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A Sensitive Latex Agglutination Method for the Detection of Soluble Fibrin in Plasma

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Summary. A sensitive method is described for the rapid detection of soluble fibrin in human plasma. It is based on the agglutination of Latex particles suspended in a solution of purified fibrin monomers and Blue Dextran 2000. Photometric registration of the decrease in optical density (OD) during agglutination allows the results to be quantitated. The ΔOD depends on the ratio of fibrin monomers to fibrinogen in the tested plasma rather than on the absolute amount of fibrin monomers present.

Key words: Soluble fibrin – Latex agglutination – Fibrin monomers – Fibrinogen – Blue Dextran.

Zusammenfassung. Wir beschreiben eine empfindliche Methode für den schnellen Nachweis von löslichem Fibrin in menschlichem Plasma. Sie beruht auf der Agglutination von Latexpartikeln, die in einer Lösung von gereinigtem Fibrin und Blue Dextran 2000 suspendiert sind. Die photometrische Aufzeichnung des Abfalls der optischen Dichte (OD) während der Agglutination führt zu quantitativen Resultaten. ΔOD ist abhängig vom Verhältnis der Fibrin-Monomere zum Fibrinogen im getesteten Plasma und nicht vom absoluten Gehalt an Fibrin-Monomeren.

Schlüsselwörter: Lösliches Fibrin – Agglutination von Latexpartikeln – Fibrin-Monomere – Fibrinogen – Blue Dextran.

Introduction

Thrombin splits negatively charged polypeptides from the fibrinogen molecule [26]. The resulting fibrin monomers aggregate to form fibrin. However, in the presence of fibrinogen, small quantities of fibrin monomers may combine with intact fibrinogen to form soluble complexes of high molecular weight [26]. In

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view of the wellknown difficulties in the diagnosis of thromboembolic disorders, and particularly of "compensated" states of intravascular fibrinogen-fibrin conversion, many attempts have been made to develop methods for the detection of the circulating products of in vivo thrombin action. A clinically useful method should not only be specific such as assays of fibrinopeptide A [23,25], the C^{14} glycine ethyl ester incorporation into fibrin clots [14] or the gel chromatographic demonstration of soluble fibrinogen-fibrin complexes in plasma [7]. For clinical purposes it should be rapid such as the ethanol gelation test [5,9] and the protamine test [20]. Unfortunately the latter methods, which are based on milieu alterations, suffer from rather low sensitivity and give only qualitative results.

In an attempt to specifically demonstrate the soluble fibrin monomers in plasma we have used fibrin monomer coated particles which, if suspended in plasma, are agglutinated when trace amounts of fibrin monomers link them together, but which remain well suspended when added to normal plasma or serum. This approach proved successful using glutaraldehyde-treated human erythrocytes coated with covalently and irreversibly attached fibrin monomers [17], where quantitation of the results was based on differences in agglutination times.

In the present study we report on a modification of the method. The use of Latex particles instead of erythrocytes allows the photometric registration and quantification of the agglutination and has the advantage of circumventing difficulties in end-point detection encountered with red cell agglutination techniques.

Materials and Methods

Materials

Fibrinogen, human, lyophilized, was purchased from AB Kabi, Stockholm, Sweden.

^{125}I-fibrinogen, 90% clottable protein, batches 193 and 210, was obtained from the Radiochemica Center, Amersham, England.

^{131}I-fibrinogen, 90% clottable protein, was provided by EIR, Würenlingen, Switzerland.

Thrombin, bovine 50 NIH units/mg, was obtained from F. Hoffmann-La Roche, Basel, Switzerland.

Heparin (Liquemin) 5000 NIH units/ml, was obtained from F. Hoffmann-La Roche, Basel, Switzerland.

Reptilase®, 6.4 thrombin-units/ml was kindly supplied by Pentapharm AG, Basel, Switzerland.

A polyvalent *snake venom antiserum* capable of neutralizing 0.064 thrombin-like units Reptilase per ml was obtained from Behringwerke, Marburg, Germany. Is was diluted in isotonic saline.

Streptokinase (Kabikinase), 750.000 IU per vial, was obtained from AB Kabi, Stockholm, Sweden.

Dextran T 2000 and *Blue Dextran 2000* (both with a mean molecular weight of 2.000.000) were obtained from Pharmacia, Uppsala, Sweden.

The *Latex* suspension containing 35% pure Latex, 60% water, 2% protein 1–2% resins, 1–2% sugar and 0.5% slat was kindly provided by Semperit, Dietikon, Switzerland.

Citrate phosphate buffer, pH 6.2, 0.15 M, was prepared by adding 0.1 M citric acid to 0.2 M disodium phosphate to give a pH of 6.2.

Acetate buffer, pH 4.6, 0.05 M was prepared by adding 0.1 M sodium acetate to 0.1 M acetic acid to give a final pH of 4.6.

Radioactivity measurements were done in a well-type scintillation counter (Twinscaler Picker Nuclear). For double-labeled samples the ^{125}I cpm were corrected for ^{131}I.

