mation of single polypeptides. The lysins in turn cleave the polypeptide chains causing formation of envelope fragments with intact S-S bonds. Those can be further reduced by dithiothreitol to form smaller fragments.

The exact site of lysin cleavage and the possible differences among the various lysis products await further studies.

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Reactivity of Fibrinogen and Fibrinopeptide A Containing Fibrinogen Fragments with Antisera to Fibrinopeptide A†

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ABSTRACT: Two antisera used in the radioimmunoassay for human fibrinopeptide A (FPA) which appear to have different immunochemical specificities have been tested for cross-reactivity with fibrinogen and with three fragments of fibrinogen which contain the FPA sequence. These fragments were the three-chain, NH_2-terminal disulfide knot (N-DSK) produced by CNBr cleavage of fibrinogen, the reduced, carboxymethyl Aα chain portion of the N-DSK, and fragment E produced by plasmin digestion of fibrinogen. One antiserum (R-2) showed high specificity for free FPA and less than 2% cross-reactivity with fibrinogen or the FPA-containing fragments. The other antiserum (R-33) possessed a much higher degree of cross-reactivity with the FPA-containing fragments. Synthetic and native fibrinopeptides were found to be indistinguishable in the assay system with either antiserum. As a result of these studies, an hypothesis has been developed concerning the nature of the antigenic determinants on FPA which favor measurement of free FPA and limit cross-reactivity with larger, FPA-containing peptides.

Thrombin cleaves the Aα chain of fibrinogen to release 2 mol of fibrinopeptide A (FPA[1]) per mole of fibrinogen to initiate fibrin aggregation (Blombäck and Vestermark, 1958; Blombäck and Yamashina, 1958). Thrombin also cleaves the Bβ chain to release fibrinopeptide B (FPB) at a slower rate than FPA (Blombäck and Vestermark, 1958; Bilezikian et al., 1975). A radioimmunoassay for FPA in plasma has been developed to serve as a quantitative index of thrombin action in vivo (Nossel et al., 1971), and this assay has been applied to measurement of FPA levels in normal plasma and in patients with thrombotic diseases (Nossel et al., 1974; Gerrits et al., 1974; Hardin et al., 1975). Since the validity of the assay as an index of thrombin action depends on the estimation of the free, circulating, 16-amino acid fibrinopeptide and not fibrinogen or other FPA-containing polypeptides such as those generated by plasmin, it is important to study the antisera used in the radioimmunoassay for cross-reactivity with fibrinogen and its degradation products. Two antisera that had sufficiently high titers of anti-FPA antibodies to be used in the routine plasma radioimmunoassay were studied. These antisera were also of special interest concerning immunochemical specificity, because they gave different values for FPA con-

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[1] Abbreviations used are: FPA, fibrinopeptide A; FPB, fibrinopeptide B; N-DSK, NH_2-terminal disulfide knot; PAGE, polyacrylamide gel electrophoresis; RCM, reduced, *S*-carboxymethylated; Pth, 3-phenyl-2-thiohydantoin.

