Latex Immunoturbidimetric Assay for Soluble Fibrin Complex

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Background: Soluble fibrin complex (SFC), composed of fibrin monomer and fibrinogen derivatives, is known to exist in the circulating blood in patients with thrombosis. Its detection and quantification are useful for obtaining information about the condition and degree of intravascular coagulation in early-stage thrombosis, but there is no rapid method to measure SFC in plasma for clinical use.

Methods: We obtained a monoclonal antibody that specifically reacts with SFC, with desAA-fibrin as the immunogen, and developed a rapid and sensitive latex immunoturbidimetric assay (LIA) using latex-immobilized anti-SFC monoclonal antibody. The assay system was based on the increase in turbidity induced by the reaction of the latex-immobilized anti-SFC monoclonal antibody with SFC in plasma, and the assay procedure was fully automated on a Hitachi 911 analyzer.

Results: The method had an analytical range of 3–300 mg/L. Intra- and interassay precision studies indicated that this system provided reproducible data (CVs <3.0% and <2.0%, respectively). The assay detection limit was <0.5 mg/L. There was no interference from bilirubin (up to 440 mg/L), hemoglobin (up to 9.6 g/L), Intralipid (up to 10%), D-dimer (up to 200 mg/L), and rheumatoid factor (up to 470 000 IU/L). SFC concentrations in plasma from patients with thrombotic diseases [mean (SD), 48.9 (57.6) mg/L; n = 160] were significantly higher than those in plasma from healthy individuals [1.8 (2.1) mg/L; P <0.001; n = 304].

Conclusion: In terms of linearity, precision, and sensitivity, the LIA, performed on a Hitachi 911 automated analyzer, may be useful for measurement of SFC in plasma.

Fibrin monomer (FM)3 and its derivatives in blood, produced by the cleavage of one or both A peptides and both A and B peptides from fibrinogen by thrombin, are found in early-stage thrombosis (1). When produced in blood, they form complexes with fibrinogen and exist as soluble complexes called soluble fibrin complex (SFC) (2, 3). Because increased SFC concentrations in plasma indicate that thrombin has converted fibrinogen to fibrin, increased SFC is considered to be a molecular marker of imminent thrombotic events (4–7). Other hemostatic molecular markers, such as D-dimer and thrombin-antithrombin (TAT) complexes, have been developed for the diagnosis of disseminated intravascular coagulation (DIC). These markers are very sensitive but not specific for the diagnosis of DIC. D-Dimer reflects the combined effect of both coagulation and fibrinolysis. Similarly, TAT complexes may reflect thrombin generation but not the actual thrombin activity in vivo. Previously, several methods have been described for the specific detection of SFC by use of monoclonal antibodies (MoAbs) to FM or SFC (8–11). Sheefers-Borchel et al. (8) first reported a MoAb to the newly exposed NH2 terminus of the fibrin α-chain, obtained by use of α-chain N-terminal peptides as the immunogen, and established an ELISA method using that MoAb. Thereafter, Dempfle et al. (9) also established a MoAb to FM in the same manner and developed an ELISA method for the detection of FM in human plasma, which involved pretreatment of the samples with sodium thiocyanate (NaSCN) (10). However, NaSCN pretreatment, used to expose the SFC sites recognized by the antibody, also exposed sites of fibrin degradation prod-

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3 Nonstandard abbreviations: FM, fibrin monomer; SFC, soluble fibrin complex; TAT, thrombin-antithrombin; DIC, disseminated intravascular coagulation; MoAb, monoclonal antibody; LIA, latex immunoturbidimetric assay; BSA, bovine serum albumin; and RF, rheumatoid factor.
ucts, such as cross-linked fibrin degradation products (12).

To solve these problems, we successfully obtained a MoAb that specifically reacts with FM, with desAA-fibrin as the immunogen, in the presence of an antipolymerant peptide (Gly-Pro-Arg-Pro) (13). We also found that the obtained MoAb reacted with SFC without pretreatment with NaSCN (13). Here we report a rapid and sensitive latex immunoturbidimetric assay (LIA) that uses latex-immobilized SFC-specific antibody and performed on a Hitachi 911 automated analyzer. In addition, we measured the SFC concentrations in human plasma and compare SFC with TAT and D-dimer by ROC analysis.