Methods

9 ml of blood was collected from antecubital veins directly into siliconized glass tubes containing 1 ml 0.1 M sodium citrate. The *fibrinogen concentration* was measured with the method of Clauss [6] or using a biuret assay on washed clots. The *thrombin time* and the Reptilase time were measured according to Funk et al. [8]. The *ethanol gelation test* was done according to Godal and Abildgaard [9]. *Fibrinogen/fibrin degradation products* (FDP) were quantitatively assayed with the method of Merskey et al. [21].

Preparation of the Fibrin-Blue-Dextran-Latex Stock Suspension

A solution of fibrin monomers in acetate buffer (pH 4.6) was mixed under constant stirring with Blue Dextran 2000 in phosphate citrate buffer (pH 6.2) at room temperature to give a concentration of aggregable protein of 50–400 mg/100 ml and a concentration of Blue Dextran 2000 of 1 g/100 ml. To 1 vol of this solution 1 vol of a suspension of Latex particles in distilled water (3.5 g/100 ml) was added. This stock suspension, containing between 25 and 200 mg/100 ml fibrin monomers, 500 mg/100 ml Blue Dextran 2000 and 1.75 g/100 ml Latex particles was dialyzed at 4°C against three times 250 vol of phosphate citrate buffer (pH 6.2) and could be stored at 4°C for a month without apparent loss of aggregability. After preliminary studies the concentration of aggregable protein in the stock suspension giving optimal results in the test (see below) was found to be between 25 and 50 mg/100 ml. By pretesting several such stock suspensions in the test system, the optimal concentration for further studies was established.

Test Procedure

The stock suspension was diluted with 35 vol of phosphate citrate buffer (pH 6.2). This dilute suspension could be kept at 4°C for 12 hours without loss of aggregability. 0.1 ml was added to 0.5 ml plasma in a photometer cuvette and after brief shaking was incubated at 37°C for 10 min. The pH of the incubation mixture was 6.9 and was stable during the procedure. After thorough mixing on a Vortex shaker for 10 sec the optical density (OD) was registered at 22°C under constant stirring in an EEL 169 aggregometer (Evans Electroselenium Ltd., Halstead, Essex, England) connected to a recorder (Labograph E 478, Metrohm, Herisau, Switzerland). Agglutination of the Latex particles led to a decrease of the optical density. The difference of OD was used for quantitation of the reaction. A registration time of 8 min allowed the most rapid processing of several samples on one aggregometer, the limiting factor being the incubation time. When difficulties were encountered in defining the zero value, the straight part of the agglutination curve was extrapolated to the zero time axis. Difference in optical density (ΔOD) is given as absolute change in absorbance of white light.

Preparation of Fibrin Monomers [3, 27]

1 g of dry fibrinogen was dissolved in 2 l of isotonic saline and incubated with 50 NIH units of thrombin for 90 min at 37°C. The clot was collected on a glass rod, washed three times with 500 ml of isotonic saline and dissolved within 30 min in 20 to 50 ml of 5 M urea. After centrifugation at 3000 RPM during 15 min at 22°C, dilution of the clear supernatant with 15 vol of isotonic saline led to clotting. This procedure (clotting, washing, dissolving) was repeated three times. Finally the urea-fibrin solution was diluted with 1 vol of 0.05 M acetate buffer, pH 4.6, and dialyzed at 4°C against three times 500 vol of the same acetate buffer for 36 hours. This fibrin monomer solution was stored at 4°C after addition of merthiolate 2.5 μg/ml and could be used during several months.

For the preparation of *^{125}I labeled fibrin monomers* 5 ml of labeled fibrinogen with a protein concentration of 20 mg/100 ml and a specific activity of 100 μC/mg were added to 1 g of unlabeled dissolved fibrinogen and the mixture treated according to the above procedure.

The aggregability (clottability) of the purified fibrin monomers was established as per cent protein clottable after neutralization of 1 vol of fibrin monomer solution in acetate buffer, pH 4.6, by 1 vol sodium acetate 2 M and 6 vol of distilled water as to give a final pH of 6.3 and a final ionic strength of 0.15. Protein measurements were done at 280 nm. Fibrin monomer concentrations given under results are concentrations of aggregable protein.

Fibrin monomer-containing normal plasma (F-plasma) was produced by either thrombin treatment or addition of purified fibrin monomers to normal plasma.

- *Thrombin-treated Plasma (F-plasma).* 0.03 NIH units of thrombin in 0.1 ml isotonic saline was added to 1 ml of normal plasma at 37° C. After 10 to 15 min incubation the ethanol gelation test was positive and cryofibrinogen was demonstrable by storing the plasma overnight at 4° C. Neither at 37° C nor at room temperature was clotting observed during 24 hours. Plasma treated in this manner contains soluble fibrinogen-fibrin intermediates [9] and is subsequently referred to as F-plasma, in contrast to untreated normal or N-plasma, to which for control purposes 1/10 vol isotonic saline was added.

