Quantitative *N*-Terminal Analysis of Fibrinogen–Fibrin-Related Antigen [FR Antigen] From Human Plasma

By Joel U. HARRIS,* Alan J. JOHNSON,* Clarence MERSKEY,† Mei-Tai WANG† and David ROBINSON*

* Department of Medicine, New York University School of Medicine, New York, NY, U.S.A., and

† Department of Medicine and Laboratory Medicine, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, U.S.A.

(Received 21 March 1979)

Fibrinogen–fibrin-related antigen (FR antigen) was isolated from as little as 1 ml of human plasma by immuno-affinity chromatography with agarose-bound antibody to human fibrinogen. *N*-Terminal analysis was performed to determine the nature and extent of proteolytic degradation of the FR antigen in patients with disseminated intravascular coagulation and in normal subjects. Thrombin cleavage of the A- and B-peptides from fibrinogen *in vitro* was monitored by the appearance of *N*-terminal glycine, and an increase in glycine was shown in the FR antigen of patients with disseminated intravascular coagulation. As plasmin progressively degraded fibrinogen, increases in *N*-terminal alanine, aspartic acid and lysine were observed, corresponding to the known plasmin-cleavage points of fibrinogen; increases in these *N*-terminal amino acids were also found in the patients' FR antigen. Thrombin treatment *in vitro* was used to remove fibrinopeptide A (*N*-terminal alanine) from the samples and to reflect specifically the *N*-terminal alanine at the plasmin-cleavage point (Arg-42–Ala-43) of the B β -chain on assay; this alanine was increased progressively in the FR antigen of a patient during urokinase therapy, and was high in other patients when the FR antigen was examined by this procedure.

The fibrinogen–fibrin-related antigen (FR antigen) from the plasma of patients with a wide variety of clinical states associated with the syndrome of disseminated intravascular coagulation (Merskey, 1973; Hamilton *et al.*, 1978) is known to be proteolytically altered or degraded. Determination of the FR antigen in serum has proved helpful in the diagnosis of this syndrome (Merskey *et al.*, 1966).

To characterize the FR antigen structurally as a partially degraded substrate, it appeared essential to isolate it quantitatively from plasma so that the amount obtained would reflect the amount actually present, and only alterations in its structure *in vivo* would be found. Any structural changes thus detected would be interpreted as being due to one or more specific physiological enzymes.

Generally, only small volumes of plasma (1–2 ml) were available for serial samples from patients with defibrination, but with immuno-affinity-column chromatography it was possible to achieve adequate recovery of the FR antigen from these samples and those from normal subjects (Merskey *et al.*, 1978). Purity of the FR antigen was established by SDS/polyacrylamide-gel electrophoresis and quantitative *N*-terminal analysis, the approach used to study the plasma FR antigen from a series of patients with florid disseminated intravascular coagulation

(Merskey *et al.*, 1979). The present paper will show the application of *N*-terminal analysis to this problem.

Experimental

Materials

Guanidine hydrochloride, pyridine, benzene, acetone, ethyl acetate, and phenylisothiocyanate (all Sequanal grade), amino acid phenylthiohydantoin derivatives and acetic acid (pHIX buffer grade) were from Pierce Chemical Co., Rockford, IL, U.S.A. Acetonitrile was from Burdick and Jackson, Muskegon, MI, U.S.A., Sepharose CL-4B from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A., bovine serum albumin from Sigma Chemical Co., St Louis, MO, U.S.A., polyelectrolyte E-100 was obtained from the Monsanto Co., St. Louis, MO, U.S.A., soya-bean trypsin inhibitor from Worthington Biochemical Corp., Freehold, NJ, U.S.A., aprotinin (Trasylol) from FBA Pharmaceuticals, New York, NY, U.S.A., and γ -globulin (human immune serum globulin; U.S. Pharmacopeia) was from Hyland Laboratories, Costa Mesa, CA, U.S.A. [¹²⁵I]Iodine (protein-iodination grade) was from New England Nuclear, Boston, MA, U.S.A., and lactoperoxidase was from Calbiochem, San Diego, CA, U.S.A. Poly(ethylene glycol) (4000 mol.wt.) was obtained

Abbreviation used: SDS, sodium dodecyl sulphate.

from the Union Carbide, Tarrytown, NY, U.S.A.; fibrinogen (Grade L) was from A.B. Kabi, Stockholm, Sweden. Human thrombin (lot 56C; 12432 National Institutes of Health units/ml; 2800 units/mg) was a gift from Dr. John Fenton, II (New York State Department of Health, Albany, NY, U.S.A.). Human albumin labelled with iodo[^{14}C]acetamide was kindly prepared by Dr. T. H. Finlay in our laboratory at New York University Medical Center. Plasma samples from a patient before, during and after urokinase therapy were kindly supplied by Dr. Grant Barlow (Abbott Laboratories, Waukegan, IL, U.S.A.).

Methods

Isolation of fibrinogen-fibrin-related antigen. Methods of blood collection, preparation of plasma, and isolation of human FR antigen by using immuno-affinity columns, have been described (Merskey *et al.*, 1979). In brief, antibody to fibrinogen was purified by absorption on, and elution from, purified human fibrinogen coupled to agarose (Sephacrose CL-4B) by the CNBr-titration method (Parikh & Cuatrecasas, 1977). The antiserum was applied directly to this column, washed extensively and eluted with 0.1 M-citrate, pH 2.7, containing 0.5 M-NaCl. It was then coupled to agarose by CNBr as described above. The specificity of the antibody was demonstrated by immunodiffusion and by immunoelectrophoresis (Merskey *et al.*, 1966). In addition, formalin-treated tanned sheep cells coupled to fibrinogen, and to Fragment D and Fragment E respectively, reacted with the antiserum when diluted to at least 1:2000 in each instance.

