Detection of Soluble Intermediates of the Fibrinogen–Fibrin Conversion Using Erythrocytes Coated With Fibrin Monomers

By Remo Largo, Verena Heller, and P. Werner Straub

The presence of minimal amounts of fibrinogen–fibrin intermediates in human plasma was visualized by an agglutination reaction of glutaraldehyde-treated human erythrocytes coated with purified fibrin monomers. A degree of monomer coating was established which produced erythrocytes not agglutinated by normal plasma but by plasma containing minimal amounts of soluble complexes of fibrinogen with fibrin monomers. Under standardized conditions of coating, erythrocyte concentration, temperature, pH, and incubation time, the agglutination time varied with the ratio of soluble fibrin to fibrinogen in plasma. The test was sensitive down to a soluble fibrin concentration of 0.675% of the plasma fibrinogen concentration. Early fibrinogen and fibrin degradation products (FDP) in the plasma led to a prolongation of the agglutination time at a concentration of more than 16 mg/100 ml. Late FDP in a concentration of 100 mg/100 ml did not convert a positive test to negative. The test was not affected by heparin and protamine at concentrations of up to 12.5 and 50 NIH units/ml, respectively.

IN THE ABSENCE of hypofibrinogenemia, the diagnosis of low-grade intravascular coagulation usually depends on the demonstration of secondary effects such as an accumulation of plasmic fibrinogen and fibrin degradation products (FDP) or a decrease in plasminogen. In order to detect the results of the primary event, namely thrombin action on circulating fibrinogen, various methods have been developed. The ethanol gelatin test and the protamine test are based on alterations in the milieu leading to precipitation or gelation of fibrinogen–fibrin intermediates. Fletcher has taken advantage of the complex formation of these intermediates for their demonstration using gel columns. In an attempt to specifically demonstrate the result of thrombin action, Nossel and others have developed assays for fibrinopeptide A, while Kisker has proposed to assay circulating fibrin by its ability to incorporate 14C-glycine ethyl ester. In order to demonstrate soluble fibrin in a rapid, specific, and sensitive way, we have developed an agglutination method using fibrin monomer-coated erythrocytes that are not agglutinated by plasma fibrinogen or FDP but by soluble fibrin present in plasma.

In this study the test conditions and some characteristics of the underlying reaction mechanism are described. Clinically important modifications of the test plasma have been studied, such as the effect of soluble fibrin and/or FDP as produced in vitro and in vivo, and the addition of heparin and protamine. Finally, the test system has been used for the semiquantitative demonstration.

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of circulating soluble fibrinogen-fibrin complexes produced by intravenous infusion of the thrombin-like snake venom enzyme, Reptilase, in a volunteer.

MATERIALS AND METHODS

Materials
To obtain normal citrated plasma, blood was collected directly into siliconized glass tubes containing 0.1 volume of 0.124 M trisodium citrate and centrifuged for 15 min at 2000 g at room temperature. The supernatant platelet-poor plasma was stored at -20°C. Fibrinogen, clottability 95%, was purchased from AB Kabi, Stockholm, Sweden. 125I and 131I-fibrinogen with a protein concentration of 2 mg/ml and a specific activity of 0.012 mCi/ml, or 1.6 mg/ml and 0.06 mCi/ml, respectively, clottability 95%, were prepared with the iodine monochloride method by EIR, Würenlingen, Switzerland. Bovine thrombin, 50 NIH units/mg of dry powder, was obtained from Hoffmann-La Roche, Basel, Switzerland. Reptilase, Defibrase, 900 NIH thrombin units/mg was kindly supplied by Pentapharm, Basel, Switzerland. Urokinase, 250,000 CTA (Committee on Thrombolytic Agents) units/vial, was kindly provided by Dr. F. Duckert, Bürgerspital Basel, Switzerland. Heparin, 5000 NIH units/ml, and protamine chloride, 10 mg/ml, were obtained from Hoffmann-La Roche. Glutaraldehyde, 30%, was obtained from Fluka, Buchs, Switzerland. ethylenediaminetetraacetate (EDTA). Komplexon III, was purchased from Siegfried, Zofingen, Switzerland. Aprotinin, Trasylol, 5000 KI (kallikrein inhibitor) units/ml was obtained from Bayer, Leverkusen, West Germany. Buffer-saline was prepared from 1 vol of isotonic NaCl and 1 vol of 0.15 M phosphate buffer, pH 7.2. Citrate phosphate buffer 0.16 M, pH 6.2, was prepared by addition of 0.2 M disodium phosphate to 0.1 M citric acid. Adsorbed serum was made from normal serum containing 0.1 volume of 0.124 M trisodium citrate, agitated with 50 mg/ml Al(OH)3 (BDH Laboratory Chemicals Division, England) for 10 min at room temperature and subsequently centrifuged at 2000 g for 15 min at room temperature. Adsorption was repeated once. After incubation at 56°C for 10 min and subsequent centrifugation, the pH was adjusted to 7.3 by addition of citrate phosphate buffer, pH 7.2, 0.16 M.