  Concentrations of fibrin monomers in F-plasma obtained with this method were not determined.
- *Preparation of F-plasma by Addition of Fibrin Monomers to Plasma.* Purified fibrin monomers in acetate buffer (pH 4.6) could be added to 9 vol of plasma (fibrinogen concentration > 85 mg/100 ml) up to a final fibrin monomer concentration of 6 mg/100 ml without visible precipitation. Controls contained acetate buffer. When a concentration of fibrin monomers in F-plasma is given under results, F-plasma was obtained with this method.
- *F-plasma Containing ^{125}I labeled Fibrin Monomers and ^{131}I labeled Fibrinogen.* 1 vol of ^{125}I fibrin monomers was added to 9 vol of plasma and after thorough mixing 1 vol of 0.05 mg/100 ml ^{131}I fibrinogen in isotonic NaCl was added. A control contained acetate buffer instead of fibrin monomers. No precipitation was visible.

Defibrinated Plasma. N-plasma was heated during 30 min at 56° C and was subsequently centrifuged twice during 10 min with 3000 RPM. For control purposes 1/10 vol of isotonic saline was added.

Results

1. Concentration of Fibrin Monomers in the Test System

In order to establish the optimal concentration of fibrin monomers in the stock suspension of Latex particles, suspensions were prepared with various dilutions of fibrin monomers as to give final concentrations ranging from 25 to 200 mg/100 ml in the stock suspension. Subsequently the test was carried out in the usual manner with F-plasma, N-plasma and with heat-defibrinated plasma (Fibrinogen concentration in F- and N-plasma 150 mg/100 ml). With increasing fibrin monomer concentration in the stock suspension an agglutination of the particles was first observed with F-plasma, later with defibrinated plasma and ultimately also with N-plasma (Table 1). For use in the finally adopted test system a fibrin monomer concentration has been chosen which gives an agglutination with F-plasma but none with defibrinated plasma. This concentration usually varies from 25 to

Table 1. Protein (fibrin monomer) concentration of the stock suspension

Protein concentration in stock suspension, mg/100 ml	ΔOD F-plasma	ΔOD defibrinated plasma	ΔOD N-plasma
25	0	0	0
37.5	0.025	0	0
50	0.0425	0.015	0
75	0.0525	0.040	0
150	0.170	0.1375	0
200	0.195	0.30	0.025

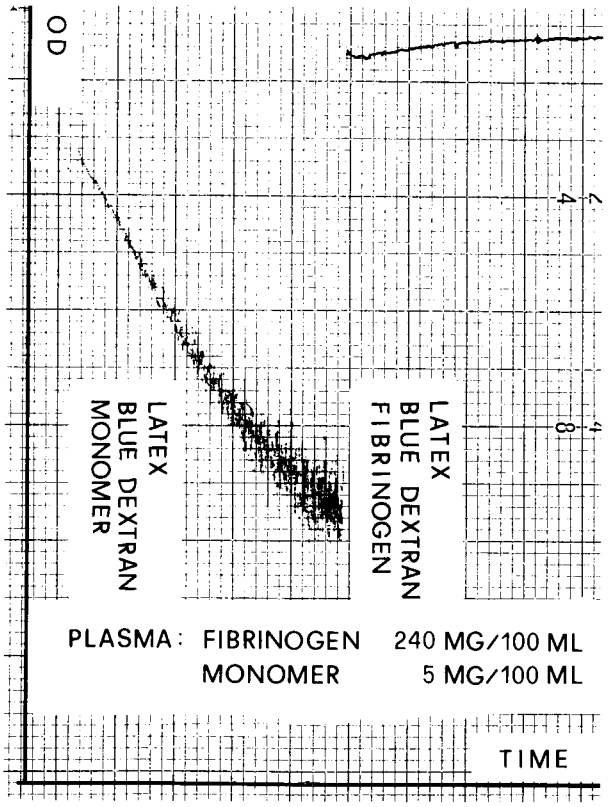

Fig. 1. Comparison of test with the same F-plasma with:
Left: Test solution from stock suspension containing 50 mg/100 ml fibrin monomers
Right: Test solution from stock suspension containin 50 mg/100 fibrinogen

50 mg/100 ml, depending on the functional integrity of the fibrin monomers. The latter was assessed by determination of the percentage of protein in the acid fibrin monomer solution clottable by increasing the pH to 6.3 (see Methods).

When the fibrin momomers in the Latex stock suspension were replaced by an equal (50 mg/100 ml) amount of fibrinogen which had been previously dialyzed against the sodium acetate buffer, pH 4.6, no agglutination was observed either with 3 different F-plasmas, as shown in Fig. 1 for one of those F-plasmas, or with N-plasma.