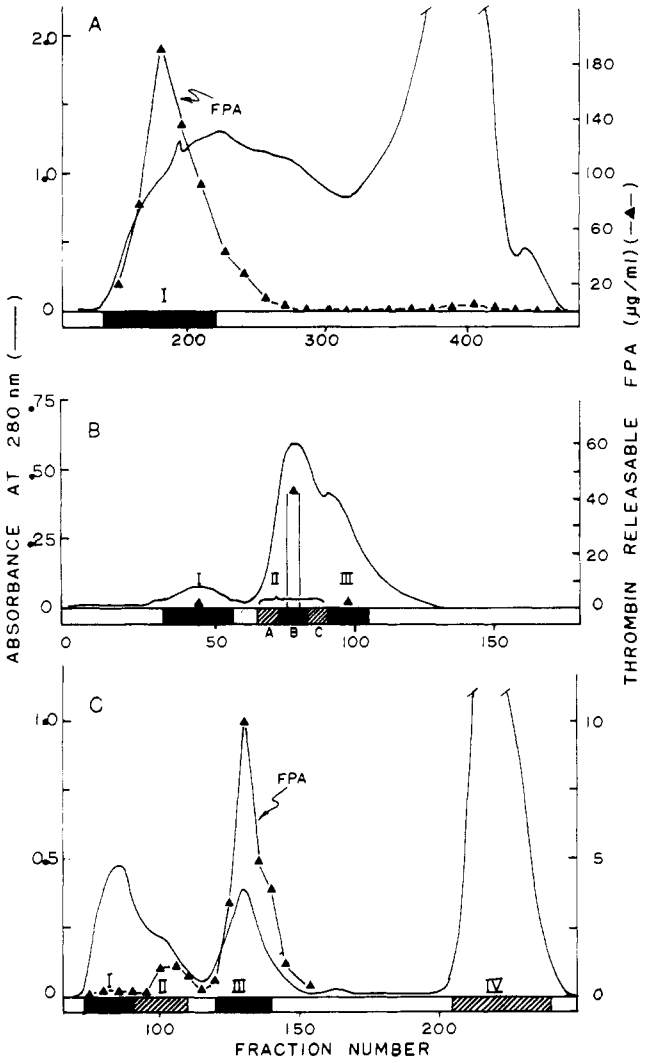

FIGURE 1: Purification of fragments produced by CNBr cleavage of human fibrinogen. (A) Gel filtration of 3 g of cleavage products on Sephadex G-100 in 10% acetic acid at room temperature. Aliquots of selected fractions were assayed for thrombin-releasable FPA as shown by the black triangles. Fractions were pooled as indicated by the bar (I) and lyophilized. (B) Ion-exchange chromatography at 4 °C of 500 mg of the product from gel filtration on DEAE-cellulose. Fractions were pooled as shown by the bars and lyophilized. Pools I, II, and III were studied for thrombin-releasable FPA and also by PAGE (see Figure 2). The results indicated that pool II contained N-DSK and, subsequently, this region was divided into pools IIA, IIB, and IIC as shown. (C) Gel filtration over Sephadex G-50 of 100 mg of RCM-N-DSK in 10% acetic acid at room temperature. Aliquots were tested for thrombin-releasable FPA as shown. Fractions were pooled as indicated by the bars and lyophilized.

tent in plasma specimens treated with streptokinase to activate plasminogen, which would be expected to produce fibrinogen fragments larger than FPA but small enough to appear in the dialysate with FPA (Nossel et al., 1974).

This communication describes the relative reactivities of these antisera with FPA, fibrinogen, and three polypeptides which contain the FPA structure. As a result of these studies, an hypothesis has been developed concerning the nature of the antigenic determinants on FPA which favor measurement of free FPA and limit cross-reactivity with larger, FPA-containing peptides. Evidence concerning this hypothesis is presented in the accompanying paper (Wilner et al., 1976) in which the immunoreactivity of synthetic peptides which correspond to portions of the FPA molecule were tested.

Materials and Methods

Human fibrinogen, grade L, was purchased as a lyophilized powder from Kabi, Stockholm. Bovine thrombin (crude) from Parke Davis was used. Hirudin, with an activity of 2000 U/ampoule, was obtained from Koch-Light Laboratories Ltd., Colnbrook-Bucks, England. Human plasminogen was isolated by affinity chromatography (Deutsch and Mertz, 1970; Liu and Mertz, 1971) from a Cohn Fraction kindly supplied by Dr. Carlos Ehrich of the New York Blood Center. It was dissolved in 0.1 M NH_4HCO_3 and stored at −20 °C. Streptokinase was streptokinase–varidase from Lederle and was dissolved in H_2O and stored at 2 °C. DEAE-cellulose (Whatman DE-32) was purchased from H. Reeve Angel.

Antigens. (1) Fibrinopeptide A: Both synthetic FPA, purchased from Schwarz/Mann (No. N-2072), and native FPA were employed as standards. The native FPA was prepared as described by Blomback et al. (1966). The concentration of a standard solution containing each of the fibrinopeptides was determined by quantitative amino acid analysis employing norleucine as an internal standard.

(2) Fibrinogen: The Kabi grade L product was purified by chromatography on DEAE-cellulose according to the method of Finlayson and Mosesson (1963) and was greater than 95% clottable. Following chromatography, the product was dialyzed against 0.3 M NaCl, lyophilized, and stored at −20 °C.

(3) NH_2-Terminal Disulfide Knot (N-DSK) Fibrinogen (Kabi grade L) was treated with CNBr, lyophilized, and filtered on Sephadex G-100 in 10% acetic acid as described by Blombäck et al. (1972). Thrombin-treated aliquots (50 U/ml for 4 hr at room temperature) of the eluted fractions were assayed for FPA content (Nossel et al., 1971). The fractions in the eluate containing the largest quantities of thrombin-releasable FPA were pooled and lyophilized (Figure 1A). The yield from 3 g of fibrinogen, representing N-DSK plus contaminating peptides, was 940 mg.

Further purification was accomplished by ion-exchange chromatography over DEAE-cellulose. In a typical experiment, 500 mg of the product from pool I (Figure 1A) was dissolved in 100 ml of 0.05 M $(NH_4)_2CO_3$ and applied to a 2.5 × 25 cm column of Whatman cellulose DE-32. The column was developed with a gradient using a four-chamber varigrad containing 250 ml of $(NH_4)_2CO_3$ in each chamber in concentrations of 0.05, 0.1, 0.2, and 0.5 M. The eluate was collected in 5-ml fractions. In an early experiment, the eluate was divided into pools I, II, and III, as indicated in Figure 1B, and these were assayed for FPA content by the method described above. Since the results indicated that virtually all of the thrombin-releasable FPA was in pool II, which was therefore presumed to contain DSK, the eluate in this region was divided as shown at the bottom of Figure 1B into pools IIA, IIB, and IIC in subsequent experiments. In a typical experiment in which the column load was 500 mg, the yields of the pools were as follows: I, 45 mg; IIA, 90 mg; IIB, 101 mg; IIC, 108 mg; and III, 120 mg. Each of the products was studied by PAGE. Since the material in pool IIA migrated as a single band, it appeared to contain N-DSK, essentially free of contaminants. This product was further characterized by amino acid analysis and by NH_2-terminal sequence determination and was employed in the immunoreactivity experiments.

