 for these two proteins from cross-reactivity assays. K_d for intact fibrinogen and its Aα-chain are similar, ie, 6×10^{-9} mol/L and 2×10^{-9} mol/L, respectively.

It is our experience and that of Soria et al[25] that some antigens tend to react with a monoclonal antibody only when they are adsorbed to a plastic wall and not in solution. In order to exclude this possibility for Mab-Y18, we performed cross-reactivity assays (CRAs) as described by Soria et al,[25] to assess the competition between fragment Y, adsorbed to plastic walls, and the antigens summarized in Table 3, in solution.

In this assay, we tested antigens for their cross-reactivities both before and after treatment with thrombin or arvin. Thrombin cleaves off fibrinopeptides A and B, whereas arvin is capable of releasing only fibrinopeptides A.

The results of the CRAs are summarized in Table 3. These results confirm and extend those of Table 2.

Fibrinogen and fibrinopeptide A-containing fibrinogen fragments in solution compete with adsorbed fragment Y. Furthermore, these fragments lose their reactivity with Mab-Y18 upon treatment with thrombin or arvin, showing that fpA is an essential component of the epitope, recognized by Mab-Y18. The Bβ-chain preparation shows cross-reactivity, although less than the Aα-chain. However, this cross-reactivity of the Bβ-chain is lost upon treatment with either thrombin or arvin. As arvin can cleave only off fpA, these results show clearly that the Bβ-chain preparation is contaminated with Aα-chain. This was confirmed with high-performance liquid chromatography (HPLC) experiments, in which we could show that fpA was released from this Bβ-chain preparation by thrombin or arvin. However, the Bβ-chain preparation showed only one band on SDS-PAGE; therefore, it is conceivable that it contains partly degraded Aα-chains with the same mol wt as intact Bβ-chains.

Free fpA is not reactive with Mab-Y18 either in the ELISA or in the CRA. This could indicate that fpA does not comprise the full epitope, but is rather an essential part of it. As judged from the strong cross-reactivity of Aα 1-51, the rest of the epitope would be localized in that case between residues Aα 17 and 51. An alternative possibility is that fpA contains the full Mab-Y18 epitope but that the extra negative charge on Aα 16 in *free* fpA has an adverse effect on the complex formation between Mab-Y18 and fpA.

More information about the epitope can be obtained by studying the cross-reactivities of fibrinogens of other species and those of genetically abnormal human fibrinogens. The results of such experiments are summarized in Tables 4 and 5.

The results in Table 4 show the specificity of Mab-Y18 for human fibrinogen. None of the other fibrinogen species tested exhibits any cross-reactivity with human fragment Y. Especially the carboxy-terminal halves of the fpAs are homologous, and in the case of pig fpA Aα 9-16 are even identical with human Aα 9-16. This suggests that a part of the Mab-Y18 epitope must be located between Aα 1 and 8. However, the results in Table 5 emphasize again a possible involvement of Aα 16 as well, since fibrinogen Metz (Aα 16

Table 3. Cross-reactivity of Different Derivatives of Fibrinogen With Fragment Y Adsorbed to Plastic Plates, Both Before and After Treatment With Thrombin or Arvin

Antigen Tested for Cross-reactivity	Cross-reactivity of the Antigen		
	Nontreated	Thrombin-treated	Arvin-treated
Normal human fibrinogen	+ + +	−	−
Fragment Y	+ + +	−	−
Aα chain	+ + +	−	−
Bβ chain	+ +	−	−
γ chain	−	−	−
Aα 1-51*	+ + +	−	−
fpA	−	−	−
fpB	−	−	−

−, No cross-reaction; + + +, 50% inhibition with $\leq 10^{-8}$ mol; + +, 50% inhibition with $\sim 10^{-7}$ mol/L; +, more than 10^{-6} mol/L required for 50% inhibition.

*Either prepared from NDSK (fbg) or from Aα chain.

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Table 4. Mab-Y18 Cross-reactivities of Fibrinogens From Different Species With Fragment Y

Fibrinogen Species	Cross-reactivity	Sequence fpA*
Normal human fpA	+ + +	ADSG-EGDFLAEGGGVR
Pig	–	AEVQDKGEFLAEGGGVR
Rat	–	ADTGTTSEFIEAGGDIR
Cat	–	GDVQ-EGEFIAEGGGVR
Cow	–	EDGSDPP-SGDFLTEGGGVR

Sequences of the fibrinopeptide A are given for comparison.
*From reference 21.

arginine replaced by cysteine[6]) cross-reacts only slightly. The (normal) fpAs of fibrinogen Milan II are released at a slow rate as a result of a decreased thrombin binding.[5] If this decreased thrombin binding is caused by mutations of amino acids outside fpA, these mutations do not destroy the immunoreactivity between Mab-Y18 and fibrinogen Milan II.

Only one fpA per molecule of fibrinogen Amsterdam (heterozygous) can be released by thrombin or arvin.[6] The defect in fibrinogen Amsterdam is not known in detail, but our results suggest that the defect has no effect on the immunoreactivity with Mab-Y18. This seems to be true also for the 50% abnormal molecules, since cross-reactivity is maintained even after thrombin or arvin treatment, when the normal fibrinogen molecules lose their fpAs.

The defect in fibrinogen Kassel is not known at present, but the results in Table 5 may be explained, assuming that its immunoreactivity with Mab-Y18 is normal and that all fpA can be cleaved off by thrombin and arvin. An alternative explanation at this stage would be that fibrinogen Kassel is heterozygous and that the population of abnormal molecules has no reactivity at all with Mab-Y18.