2. Binding of the Fibrin Monomers to the Latex Particles

Centrifugation at 25000 G for 20 minutes at 4° C of a stock suspension containing 150 mg/100 ml ^{125}I labeled fibrin monomers as prepared by addition of ^{125}I fibrinogen to the original fibrinogen solution used for the monomer production, allowed the separation of the Latex particles from the heavier suspending medium. While this medium contained 38% of the total radioactivity, subsequent consecutive washings of the separated Latex particles led only to a minor liberation of radio-

Table 2. Binding of fibrin monomers to Latex particles

Washing	% ^{125}I fibrin monomer radioactivity eluted from the Latex particles by consecutive washings in 5 ml of		
	phosphate citrate buffer pH 6.2	Blue Dextran 2000 500 mg/100 ml in phosphate citrate buffer pH 6.2	Urea, 5 M
1	3.6	4.2	2.8
2	0.9	2.1	0.62
3	0.2	0.5	0.4
4	0.5	0.4	0.2
5	0.1	0.3	0.2
total	5.3	7.5	4.2

activity from the particles (Table 2), regardless of whether they were washed in the original suspending medium, in buffer alone, or even in 5 M urea.

3. Influence of Various Dextran Concentrations

The addition of Blue Dextran 2000 to the stock suspension was found to be of crucial importance in our test system. It could not be replaced by Dextran T 2000 of the same molecular weight distribution. In order to check, if this difference is due to differences in the effect on fibrin aggregation per se, acid fibrin monomer solutions (75 mg/100 ml) in various concentrations of each Dextran were neutralized to a pH of 6.3 by addition of 1 vol of 2 M sodium acetate at room temperature.

Fibrin aggregation was assessed visually 20 hours after neutralization. While Blue Dextran 2000 in concentrations below 87.5 mg/100 ml precipitated fibrin monomers (75 mg/100 ml) before neutralization at a pH of 4.6, higher concentrations prevented aggregation at pH 4.6 and 6.2. Dextran T 2000 was not able to

Table 3. Influence of Dextran F 2000 and Blue Dextran 2000 on the solubility of fibrin monomers

Final Dextran concentration mg/100 ml		Visible precipitation of fibrin at pH	
Blue Dextran 2000		4.6	6.2
	1400	—	—
	700	—	—
	350	—	—
	175	—	—
	87.5	+	+
	43.7	+	+
Dextran T 2000	1400	—	+
	700	—	+
	350	—	+
	175	—	+
	87.5	—	+
	43.7	—	+
Buffer		—	+

Table 4. Influence of Dextran T 2000 or Blue Dextran 2000 on thrombin- and Reptilase time of normal plasma

Mixture of 1 vol N-plasma + 1 vol of	Thrombin time 5 NIH u/ml sec	Thrombin time 2.5 NIH u/ml sec	Reptilase time 4 thrombinlike u/ml sec
Dextran T 2000, 1 g/100 ml in phosphate citrate buffer, pH 6.2	19.9 ± 0.63	26.8 ± 1.34	19.6 ± 0.71
Blue Dextran 2000, 1 g/100 ml in phosphate citrate buffer, pH 6.2	19.4 ± 0.19	25.3 ± 1.65	20.1 ± 0.6
Phosphate citrate buffer, pH 6.2	23.3 ± 1.12	31.2 ± 1.69	24.2 ± 0.54

prevent aggregation after neutralization at a pH of 6.2 even in concentrations up to 1400 mg/100 ml (Table 3). In contrast, when Blue Dextran 2000 and Dextran T 2000 were compared with regard to their effect on thrombin and Reptilase-times of normal plasma, no difference was found (Table 4).

4. Mechanism of Agglutination

Since under conditions adopted for the test system Latex agglutination only occurs with F-plasma but not with N-plasma or defibrinated plasma, it appears likely that fibrin monomers must be involved, possibly by linking monomer-coated particles together in a fibrin aggregation reaction similar to what is known for normal fibrin clot formation. Thus, experiments were done to establish whether ^{125}I labeled fibrin momomers of the F-plasma show a higher affinity toward the fibrin coated Latex particles than the ^{131}I labeled fibrinogen of the same F-plasma (see Methods). After completion of a normal agglutination assay and subsequent centrifugation, the medium contained relatively more fibrinogen-bound radio-activity than fibrin monomer-bound radioactivity, i.e. the fibrin-coated particles extracted a greater proportion of the fibrin monomers than of the fibrinogen present in F-plasma (Table 5).

Table 5. Extraction of ^{125}I fibrin monomers versus ^{131}I fibrinogen from the test plasma by the fibrin coated Latex particles

Test plasma Fibrin monomer mg/100 ml	Fibrinogen mg/100 ml	Radioactivity ^{125}I fibrin monomer total cpm	residual cpm	%	^{131}I fibrinogen total cpm	residual cpm	%
6	220	3208	2507	78	1045	973	93
3	220	1447	1195	83	1006	941	94
1.5	220	758	638	84	1070	1032	96
0	220	0	0	0	975	878	90

Radioactivity was determined in the incubation mixture (total) and after completion of the test and subsequent removal of the Latex agglutinates (residual)

5. Modification of the Test Plasma

a) Fibrin Monomer Comcentration in F-Plasma. As shown in Fig. 2, increasing the fibrin monomer concentration at a constant fibrinogen concentration of 235 mg/100 ml leads to a progressive increase of the ΔOD. Similarly, if F-plasma is diluted with N-plasma, which reduces its fibrin monomer content while maintaining the normal fibrinogen concentration, the ΔOD again decreases with decreasing fibrin monomer concentration (Table 6).

