Inhibition of Platelet Prothrombinase Activity by a Lupus Anticoagulant

By Björn Dahlbäck, Inga Marie Nilsson, and Birgitta Frohm

Lupus anticoagulants are spontaneously occurring antibodies with specificity for negatively charged phospholipids. The plasma of a patient with such a polyclonal antibody of IgM type demonstrated low levels of factor VIII coagulant activity (VIII:C) and factors IX, XI and XII when analyzed by biologic clotting assays, whereas in immunochemical assays, normal levels of VIII coagulant antigen and factor IX were obtained. After immunoadsorption of patient plasma with anti-IgM Sepharose, normal biologic activities were demonstrated in clotting assays for VIII:C, factors IX, XI, and XII. The addition of the patient’s isolated IgM to normal plasma resulted in grossly abnormal results in these coagulation assays, and a pattern similar to that of the patient’s plasma was obtained. The inhibitory effect of the patient’s lupus anticoagulant on blood coagulation was demonstrated also in platelet-rich plasma. The results of the clotting assays indicated that the anticoagulant inhibited several of the reactions in the blood coagulation cascade. The availability of purified components made it possible to demonstrate an inhibiting effect on the activation of prothrombin by factor Xa in the presence of isolated platelets, as well as in a system where purified factor V and well defined phospholipid vesicles were substituted for the platelets.

Occasionally it happens that subjects, though previously normal with respect to blood coagulation, develop antibodies against individual coagulation factors. This usually leads to severe hemostatic problems. Another type of spontaneously occurring antibodies has also been described, the so-called lupus anticoagulant. In contrast to the first type, patients with these antibodies usually have no bleeding tendency. Experiments using fractionated or whole plasma systems have suggested that lupus antibodies inhibit the activation of prothrombin due to a specificity for phospholipids.

In recent years, our knowledge of the role of phospholipids has considerably increased. Thus, negatively charged phospholipids are important for several of the reactions involved in the blood coagulation cascade, of which the activation of prothrombin by factor Xa is the best characterized. Factor V is an important nonenzymatic cofactor for the reaction. Factor Xa and thrombin-modified factor V (factor Va) bind to the phospholipid surface, forming the prothrombinase complex that converts prothrombin to thrombin. Prothrombin and factor Xa interact with the phospholipid surface via their vitamin-K-dependent γ-carboxyglutamic acid residues. This reaction is dependent on the presence of calcium ions, whereas the binding of factor Va is not calcium dependent. Factor Va also binds with high affinity to a limited number of sites on the platelet surface. Platelet-bound factor Va constitutes the factor Xa platelet receptor. Factor Xa bound to these receptors catalyzes the activation of prothrombin efficiently. It was recently reported that factor Va bound equally well to unstimulated (e.g., by thrombin) platelets and that the platelet release reaction was not a prerequisite for a rapid prothrombin activation on the platelet surface. These results raised the question of whether negatively charged phospholipids are of importance in the reactions on the platelet surface, since the platelet phospholipids are asymmetrically distributed in the membrane and the negatively charged “procoagulant” phospholipids (mainly phosphatidylerine) are almost exclusively localized to the inner membrane layer.

Recently, Thiagarajan et al. described a patient who had a monoclonal IgM antibody with anticoagulant activity. In an Ouchterlony system, the isolated IgM precipitated several different negatively charged phospholipids. Furthermore, the isolated IgM inhibited the binding of factor X and prothrombin to phospholipid vesicles. However, the inhibitor had no activity in the presence of platelets, and the isolated IgM did not inhibit the binding of factor Xa to platelets.

We report a case with a polyclonal lupus anticoagulant of IgM type. The inhibitor disturbed several of the coagulation assays and was also active in the presence of platelets. The effect of the isolated inhibitor on the activation of prothrombin by factor Xa was studied in purified systems. The inhibitor was found to have an inhibitory effect both in the presence of factor V and phospholipids and in the presence of platelets.

From the Department for Coagulation Disorders and the Department of Clinical Chemistry, University of Lund, Malmö General Hospital, Malmö, Sweden.

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Address reprint requests to Professor Inga Marie Nilsson, Department for Coagulation Disorders, Malmö General Hospital, S-214 01 Malmö, Sweden.

