Plasma Thromboplastin Antecedent (PTA, Factor XI): A Specific and Sensitive Radioimmunoassay

By Hidehiko Saito and George H. Goldsmith, Jr.

A specific, sensitive, and reproducible radioimmunoassay for human plasma thromboplastin antecedent (PTA, factor XI) has been developed with purified PTA and monospecific rabbit antisera. Precise measurements of PTA antigen were possible for concentrations as low as 0.3% of that in normal pooled plasma. Normal plasma contained approximately 6 μg PTA/ml. A good correlation (correlation coefficient 0.68) existed between the PTA procoagulant assays and radioimmunoassays among 50 normal adults (25 males and 25 females). PTA antigen was markedly reduced in plasma of 13 patients with congenital homozygous PTA deficiency (range <0.003-0.128 U/ml) and 9 patients with hepatic cirrhosis (0.35 ± 0.17 U/ml), but was normal in those of 9 patients under treatment with warfarin, 8 patients with disseminated intravascular coagulation and 16 patients with other congenital clotting factor abnormalities, including prekallikrein deficiency (Fletcher trait) and high molecular weight kininogen deficiency (Fitzgerald trait).

PLASMA THROMBOPLASTIN ANTECEDENT (PTA, factor XI) is a plasma protein functionally deficient in plasmas of patients with congenital PTA deficiency. This agent participates early in the intrinsic pathway of blood coagulation. The titer of PTA has been conventionally assayed by measuring the degree to which a test sample reduces the prolonged partial thromboplastin time of known PTA-deficient plasma. This functional assay is dependent upon the whole clotting sequence of the intrinsic pathway and may give an erroneous result if the test sample contains an activated clotting factor participating at a point beyond that at which PTA functions or if the sample contains an anticoagulant such as heparin that interferes with coagulant assays.

Recent advances in the preparation of purified clotting proteins and their specific antibodies have made it possible to measure the concentration of clotting factors by immunologic means. Earlier studies from this laboratory reported the development and application of quantitative immunoassays of antihemophilic factor (AHF, factor VIII), Hageman factor (factor XII), and Fletcher factor (plasma prekallikrein). The present paper will describe a specific, sensi-
tive, and reproducible radioimmunoassay for PTA and report the titer of PTA antigen in some clinical conditions.

MATERIALS AND METHODS

PTA Preparations

Purified human PTA, isolated as reported earlier, was further purified by filtering through a column of Sepharose-attached goat anti-human IgG. This step removed contaminating IgG in the PTA preparations. The batches used had a specific activity of 160–200 U/mg protein, 1 unit being arbitrarily defined as that amount present in 1 ml of a standard pool of normal plasma. These preparations represented 11,000–14,000-fold purification over the starting plasma. The purified PTA preparations were stored in barbital-saline buffer at –70°C. 125I-labeled PTA (125I-PTA) was prepared by enzymatic iodination of purified PTA with Na125I, using lactoperoxidase as described previously. The specific activity of 125I-PTA was approximately 4 x 10^6 cpm/μg protein. 125I-PTA was diluted in crystalline bovine albumin (Pentex, Kankakee, Ill.), 4 mg/ml of barbital-saline buffer, and was stored in small aliquots at –70°C and used over a span of several months.

Antiserum Preparation

Monospecific rabbit antibody to human PTA was prepared against purified PTA and the crude IgG fraction was isolated as reported earlier. Upon immunodiffusion, this antiserum formed a single precipitin line with purified PTA (25 μg/ml), but formed no line with normal plasma or serum. The unabsorbed antiserum, diluted fivefold, inactivated more than 99% of PTA activity of an equal volume of normal pooled plasma in 1 hr at 37°C, but did not inactivate the coagulant activities of Hageman factor, plasma prekallikrein, high molecular weight kininogen (Fitzgerald factor), Christmas factor (factor IX), or AHF. Antiserum which had not been absorbed with PTA-deficient plasma was used in this study.

Assays of Activity of Factors

PTA clot-promoting activity, activated PTA activity, and the procoagulant activities of HF, prekallikrein, high molecular weight kininogen, Christmas factor, and AHF were assayed as described previously. PTA activity was measured by incubating 0.1 ml of appropriately diluted samples with 0.1 ml congenital PTA-deficient plasma and 0.1 ml kaolin-Centrolex “0” (a mixture of 10 mg kaolin/ml Centrolex “0”) for 8 min at 37°C in 10 x 75 mm glass tubes. The mixture was then recalculated with 0.1 ml 0.025 M CaCl2 and the clotting time was estimated at 37°C. The clotting time was converted to arbitrary units by comparison with the clotting activity of serial dilutions of a standard pool of plasma. A linear relationship existed between the clotting time and the concentration of PTA when plotted on full logarithmic paper. Activated PTA activity was assayed by incubating suitably diluted samples with Centrolex “0” and PTA-deficient plasma in 10 x 75 mm polystyrene tubes at 37°C for 1 min. The contents were immediately recalculated, and the clotting time was measured at 37°C.

