Inhibition of Lupus Anticoagulant Activity by Hexagonal Phase Phosphatidylethanolamine in the Presence of Prothrombin

Joyce Rauch¹, Marion Tannenbaum¹, Carolyn Neville¹, ², Paul R. Fortin¹, ²

From the ¹Divisions of Rheumatology and ²Clinical Epidemiology, Department of Medicine, The Montreal General Hospital Research Institute, McGill University, Montreal, Quebec, Canada

Summary

We have previously demonstrated that lupus anticoagulant antibodies from patients with systemic lupus erythematous (SLE) specifically recognize hexagonal (II) phase phosphatidylethanolamine (PE), but not bilayer PE (Thromb Haemost 1989; 62: 892). In those studies, the involvement of proteins in this recognition was not evaluated. To address this issue, we have isolated IgG lupus anticoagulant antibodies from the plasma of SLE patients and evaluated the inhibition of lupus anticoagulant activity by hexagonal (II) phase PE in the presence and absence of purified plasma proteins. All six of the IgG lupus anticoagulant antibodies tested were inhibited by hexagonal (II) phase PE in the presence, but not the absence, of human prothrombin. In contrast, little or no inhibition was observed with prothrombin alone or with PE in combination with either β₂-glycoprotein I or annexin V. These data indicate that, for certain lupus anticoagulant antibodies, inhibition by hexagonal (II) phase PE is dependent on prothrombin, suggesting that these antibodies recognize a complex of PE and prothrombin.

Introduction

The nature of the antigens recognized by lupus anticoagulant antibodies remains unclear. In recent years, focus has been placed on the role of phospholipid-binding proteins in this recognition (reviewed in 1, 2). Several candidate proteins have been proposed, but the involvement of prothrombin appears to be clear for some lupus anticoagulant antibodies. Prothrombin was first proposed as a possible cofactor for lupus anticoagulant antibodies by Loeliger in 1959 (3). In 1991, Bevers et al. (4) demonstrated that some lupus anticoagulant antibodies do, indeed, recognize a complex of phospholipid-bound human prothrombin. In the latter study, two lupus anticoagulant IgG antibodies were shown to inhibit thrombin formation (prothrombinase activity) only in the presence of phospholipids and human prothrombin. No inhibition was observed if human prothrombin was replaced by bovine prothrombin or if the phospholipid was omitted from the prothrombinase assay. Oosting et al. (5) also studied the specificity of four IgG antibodies that inhibited prothrombinase activity and found that these antibodies recognized a complex of prothrombin and phospholipid.

Antibodies to prothrombin have been found in many patients with lupus anticoagulant, both by crossed immunoelectrophoresis (6, 7) and, more recently, by enzyme-linked immunosorbent assay (ELISA) (8-10). As these antibodies are usually detected in plasma or sera, it is difficult to identify their overlap with lupus anticoagulant antibodies. Permpikul et al. (8) recently demonstrated that many lupus anticoagulant-positive IgG fractions react with phospholipid-free prothrombin on Western blots and affinity columns. This group of investigators has also shown that the binding of prothrombin to immobilized phospholipid and to cultured human umbilical vein endothelial cells is enhanced in the presence of lupus anticoagulant IgG antibodies (9).

We have previously demonstrated that lupus anticoagulant antibodies from patients with systemic lupus erythematous (SLE) specifically recognize hexagonal (II) phase phosphatidylethanolamine (PE) (10). The role of phospholipid-binding proteins in this recognition was not evaluated. In the present study, we have investigated the involvement of plasma proteins in this recognition. Using lupus anticoagulant containing-IgG fractions isolated from the plasma of patients with SLE, we have demonstrated that inhibition of lupus anticoagulant activity by hexagonal (II) PE occurs in the presence, but not the absence, of plasma. We evaluated the ability of three purified plasma proteins, which have been suggested to be involved in lupus anticoagulant activity (reviewed in 1, 2), to replace the missing plasma component(s). Lupus anticoagulant activity was inhibited by hexagonal (II) phase PE in the presence of human prothrombin, but not in the presence of human β₂-glycoprotein I (β₂-gpI or annexin V. These data suggest that inhibition of lupus anticoagulant antibodies by hexagonal (II) phase PE can be dependent on prothrombin, suggesting that these antibodies recognize a complex of PE and prothrombin.