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MATERIALS AND METHODS

Ultrogel AcA 22 was from LKB, Bromma, Sweden. L-α-Phosphatidyl-l-serine (approximately 98% pure) and L-α-phosphatidylcholine (type V-E) prepared from bovine brain and egg yolk, respectively, were from Sigma Chemical Co., St. Louis, MO. Anti-IgM and anti-IgA Sepharose were provided by Professor C.-B. Laurell, Dept. of Clinical Chemistry, Malmö, Sweden.

Protein Purification

Bovine factor X and prothrombin were purified as described earlier. Factor X was activated with the factor X activator isolated from Russell's viper venom and purified by DEAE-Sephadex chromatography, as described by Jesty and Nemerson. Bovine factor V was purified as described by Dahlbäck; it had a specific activity of 150 U/mg in a factor V assay, when human plasma was chromatography, as described by Jesty and Nemerson. Bovine factor V was purified as described by Dahlbäck; it had a specific activity of 150 U/mg in a factor V assay, when human plasma was chromatography, as described by Jesty and Nemerson. Bovine factor V was purified as described by Dahlbäck; it had a specific activity of 150 U/mg in a factor V assay, when human plasma was chromatography, as described by Jesty and Nemerson. Bovine factor V was purified as described by Dahlbäck; it had a specific activity of 150 U/mg in a factor V assay, when human plasma was chromatography, as described by Jesty and Nemerson. Normal and patient IgM were purified from normal and patient plasma, respectively. The 0%-40% ammonium sulfate precipitate, from approximately 25 ml plasma, was dissolved in 50 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4, and applied to a column (2.5 x 95 cm) packed with Ultrogel AcA 22 in the same buffer (Fig. 1). The column was run at room temperature and a flow rate of 14 ml/hr, and 5-ml fractions were collected. The fractions were monitored immunologically with antisera against IgM, IgG, IgA, α-macroglobulin, fibrinogen, and factor-VIII-related antigen (VIII:Ag). The fractions were also analyzed by agarose gel electrophoresis, and the anticoagulant was localized by a plasma recalcification assay system (see below). The fractions containing the anticoagulant were pooled and concentrated by Amicon ultrafiltration on UM 10 filter.

The purified proteins were quantitated spectrometrically using the following ELISA at 280 nm: factor X, 12.4;29 prothrombin, 14.6. The purified IgM was quantitated by electroimmunoassay.

Electrophoretic and Immunochemical Methods

Agarose gel electrophoresis, electroimmunoassay, and Ouchterlony analysis were performed by standard methods; for references, see Stenflo. The patient plasma was subjected to immunoadsorption, using either anti-IgM or anti-IgA Sepharose. To patient or control plasma (3.5 ml) were added approximately 2–3 ml of either the anti-IgG or the anti-IgA Sepharose, both equilibrated in 50 mM Tris-HCl, 0.5 M NaCl, pH 7.4, containing 10 mM trisodium citrate. After 15 min gentle mixing at room temperature, the plasma was collected by filtration on a glass filter funnel, and the gels were then washed with approximately 25 ml equilibration buffer. The nonsorbed plasma and the washing buffer were pooled (30 ml), diluted 1/2 to 1/10 in distilled water, and immediately analyzed in factor VIII, IX, XI, and XII assays and also assayed for anticoagulant activity by a plasma recalcification system (see below). The absorbed proteins were eluted with 0.1 M glycine-HCl, pH 2.5. After immediate neutralization of its pH with 1 M Tris, the eluate was assayed for anticoagulant activity.

Coagulation Studies

Citrated platelet-rich and platelet-poor plasma were prepared as described previously. Plasma samples were immediately tested for fibrinolytic activity. Samples for coagulation studies were stored at −70°C until used. Citrated plasma from 20 normal individuals was pooled and used as a referent in the coagulation assays.

