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A Study of Fibrinogen and Fibrinolysis in 10 Adults with Nephrotic Syndrome

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Key words

Nephrotic syndrome – Fibrinolytic system – Fibrinogen – Albumin

Summary

In 10 patients with nephrotic syndrome (NS), the coagulation inhibitors, the fibrinolytic system and several functions of the fibrinogen-fibrin molecule were studied.

Among the coagulation inhibitors, only antithrombin III (AT III) was found decreased and correlated with serum-albumin levels. Venous occlusion test provoked a normal tissue plasminogen activator (tPA) release in all patients. The plasminogen activator inhibitor (PAI) had an increased activity in 5 out of the 10 patients.

Thrombin and reptilase times were found abnormal in most patients. The thrombin time (TT) prolongation correlated with serum albumin levels and was corrected by adding purified albumin.

The fibrinogen was purified from each of the 10 patients' plasma. Only 2 of them showed abnormal polymerization in purified system, suggesting dysfibrinogenaemia. Other functions (thrombin binding, tPA stimulating activity, lysis by purified plasmin) were found normal except in one of the 2 patients with dysfibrinogenaemia whose fibrinogen lysis by plasmin was delayed.

It is concluded that an abnormal fibrinogen molecule is not the most frequent explanation for thrombin time prolongation in NS.

Introduction

Since the publication by Kanfer et al. (1) of various disorders of the haemostatic system in nephrotic syndrome (NS), many studies have attempted to relate thromboembolism – a well-known complication of NS (2, 3) – to the so-called hypercoagulability state. Several mechanisms have been found to play a key role in the regulation of the coagulation system: (a) antithrombin III (AT III) and other protease inhibitors (4); (b) protein C-protein S system (5); (c) the fibrinolytic enzyme plasmin. One of the main components of this system is fibrin itself which presents numerous specific sites for plasmin attack allowing degradation in small fragments (6) and plays a key role in plasmin formation by increasing the rate of plasminogen activation by tissue-plasminogen activator (tPA). Furthermore fibrin restricts and localizes the clotting process by binding large amounts of thrombin (7). As prolongation of the fibrin polymerization time is frequent in NS, it has been suggested that thrombosis may result from an abnormality of the fibrinogen molecule (8). We have purified fibrinogens from 10 patients with the characteristic feature of NS and measured, beside the ability of the molecule to polymerize, its different natural antithrombotic functions. We also measured in the same patients the in vivo release of tPA by venous occlusion test and the concentration of the main coagulation and fibrinolysis inhibitors including plasminogen activator inhibitor (PAI). One of the most interesting finding of this study was that in most patients the prolonged thrombin time (TT) resulted from the low albumin concentration except in two of them who present some feature of a dysfibrinogenaemia.

Patients, Materials and Methods

Ten consecutive patients with NS clinical and biochemical features entered the study between January 1986 and June 1987. Were excluded the patients with impaired renal function (serum creatinine >210 µmol/l), diabetes mellitus, systemic lupus erythematosus, and the patients receiving steroid therapy or immuno-suppressive drugs.

Blood samples for haemostasis tests were obtained by venipuncture in 0.11 M natrium citrate (1:9) after a 30 min-resting period. The venous occlusion test (VO) was performed by applying at one arm a sphygmomanometer cuff and inflating the cuff to maintain a pressure midway between the diastolic and the systolic pressure during 10 min. The second blood sample was drawn just before deflating the cuff. Blood was kept at +4° C, centrifuged within 10 min during 20 min at +4° C, 2,300 × g and immediately frozen in aliquots at −80° C except for routine haemostasis tests including euglobulin lysis time (ELT), plasma thrombin time and fibrinogen determination which were performed immediately.

Fibrinogen was measured by functional assay according to Clauss (9). Prothrombin time (PT) was performed using thromboplastin bioMérieux (Marcy l'Etoile, France) and activated partial thromboplastin time (APTT) using Actimat (bioMérieux).