The results described above all strongly suggest that fpA is an essential constituent of the Mab-Y18 epitope and that Aα 16, ie, the thrombin and arvin cleavage site, plays an important role. It would be anticipated then that Mab-Y18 affects the interactions of thrombin and/or arvin with fibrinogen, the thrombin and arvin clotting times of fibrinogen, and the fpA release.

Figure 1 shows the effect of Mab-Y18 on the thrombin and arvin clotting times of normal human fibrinogen. A strong concentration-dependent effect of Mab-Y18 is seen both for the thrombin and for the arvin clotting times. However, the profiles are different, ie, at low Mab-Y18 concentrations the increase of the arvin clotting times is

Table 5. Mab-Y18 Cross-reactivities of Some Genetically Abnormal Human Fibrinogens With Fragment Y of Normal Human Fibrinogen

	Cross-reactivity of Fibrinogen		
Abnormal Fibrinogen	Nontreated	Thrombin-treated	Arvin-treated
Milan II	+ + +	–	–
Metz	+/–	+/–	+/–
Amsterdam	+ + +	+ +	+
Kassel	+ + +	–	–

–, No cross-reactivity; + + +, 50% inhibition at 10^{-8} mol/L; + +, 50% inhibition at approximately 10^{-7} mol/L; +, 50% inhibition at 10^{-6} mol/L; +/–, inhibition observed; too much fibrinogen needed to reach 50% inhibition in cross-reactivity assay.

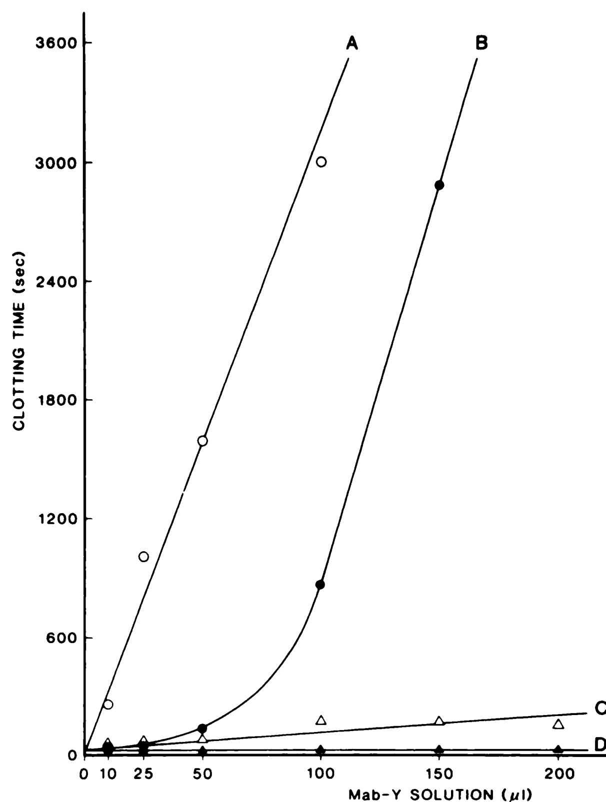

Fig 1. Influence of Mab-Y18 on thrombin and arvin clotting times: curve A: dependence of arvin clotting time (human fibrinogen) upon Mab-Y18 concentration; curve B: dependence of same for thrombin; curve C: thrombin time of rat fibrinogen; and curve D: thrombin time of normal human fibrinogen in the presence of an anti-tissue-type plasminogen activator monoclonal antibody.

much more pronounced than that of the thrombin clotting times. This may be due to the fact that thrombin can cleave off both fibrinopeptides A and B. Mab-Y18 inhibits fpA release but leaves the fpB release intact, thus favoring formation of des BB-fibrin, which forms weak clots.[29] A monoclonal antibody directed against tissue-type plasminogen activator, used as a control, has no effect on the clotting times (Fig 1). As expected from the lack of immunoreactivity of Mab-Y18 with rat fibrinogen (Table 4), Mab-Y18 had no effect on the thrombin and arvin (latter not shown) clotting times of rat plasma (Fig 1, curve C).

Fibrinopeptide A release by thrombin from normal human fibrinogen after incubation with Mab-Y18, as measured by HPLC, was decreased, whereas fpB release was normal. After 90 minutes' incubation with thrombin or arvin only about 1 mol of fpA per mol of Mab-Y18 treated fibrinogen had been released, whereas the release of fpA from the same fibrinogen after preincubation with anti-tissue-type plasminogen activator was 2 mol of fpA per mol of fibrinogen. The rates of release of the different forms of fibrinopeptides of normal human fibrinogen, ie, of fibrinopeptides A, AP (phosphoserine at Aα 3) and AY (lacking Aα 1) were affected to the same extent, indicating that alanine at Aα 1 and serine at Aα 3 are not essential constituents of the Mab-Y18 epitope.

Mab-Y18 can be a valuable tool in the elucidaton of the

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defects in genetically abnormal fibrinogens. In combination with a (catching) antibody, which we recently produced (manuscript in preparation) and which is reactive with degradation products both of fibrinogen and fibrin and not with intact fibrinogen, Mab-Y18 has enabled us to develop an assay to discriminate and quantitate, in plasma, degradation products derived from fibrinogen (primary fibrinolysis) and those derived from fibrin in plasma, without interference of free fibrinopeptide A. Furthermore, immunohistochemical discrimination between fibrinogen and fibrin deposits in tissue samples is possible. Such studies are currently in progress.

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