The following coagulation tests were used: platelet count, Ivy bleeding time, platelet adhesiveness, whole blood clotting time, recalcification time of plasma, manual and automated activated partial thromboplastin time (APTT) (APTT, General Diagnostics, Morris Plains, NJ, or Cephotest, Nyegaard and Co., Oslo, Norway); nonactivated thromboplastin time (Thromb infix, Ortho Diagnostics, Raritan, NJ), one-stage prothrombin time, Russell's viper venom time (RVV time) (Wellcome Foundation Ltd., London, UK); thrombin time, Owren's P&P (prothrombin, factor VII, factor X), factor X, factor VII, factor V, fibrinogen; factor XIII, euglobulin clot lysis time, fibrinolytic activity of plasma and resuspended euglobulin precipitate, fibrinogen degradation products (FDP), and antithrombin III and α-antiplasmin. The procedures have been described elsewhere. Factor VIII coagulant activity (VIII:C) and factor IX clotting activity, factor XI, and XII were measured in one-stage systems using platelet-rich congenitally deficient plasmas as test bases. No partial thromboplastin was added in these test systems. The assays were performed using 1:10, 1:20, and 1:50 dilutions of patient or standard normal plasma, though assay results are based on the 1:20 dilution only; patient plasma dilution curves were not parallel to the standard curve. Factor-VIII-related antigen (VIII:Ag) was determined as described by Holmberg and Nilsson.
son. Factor VIII clotting antigen (VIII:CAg) and IX clotting antigen (IX:CAg) were determined by two-site solid-phase immunoradiometric assay. Prothrombin and factor XII were determined by electroimmunoassay.

A plasma recalcification system was used to measure anticoagulant activity. Normal citrated plasma (0.2 ml) was incubated in glass tubes with 0.2 ml sample diluted in 50 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4. After 3-min incubation at 37°C, 0.2 ml 30 mM calcium chloride was added and the clotting time measured.

**Activation of Prothrombin**

All studies were done at 37°C in 50 mM Tris-HCl, 0.15 M NaCl, 2 mM CaCl$_2$, pH 7.5, containing 5 mg bovine serum albumin and 1 mg glucose/ml. Prothrombin (0.1 mg/ml) was incubated with platelets (0.5 x 10$^9$/ml) or phospholipid vesicles (PCPS) (4 mg/ml) and/or factor V (0.05-2 U/ml). The prothrombin activation was started by the addition of factor Xa (1 ng/ml - 50 µg/ml). Unless noted, the isolated IgM was added at the same time as factor Xa. Aliquots of the reaction mixtures were removed, and the generated thrombin was determined in a Fibrometer coagulation timer (BBL), using the method described by Fenton and Fasco. The phospholipid vesicles (PCPS), composed of 75% phosphatidylcholine and 25% phosphatidylyserine, were prepared by a method previously described. The platelets were isolated and incubated with C$^14$-serotonin by a modification of the method of Tollefsen et al. The isolated platelets had a normal shape when inspected in the phase-contrast microscope and demonstrated a normal platelet release reaction (measured as the liberation of C$^14$-serotonin) and a normal shape change reaction upon incubation with a small amount of thrombin (1U/ml).

**CASE REPORT**

The patient was an 83-yr-old senile man. At the age of 70 he had a coronary infarction, resulting in a persisting moderate heart decompensation. He was treated with Aldactone (Searle), 50 mg daily. At the age of 80 he was admitted to hospital because of gastrointestinal bleeding. On this occasion a malignant tumor in the urinary bladder was discovered. The patient declined further treatment. Otherwise, he had no past history of excessive bleeding or thrombosis. In September 1981 (aged 83), he was readmitted to the hospital because of a spontaneously occurring large subcutaneous bleeding in the right arm and part of the chest. Physical examination was otherwise normal. Blood pressure was 160/80 mm Hg. His blood count showed hemoglobin 90 g/liter, platelets 200 x 10$^9$/liter, and leukocytes 7.8 x 10$^9$/liter. The blood film was normal. ESR was 15 mm in 1 hr; creatinine 119 µmole/liter; and serum bilirubin and liver function tests were normal. The serum immunoglobulin levels were (g/liter): IgA 3.2, IgM 3.4, and IgG 12; there was no paraprotein band. He was treated with tranexamic acid, 1.5 g 3 times daily. During 6 mo observation, he had no further bleeding problems and no signs of thromboses.

**RESULTS**

**Coagulation Assays**

The results of screening coagulation tests, and those of more specific assays, are given in Tables 1 and 2. The results indicate that the patient plasma contained an anticoagulant, affecting several of the assay systems used. The pattern was compatible with the presence of a lupus anticoagulant, which disturbed several of the clotting assays comprising phospholipids. This explains the observed discrepancy between results obtained in biologic and in immunologic assays. From Table 2 it appears that the assays for factors XII, XI, IX, and VIII:C were more affected by the anticoagulant than the assays for factors II, V, and X. This may be explained by the fact that the two groups of assays utilize different phospholipid sources. Brain thromboplastin was used in the latter assays, whereas disintegrated platelets was the lipid source in the assays of factors XII, XI, IX, and VIII:C. Normal values were obtained in the following assays: VIIIIR:Agr, factor XIII, antithrombin III, and platelet adhesion (results not given).

