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Radioimmunoassay of Human Fibrinopeptide A

(fibrinogen/blood coagulation/peptide synthesis/pmoles detected/dysfibrinogenemia)

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ABSTRACT **A radioimmunoassay capable of measuring 1 pmole of human fibrinopeptide A has been developed, and should prove useful to detect the release of this peptide from fibrinogen during the coagulation process. Antibodies to fibrinopeptide A were produced by injecting New Zealand white rabbits with a mixture of Freund's adjuvant and native fibrinopeptide coupled to human albumin. *N*-Tyrosyl fibrinopeptide A was synthesized by the solid-phase method, and was iodinated with ^{125}I by the Chloramine-T method. 48–73% of the radiolabeled peptide could be bound by the serum of a rabbit immunized with the fibrinopeptide–albumin preparation. Antibody-bound peptide was precipitated by dioxane and was thus separated from unbound peptide. The addition of excess native fibrinopeptide to the radiolabeled material prevented its binding to serum. Native fibrinopeptide A and synthetic fibrinopeptide A were identical in their ability to prevent binding, whereas fibrinogen was from 1/25,000 to 1/50,000 as effective on a weight basis. Plasma filtered through a membrane relatively impermeable to molecules larger than a molecular weight of 34,000 showed no fibrinopeptide reactivity, whereas a similar filtrate of serum gave quantitative recovery of fibrinopeptide reactivity.**

Visible coagulation of blood is due to polymerization of monomeric fibrin after removal of fibrinopeptide A from fibrinogen (1). The fibrinogen molecule is thought to consist of a dimer of three pairs of peptide chains, designated alpha, beta, and gamma (2). Thrombin removes the fibrinopeptides from the *N*-terminal ends of fibrinogen by cleavage of four arginylglycine bonds to release two A peptides from the alpha chains and two B peptides from the beta chains of a single molecule (2–9). A sensitive method of specifically measuring these peptides may prove useful in studying coagulation *in vivo* and thrombin action *in vitro*. We report here the development of a radioimmunoassay capable of measuring 1 ng/ml of fibrinopeptide A. A feature of the assay is the use of synthetic *N*-tyrosyl fibrinopeptide A, prepared by the solid phase technique of Merrifield (10), as the radioactive antigen.

MATERIALS AND METHODS

Native fibrinopeptide A was isolated by the method of Blomback and coworkers (9), except that Cohn Fraction I, rather than plasma served as starting material. Synthetic *N*-tyrosyl fibrinopeptide A and fibrinopeptide A with tyrosine substituted for valine-15 were synthesized by the solid-phase method (G. Wilner, in preparation). Synthetic fibrinopeptide A was obtained from Schwarz Biochemicals, Orangeburg, New York. Amino acid analyses of these preparations are given in Table 1. A crude preparation containing fibrinopeptide A was used for immunization and was prepared as follows: 2g of Cohn fraction I from human plasma (Nutritional Biochemicals Corporation, Cleveland, Ohio) was dissolved in 200 ml of 0.3 M ammonium acetate buffer (pH 6). The pH was adjusted to 6.4 with NH_4OH and the solution was diluted to 400 ml with distilled water. 200 units of human thrombin (fibrindex, Ortho Pharmaceuticals, Raritan, N. J.) was added to the Cohn fraction I solution, which was stirred until clotting occurred. After 6 hr at 25°C, the clot was broken up with a glass rod and centrifuged for 10 min at 6000 × *g* at 4°C. The supernatant solution was lyophilized, dissolved in 10 ml of 0.1 M NH_4HCO_3, and fractionated by gel filtration on Sephadex G-25 (Fig. 1). Tubes comprising each protein peak were pooled and subjected to amino acid analysis. The pooled contents of tubes 41–45 (amino acid analysis in Table 1) was lyophilized and used for immunization as follows: 15 mg was dissolved in 1 ml of distilled water and added to 30 mg of human albumin (3 times crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio). 300 mg of carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl, Ott Chemical Co., Muskegon, Mich.] was dissolved in 1 ml of distilled water and immediately added to the peptide–albumin mixture (11). After it was stirred for 1-hr at room temperature, the solution was dialyzed against distilled water for 24 hr and lyophilized. 32 mg of material was obtained, and 9.5 mg was dissolved in 4.75 ml of 0.15 M saline; 4.75 ml of complete Freund's adjuvant mixture was added. The solution was