**Materials and Methods**

**CHEMICALS AND REAGENTS**

Polystyrene latex particles, bovine serum albumin (BSA), and skim milk were purchased from JSR Corporation, Sigma, and Difco, respectively. The TAT and D-dimer tests were obtained from International Reagents Corporation. All chemicals and reagents were of the highest available grade.

**SAMPLE COLLECTION**

For method validation, we selected citrate-anticoagulated human plasma from 304 healthy volunteers (age range, 18–74 years) and 160 patients with DIC, which was diagnosed on the basis of the DIC score according to the DIC scoring guidelines proposed by the DIC Study Group of the Japanese Ministry of Public Welfare in 1987 (14). The Japanese scoring system takes into account underlying disease, bleeding symptoms, organ failure, and global coagulation tests (fibrinogen, fibrin degradation products, platelet count, and prothrombin time). Patients with DIC were as follows: no hematopoietic tumor or infectious diseases (n = 16); sepsis (n = 7); gastric cancer (n = 20); lung cancer (n = 19); hematopoietic tumor or acute myeloblastic leukemia (n = 38); promyelocytic leukemia (n = 23); acute lymphoblastic leukemia (n = 26); and myelodysplastic syndrome (n = 11). Blood samples were collected in plastic tubes containing 0.109 mol/L sodium citrate in the ratio of nine parts blood to one part sodium citrate, and were centrifuged for 10 min at 2000g. All plasma samples collected were stored at −80 °C before analysis.

MoAb

We used the MoAb designated as F405 (IgG1 isotype and κ light chain) for the preparation of latex-immobilized antibody. This was established previously in our laboratory (13). F405, obtained with use of desAA-fibrin as the immunogen in the presence of the antipolymerant peptide (Gly-Pro-Arg-Pro), was reactive to desAA-fibrin and SFC but not to fibrinogen. Briefly, the reactivity of F405 to fibrinogen, fibrin, and their derivatives was as follows: F405 was reactive to FM and thrombin-treated fibrin fragments X, Y, and E but not to fibrinogen; the fibrinogen-derived fragments X, Y, D, and E; or D-dimer. Furthermore, when fibrinogen, with or without thrombin treatment, was subjected to sulfate-polyacrylamide gel electrophoresis under reducing conditions followed by immunoblotting, only the α-chain reacted with F405. These results indicated that the epitope recognized by F405 was in the α-chain N-terminal region exposed by removal of the A peptide from the NH$_2$ terminus of the A α-chain.

The IgG fraction of the MoAb was prepared from mouse ascitic fluid obtained by intraperitoneal injection of hybridoma cells into BALB/c mice primed with Pristane. The ascitic fluid was treated with 50% ammonium sulfate, and the antibody fraction was further purified on a DE-52 cellulose column (Whatman) with 0.05 mol/L Tris buffer (pH 8.0) containing 0.1 mol/L NaCl as the eluent. The protein concentration in the IgG fraction was estimated based on the absorbance at 280 nm.

**PREPARATION OF LATEX-IMMOLIZED ANTIBODY REAGENT**

F405 (1.0 g/L of IgG1) and polystyrene latex (1.0 g/L; mean diameter, 190 nm) were incubated in 0.01 mol/L MES monohydrate-buffered saline (pH 6.5) for 2 h at 37 °C, followed by the addition of 0.01 mol/L MES-buffered saline containing 100 g/L skim milk, at a ratio of 0.1 volume to 1 volume of latex suspension. After 1 h, the latex-immobilized antibody was washed twice by centrifugation and then suspended in 0.01 mol/L TES-buffered saline (pH 7.5) containing 1 g/L BSA. We used 1 g/L of latex suspension for the assay.