Radioimmunoassay

Radioimmunoassay of PTA antigen was performed by a double antibody method (Fig. 1). The assay mixture consisted of 0.4 ml bovine serum albumin (1 mg/ml in barbital-saline buffer, pH 7.4), 0.1 ml barbital-saline buffer, 0.02 ml 125I-PTA (usually 15,000–30,000 cpm), 0.1 ml test
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Sample diluted appropriately in barbital-saline buffer, and 0.1 ml of a 1:2000 dilution of unabsorbed anti-PTA serum, in 10 x 75 mm polystyrene tubes. After incubation at 4°C for 3 days, 0.1 ml of a 1:40 dilution of normal rabbit serum and 0.1 ml of a 1:8 dilution of goat anti-rabbit IgG serum (Antibodies Inc., Paris, Calif.) were added to the mixture. Normal rabbit serum was used as a carrier to increase the volume of the precipitates formed. After incubation overnight at 4°C, the tubes were centrifuged at 1800 g for 10 min at 2°C, the supernatant fluids were aspirated, and the radioactivity of the precipitates was measured in a Nuclear-Chicago γ-Counter (model 1085).

The values obtained were expressed as percentage of bound (precipitated) radioactivity relative to that of total radioactivity added to the tube initially. Maximum binding, determined from a mixture containing all reagents except the sample, was usually between 30%-55%. Nonspecific binding (precipitation) of 125I-PTA, as determined by substituting normal rabbit IgG for anti-PTA IgG, was consistently less than 10%. Duplicate determinations were performed for each test sample and serial dilutions of purified PTA or normal pooled plasma were included in each experiment. A standard curve was prepared by plotting the percentage of bound radioactivity versus the logarithm of the concentration of purified PTA or normal pooled plasma. Citrated plasmas were used for all routine assays.

Plasmas

A standard pool of 24 normal male plasmas was prepared by an earlier method, and was used as the standard for measurement of both PTA clot-promoting activity and PTA antigen. The pooled plasma was arbitrarily said to contain 1.0 unit of clot-promoting activity per ml and 1.0 unit of antigen per ml. It should be noted that there was no reason to assume that the unitages of functional and antigenic PTA were identical. Citrated plasmas from normal individuals and from patients with congenital clotting factor deficiencies were prepared as described earlier. Serum was isolated 3 hr after whole blood was clotted in glass tubes at 37°C. Prekallikrein-deficient plasmas were purchased from George King Bio-Medical, Salem, N.H. High molecular weight kininogen-deficient plasma was obtained through the courtesy of Dr. R. Waldmann, Henry Ford Hospital, Detroit.

Plasmas from patients with advanced hepatic cirrhosis, patients under treatment with warfarin, and those with suspected disseminated intravascular coagulation (DIC) were obtained at the University Hospitals of Cleveland. The diagnosis of DIC was inferred from the presence of a prolonged thrombin time, reduced plasma fibrinogen, decreased platelet count, and a positive test for fibrinogen-related antigen (so-called fibrin degradation products) as detected by the Thrombo-Welco test (Wellcome Reagents, England). Venepuncture in patients and in normal subjects was performed after informed consent for the procedure was obtained and in accordance with the principles of the Declaration of Helsinki.

Gel Filtration

Gel filtration of Hageman trait plasma was performed at 4°C on a 1.5 x 75 cm column of Sephadex G 150 (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with barbital saline buffer containing 0.0001 M disodium ethylenediaminetetraacetic acid (EDTA). One ml of plasma was applied and 2-ml fractions were collected at a flow rate of 12 ml/hr.

Recovery of PTA

The recovery of PTA from PTA-deficient plasma was studied by adding different amounts of normal pooled plasma to a severe PTA-deficient plasma and testing the mixtures by radioimmunoassay.