The FR antigen eluted quantitatively in about 10 ml of 8 M-urea/0.05 M-citrate buffer, pH 4.5, and was less than 10% contaminated with other proteins, as shown by SDS/polyacrylamide-gel electrophoresis. When the protein concentration was less than 120 $\mu\text{g}/\text{ml}$ in the eluate, succinyl-lysozyme (100 $\mu\text{g}/\text{ml}$), a low-molecular-weight protein with blocked N-terminal residues (Weiner *et al.*, 1972), was added to facilitate concentration by precipitation. The proteins were then precipitated for 1 h at 0°C in 10% (w/v) trichloroacetic acid, washed with the same solution and extracted with cold ether, dried under N_2 and stored at -20°C in the deep-freeze pending N-terminal analysis. Good recovery of fibrinogen and Fragments X, Y and D, usually about 80%, was obtained from the immuno-affinity columns. However, most of the Fragment E was not bound. The primary contaminant proved to be γ -globulin (mol.wt. 160000); no proteolytic degradation of the FR antigen *in vitro* was demonstrable (Merskey *et al.*, 1979).

Standard human fibrinogen. Human fibrinogen (95% clottable, purity confirmed by SDS/polyacrylamide-gel electrophoresis and N-terminal analysis)

was isolated from fresh frozen plasma by a method developed in our laboratory and described by Lackner *et al.* (1970), with the following modifications: poly(ethylene glycol) 4000 was used in place of poly(ethylene glycol) 6000 and the redissolved cryoprecipitate was absorbed with $\text{Al}(\text{OH})_3$ gel before two precipitations with 6% (w/v) poly(ethylene glycol) and one with 4% poly(ethylene glycol) at pH 6.0.

Fibrinogen and fibrin digested with plasmin. Fibrinogen (1 g) was reconstituted with 50 ml of water and used for all the following digests. (a) Early digest. The mixture contained 4.5 ml of fibrinogen solution, 1.2 ml of 1 M-Tris/0.9% NaCl buffer, pH 7.3, 0.12 ml of 2% sodium azide, and 0.2 CTA (Committee on Thrombolytic Agents) unit of plasmin. The mixture was shaken overnight at room temperature and 6-aminohexanoic acid was added to a final concentration of 0.2 M to stop the reaction (Marder *et al.*, 1969).

(b) Intermediate digest. The same as (a), except for the addition of 2 CTA units of plasmin and, after 2 h of shaking at room temperature, 100 KI (kallikrein-inhibitor) units/ml of aprotinin.

(c) Late digest. The same as (b), except that the mixture was shaken overnight before addition of the aprotinin.

(d) Fibrin digested with plasmin. A terminal digest was prepared by the method of Pizzo *et al.* (1973).

Radiolabelled γ -globulin. Radiolabelled human γ -globulin was prepared with ^{125}I by the lactoperoxidase procedure of Thorell & Johansson (1971); a specific radioactivity of 3.9 $\mu\text{Ci}/\text{mg}$ was generally achieved.

Radioactivity measurements. A Packard Tri-Carb liquid-scintillation spectrometer (model 3320) was used for ^{14}C counting (efficiency uncalibrated) and an Abbott Logic gamma counter (model 211) for ^{125}I counting (efficiency uncalibrated).

Analysis of plasma FR antigen by SDS/polyacrylamide-gel electrophoresis. FR antigen isolated by immuno-affinity chromatography was run in the unreduced state by the method of Weber & Osborn (1969) in 3.5% (w/v) polyacrylamide gel and, after reduction by dithiothreitol, in 7.0 or 7.5% gels. When run through the affinity column, the FR antigen of normal plasma was indistinguishable from our standard human fibrinogen.

Quantitative N-terminal analysis of FR antigen. N-Terminal derivatives. 3-Phenyl-2-thiohydantoin derivatives of N-terminal amino acids of fibrinogen were prepared by Edman's (1970) procedure modified by Collen *et al.* (1975) and further modified as described below. The protein, 0.35–2 mg, was dissolved in 0.1 ml of 6 M-guanidine/0.15 M-Tris buffer, pH 9.5, and placed in 4 ml glass-stoppered conical tubes; 0.2 ml of pyridine was added, and the protein was coupled with 5 μl of phenyl isothiocyanate. A manifold with heating bath (N-Evap; Organomation,

Shrewsbury, MA, U.S.A.) was used for N₂ flushing and evaporation. The tubes were heated to 50°C in a heating block (Pierce) during coupling and hydrolysis. The precipitated protein was dispersed by 5 min sonication (model no. 83 sonicator; Heat System Ultrasonics, Plainview, NY, U.S.A.) during each of five extractions with 1.2 ml of benzene. After benzene extraction, 2 mg of bovine serum albumin was added, and the proteins were precipitated by mixing with 2 ml of acetone followed by 0.15–0.2 ml of water. Precipitation was complete at 0°C after 1 h, although it was sometimes more convenient to precipitate overnight at 4°C. After two aqueous-acetone extractions (1.6 ml of acetone and 0.2 ml of water) and freeze-drying of the precipitated protein, two ethyl acetate extractions were carried out, facilitated by sonication. The solvent was removed in a heated vacuum desiccator (Precision Scientific, Chicago, IL, U.S.A.).