Performance of the Agglutination Test for the Demonstration of Soluble Fibrin in Plasma
Fibrin monomer-coated erythrocytes were prepared each day using the stock glutaraldehyde-treated erythrocytes, and the stock solution of fibrin monomers (see below). One-tenth milliliter of the 2.5% suspension of fibrin monomer-coated erythrocytes (0.27 mg fibrin monomers/ml packed cells) was added to 0.2 ml of citrated test plasma and citrated normal plasma (N-plasma), respectively, in 4 x 1-cm glass tubes at 37°C. The final pH of the incubation mixture was 6.9 ± 0.05. It remained stable for 30 min. After rapid shaking, the suspension was incubated for 10 min at 37°C without stirring. The suspension was then mixed using a Vortex shaker (Scientific Industries, Inc., Springfield, Mass.) and the agglutination time was determined visually using gentle tilting on a Coombs tile rocker (Merz and Dade, Switzerland) at about 37°C. The agglutination time was measured with a stopwatch, the endpoint being defined as the time when a clear-cut difference became apparent between thrombin-treated test plasma and N-plasma (Fig. 1). Beyond an observation time of 600 sec, agglutination was never observed.

Preparation of the Glutaraldehyde-treated Erythrocytes
Four hundred milliliters venous blood were drawn from the antecubital vein of one blood group O, Rh-negative healthy donor, and collected directly into 0.1 volume of 4% EDTA. The blood was immediately centrifuged at 4°C and 180 g for 15 min and the platelet- and WBC-rich plasma was removed. One hundred twenty milliliters of the packed red cells were washed eight times at 4°C with 10 volumes of 0.11 M phosphate buffer, pH 7.2. To an 8% erythrocyte suspension in phosphate buffer 0.11 M, pH 7.2, an equal volume of 3% glutaraldehyde in the same buffer was added dropwise, with constant stirring and continual adjustment of the pH to 7.2 by addition of 0.1 N NaOH at room temperature. The suspension was further stirred at 22°C for 24 hr. The erythrocytes were then washed six times with 10 volumes of 0.11 M phosphate buffer, pH 7.2, and once with buffer-saline, and were stored at 4°C as a 10% suspension in buffer-saline containing merthiolate 0.25 mg/100 ml.
Preparation of Fibrin Monomers

Thrombin fibrin monomers. In order to prevent possible crosslinking by traces of factor XIII in Kabi-fibrinogen, the following procedure was performed in the complete absence of calcium ions, using glassware previously rinsed with a 5°, EDTA solution. One gram of dry fibrinogen was dissolved in 2000 ml isotonic NaCl 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 NaCl, dried between filter papers and dissolved within 30 min in 20-50 ml of 5 M urea. Some insoluble material was removed by centrifugation at 2000 g for 15 min at 22°C. Subsequent dilution of the clear supernatant with 15 volumes of isotonic NaCl led to clotting. The fibrin was again washed with isotonic NaCl, dried, redissolved, and reclotted three times. Finally, the urea-fibrin solution was diluted with 1 volume of 0.05 M acetate buffer, pH 4.6, and dialyzed against the same buffer at 4°C for 24 hr. The protein content of this fibrin monomer stock solution was 500-1000 mg/100 ml, as measured with a biuret method and the solution was stored at 4°C after addition of merthiolate 0.25 mg/100 ml.

For the preparation of 125I-labeled fibrin monomers, 5 ml of labeled fibrinogen with a protein concentration of 2 mg/ml and a specific activity of 0.012 mCi/mg were added to 1 g of dissolved unlabeled fibrinogen. The mixture was treated as described above for thrombin fibrin monomers.
Reptilase fibrin. The procedure was as for thrombin fibrin, using amounts of Reptilase equivalent to 50 NIH units of thrombin in terms of clotting time of 0.2 ml human citrated plasma with 0.1 ml of thrombin or Reptilase, respectively, and processed as described above.