Relationship Between Clotting Assay and Radioimmunoassay

The titers of PTA clot-promoting activity and of PTA antigen present in 50 normal adults (25 males and 25 females) were estimated. PTA activity was plotted against PTA antigen and the regression line was calculated by the method of least squares. The 95% and 99% confidence limits for individual observations were calculated by a standard formula.
Comparison of PTA and Activated PTA by Radioimmunoassay

This comparison was performed as follows: 0.3 ml Christmas factor-deficient plasma was incubated with 0.018 ml 0.5 M sodium citrate (pH 5.0) in a 12 x 75 mm polystyrene tube at 37°C for 2 min. Then 0.6 ml prewarmed 0.0001 M ellagic acid (synthesized by Dr. J.D. Crum, Case Western Reserve University, Cleveland, Ohio) in barbital-saline buffer or barbital-saline buffer alone was added and the tube was incubated at 37°C. At intervals, 0.1-ml aliquots were transferred into (1) 10 x 75 mm polystyrene tubes containing 0.1 ml of a mixture of soybean trypsin inhibitor (SBTI, 0.1 mg/ml) and lima bean trypsin inhibitor (LBTI, 0.1 mg/ml) in barbital-saline buffer for use in assaying PTA antigen (SBTI and LBTI were used to stop any further activation of PTA), and (2) 10 x 75 mm polystyrene tubes containing 0.1 ml PTA-deficient plasma, 0.1 ml Centrolex "O", and 0.1 ml 0.05 M CaCl₂ for assay of activated PTA. SBTI and LBTI were purchased from Worthington Biochemical Co., Freehold, N.J.

Barbital-saline buffer, pH 7.4, contained 2.76 g of barbital, 7.3 g of sodium chloride, and 2.06 g of sodium barbital per liter.

Centrolex "O", crude soybean phosphatides, was a gift of Central Soya Co., Chicago, Ill.

RESULTS

Development of a Radioimmunoassay for PTA

When increasing concentrations of normal pooled plasma or purified PTA were tested in the assay system described in Fig. 1, the percentage of bound (precipitated) radioactivity progressively decreased (Fig. 2). The displacement curves obtained with normal plasma and purified PTA were similar. In contrast, the addition of severe PTA-deficient plasma at any concentration did not appreciably decrease binding of radioactive PTA to the antiserum. The effect of addition of PTA-deficient plasma was similar to that obtained when buffer was substituted for plasma. Thus, displacement of radioactive PTA occurred upon the addition of normal plasma or purified PTA, but not upon addition of PTA-deficient plasma. The addition of Hageman trait, Fletcher trait, or other congenital clotting factor-deficient plasmas showed a similar displacement curve to that of normal plasma. The PTA antiserum used in this experiment had not been absorbed with PTA-deficient plasma.

The linear portion of the curves obtained with normal plasma or purified PTA could be used as a standard curve for the assay of unknown samples. The concentration of PTA present in normal pooled plasma was estimated to be approximately 6 μg protein/ml as read by comparison to a standard curve using
purified PTA (specific activity: 160 U/mg protein). Normal pooled plasma from 24 normal males was used routinely to make a standard curve. The relationship of the percentage of bound radioactivity (cpm) to the logarithm of the concentration of PTA added was essentially linear between 0.1 U/ml and 0.003 U/ml. Test materials were diluted as necessary to give values falling within this range. The arithmetic mean and SD for five standard curves on separate days are shown in Fig. 3. The minimum concentration of PTA detectable quantitatively by this assay was approximately 0.003 U/ml, that is, 0.3% of that in normal pooled plasma. When Hageman trait plasma was filtered through a column of Sephadex G-150, PTA clot-promoting activity and PTA antigen detected by this assay eluted identically.

Reproducibility and Accuracy of the Assay

Eleven determinations of the same samples on the same day were performed at 1:20 dilution. The mean ± SD was 1.24 ± 0.08 U/ml. Two normal plasma specimens were frozen at −70°C in aliquots and each aliquot was tested on 10 different occasions over 12 mo. The means ± SD for these two specimens were 0.99 ± 0.15 and 1.17 ± 0.13 U/ml respectively.

Recovery of Added PTA From PTA-deficient Plasma

When different amounts of normal pooled plasma were added to severe PTA-deficient plasma and the mixtures were then assayed for PTA, recovery was excellent (Table 1).

