Fibrinopeptide A in Plasma of Normal Subjects and Patients with Disseminated Intravascular Coagulation and Systemic Lupus Erythematosus

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ABSTRACT A radioimmunoassay for fibrinopeptide A (FPA) has been developed. This assay uses rabbit antibodies induced by injection of native FPA-human serum albumin conjugates and ^{125}I introduced into tyrosine-FPA synthesized in our laboratory. Plasma FPA is separated from fibrinogen by TCA extraction. The assay is capable of detecting as little as 50 pg/ml of FPA.

In 20 normal donors this assay revealed a mean concentration of 0.9 ng/ml (0.3 SD). In five patients with disseminated intravascular coagulation, FPA concentrations ranged from 13.0 to 346 ng/ml. Two groups of patients with systemic lupus erythematosus (SLE) whose disease had achieved complete remission were studied; one consisted of four patients with no history of lupus nephritis and another with a history of nephritis. Mean FPA concentrations of 1.5 ng/ml (range, 0.7–1.8 ng/ml) and 2.7 ng/ml (range, 1.1–5.6 ng/ml) were found in these two groups, respectively. Another group of nine patients with active SLE, but without evidence of lupus nephritis, had a mean FPA concentration of 4.5 ng/ml (range, 2.4–7.8 ng/ml). Finally, a group of seven patients with active SLE, including active nephritis, had a mean FPA concentration of 10.2 ng/ml (range, 5.3–17.0 ng/ml).

A positive correlation was found between the concentration of plasma FPA and serum DNA-binding activity and an inverse correlation was found between plasma FPA and the concentration of serum C3. No correlation existed between plasma FPA and concentration of serum creatinine. Several possibilities for the origin of plasma FPA in patients with SLE were considered; at present it seems most likely that FPA arises through the action of thrombin on fibrinogen.

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INTRODUCTION

Thrombin is a proteolytic enzyme which acts on the Aα and Bβ chains of fibrinogen to release 2 mol each of fibrinopeptide A (FPA)[1] and fibrinopeptide B (FPB) and 1 mol of fibrin monomer (1). The release of FPA is more rapid than the release of FPB, and clotting occurs as soon as FPA has been cleaved from fibrinogen (2, 3). Thus the determination of FPA release is a direct measure of the conversion of fibrinogen to fibrin. Nossel et al. (4, 5), Gerrits et al. (6), and Budzynski et al. (7) have recently developed radioimmunoassays for the detection of FPA. We are reporting the independent development of such an assay which uses an improved method for separating FPA from antigenically related material in plasma. This assay has been used to determine the level of FPA in normal donors, patients with disseminated intravascular coagulation (DIC), and patients with systemic lupus erythematosus (SLE). In the latter group, close correlation was observed between plasma FPA levels and other parameters of disease activity.

METHODS

Preparation of immunogen and antiserum. Human fibrinogen was prepared from fresh plasma by cold ethanol fractionation, followed by DEAE-cellulose chromatography according to the method of Laki (8). Fibrinogen (1-g portions) was clotted by the addition of 100 U of bovine thrombin (Parke Davis & Co., Detroit, Mich.). After 2 h, the supernate and the first wash of the homogenized clot were combined and concentrated by lyophilization. The peptide fraction was isolated by chromatography on Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in 0.1

[1] *Abbreviations used in this paper:* FPA, fibrinopeptide A; FPB, fibrinopeptide B; GuHCl, guanidine hydrochloride; DIC, disseminated intravascular coagulation; SLE, systemic lupus erythematosus; TCA, trichloroacetic acid.

M NH_4HCO_3, and was further purified by preparative high-voltage electrophoresis on Whatman 3 MM paper at pH 6.5 in pyridine : acetic acid : water (25 : 1 : 225) at 50 V/cm. Several ninhydrin-positive bands were found. After elution of the bands, the fraction having an amino acid composition corresponding to that of FPA was used for synthesis of immunogen. Purified FPA (11 mg) and human serum albumin (22 mg) in water (1 ml) were coupled by reaction with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide (200 mg) for 1 h at room temperature. Reagents were removed by threefold dialysis in 2 liters of water. The immunogen was diluted with water to a final concentration of 2 mg FPA/ml of water. This solution was emulsified with an equal volume of Freund's adjuvant (Difco Laboratories, Detroit, Mich.) plus added tubercle bacilli (10 mg/ml). The emulsion was injected into the toe pads or into multiple intradermal sites of New Zealand white rabbits. Injections were repeated at intervals of 2–4 wk., a total of two to six injections were administered.