(4) RCM-Aα 1-51 Fragment. Fraction IIB (100 mg; Figure 1B), which contained N-DSK but also showed a

Table I: Amino Acid Compositions of Standard Antigen Solutions.

Antigen:	Native FPA		N-DSK		RCM-Aα 1–51		Fragment E	
Concentration:	1.28 μmol/ml		0.0684 μmol/ml		0.426 μmol/ml		0.0556 μmol/ml	
Residue	μmol[a]	Residues[b]	μmol[a]	Residues[c]	μmol[a]	Residues[d]	μmol[a]	Residues[e]
SCMC					0.0784	2.1 (4)		
Asp	0.513	2.00 (2)	0.453	61.7 (60)	0.2388	6.4 (7)	0.282	69.8
Thr		(0)	0.157	21.4 (20)	0.0700	1.9 (0)	0.078	19.3
Ser	0.252	0.98 (1)	0.288	39.2 (40)	0.1800	4.8 (5)	0.150	37.1
Glu	0.528	2.06 (2)	0.500	68.1 (62)	0.1885	5.0 (5)	0.253	62.6
Pro		(0)	0.244	33.2 (34)	0.1015	2.7 (3)	0.081	20.1
Gly	1.274	4.97 (5)	0.277	37.8 (38)	0.2621	7.0 (7)	0.105	26.0
Ala	0.514	2.00 (2)	0.264	36.0 (38)	0.0923	2.5 (3)	0.087	21.5
Cys		(0)	0.128	17.4 (22)		(0)	0.084	20.8
Val	0.250	0.97 (1)	0.195	26.6 (24)	0.1247	3.3 (3)	0.111	27.5
Met		(0)		(0)		(0)	0.022	5.4
Ile		(0)	0.126	17.1 (14)	0.0422	1.1 (0)	0.064	15.8
Leu	0.253	0.99 (1)	0.270	36.8 (34)	0.0721	1.9 (1)	0.165	40.8
Tyr		(0)	0.103	14.0 (14)	0.0529	1.4 (1)	0.069	17.1
Phe	0.260	1.02 (1)	0.095	12.7 (14)	0.0601	1.6 (2)	0.062	5.3
His		(0)	0.057	7.8 (8)	0.0237	0.6 (1)	0.031	7.7
Lys		(0)	0.227	30.9 (28)	0.0932	2.5 (2)	0.126	31.2
Trp		(0)		5.6[f] (4)		(2)		
Arg	0.254	0.99 (1)	0.220	30.0 (30)	0.1326	3.5 (4)	0.085	21.0
Hse + Lactone			0.047	6.4 (6)	Trace	(1)		

[a] Based on average or extrapolated values derived from duplicate 24, 48, and 72 h hydrolyses. [b] Based on Asp = Ala = 2.00. Figures in parentheses taken from Blombäck et al. (1966). [c] Based on Arg = 30.0. Figures in parentheses taken from Wallen (1971). [d] Based on Gly = 7.0. Figures in parentheses taken from Wallen (1971). [e] Based on Gly = 26.0. Mol wt = 50 000 daltons. [f] Determined by $MeSO_3H$ hydrolysis.

faint contaminating band on gel electrophoresis, was reduced in 10 M urea and alkylated with [^{14}C]iodoacetic acid as described by Crestfield et al. (1963). The mixture was filtered over a 2.5 × 200 cm column of Sephadex G-50 in 10% acetic acid as shown in Figure 1C. Selected fractions were assayed for thrombin-releasable FPA as described earlier.

(5) Fragment E. Fibrinogen was incubated at 0 °C with 15 000 U of streptokinase and 100 U of plasminogen in a total volume of 150 ml of 0.1 M NH_4HCO_3, pH 7.7. After 3 h the solution was filtered at 4 °C over an 8 × 105 cm column of Sephadex G-100 (Figure 3A). The products were characterized by sodium dodecyl sulfate, PAGE. Fractions I–VII were heterogeneous and further purified by ion-exchange chromatography on QAE Sephadex A-50 (Figure 3B). The material in pool IV, shown in Figure 3B, was fragment E.

Antisera. Two antisera that exhibited relatively high titers of immunoreactivity against FPA were employed in this study. The first (R-2) was prepared by immunization of a rabbit with native FPA coupled to human serum albumin by the carbodiimide method (Goodfriend et al., 1964). The second (R-33) was obtained by immunization of a rabbit with synthetic FPA coupled to bovine serum albumin by the glutaraldehyde method (Nossel et al., 1974).