Fibrin Coating of Erythrocytes

One-tenth milliliter of stock packed glutaraldehyde-treated erythrocytes were washed twice with 100 volumes of 0.05 M acetate buffer, pH 4.6. The cells were then suspended in 5 ml of the same acetate buffer, and incubated with 0.2 ml diluted fibrin monomer stock solution (0.5-0.9 mg/100 ml acetate buffer) for 15 min at 22°C. They were then washed once with 100 volumes of 5 M urea, and once with 100 volumes of 0.16 M citrate phosphate buffer, pH 6.2. After centrifugation at 180 g for 5 min at 22°C, the packed cells were suspended in 0.16 M citrate phosphate buffer, pH 6.2, to yield 4 ml of a 2.5% erythrocyte suspension.

The fibrin monomer concentration, which after coating produced erythrocytes agglutinated by thrombin-treated unclotted normal plasma (F-plasma, preparation see below), but not by untreated normal plasma (N-plasma), had to be established for each new lot of fibrin monomers using a serial dilution of monomers and subsequent testing of the coated erythrocytes with F-plasma and N-plasma. The ideal concentration of 0.25-0.45 mg fibrin monomers/ml packed cells was obtained when the glutaraldehyde-treated erythrocytes were suspended in a fibrin monomer solution with 0.5-0.9 mg/100 ml acetate buffer.

Preparation of Thrombin-treated Plasma (F-Plasma)

When platelet-poor citrated plasma was incubated with thrombin, 0.03 NIH units/ml at 37°C, no coagulation occurred during 24 hr. The ethanol gelation test was positive within 15 min and cryofibrinogen was invariably present. For this thrombin-treated plasma, which probably contains intermediate products of the fibrinogen-fibrin conversion, the abbreviation F-plasma will be used, in contrast to N-plasma for untreated normal plasma.

Detection of Traces of Thrombin in Test Plasma

In order to detect residual thrombin traces in unclottable test solutions previously treated with thrombin, the test solutions were incubated for 24 hr at 22°C with an equal volume of fibrinogen (500 mg/100 ml in buffered saline, pH 7.2). Absence of clotting indicated a negligible amount of residual thrombin. For comparative purposes, it should be noted that 0.0001 NIH units of thrombin/ml of the above fibrinogen led to clotting after 24 hr, whereas a thrombin concentration of 0.00005 NIH units/ml did not.

Preparation of F-Plasma Containing \( ^{125}I \)-labeled Soluble Fibrin and \( ^{131}I \)-labeled Fibrinogen

One-half milliliter of \( ^{125}I \)-labeled fibrin monomers (500 mg/100 ml in acetate buffer 0.05 M, pH 4.6; preparation described above) were mixed with 0.5 ml of \( ^{131}I \)-labeled fibrinogen (500 mg/100 ml in the same acetate buffer). The \( ^{131}I \)-fibrinogen solution was previously dialyzed against 5 M urea and subsequently against the above acetate buffer. The pH of the fibrin monomer-fibrinogen mixture was increased to 6.0 by adding 0.3 ml citrate phosphate buffer, 0.16 M, pH 6.2. Subsequently, 2 ml citrated plasma with a fibrinogen content of 140 mg/100 ml were added. Precipitated fibrinogen-fibrin material was eliminated by centrifugation at 2000 g for 15 min at room temperature. The final pH of this double-labeled F-plasma was 7.3, and the fibrinogen concentration 150 mg/100 ml. As calculated from its \( ^{125}I \)-radioactivity, 5.4% of the total clottable protein was present as soluble fibrin.