Thrombin time was realized on plasma or purified fibrinogen, as described (10) and reptilase time was measured according to the manufacturer's recommendations using reptilase (FTH 50, Diagnostica-Stago, Asnières, France). The results were expressed as the ratio of the time obtained for the patients' plasma or fibrinogen to the time obtained for a normal subject in the same series.

AT III and plasminogen were measured using functional chromogenic assays (11) and protein C using an immunoenzymatic technique (12) with a kit from Diagnostica-Stago. Protein S and α_2-antiplasmin (α_2-AP) were measured using rocket immunoelectrophoresis and respectively an immune serum from American Diagnostics (Greenwich, USA) and from Diagnostica-Stago. α_2-macroglobulin (α_2-M) was evaluated with a Norpartigen α_2-M kit from Behringwerke (Marburg, Germany). Heparin cofactor II (HC II) was measured as dermatan sulfate cofactor according to Toulon et al. (13).

Techniques used for specific studies on purified fibrinogen have been described elsewere (14). They all derive from published methods: fibrinogen purification was performed according to Kazal et al. (15), tPA fibrin interaction according to Anglés-Cano (16), thrombin fixation on fibrin according to Haverkate et al. (17), fibrinogen degradation by plasmin according to Mosesson et al. (18). Fibrinogen sialic acid content was measured according to Aminoff (19).

ELT was measured using the technique of Kluft (20). tPA was measured in euglobulins using the solid phase fibrin tPA activity (SOFIA) assay described by Anglés-Cano (16, 21).

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Table 1 Main clinical data, standard laboratory investigations and concentration of coagulation inhibitors in 10 patients with NS

Patients	Age	Renal disease	Protein-uria g/24 h	Serum albumin g/l	Alpha 2-M %	Fibri-nogen g/l	AT III %	HC II %	Protein C %	Protein S %
1	16	MC	5.10	11.5	384	6.8	73	123	140	108
2*	82	ND	9.20	24.5	210	5.5	71	104	97	110
3	68	Am-MF	6.70	10.0	201	5.6	66	95	93	150
4**	45	ND-MF	ND	24.5	168	7.6	100	155	38	56
↙5*	46	MC	8.30	13.0	158	10.15	52	151	105	150
6	78	Am	2.55	26.2	148	5.5	95	108	78	160
7	29	MGN	12.80	8.5	ND	8.4	75	103	110	126
8	80	MGN	3.40	22.5	168	6.2	88	80	110	148
9	56	Am	2.75	19.5	110	6.8	78	120	95	200
10	81	MGN	3.90	27.5	138	4.0	89	70	120	148
Normal ranges			0	41–49	52–144	2–4	82–122	65–145	70–122	70–130

↙ = thrombotic manifestation; ND = not determined; Am = amylosis; MC = minimal change; MGN = membranous glomerulonephritis; MF = Mediterranean fever.
* patients under heparin therapy; ** patients under antivitamin K therapy.

Table 2 Plasma thrombin and reptilase times expressed (as a ratio of the patients time to a normal subject time performed at the same day)

Patients	Thrombin time (ratio)	Reptilase time (ratio)
1	1.52	1.27
2*	18.90	1.65
3	2.25	2.30
4	1.41	2.21
5*	2.30	2.42
6	1.16	1.60
7	1.58	2.10
8	1.50	1.57
9	1.37	1.95
10	1.05	1.63
Normal value	≤1.25	≤1.25

* Patients under heparin therapy .

PAI activity was measured by incubating a known amount of tPA with plasma diluted in PAI depleted plasma to minimize other inhibitors influence according to Contant et al. (22) using reagents kindly provided by G. Contant (Serbio, Asnieres, France). The results were expressed in IU of tPA inactivated.

For the following parameters: albumin, α_2-M, fibrinogen, thrombin time, reptilase time, the normal range or normal limits were the one being used in the laboratory since many years for clinical diagnosis.

AT III, HC II, protein C, protein S, plasminogen and α_2-AP were measured by comparison with a pool of a normal plasma and expressed as percentages. The normal ranges (mean ± 2 SD) used for those parameters were obtained by testing at least 30 healthy volunteers. They are consistant with the ranges published in the literature.