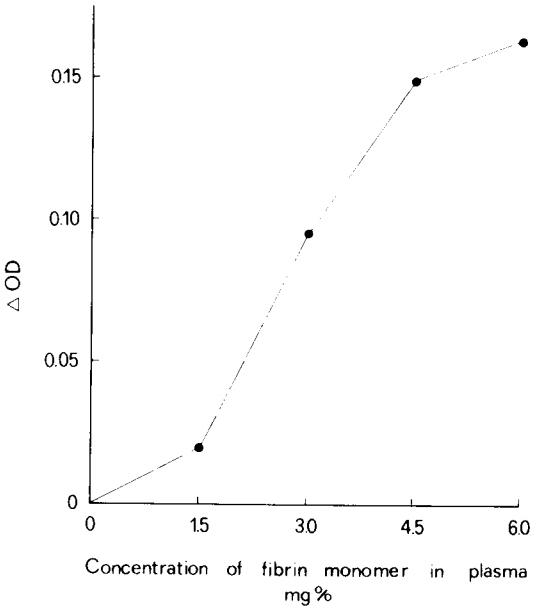

Fig. 2. Increase of ΔOD as a function of the amount of fibrin monomer added to normal plasma (fibrinogen concentration 235 mg/100 ml)

Table 6. Dilution of fibrin monomers with constant fibrinogen concentration (A) and with simultaneously decreasing fibrinogen concentration (B)

Vol F-plasma	+ Vol diluent	Dilution of F-plasma with A N-plasma Fibrinogen mg/100 ml*	Ethanol gelation test	ΔOD	B defibrinated plasma Fibrinogen mg/100 ml[a]	Ethanol gelation test	ΔOD
1	0	200	+	0.050	200	+	0.050
1	1	200	—	0.0300	100	—	0.055
1	3	200	—	0.010	50	—	0.042
1	7	200	—	0	25	—	0.037
1	15	200	—	0	12.5	—	0.001
1	31	200	—	0	6.25	—	0.001
1	63				3.125	—	0
0	1	200	—	0	0	—	0

Fibrinogen concentration mg/100 ml; F-plasma 200; N-plasma 200; defibrinated plasma 0
[a] Calculated values

Table 7. Influence of fibrinogen concentration in plasma

Mixture Acetate buffer pH 4.6	Fibrin monomer 50 mg/100 ml	N-plasma	Defibrinated plasma	Final concentration Fibrin monomer mg/100 ml	Fibrinogen mg/100 ml	ΔOD
—	0.2	2.0	—	4.5	170	0.017
—	0.2	1.2	0.6	4.5	120	0.030
—	0.2	1.0	1.0	4.5	90	0.067
0.2	—	2.0	—	0	170	0
0.2	—	—	2.0	0	0	0

The fibrin monomer concentration was kept constant. Since in plasma with low fibrinogen concentrations solubility of fibrin monomer decreases, fibrinogen concentrations below 90 mg/100 ml were not used

b) Fibrinogen Concentration in F-plasma. When identical amounts of fibrin monomers are added to plasmas with various fibrinogen concentrations obtained by mixing normal plasma with heat-defibrinated plasma (Table 7), the ΔOD increases with decreasing fibrinogen concentration.

c) Simultaneous Reduction of Fibrin Monomer and Fibrinogen in F-plasma. When F-plasma is diluted with heat-defibrinated plasma, ΔOD decreases with decreasing fibrin monomer and fibrinogen concentration. However, this decrease occurs less rapidly than when fibrin monomer is diluted without simultaneous dilution of fibrinogen (Table 6).

d) Reptilase Addition to Plasma in Vitro. Reptilase, 0.001 thrombin-like units in 0.1 ml citrate phosphate buffer, pH 6.2, was added to 0.9 ml plasma, the mixture was incubeted at 37° C and the snake venom enzyme blocked after various intervals

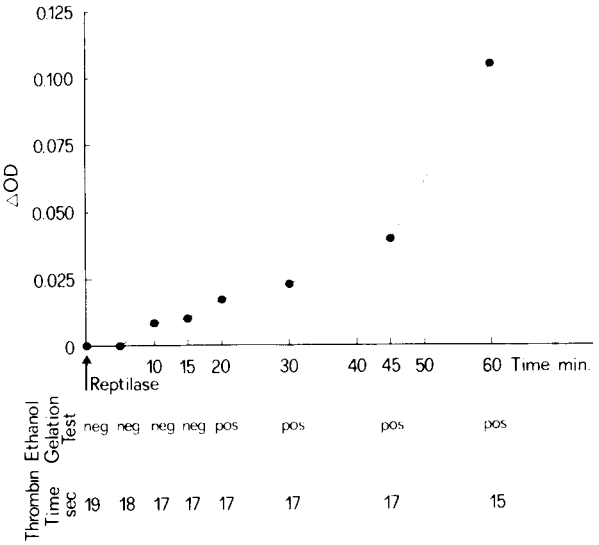

Fig. 3. Influence of Reptilase (0.001 thrombin-like u/ml) on the Latex agglutination test (ΔOD), on the ethanol gelation test and on the thrombin time of normal plasma in vitro. First threads of fibrin after 90 min