Routine Coagulation Tests

The partial thromboplastin time (PTT) was performed by incubation of 0.2 ml citrated plasma with 0.2 ml PTT-reagent (sonicated human platelets and kaolin, Behringwerke AG, Marburg, West Germany) for 2 min at 37°C and subsequent recalcification with 0.2 ml 0.025 M CaCl\(_2\) (normal values: 40-55 sec). The prothrombin time and individual clotting factors were measured by the methods of Duckert.\(^\text{12}\) Fibrinogen (total clottable protein) was determined with a biuret method.\(^\text{10}\) The thrombin time was measured according to Funk et al.\(^\text{13}\) plasminogen according
Table 1. Fibrin Monomer Binding to Erythrocytes

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>Coating*</th>
<th>Fibrin Monomer Attachment to Erythrocytes</th>
<th>After Centrifugation</th>
<th>Coated, Washed Erythrocytes Incubated in 5 M Urea</th>
<th>After Centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Fibrin Monomer Concentration (mg/100 ml)</td>
<td>cpm</td>
<td>Erythrocyte Supernatant</td>
<td>Erythrocyte Pellet</td>
<td>mg Fibrin/ml Packed Cells</td>
<td>cpm</td>
</tr>
<tr>
<td>4.8</td>
<td>9588</td>
<td>2285</td>
<td>7208</td>
<td>1.54</td>
<td>5501</td>
</tr>
<tr>
<td>2.4</td>
<td>4718</td>
<td>380</td>
<td>4314</td>
<td>0.99</td>
<td>3411</td>
</tr>
<tr>
<td>1.2</td>
<td>2201</td>
<td>68</td>
<td>2048</td>
<td>0.57</td>
<td>1937</td>
</tr>
<tr>
<td>0.6</td>
<td>1325</td>
<td>45</td>
<td>1201</td>
<td>0.27</td>
<td>1121</td>
</tr>
</tbody>
</table>

Background: 58 cpm.

*For coating, the erythrocytes (final concentration 1%) were incubated with various concentrations of 125I-fibrin monomers and the amount of attachment established.

To ascertain the irreversibility of attachment, the coated erythrocytes were incubated in 50 volumes of 5 M urea for 1 hr at room temperature. The lowest degree of coating corresponded to coating used in the standard assay system. After centrifugation, the radioactivity of the supernatant was less than 3% of the total erythrocyte-bound radioactivity.

RESULTS

Test System

In preliminary experiments glutaraldehyde-treated erythrocytes were used without attachment of protein, or were coated either with purified human fibrinogen or purified human fibrin monomers. Whereas fibrinogen-coated or uncoated cells were not agglutinated by both F-plasma (thrombin-treated unclotted normal plasma) and N-plasma (untreated normal plasma), fibrin monomer-coated erythrocytes were agglutinated by F-plasma but not by N-plasma under the experimental conditions as finally adopted.

Amount and Nature of Fibrin Monomers Attached to Red Cells

When erythrocytes were coated with 125I-fibrin monomers (Table 1), all of the fibrin monomers in the incubation mixtures with a final fibrin monomer concentration of up to about 1.2 mg/100 ml were bound to the erythrocytes, corresponding to a coating of 0.57 mg/ml packed cells. These erythrocytes showed a loss of bound fibrin radioactivity of less than 3% during 24 hr of standing at room temperature and also after 1 hr incubation in 5 M urea (Table 1). Fibrin concentrations higher than 0.6 mg/ml packed cells led to agglutination by F-plasma and also by N-plasma. On the other hand, fibrin monomer concentrations of 0.25-0.6 mg/ml packed cells gave a definite difference in agglutination times between N-plasma and F-plasma. Below 0.45 mg fibrin monomers/ml packed cells, N-plasma was no longer able to agglutinate these cells. Uncoated glutaraldehyde-treated erythrocytes were neither agglutinated by N-plasma nor by F-plasma. The results were identical using erythrocytes...
loaded with either fibrin monomers produced with thrombin, and thus devoid of fibrinopeptides A and B, or with fibrin monomers produced with Reptilase, and thus devoid of only fibrinopeptide A.

**Soluble Fibrin Versus Fibrinogen in Red Cell Interaction**

In order to determine whether the coated erythrocytes were predominantly agglutinated by soluble fibrin or fibrinogen, fibrin monomer-coated erythrocytes were exposed to F-plasma containing $^{131}$I-labeled fibrinogen and $^{125}$I-labeled soluble fibrin (preparation described in Materials and Methods). After centrifugation the erythrocyte pellet contained $11\%$ of the original fibrinogen radioactivity and $45\%$ of the original soluble fibrin radioactivity. In contrast, when fibrinogen-coated erythrocytes ($0.25$ mg/ml packed cells) were incubated with F-plasma containing labeled fibrinogen and fibrin, $9\%$ of the initial fibrinogen radioactivity and $7\%$ of the initial soluble fibrin radioactivity were found in the erythrocyte pellet.