The phenylthiocarbamoyl-protein was cleaved and converted into amino acid phenylthiohydantoin derivatives at 100°C for 1 h with 1 M-HCl, followed by extraction into ethyl acetate of the final product, which was dried under N₂.

Quantification of amino acid phenylthiohydantoin derivatives. The derivatives were analysed by high-pressure liquid chromatography (h.p.l.c.) with a Waters Associates (Milford, MA, U.S.A.) modular system including two model-6000 A solvent-delivery pumps, a model-660 solvent programmer, a model-U6K injector, a model-440 absorbance detector, and

a model-7132A two-channel recorder (Hewlett Packard, Paramus, NJ, U.S.A.). For most analyses a program modified from that of Downing & Mann (1976) was used. The conditions were as follows: a Bondapak C18 (Waters) column was used with a 30 min gradient and a 2 ml/min flow rate. 'Curve 7' of the solvent programmer was used; the initial buffer was 10% (v/v) buffer B; the final buffer was 90% buffer B; buffer A was 90% 0.02 M-sodium acetate (pH 3.7)/10% (v/v) acetonitrile; buffer B was 10% (v/v) acetate buffer (as above)/90% acetonitrile. In some instances the results were confirmed with a different program on a column (0.45 cm × 25 cm) of Zorbax ODS obtained from Dupont (Zimmerman *et al.*, 1977). In either case, 0.1 nmol or more of each amino acid derivative was analysed. Samples were injected in 0.02 ml of 90% (v/v) acetic acid. The 'peak-height' method or planimetry was usually used to quantify large peaks, and the 'cut-and-weight' method for small peaks; calibration was always performed with standard solutions of each amino acid phenylthiohydantoin derivative.

Thrombin treatment of FR antigen. FR antigen samples (0.5–2 mg) of protein were placed in 13 mm × 100 mm Teflon-capped tubes (Kimble Products, distributed by Fisher Chemicals, Springfield, NJ, U.S.A.) and precipitated with aqueous acetone overnight at 4°C to remove the previous buffer, in a manner similar to the Edman procedure (but without the addition of carrier albumin or acetone washes), and were then freeze-dried. The precipitate was

Table 1. *N*-terminal amino acids of human fibrinogen, fibrin and plasmin-digested fibrinogen

N-Terminal residues of products from plasmin-digested fibrin are not shown. The D-D dimer from cross-linked fibrin is assumed to have *N*-terminal residues similar to those of Fragment D. Fibrin Fragment E would have glycine-17 of the A α -chain in place of alanine-1, and glycine-15 of the B β -chain in place of pyroglutamic acid (Pyr)-1. Abbreviations used: T, thrombin; P, plasmin; Fg, fibrinogen; Fn, fibrin.

Chain	Enzyme	<i>N</i> -Terminal amino acid*	Presence (+) or absence (–) in:					
			Degradation products				Normal	Normal
			X	Y	D	E	Fg	Fn
A α		Ala (1)	+	+	–	+	+	–
	T	Gly (17)	–	–	–	±†	–	+
	P	Val (20)	–	–	–	±	–	–
	P	Asp (102)	+	+	+	–	–	–
B β		Pyr (1)	–	–	–	–	+	–
	T	Gly (15)	–	–	–	–	–	+
	P	Ala (43)	+	±	–	–	–	–
	P	Lys (54)	–	±	–	+	–	–
	P	Asp (134)	±	+	+	–	–	–
γ		Tyr (1)	+	+	–	+	+	+
	P	Ala (120)	–	–	±	–	–	–
	P	Ser (143)	–	–	±	–	–	–

* The position in sequence (given in parentheses) of intact fibrinogen chain given here is derived from Takagi & Doolittle (1975) and Collen *et al.* (1975).

† Fragments in the same group may have alternative *N*-terminal residues.

dissolved in 0.25 ml of deionized 8M-urea and 0.5 ml of 0.3M-NaCl/0.04M-EDTA/0.1M-Tris buffer, pH 8.0, and aprotinin [1000KI (kallikrein-inhibitory) units] in 0.25 ml of water was added. Highly purified α -thrombin [20NIH (National Institutes of Health) units] was added, and the tube was placed in a rotary shaker and gently agitated for 1 h at room temperature. An equal volume of chilled 20% trichloroacetic acid was added, and the sample mixed and incubated for 1 h at 0°C; it was then centrifuged, the supernatant was removed and the precipitate washed with 5% trichloroacetic acid. The proteins were finally washed with ether, dried, and dissolved in 0.1 ml of 6M-guanidine/0.15M-Tris buffer, pH 9.5, in which they were stored pending *N*-terminal analysis.

Results

The fibrinogen *N*-termini and those resulting from the action of thrombin and plasmin are shown in Table 1 and Fig. 1. Thrombin action is shown to be readily detected by *N*-terminal analysis, since, on cleavage of the fibrinopeptides by thrombin, glycine becomes the *N*-terminus of both the α - and β -chains. Early plasmin action also results in a cleavage in the B β -chain, with a change in the *N*-terminus (Lahiri *et al.*, 1970) and release of the B β -chain peptide 1–42 (Fig. 1). Thus a new *N*-terminal residue, alanine, is found in place of pyroglutamic acid. Later plasmin action results in a striking increase in *N*-terminal aspartic acid, two aspartic acid residues being present in each D-fragment.