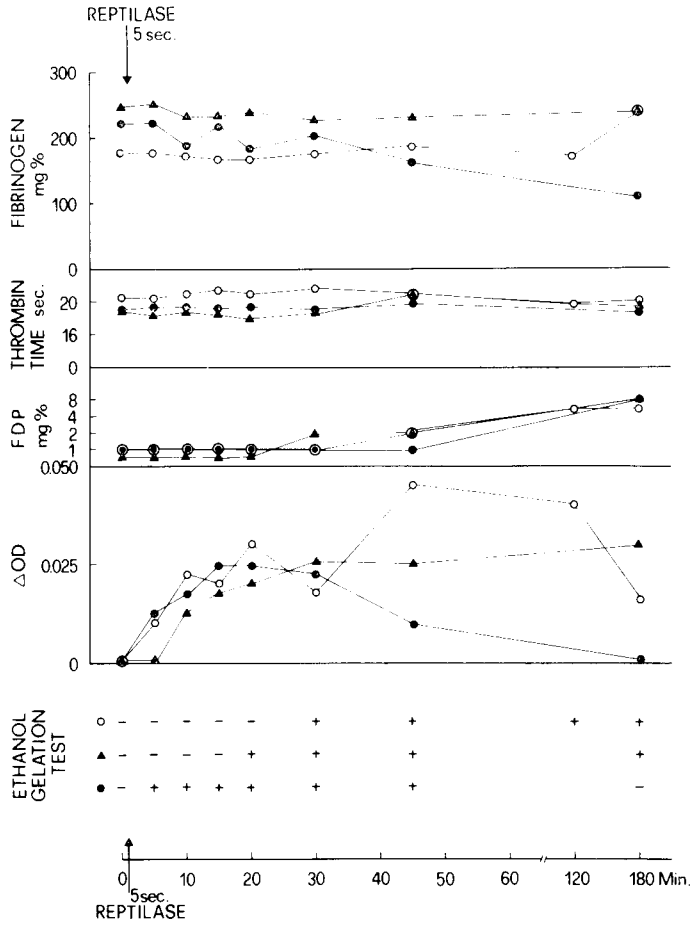

Fig. 4. Influence of an infusion of Reptilase (0.33 μg/kg in 5% glucose) in vivo on various parameters of plasma

by addition of 0.1 ml of a 1 in 7 dilution of antivenom. In the control assay, Reptilase and antivenom were mixed prior to their addition to the same plasma. As shown in Fig. 3, Reptilase led to a progressive increase of the ΔOD and finally to clotting of the plasma. No change of ΔOD was found with the control plasma.

e) Reptilase Infusion in Vivo (Fig. 4). In 3 volunteers a 5-minute infusion of a non-defibrinating dose of 0.33 μg/kg body weight of Reptilase (Defibrase), started 5 seconds after collection of the preinfusion samples, led to an increase of ΔOD, to a positive ethanol gelation test and to an increase of FDP.

f) Streptokinase in Vitro (Fig. 5). In order to get some information as to the effect of fibrinogen/fibrin degradation products (FDP), F-plasma was subjected to fibrinolysis activation by Streptokinase. Kabikinase was added to give a final concentration of 100 IU/ml. Fibrinolysis is documented by a drop of fibrinogen and an increase of FDP and of the thrombin time. The Latex agglutination test became negative only at a fibrinogen concentration of below 14 mg/100 ml. It

must be noted that fibrinogen values obtained with the Clauss method probably represent fibrinogen plus fibrin monomers. At higher fibrinogen concentrations FDP of up to 32 mg/100 ml did not prevent Latex agglutination although the ethanol gelation test reverted to negative. N-plasma subjected to the same fibrinoly-