**Test Plasma**

**Variation of the Concentration of Soluble Fibrin and Fibrinogen in F-Plasma**

The sensitivity of the method could be established using F-plasma (fibrinogen 140 mg/100 ml) containing $^{125}$I-labeled soluble fibrin (Table 2). With decreasing concentrations of soluble fibrin as obtained by dilution of F-plasma with N-plasma (fibrinogen 140 mg/100 ml) the agglutination time increased and became infinite at a dilution beyond 1:8, corresponding to a concentration of less than $0.675\%$ of soluble fibrin relative to fibrinogen. In contrast, the ethanol gelation test was positive down to a 1:4 dilution at best.

Simultaneous variation of both fibrinogen and soluble fibrin concentration in F-plasma was obtained by dilution of F-plasma with citrated adsorbed serum. As shown in Table 2, agglutination times were prolonged only with much higher dilutions than when only the fibrin monomer concentration was reduced by dilution with normal plasma.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Mixtures of Labeled F-Plasma With</th>
<th>N-plasma</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fibrin in % of Fbg.</td>
<td>Agglutination Time (sec)</td>
<td>Fibrin in % of Fbg.</td>
</tr>
<tr>
<td>1:1</td>
<td>5.4</td>
<td>44</td>
<td>5.4</td>
</tr>
<tr>
<td>1:2</td>
<td>2.7</td>
<td>70</td>
<td>5.4</td>
</tr>
<tr>
<td>1:4</td>
<td>1.35</td>
<td>150</td>
<td>5.4</td>
</tr>
<tr>
<td>1:8</td>
<td>0.675</td>
<td>400</td>
<td>5.4</td>
</tr>
<tr>
<td>1:16</td>
<td>0.38</td>
<td>&gt;600</td>
<td>5.4</td>
</tr>
<tr>
<td>1:32</td>
<td>0.19</td>
<td>&gt;600</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Table 2. Sensitivity of the Test System and Influence of the Soluble Fibrin/Fibrinogen Ratio

F-plasma with labeled soluble fibrin (see Materials and Methods) was diluted with N-plasma (changing the soluble fibrin/fibrinogen ratio) or citrated adsorbed normal serum (without changing the soluble fibrin/fibrinogen ratio). Fibrinogen concentration of both N-plasma and F-plasma was 140 mg/100 ml.
Influence of Fibrinogen–Fibrin Degradation Products (FDP)

In vitro studies. Fibrinogen and fibrin degradation products (FDP) were produced by urokinase activation of the endogenous plasma fibrinolytic system. Early FDP are defined as those observed from the start of urokinase incubation up to a time when the thrombin time is three times the initial value, immunoelectrophoresis showing fragments of X and small amounts of Y. Late FDP are defined as those observed beyond incoagulability of digested plasma or complete dissolution of a plasma fibrin clot, immunoelectrophoresis showing fragments Y, D, and E.

Effect of early plasmin digestion products. N-plasma was incubated with urokinase 250 CTA units/ml at 37°C during 2 hr (fibrinogen degradation products), and digestion was blocked with Aprotinin 250 KI units/ml after intervals of 1, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min. The agglutination test and the ethanol gelation test were performed on each sample. During incubation, fibrinogen was reduced from 185 to 130 mg/100 ml, the thrombin time increased from 15 to 81 sec, and FDP increased from 0 to 16-32 mg/100 ml. Fibrinogen degradation products did not lead to a positive agglutination test.

N-plasma was incubated with thrombin 5 NIH units/ml. After 4 hr at 37°C, the clot supernatant had no residual thrombin activity as tested with purified fibrinogen (see Materials and Methods). Subsequently, the clotted plasma was incubated with urokinase and digestion was blocked after various intervals as for N-plasma (fibrin degradation products). During the same incubation time FDP increased from 0 to 32 mg/100 ml. Fibrin degradation products also did not lead to a positive agglutination test.

F-plasma was incubated with urokinase (Table 3) and digestion blocked as for N-plasma. Fibrinogen decreased from 180 to 98 mg/100 ml, the thrombin time increased from 14 to 92 sec, and FDP increased from 0 to 16-32 mg/100 ml.