Polyacrylamide Gel Electrophoresis (PAGE). PAGE was performed as described by Gabriel (1971) using 5% gels and staining with Amido Black. Sodium dodecyl sulfate PAGE was performed as described by Weber and Osborn (1969) using 5 M urea in the incubation solution and staining with Coomassie brilliant blue. No reducing agent was used.

Amino Acid Analysis. Amino acid analyses were performed using a Beckman Automatic Amino Acid Analyzer Model 121 and a one-column system. Duplicate samples were hydrolyzed in vacuo in 6 N HCl at 109 °C for 24, 48, and 72 h.

Amino Acid Sequence Determination. NH_2-terminal amino acid sequences were determined using the automated phenyl isothiocyanate degradation method (Edman and Begg, 1967; Edman, 1970) for the larger fragments and by the manual method for FPA (Peterson et al., 1972). The Pth derivatives were identified by thin-layer chromatography. In some cases identifications were confirmed by gas chromatography (Pisano and Bronzert, 1969). The aqueous phases from the final Pth extractions were examined by means of specific color reactions for Pth-His (Edman, 1970) and Pth-Arg (Easley et al., 1969).

Radioimmunoassay Technique. The assay was performed as previously described (Nossel et al., 1974) except the buffer contained 0.01 U/ml of freshly reconstituted hirudin to inhibit thrombin in the antiserum. All assay points were determined in replicates of five, and the data were plotted employing the linearizing logit transforms described by Rodbard et al. (1969). The concentration of antigen required to produce 50% displacement of tracer from antibody was read directly from the abscissa. In every experiment to determine molar reactivity, a control was included on the same day to measure the immunoreactivity of the FPA standard with the particular antiserum employed.

Results

A. Purification and Characterization of Antigens. (1) Fibrinopeptide A. The amino acid composition of a standard preparation of FPA isolated from human fibrinogen is listed in Table I and compared with the composition reported by Blombäck et al. (1966). The NH_2-terminal sequence is given in Table II.

(2) NH_2-Terminal Disulfide Knot. The results of gel filtration and ion-exchange chromatography for the purification of the N-DSK are illustrated in Figure 1. Thrombin-releasable FPA emerged from the gel filtration column among the largest of the cyanogen bromide (CNBr) cleavage products (Figure 1A) in agreement with Blombäck et

Table II: NH_2-Terminal Amino Acid Sequences of Purified Fibrinogen Fragments.[a]

Material Analyzed	Chain	Amino Acid Sequence
		1 2 3 4
Figure 1B, pool IIA (8.5 mg) (N-DSK)	Aα	Ala-Asp-Ser-Gly
		(Asp)
		1 2 3 4
	γ	Tyr-Val-Ala-Thr
Figure 1C, pool I (3.3 mg) (RCM Bβ 1–115 fragment of N-DSK)	Bβ	No detectable NH_2-terminal amino acid
		1 2 3 4
Figure 1C, pool II (3.0 mg) (RCM γ 1–78 fragment of N-DSK)	γ	Tyr-Val-Ala-Thr
Figure 1C, pool III (2.8 mg) (RCM Aα 1–51 fragment of N-DSK)	Aα	1 2 3 4 Ala-Asp-Ser-Gly (Asp)
Figure 3B, pool IV (10 mg) (fragment E)		1 2 3 4
	Aα	Ala-Asp-Ser-Gly-
		54 55 56 57
	β	Lys-Val-Glu-Arg-
		1 2 3 4
	γ	Tyr-Val-Ala-Thr
		1 2 3 4
Native FPA	Aα	Ala-Asp-Ser-Gly (Asp)

[a] Where mixtures of amino acids were found at each step because of the presence of multiple chains, the results have been aligned by comparison with known primary structures. Asp is known to occur in the AY of FPA (Blombäck et al, 1966).

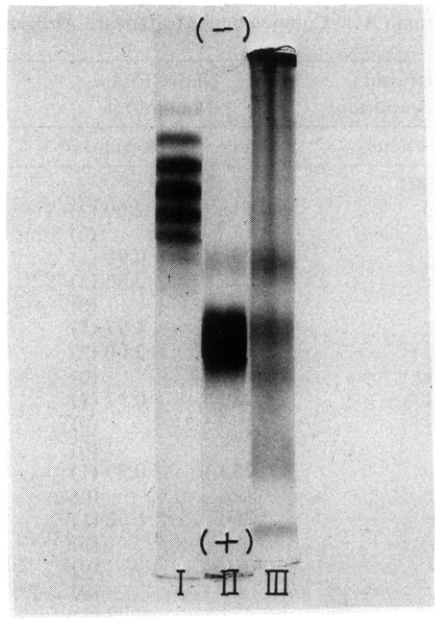

FIGURE 2: Polyacrylamide gel electrophoresis of material from pools I, II, and III from Figure 1B. The N-DSK band in II is broad, because of charge heterogeneity in the homoserine residues.