Fig. 1. Basis for estimation of soluble fibrin and early Fragment X in FR antigen

Broken lines indicate disulphide-bond linkage of chains (solid horizontal lines). *N*-Terminal residues and the changes resulting from thrombin and limited plasmin action are shown. Pathway (a): soluble fibrin in FR antigen samples is estimated from the *N*-terminal glycine. Pathway (b): Fragment X in sample is estimated from the *N*-terminal alanine after thrombin treatment *in vitro* to remove A α -chain alanine. Abbreviation used: Pyr, pyroglutamic acid.

N-Terminal analysis of native fibrinogen cannot discriminate between the normal alanine at the *N*-terminus of the A α -chain and low amounts of B β -chain alanine-43. To measure the early *N*-terminal effects of plasmin in patients with disseminated intravascular coagulation, we removed the fibrinopeptides *in vitro* with thrombin, permitting the specific estimation of B β -chain alanine-43 in the core protein (Fig. 1). All samples were analysed for *N*-terminal residues without thrombin pretreatment; some parallel samples were also analysed after thrombin treatment.

The results of *N*-terminal analysis of the FR antigen in patients' plasma are presented as the molar ratios of each amino acid compared with tyrosine, the *N*-terminal amino acid of the γ -chain, in which little or no thrombin or early plasmin cleavage has been observed (Takagi & Doolittle, 1975). Those authors provided a diagrammatic representation of the fibrinogen molecule and its plasmin degradation products including the *N*-terminal residues at the cleavage points.

N-Terminal analysis of fibrinogen and plasma FR antigen

The molar ratio of the *N*-terminal amino acids, relative to tyrosine, of standard human fibrinogen and normal human plasma FR antigen are shown in Table 2. When fibrinogen was also passed through the column, the amounts of *N*-terminal amino acids did not change significantly. However, amounts of *N*-terminal aspartic acid and glutamic acid of normal plasma FR antigen were increased compared with those of fibrinogen, depending on the total amount of γ -globulin coupled to the affinity column. FR

antigen isolated from columns with the least amount of agarose-linked γ -globulin yielded results of *N*-terminal analysis closest to those of the standard fibrinogen.

The differences in the molar ratios of aspartic acid as well as glutamic acid to tyrosine between standard fibrinogen (Table 2, item 2) and normal FR antigen (Table 2, item 3) were both 0.07. The potential contribution of contaminating γ -globulin and albumin to the aspartic acid/tyrosine or glutamic acid/tyrosine ratios was calculated on the basis of recovery of labelled protein and known amounts of γ -globulin (7.4 mg/ml), albumin (40.4 mg/ml) and fibrinogen (3.4 mg/ml) in normal plasma (Tiselius, 1937), as well as their molecular weights per *N*-terminal residue. If the FR antigen were contaminated by 1% of the γ -globulin in 1 ml of plasma, the aspartic (glutamic) acid/tyrosine ratio would be increased by 0.05, a value comparable with the observed difference between standard fibrinogen and plasma FR antigen.

On SDS/polyacrylamide-gel electrophoresis of normal plasma FR antigen, a band corresponding to γ -globulin (mol.wt. 160000) was occasionally seen.

Determination of N-terminal alanine in the plasmin-degraded B β -chain

When purified fibrinogen was incubated with various amounts of highly purified standard thrombin, 2 NIH units/ml were required to remove fibrinopeptide A, whereas 20 units/ml or more removed both fibrinopeptides (Fig. 2).

To simulate limited plasmin action of fibrinogen in plasma, standard fibrinogen was exposed to various amounts of plasmin for 10 min, the plasmin was inhibited, and the heat-precipitable protein analysed

Table 2. *N*-Terminal analysis of fibrinogen and normal plasma FR antigen

Analyses were performed on the following samples: (1) the fibrinogen standard; (2) the same standard (2.6 mg of fibrinogen per run) carried through the immuno-affinity column procedure used in (3); (3)–(5) FR antigen after immuno-affinity chromatography of 2 ml of plasma on columns with various amounts of agarose-linked γ -globulin. For the columns in (3), the antibody was isolated from donkey antiserum by means of a fibrinogen-coupled agarose column (Merskey *et al.*, 1979); for the columns in (4) and (5), γ -globulin was isolated from antiserum by the polyelectrolyte E-100 method (Johnson *et al.*, 1978). Column resin was determined for protein by acid hydrolysis followed by amino acid analysis; all columns were 1.0 cm $\times$ 30 cm. The s.d. is given in parentheses; * $P < 0.05$ [between (3) and (4)]; ** $P < 0.01$ [between (3) and (5)].