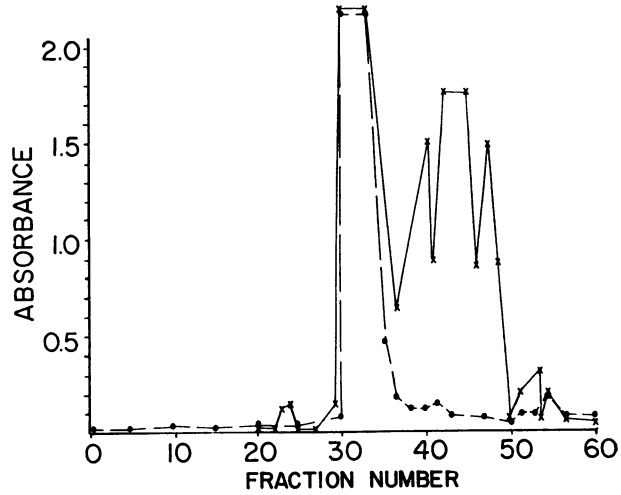

FIG. 1. Sephadex G-25 filtration of the supernatant of Cohn fraction I clotted with thrombin. Column size was 150 × 2.5 cm, the buffer used was 0.1 M NH_4HCO_3, and 8-ml fractions were collected at 25°C. ●—● 280 nm absorbance; ×---× 570 nm absorbance (ninhydrin).

TABLE 1. *Amino acid analyses of fibrinopeptide preparations*

	A peptide (9)	B peptide (9)	Native peptide	Synthetic peptides			Peptide fraction used for immunization
				N-tyrosyl	Tyrosine substituted for valine-15	Schwarz	
Asp	2	1 (2 Asn)	2.1	1.8	2.3	2	2.7
Thr	—	—	—	—	—	—	0.6
Ser	1	1	0.9	0.6	0.8	0.9	1.6
Glu	2	3	2	1.6	1.7	2.1	3
Gly	5	2	5	5	5	5	5
Ala	2	1	1.9	1.7	2.2	2.3	2.6
Val	1	1	0.9	1	—*	0.85	2
Leu	1	—	0.9	1	1.2	1	1.2
Phe	1	2	1	1	1.1	1	1.4
Tyr	—	—	—	0.3	0.6	—	—
Lys	—	—	—	—	—	—	0.8
His	—	—	—	—	—	—	0.2
Arg	1	1	1	1	0.8	1	1.4

* Means only trace amounts detected.

thoroughly mixed with a syringe till it had thickened. After we obtained preimmunization sera, white New Zealand rabbits were injected with the mixture on the following schedule: 0.6 ml into toe pads at 1-week intervals for a total of 3 injections, then 0.4 ml intramuscularly in the haunches at weekly intervals for 10 injections, thereafter at 2- or 3-week intervals. Significant binding of radiolabeled tracer occurred after 3 months. Control sera were obtained from rabbits immunized in a similar manner with bovine-serum albumin and human-serum albumin, respectively.

Radiolabeling of Fibrinopeptide A. Fibrinopeptide A lacks tyrosine; in order to label it with radioactive iodine, tyrosine was covalently coupled to it by two different methods. Desaminotyrosine was coupled to native or synthetic peptide (obtained from Schwarz Biochemicals) as follows: 4 mg of peptide A was mixed with 25 mg of 3-(*p*-hydroxyphenyl) propionic acid-*p*-nitrophenyl ester (Cyclo Chemical Corp., Los Angeles, Calif.) and 3 ml of dimethylformamide (Mallinckrodt) was added (12). The mixture was stirred constantly for 18 hr at 37°C, then 2 ml of distilled water and 0.2 ml of 10% aqueous acetic acid were added. The mixture was extracted twice with 7 ml of chloroform–ethyl ether 1:1. After centrifugation at 2,500 rpm for 5 min, the aqueous phase was lyophilized. The lyophilized peptide was dissolved in 4 ml of 0.1 M NH_4HCO_3, filtered over a 20 × 1.2 cm Biogel P2 column (Calbiochem, equilibrated with 0.1 M NH_4HCO_3), and eluted with the same buffer. The contents of the fractions showing maximal ninhydrin reactivity and maximal absorbance at 280 nm were pooled and used for radiolabeling. From the 280 nm and 570 nm (ninhydrin) absorbance, we estimated the coupling of tyrosine at 0.9–1 molecule of desaminotyrosine per molecule of peptide, for both the native- and synthetic-peptide preparations. Tyrosine was coupled to the peptide by the synthetic process at the N-terminal alanine and in place of valine-15 (Table 1).