**PREPARATION OF SFC CALIBRATOR**

Solubilized FM was prepared by modifying the method of McCarron et al. (12). Briefly, a mixture of 1 g/L fibrinogen and 1 U/mL human thrombin (Mochida) in 0.05 mol/L Tris-buffered saline, pH 7.4, was incubated for 1 h at 37 °C and then allowed to stand overnight at 4 °C. After the addition of hirudin at a final concentration of 2.5 IU/mL (Sigma), the clot obtained was centrifuged and washed with physiologic saline. The pellet was solubilized by treatment with 0.02 mol/L acetic acid. The concentration of solubilized FM was determined according to the following equation: absorbance at 280 nm/1.51 = g/L. The polypeptide chain composition of fibrinogen, fibrin, and their derivatives was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

We prepared artificial SFC by adding known concentrations of solubilized FM to SFC-negative human plasma (i.e., SFC concentration below the detection limit of the assay), and the obtained SFC was used as the first calibrator. Human plasma containing desAA-fibrin (Bipod) was used as the working calibrator, and the values of the working calibrators (0, 7, 41, 101, 202, and 312 mg/L) were determined by use of the calibration curve prepared from a series of the diluted first calibrator. Stock working
calibrators were freeze-dried and prepared by diluting the stock with distilled water before use.

ASSAY PROCEDURE
Sample processing, pipetting steps, and measurements were performed automatically on a Hitachi 911 automated analyzer (Hitachi Instruments Engineering). Briefly, 5 μL of a plasma sample and 200 μL of reagent 1 (0.01 mol/L MES buffer, pH 6.5, containing 0.25 mol/L NaCl and 1 g/L BSA), which was used for stabilizing the sample blank, were injected into a reaction cuvette. After a 5-min incubation at 37 °C, 85 μL of reagent 2 (latex-immobilized anti-SFC antibody suspension in 0.01 mol/L TES-buffered saline, pH 7.5, containing 1 g/L BSA) was added to the cuvette. After another 5 min, the SFC concentration was calculated from the difference in absorbance between the two signals (the absorbance at 5 and 10 min) at two wavelengths (primary wavelength, 576 nm; secondary wavelength, 800 nm). A nonlinear six-point calibration curve prepared with the SFC calibrators was used to calculate the values of the plasma samples.

STATISTICS
P values <0.01 were considered significant. Regression analysis was used to investigate the effect of sample treatment with and without NaSCN in our assay system. Diagnostic accuracy was assessed by ROC analysis using commercially available statistical software (STAT FLEX®, Ver. 4). The ROC area was used to provide an overall estimate of the test’s diagnostic performance in differentiating between healthy volunteers and patients with DIC as well as differences in diagnostic accuracy among the three test methods.

Results and Discussion

REACTIVITY OF ANTI-SFC ANTIBODY (F405)
We examined the effect of sample treatment with and without NaSCN in our assay system, using 50 samples from patients with DIC. The sample treatment with NaSCN was carried out according to the method of Lill et al. (10), and the result was compared with that obtained by the sample treatment without NaSCN. Regression analysis of the data gave the following equation: $y = 0.941x + 1.59$ mg/L ($r = 0.995$). Because F405 can react with both FM and SFC, but not with D-dimer, sample treatment with and without NaSCN did not affect our assay method using latex-immobilized F405. This indicated that the LIA is suitable for the Hitachi 911 automated analyzer. We therefore used F405 to prepare a latex reagent. The following data were obtained with the latex-immobilized F405 reagent (reagent 2).

REAGENT 1
Because reagent 1 affects the sensitivity of the assay and the stabilization of the sample blank, we examined the optimum pH of reagent 1, using three buffers (0.01 mol/L MES, 0.01 mol/L TES, and 0.01 mol/L Tris-HCl) in the pH range 5.5–8.5. We measured a series of working calibrators, using assay conditions for the Hitachi 911. The absorbance produced by the latex agglutination reaction increased with decreasing pH of reagent 1, but fibrinogen in plasma precipitated at pH values below 6.0 (Fig. 1).

