The Generation of Fibrinopeptide A in Clinical Blood Samples

EVIDENCE FOR THROMBIN ACTIVITY

H. L. NOSSEL, M. TI, K. L. KAPLAN, K. SPANONDIS, T. SOLAND, and V. P. BUTLER, JR.

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York 10032

ABSTRACT Plasma fibrinopeptide A (FPA) concentrations were measured in clinical blood samples incubated in the collecting syringe for different time periods before addition to heparin and Trasylol, and the rate of in vitro generation of FPA was calculated as the mean increment in FPA concentration per minute over the linear portion of the generation curve. 36 normal individuals had a mean plasma FPA level of 0.64±0.56 pmol/ml and an FPA generation rate of <0.5 pmol/ml per min. Clinical samples with elevated plasma FPA levels manifested slow (<1 pmol/ml per min) (28 patients) or rapid FPA generation (>1 pmol/ml per min) (33 patients). Slow FPA generation was found in 10/10 patients with venous thrombosis, in 4/4 with aortic aneurysm, and in several patients with acquired hypofibrinogenemia. In one such patient, addition of fibrinogen resulted in rapid FPA generation whereas thrombin addition was without effect. Rapid FPA generation was generally linear, was usually associated with slower fibrinopeptide B generation and was inhibited by parenteral or in vitro heparin. It is thought to reflect increased thrombin activity and was seen in patients with pulmonary embolism, active systemic lupus erythematosus, renal transplant rejection, and after infusion of prothrombin concentrates. The initial rate of FPA cleavage by thrombin at fibrinogen concentrations from 0.05 to 4 mg/ml showed little change between 2 and 4 mg/ml with a K_m of 2.99 μM. At a fibrinogen concentration of 2.5 mg/ml the FPA cleavage rate was 49.2±1.6 nmol/ml per min per U of thrombin. Exogenous thrombin added to normal blood generated 21.7 nmol/ml per U of thrombin FPA in the first minute with a nonlinear pattern reflecting inactivation of thrombin and the presence of alternative substrates. Hence, the thrombin concentration in the blood cannot be calculated from the FPA generation rate. The FPA generation rates in clinical samples with rapid generation (1–28 pmol/ml per min) could be produced by 2×10^{-5} to 5.6×10^{-4} thrombin U/ml acting on purified fibrinogen at physiological conditions of pH, ionic strength, and temperature.

INTRODUCTION

Thrombin specifically cleaves fibrinopeptides A and B (FPA and FPB)[1] from fibrinogen to initiate blood clotting (1). Teger-Nilsson demonstrated the presence of the fibrinopeptides chemically in the blood of dogs infused with thrombin (2). The development of a radioimmunoassay (3) has permitted measurement of FPA levels in clinical blood samples as an index of thrombin action in vivo (4–9). A radioimmunoassay for FPB has also been developed and together with the FPA assay, used to measure the relative cleavage rates of small fibrinopeptide containing fractions from fibrinogen by different enzymes in buffer solutions (10).

In the present paper the rate of in vitro generation of FPA was studied by measuring plasma FPA levels in blood incubated in the collecting syringe. At 1-min intervals for a total of 4 min aliquots of blood were mixed with heparin and Trasylol. The plasma FPA level was then measured and generation rates were obtained by expressing FPA levels as a function of incubation time. In patients with elevated FPA levels, two different patterns of FPA generation in vitro have been distinguished; a relatively low rate

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[1] *Abbreviations used in this paper*: FPA, fibrinopeptide A; FPB, fibrinopeptide B.

of generation (<1 pmol/ml per min) and a relatively high rate (>1 pmol/ml per min). Evidence will be presented to indicate that a relatively high rate of FPA generation is due to thrombin activity in the blood.

METHODS

Native fibrinopeptides A and B prepared as described by Blombäck et al. were used as standards throughout these studies (11). Preparation of the antisera to the fibrinopeptides has been previously described (3, 4, 10). Hirudin was obtained from K and K Laboratories, Inc., Plainview, N. Y. Human thrombin purified by Dr. Kent Miller (12) was given to us by Dr. D. Aaronson of the Division of Biological Standards, Bethesda, Md.; the sp act of this material is 2,500 U/mg. Heparin for in vitro use was obtained from Hynson, Westcott & Dunning, Inc., Baltimore, Md. Trasylol was purchased from FBA Pharmaceuticals, Inc., New York. Anhydrous denatured ethanol (Matheson Gas Products, East Rutherford, N. J.) was used to precipitate fibrinogen from plasma samples; syringes and needles for blood collection were as previously described (4). Fibrinogen (Kabi, grade L) was dialyzed against a large volume of barbital-buffered saline (pH 7.4) for 72 h to remove dialyzable FPA immunoreactivity before use. Ovalbumin was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.