Synthesis of peptides. Tyrosylalanylaspartylserylglycylglutamylglycylaspartylphenylalanylleucylalanylglutamylglycylglycylglycylvalylarginine (Tyr-FPA) and alanylaspartylserylglycylglutamylglycylaspartylphenylalanylleucylalanylglutamylglycylglycylglycylvalylarginine (FPA) were synthesized by using the solid-phase method of Merrifield (9, 10). Boc-Arg (Tos) was esterified to Biobeads SX·1 (Bio-Rad Laboratories, Richmond, Calif.), 0.69 meq Cl/g, by heating equimolar amounts of material under reflux conditions in ethanol containing 0.9 mol eq of triethylamine. After a standard purification procedure (10), amino acid analysis showed 80.7 μmol arginine/g support (13%). Synthesis of the peptides was completed by using the cycle of synthetic operations described previously (11). Amino acids were coupled using the N^{α}-*tert*-butoxycarbonyl derivatives (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif). Side-chain protection was: arginine, N^{G}-tosyl, tyrosine, *O*-benzyl; serine, *O*-benzyl; aspartic acid, β-benzyl ester, and glutamic acid, γ-benzyl ester. The molar ratio of added amino acid to the growing peptide was 3.5 : 1. On completion of the synthetic operations, 1.5 g of protected peptidyl resin was treated with HF-10% anisole (vol/vol) for 90 min at 0°C. After evaporation, the residue was extracted with 1 M acetic acid, filtered, and lyophilized to yield 135 μmol of product.

In separate purifications, portions of the products (40 μmol) were dissolved in 6 ml of 1 M acetic acid and fractionated on a 1.2 × 290-cm column of Sephadex G-25 in 1 M acetic acid. Single asymmetrical peaks were detected by their absorbance at 260 nm. The fractions comprising the ascending portion of these peaks were pooled; the trailing, descending portion of the peaks were discarded. The pooled material was applied to a 1.2 × 25-cm column of sulfopropyl Sephadex equilibrated with 0.1 M sodium citrate, pH 3.5, and was developed with a linear gradient of sodium citrate, from 0.1 to 0.5 M at pH 3.5. Both FPA and Tyr-FPA were eluted as the dominant peak midway through the gradient. The fractions constituting this peak were pooled, lyophilized, and desalted on a Sephadex G-10 column in water. The purity of both FPA and Tyr-FPA was assessed by amino acid analysis, high voltage paper electrophoresis under conditions previously described, thin-layer chromatography on silica gel G in three different solvent systems (ethyl acetate : benzene : : 164 : 36; *n*-butanol : 3% NH_3 : : 150 : 66; 1-butanol : pyridine : acetic acid : water : : 75 : 50 : 15 : 60), and electrophoresis at pH 10 in 16% polyacrylamide gels (12). Both FPA and Tyr-FPA were judged to be homogeneous by these methods.

Synthetic FPB was purchased from Schwarz/Mann, Orangeburg, N. Y.

Iodination. 1.8-μg aliquots of Tyr-FPA were iodinated with 1 mCi of carrier-free ^{125}I-Na (New England Nuclear, Boston, Mass.) by using the chloramine-T method of Hunter and Greenwood (13). The iodinated peptide was separated from unreacted iodine and peptide degradation products by chromatography on a 0.9 × 60-cm column of Sephadex G-10 in a buffer composed of 0.01 M KH_2PO_4, pH 7.4, 0.15 M NaCl, 0.01% thimerosal, and 0.1% bovine serum albumin. The peptide incorporated 25–50% of the isotope, and specific activities of 210–420 Ci/mmol of FPA were obtained in different experiments.

Immunoassay. The immunoassay solution consists of 0.2 μl antiserum R17, 4 pg ^{125}I-Tyr-FPA (approximately 10,000 cpm), varying amounts of FPA standard or sample, 1 mg bovine γ-globulin[2] (as "protective protein" and coprecipitant), and 10 μmol EDTA and 10 μmol 2,3-dimercapto-1-propanol (as protease inhibitors); the mixture is brought to a final volume of 1 ml with phosphate-buffered saline. In control tubes, either FPA or antiserum was omitted. The mixture was incubated overnight at 4°C, and an equal volume of saturated ammonium sulfate at pH 8 was added. The tubes were kept in an ice bath for 30 min, and the precipitate was separated by centrifugation. Radioactivity was measured in a crystal scintillation spectrometer. The control that lacked antiserum bound approximately 10% of the added counts. After the precipitate was washed with 50% saturated ammonium sulfate, the background radioactivity could be reduced to 3–4%, indicating that the majority of the isotope remaining in the control tubes was entrapped in the precipitate. Correction for entrapped counts was made without washing because these values were very reproducible. The control tubes lacking FPA contained 35–45% of the isotope in the precipitate. Radioimmunoassay binding curves were analyzed and sample values were determined by using a computer-assisted, weighted least-squares fit to a logit transform of the binding data (14); FPA standards and control tubes were included in every group of analyses, and were used in computation of the standard curve.

Plasma digestion. Endogenous plasminogen in blood plasma and fibrinogen solutions (A. B. Kabi, Stockholm, Sweden) was activated by the addition of 100 U/ml of streptokinase-streptodornase (Lederle Laboratories, Pearl River, N. Y.) to each sample. Samples were removed from the digestion mixtures at intervals, chilled in an ice-water bath, and mixed with an equal volume of cold 20% TCA. After centrifugation, extraction of the TCA-soluble FPA-containing fraction was performed as described below. FPA immunoreactivity was determined by radioimmunoassay. The plasma used in these experiments was freshly obtained from a normal volunteer, and contained heparin, 70 U/ml, and EDTA, 10 mM. Fibrinogen was dissolved in phosphate-buffered saline at 37°C and diluted to a concentration of 2.5 mg/ml as determined spectrophotometrically at 280 nm using $\epsilon_{1\,cm}^{0.1\%} = 1.39$.