al. (1972). Subsequent chromatography on DEAE-cellulose of the material from gel filtration yielded three major peaks (Figure 1B). Peak II, which contained virtually all the thrombin-releasable FPA, exhibited one major band and a slight contaminant upon examination by PAGE as shown in Figure 2. This portion of the eluate was divided into IIA, IIB, and IIC, and examination by PAGE showed that pool IIA had no detectable contaminant so this preparation of N-DSK was used in the immunologic studies. The amino acid composition of pool IIA is given in Table I and is compared with the composition reported by Blombäck et al. (1972) for N-DSK. The NH_2-terminal sequence of N-DSK is given in Table II and is compared with that expected for the Aα and γ chains of fibrinogen (Wallén, 1971; Blombäck et al., 1973). The NH_2-terminal residue of the Bβ chain is pyroglutamic acid, which is unreactive in the Edman degradation. Radioimmunoassay studies, following thrombin treatment of N-DSK, indicated that 1.8 mol of FPA and 1.5 mol of FPB were released per mole of N-DSK.

(3) RCM-Aα 1–51 Fragment. Following reduction and carboxymethylation of the N-DSK (pool IIB), gel filtration on Sephadex G-50 gave the effluent pattern shown in Figure 1C. The thrombin-releasable FPA appeared in pool III indicating that it contained the RCM-Aα 1–51 fragment. The amino acid composition of material from this peak, compared with that reported by Wallén (1971), is given in Table I. Pool I had the amino acid composition expected for the Bβ 1–115 fragment and gave a negative result in the Edman degradation which is consistent with an NH_2-terminal pyroglutamic acid. Pool II had the amino acid composition and NH_2-terminal sequence expected for the γ 1–78 fragment. Pool IV contained salt, urea, and β-mercaptoethanol.

(4) Fragment E. Gel filtration over Sephadex G-100 of a plasmin digest of fibrinogen gave the effluent pattern shown in Figure 3A. The results of sodium dodecyl sulfate PAGE on fractions isolated from the first peak containing large-molecular-weight products showed that they all contained mixtures of fragments corresponding to fragments D and E. Therefore, pools I through VII were combined and subjected to ion-exchange chromatography with the results illustrated in Figure 3B. Sodium dodecyl sulfate PAGE showed that pools I, II, and III contained heterogeneous mixtures of material whose molecular weight ranges corresponded to those of fragment D, while pool IV contained a species whose molecular weight was in the correct range for fragment E, i.e., 50 000 daltons. The amino acid composition of pool IV is included in Table I, and the results of the NH_2-terminal sequence determination, given in Table II, are those expected for fragment E, based on the structure of "E-knot" reported by Kudryk et al. (1974), indicating that the NH_2 terminus of the Aα chain and of the γ chain was intact but that the β chain had been cleaved by plasmin at residue 53. Radioimmunoassay studies of fraction IV for thrombin-releasable FPA and FPB immunoreactivity were consistent with this interpretation, indicating a yield of 1.85 mol of FPA per mole of fragment E but no detectable FPB, i.e., <0.003 mol.

B. Immunochemical Studies. Figures 4A and 4B give the results of assays with native and synthetic FPA on the same day and showed indistinguishable inhibition curves with the R-2 antiserum. Similar results have been obtained with the R-33 antiserum. The immunoreactivity of fibrinogen was compared with that of FPA using R-2 antiserum in the presence of different concentrations of the thrombin inhibitor, hirudin (Figure 4C).

A comparison of all the data obtained with the two antisera is given in Figure 5, which indicates that the R-2 antiserum is capable of discriminating between free FPA and larger molecular species such as fibrinogen itself or the RCM-Aα 1–51 fragment, the N-DSK, or fragment E, all of which contain the FPA sequence. The immunoreactivity for each of these polypeptides was in the order of 1–2% of that of FPA. The R-33 antiserum, on the other hand, exhibited a