The FPA radioimmunoassay was performed as previously described (4). Antiserum R2 diluted in Tris buffer, pH 8.6, (0.05 M Tris hydrochloride, 0.1 M NaCl) containing heparin 15 U/ml was used throughout. The sensitivity of the assay has increased from that previously described so that at present 0.05 pmol FPA inhibits binding of the tracer by 50% and 0.013 pmol/ml inhibits binding by 20%.

The FPB assay was performed as described for the FPA assay except that FPB antisera RB22 and RB 29 and ^{125}I-FPB analogue tracer were used (10).

The results of preliminary experiments indicated that FPB immunoreactivity increased markedly in plasma samples stored at −40°C before being tested. This effect may have resulted from the action of plasmin bound to α_2-macroglobulin which was still active despite the high concentration of Trasylol (13). For the experiments in this study, plasma samples were precipitated with ethanol within ½ h of collection to prevent in vitro generation or digestion of FPB immunoreactivity. Blood samples were collected and mixed with heparin and Trasylol as previously described (4). The tubes were placed in an ice-water bath and within 30 min of collection the cells were separated by centrifugation at 1,700 *g* at 4°C for 20 min. 2 ml of plasma was pipetted into a new 15-ml conical polycarbonate tube (VWR Scientific Div., UNIVAR, San Francisco, Calif.), 2 ml of ethanol was added, and the contents were mixed on a Vortex-genie mixer. After 30 min in an ice bath, the protein precipitate was deposited by centrifugation at 3,000 *g* at 4°C for 15 min. The supernatant fluid was pipetted into a new conical tube and after 30 min in an ice bath was centrifuged at 3,000 *g* for 15 min. The supernatant 2 ml was evaporated to dryness at 40°C in a Brinkmann SC/48 evaporator (Brinkmann Instruments, Inc., Westbury, N. Y.), reconstituted in 1 ml distilled water and dialyzed against 6 ml Tris-buffered NaCl, pH 8.6, with 1 mg/ml of ovalbumin in 13 × 100-mm glass tubes. Visking dialysis tubing (Union Carbide Corp., New York), ¼ inch inflated, was used for dialysis. The dialysis tubes were gently agitated (1 rotation/7 min) at 4°C on a Bellco Glass, Inc. (Vineland, N. J.) roller drum set at an angle so that air bubbles did not agitate the fluid. The FPA level was measured in the dialysis fluid after 24 h. ^{125}I-labeled FPA was dialyzed simultaneously in separate tubes, and the FPA concentration in the dialysis fluid was multiplied by the reciprocal of the percentage tracer present in the dialysate. With this technique, recovery of FPA added to plasma or whole blood in vitro was 100 ±10%.

FPA generation in vitro. The rate of FPA cleavage in blood after sampling was tested as follows: a 19-gauge needle attached to a polyethylene syringe (Sherwood Medical Industries, Inc., St. Louis, Mo.) was inserted into a forearm vein and a stopwatch was started as blood began to enter the syringe. After collection of the sample of blood required (18 ml), the needle was withdrawn from the vein and removed from the syringe. The blood in the syringe was mixed by inversion and 4.5 ml blood was added at 1-min intervals to each of four tubes containing 0.5 ml of 0.15 M NaCl solution with 500 U of heparin and 500 U of Trasylol. The plasma was then processed as described above and the fibrinopeptide levels were measured. When FPA generation was tested in blood collected in two syringes, a 19-gauge butterfly needle was inserted into a vein, a 35-ml polyethylene syringe was attached, and 18 ml of blood was withdrawn and processed as described above. As soon as the first syringe was withdrawn from the needle the second syringe was attached, and a further 18 ml of blood was collected and treated in exactly the same way as the first 18 ml of blood. The influence of added fibrinogen on fibrinopeptide generation was tested by pipetting 2 ml of fibrinogen solution (5 mg/ml) into a blood collection syringe and collecting 18 ml of blood into the syringe in the usual manner. The fibrinogen and blood were mixed thoroughly and the generation rate of fibrinopeptides was determined. The influence of exogenous thrombin on fibrinopeptide generation was tested as for fibrinogen except that a thrombin solution in Tris-buffered saline with 0.1% ovalbumin was pipetted into the syringe in place of fibrinogen.

The effect of EDTA on the in vitro generation of FPA was tested by measuring plasma FPA levels in each of four tubes of blood. Tubes 1 and 2 contained 0.5 ml of 0.15 M NaCl with 500 U of heparin and 500 U of Trasylol. Tube 3 contained 0.5 ml of 0.15 M NaCl with 500 U of heparin, 500 U of Trasylol, and 5 μg of EDTA. Tube 4 contained 250 μl of 0.15 M NaCl with 5 μg of EDTA. 18 ml of blood was collected, and 4.5 ml was rapidly (within 1 min) added to tubes 1, 3, and 4, the contents of which were mixed by inversion. At 3 min, 4.5 ml of blood was added to tube 2, and 0.25 ml of 0.15 M NaCl with 500 U of heparin and 500 U of Trasylol was added to tube 4 and mixed. The blood in each tube was then processed and assayed for FPA content. The protocol for this experiment is given in the legend to Fig. 3.