The patient also had signs of increased fibrinolytic activity. This was evident from shortened euglobulin clot lysis times and increased lysis on fibrin plate assays of patient plasma. The patient plasma also showed a low level of α$_2$-antiplasmin (44%-57%). No increased amount of fibrin degradation products could, however, be demonstrated.

**Classification of the Anticoagulant**

The addition of diluted patient plasma to normal plasma resulted in marked prolongation of nonactivated and activated partial thromboplastin times, and of the recalcification time. The latter proved to be the

<table>
<thead>
<tr>
<th>Table 1. Screening Coagulation Tests</th>
</tr>
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<tbody>
<tr>
<td><strong>Assay</strong></td>
</tr>
<tr>
<td>Bleeding time, Ivy method (min)</td>
</tr>
<tr>
<td>Platelet count (10$^9$/liter)</td>
</tr>
<tr>
<td>Recalcification time of plasma (sec)</td>
</tr>
<tr>
<td>Activated partial thromboplastin time (sec)</td>
</tr>
<tr>
<td>One-stage prothrombin time (sec)</td>
</tr>
<tr>
<td>Thrombin time (sec)</td>
</tr>
<tr>
<td>P &amp; P (%)</td>
</tr>
<tr>
<td>Fibrinogen (g/liter)</td>
</tr>
<tr>
<td>Fibrin degradation products (mg/liter)</td>
</tr>
</tbody>
</table>

*Result obtained with a control plasma.

Table 2. Coagulation Factor Analysis on the Patient's Plasma

<table>
<thead>
<tr>
<th>Clotting Assay</th>
<th>Immunologic Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>XII (%)</td>
<td>8</td>
</tr>
<tr>
<td>XI (%)</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>IX (%)</td>
<td>19</td>
</tr>
<tr>
<td>VIII:C (%)</td>
<td>9</td>
</tr>
<tr>
<td>X (%)</td>
<td>70</td>
</tr>
<tr>
<td>VII (%)</td>
<td>30</td>
</tr>
<tr>
<td>V (%)</td>
<td>80</td>
</tr>
<tr>
<td>II (%)</td>
<td>89†</td>
</tr>
</tbody>
</table>

*Normal values in clotting assays for factors XII, XI, IX, VIII:C were 60%-160% and for factors X, VII, V, and II were 80%-120%.
†Determined with P&P method.
‡Determined by electroimmunoassay according to Hedner.
ND, not determined.
INHIBITION OF PLATELET PROTHROMBINASE ACTIVITY

The most sensitive assay system of the three, and an inhibitory effect of patient plasma was observed even when it was added in dilutions of 1:400 and 1:800. The recalcification assay system was used to localize the lupus antibody during its purification. Figure 1 illustrates the elution of anticoagulant activity on a gel filtration chromatography on Ultrogel AcA 22. A 0%–40% ammonium sulfate fraction of patient plasma was applied to the column. The anticoagulant eluted at a position corresponding to that of IgM, indicating that the antibody was of IgM type. This was confirmed by the observation that the anticoagulant in the patient’s plasma was adsorbed to an anti-IgM Sepharose (Table 3). After gel filtration chromatography, the patient’s IgM was approximately 80%–90% pure, as judged by agarose gel electrophoresis, but still contained immunochemically detectable amounts of other high molecular weight proteins like VIII:Ag, α2-macroglobulin, and IgA. The addition of the purified patient IgM to normal plasma yielded grossly abnormal results in several coagulation assays (Table 4) and the pattern obtained was similar to that observed when plasma was tested.