Statistical analysis for comparing values observed in normal and patients' purified fibrinogen was performed using the Mann and Whitney test.

Results

Clinical Data and Standard Laboratory Investigation

Results are presented in Table 1.

The underlying renal disease was amyloidosis in 3 patients (patients Nos. 3 and 4 having Mediterranean fever), minimal glomerular lesions in 2 patients and membranous glomerulonephritis in 3 of them. Patient No. 5 presented a myocardial infarction soon after the onset of the NS. Only in patient No. 4 was the NS complicated by deep venous thrombosis. APTT and PT were normal in all patients, except patients Nos. 2 and 5 who were receiving heparin and patient No. 4 who was treated by antivitamin K. Fibrinogen concentrations were elevated in all patients. α_2-M was found increased in 8 of them.

AT III was below the normal limit in 4 out of the 8 patients who were not under heparin therapy. A significant correlation

Table 3 In vitro correction of plasma thrombin and reptilase time prolongations in patients with NS by purified albumin

		Thrombin time (ratio)			Reptilase time (ratio)	
		Serum albumin g/l	Native plasma	Plasma supplemented with albumin[1]	Native plasma	Plasma supplemented with albumin[1]
Patients entering the study	NS3	10.0	3.86	1.28	2.38	1.50
	NS4	22.6	1.62	1.16	1.48	1.25
	NS6	20.0	1.43	1.00	1.33	0.88
	NS7	11.2	1.81	1.08	1.73	1.30
	NS8	22.5	1.22	0.95	1.21	1.01
	NS9	17.7	2.04	1.32	1.37	1.07
Other patients with NS	A	17.2	1.42	0.95	1.50	1.02
	B	24.6	2.04	1.32	1.37	1.07
	C	7.8	1.63	0.85	2.16	1.33
	D	21.1	1.30	0.85	1.33	1.00
	E	28.2	1.15	0.95	1.12	0.87
	F	15.7	2.51	1.29	1.54	1.25

[1] Bovine albumin (Sigma) final concentrations 40 g/l

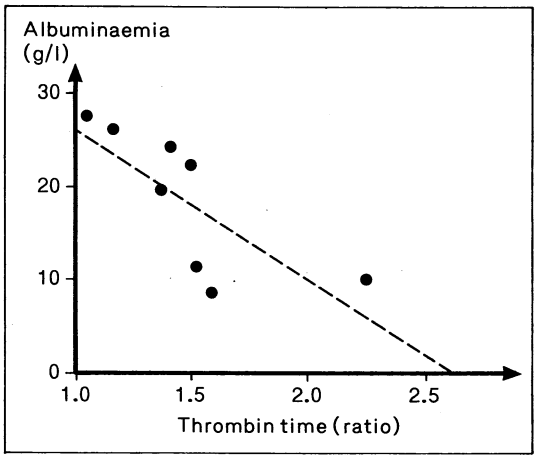

Fig. 1 Correlation between the thrombin time prolongation (ratio) and the serum-albumin levels