Two individuals collected all the blood samples, and the interval between the first entry of the blood into the syringe and the mixing of the blood with the anti-coagulant mixture was timed with a stopwatch. This time was less than 50 s. If the blood did not flow freely, a fresh sample was collected. Care was taken to avoid drawing blood from patients who were receiving intravenous infusions. Although in normal individuals an intravenous needle was not associated with a detectable increment in the plasma FPA level in blood drawn from the opposite arm, blood drawn through the intravenous needle did have an elevated plasma FPA level.

FPA generation in the clinical blood samples was generally linear or in some instances accelerating at 4 min, and the rate of FPA generation in vitro in the syringe was calculated by averaging the mean of the generation rates per

TABLE I

Individuals with Insignificant FPA Generation (<1 pmol/ml/min)

Condition	Number	Initial plasma FPA level	Fibrinogen level	FDP level	Thrombin time
		pmol/ml	*mg/100ml*	*μg/ml*	*s*
(a) Initial plasma FPA level in the normal range (<*1.5 pmol/ml*)					
Normals	24	0.64 (0.07–1.2)	200–400	<2	15–16
Clinically suspected thrombophlebitis (not present on phlebography)	3	1.07 (0.9–1.4)	287	0	16. 2 (13.4–20.1)
Carotid artery thrombosis (16 days previously)	1	0.72	490	0	17.7
Septicemia-treated	2	1.25 (1.1–1.4)	335 (240–430)	0	22.1 (15.3–28.9)
Renal transplantation without rejection	3	0.87 (0.8–0.9)	363 (240–480)	0	14.3 (12.3–15.5)
Hepatic cirrhosis	2	1.22 (1.14–1.3)	272 (255–290)	0	22.1 (15.3–28.9)
Congenital dysfibrinogenemia	1	0.78	160	0	32.9
Suspected pulmonary embolism (not present on lung scan)	5	1.34 (1–1.5)	373 (330–440)	0	15.5 (13–17.4)
Systemic lupus erythematosus (inactive)	2	1.25 (1.2–1.3)	337 (270–405)	0	18.4 (15.2–21.6)
(b) Initial plasma FPA level elevated (>*1.5 pmol/ml*)					
Thrombophlebitis	10	7.0 (2.3–16.6)	358 (200–485)	3.6 (0–20)	18.4 (14.9–25)
Aortic aneurysm	4	4.9 (2.3–8.9)	503 (380–740)	1.7 (0–5)	17.6 (17.6–18.3)
Systemic lupus erythematosus	3	3.3 (2–4.2)	368 (340–395)	7 (0–21)	14.3
Defibrination	3	14.4 (4–32.7)	(See Table II)		
Neoplasia	3	3.0 (2.2–4)	567 (230–870)	1.7 (0–5)	20.8 (17.9–25.9)
Sepsis	4	4.1 (1.7–7.1)	555 (240–820)	3.8 (0–10)	20.4 (15.7–26.2)
Ruptured varix (lower limb)	1	2.5	340	—	—

minute over the linear part of the curve. FPA generation in normal blood with added exogenous thrombin decreased with time and was not linear.

The rate of fibrinopeptide cleavage from fibrinogen by thrombin vitro was determined as follows: a 900-μl fibrinogen (0.05–4 mg) solution was pipetted into 15.0-ml conical polypropylene centrifuge tubes in a waterbath (Eberbach Corp., Ann Arbor, Mich.) at 37°C. 100 μl of thrombin (1×10^{-5} to 2×10^{-2}U) was added to each tube, the contents of which were thoroughly mixed. At intervals, 50 μl of hirudin (1 U/ml in saline) was added to each tube, the contents were mixed and placed in an ice-bath, and 1 ml of

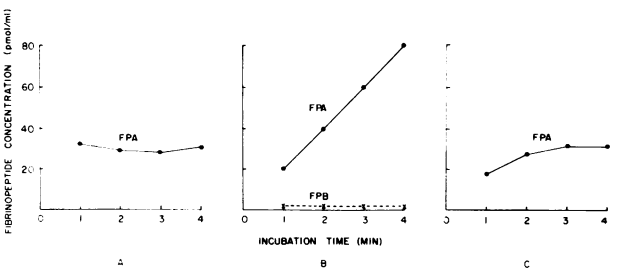

FIGURE 1 (*A*) Plasma FPA levels in patient IV3. Blood mixed with heparin and Trasylol at various time intervals. The incubation time represents the time interval between the initial entrance of the blood into the syringe and its mixture with heparin and Trasylol. (*B*) Plasma fibrinopeptide levels in patient XII 19 with systemic lupus erythematosus and inferior vena caval thrombosis. The technique was as in (*A*). ● ——— ●, FPA; × - - - × FPB. (*C*) Plasma FPA levels in patient XII 19. The technique was as in (*A*) except that 4.5 ml of blood was added to four tubes each containing 250 μl of EDTA (5 μg) immediately after collection (at 50 s) and 250 μl of heparin and Trasylol was added at 1, 2, 3, and 4 min.

ethanol was added. The contents of each tube were then processed as described for the clinical blood samples and assayed for fibrinopeptide content. With 0.02 U thrombin/ml, samples were tested at 1-min intervals for the first 4 min; for the lower thrombin concentrations samples were tested at 0 time and at 5, 10, 15, 20, 30, and 60 min after addition of thrombin.