Effects of Phospholipids and Platelets on Coagulation Assays

In contrast to the patient described by Thiagarajan et al;23 our patient showed markedly prolonged coagulation times of whole blood and of platelet-rich plasma, indicating that the antibody had an inhibitory effect also in the presence of platelets. Furthermore, the presence of disintegrated normal platelets (frozen and thawed) failed to normalize the activated partial thromboplastin time (results not shown). The effects of different phospholipids and of platelets on partial thromboplastin and on RVV times are shown in Table 5. Although not totally normalized, both were shortened by the presence of platelets. The partial thromboplastin time of the patient’s plasma, measured in the presence of a high concentration of phospholipid vesicles (PCPS) (approximately 1 mg/ml), was markedly prolonged, whereas under similar conditions, the RVV time was equal to that of normal plasma. However, at lower concentrations of PCPS, the RVV times were prolonged. These results indicated that the patient’s anticoagulant not only disturbed the activation of prothrombin, but also had an inhibitory effect on earlier reactions in the intrinsic pathway of blood coagulation. The inhibitory effect was presumably due to an affinity of the anticoagulant for the phospholipid surface, resulting in a disturbance in the assembly of clotting factors. However, it was not possible to demonstrate precipitation of phospholipid vesicles by patient IgM in an Ouchterlony system in a way similar to that described by Thiagarajan et al.23 This is presumably due to the fact that the IgM we studied was polyclonal, and therefore, the concentration of specific antibodies was too low to be able to precipitate the vesicles.

Inhibitory Effect of Patient IgM on Prothrombin Activation in the Presence of Phospholipid

The activation of prothrombin has been studied in detail in systems using highly purified bovine components.10,11 Normally, the rate of prothrombin activation by factor Xa is enhanced by the addition of negatively charged phospholipid vesicles. This is due to binding of both components to the phospholipid surface, resulting in high local concentrations of enzyme and substrate. As illustrated in Fig. 2B, this reaction apparently was

<table>
<thead>
<tr>
<th>Assay</th>
<th>Before Adsorption</th>
<th>Anti-IgA</th>
<th>Anti-IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII:C (%)</td>
<td>35</td>
<td>23</td>
<td>140</td>
</tr>
<tr>
<td>IX (%)</td>
<td>18</td>
<td>20</td>
<td>140</td>
</tr>
<tr>
<td>XI (%)</td>
<td>&lt;2.5</td>
<td>&lt;2.5</td>
<td>52</td>
</tr>
<tr>
<td>XII (%)</td>
<td>40</td>
<td>17</td>
<td>80</td>
</tr>
</tbody>
</table>

*Prepared from 75% phosphatidylcholine and 25% phosphatidylserine; concentration used was approximately 1 mg/ml.
†Thrombofax (Nyegaard and Co.) diluted according to manufacturer’s instructions.
‡Approximately 3 x 10⁹/ml.

Table 4. Analysis of Factors VIII, IX, XI, and XII in Normal Plasma After Addition of Patient and Normal IgM

<table>
<thead>
<tr>
<th>Assay</th>
<th>Buffer</th>
<th>Control IgM*</th>
<th>Patient IgM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII:C (%)</td>
<td>45</td>
<td>60</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>IX (%)</td>
<td>45</td>
<td>58</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>XI (%)</td>
<td>60</td>
<td>80</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>XII (%)</td>
<td>40</td>
<td>60</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>53</td>
<td>43</td>
<td>88</td>
</tr>
</tbody>
</table>

*The final IgM concentration was 2.3 mg/ml; the plasma was diluted ½ by the addition of buffer or IgM. The IgM was purified as described under Materials and Methods.

Table 5. Effect of Different Phospholipids and Platelets on Coagulation Tests

<table>
<thead>
<tr>
<th>Test</th>
<th>Patient’s Plasma</th>
<th>Control Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial thromboplastin time (sec)</td>
<td>349</td>
<td>117</td>
</tr>
<tr>
<td>Phospholipid (PCPS)*</td>
<td>145</td>
<td>80</td>
</tr>
<tr>
<td>Normal platelets†</td>
<td>108</td>
<td>48</td>
</tr>
<tr>
<td>Patient’s platelets‡</td>
<td>89</td>
<td>38</td>
</tr>
<tr>
<td>Russel’s viper venom time (sec)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Phospholipid (PCPS)*</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Normal platelets†</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td>Patient’s platelets‡</td>
<td>18</td>
<td>11</td>
</tr>
</tbody>
</table>

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inhibited by the presence of patient IgM. The addition of increasing concentrations of patient IgM resulted in gradually decreased thrombin generation rates. In contrast to this, even a high concentration of normal IgM had no inhibitory effect on the rate of prothrombin activation (Fig. 2B). In the absence of phospholipids (Fig. 2A), no inhibitory effect on thrombin generation was observed by addition of IgM from the patient. This indicated that the antibody did not inhibit the interaction between factor Xa and prothrombin in fluid phase. Furthermore, the anticoagulant obviously did not inhibit the enzymatic activity of thrombin. Presumably, the antibody disturbed the interaction between factor Xa and prothrombin on the phospholipid surface by inhibiting the binding of one or both components to the surface.