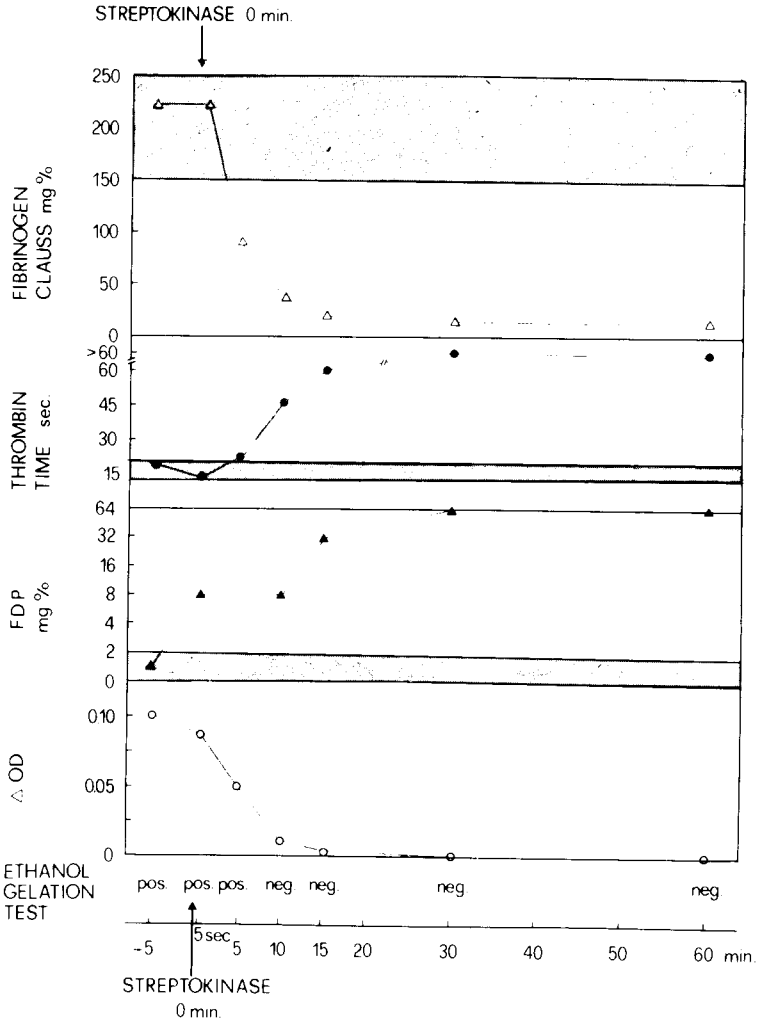

Fig. 5. Influence of fibrinolysis as induced by streptokinase addition on various parameters of thrombintreated plasma (F-plasma)

Table 8. Influence of heparin

To F-plasma with 4 mg/100 ml final concentration of fibrin monomers 1/10 vol of heparin in isotonic saline was added, and the test performed as usual

Heparin concentration U/ml plasma	ΔOD
5	0.100
2	0.087
0	0.097

sis by Streptokinase over 30 min (fibrinogen 72 mg/100 ml) gave negative results in the test. Plasmas of patients subjected to therapeutic fibrinolysis with Streptokinase always gave negative results.

g) Heparin. Heparin, added to F-plasma in concentrations that may be therapeutically achieved, does not affect the ΔOD (Table 8).

Discussion

Test System

In plasma, i.e. in the presence of fibrinogen, fibrin monomers generated by the action of thrombin, aggregate to visible fibrin, if their concentration exceeds a certain threshold value [26]. Abildgaard [1] showed, that about 6 percent of fibrinogen has to be converted to fibrin before visible coagulation takes place. Fibrin monomers in concentrations below this threshold value form complexes with fibrinogen ("cryoprofibrin", [26]), which can be demonstrated by gel chromatography [7]. Addition of purified fibrin monomers to a plasma already containing soluble fibrin should therefore lead to dissociation of those complexes and to formation of visible fibrin. A solution of purified fibrin monomers, however, will aggregate even in low concentrations at a pH above 5.6 [18]. By addition of Blue Dextran 2000 in adequate concentration it was possible to keep monomers in solution at a pH of 6.2. If such a solution of fibrin monomers in Blue Dextran was added to F-plasma containing grossly 10 times more soluble fibrin, small aggregates precipitated. This did not occur after addition of the same fibrin monomer solution to normal plasma. The precipitated aggregates were difficult to see, however, and end point determination was troublesome. Addition of Latex particles to the solution of fibrin monomers and Blue Dextran 2000 made the aggregation clearly visible and registerable as a decrease in optical density of the mixture with an aggregometer.

Attempts to separate the Latex particles by centrifugation from the fibrin monomers showed, that a considerable part of the monomers had been bound to the particles and that they could not be separated from them even by 5 M urea. It appears therefore, that in this test system Latex particles carrying fibrin monomers are agglutinated when added to plasma treated with thrombin or another coagulating enzyme and containing soluble fibrin. The system is comparable to the red cell agglutination test of Largo et al. [17], in which fibrin momomer-coated erythrocytes are agglutinated by plasma containing soluble fibrin.

The influence of dextran on the fibrinogen-fibrin conversion has been examined for dextrans with molecular weights up to 500.000 [2, 12, 15, 19, 24]. We found, that Blue Dextran 2000 and Dextran T 2000, both with a molecular weight of 2 millions, accelerated the clotting time in the thrombin time and the Reptilase time assay. Blue Dextran 2000, in contrast to Dextran T 2000, in sufficient concentrations, was able to prevent fibrin aggregation at a pH of 6.2, but in lower concentrations precipitated fibrin monomers even at an acid pH. The large negative charge of the dye of Blue Dextran 2000 [28] may explain this difference between the two dextrans, which both have a molecular weight of 2 millions.