The partial thromboplastin time, prothrombin time, and thrombin time were measured as previously described (14); fibrinogen was measured by the Ellis and Stransky (15) and Ratnoff and Menzie (16) methods. Serum was prepared and fibrinogen antigen was measured by the tanned erythrocyte hemagglutination inhibition test as described by Merskey et al. (17).

For control purposes, blood was obtained from normal, healthy laboratory personnel and student donors. The patients studied were all examined by a single physician (Dr. M. Ti) participating in this study. This physician followed each patient during the hospital course. After discharge, the overall record was reviewed by three physicians to assign a final diagnosis. The principal evidence for pulmonary embolism was radiological assessment of lung scans with radiolabeled macroaggregated albumin and chest X ray as well as the hospital course and response to heparin therapy. The diagnosis of venous thrombosis was confirmed by venography in seven patients and was based on clinical criteria in the other three.

RESULTS

Since tissue thromboplastin derived from the venipuncture might influence the results, the rate of FPA cleavage was studied in each of 12 blood samples (10 normal individuals and 2 patients with elevated FPA levels) collected with the 2-syringe technique. No consistent or significant difference in initial plasma FPA level or rate of generation was found in the sample collected into the first syringe (mean initial level 1.2 pmol/ml and mean generation rate 0.2 pmol/ml) as compared with the sample collected into the second syringe (mean initial level 1.2 pmol/ml and mean generation rate 0.2 pmol/ml). For subsequent studies, blood collected into a single syringe was studied.

The ranges of plasma FPA levels (mean 0.64 pmol/ml), and the in vitro generation rate (<0.5 pmol/ml per min) in 24 normal individuals are shown in Table Ia. The results in 19 hospitalized patients in whom the initial FPA levels fell into the normal range are also shown in Table Ia; mean initial level 1.13 pmol/ml, and in vitro generation rate <0.7 pmol/ml per min. Plasma fibrinogen and FDP levels and thrombin clotting time results are also shown in the table.

The remaining studies concern patients with elevated plasma FPA levels. These patients were divided into groups depending on whether the FPA generation rate was less than or greater than 1 pmol/ml per min. In 28 patients, the FPA generation rate was <1 pmol/ml per min (Table Ib, Figs. 1a and 2). The diseases for which this pattern was consistent were thrombophlebitis (10/10) and aortic aneurysm (4/4). All of the patients studied with the exception of those listed in Table II had normal or elevated fibrinogen levels. The coagulation findings in five patients thought to have excessive intravascular coagulation were examined in more detail (Table II). Four of the patients had low fibrinogen levels. Three of the patients (in-

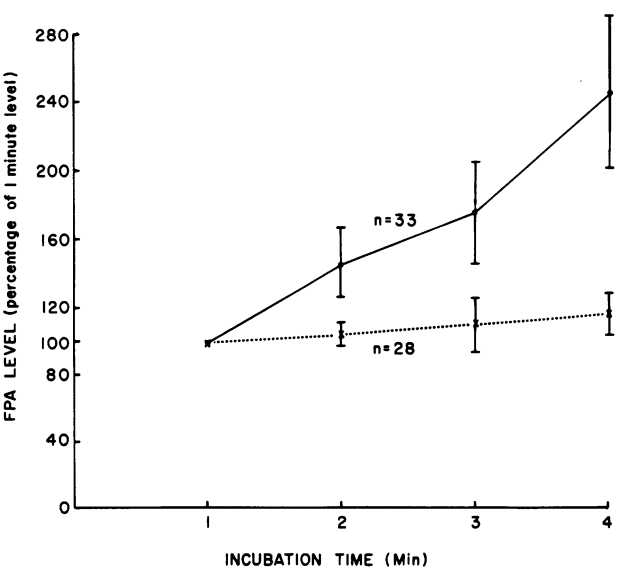

FIGURE 2 Plasma FPA levels at 1–4 min after blood started to enter syringe. The results are expressed as a percentage of the 1-min level and are plotted as mean±1 SD. ● ——— ●, mean of 33 patients in Table IV, × - - - ×, mean of 28 patients in Table Ib. The mean FPA levels (expressed in picomoles per milliliter) for the rapid generation group (● ——— ●) were: 1 min, 9.2; 2 min, 13.5; 3 min, 16.6; and 4 min, 22.7; and for the slow generation group were: 1 min, 6.1; 2 min, 6.5; 3 min, 6.9; and 4 min, 7.1.