Factor V (factor Va) is an important cofactor for factor Xa-mediated activation of prothrombin. Therefore, the effect of patient IgM on prothrombin activation by factor Xa (2 µg/ml) in the presence of factor V (1–2 U/ml), was investigated. In an initial experiment, we observed a 4–5-fold decrease in thrombin generation rates by the addition of patient IgM (0.5 mg/ml), whereas normal IgM had no such effect. This result was difficult to explain, since the results of coagulation assays (Table 2) indicate that the antibody was not directed against factor V. Furthermore, the factor V preparation used was from bovine plasma. It was recently reported that factor IX prothrombin concentrates purified from human plasma contain trace amounts of procoagulant phospholipids. The presence of contaminating phospholipids in our factor V preparation could not be excluded. Therefore, the same experiment was performed in the presence of 1% (v/v) of the nonionic detergent Triton X-100. Under these experimental conditions, no inhibitory effect of patient IgM was observed. The addition of Triton, per se, resulted in an inhibition of the rate of prothrombin activation equal to that observed in the initial prothrombin activation experiment with factor V and patient IgM. To investigate whether Triton X-100 affected the factor V molecule, factor V (approximately 10 U/ml) was incubated with 1% Triton X-100 at room temperature for 10 min, then diluted 1/1,000 and immediately assayed in factor V assay. No inhibitory effect on factor V activity was observed after incubation with Triton X-100. Similar results were obtained after incubation of a thrombin-activated factor V (factor Va) with Triton X-100. The experimental data indicated that the inhibitory effect of the patient’s IgM on prothrombin activation in the presence of factor V, which had been observed initially, was due to the presence of trace amounts of contaminating phospholipids. Thus, it was demonstrated that the patient antibody did not inhibit any of the following interactions: factor Xa–factor Va, factor Va–prothrombin, and factor Xa–prothrombin.

Inhibitory Effect of Patient IgM on Activation of Prothrombin in the Presence of Platelets

The addition of washed platelets catalyzes the activation of prothrombin by factor Xa. After addition of factor Xa to a reaction mixture containing prothrombin and platelets, thrombin is rapidly generated. This is preceded by a short lag phase, during which the
platelet release reaction (e.g., measured by liberation of ¹⁴C-serotonin) is induced, presumably by a small amount of thrombin formed on the surface of disintegrated platelets.¹⁹ ²¹ During the platelet activation, factor V is released and then bound to the platelet surface, thus constituting the factor Xa platelet receptor.¹⁵ ²¹ The described experimental model has been widely used, and it was interesting, therefore, to investigate whether the isolated IgM from the patient could influence the reactions. The results observed in the platelet system were compared with those obtained in a system using purified factor V and phospholipid (Fig. 3). In both systems, the presence of the patient’s IgM resulted in pronounced inhibition of the rates of thrombin generation. The effect of the anticoagulant in the two systems was similar. The effect observed in the platelet system was not due to an inhibition of the platelet release reaction as judged by the liberation of ¹⁴C-serotonin (not shown in figure). In the experiments illustrated in Fig. 3, the IgM was added at the same time as factor Xa. If, instead, the IgM was added 5–10 min after the reactions had been started by factor Xa, all further thrombin activation was effectively inhibited (not shown in figure). This was observed both in the presence of platelets and in the presence of factor V and phospholipid. When, after induction of the platelet release reaction by a small amount of thrombin, the platelet suspension was incubated with the patient’s IgM for 5–10 min, no thrombin generation could be detected upon addition of prothrombin and factor Xa. In part, this effect was presumably due to a displacement of factor Va from the surface of released platelets by the patient antibody. In the experiments discussed, the platelet concentration was relatively low (0.5 × 10⁸/ml). At higher concentrations of platelets (more than 1 × 10⁸/ml), the activation of prothrombin was very rapid also in the incubation mixture containing the patient’s IgM, and no inhibitory effect could be demonstrated. The concentration of factor V used in the experiment described in Fig. 3 was relatively low (0.05 U/ml or approximately 1 μg/ml). However, early during the prothrombin activation, factor V was activated to factor Va (15–20-fold more active than factor V) by the first thrombin formed. This in part explains the lag phase observed in Fig. 3B. In analogy with results obtained in the platelet system, at higher concentrations of phospholipid, the inhibitory effect by the lupus anticoagulant gradually decreased. In the experiments described, the activation of prothrombin never did go to completion, even in the absence of the lupus inhibitor. Theoretically, 0.1 mg/ml prothrombin can give rise to 120–140 U of thrombin/ml, whereas here only 20–40 U were observed, even after prolonged incubation. The main explanation for this is that the rate of prothrombin activation was relatively low, which allowed degradation of prothrombin to prothrombin-1 by thrombin formed.¹⁰ Prethrombin-1 lacks the phospholipid binding part of the molecule and is therefore only very slowly activated to thrombin in the presence of factor V and phospholipid as well as in the presence of platelets.¹⁰ ²¹