Specimen	No.	Agarose-linked protein/column bed volume (mg/ml)	Molar ratio (relative to tyrosine = 1)				
			Alanine	Glycine	Aspartic acid	Glutamic acid	Valine
(1) Fibrinogen	16	—	0.74 (0.09)	0.05 (0.02)	0.14 (0.03)	0.06 (0.02)	0.13 (0.03)
(2) Fibrinogen	5	0.4	0.72 (0.07)	0.08 (0.01)	0.15 (0.04)	0.05 (0.01)	0.13 (0.02)
(3) Normal plasma FR antigen	37	0.4	0.77 (0.10)	0.08 (0.02)	0.22 (0.05)	0.12 (0.05)	0.10 (0.03)
(4) Normal plasma FR antigen	10	1.6	0.80 (0.09)	0.08 (0.02)	0.26* (0.05)	0.15 (0.08)	0.10 (0.02)
(5) Normal plasma FR antigen	8	3.6	0.77 (0.04)	0.07 (0.01)	0.36** (0.04)	0.20 (0.06)	0.10 (0.02)

for *N*-terminal residues. Under the conditions described in Table 3 (Takagi & Doolittle, 1975), as little as 0.05 CTA unit of plasmin had a significant effect, compared with the control. The control amount of *N*-terminal alanine was 0.68 mol/mol of tyrosine; Blombäck & Yamashina (1957) found 0.8 mol/mol after correction for loss of alanine phenylthiohydantoin derivative during derivatization. Most

Fig. 2. *N*-Terminal residues of fibrinogen after treatment with various amounts of thrombin

A mixture of 1.0–2.0 mg of human fibrinogen and 100 KI units of aprotinin/ml, 0.15 M-NaCl, 0.02 M-EDTA and 2 M-urea in 0.05 M-Tris, pH 8.0, was incubated for 1 h at room temperature with the indicated amount of thrombin.

Table 3. Effect of thrombin on *N*-terminal amino acids of fibrinogen degraded by varying amounts of plasmin. Plasmin digest mixtures contained 3 mg of standard fibrinogen/ml, various amounts of plasmin and 0.9% NaCl in 0.1 M-Tris, pH 7.3, at 22°C; digestion was stopped after 10 min by the addition of soya-bean trypsin inhibitor. The fibrinogen and high-molecular-weight core fragments of plasmin digestion were precipitated at 60°C for 30 min, washed with water, and analysed for *N*-terminal residues with and without thrombin treatment.

Plasmin (CTA unit/ml)	Molar ratio			
	Without thrombin		With thrombin	
	Gly/Tyr	Ala/Tyr	Gly/Tyr	Ala/Tyr
(1) 0	0.09	0.68	2.3	0.03
(2) 0.05	0.06	0.86	2.0	0.28
(3) 0.1	0.06	1.17	1.7	0.68
(4) 0.2	0.05	1.33	1.06	0.73

laboratories (Kierulf & Abildgaard, 1971, and references cited therein) have also found amounts of *N*-terminal aspartic acid (per mol of tyrosine) in fibrinogen comparable with those found here. This is generally considered to be A α -chain aspartic acid-2 of the AY variant (Blombäck & Blombäck, 1972). The sample treated with 0.2 CTA unit of plasmin had double the amount of alanine in the control; it is assumed that, as the amounts of plasmin were increased, the newly formed *N*-terminal alanine was the B β -chain alanine-43 (Table 1). SDS/polyacrylamide-gel electrophoresis of the dithiothreitol-reduced samples (Table 3 and Fig. 3) showed simultaneous loss of intact A α - and B β -chains and the appearance of a chain of 52000 mol.wt., probably the B β -chain lacking peptide 1–42, as well as 26000- and 18000-mol.wt. chains probably derived from the A α -chain. When the samples were treated with thrombin, an increase in *N*-terminal alanine was still evident in the plasmin-digested samples, although there was virtually none in the control.

Plasmin-degradation products of fibrinogen and FR antigen from normal subjects and four patients with disseminated intravascular coagulation

(a) *Polyacrylamide-gel electrophoresis.* FR antigen samples from standard fibrinogen (with and without plasmin digestion), plasma from control subjects, and plasma from patients with disseminated intra-

Fig. 3. SDS/polyacrylamide-gel (7.5%, w/v, acrylamide) electrophoresis of reduced fibrinogen with and without plasmin digestion

Std., standard fibrinogen. (1)–(4), Fibrinogen exposed to various concentrations of plasmin corresponding to Table 3. Samples in (2)–(4) were run in duplicate in different amounts. A α -, B β - and γ -chains of fibrinogen of known molecular weights were used to calibrate the gel.

vascular coagulation, were isolated and purified by immuno-affinity chromatography, and analysed by SDS/polyacrylamide-gel electrophoresis (Figs. 4 and

Fig. 4. SDS/polyacrylamide-gel (3.5%, w/v, acrylamide) electrophoresis of unreduced FR antigen after immuno-affinity chromatography

(a) Standard fibrinogen. (b), (c) and (d) Early, intermediate and late plasmin digests (respectively) of fibrinogen. (e) Marker mixture containing an intermediate plasmin digest of fibrinogen plus added Fragment E. (f) Plasma FR antigen from control. (g), (h), (k) and (l) Plasma FR antigen from patients with disseminated intravascular coagulation due to (respectively) acute myeloblastic leukaemia, aortic aneurysm, unknown cause, and multiple trauma and shock. (i) Plasmin digest of fibrin (major band is D-D dimer). (j) Bovine serum albumin (monomer is of mol.wt. 68000). The bands at the bottom of gels (k) and (l) are from succinyl-lysozyme added to enhance precipitation in urea/trichloroacetic acid.

Fig. 5. SDS/polyacrylamide-gel (7.0%, w/v, acrylamide) of reduced FR antigen after immuno-affinity chromatography

As for Fig. 4, except that (e) and (j) were omitted.