TABLE II

Studies in Patients Thought to Have Excessive Intravascular Coagulation

Patient	Diagnosis	Fibrinogen	Thrombin clotting time	Fibrinogen degradation products	Platelets	Plasma FPA level	FPA generation rate	FPB generation rate
		mg/100 ml	*s*	*μg/ml*	*per μl*	*pmol/ml*	*pmol/ml/min*	*pmol/ml/min*
	Normal	200-400	15-16	<2	200,000–400,000	0.64 (0.07–1.5)	<1	
IV1	Chloroma	60	>60	5	37,000	4	<1	0
IV2	Staphylococcal septicemia	80	28.8	21	100,000	3.2	1.1	0.17
IV3	Acute promyelocytic leukemia	350	17.4	166	35,000	32.7	<1	3.2
IV4	Kasabach Merritt syndrome	55	>180	20	80,000	6.6	<1	1.3
IV5	Prostatic carcinoma with metastases	100	>60	20	28,000	40	2.8	0

cluding one with a normal fibrinogen level) had in vitro FPA generation rates in the normal range. Patient IV4 was in a relatively steady state and similar plasma FPA levels and generation rates were found on six occasions over a 5-yr period. Addition of thrombin to this patient's blood did not produce a detectable increase in the rate of FPA generation. Addition of fibrinogen however, increased FPA generation markedly (Table III). Patient IV3 generated significant FPB immunoreactivity in vitro.

33 patients with elevated plasma FPA levels (mean 9.2 pmol/ml) had relatively rapid FPA generation (>1 pmol/ml per min) Figs. 1b and 2, and Table IV). To test the postulate that in vitro FPA release resulted from thrombin action, FPA and FPB cleavage rates were compared in 19 blood samples. FPA release reflects thrombin action, and FPB immunoreactivity release probably reflects plasmin action (10). In 17 patients the generation of FPA was significantly greater than that of FPB (Table V). In two patients (nos. 10 and 12) generation of FPB was more rapid than that of FPA. Further evidence consistent with FPA cleavage in the blood samples being due to thrombin is the reduction of plasma FPA levels to the normal range and virtual abolition of in vitro FPA generation after 1 day of heparin therapy (Table VI). Addition of heparin in vitro significantly decreased in vitro FPA generation (Fig. 3). EDTA added in vitro did not affect the initial plasma FPA level but reduced in vitro FPA generation in normals and in patients with elevated FPA levels (Fig. 3). FPA generation in the presence of EDTA decreased with time and was nonlinear (Fig. 1c).

TABLE III

The Influence of Thrombin and Fibrinogen on FPA Generation in Patient IV4

Blood sample	Plasma FPA concentration, *pmol/ml*			
	1 min	2 min	3 min	4 min
Patient IV4 + buffer	4.9			4.5
+ thrombin*	4.9			5.3
+ fibrinogen‡	4.9	6.1	38.7	62.3
Control + buffer	0.5	0.4	0.4	0.3
+ thrombin*	0.4	3.7	5.1	5.3
+ fibrinogen‡	1.6	1.5	1.5	1.4

* Added thrombin was 0.00016 U/ml plasma.

‡ Added fibrinogen was 1 mg/ml plasma.

The effect of thrombin on FPA generation in normal blood was examined. The FPA generation pattern was nonlinear, and FPA levels were constant between 3 and 4 min (Fig. 4). In the 1st min after addition of thrombin, 21.7 nmol FPA were cleaved per ml/U of thrombin. Initial FPA cleavage rates from fibrinogen by thrombin were studied at varying fibrinogen concentrations. There was relatively little change in cleavage rate at fibrinogen concentrations of 2–4 mg/ml (Fig. 5). The Michaelis constant (K_m) calculated from this data was 2.99 μM. When the fibrinogen concentration was 2.5 mg/ml the rate of FPA cleavage was proportional to the thrombin concentration. The rate was calculated to be 49.2±1.6 nmol FPA/ml per min per U of thrombin (Fig. 6).

TABLE IV
Plasma FPA Level Elevated with Generation Rate (>1 pmol/ml/min)