To precisely measure SFC in plasma samples, the absorbance of the sample blank in reagent 1 must reach a plateau within the first 5 min. We examined the stabilization of the sample blank by measuring 100 random plasma samples in the above buffers containing additives according to Hitachi 911 assay conditions. The obtained result indicated that the sample blank stabilized in proportion to the amount of NaCl, but NaCl concentrations >0.3 mol/L caused an abrupt decrease in assay sensitivity. On the other hand, although BSA was less effective than NaCl for stabilization of the sample blank, the abrupt decrease in assay sensitivity was not observed at BSA concentrations up to 3 g/L. On the basis of these results, we used 0.01 mol/L MES-buffered saline (pH 6.5) containing 0.25 mol/L NaCl and 1 g/L BSA as reagent 1.

CALIBRATION CURVE
A representative calibration curve obtained with a series of SFC working calibrators (six points) is shown in Fig. 2. We assayed a series of SFC working calibrators and plotted the absorbances against theoretical values. The resulting calibration curve showed direct proportionality up to 300 mg/L with deviations ≤5% from theoretical values.

LINEARITY
We evaluated assay linearity, using serial dilutions of a plasma sample (300 mg/L). The results of the study are shown in Fig. 3. Within the measuring range of 3–300 mg/L, the deviations from theoretical values did not exceed 5%, indicating no lack of parallelism.
ANTIGEN EXCESS
We examined the antigen excess phenomenon by assaying a series of diluted SFC solutions (300 mg/L to 1 g/L) on a Hitachi 911 automated analyzer. The absorbance derived from SFC concentrations reached a plateau at a concentration of 500 mg/L, and the plateau was maintained up to 1 g/L. The result indicates that LIA is suitable for diagnostic purposes.

DETECTION LIMIT
We measured physiologic saline in 21 replicates and calculated the SD of the measured values. The detection limit, defined as the mean + 3 SD, was <0.46 mg/L.

PRECISION
We assessed intraassay precision by performing 21 replicate analyses of three samples of ~8.8, 30.0, and 85.7 mg/L. As shown in Table 1, the CVs of the method were in the range of 0.4–2.9%. We assessed interassay precision by analyzing samples in the same manner on 10 consecutive days, and the CVs of the method were 0.5–2.0% (Table 1).

We also assayed three different samples (~7.8, 19.0, and 86.0 mg/L) with three separately prepared batches of our reagents, and the CVs of the results were 0.4–2.2% (Table 1).

RECOVERY
We assessed recovery by adding low, medium, and high concentrations (10–100 mg/L) of SFC to three different samples. The percentage recovery of SFC at each concentration ranged from 91.2% to 103% (mean recovery, 97.8%).

INTERFERENCE
We examined the effects of free bilirubin, ditaurobilirubin, hemoglobin, Intralipid, D-dimer, and rheumatoid factor (RF) on SFC measurement, using three different plasma samples (~8.8, 30.0, and 85.7 mg/L). The results showed that free bilirubin and ditaurobilirubin of up to 400 and 440 mg/L, respectively, did not affect assay precision. Similarly, hemoglobin of up to 9.6 g/L, Intralipid of up to 10%, and D-dimer of up to 200 mg/L did not alter assay precision. It is known that RF affects immunoassays based on the agglutination reaction because RF accelerates nonspecific agglutination via hydrophobic binding. However, RF concentrations of up to 470 000 IU/L did not affect assay precision.

CLINICAL BASELINE (CUTOFF VALUE)
To establish a clinical baseline value in our assay method, we analyzed plasma samples from 304 healthy volunteers (age range, 18–74 years) and 160 patients with DIC for SFC. We obtained mean (SD) SFC concentrations of 1.8 (2.1) mg/L for healthy volunteers and 48.9 (57.6) mg/L for patients with DIC. The SFC concentrations in the plasma from patients with DIC were significantly higher than those in the plasma from healthy volunteers ($P < 0.001$). Furthermore, the result of ROC analysis

### Table 1. Assay precision.

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD), mg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraassay$^{a}$</td>
<td>8.78 (0.25)</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>30.08 (0.19)</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>85.71 (0.36)</td>
<td>0.42</td>
</tr>
<tr>
<td>Interassay$^{a}$</td>
<td>8.75 (0.16)</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>30.23 (0.17)</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>84.18 (0.45)</td>
<td>0.54</td>
</tr>
<tr>
<td>Lot to lot$^{a,b}$</td>
<td>7.83 (0.19)</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>18.94 (0.21)</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>86.08 (0.37)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

$^{a} n = 21.$

$^{b}$ Three different lots.
showed that, in the range of clinical baseline values from 6.1 to 6.7 mg/L, diagnostic specificity remained at 0.977, whereas diagnostic sensitivity decreased from 0.756 to 0.718 (Table 2). We therefore defined 6.1 mg/L as the clinical baseline value. This value agreed well with the mean (6.0 mg/L) that was obtained from healthy volunteers.