Each of the four preparations was labeled (13) as follows: 30 μl of 0.3 M phosphate buffer (pH 7.4) and 20 μl of carrier-free ^{125}I (5 mCi) were mixed, and 15 μl of tyrosylated peptide A (5 μg) was added. 25 μl of Chloramine T (100 μg) and, after 30 sec, 50 μl of sodium metabisulfite (120 μg) were added and, after a further 30 sec, 50 μl of ovalbumin (2 mg) was added as a carrier. The mixture was passed over a Sephadex G-10 column (1.2 × 10 cm) equilibrated with 0.05 M Tris–0.1 M NaCl (pH 7.5). 0.5-ml fractions were collected and radioactivity was counted. The early peak of radioactivity was used as the radiolabeled antigen, being diluted so as to give about 12,000 cpm per 50 μl (usually a 1:5,000 dilution). Radioimmunoassay was performed as follows: the assay tubes contained, in 0.5 ml final volume, the following, in order of addition: 250 μl buffer, 50 μl [^{125}I]tyrosyl-fibrinopeptide A, 100 μl of the sample to be assayed, and 100 μl of antiserum diluted 1:1,000 (a dilution that binds about 30% of the total counts). Tubes were incubated for 1 hr at room temperature. Antibody-bound [^{125}I]tyrosyl-fibrinopeptide A was separated from unbound radioactivity by precipitation of the bound peptide by the addition of 2.0 ml of dioxane–water 9:1 (14) and immediate centrifugation (6,000 × *g* for 30 min) The supernatant fluid was decanted and the precipitate was counted with a Packard Auto-Gamma Spectrometer. Tris–saline buffer (0.15 M, pH 8.5) with 1 mg/ml ovalbumin, was used as a diluent for all reagents.

Plasma was derived from blood collected and prepared as described (15). The blood was prevented from coagulation with 0.1 volume of 4% trisodium citrate or 0.1 volume of 0.05% heparin. Serum was prepared by addition of bovine thrombin (Parke Davis Co., Detroit, Mich.), 20 units per ml, to citrated plasma and incubation for 2 hr at 37°C. Plasma and serum were filtered through XM 50 membranes (a membrane that retains molecules of molecular weight 34,000 and above) with apparatus from the Amicon Corp. (Lexington, Mass.). Three fibrinogen preparations were used, all containing at least 90% clottable protein. One preparation made by polyethylene-glycol precipitation of plasma was the gift of Dr. Alan J. Johnson, Department of Medicine, New York University School of Medicine, a second preparation, obtained from Kabi Co., Stockholm, was prepared by alcohol fractionation (16), and the third sample was prepared by alcohol fractionation and DEAE-cellulose ion-exchange chromatography. For all experiments in which fibrinogen or fragments of the fibrinogen molecule might be present, 30 units/

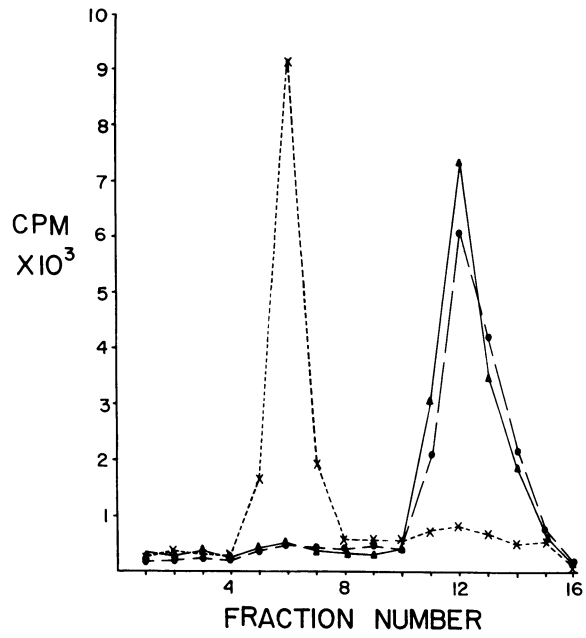

FIG. 2. Gel filtration of labeled peptide. Peptide A was filtered over 22 × 0.8 cm Sephadex G-100 columns with 0.1 M NH_4HCO_3 as eluent. The following mixtures were incubated at room temperatures for 2 hr before passage over the columns:

Tube 1: 100 μl of labeled peptide (4 ng) and 400 μl of 0.05 M veronal buffer, pH 8.6 (▲—▲).