Condition	Number	Initial FPA level	FPA generation rate	Fibrinogen level	FDP level	Thrombin time
		pmol/ml	*pmol/ml/min*	*mg/100 ml*	*μg/ml*	*s*
Post-prothrombin complex infusion	7	3.0 (2.2–5)	2.0 (1–3.4)			
Pulmonary embolism	9	14.9 (2–52.7)	4.05 (1.3–28)	554 (290–610)	4.1 (0–21)	16.6 (13.6–19.2)
Arterial thrombosis	3	8.4 (2.1–12.7)	2.5 (2.44–2.6)	500 (200–670)	2.3 (0–5)	15.6 (15.3–16)
Transplant rejection	3	4.5 (2.5–6.3)	1.26 (1.0–1.7)	230 (200–280)	28 (2–41)	20.3 (17.8–23)
Systemic lupus erythematosus	4	8.2 (2.1–22)	6 (1.1–18.9)	639 (380–905)	11.5 (0–41)	17.7 (15.8–20.5)
Defibrination	2	21.6 (3.2,40)	1.95 (1.1,2.8)	(See Table IV)		
Neoplasia	3	4.9 (2.7–8.6)	2.88 (1.24–4.5)	681 (405–960)	5 (0–10)	23.4 (21.8–25.1)
Sepsis	2	9.1 (2.3,15.9)	1.11 (1,1.22)	685 (600,770)	5 (0,10)	16.7 (16.5,16.9)

DISCUSSION

The results of the FPA generation tests on the clinical blood samples demonstrate three different patterns: (*a*) Normal plasma FPA level and slow generation, (*b*) Elevated plasma FPA level and slow generation, and (*c*) Elevated plasma FPA level and rapid FPA generation. The validity of the different patterns as reflecting differences in the blood, rather than differences in tissue thromboplastin released into the blood by the venipuncture is supported by a number of observations; the different findings in various disease states, the reproducible findings in a patient with a steady state of intravascular coagulation over a period of 3 yr, and the similar results in blood collected sequentially into two syringes when blood flow is free. A poor venipuncture would promote thrombin formation and increase the FPA level and generation rate.

When the clinical findings are considered, the mean plasma FPA level in 24 normal individuals was 0.64 pmol/ml (0.07–1.2) which is somewhat higher than our previous finding of 0.36 pmol/ml (0.07–1.3) (4); the discrepancy is thought to result from the lack of correction for incomplete FPA extraction from the blood in our previous study, whereas in the present study the levels represent complete FPA recovery. The

TABLE V
Fibrinopeptide A and B Generation Rates In Vitro

	Fibrinopeptide A generation rate	Fibrinopeptide B generation rate
	pmol/ml/min (normal 0.23±0.23)	*pmol/ml/min*
(1) Post-proplex infusion	1.9	0
(2) Post-proplex infusion	1.3	0
(3) Post-proplex infusion	1.9	0
(4) Post-proplex infusion	2.9	0
(5) Post-proplex infusion	1.4	0
(6) Post-proplex infusion	3.4	0.15
(7) Post-proplex infusion	1.0	0.04
(8) VI1 Breast carcinoma	4.3	1.4
(9) VI3 Pulmonary carcinoma with metastases	0.8	0.4
(10) VI6 Metastic carcinoma	2.7	2.9
(11) VII7 *Escherichia coli* septicemia	4.9	0.1
(12) IV3 Acute promyelocytic leukemia	0	3.2
(13) X4 Pulmonary embolism	28	1.3
(14) X9 Pulmonary embolism	4.8	0.2
(15) X11 Pulmonary embolism	3.4	0
(16) XII2 Systemic lupus	1.5	0.3
(17) XII5 Systemic lupus	1	0
(18) XII6 Systemic lupus	1.86	0
(19) XII9 Systemic lupus	17.3	0.33

TABLE VI
Plasma FPA Levels and In Vitro Generation Rates before and after Heparin Therapy

Patient	Before therapy		After heparin therapy for 1 day	
	FPA level	In vitro generation rate	FPA level	In vitro generation rate
	pmol/ml	*pmol/ml/min*	*pmol/ml*	*pmol/ml/min*
II3 Thrombophlebitis	10.6	<1	0.87	<1
II5 Thrombophlebitis	5.3	<1	0.1	<1
X1 Pulmonary embolism	14.1	1.3	1.1	<1
X3 Pulmonary embolism	1.5	<1	0.98	<1
X4 Pulmonary embolism	38.7	28	1.3	<1
X11 Pulmonary embolism	6.0	3.4	1.26	<1
X12 Pulmonary embolism	8.9	3.2	1.7	<1
Mean	12.2	5.4	1.0	<1

mean level in patients at bed rest with FPA levels in the normal range was 1.1 pmol/ml (0.8–1.5) (Table Ia). The findings in 63 patients with elevated plasma FPA levels are shown in Tables Ib, II, and IV. The relatively slow FPA generation pattern indicates low effective thrombin activity. This could be due to low thrombin activity, the presence of inhibitors, or insusceptibility of the fibrinogen to thrombin. It is proposed that the relatively low in vitro FPA generation pattern in patients with normal or elevated fibrinogen levels reflects a relatively low concentration of thrombin in the arm vein blood. This could be associated with an elevated plasma FPA level if thrombin formation was predominantly local. Such a pattern was consistently found in patients with venous thrombosis (10/10) and with aortic aneurysm (4/4). In the patients with hypofibrinogenemia, the low FPA generation rate could be due in part to low substrate concentration. This possibility was tested by adding thrombin and fibrinogen to the blood of patient IV4. Thrombin added to her blood did not generate FPA normally whereas FPA generation was rapid in the presence of added fibrinogen. The results are consistent with the interpretation that hypofibrinogenemia is at least partly responsible for slow FPA generation. This explanation cannot apply in the case of patient IV3 who had a normal fibrinogen level. In this patient, FPB was cleaved rapidly without FPA generation, unlike the cleavage pattern resulting from thrombin action (10). Plasmin (10) or possibly a leukocyte protease (18) may have been acting in this patient.