Collection and preparation of plasma samples. Blood samples (9 ml) were collected with heparinized 19-gauge scalp-vein needles and polypropylene syringes containing 500 U of heparin. Samples were immediately transferred

[2] Although one lot of bovine γ-globulin was found to be free of cross-reacting bovine FPA, other lots were unsatisfactory. Therefore, in the standard procedure adopted after these original determinations were performed, 2.5 mg of ovalbumin was substituted.

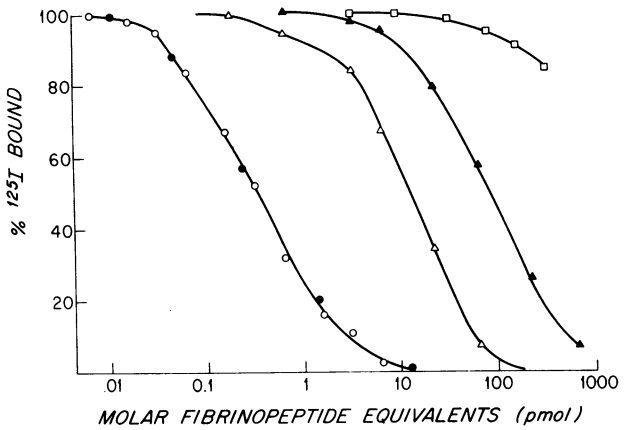

FIGURE 1 Binding of fibrinopeptides to antisera. Antisera and a tracer dose of ^{125}I-Tyr-FPA (4 pg; 10,000 cpm) were incubated with the indicated dose of fibrinopeptide (open symbols) or fibrinogen containing the indicated molar equivalent of fibrinopeptide (closed symbols). Incubation conditions and methods for determining the number of radioactive counts bound to antiserum were as described in Methods. Antiserum R17, 1:5,000 dilution, incubated with FPA (○); antiserum R17, 1:5,000, incubated with fibrinogen (●); antiserum R10, 1:2,000, incubated with FPA (△); antiserum R10, 1:2,000, incubated with fibrinogen (▲); antiserum R17, 1:5,000, incubated with FPB (□).

to new polypropylene tubes containing 1.0 ml of anticoagulation solution that contained 38 mg trisodium citrate, 0.5 mg soy bean trypsin inhibitor, 1 mmol ε-aminocaproic acid, and 10 μmol Na_2EDTA. The samples were centrifuged for 15 min at 2,000 *g* in the cold, and the plasma was transferred with a disposable polyethylene pipet to another new polypropylene tube and stored at −20°C until tested. A 2-ml sample of plasma was precipitated with an equal volume of ice-cold 20% TCA in 16 × 100-mm glass tubes in an ice bath. Samples were centrifuged immediately, and the supernates were decanted into 16 × 100-mm test tubes. The supernates were extracted three times with 3 ml of ether using a Vortex mixer to combine the fluid phases and centrifugation to separate them. After three extractions, the pH of the aqueous phase, which contains the FPA, was above 3. A drop of mixed methyl red indicator (15) was added, and the samples were titrated to neutrality by adding 10 N NaOH with a syringe microburet equipped with a finely pulled glass tip. Neutralization of the extract of a 2-ml plasma sample required 25–30 μl of 10 N NaOH, and resulted in dilution of the sample by about 0.01 vol or less. The samples were heated to 50°C in a water bath, then removed from the bath and placed under water aspirator vacuum until they began to boil. The vacuum was quickly released and the evacuation process repeated five times. Incomplete removal of ether results in falsely elevated levels in the calculated plasma concentration of FPA.

Assays for DNA-binding activity and C3 concentration. DNA-binding activity was measured by the Millipore filter technique (16). KB cell DNA labeled with ^{125}I (Electro-Nucleonics Laboratories, Bethesda, Md.) and nonradioactive calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) were combined in 0.15 M Tris buffer pH 7.5 at a final concentration of 1.0 μg/ml (~2×10^4 cpm/μg). In the assay 0.1 μg ^{125}I-DNA and 25 μl of test serum were incubated at 37°C for 30 min and subsequently filtered through a HAWP Millipore filter (Millipore Corp., Bedford, Mass.). After washing the filter, the radioactivity retained on the filter was measured in a crystal scintillation spectrometer; binding activity was expressed as a percentage of the total radioactivity added to serum. For normal sera the mean percentage of binding plus 2 SDS was 30%.

The serum concentration of C3 was estimated by using commercial immunodiffusion plates (Behring Diagnostics, Somerville, N. J.).