<table>
<thead>
<tr>
<th>Table 1. Recovery of PTA Added to Severe PTA-deficient Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PTA Radioimmunoassay</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>Test Mixtures</td>
</tr>
<tr>
<td>PTA-deficient Plasma (%)</td>
</tr>
<tr>
<td>Plus Normal Plasma (%)</td>
</tr>
<tr>
<td>Added (U/ml)</td>
</tr>
<tr>
<td>Found (U/ml)</td>
</tr>
</tbody>
</table>

Different amounts of normal pooled plasma were added to severe PTA-deficient plasma and the mixtures were tested by radioimmunoassay. The results were read by interpolation into a standard curve of dilutions of normal pooled plasma.
Comparison of PTA and Activated PTA by Radioimmunoassay

Table 2. Comparison of PTA and Activated PTA by Radioimmunoassay

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>Time (sec)</th>
<th>PTA Activity (U/ml)</th>
<th>Antigen (Antigen U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Christmas factor-deficient plasma + EA</td>
<td>10 sec</td>
<td>133</td>
<td>0.72</td>
</tr>
<tr>
<td>Christmas factor-deficient plasma + EA</td>
<td>4 mm</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Christmas factor-deficient plasma + EA</td>
<td>8 mm</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Christmas factor-deficient plasma + buffer</td>
<td>10 sec</td>
<td>&gt;200</td>
<td>&lt;0.003</td>
</tr>
</tbody>
</table>

See Materials and Methods for experimental details.

*EA: ellagic acid.

Comparison of PTA in Plasma and Serum

The concentration of PTA in citrated plasma and serum correlated well in nine normal persons (correlation coefficient 0.89). This experiment suggests that clotting in vitro does not significantly alter the level of PTA antigen and that serum samples can be used in the assay described.

Comparison of PTA and Activated PTA

To examine whether the radioimmunoassay was able to detect activated PTA as well as PTA, PTA in Christmas factor-deficient plasma was activated by ellagic acid and the titers of activated PTA activity and PTA antigen were followed by activated PTA assay and radioimmunoassay respectively (Table 2). When Christmas factor-deficient plasma was incubated with ellagic acid, activated PTA activity evolved with time. The titer of PTA antigen, however, stayed constant throughout at a level comparable to that of Christmas factor-deficient plasma incubated with buffer. This experiment suggests that this radioimmunoassay detects activated PTA as well as PTA.
Clinical Studies

Simultaneous clotting and radioimmunoassay for PTA were performed on plasmas from 50 healthy adults (25 males and 25 females, ranging in age from 22 to 45 yr). There was good correlation between both assays (correlation coefficient 0.68; Fig. 4). The mean ± SD for PTA antigen in males was 0.98 ± 0.23 U/ml, and in females it was 1.02 ± 0.17 U/ml, indicating that there was no sex difference (Table 3).

Both PTA antigen and activity were greatly reduced in plasmas of 9 patients with liver cirrhosis ($p < 0.001$), but were normal in plasmas of 9 patients treated with warfarin. The level of PTA antigen in the plasmas of 8 patients with suspected DIC showed a relatively wide range (0.44 - 2.0 U/ml), but there was no significant difference as a group from that in normal plasma. PTA antigen was significantly lower in 11 cord serums than in serums of normal adults ($p < 0.001$).

The plasmas of 13 individuals with congenital homozygous PTA deficiency contained very low titers of PTA antigens as detected by this method (Table 4). The level of PTA antigen in this disorder was in good agreement with that of PTA clot-promoting activity. Normal amounts of both PTA antigen and PTA clotting activity were present in other plasmas tested (Table 5), including the plasmas of 8 individuals with Hageman trait, 5 patients with Christmas factor deficiency, 1 patient with plasma prekallikrein deficiency, and 1 patient with high molecular weight kininogen deficiency.

**Table 3. PTA Radioimmunoassay in Various Conditions**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of Subjects</th>
<th>Activity (U/ml)</th>
<th>Antigen (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal men</td>
<td>25</td>
<td>0.96 ± 0.27</td>
<td>0.98 ± 0.23</td>
</tr>
<tr>
<td>Normal women</td>
<td>25</td>
<td>1.00 ± 0.20</td>
<td>1.02 ± 0.17</td>
</tr>
<tr>
<td>Hepatic cirrhosis</td>
<td>9</td>
<td>0.30 ± 0.09</td>
<td>0.35 ± 0.17</td>
</tr>
<tr>
<td>Warfarin treatment</td>
<td>9</td>
<td>0.80 ± 0.13</td>
<td>0.91 ± 0.20</td>
</tr>
<tr>
<td>DIC</td>
<td>8</td>
<td>0.94 ± 0.56</td>
<td>0.89 ± 0.50</td>
</tr>
<tr>
<td>Cord serum</td>
<td>11</td>
<td>—</td>
<td>0.25 ± 0.07</td>
</tr>
</tbody>
</table>

Mean ± SD.