The uniform finding (7/7) of rapid in vitro FPA generation after prothrombin complex infusion is thought to reflect activation of prothrombin by activated factors in the infused preparation (19). These FPA generation curves showed an acceleration at 4 min and are largely responsible for the nonlinearity at 4 min evident in Fig. 2. The mechanism for activation of coagulation and rapid FPA generation in systemic lupus erythematosus is unknown. Rapid FPA generation in the patients with pulmonary embolism may reflect activation of coagulation secondary to tissue damage produced by the embolism consistent with the finding that experimental pulmonary embolism is followed by fibrin deposition and lysis (20).

The rapid FPA generation pattern was investigated further. In 17 of 19 patients, FPA generation was more rapid than FPB generation, consistent with thrombin action; however, in 2 patients, FPB generation was more rapid, suggesting the action of another enzyme, possibly plasmin (10). The reduction in FPA level and generation rate after parenteral heparin is also consistent with thrombin action (Table VI). Heparin added to blood in vitro markedly decreased FPA generation (Fig. 3) as would be expected if thrombin were responsible.

Thrombin added to normal blood in vitro produced non-linear FPA generation which progressively slowed, reflecting rapid inactivation of the thrombin. With isolated components thrombin is inactivated by antithrombin with a $t_{\frac{1}{2}}$ of about 40 s (20, 21). The FPA generation curve in the patients listed in Table IV was linear or increasing at 4 min (Fig. 2) and therefore different from that produced by adding thrombin to normal blood. This finding implies a constant or increasing thrombin concentration. Possible reasons for the discrepancy from the results of adding exogenous thrombin to normal blood include defective inhibition of the thrombin in the clinical samples or a balanced rate of thrombin formation and

inactivation. The conversion of the FPA generation curve in Fig. 1b to that in Fig. 1c by addition of EDTA is consistent with the latter possibility. EDTA chelates calcium ions and thereby inhibits prothrombin activator conversion of prothrombin to thrombin. In further experiments, EDTA added in vitro reduced but did not abolish FPA generation (Fig. 3). Balanced formation and inactivation of thrombin in the circulating blood would imply the presence of prothrombin activator in the blood.

On the basis of these findings, it is postulated that the blood of certain patients contains elevated

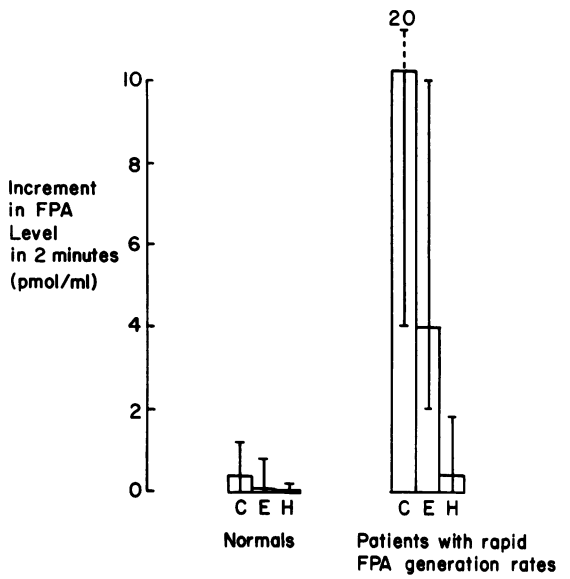

FIGURE 3 Effect of EDTA and heparin on the generation of FPA. 11 normals were tested and 13 patients with elevated FPA generation rates. C gives the 2-min increment in FPA level in the absence of EDTA or heparin; E, in the presence of EDTA; H, in the presence of heparin. The ranges given include all the data.

		Protocol		
Tube	Anti-coagulant mixture	1 min	3 min	Information
(1)	Heparin + Trasylol	blood		Initial plasma FPA level without EDTA.
(2)	Heparin + Trasylol		blood	Plasma FPA level after 2 min without anti-coagulant.
(3)	Heparin, Trasylol, + EDTA	blood		Initial plasma FPA level with EDTA.
(4)	EDTA	blood	heparin	Plasma FPA level after 2 min in presence of EDTA.