Materials and Methods

Patients

Patients with SLE and confirmed lupus anticoagulant antibodies were selected from The Montreal General Hospital Lupus Registry. All patients (indicated by code numbers 1-6) fulfilled the American College of Rheumatology (ACR) criteria for the classification of SLE (12). The clinical laboratory tests performed for each patient included: anti-cardiolipin IgG and IgM (Kallestad anti-cardiolipin microplate ELA, Sanofi Diagnostics Pasteur, Inc., Chaska, MN); ANA; anti-DNA; direct and indirect Coombs’ tests; cell counts; prothrombin time; partial thromboplastin time (PTT); and dilute activated PTT (APTT).

Materials

Unless stated otherwise, all chemicals were obtained commercially and used without further purification. Human β₂-gpI was purchased from Crystal Chem (Chicago, IL) and phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Purified human prothrombin and annexin V (13) were generously provided by Dr. Kenneth Mann (University of Vermont, Burlington, VT) and Dr. Jonathan Tait (University of Washington, Seattle, WA), respectively.
Preparation of Phospholipids

Egg PE (hexagonal II at 37° C) and dielaidoyl PE (lamellar at 37° C) were prepared as follows. A volume containing 1.5 mg of phospholipid in chloroform was placed in a round bottom glass tube and the chloroform removed by evaporation under dry nitrogen. The dried lipids were resuspended in 0.5 ml of 20 mM HEPES buffer, pH 7.4, containing 15 mM NaCl, and hydrated for 15 min at 37° C and 45 min at 25° C, with vigorous vortexing initially and every 15 min.

Isolation of Immunoglobulin from Plasma

IgG was isolated from 3 ml of plasma from individual patients using Protein A Sepharose affinity columns (Pharmacia, Uppsala, Sweden). The IgG was eluted from the column with 0.1 M glycine-HCl buffer, pH 2.3, neutralized with 1 M Tris, and dialyzed against four changes of 0.01 M phosphate-buffered saline, pH 7.3. In some cases, plasma samples from several dates had to be pooled. Normal immunoglobulin was isolated in the same manner from freshly reconstituted Verify 1 (normal human coagulation control) (Organon Teknika Corp., Durham, NC). The purified IgG contained <1.0 µg/ml prothrombin and <1.0 µg/ml β2-gPI, as measured by ELISA.

Dilute Activated Partial Thromboplastin Time (APTT) Assay

Plasmas or isolated immunoglobulin fractions were tested using a modified dilute APTT assay, similar to that described previously (11). The term, APTT, will be used to refer to this dilute APTT assay. Plasma or purified IgG (1.8 mg/ml) was diluted with an equal volume of freshly reconstituted Verify 1 (normal human coagulation control) (Organon Teknika Corp.) in an ST4 disposable cuvet (Diagnostica Stago, Murex Diagnostics – Canada, Guelph, ON). One hundred microliters of Automated APTT reagent (Organon Teknika Corp.), diluted 1/10 in 20 mM HEPES buffer, pH 7.4, containing 15 mM NaCl and prewarmed to 37° C, was added and the mixture incubated for 5 min at 37° C. One hundred microliters of prewarmed 25 mM CaCl2 was added and the clotting time monitored by a ST4 BIO clot detection instrument (Diagnostica Stago, Murex Diagnostics – Canada). A sample was considered to be positive if the APTT exceeded the value of the negative control (Verify 1 normal control plasma or isolated IgG) by 6 sec or greater. Inhibition of the prolonged APTT by hexagonal phase egg PE was performed by premixing the purified IgG with egg PE (3 mg/ml by weight), prior to performing the APTT assay, as previously described (11,14). To evaluate inhibition by PE in the absence or presence of individual phospholipid-binding proteins, the IgG was mixed with either PE with buffer or PE with protein and incubated for 10 min at 37° C. The final concentrations of the purified proteins used were 200 µg/ml for human β2-gPI, 100 µg/ml for human prothrombin, and 4 µg/ml for human annexin V, based on the physiological concentrations of these proteins in human plasma. The six IgG fractions were also tested for inhibition by dielaidoyl PE in the presence of human prothrombin. Controls included incubation of the IgG with HEPES buffer, PE, or phospholipid-binding protein only. All competitions were performed in buffer containing a final concentration of 1.25 mM CaCl2, unless otherwise indicated. In order to remove the phospholipid or phospholipid/protein complex prior to testing in the APTT assay, the IgG/Competitor mixture was filtered through a 0.22 µm hydrophobic Durapore membrane (Millipore, Bedford, MA) that had been prewetted with HEPES buffer containing 0.2% bovine serum albumin (99% fatty acid free, Sigma, St. Louis, MO), as described previously (14). The filtrate (50 µl) was then added to 50 µl of Verify 1 normal plasma and the APTT assay performed as described above. To evaluate inhibition by PE in the absence or presence of normal plasma, the IgG was mixed with PE and the mixture was filtered as described above (for absence of plasma) or not filtered (for presence of plasma) prior to adding it to 50 µl of Verify 1 normal plasma for testing in the APTT assay. The ability of PE and/or phospholipid-binding protein (“competitor”) to inhibit lupus anticoagulant activity was calculated using the following formula:

\[ \text{% Inhibition} = \frac{\text{APTT (plasma + buffer)} - \text{APTT (plasma + competitor)}}{\text{APTT (plasma + buffer)}} \times 100\% \]

Results

Clinical and Laboratory Finding of Patients

All six patients from whom lupus anticoagulant antibodies were isolated were women who fulfilled the American College of Rheumatology criteria for the diagnosis of SLE. The ages of these patients ranged from 27 to 66 years (Table 1). Two patients (#2 and #6) had no clinical features associated with the presence of the anti-phospholipid antibodies. The other four developed the following manifestations during the course of their disease: fetal loss (patient 1); coronary artery disease with myocardial infarction and thrombocytopenia (patient 3); deep venous thrombosis (patient 4); and transient ischemic attack and fetal loss (patient 5). Patient 1 subsequently developed Coombs’ positive haemolytic anaemia. Laboratory findings, concomitant (or closest in time) to the dates on which blood was obtained for isolation of immunoglobulin, were as follows: leucopenia (< 4,000/mm³) in patients 1 and 6; lymphopenia (<1,500/mm³) in patients 1, 3, 4, and 6; non-haemolytic anaemia (<120 g/l) in patients 1 and 3; and negative direct and indirect Coombs’ tests in all patients. All six plasmas were tested by dilute APTT in the presence of a 1:1 dilution with normal plasma and found to be significantly elevated above the normal control. Patients 4 and 5 were on coumadin at the time of these blood tests.
Anti-cardiolipin IgG antibodies were present in the sera of patients 2, 3, and 4, and IgM antibodies were also found for patients 2 and 4. Patients 1 and 5 were negative for anti-cardiolipin antibodies on the dates shown, but expressed low levels of anti-cardiolipin IgG antibodies at other times. Patient 6 was negative for anti-cardiolipin antibodies.

**Inhibition of Lupus Anticoagulant Activity in the Presence and Absence of Plasma**

IgG isolated from the plasma of all six patients showed significant prolongation of the dilute APTT assay in the presence of a 1:1 dilution with normal plasma, when compared to IgG isolated from normal plasma (Table 2). Hexagonal (II) phase PE was added to these IgG fractions both in the presence and absence of normal plasma to confirm the lupus anticoagulant nature of these antibodies and to determine whether this interaction required plasma components. Prolongation of the APTT by the IgG fractions was inhibited by egg PE (53.8-74.9%) incubated in the presence of normal plasma, but not by PE that was not permitted to interact with plasma (0-7.1%). These results indicate that interaction of these lupus anticoagulant antibodies with PE requires one or more plasma components.

In order to determine the nature of the plasma constituent(s) required for PE inhibition, the six lupus anticoagulant-containing fractions were incubated with egg PE in the presence of human prothrombin, β2-gpI, or annexin V, three plasma proteins that have been suggested to be involved in lupus anticoagulant reactivity. Controls for these experiments included the IgG fractions incubated with buffer, PE alone, or the protein alone. As shown in Table 3, only IgG fractions incubated with PE mixed with prothrombin significantly inhibited APTT activity (51-81%). In contrast, lupus anticoagulant IgG incubated with PE or prothrombin, alone, showed little or no inhibition (0-5.7%). Similarly, incubation of the lupus anticoagulant antibodies with β2-gpI or annexin V, alone or in combination with PE, resulted in little or no inhibition of the prolonged APTT (0-11.5%). In addition, there was little or no inhibition (0-19.3%) of lupus anticoagulant activity by a lamellar form of PE (dilaidoyl PE) in the presence of prothrombin, indicating specific interaction of the antibodies with hexagonal (II) phase PE and prothrombin (Table 3).