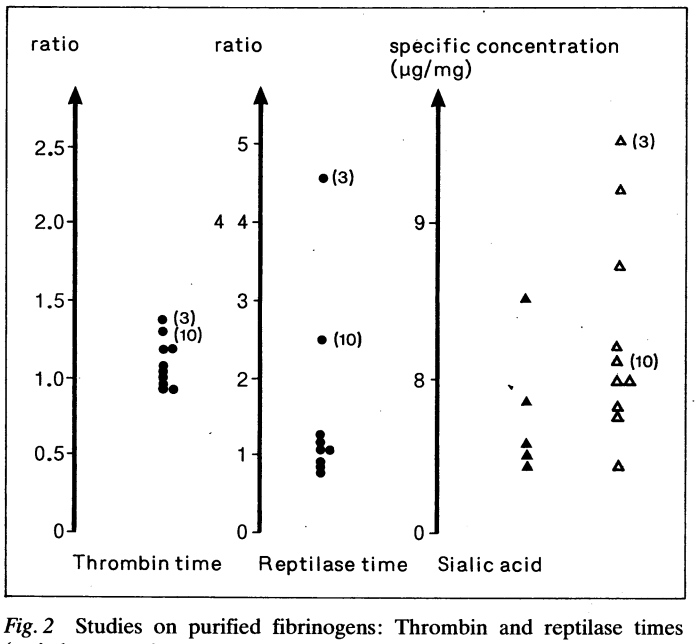

Fig. 2 Studies on purified fibrinogens: Thrombin and reptilase times (ratio between the value obtained with the fibrinogen from the patient and the mean value from 3 normal fibrinogens tested in the same series); Sialic acid: ▲ normal fibrinogens; △ patients' fibrinogens; (3) and (10) = patients Nos. 3 and 10

(r = 0.88, $p < 0.05$) was found between AT III and serum albumin concentrations.

HC II was normal in 8 patients, slightly elevated in 2 of them.

Protein C was within the normal range in all patients but one (patient No. 4, who was under oral anticoagulant). Protein S was above the upper limit in 6 out of the 10 patients. No correlation was found between these 2 proteins and serum albumin.

Plasma thrombin times were prolonged in all patients but 2 (Nos. 6 and 10) (Table 2). Reptilase time (which is unsensitive to the presence of heparin) was prolonged in all patients including the 2 patients receiving heparin (Table 2). A significant correlation was found between the thrombin time prolongation and serum albumin levels (r = −0.75, $p < 0.05$, the 2 patients under heparin being excluded) (Fig. 1).

We have tested retrospectively the hypothesis of an in vitro influence of serum albumin levels on plasma thrombin and reptilase time using plasmas from 6 patients of the series who

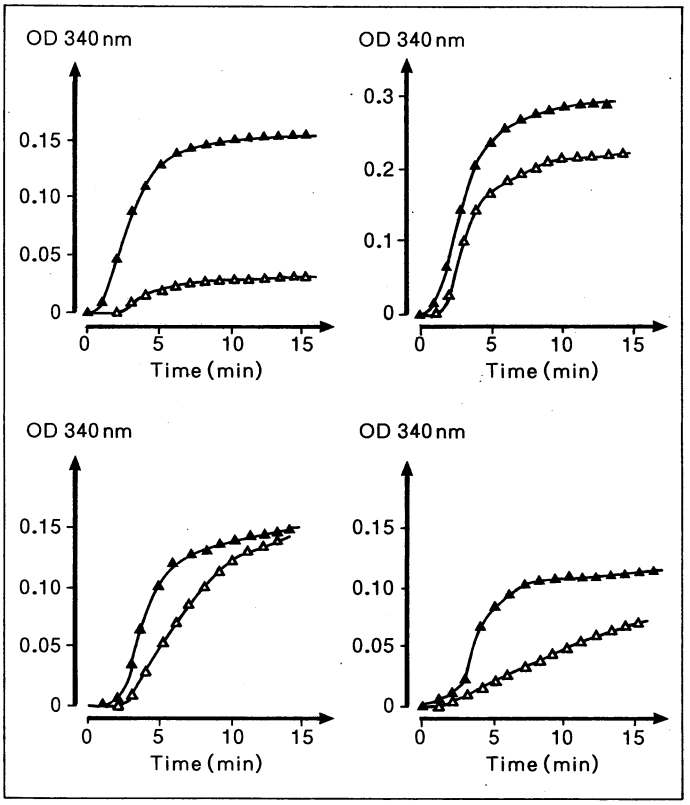

Fig. 3 Polymerization curves obtained after adding thrombin (0.1 U/ml) or reptilase, to purified fibrinogens (0.15 mg/ml): Upper panels: polymerizations with thrombin: ▲ mean of 4 normal fibrinogens; left panel: △ patient No. 3' fibrinogen; right panel: △ patient No. 10' fibrinogen. Lower panels: polymerizations with reptilase: ▲ mean of 4 or 2 normal fibrinogens; left panel: △ patient No. 3; right panel: △ patient No. 10