Selection of subjects. Plasma samples were obtained from 20 healthy donors of both sexes whose ages ranged from the 2nd–5th decades. Five patients with DIC who fulfilled previously published criteria for the diagnosis of this disorder (17) were studied; in four patients DIC was attributed to sepsis and in one patient was of unknown etiology. Patients with SLE (28 females, 1 male) were selected from the inpatient and outpatient services of the Massachusetts General Hospital and the Clinical Center of the National Institutes of Health. These patients met the preliminary criteria of the American Rheumatism Association for the diagnosis of SLE (18). The following criteria led to exclusion from the study: recent surgery, hemodialysis, major infection, presence of indwelling venous or arterial catheters, or platelet counts less than 100,000. There were 29 patients with SLE available for study. These patients were categorized as having active SLE if, in the absence of other causes, one or more of the following conditions were present: alopecia, arthritis, active skin lesions, pleuritis, pericarditis, peritonitis, hepatitis, pneumonitis, myocarditis, or unexplained fever. Lupus nephritis was considered to be present in an active form in patients with one or more of the following conditions: red cell casts present in freshly voided urine, granular casts associated with hematuria equal to or greater than 20 erythrocytes/high power field, pyuria equal to or greater than 20 leukocytes/high power field and not accounted for by infection, or proteinuria equal to or greater than 1 g/24 h.

Statistical treatment. Data from control and patient groups were analyzed by the chi-square test with Yates' correction.

RESULTS

Characterization of antibody. 10 rabbits were immunized with FPA-human serum albumin conjugates; of these, two produced antibodies with sufficiently high binding affinities and titers to be useful in the assay. At high concentration, antisera R10 and R17 bound up to 90% of a tracer dose of ^{125}I-Tyr-FPA (4 pg; 10,000 cpm). A 1:2,000 dilution of antiserum R10 bound 37% of the tracer dose; of this amount, 50% was displaced by 22 ng of unlabeled FPA (Fig. 1). A 1:5,000 dilution of antiserum R17 bound 47% of the tracer dose; of this amount, 50% was displaced by 0.5 ng of FPA. Synthetic FPA, natural FPA, and fibrinogen containing equivalent amounts of FPA were equipotent in the displacement of bound ^{125}I-Tyr-FPA from antiserum R17. In contrast, 50% displacement of the label from antiserum R10 required a 6.5-fold molar excess of FPA equivalents as fibrinogen, in comparison to synthetic FPA (Fig. 1). The binding of FPB to antiserum R17 was much less than that of FPA; 500 ng of FPB (mol

wt, 1,535) displaced a tracer dose of ^{125}I-Tyr-FPA to the same extent as 0.1 ng of FPA (mol wt, 1,537).

Despite the increased cross-reactivity with fibrinogen of antiserum R17 compared to antiserum R10, R17 was routinely used at a dilution of 1:5,000 because of the greater sensitivity of the assay performed under this condition.

Reproducibility and accuracy of method. 20 replicates of the same plasma sample from a normal donor were processed as described. The mean FPA concentration was 0.88 ng/ml, with an SD of 0.31 ng/ml. In another experiment, varying quantities of FPA were added to replicate plasma samples, which were then processed and assayed. The results, shown in Table I, indicate reproducible recovery of the added peptide. The measured recovery usually exceeded the amount of FPA that was added by approximately 10%, which reflects the reduction in volume caused by ether extraction of the TCA supernate. Assay values reported subsequently were corrected for this volume change.

A high-SD of the mean was observed in replicate determinations of samples containing low concentrations of FPA. In terms of absolute nanograms per milliliter, however, the deviations are low, and the percentage deviation ([SD/mean]100) decreases as FPA concentration increases. The data in Table II demonstrate a similar decrease in error as the FPA concentration increases.

Three investigations were performed to determine whether immunoreactive substances other than FPA were present in plasma extracts. First, extracts prepared from the plasma of several patients with high concentrations of FPA were pooled and serial dilutions prepared in phosphate-buffered saline and bovine serum albumin. The FPA concentration of each diluted sample was determined (Table II). The results indicated that the measured concentration of FPA in each dilution corresponded to the calculated concentration in the diluted sample. These findings indicate that the plasma extracts did not contain materials that affected antibody binding in a manner different from FPA.

TABLE I
Recovery of FPA Added to Normal Plasma

FPA added	Total FPA concentration determined by RIA*	Added FPA recovered	
ng/ml	*ng/ml*	*ng/ml*	% (± *range*)
0	1.94		
1	3.28	1.34	134 (±32)
5	7.43	5.49	110 (±7)
10	13.30	11.20	112 (±6)
50	57.70	55.70	111 (±10)

Table entries are the means of quadruplicate determinations. A single plasma sample was used for all tests. After addition of a known quantity of synthetic FPA, samples were processed as described. The amount of added FPA was based on amino acid analysis of a stock solution.
* Radioimmunoassay.

TABLE II
FPA Immunoreactivity in Serial Dilutions of a Plasma Extract

Dilution	FPA		
	Calculated	Measured	
	ng/ml	*ng/ml ±1 SD*	*n**
None	—	5.56±0.40	(15)
1:2	2.78	2.64±0.34	(8)
1:4	1.39	1.43±0.16	(8)
1:8	0.70	0.77±0.22	(8)
1:16	0.35	0.32±0.15	(8)
1:32	0.17	0.14±0.10	(8)

A pool of plasma extract was serially diluted 1:2 in phosphate-buffered saline and bovine serum albumin, and FPA immunoreactivity was determined in each dilution.
* Number of determinations performed with each dilution in parentheses.