Table 3. Influence of Early Plasmin Digestion Products as Produced by Urokinase Treatment of F-Plasma

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>Aggl. Ethanol Gel.</th>
<th>Fbg. (mg/100 ml)</th>
<th>Thrombin Time (sec)</th>
<th>FDP (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>120 Pos.</td>
<td>180</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>120 Pos.</td>
<td>205</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>160 Pos.</td>
<td>167</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>180 Neg.</td>
<td>185</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>15</td>
<td>300 Neg.</td>
<td>145</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>20</td>
<td>300 Neg.</td>
<td>122</td>
<td>61</td>
<td>16</td>
</tr>
<tr>
<td>30</td>
<td>300 Neg.</td>
<td>128</td>
<td>70</td>
<td>16</td>
</tr>
<tr>
<td>45</td>
<td>450 Neg.</td>
<td>124</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>60</td>
<td>&gt;600 Neg.</td>
<td>120</td>
<td>83</td>
<td>16</td>
</tr>
<tr>
<td>90</td>
<td>&gt;600 Neg.</td>
<td>146</td>
<td>81</td>
<td>16-32</td>
</tr>
<tr>
<td>120</td>
<td>&gt;600 Neg.</td>
<td>98</td>
<td>92</td>
<td>16-32</td>
</tr>
<tr>
<td>Normal range</td>
<td>&gt;600 Neg.</td>
<td>150-500</td>
<td>13-18</td>
<td>0-1</td>
</tr>
</tbody>
</table>

F-plasma was incubated with urokinase 250 CTA units/ml at 37°C. Assays were performed on aliquots removed at various intervals and after blockage of proteolysis with Aprotinin 250 KI units/ml.
100 ml. The ethanol gelation test became negative in an early digestion stage when the thrombin time was 22 sec and FDP 4 mg/100 ml. In contrast, the agglutination test remained positive up to a thrombin time of 80 sec and FDP of 16 mg/100 ml.

**Effect of late plasmin digestion products.** When N-plasma (fibrinogen 200 mg/100 ml) was digested with urokinase, 5000 CTA units/ml, and plasminogen, 2.5 CU*/ml, and digestion blocked with Aprotinin, 5000 KI units/ml, after 6 hr at 37°C, incoagulability and late *fibrinogen* degradation products on immunoelectrophoresis were obtained at 4 hr. Both the agglutination and the ethanol gelation tests remained negative. Mixtures of this digest with an equal volume of F-plasma showed a thrombin time of 250 sec and negative results in the ethanol gelation test. The agglutination test was positive despite the presence of a calculated concentration of 100 mg/100 ml of late fibrinogen digestion products.

N-plasma was clotted with thrombin, 5 NIH units/ml, for 4 hr at 37°C, and digested with urokinase and plasminogen and digestion blocked with Aprotinin as above. After complete clot dissolution, no residual thrombin activity was detectable (see Materials and Methods) and immunoelectrophoresis showed late *fibrin* degradation products. The agglutination and the ethanol gelation tests remained negative. Mixtures of this digest with F-plasma showed a thrombin time of 95 sec. Both the agglutination and the ethanol tests remained positive in a mixture with an equal volume of F-plasma.

**In vivo study.** Agglutination and ethanol gelation tests were performed on blood samples taken from a 57-yr-old patient with myocardial infarction during thrombolytic therapy with urokinase, 5 million CTA units for 18 hr. Results were negative at 4, 8, 12, and 18 hr and over the whole range of fibrinogen levels from 290 to 74 mg/100 ml. The prolongation of the thrombin time from 17 to 38 sec documented that large amounts of fibrinogen breakdown products were present.

**Influence of Des-A-Fibrin Monomers**

**In vitro studies.** Incubation of N-plasma with Reptilase, 0.0003 NIH thrombin units/ml at 37°C, led to the appearance of the first threads of fibrin within 110 min. During the whole incubation period, fibrinogen and thrombin time did not show significant changes. PTT and prothrombin time showed a shortening from 48 to 38 sec and from 12.0 to 11.4 sec, respectively. The agglutination test became positive at 10 min, while the ethanol gelation test was positive at 20 min.

**In vivo study.** In order to simulate a condition of low-grade intravascular coagulation, a small, nondefibrinating dose of Reptilase was infused in an informed volunteer medical student (Table 4). A shortening of the PTT and of the prothrombin time indicated a transient hypercoagulability. This stage was followed by a moderate drop of fibrinogen. No systemic fibrinolysis was demonstrable; however, a slight increase of FDP was observed. The agglutination test was positive during the entire 3-hr study period, the ethanol gelation test only for a limited period.