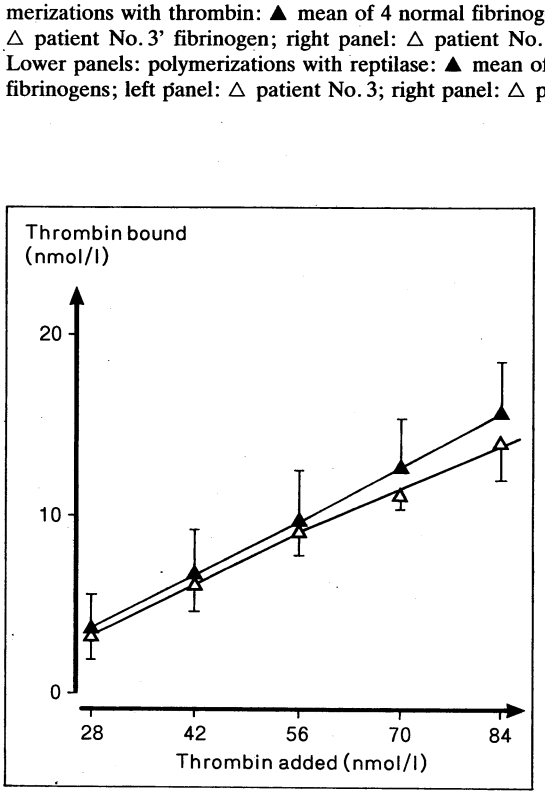

Fig. 4 Thrombin fixation on fibrin. Increasing amounts of thrombin were added to fibrinogen solution (final concentration 0.5 mg/ml) in presence of calcium chloride (2.5 mM). After 30 min incubation at 37° C, reptilase was added. After a further 30 min incubation at 37° C, clots were removed by centrifugation and the residual thrombin activity was measured with a substrate: H-D-Phe-Pip-Arg-PNA (S 2238, Kabi-Flow). ▲ 6 normal subjects (mean ± SD); △ 10 patients with nephrotic syndrome (mean ± SD)