In the second experiment, endogenous plasminogen was activated by the addition of streptokinase-streptodornase to plasma or fibrinogen solutions (Table III). The results show that in the presence of EDTA, FPA immunoreactivity increases slowly. In contrast, in the absence of EDTA, there is a significant increase in FPA immunoreactivity after 60 min of digestion time. Gel electrophoresis of the fibrinogen solutions digested in the presence of EDTA showed the appearance of X and Y fragments during the 30- to 240-min incubation period; D and E fragments did not appear until 24 h of digestion time, corresponding to a stage 3 digest (7). In the absence of EDTA, 60 min of digestion results in a stage 3 digest (7). These experiments indicate that FPA immunoreactivity is not markedly increased in fibrinogen solutions until extensive plasmin digestion has occurred, and that digestion occurs at a slow rate in plasma.

In a third experiment, 25 ml of plasma from a patient with DIC was processed in the conventional manner, except that the extracted material was lyophilized, desalted on Sephadex G-10, relyophilized, and the dried residue dissolved in 3 ml of 6 M guanidine HCl (GuHCl). The solution was applied to a 1.2 × 100-cm column of Sephadex in 6 M GuHCl. The FPA activity

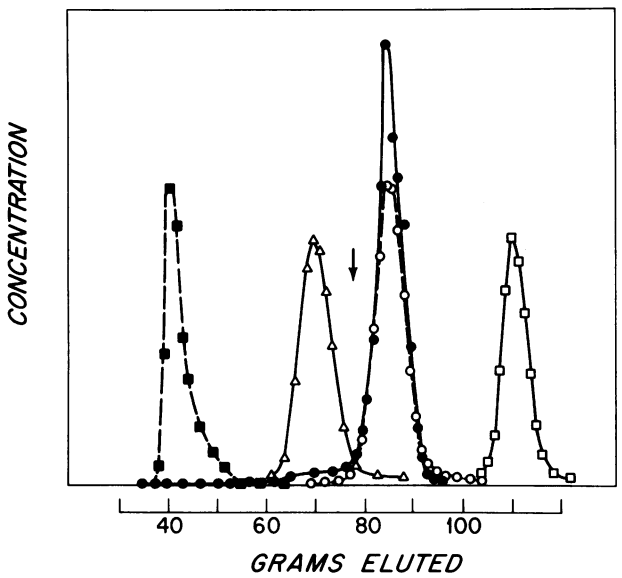

FIGURE 2 Chromatography of FPA immunoreactivity on Sephadex G-50 in 6 M GuHCl. A TCA plasma extract from a patient with DIC was applied to the column (see text). Elution positions were determined by weight in tared tubes. FPA immunoreactivity was determined by assays of small samples (5–50 μl) of the column effluent. Assays were performed as described in Methods except that all standard and control tubes contained a volume of 6 M GuHCl equal to that present in the samples. FPA immunoreactivity from the DIC extract (●); FPA immunoreactivity of native FPA, determined separately (○). In addition, the column was calibrated separately with immunoglobulin heavy chain (■), detected by absorbance at 280 nm, and with reduced and carboxymethylated [^{14}C]insulin B chain (△), detected by liquid scintillation spectrometry. All samples contained 0.1 μCi of $^{3}H_2O$ as a calibration standard (□), detected by liquid scintillation spectrometry. The radioactivity peak of the $^{3}H_2O$ marker was eluted within 1 g of the same position in all experiments. The arrow indicates the calculated elution position of a 23 residue peptide based on the hydrodynamic size relationship given in reference 23.

was eluted from this column as a single, symmetrical peak in a position identical with that of native FPA (Fig. 2). The elution profile of synthetic FPA (not shown in Fig. 2) was also determined in a separate experiment with the same column and was identical with that of native FPA. The elution volume of insulin B chain (30 amino acid residues) was clearly differentiated from that of FPA (16 amino acid residues). This experiment demonstrates that there are no detectable immunoreactive substances differing in size from native FPA in plasma from a patient with a diffusely activated coagulation mechanism. The smallest cross-reacting peptide expected is the 1-23Aα plasmin digestion product, which would be eluted in the position indicated by the arrow in Fig. 2.

FPA in normal donors. The concentration of FPA in the blood of 20 healthy donors was found to range from 0.6 to 1.9 ng/ml with a mean of 0.9 ng/ml and an SD of 0.3 ng/ml. Blood was obtained daily from the same antecubital vein in three normal donors to test the stability of plasma FPA values, as well as the effect of repeated phlebotomy on the concentration of FPA. The results of these determinations are shown in Table IV. With three exceptions, all values fell in the normal range. The markedly elevated value in donor 1 on day 2 was probably consequent to a traumatic venipuncture. The other two elevated values were not associated with venipunctures that were recognized to be unusual. Although samples were routinely obtained with a 19-gauge scalp-vein needle (to facilitate collection of multiple samples), no significant difference was seen in the FPA concentration of samples collected with disposable needles attached directly to heparinized syringes.