**DISCUSSION**

The presence of a lupus anticoagulant does not usually cause a bleeding tendency, unless combined
with a second abnormality, such as thrombocytopenia or hypoprothrombinemia. Our patient had an adequate primary hemostasis with a normal platelet count and a normal bleeding time. He had a shortened euglobulin clot lysis time and increased lysis of fibrin plates, but the fibrinogen and FDP levels were normal. It is therefore hardly possible to explain his large subcutaneous hematomas as a consequence of fibrinolysis. Thus, the abnormal bleeding in this patient may be related to the lupus anticoagulant.

It has previously been suggested that a lupus anticoagulant inhibits the interaction of formed prothrombin activator (i.e., the prothrombinase complex) with prothrombin and that the antibody is directed against the phospholipid component of the complex. Although no direct evidence for this concept has been demonstrated. In our patient, as in other reports, we found a prolongation of phospholipid-dependent coagulation tests, but we could also demonstrate that the IgM of the patient inhibited the activation of prothrombin by factor Xa in purified systems. The complexity of the protein–protein and the protein–phospholipid interactions in the conventional coagulation assays makes it difficult to draw valid conclusions about the effects of the patient IgM on earlier reactions in the coagulation cascade. However, the results indicate that several of these reactions were inhibited by the anticoagulant. This could not be further studied due to the unavailability of purified components.

Thiagarajan et al. provided the first direct demonstration that a lupus antibody was specifically directed against phospholipids. The monoclonal IgM they purified from a patient with Waldenström's disease immunoprecipitated well defined phospholipid vesicles. However, the isolated IgM precipitated vesicles prepared from several structurally different phospholipids, whose only common attribute was that they were negatively charged. This indicated that the antibody specificity was low. Presumably, the affinity constants for the binding between the different phospholipids and the IgM were also relatively low, but they were not reported. In 1975, Riesen et al. demonstrated a human monoclonal IgM with specificity against phosphatidylcholine. However, it was not studied if this IgM had anticoagulant activity. The affinity constant for the binding between phosphatidylcholine and the IgM was found to be $6.4 \times 10^4 M^{-1}$ at 25°C. This value equals the value obtained with a mouse IgA myeloma protein of the same specificity (i.e., against phosphatidylcholine). Compared to other antigen–antibody reactions, these values are low—in fact, approximately 10 times lower than the affinity constants measured for factor Xa or factor Va binding to platelets. This may explain the observed inability of the IgM, isolated by Thiagarajan et al., to inhibit the binding of factor Xa to platelets.

Owing to the low concentration of specific antibodies in our immunoglobulin preparation, the specificity of the antibody could not be established by binding experiments. In contrast to the experiments by Thiagarajan et al., who had monoclonal antibody, our antibody inhibited the activation of prothrombin by factor Xa in the presence of platelets. Our results thus support the notion that negatively charged phospholipid is important for the activation of prothrombin on the surface of platelets. They are also in line with previous results, showing that the vitamin-K-dependent parts of prothrombin and factor X are required for normal interaction with platelets.

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Inhibition of platelet prothrombinase activity by a lupus anticoagulant

B Dahlback, IM Nilsson and B Frohm