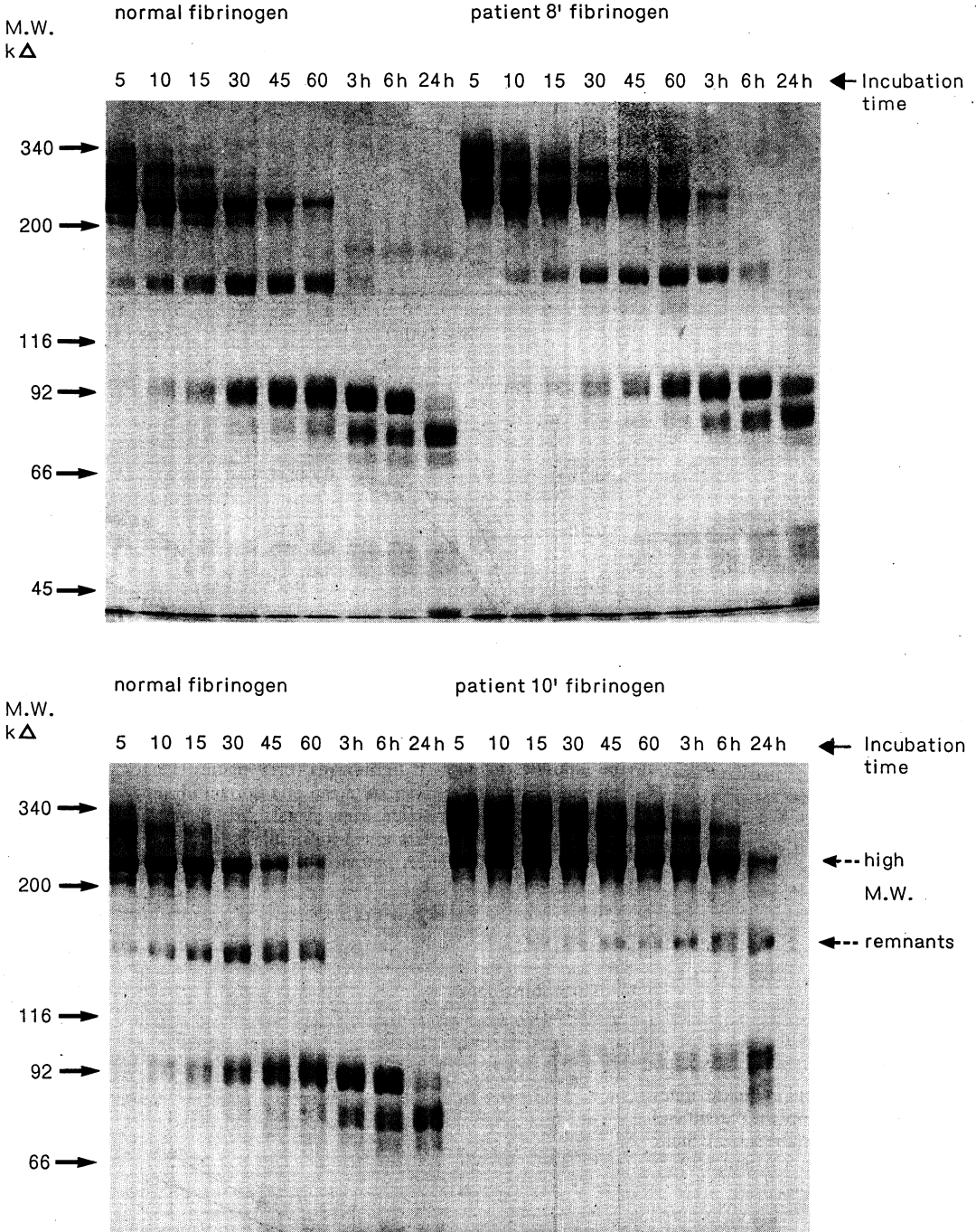

Fig. 5 Fibrinogen digestion by plasmin. To normal or nephrotic syndrome fibrinogen (1.5 mg/ml in 0.1 M, pH 8.6, phosphate buffer) plasmin was added at a final concentration of 0.2 CU/ml. At various times (1, 5, 10, 15, 30, 45, 60 min, 3, 6, and 24 h), a sample of the fibrinogen-plasmin mixture was drawn and digestion was stopped by adding an aliquot of aprotinin (2,000 UIK/ml). Each sample was then submitted to SDS-polyacrylamide 7.5% gel electrophoresis. ——▶ HMW standards; - - -▶ HMW remnants observed in the case of patient No. 10

were still available and plasmas from 6 patients with characteristic NS but excluded of the series because of steroid treatment or admission after the end of the study. There was a significant shortening of both thrombin and reptilase times following addition of serum albumin. Only in patient No. 3, the reptilase time remained unambiguously prolonged (Table 3).

Studies on Purified Fibrinogen

In a purified system, thrombin and reptilase times were prolonged in only two cases: patients Nos. 3 and 10 (Fig. 2).

The sialic acid specific concentrations observed with the patients' fibrinogen were compared with a series of 5 fibrinogens purified from normal healthy volunteers. No significant difference was found ($p > 0.05$). However, patient No. 3' fibrinogen showed the highest sialic acid concentration (Fig. 2).

The polymerization was followed by the increase in optical density at 350 nm after addition of thrombin or reptilase to 0.15 mg/ml fibrinogen solutions. Only patients Nos. 3 and 10 presented delayed and abnormal polymerization (Fig. 3). The other patients had normal patterns (not shown).

The ability of fibrin clots to bind thrombin was measured using increasing thrombin concentrations. Fibrin clots prepared from the 10 patients' fibrinogens bound normal amounts of thrombin when compared to 6 normal subjects' fibrinogens (Fig. 4).