FPA in patients with DIC. DIC is characterized by the rapid consumption of clotting factors and the generation of fibrin within the circulation (17, 19). To determine whether patients with DIC also manifested elevated levels of FPA, plasma from five patients with this disorder was tested (Table V). FPA values ranged from 13 to 346 ng/ml. None of these patients had been treated with heparin before sampling.

FPA in patients with SLE. The concentration of FPA was determined in plasma samples from 29 patients with SLE; values ranged from 0.7 to 17.0 ng/ml. There was a significantly greater concentration of FPA in plasma from patients with SLE compared to controls ($\chi^2 = 4.1$, $P < 0.001$). The patients were assigned to four subgroups on the basis of clinical criteria listed in

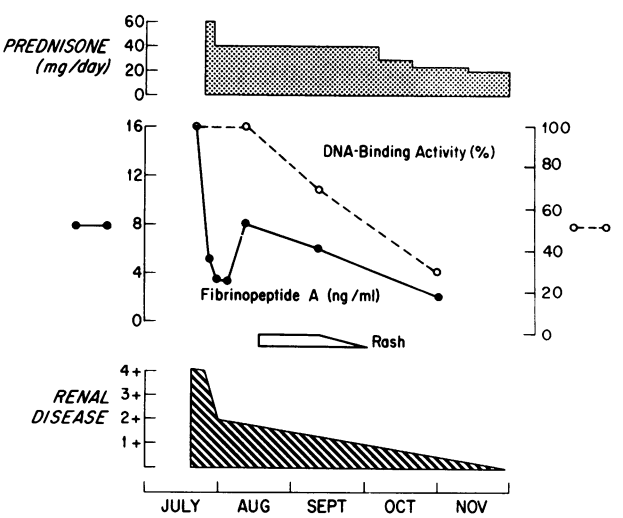

FIGURE 3 Relationship of the concentration of plasma FPA and serum DNA-binding activity to the clinical course and treatment of a patient with SLE. The designation 4+ refers to active nephritis with serum creatinine greater than 1.5 mg/100 cm^3; 2+ refers to active nephritis and a normal serum creatinine level.

Methods (Table VI). FPA values shown are those obtained on the first sample from each patient. 13 patients were in remission; of these, 4 had been free of renal disease while 9 previously had nephritis. 15 patients had active disease; of these, 9 were currently free of renal disease while 7 had active nephritis. The FPA values of patients with active disease were significantly greater than those of patients with inactive disease ($\chi^2 = 7.96$, $P < 0.01$). It is of interest that among patients with active disease, FPA values were elevated in those with and without renal involvement.

The DNA-binding activity of serum from 28 of the patients with SLE was determined and compared with the plasma level of FPA. The FPA levels of patients with DNA-binding activity greater than 30% of the labeled DNA added were significantly greater than that of patients with DNA-binding activity less than 30% ($\chi^2 = 5.17$, $P < 0.05$).

FPA was measured in several plasma samples from one patient with SLE whose active disease was rapidly controlled by corticosteroid therapy (Fig. 3). The plasma FPA concentration decreased rapidly after treatment was begun and it paralleled clinical improvement. During convalescence a rise in FPA concentration was observed; this change was followed by recurrence of a skin rash. The FPA concentration again declined with subsidence of these lesions. During this entire episode, the DNA-binding activity gradually declined; no increase in binding activity in relationship to the skin rash was observed.

The concentration of C3 in serum from 17 patients was compared with the concentration of FPA determined on a corresponding plasma sample (Fig. 4); in the 12 other patients with SLE, simultaneous C3 and FPA values were not available. 8 patients had normal concentrations of C3; of these, FPA values were normal in 4 patients, slightly elevated in 2 patients (2.2 ng/ml), and moderately elevated in 2 patients (3.1 and 4.8 ng/ml). With one exception, patients with a decreased concentration of C3 had elevated levels of FPA.

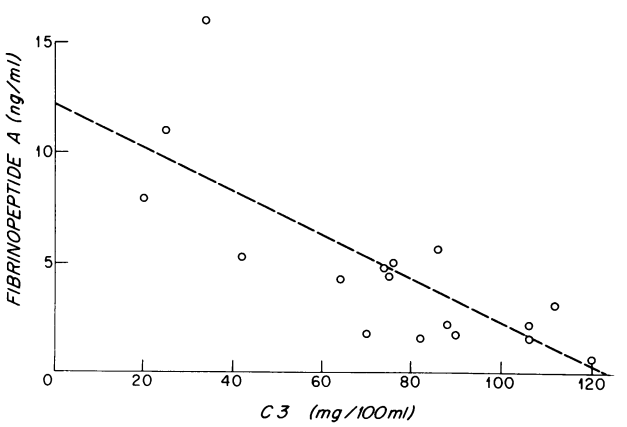

FIGURE 4 Inverse relationship between the concentration of plasma FPA and concentration of serum C3 in patients with SLE. The $r = -0.764$ ($P < 0.01$); the regression line is included in the figure ($y = -0.099\ X + 12.2$).

TABLE III

Percentage of Total FPA Equivalents Appearing in TCA Extract of Endogenous Plasmin-Digested Samples

Digestion time	Sample: Plasma with heparin and EDTA	Sample: Fibrinogen in PBS* and EDTA	Sample: Fibrinogen in PBS*
0 min	0.014	3.8	1.0
30	0.21	4.4	1.4
60	0.23	5.2	10.1
120	0.24	—	48.9
24 h	0.48	7.9	80.0

* Phosphate-buffered saline.