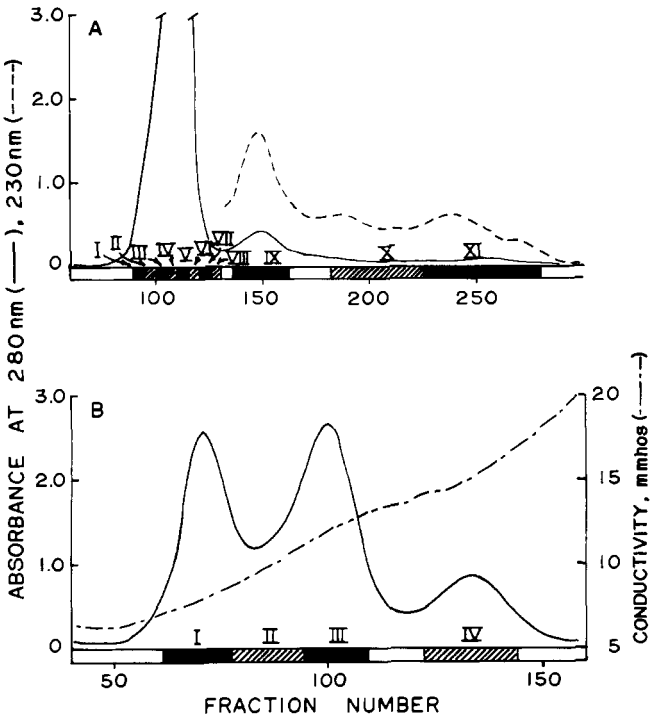

FIGURE 3: Purification of fragment E from human fibrinogen. (A) Gel filtration of a plasmin digest of 1.5 g of fibrinogen on Sephadex G-100 in 0.1 M NH_4HCO_3, pH 7.7, at 4 °C. Fractions were pooled as indicated by the bars and lyophilized. (B) Ion exchange chromatography of 2 g of the large-molecular-weight products from gel filtration (fractions I–VII, Figure 3A) on QAE Sephadex A-50 equilibrated with 0.3 M Tris-HCl, pH 8.6. The column was developed with a three-chamber varigrad containing 1200 ml in each chamber of the following solutions: (i) 0.3 M Tris-HCl, pH 8.6; (ii) 0.47 M Tris-HCl, pH 8.6; (iii) 0.8 M Tris-HCl, pH 7.8. The eluate was collected in fractions of ~20 ml. Fractions were pooled as indicated by the bars, dialyzed against 0.1 M NH_4HCO_3, and lyophilized.

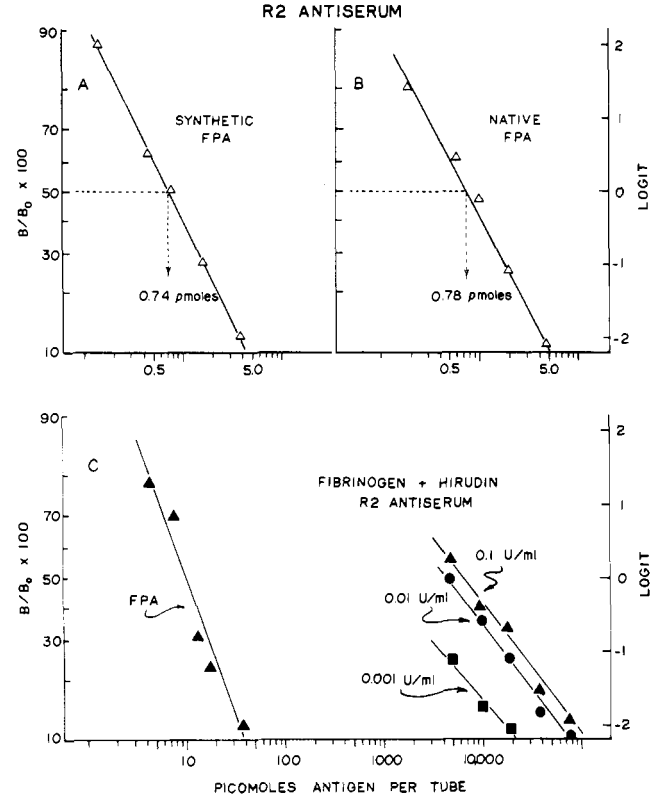

FIGURE 4: Radioimmunoassays employing the R-2 antiserum. The upper panels show the results with synthetic FPA (A) and native FPA (B), assayed under identical conditions on the same day. The lower panel (C) illustrates data from an experiment in which synthetic FPA was compared with human fibrinogen assayed in the presence of different concentrations of hirudin.

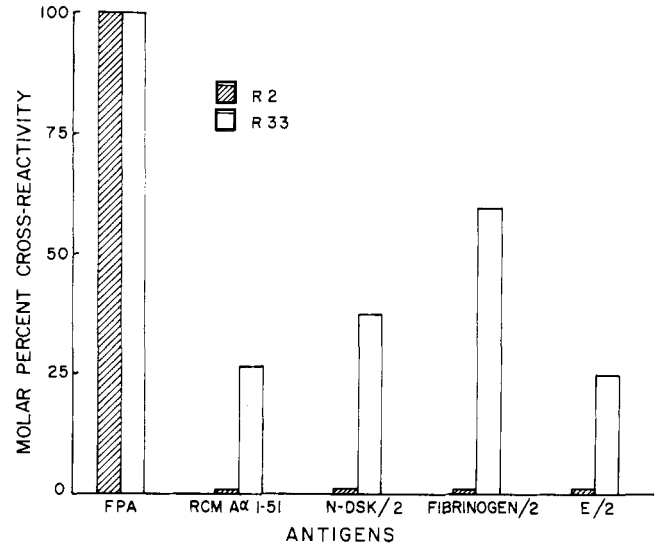

FIGURE 5: Comparison of the immunoreactivity of the R-2 and R-33 antisera with FPA and with four larger antigens that contain the FPA sequence. The ratio of immunoreactivity of a particular antigen with respect to FPA was calculated by dividing the number of picomoles of FPA required to achieve 50% displacement of tracer by the number of picomoles of the other fragments that were required to achieve the same displacement. N-DSK, fragment E, and fibrinogen are dimers with 2 mol each of FPA. The results are expressed as the percent of cross-reactivity defining the immunoreactivity with FPA as 100%.

greater degree of immunoreactivity with all four FPA containing species.