Test Plasma

At a constant fibrinogen concentration the change in optical density is a function of the *concentration of fibrin monomers* in plasma. This could be shown both in vitro by addition of fibrin monomers to normal plasma and in vivo after infusion of a low dose of Reptilase which we have earlier shown to produce circulating fibrinogen-fibrin complexes demonstrable with gel chromatography [4] and ultracentrifugation [10].

The *fibrinogen concentration* of the test plasma is of crucial importance for the interpretation of results obtained in the present test system. This is in agreement with studies of Shainoff and Page [26] who have clearly demonstrated that more fibrin monomer is soluble the higher the fibrinogen concentration in the medium. Accordingly, at a constant fibrin monomer concentration, we have found a progressive increase of the agglutination velocity with decreasing fibrinogen concentration. From these observations and from the comparison of dilutions of F-plasma with either N-plasma or defibrinated plasma it must be concluded that the velocity of agglutination is not so much a function of the absolute amount of fibrin monomers in the test plasma but rather of the ratio of fibrin monomer to fibrinogen. It is therefore mandatory to use defibrinated plasma in addition to normal plasma for the establishment of the optimal fibrin monomer concentration in the test suspension, in order to prevent false positive results with test plasmas of low fibrinogen concentration. We have preferred heat defibrinated plasma to serum because the latter may contain some thrombin and possibly also some FDP [22]. For quantitation of fibrin monomers the effect of the fibrinogen concentration will have to be taken into account by establishment of calibration curves for different ranges of fibrinogen concentration.

This is complicating the clinical use of the test to some extent. However, for theoretical reasons any procedure for the assay of circulating fibrin will be influenced by the fibrinogen concentration as long as no specific test is available for the estimation of fibrin monomers irrespective of their binding to fibrinogen.

The *sensitivity* of this test system is obviously greater than that of the ethanol gelation test. Indeed, clearcut differences in optical density were found with an estimated fibrin monomer concentration of 1.5 mg/100 ml in a plasma sample with a fibrinogen content of 235 mg/100 ml, i.e. when the concentration of fibrin relative to fibrinogen amounted to approximately 0.6%. This concentration may not be detected with ethanol addition [9]. The greater sensitivity was particularly found at low fibrinogen concentrations, suggesting that the assay detects fibrin monomers rather than fibrinogen-fibrin complexes as precipitated in the ethanol gelation test.

As to the influence of *fibrinogen/fibrin digestion products* (FDP) further studies will be needed to assess the effect of various isolated FDP from either fibrinogen or fibrin. Preliminary results obtained during in vitro fibrinolytic digestion of F-plasma indicate that the sensitivity of the Latex test at low fibrinogen concentrations is great enough to overcome the aggregation-inhibiting effect of as much as 32 mg/100 ml of FDP. At this stage of digestion the ethanol test is negative, probably due to its low sensitivity at low fibrinogen concentrations [9, 13].

To check the possibility of a positive test with a plasma containing fibrinogen

and FDP but no fibrin monomers, normal plasma was subjected to fibrinolysis in vitro and several plasmas of patients undergoing therapeutic fibrinolysis with Streptokinase were tested. FDP in absence of fibrin monomers whether produced in vitro or in vivo, never gave a positive result in this test system.

Heparin in therapeutic concentrations does not affect the results.

Mechanism of Agglutination

The analogy between Latex agglutination and normal fibrin aggregation [26] with respect to the inhibiting effect of incereasing fibrinogen concentrations is an indirect argument in favor of our assumption that the process responsible for Latex agglutination in our system is predominantly one of fibrin aggregation, i.e. formation of hydrogen bonds (fibrin-s) [16]. Another argument is the finding that relatively more labeled fibrin monomers than labeled fibrinogen is extracted from F-plasma by the Latex agglutinates. A mechanism of fibrinogen-fibrin complex formation may account for the fact that fibrinogen apparently also takes part in the reaction.

The present test system fulfills several of the criteria for a routine test to detect soluble fibrin in plasma. In contrast to the gelation or precipitation tests [9,20] it is more specific for fibrin monomers, and more sensitive. In contrast to elaborate and time-consuming test systems such as gel chromatography [7] for high-molecular weight fibrinogen-fibrin complexes, the use of insolubilized fibrinogen on colums [11], the ^{14}C-glycine-ethyl ester incorporation method [14] or the radioassay for fibrinopeptide A [25] it is rapid to perform.

Results obtained with plasma containing different concentrations of fibrin monomers added as purified solution or produced in vitro or in vivo by Reptilase in plasma indicate that there is a correlation between concentration of monomers in the test plasma and the decrease of OD in the test mixture during the test.

A calibration, however, will have to rely on methods for exact measurement of fibrin monomers in plasma, and the influence of fibrinogen will have to be taken into account. Studies to obtain a better calibration with the help of the fibrinopeptide A radioimmunoassay [25] and the N-terminal amino acid analysis of fibrinogen have been initiated.

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