In 12 patients with SLE, simultaneous creatinine and FPA levels were available for comparison. Within this group a single patient had an elevated serum creatinine of 2.2 mg/dl, but a near normal FPA level of 1.8 ng/ml. Four patients had simultaneous elevations of FPA and creatinine. The remaining 7 patients had normal creatinine levels but FPA values ranged from 0.7 to 11.0 ng/ml. Among these 12 patients there was no significant relationship ($\chi^2 = 0$; $P > 0.05$) between the serum creatinine and the measured FPA level.

DISCUSSION

A major requirement in the development of a radioimmunoassay for FPA is for discrimination between free FPA and the FPA sequence of fibrinogen and certain fragments of fibrinogen. One approach is the production of antisera that are capable of recognizing determinants present only on free FPA. In the present experiments, immunogen was prepared by coupling purified native FPA to human serum albumin with carbodiimide. The antisera produced in response to this immunogen, however, failed to distinguish between free FPA and the FPA sequence of native fibrinogen (R17), or it showed only moderate preference for the free FPA

TABLE IV

FPA during Serial Phlebotomies in Normal Donors

Donor	Days...	FPA, ng/ml: 1	2	3	4	5
1		1.0	7.1*	2.3	1.5	1.4
2		0.6	0.8	1.0	2.3	0.8
3		0.8	1.1	1.1	1.2	0.8

* Required several attempts to obtain specimen of blood.

TABLE V
FPA Concentration in Patients with DIC

Patient no.	FPA
	ng/ml
1	13.0
2	17.0
3	40.0
4	200.0
5	346.0

(R10). An alternate approach is the quantitative separation of FPA from fibrinogen and other cross-reacting plasma constituents. Precipitation of plasma proteins with TCA provided a reliable means for separation. This technique was validated by experiments that demonstrated the reproducible determination of FPA in replicate samples and adequate recovery of various increments of FPA added to normal plasma (Table I).

Nossel et al. (5) have previously reported a different separation technique based on ethanol precipitation of proteins followed by dialysis of the supernate to recover FPA. While the two methods have not been compared directly, TCA precipitation seems more rapid and allows a larger number of samples to be processed by one technician.

Three experiments were performed to determine whether any immunoreactive substances other than FPA might be present in TCA extracts of blood plasma. In the first of these (Table II), measured values of FPA conformed with calculated values in dilutions of a single pool of TCA extract of plasma. Immunoreactive substances containing the FPA sequence would be expected to show binding curves identical with those of native FPA, and this prediction holds for native fibrinogen (Fig. 1). Nonspecifically cross-reacting substances, however, are very unlikely to have the same binding properties as the native FPA determinant, and therefore the binding curve would be altered. The accuracy with which FPA concentrations were predicted in the diluted extract therefore demonstrates that cross-reacting substances were not isolated in any significant quantities during TCA extraction of FPA from plasma.

In the second experiment (Table III) it was shown that extensive plasmin digestion of fibrinogen is required before FPA immunoreactivity increases in TCA extracts of fibrinogen solutions. The results of these experiments corroborate those of Budzynski et al. (7) who also noted that increase of FPA immunoreactivity was associated with the appearance of plasmin fragments of the D and E type. Based on molecular size, fragments D and E would be separated from FPA by either the TCA fractionation method here described or by the ultrafiltration system used by Budzynski et al. (7). The increase in immunoreactivity, therefore, is not due to the presence of fragment E in the extracts, nor is it due to increase in FPA, since plasmin does not cleave the 16–17 peptide bond of the Aα chain, which contains the FPA sequence (20). Low molecular weight fragments begin to appear only after the Aα chain has been extensively degraded, as signaled by the appearance of D and E fragments. A small peptide, the 1-23 Aα fragment, is produced late in plasmin digestion of fibrinogen, but at a low generation rate (20).

TABLE VI
Plasma Concentration of FPA in Patients with SLE

Groups							
I. Inactive disease, no history of renal involvement		II. Inactive disease, history of renal involvement		III. Active disease without renal involvement		IV. Active disease with renal involvement	
Patient	FPA level	Patient	FPA level	Patient	FPA level	Patient	FPA level
	ng/ml		*ng/ml*		*ng/ml*		*ng/ml*
C. M.	0.7	F. C.	1.8	M. S.	4.8	L. B.	7.9
R. R.	1.7	E. B.	2.1	M. M.	4.4	T. W.	16.0
B. C.	1.6	T. W.	5.6	A. F.	6.8	S. S.	10.0
M. B.	1.8	C. M.	1.6	F. C.	4.0	G. B.	17.0
		S. K.	2.0	M. G.	2.4	C. R.	8.3
		C. V.	2.6	R. Z.	5.0	F. B.	7.8
		C. G.	4.6	D. G.	3. 0	L. P.	5.3
		L. L.	3.3	D. S.	7.8		
		M. B.	1.1	D. D.	2.2		
Mean	1.5		2.7		4.5		10.2
Median	1.65		2.1		4.4		8.3