Tube 2: 100 μl of labeled peptide, 350 μl of veronal buffer, and 50 μl of serum (rabbit R2) (×- - -×).

Tube 3: 100 μl of labeled peptide, 25 μl of unlabeled peptide, (400 ng), 325 μl of buffer, and 50 μl of serum (rabbit R2) (●- - -●).

0.5 ml of buffer was added to each tube and the contents were applied to separate columns and eluted with 0.1 M NH_4HCO_3. 1-ml fractions were collected and radioactivity was measured.

ml of the antithrombin, hirudin (K & K Laboratories, Plainview, Long Island, N. Y.), was added to the diluted antiserum and 200 units/mg of fibrinogen to the fibrinogen-containing solution. The hirudin preparation did not affect either binding of the radioactive peptide by antiserum or subsequent displacement by the peptide.

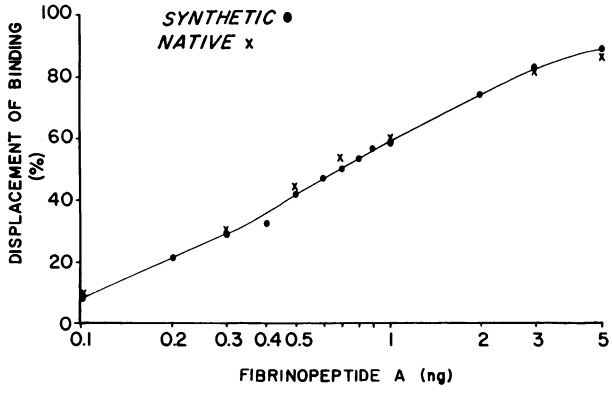

FIG. 3. Inhibition of binding of ^{125}I-labeled tyrosinated-peptide A by native and synthetic fibrinopeptide. 100 μl that contained various concentrations of native fibrinopeptide A (●) or synthetic fibrinopeptide A (×) (*amount shown on abscissa*) were tested.

TABLE 2. *Binding of peptide by various sera*

Rabbit No.	Nature of serum	Dilution	Radioactivity, bound, %
R1	Preimmunization	1/10	3
		1/100	<1
R2	Preimmunization	1/10	<1
		1/100	<1
R3	Preimmunization	1/10	1
		1/100	<1
R1	Immunized with fibrinopeptide-human albumin	1/10	48
		1/100	30
		1/500	11
R2	Immunized with fibrinopeptide-human albumin	1/10	73
		1/100	47
		1/500	39
		1/1000	32
		1/5000	14
R3	Immunized with fibrinopeptide-human albumin	1/10	33
		1/100	20
		1/500	10
R4	Anti-bovine serum albumin	1/10	1
R5	Anti-human serum albumin	1/10	<1
	Anti-fibrinogen (Hyland Laboratories #8108H003B1)	1/10	4
55	Anti-fibrinogen (from C. Merskey)	1/10	8
		1/100	<1

RESULTS

Each of the four radiolabeled tracers was bound specifically by R1, R2, and R3 rabbit sera after immunization with fibrinopeptide–albumin. The synthetic preparation with *N*-tyrosyl-fibrinopeptide A (Table 1) gave the best results, in terms of % binding and sensitivity of the assay, and it was used for all subsequent experiments. Postimmunization sera bound significantly more radioactivity than the preimmunization sera (Table 2). Maximal binding ranged from 48–73% of the radioactivity. R2 serum was the most active and was used in all subsequent experiments. Antisera to human- and bovine-serum albumin, and two antisera to human fibrinogen bound insignificant amounts of radioactivity (Table 2). Binding was demonstrated by two methods: displacement of the Sephadex G-100 elution pattern of the labeled tracer (Fig 2), and precipitation with dioxane. The dioxane method gave reproducible results and, because of its rapidity and convenience, was used as our standard method for separating bound from free radioactive antigen.