**Table 4. PTA Activity and Antigen in Plasmas of 13 Patients With Homozygous PTA Deficiency**

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Activity (U/ml)</th>
<th>Antigen (U/ml)</th>
<th>Subject No.</th>
<th>Activity (U/ml)</th>
<th>Antigen (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;0.01</td>
<td>&lt;0.003</td>
<td>8</td>
<td>0.040</td>
<td>0.035</td>
</tr>
<tr>
<td>2</td>
<td>&lt;0.01</td>
<td>&lt;0.003</td>
<td>9</td>
<td>0.045</td>
<td>0.042</td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.01</td>
<td>&lt;0.003</td>
<td>10</td>
<td>0.047</td>
<td>0.055</td>
</tr>
<tr>
<td>4</td>
<td>&lt;0.01</td>
<td>&lt;0.003</td>
<td>11</td>
<td>0.080</td>
<td>0.128</td>
</tr>
<tr>
<td>5</td>
<td>&lt;0.01</td>
<td>&lt;0.003</td>
<td>12</td>
<td>0.082</td>
<td>0.090</td>
</tr>
<tr>
<td>6</td>
<td>&lt;0.01</td>
<td>&lt;0.003</td>
<td>13</td>
<td>0.092</td>
<td>0.105</td>
</tr>
<tr>
<td>7</td>
<td>0.037</td>
<td>0.039</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5. PTA Radioimmunoassay in Some Congenital Deficient Plasmas

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of Subjects</th>
<th>Activity (U/ml)</th>
<th>Antigen (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>50</td>
<td>0.98 ± 0.24*</td>
<td>1.00 ± 0.20*</td>
</tr>
<tr>
<td>Hageman trait</td>
<td>8</td>
<td>0.99 ± 0.31</td>
<td>0.98 ± 0.40</td>
</tr>
<tr>
<td>Christmas factor deficiency</td>
<td>5</td>
<td>0.75 ± 0.15</td>
<td>0.81 ± 0.19</td>
</tr>
<tr>
<td>Prekallikrein deficiency</td>
<td>1</td>
<td>1.04t</td>
<td>1.00t</td>
</tr>
<tr>
<td>High molecular weight kininogen deficiency</td>
<td>1</td>
<td>0.84t</td>
<td>0.80t</td>
</tr>
</tbody>
</table>

*Mean ± SD.
†Individual data given.

DISCUSSION

The specificity of the radioimmunoassay for PTA that is described is supported by the following lines of evidence. First, displacement of the radioactive PTA occurred upon the addition of normal, Hageman trait, Fletcher trait, or other congenital clotting factor-deficient plasmas, but not upon addition of severe PTA-deficient plasma. Second, the slopes of the displacement curves for normal plasma and purified PTA were similar (Fig. 2). Third, the PTA clot-promoting activity and PTA antigen of Hageman trait plasma eluted identically on gel filtration, as if they were properties of the same molecule. Fourth, the amount of PTA antigen in normal plasma correlated well with the amount of PTA clot-promoting activity (Fig. 4).

The sensitivity of this method is excellent, since it can quantitatively detect as little as 0.003 U/ml, 0.3% of that present in normal pooled plasma. The assay is reproducible, with little day-to-day variation. Thus, the present radioimmunoassay is highly specific, sensitive, and reproducible.

The concentration of PTA in normal plasma was found to be approximately 6 μg/ml. This level was much less than that of Hageman factor (40 μg/ml) and of Fletcher factor (40 μg/ml), and may explain why no precipitin line was seen between normal plasma and PTA antiserum.

The present assay was unable to differentiate activated PTA from PTA, probably because activated PTA is very close to PTA in its size and charge and rabbit antiserum made against PTA may have many common sites against activated PTA.

The amount of PTA antigen was markedly decreased in plasmas of 13 patients with congenital PTA deficiency, in proportion to their low PTA activity. This finding implies that the deficiency of PTA in congenital PTA deficiency is due to a decreased synthesis of this protein. Forbes and Ratnoff have previously reported similar findings in 10 PTA-deficient plasmas using antibody neutralization assays, an observation that has recently been confirmed. Some homozygous PTA-deficient plasmas contain small amounts of PTA (5'-10% of normal pooled plasma). It would be interesting to study the size and charge of PTA present in such PTA-deficient plasmas with this sensitive radioimmunoassay.

PTA antigen was greatly reduced in plasmas from patients with hepatic
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cirrhosis, but it was normal in patients under treatment with warfarin. Although this reduced PTA clotting activity in hepatic cirrhosis has been known since the report by Rapaport, this was the first demonstration that PTA antigen is also decreased. This observation supports the hypothesis that the liver is a site of production of this clotting factor. The low PTA antigen found in the cord serum is consistent with a previous report, and may be a reflection of immature liver function in the neonate.

ACKNOWLEDGMENT

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REFERENCES

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