The third experiment indicates that low molecular weight, plasmin-derived fragments containing FPA were not produced at a sufficient rate to interfere with the measurement of FPA. Gel-filtration chromatography under dissociating conditions was performed with a TCA extract of plasma from a patient with DIC (Fig. 2). In DIC appreciable amounts of plasmin-produced fragments cross-reactive with FPA might appear in plasma as a consequence of activation of the clotting and fibrinolysin systems. Of chief concern was the possibility that the 1-23 Aα-chain fragment produced by the action of plasmin on fibrinogen (20) might be present in significant amounts and extracted from such samples together with FPA. Gel filtration of such a plasma TCA extract showed a single peak of FPA activity corresponding to the elution position of native FPA (Fig. 2). Insulin B chain (30 amino acid residues) was readily resolved from FPA on this column. The elution position of each peptide is a function of its hydrodynamic size. In the strong dissociating solvent used in elution, hydrodynamic size is a function of the number of residues in the peptide chain (21). The hydrodynamic size of the 1-23 Aα-chain fragment was calculated from data in reference 21. Based on these calculations, the fragment would appear at the position indicated by the arrow in Fig. 2; little immunoreactive material was detected at this position. Thus it appears that no significant cross-reacting substance was detectable in the plasma extract. Nossel et al. (5) also concluded that the immunoreactive substance in the plasma of patients with DIC was FPA.

The FPA assay was applied to an examination of the role of the clotting system in SLE. Fibrin deposition in the renal glomerulus is believed to be important in the pathogenesis of lupus nephritis (22–24). The deposition of antigen–antibody-complement complexes in the glomerulus may result in localized intravascular coagulation with consequent fibrin deposition (25). Evidence for the occurrence of low-level DIC in SLE has been provided by studies based chiefly on the detection of fibrinogen/fibrin degradation products in serum (22–24). Since production of fibrinogen/fibrin degradation products generally depends on clotting followed by fibrinolysis, detection provides only an indirect estimate of clotting. Some investigators have also reported abnormal coagulation studies (23), while others have failed to find consistent changes (26). Since the FPA assay measures the initial alteration of the fibrinogen molecule in the clotting process and does not depend on depletion of clotting factors or the secondary release of fibrin degradation products, its application to studies of patients with SLE may directly define the rate of intravascular coagulation.

In the present study, FPA values above the normal range were detected in plasma from 22 of 29 patients with SLE. These patients were classified in four categories on the basis of the activity of their disease and the presence or absence of renal involvement (Table VI). Patients with inactive disease tended to have plasma FPA values within the normal range, although in group II, four patients with a history of renal involvement had clearly elevated FPA levels. Patients with active disease without renal involvement (group III) had a mean FPA level of 4.5 ng/ml. The major sites of involvement in these patients included the joints, skin, serosal surfaces, lung, and liver. There was no consistent relationship between any one manifestation of disease activity and a unique plasma FPA value. Patients with active renal disease (group IV) had the highest FPA levels; a mean concentration of 10.2 ng/ml was found in this group. Four of the seven patients in group IV had elevated levels of serum creatinine. It is possible that renal impairment contributed to the high levels of FPA observed in these patients. Stiehm and Trygstad (20), as well as others (27), have noted that patients with uremia invariably had high levels of fibrinogen/fibrin degradation products. It should be noted, however, that among the present group, elevated levels of FPA were seen in the absence of uremia in patients with active SLE. Thus, factors other than impaired renal clearance can lead to elevated FPA levels in patients with active SLE.

The capacity of serum to bind DNA and the concentration of serum complement reflect disease activity in SLE. An increase in DNA binding, a fall in concentration of C3, or both, often heralds or accompanies an increase in disease activity (28, 29). In this study, a positive correlation was found between levels of plasma FPA and DNA-binding activity, and an inverse correlation was found between plasma FPA and concentration of C3 (Fig. 4). Since both enhanced DNA-binding activity and complement depletion are closely related to the pathogenesis of the renal lesions in SLE, the generation of FPA may also be related to this process. Additionally, FPA release may occur at other sites of conversion of fibrinogen to fibrin in diverse, inflamed organs, such as the skin (30).

While it seems likely that the FPA measured in this study results from the action of thrombin on fibrinogen, other proteolytic enzymes may also be responsible. Plow and Edgington (31) have recently described a leukocyte protease that cleaves fibrinogen at physiologic pH. If FPA were one of the products of this enzyme, then a cell type that is closely related to inflammation might account for the relationship between disease activity and FPA generation that is observed in patients with SLE.

Finally it must be considered that the increased FPA levels observed in SLE may reflect the increased metabolic turnover of fibrinogen. Patients with SLE tend

to have elevated plasma fibrinogen (32). Few turnover studies in patients with SLE have been reported (33). In rheumatoid arthritis, greatly increased catabolic and synthetic rates were found; the increased synthetic rate was roughly proportional to the severity of the disease process (34). Direct comparisons between FPA levels and fibrinogen turnover will be required to test the possibility that elevated FPA in SLE reflects enhanced metabolism of fibrinogen.

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