Inhibition of binding was also demonstrated by inhibition of precipitation with dioxane (Fig 3). Binding of all four labeled antigens was specifically inhibited by native and synthetic peptides, to exactly the same extent (Fig. 3).

The purified fibrinogen preparations displaced binding significantly (Table 3); a fraction of the displacing activity, presumed to be due to fragments of the fibrinogen molecule containing fibrinopeptide A, could be filtered through the XM 50 membrane (Table 4). On a weight basis, purified fibrinogen in buffer was 1/25,000–1/50,000 as effective as fibrinopeptide A in displacing binding. Fibrinogen in plasma displaced binding less than 1/100,000 as effectively as fibrinopeptide A on a weight basis. The reason for this discrepancy in the behavior of purified fibrinogen in plasma is unknown, but we suspect there was alteration of the molecule due to partial proteolysis

TABLE 3. *Inhibition of binding of labeled fibrinopeptide by various materials*

Substance	Quantity of material Causing 50% inhibition of binding (μg)	Comparison of inhibitory activity on a molar basis
Fibrinopeptide A (*native*)	0.0008	1
Fibrinopeptide A (*synthetic*)	0.0008	1
Fibrinogen (Johnson) in buffer	20	1:112
Fibrinogen (Kabi) in buffer	20	1:112
Fibrinogen (DEAE-cellulose) in buffer	40	1:224
Fibrinogen in plasma (*citrated*)	>30 g*	<1:1500
Fibrinogen in plasma (*heparinized*)	>30 g*	<1:1500
Cohn fraction II	1000	
Cohn Fraction III	1000	
Cohn Fraction IV-4	2000	
Cohn Fraction V	1500	
Human albumin	4000 g†	
Bovine albumin	1000 g†	
Lysozyme	1000 g†	
Bradykinin	5 g†	
Insulin	1 g†	
Fibrinopeptide B(native)‡	0.0300†	

* Less than 10% inhibition at this concentration.
† No inhibition at this concentration.
‡ Fibrinopeptide B was the gift of Drs. B. & M. Blomback.

during purification or by the plasma environment. Several other proteins, including dialyzed albumin, did not inhibit binding of the labeled peptide. Dialyzed Cohn fractions had slight inhibitory activity, possibly because of their fibrinogen content (Table 3).

The filtrate of normal plasma through an XM 50 membrane showed no displacing activity. When fibrinopeptide was added to normal plasma, 80% was recovered in the ultrafiltrate (Table 4).

DISCUSSION

The data indicate that a specific and sensitive radioimmunoassay for human fibrinopeptide A can be performed with the use of *N*-tyrosyl-fibrinopeptide. A detailed account of the synthetic preparations and of their binding is in preparation (G. Wilner). The specificity of the assay for the fibrinopeptide is high in comparison with other proteins. Cross-reactivity with fibrinogen is relatively low but, in view of the high plasma content of fibrinogen, physical separation of fibrinogen will be necessary for fibrinopeptide measurement in plasma. Filtration through an XM 50 Amicon membrane satisfactorily separates peptide and fibrinogen. The sensitivity of the assay is far higher than previously used chemical methods for quantitating fibrinopeptide (2).

When possible applications of this test are considered, the kinetics of the release of peptide A should be readily studied in normal and abnormal plasma samples, such as those from patients with dysfibrinogenemia (17, 18) and conditions with long thrombin-clotting times, such as severe liver disease (19).

TABLE 4. *Quantity of material required to be filtered against an XM 50 membrane filter so that the filtrate gave 50% inhibition of binding of labeled peptide*

Substance	Quantity of material, μg
Fibrinopeptide A in buffer	0.0008
Fibrinopeptide A added to heparinized plasma	0.0010
Fibrinogen & thrombin	0.0900
Fibrinogen (Johnson)	60
Fibrinogen (Kabi)	>180
Fibrinogen (DEAE-cellulose)	180
Citrated plasma	—*
Heparinized plasma	—*

In each instance, 100 μl of a 1/5 dilution of the filtrate was used.
* No inhibition.

Of special interest is the possibility of measuring fibrinopeptide A concentrations in circulating blood to assess the effect of metabolic alterations and pharmacologic intervention on intravascular clotting. In order to achieve these objectives, significant improvement in sensitivity will still be required, as well as a thorough study of the effects of enzymes, such as plasmin, on the ability of fibrinogen to inhibit binding of the labeled peptide.

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