Discussion

The radioimmunoassay for FPA can serve as a diagnostic test and as a monitor of the effectiveness of therapy in slowing or eliminating thrombin action in vivo (Nossel et al., 1974). In such studies it is critical to define the specificity of the antisera employed in the radioimmunoassay and to determine to what extent a given antiserum cross-reacts with larger fragments of the fibrinogen molecule that also contain the FPA portion. Such fragments may arise as a result of plasmin-induced proteolysis. Toward this end we have purified and characterized fibrinogen and three fragments of the fibrinogen molecule that contain the FPA primary structure for study of immunoreactivity with anti-FPA antisera.

Treatment of fibrinogen with CNBr produces a large fragment, the N-DSK, consisting of the NH_2-terminal portions of all three chains, and Blombäck et al. (1972) have shown that its structure includes residues 1–51 of the Aα chain, 1–115 of the Bβ chain, and 1–78 of the γ chain, held together as a dimer by disulfide bridges. Upon reduction and carboxymethylation, the N-DSK can be separated into its component chains and the RCM-Aα 1–51 portion contains the FPA sequence in its first 16 residues. Plasmin digestion of fibrinogen yields a large amino terminal fragment, designated fragment E, which has been shown to contain residues 1–86 of the Aα chain, 54–119 of the β chain, and 1–58 of the γ chain, again held together as a dimer by disulfide bonds (Kowalska-Loth et al., 1973). Thus these three fragments, namely N-DSK, RCM-Aα 1–51, and E,

as well as fibrinogen itself, contain the FPA sequence as part of their structure and they seemed to be appropriate chemically defined models for study of cross-reactivity with FPA antisera.

In general each antiserum was consistent in the pattern of reactivity. The R-2 antiserum exhibited less than 2% of the immunoreactivity, on a molar basis, with all of the molecules tested when compared with the immunoreactivity against the smaller FPA fragment (Figures 4 and 5). Thus it seems reasonable to conclude that this antiserum combines with antigenic determinants of FPA that are accessible when it exists in solution as a small peptide but are inaccessible when the 16 amino acids are part of a larger molecule. It seems likely that hydrophobic side chains reacting with the antibody recognition site could account for this phenomenon since the FPA sequence probably does not provide an adequate chain length to allow hydrophobic side chains to be sequestered from the aqueous solvent in a stable structure. In larger molecules, however, there could be sufficient tertiary structure to ensure that the Phe-Leu pair at positions 8 and 9, for example, would have their side chains buried in the interior of the molecule. While the R-33 antiserum was not fully as reactive with the molecules tested as it was with FPA, there was a 30–50 times greater degree of cross-reactivity than that exhibited by the R-2 antiserum (Figure 5). This suggests that R-33 is combining with one or more hydrophilic sites on FPA that are also accessible at the surface of the larger molecules.

These differences have provided several useful applications. First it is clear that the R-2 antiserum is the more specific reagent to use in the radioimmunoassay for FPA in plasma. As noted earlier, if FPA immunoreactivity in a plasma specimen is much greater with the R-33 antiserum than with R-2, it is likely that significant fibrinogenolysis has occurred. For example, in initial clinical studies, FPA was separated from fibrinogen by dialysis of plasma. Markedly different FPA immunoreactivity was found by testing the dialysates with the R-2 and R-33 antisera. Similar differences between R-2 and R-33 immunoreactivity were obtained when the dialysate of streptokinase treated plasma was tested (Nossel et al., 1974). In keeping with the observations reported here, these results were interpreted as reflecting plasmin digestion of fibrinogen during dialysis yielding a number of smaller molecular weight, dialyzable Aα chain fragments that reacted with the R-33 antiserum but not with R-2. When in vitro fibrinogenolysis was prevented by first precipitating the fibrinogen in plasma of normal individuals with ethanol, dialysates of the supernatant showed indistinguishable FPA immunoreactivity with both antisera.

Another practically important observation is the finding that small quantities of hirudin lower the apparent cross-reactivity of fibrinogen with R-2 antiserum presumably by neutralizing small amounts of thrombin present in the antiserum and inhibiting release of FPA. Therefore, all of the radioimmunoassays reported here were conducted in the presence of hirudin.