5). A higher-molecular-weight band seen above the fibrinogen in Fig. 4(a) and in some of the other samples, was probably fibronectin (mol.wt. 450000, N-terminal pyroglutamic acid), which is commonly co-purified with fibrinogen, and would not affect the interpretation of gel and N-terminal-analysis results.

As Fig. 4 shows, the early plasmin digest (b) had primarily Fragment X and superficially degraded fibrinogen (fibrinogen band III, Fig. 4); the intermediate digest (c) had Fragments X, Y and D, and the late digest (d) had only Fragment D. The terminal digest of fibrin (i) had D-D dimer, and some Fragments D and E. The FR antigen samples from a control subject (f) had only fibrinogen, whereas disseminated-intravascular-coagulation patients (g, h, k and l) had bands with mobilities similar to marker Fragments X, Y, D and D-D dimer. Gels (not shown) of fibrinogen and its early and intermediate plasmin-degradation products before immuno-affinity chromatography were similar to the corresponding gels of Fig. 4. When the samples were reduced and run on 7% gels (Fig. 5), alterations were noted in the polypeptide chains of various patients' FR antigens, e.g., γ - γ dimers and other lower-molecular-weight bands probably corresponding to degradation of the α -, β -, and γ -chains.

(b) *N-Terminal analysis.* The N-terminal residues of the FR antigen in these samples were analysed before and after thrombin treatment (Table 4). As expected, alanine was increased in the 'early' digest compared with the control, and the amount of alanine in the thrombin-treated samples suggested that one-third of the molecules lacked the B β -chain peptide 1-42. The 'intermediate' digest contained approximately equimolar amounts of glycine, alanine and tyrosine after thrombin treatment, indicating complete loss of B β -chain peptide 1-42; the appearance of aspartic acid and lysine represented N-terminal residues characteristic of late Fragment X and Fragments Y and D (Table 1). The N-terminal residues of the 'late' digest resembled Fragment D, rather than a mixture of Fragments D and E, indicating that the E Fragment was bound poorly by the immuno-affinity column.

Low amounts of N-terminal alanine were still present after either standard fibrinogen or normal plasma FR antigen was treated with thrombin (Table 4, items 1 and 5); the reason for this was not clear. [However, these results were all derived from analyses using the Bondapak C18 column. It was subsequently shown that low molar ratios (0.15 or less, alanine/tyrosine) are more sensitively measured by the Zorbax ODS column procedure, owing to the production of sharper peaks and consequently greater discrimination between peaks and background. Thus recent results in our laboratory suggest that the values for fibrinogen can be lowered still further by using a Zorbax ODS column.] Fibrinogen has been shown to be partially degraded in normal individuals,

Table 4. *N*-Terminal analysis of FR antigen [fibrinogen and plasmin degradation products, normal and disseminated-intra-vascular-coagulation (DIC)-patient plasma samples] with and without thrombin treatment

The preparation of the plasmin digests of fibrinogen is described in text. Samples (2), (3), (4), (6), (7), (8) and (9) correspond to Fig. 4 gels (b), (c), (d), (g), (h), (k) and (l) respectively. The immuno-affinity columns used to isolate the samples were similar to those in Table 2, set 3. *n* Denotes the number of samples analysed when more than one.

Source of FR antigen	Amount of amino acid (nmol)*													
	Without thrombin treatment							With thrombin treatment						
	Ala	Gly	Asp	Glu	Val	Lys	Tyr	Ala	Gly	Asp	Glu	Val	Lys	Tyr
(1) Fibrinogen standard (<i>n</i> = 5)	0.72 0.07†	0.08 0.01	0.15 0.01	0.05 0.01	0.13 0.02	0.02	1.0	0.10 0.02	1.8 0.2	0.10 0.06	0.07 0.03	0.30 0.03	0	1.0
(2) Early plasmin digest (some X)	0.88	0.16	0.16	0.08	0.06	0.07	1.0	0.23	1.3	0.08	0.07	0.10	0.03	1.0
(3) Intermediate plasmin- digest Fragments X, Y and D	1.5	0.15	1.4	0.19	0.06	0.17	1.0	0.78	0.76	1.1	0.15	0.09	0.21	1.0
(4) Late plasmin digest (Fragment D)	0.71	0.11	3.0	0.17	0.18	0.07	0.26	0.40	0.20	2.0	0.20	0.15	0.16	0.30
(5) Normal plasma (<i>n</i> = 11)	0.74 0.06†	0.07 0.01	0.25 0.05	0.10 0.02	0.11 0.04	<0.02	1.0	0.13 0.05	1.6 0.13	0.18 0.05	0.10 0.04	0.19 0.04	0.04 0.01	1.0
(6) Acute myeloblastic leukaemia	0.71	0.32	0.28	0.18	0.13	0.13	1.0	0.24	1.5	0.26	0.12	0.14	0.07	1.0
(7) Aortic aneurysm	0.76	0.17	0.17	0.18	0.13	0.02	1.0	0.29	1.6	0.27	0.21	0.39	0.05	1.0
(8) DIC of unknown cause	0.96	1.1	4.4	1.6	0.46	0.08	1.1	—	—	—	—	—	—	—
(9) Multiple trauma	0.71	0.94	1.1	0.25	0.21	0.11	1.0	—	—	—	—	—	—	—

* The tyrosine values were normalized, except for (4) and (8).

† Numbers in the second line are s.d. values.

owing to a low extent of fibrinolysis (Mosesson & Sherry, 1966; Lipinska *et al.*, 1974), but Fragment X normally does not accumulate *in vivo*.