Fibrinogens purified from the patients were incubated with plasmin during 24 h. The reaction was stopped at different times and the rate of lysis evaluated by the appearance of lower molecular weight fibrinogen degradation fragments (FDP) on sodium dodecyl sulfate (SDS) 7.5% polyacrylamide gels. In each run a fibrinogen from a normal subject was compared to a patient's fibrinogen. The patterns obtained were normal in all patients (example on Fig. 5A), except for patient No. 10 (Fig. 5B) who presented a delayed FDP generation.

The interaction of fibrin with plasminogen and tPA was measured in the SOFIA technique described by Anglés-Cano: fibrin networks were prepared from the patients or normal fibrinogens at the surface of microplate wells. The stimulating effect of fibrin was evaluated by the amount of plasmin generated in presence of plasminogen and increasing amounts of purified tPA. We have tested the 10 patients and 8 normal subjects and found no significant differences (Fig. 6).

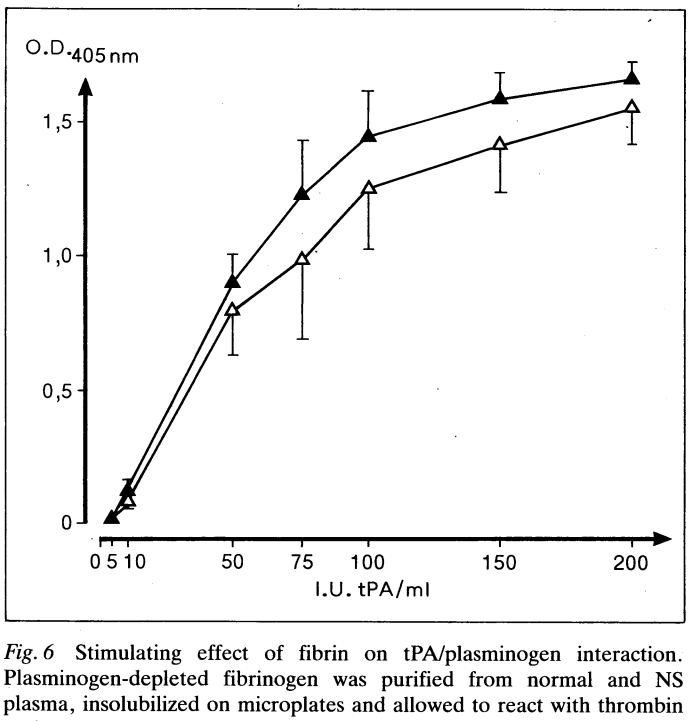

Fig. 6 Stimulating effect of fibrin on tPA/plasminogen interaction. Plasminogen-depleted fibrinogen was purified from normal and NS plasma, insolubilized on microplates and allowed to react with thrombin to form a fibrin network. Purified tPA at various concentrations (10 to 200 IU/ml) was then bound on the solid phase fibrin and reacted with plasminogen and a specific substrate for plasmin. ▲ normal subjects' fibrin (mean ± SD from 8 subjects); △ patients with NS' fibrin (mean ± SD from 10 patients)

Study on the Fibrinolytic System

The venous occlusion test gave ELT and tPA values within the normal limits in all the patients. Plasminogen was normal. α_2-AP was decreased in one patient (No. 5 who also presents the lowest level in AT III). PAI was found above the normal limit of 8 IU in 5 out of the 10 patients. No significant correlation was found between PAI and respectively serum albumin and fibrinogen levels.

Discussion

The genesis of thromboembolic complications in NS is still a matter of debate as puzzling observations of various haemostasis disorders have been done. We selected 10 patients with NS, receiving no steroid therapy, and measured, beside the coagulation inhibitors, the different components of the fibrinolytic system and several fibrinogen functions that participate to the natural antithrombotic process.

AT III was found decreased in 4 out of the 8 patients receiving no heparin, which is known to be associated with low AT III levels (23), and correlated with the albumin level. This has already been shown in several studies (24, 25, 26). HC II, which presents large homologies in structure and functions with AT III, was normal or slightly elevated.