We also compared SFC with TAT and with D-dimer in 107 plasma samples (62 of 304 healthy volunteers and 45 of 160 patients with DIC), using ROC analysis (Fig. 4). Results of the ROC analysis indicated that SFC is an excellent marker for the diagnosis of DIC [area under the ROC curve (95% confidence interval): 0.94 (0.92–0.96) for SFC; 0.84 (0.80–0.88) for TAT; and 0.88 (0.85–0.91) for D-dimer].

We have demonstrated here that the LIA established with F405 as the immobilized antibody on latex particles is sensitive and reproducible for the specific measurement of SFC concentrations in plasma samples. The quantitative measurement of SFC in plasma is complicated by the heterogeneous composition of the analyte as a result of variations in thrombin, factor XIIIa, and plasmin activity (15, 16). It has been shown that SFC in plasma from patients with DIC exists as complexes with a variety of fibrinogen derivatives, including intact fibrinogen as well as early and late fibrin degradation products. Accordingly, if the relative concentrations of the various fibrinogen derivatives present in the complex vary depending on the disease stage, the specificities of the MoAbs used for SFC immunoassays may affect the quantitative results for SFC in plasma. Thus, the LIA based on F405 seems to recognize all SFC species because its recognition site is in FM.

In conclusion, the proposed LIA, performed on a Hitachi 911 automated analyzer, is a convenient method and represents an interesting alternative to other immunoas-

says for SFC in plasma. Furthermore, because the SFC concentration reflects the thrombin-generating activity in plasma, the F405-based LIA could be a strong diagnostic tool not only for DIC but also for other suspected thrombotic disorders. We are continuing our studies to further examine its potential usefulness in predicting thrombotic events in patients, e.g., during the postoperative phase and long-term hospitalization.

References


Table 2. ROC analysis of SFC.

<table>
<thead>
<tr>
<th>Cutoff value, mg/L</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>False-positive rate</th>
<th>Test-positive rate</th>
<th>Efficiency</th>
<th>False-negative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.7</td>
<td>0.718</td>
<td>0.977</td>
<td>0.023</td>
<td>0.263</td>
<td>0.888</td>
<td>0.281</td>
</tr>
<tr>
<td>6.5</td>
<td>0.731</td>
<td>0.977</td>
<td>0.023</td>
<td>0.269</td>
<td>0.894</td>
<td>0.262</td>
</tr>
<tr>
<td>6.3</td>
<td>0.743</td>
<td>0.977</td>
<td>0.023</td>
<td>0.272</td>
<td>0.897</td>
<td>0.256</td>
</tr>
<tr>
<td>6.1</td>
<td>0.756</td>
<td>0.977</td>
<td>0.023</td>
<td>0.276</td>
<td>0.901</td>
<td>0.243</td>
</tr>
<tr>
<td>5.9</td>
<td>0.768</td>
<td>0.973</td>
<td>0.026</td>
<td>0.282</td>
<td>0.903</td>
<td>0.231</td>
</tr>
<tr>
<td>5.7</td>
<td>0.775</td>
<td>0.970</td>
<td>0.030</td>
<td>0.287</td>
<td>0.901</td>
<td>0.225</td>
</tr>
<tr>
<td>5.5</td>
<td>0.780</td>
<td>0.965</td>
<td>0.033</td>
<td>0.289</td>
<td>0.900</td>
<td>0.225</td>
</tr>
</tbody>
</table>

Fig. 4. ROC curves for SFC (○), TAT (△), and D-dimer (■) in the diagnosis of DIC. We compared SFC with TAT or D-dimer by ROC analysis, using 107 plasma samples (from 62 healthy volunteers and 45 patients with DIC).