The FR antigen from disseminated-intravascular-coagulation patients showed evidence of thrombin and plasmin with both polyacrylamide-gels (Fig. 5, samples *k* and *l*) and from *N*-terminal data (Table 4, samples 6–9). Major increases in glycine with widespread loss of fibrinopeptides were seen in the untreated samples, and abnormally high values of alanine consistent with early plasma action were seen in the thrombin-treated samples. Above-normal amounts of aspartic acid were also found, indicating later plasmin action.

The plasmin-degradation products of fibrinogen (Table 4, samples 2–4) had a higher content of *N*-terminal lysine than did the standard fibrinogen; three of the patients had elevated values (Table 4, samples 6, 8 and 9), suggesting intermediate plasmin degradation. Some *N*-terminal valine was found in all of our preparations. Its origin is uncertain, but some cleavage may have occurred in thrombin-treated samples at arginine-19 and valine-20 of the A α -chain. The increased *N*-terminal glutamic acid in the FR antigen from one patient (Table 4, sample 8) cannot readily be attributed to plasmin or thrombin; the origin of this *N*-terminal glutamic acid is not known. Since most of the FR antigen in the sample was

evidently fibrinogen Fragment D (see Fig. 4, gel *k*), which contains *N*-terminal tyrosine, the values were expressed as total nmoles.

Effect of urokinase therapy on FR antigen

A patient with pulmonary embolism underwent urokinase therapy for 12 h; sequential plasma samples were taken before, during and after therapy and the FR antigen was isolated.

In Figs. 6 and 7 it is evident that most of the circulating FR antigen was progressively degraded to Fragment X during therapy. Faint bands corresponding to Fragments Y, D and E were also found (Fig. 6); no D–D dimer was present. A α - and B β -chains were degraded to remnants characteristic of plasmin activity (Fig. 7). *N*-Terminal analysis of the isolated FR antigen samples after incubation with thrombin showed a progressive increase in *N*-terminal alanine, probably B β -chain alanine-43 (Fig. 8). These findings were consistent with the action of plasmin *in vivo*.

Discussion

By using immuno-affinity columns to isolate the FR antigen, and *N*-terminal analysis as well as polyacrylamide-gel electrophoresis to characterize the circulating fibrinogen and residual core peptides, we

Fig. 6. SDS/polyacrylamide-gel (3.5%, w/v, acrylamide) electrophoresis of unreduced samples of FR antigen from the plasma of a patient undergoing 12h urokinase therapy (a) Before therapy; (b) 2h, (c) 6h, (d) 12h, (e) 24h, (f) 72h and (g) 120h after initiation of therapy. (h) Control, normal plasma FR antigen. (i) Marker mixture from plasmin digestion of standard fibrinogen and Fragments X, Y, D and E.

Fig. 7. Plasma of patient undergoing 12h urokinase therapy: SDS/polyacrylamide-gel (7.5%, w/v, acrylamide) electrophoresis of reduced samples of FR antigen. As for Fig. 5, except that (i) is omitted. The reduced chains of intact fibrinogen, (Aα, Bβ, and γ) as well as the plasmin-degraded chain remnants (α' and β') are shown.

showed that disseminated intravascular coagulation secondary to disseminated cancer or abruptio placentae was due to the action of both the thrombin and plasmin-like enzymes (Merskey *et al.*, 1978, 1979). N-Terminal analysis was useful in those studies to demonstrate an increase in N-terminal glycine due to

Fig. 8. N-Terminal residues of FR antigen (from plasma of patient undergoing 12h urokinase therapy) treated with thrombin *in vitro*

Sequential plasma samples were taken at the times indicated. At zero time, urokinase therapy was initiated; at 12h therapy was discontinued.

the action of thrombin *in vivo*, since polyacrylamide-gel electrophoresis does not detect non-cross-linked soluble fibrin. In moderate or severe cases of defibrination, N-terminal analysis also confirmed the action, of plasmin by an increase in N-terminal alanine, presumably due to Bβ-chain alanine-43 in Fragment X and, in some cases, an increase in aspartic acid and lysine from Fragment Y and early Fragment D.

FR antigen from the immuno-affinity column was diluted to 2M-urea to permit the thrombin to cleave the fibrinopeptide A from the core residue while maintaining the antigen in a soluble form. The improved specificity of this analysis was confirmed by detecting these changes in early plasmin digests of fibrinogen, in the plasma FR antigen of patients with disseminated intravascular coagulation and in a patient undergoing urokinase therapy.

Kierulf & Abildgaard (1971) used quantitative N-terminal analysis to determine the extent of proteolysis *in vivo* of circulating clottable protein. Their procedure required large amounts of plasma (40–60ml) for each analysis, instead of the 1–2ml required here. Large amounts of plasma are not usually available from patients with disseminated intravascular coagulation, especially for serial samples during their clinical course. Kierulf & Abildgaard (1971) also used 8 or 16% (v/v) ethanol to precipitate the fibrinogen from plasma in a modified Cohn fraction I procedure. The N-terminal residues of the clottable protein were then determined by subtraction, i.e. N-

terminal residues of the preparation minus background *N*-terminal residues of the clot supernatant. In contrast, immuno-affinity-prepared FR antigen is sufficiently pure to permit direct *N*-terminal analysis. Kierulf (1973) found, as we did, an increase in *N*-terminal alanine and aspartic acid attributable to plasmin action and an increase in glycine after thrombin activity. He did not report glutamic acid in the clottable protein of normal plasma or thrombin-treated plasma, but actually found as much as 0.5 mol of glutamic acid/mol of tyrosine in the clottable protein in urokinase-treated plasma and up to 0.55 in the plasma of patients with disseminated intravascular coagulation. In common with other investigators using purified fibrinogen (Takagi & Doolittle, 1975), we failed to find large amounts of *N*-terminal glutamic acid after plasmin digestion of purified fibrinogen *in vitro*. The small amounts of glutamic acid found in all except one of our preparations (Table 4) are probably due to small amounts of contaminating γ -globulin, but the source of the larger amounts of glutamic acid in Kierulf's (1973) experiments and in one of ours remains to be determined.