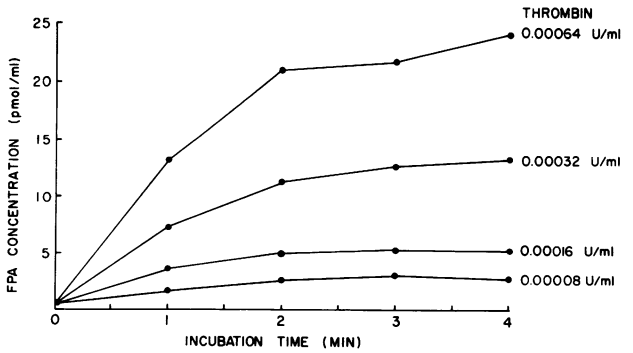

FIGURE 4 Effect of added thrombin on the in vitro generation of FPA in normal blood. The thrombin concentration is shown in units per milliliter of plasma. The results at each concentration represent the mean of experiments on three normal individuals.

levels of thrombin. Because of the multiple factors involved in whole blood (inhibitors and alternate substrates) it is not possible to calculate the thrombin concentration in the clinical blood samples from the FPA generation rates. It is possible to calculate the thrombin concentrations required to produce comparable FPA generation rates in a purified system. The FPA generation rate in normal blood (<0.5 pmol/ml plasma per min) would be produced by $<1 \times 10^{-5}$ U/ml in a purified system. The rapid FPA generation rate (1–28 pmol/ml plasma per min) would be produced by 2×10^{-5} U/ml to 5.6×10^{-4} U/min in a purified system. In a purified system the FPA generation rate was 49.2±1.6 nmol/ml per min per U of thrombin (Fig. 6). When thrombin was added to whole blood the amount of FPA generated in the 1st min was 21.7 nmol/min per U of thrombin (Fig. 4). The lesser activity of thrombin in whole blood pre-

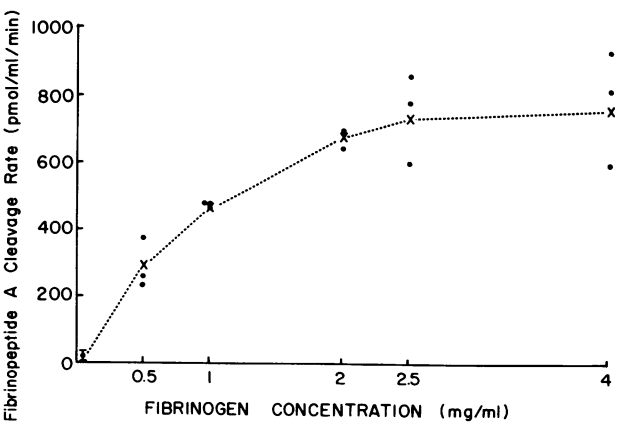

FIGURE 5 The initial rate of FPA cleavage by thrombin from fibrinogen. The thrombin concentration was 0.02 U/ml, pH 7.4, temperature 37°C, ionic strength 0.15 M, and the fibrinogen concentration is plotted on the abscissa. The results are the mean of three separate experiments.

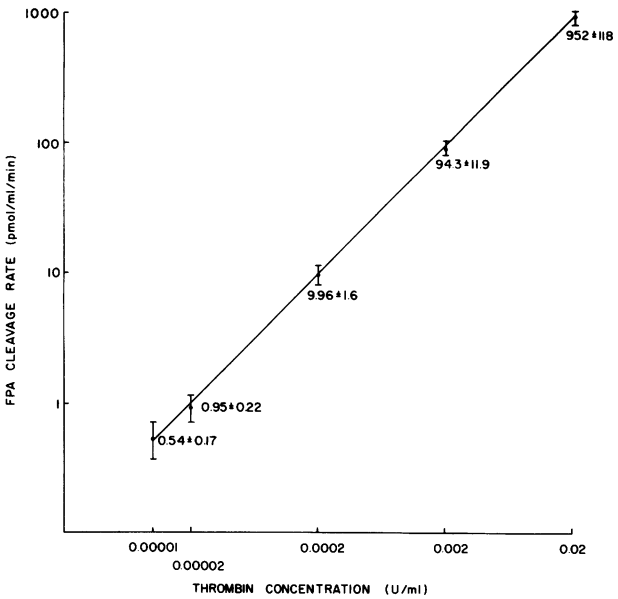

FIGURE 6 Rate of cleavage of FPA from fibrinogen by thrombin Fibrinogen concentration 2.5 mg/ml, pH 7.4, temperature 37°C, ionic strength 0.15 M NaCl, thrombin concentration as indicated on the abscissa. The points plotted are the mean and standard deviation for five experiments.

sumably reflects the effect of inhibitors (antithrombin [22, 23] and α-2-macroglobulin [24, 25]) and alternate substrates.

These findings raise many questions which require further studies for resolution. Among the questions are: What is the specificity of the different FPA generation patterns in patients with different diseases? Can the presence of circulating thrombin antigen as distinct from activity be demonstrated by immunological techniques (23)? Are activated coagulation intermediates present in the blood of patients with certain diseases?

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