*CU, casein units according to Sgouris et al., Vox Sang 5:357, 1960.*
Table 4. Influence of Circulating Des-A-Fibrin Monomers as Produced by a 5-min Intravenous Infusion of Reptilase, 0.023 μg/kg (Corresponding to 0.33 NIH thrombin units/kg), in a Normal Volunteer

<table>
<thead>
<tr>
<th>Time of Blood Sampling (min)</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTT (s)</td>
<td>49 43 37 43 43 49 40-55</td>
</tr>
<tr>
<td>Prothrombin time (sec)</td>
<td>11.9 11.9 11.5 11.4 11.9 12.2 11-12.8</td>
</tr>
<tr>
<td>Fibrinogen (mg/100 ml)</td>
<td>280 280 280 280 235 230 150-500</td>
</tr>
<tr>
<td>Thrombin time (sec)</td>
<td>16 17 17 15 16 19 13-18</td>
</tr>
<tr>
<td>Euglobulin lysis time (hr)</td>
<td>&gt;4 &gt;4 &gt;4 &gt;4 &gt;4 &gt;4 &gt;4</td>
</tr>
<tr>
<td>FDP (mg/100 ml)</td>
<td>0 0 0 0 1 8 0-1</td>
</tr>
<tr>
<td>Agglutination time (sec)</td>
<td>&gt;600 80 60 60 100 120 &gt;600</td>
</tr>
</tbody>
</table>

Influence of Heparin and Protamine

N-plasma or F-plasma were mixed with 0.1 volume of either heparin or protamine or a mixture of both. A prolongation of the agglutination time was observed only with final plasma concentrations of heparin above 12.5 NIH units/ml or of protamine above 50 NIH units/ml. When heparin and protamine were both added to F-plasma the agglutination test became negative with 6.25 NIH units/ml of each agent.

DISCUSSION

It was the aim of the present study to develop a sensitive, rapid, and specific method for the detection of soluble fibrin in plasma, based on the property of fibrin molecules to aggregate. This property was exploited in a test system in which soluble fibrin mediated an agglutination of fibrin monomer-coated erythrocytes.

Test Conditions and Sensitivity of the Method

Individual test conditions were varied so as to obtain a clear-cut agglutination of erythrocytes coated with covalently and irreversibly bound fibrin monomers exclusively in the presence of soluble fibrin. Erythrocytes in a final suspension of 0.8% and with a coating of 0.25–0.45 mg fibrin monomers/ml packed cells showed an agglutination reaction after incubation with thrombin-treated unclotted normal plasma (F-plasma), but no agglutination reaction with untreated normal plasma (N-plasma). In the test system, using a coating of 0.27 mg fibrin monomers/ml packed cells, erythrocyte-bound fibrin monomers represented about 10% of the soluble fibrin in F-plasma, a concentration which, if present in soluble form in plasma, would not lead to fibrin formation. Dilution experiments with F-plasma showed that the agglutination test was sensitive down to a 1:8 dilution at best. Using F-plasma containing 125I-labeled soluble fibrin, the lowest detectable soluble fibrin concentration was 0.675% of the fibrinogen content. For practical purposes, it is apparent from Table 2 that the agglutination test was positive down to a dilution of F-plasma resulting in a fibrinogen concentration of 25 mg/100 ml, and thus for the entire clinically relevant range of fibrinogen concentrations. Dilution curves as obtained by
mixtures of F-plasma with N-plasma or serum further demonstrated that the agglutination times were not only dependent on the absolute fibrin monomer content of plasma but that they were influenced considerably by the soluble fibrin content relative to the fibrinogen concentration. The higher the soluble fibrin/fibrinogen ratio, the shorter the agglutination times. This observation was in agreement with that of Shainoff and Page, who found that initiation of clot formation was dependent on the ratio of soluble fibrin to fibrinogen, rather than on the absolute concentration of soluble fibrin. It therefore also underlines the similarity of the agglutination mechanism with the coagulation mechanism.