A low extent of fibrinolysis probably occurs physiologically in the plasma of normal subjects, resulting in heterogeneity of the circulating fibrinogen; this is demonstrable on polyacrylamide-gel electrophoresis (Mosesson & Sherry, 1966; Lipinska *et al.*, 1974). The earliest changes *in vivo* appear to be at the C-terminus of the A α -chain (Mills & Karparkin, 1970; Cottrell & Doolittle, 1976). Pathological fibrinolytic changes also affect the fibrinogen *N*-terminus by cleavage of peptide 1–42 from the B β -chain, exposing a new *N*-terminus at alanine-43 (Takagi & Doolittle, 1975).

This work was supported by United States Public Health Service Program Project Grant no. 15596 from the National Heart, Lung and Blood Institute. We are grateful to Ms. Maria O. Longas, Ms. Santosh Swain and Ms. Pamela Schiro for technical assistance and to Ms. Elma T. Wadsworth for assistance in the preparation of the manuscript.

References

- Blombäck, B. & Blombäck, M. (1972) *Ann. N.Y. Acad. Sci.* **202**, 77–97
- Blombäck, B. & Yamashina, I. (1957) *Ark. Kemi* **12**, 299–319
- Collen, D., Kudryk, B., Hessel, B. & Blombäck, B. (1975) *J. Biol. Chem.* **250**, 5808–5817
- Cottrell, B. A. & Doolittle, B. F. (1976) *Biochem. Biophys. Res. Commun.* **71**, 754–761
- Downing, M. R. & Mann, K. G. (1976) *Anal. Biochem.* **74**, 298–318
- Edman, P. (1970) in *Protein Sequence Determination* (Needleman, S. B., ed.), pp. 211–255, Springer-Verlag, Heidelberg
- Hamilton, P. J., Stalker, A. L. & Douglas, A. S. (1978) *J. Clin. Pathol.* **31**, 609–619
- Johnson, A. J., Macdonald, V. E., Semar, M., Fields, J. E., Schuck, J., Lewis, C. & Brind, J. (1978) *J. Lab. Clin. Med.* **92**, 194–210
- Kierulf, P. (1973) *Scand. J. Clin. Lab. Invest.* **31**, 43–49
- Kierulf, P. & Abildgaard, U. (1971) *Scand. J. Clin. Lab. Invest.* **28**, 231–240
- Lackner, H., Hunt, V., Zucker, M. B. & Pearson, J. (1970) *Br. J. Haematol.* **18**, 625–636
- Lahiri, B., Bumpus, F. M. & Shainoff, J. R. (1970) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **29**, 648 (Abstr.)
- Lipinska, I., Lipinski, B. & Gurewich, V. (1974) *J. Lab. Clin. Med.* **84**, 509–516
- Lay, W. & Polgase, W. J. (1957) *Can. J. Biochem. Physiol.* **35**, 39–44
- Marder, V. J., Shulman, N. R. & Carroll, W. R. (1969) *J. Biol. Chem.* **244**, 2111–2119
- Merskey, C. (1973) *Blood* **41**, 599–603
- Merskey, C., Kleiner, G. J. & Johnson, A. J. (1966) *Blood* **28**, 1–18
- Merskey, C., Johnson, A. J., Harris, J. U. & Wang, M. T. (1978) *Int. Soc. Haematol. Congr. 17th*, 692 (Abstr.)
- Merskey, C., Johnson, A. J., Harris, J. U., Wang, M. T. & Swain, S. (1979) *Br. J. Haematol.* in the press
- Mills, D. & Karparkin, S. (1970) *Biochem. Biophys. Res. Commun.* **40**, 206–211
- Mosesson, M. W. & Sherry, S. (1966) *Biochemistry* **5**, 2829–2835
- Parikh, I. & Cuatrecasas, P. (1977) in *Immunochemistry of Proteins* (Atassi, M. Z., ed.), vol. 2, Plenum Press, New York
- Pizzo, S. V., Schwartz, M. L., Hill, R. L. & McKee, P. A. (1973) *J. Biol. Chem.* **248**, 4574–4583
- Takagi, T. & Doolittle, R. F. (1975) *Biochemistry* **14**, 940–946
- Thorell, J. I. & Johansson, B. G. (1971) *Biochim. Biophys. Acta* **251**, 363–369
- Tiselius, A. (1937) *Trans. Faraday Soc.* **33**, 524–531
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Weiner, A. M., Platt, T., & Weber, K. (1972) *J. Biol. Chem.* **247**, 3242–3251
- Zimmerman, C. L., Appella, E. & Pisano, J. J. (1977) *Anal. Biochem.* **77**, 569–573