On the basis of the data reported here, we conclude that there are significant variations in the specificity of antisera which can influence the interpretation of FPA immunoreactivity measurements in blood samples. Hence, the conformation of this FPA sequence is different when it is a part of fibrinogen than when it exists as a small polypeptide. These observations suggested that antisera raised against hydrophobic portions of the FPA molecule such as the region that includes the Phe and Leu at positions 8 and 9 are more likely to be specific for FPA and limit cross-reactivity with other FPA-containing fibrinogen fragments that may occur in plasma specimens. In the accompanying paper (Wilner et al., 1976), evidence is presented consistent with the hypothesis that such hydrophobic amino acid side chains are important determinants in interacting with the combining site of the R-2 antiserum, whereas the R-33 antigenic determinants reside in other parts of the FPA molecule.

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Immunochemical Studies of Human Fibrinopeptide A Using Synthetic Peptide Homologues†

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ABSTRACT: Previous studies have indicated that rabbit antisera R2 and R33 to human fibrinopeptide A differ markedly in terms of cross-reactivity with fibrinogen and fibrinopeptide A-containing fragments of the fibrinogen molecule. Antiserum specificity was characterized by comparison of inhibition of binding to radiolabeled tyrosyl fibrinopeptide A produced by synthetic fragments and enzymatic digests of the fibrinopeptide A molecule vs. the complete fibrinopeptide sequence (Aα 1–16). Synthetic COOH-terminal homologues through the dodecapeptide (Aα 5–16) exhibited less than 16% immunoreactivity with R33 antiserum, which cross-reacts extensively with fibrinogen and fibrinopeptide A-containing fibrinogen fragments. In contrast, the synthetic COOH-terminal decapeptide (Aα 7–16) gave 100% immunoreactivity with R2 antiserum, which cross-reacts minimally with fibrinogen and fibrinopeptide A-containing fibrinogen fragments. Synthetic homologues smaller than Aα 7–16, such as Aα 9–16 and Aα 7–11, reacted only minimally with R2 antiserum. Carboxypeptidase B digests of fibrinopeptide A retained less than 25% of their initial immunoreactivity with R2 antiserum. It is concluded that the antigenic determinants of R2 immunoreactivity reside entirely within the COOH-terminal ten-residue sequence of fibrinopeptide A, and that Phe-8, Asp-7, and Arg-16 contribute significantly to R2 immunoreactivity. The R2 antigenic determinants appear to be significantly less accessible to reaction with antibody than the R33 determinants when the fibrinopeptide is attached to its parent α chain (Canfield et al., 1976). A possible mechanism for the sequestration is discussed.

The conversion of fibrinogen to fibrin is directly related to the cleavage of fibrinopeptide A, representing the NH_2-terminal 16-residue segment of the fibrinogen α chain, by the enzyme thrombin (Blombäck et al., 1967). In order to specifically measure the action of this enzyme, a radioimmunoassay for human fibrinopeptide A (Aα 1–16) has been developed (Nossel et al., 1971) and applied to the study of clinical blood samples (Nossel et al., 1974; Gerrits et al., 1974). A number of antifibrinopeptide A sera have been prepared, and two, R2 and R33, have been used in clinical studies (Nossel et al., 1974).

Previous studies have shown that dialysates of thrombin-treated normal human plasma demonstrate equivalent immunoreactivity with R2 and R33 antisera, while dialysates of streptokinase-treated plasma showed significantly higher immunoreactivity with R33 antiserum than with R2 antiserum (Nossel et al., 1974). Following thrombin treatment of the dialysates, similar immunoreactivity was found with both R2 and R33 antisera. To explain these findings, it was postulated that fibrinopeptide A gave comparable results with the two antisera, but that larger fragments of the fibrinogen molecule which included the A peptide, as would be produced by plasmin cleavage, gave relatively lower reactivity with R2 antiserum (Canfield et al., 1976; Nossel et al., 1974). The hypothesis was advanced that R2, but not R33, antigenic determinants are immunologically hidden in the larger fibrinopeptide A-containing fragments of the fibrinogen molecule, thus permitting distinction between thrombin and plasmin proteolysis products in clinical plasma samples. This postulate has assumed a crucial role in the interpretation of fibrinopeptide A measurements in patients with various clinical disorders (Nossel et al., 1974). Detailed immunochemical studies of these antisera were therefore undertaken using synthetic fibrinopeptide homologues to define the antigenic sites of fibrinopeptide A which react with the R2 and R33 antisera.

Materials and Methods

Reagents. All chemicals were of reagent grade. N^{α}-*tert*-Butyloxycarbonyl (BOC[1]) amino acids were purchased from Fox Chemical Co., Los Angeles, California. BOC-amino acids with protected side chains were γ-benzylglutamic acid, β-benzylaspartic acid, *O*-benzylserine, and N^{g}-tosylarginine. All BOC-amino acids were of the L configuration with the exception of glycine. Purity of the BOC-

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[1] Abbreviations used: BOC, N^{α}-*tert*-butyloxycarbonyl; DFP, diisopropylfluorophosphate.