**Mechanism of the Agglutination Reaction**

Since uncoated glutaraldehyde-treated erythrocytes were not agglutinated by serum, N-plasma, or F-plasma, a nonspecific agglutination was highly unlikely. On the other hand, intense coating of the erythrocytes with fibrin monomers led to spontaneous agglutination of the coated cells that was not observed after coating with fibrinogen. Thus, despite covalent binding to the erythrocyte surface, the property of the attached fibrin monomers to aggregate appeared to be preserved. Since agglutination was possible only in the presence of soluble fibrin in the test plasma, the latter appeared to have some kind of bridging effect between the fibrin monomer-coated cells. In fact, experiments with F-plasma that contained both labeled fibrinogen and labeled soluble fibrin showed that the latter were found in the erythrocyte pellet in a concentration about four times greater than fibrinogen, relative to the respective concentrations in the F-plasma used. This differential reactivity was only found in a narrow range of pH, temperature, and degree of fibrin monomer coating of the cells. This finding was barely surprising since the molecular interaction involved was likely to be fibrin aggregation, e.g., formation of hydrogen bonds in a reaction similar to soluble fibrin (fibrin s) formation during coagulation.

Ferry and Morrison distinguished end-to-end from side-to-side aggregation of fibrin monomers. Erythrocytes coated with des-A fibrin monomers, as obtained by clotting of fibrinogen with Reptilase, behaved exactly as erythrocytes coated with des-AB fibrin monomers, as obtained by clotting of fibrinogen with thrombin. It has been postulated that Reptilase fibrin is end-to-end aggregated, while in thrombin fibrin both types of aggregates are found. Soluble fibrin in F-plasma obtained by Reptilase action in vitro or in vivo produced agglutinates under the same test conditions as soluble fibrin in F-plasma obtained with thrombin in vitro.

It may be concluded that agglutination is mediated predominantly by fibrin monomers, that cleavage of only fibrinopeptide A from fibrinogen in the test plasma is sufficient to induce agglutination, and that agglutination is due to hydrogen bonding in an “end-to-end” aggregation reaction of fibrin molecules involved in the bridging between the coated erythrocytes.

**Effect of FDP in the Test Plasma**

When N-plasma or clotted N-plasma was incubated with urokinase, the resulting early and late plasmic degradation products (fragments X, Y and D, E, respectively, of both fibrinogen and fibrin did not lead to agglutination of
the test erythrocytes. This finding was clinically confirmed in a patient with hypofibrinogenemia and prolonged thrombin time during thrombolytic therapy with urokinase.

Since, in patients with thrombosis or low-grade intravascular coagulation one usually deals with plasma containing both soluble fibrin and plasmic degradation products of either fibrinogen and/or fibrin, urokinase digestion was also performed on F-plasma. Whereas the ethanol gelation test became already negative at a thrombin time of 22 sec and at an FDP concentration of 4 mg/100 ml, the agglutination test became negative only beyond a thrombin time of 80 sec, when FDP exceeded 16 mg/100 ml. This effect must be due to early FDP since addition to F-plasma of previously prepared late FDP from normal plasma or clotted normal plasma in a calculated final concentration of up to 100 mg/100 ml did not convert the positive test to negative. It is well known that early FDP have a more pronounced inhibiting influence on the aggregation phase of fibrin formation than late FDP.22

Effect of Heparin and Protamine

Since the present test system has been devised for clinical purposes, the influence of heparin and protamine is of practical importance. In concentrations used either in vivo or in vitro neither heparin nor protamine had an effect on the agglutination times and no false positive or false negative results were produced. Addition in vitro of both agents together led to a false negative agglutination test in a concentration of 6.25 NIH units/ml of each agent.

CONCLUSION

From the clinical standpoint, a method for the detection of thrombin action in vivo should be specific, sensitive, quantitative, and rapid. Elaborate methods like the radioassay for fibrinopeptide A,4-5 the 14C-glycine ethyl ester incorporation method,6 and the gel filtration method7 have the disadvantage of being time consuming, whereas the rapid ethanol gelation8 test and the protamine test2 are rather nonspecific. The present agglutination test is not time consuming; it proved to be specific for soluble fibrin monomers, and is more sensitive than the ethanol gelation test and less influenced by the presence of proteolytic degradation products of fibrinogen and fibrin. The quantitative use of the test in the follow-up of patients should be possible in view of the results obtained sequentially in a volunteer infused with the thrombin-like enzyme Reptilase. On the basis of these advantages, the agglutination test may become a clinically useful assay for circulating fibrin.

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Detection of soluble intermediates of the fibrinogen-fibrin conversion using erythrocytes coated with fibrin monomers

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