We found no major disturbance in the protein C-protein S system. Results should be interpreted with caution for protein S, measured in our study as total immunoreactive protein: low functional protein S levels were observed in NS (27).

Our major interest was the fibrinogen abnormality, which has been suggested to participate in thrombosis genesis in NS (8), and the fibrinolytic system, which to our knowledge has not been the subject of extensive studies.

After venous occlusion, the ELT was shortened in all the 10 patients. A normal release of tPA by the vascular wall was evidenced by the increase of the fibrin dependent plasminogen activator activity observed in all patients using the SOFIA assay. Plasminogen and α_2-AP were normal in all patients but one who presented low α_2-AP. This patient (No. 5) also had low AT III level. An elevation in PAI activity was observed in five cases. This is an interesting finding, because PAI is believed to play an important role in thrombosis (28, 29, 30). However, this inhibitor varies in a wide range in many pathologic or physiologic situations (31). We found no correlation with albumin i.e. with the severity of the NS, nor with fibrinogen i.e. with an acute phase reaction, and no relationship with the underlying disease.

In 8 of the 10 patients using classical polymerization tests in purified systems, we were unable to confirm the existence of a fibrinogen molecular abnormality: thrombin and reptilase times were normal when purified fibrinogens were tested. Sialic acid specific levels, which have been shown to be increased in acquired (32) or congenital dysfibrinogenaemia (33), were normal. One explanation for the prolonged plasma thrombin and reptilase times in these patients is the in vitro influence of serum albumin levels: thrombin times were correlated with serum albumin levels and corrected by adding purified albumin to a final concentration of 40 g/l.

In 2 patients, the results obtained with purified fibrinogens suggest a molecular abnormality: reptilase times were dramatically prolonged (respectively 4.6 and 2.5 times) and the polymerization curves showed delayed and abnormal polymerization in presence of both thrombin and reptilase. One of these 2 patients (No. 3) presented Mediterranean fever, a disease known to be associated with abnormal fibrinogen derivatives, which precipitate at +4° C (34). We did not find cryofibrinogenaemia in this patient and in patient No. 4 who also had Mediterranean fever.

The 10 fibrinogens purified from the patients were also studied with respect to the different functions that participate to coagulation regulation. The only abnormality observed was a resistance to plasmin lysis of patient No. 10' fibrinogen which also presents polymerization abnormalities. The stimulating effect of fibrinogen on plasminogen activation by tPA was normal in the 10 patients explored. Also normal was the thrombin binding by fibrin clots in the 10 patients..

This study confirms that the disorders in the coagulation and fibrinolytic system bring no clear-cut explanation for the hight incidence of thrombosis in NS. In most patients, the fibrinogen polymerization abnormality was not confirmed in purified system

Table 4 Fibrinolytic system in 10 patients with NS

Patients	ELT (min)		t-PA (IU/ml) in euglobulin		Plasminogen	alpha 2-AP	PAI
	before VO	after VO	before VO	after VO	%	%	IU/ml
1	60	30	0.45	3.3	89	96	4.1
2	45	40	17.50	18	88	95	10.8
3	180	30	1.25	33.9	87	ND	3.6
4	180	20	0.80	45	94	117	10.5
5	365	50	0.65	28	106	62	5.9
6	100	15	1.60	43	90	88	5.1
7	150	35	1.40	65	88	ND	4.5
8	150	25	ND	ND	99	77	8.2
9	160	30	0.90	43	116	90	13.2
10	150	30	1.40	11.5	96	103	12.4
Normal range	<275	<62	0.1–2.4*	0.3–40*	70–128	76–141	<8.0

VO = 10 min venous occlusion; alpha 2-AP = alpha 2-antiplasmin; PAI = plasminogen activator inhibitor.
* According to Anglés-Cano et al. (21).

and seems to be due to the low albumin level. In two patients with no thrombotic complication several data suggest a molecular abnormality of which the genetic or acquired origin needs to be further assessed.

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