**Table 1** Clinical and laboratory findings for the SLE patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Dilute APTT</th>
<th>Anti-CL IgG</th>
<th>Anti-CL IgM</th>
<th>Thrombosis (arterial or venous) and pregnancy loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>103.8</td>
<td>22.0</td>
<td>2.1</td>
<td>1 fetal loss</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>88.4</td>
<td>50.1</td>
<td>17.1</td>
<td>Nil</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>87.0</td>
<td>59.7</td>
<td>0.8</td>
<td>M*</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>95.6</td>
<td>33.6</td>
<td>23.0</td>
<td>DVT**</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>74.7</td>
<td>12.2</td>
<td>5.6</td>
<td>TIA***, 1 fetal loss</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>81.3</td>
<td>12.0</td>
<td>2.3</td>
<td>Nil</td>
</tr>
</tbody>
</table>

*MI = myocardial infarction
**DVT = deep venous thrombosis
***TIA = transient ischemic attack

**Table 2** Inhibition of lupus anticoagulant activity by hexagonal (II) phase phosphatidylethanolamine in the presence and absence of plasma

**Table 3** Inhibition of lupus anticoagulant activity by phosphatidylethanolamine in combination with different phospholipid-binding proteins

*All competitions were done at buffer containing 1.25 mM CaCl₂ (final concentration).
**% Inhibition was calculated as described in Materials and Methods. Values >20% are shown in bold.

% Inhibition of APTT:

- Buffer + Buffer = 0%
- Buffer + Prothrombin = 0%
- Egg PE + Buffer = 4.4%
- Egg PE + Prothrombin = 0%
- Buffer + β2-gpI = 4.4%
- Egg PE + Annexin V = 0%
of PE and prothrombin in the presence of calcium, suggesting that the lupus anticoagulant antibodies in these fractions recognize prothrombin bound to PE. To demonstrate this reactivity by independent means, antibodies reactive with human prothrombin were assessed in the sera and isolated IgG fractions from the six SLE patients by ELISA (Table 4). For sera, reactivity was evaluated both on high-binding ELISA plates coated with prothrombin and on plates coated with PE to which prothrombin was bound. Interestingly, only one serum (patient 5) was positive in the ELISA on prothrombin-coated plates and this was for IgG antibodies only. In contrast, sera from three patients (#1, #2, and #4) contained IgG antibodies reactive with prothrombin bound to PE-coated plates. These sera also demonstrated IgM reactivity in this assay and one additional serum (patient 6) contained IgM antibodies only. Reactivity of the lupus anticoagulant-containing IgG fractions with PE-bound prothrombin was consistent with that found in the serum in all but one patient (#3). The positivity of the IgG fraction from patient 3 may be explained by the fact that the serum sample was obtained five months prior to the plasma used for IgG isolation. From the results shown in Table 4, it is clear that the IgG in these fractions reacted with prothrombin bound to PE and not with PE alone. No significant binding was observed on PE-coated wells incubated with buffer, while elevated binding was found for four of the six IgG fractions on PE-coated wells incubated with prothrombin. In contrast, incubation of PE-coated wells with β₂-gpI did not increase reactivity with PE (data not shown).

The plasma (often pooled plasma obtained on different dates) used for IgG isolation and IgG fractions were also tested for antibodies reactive with cardiolipin (in the presence of fetal bovine serum) and human β₂-gpI. All six plasma pools and IgG fractions were negative for anti-cardiolipin and anti-β₂-gpI antibodies (data not shown), although the sera of several of these patients were positive for anti-cardiolipin antibodies in the clinical laboratory test. This discrepancy is likely due to the fact that plasma was pooled on the basis of lupus anticoagulant, and not anti-cardiolipin, activity. Therefore, the plasma pools often contained both anti-cardiolipin positive and negative samples. Furthermore, there are likely differences in sensitivity and specificity between the clinical laboratory and research laboratory tests.

**Table 4** Anti-prothrombin reactivity in sera and lupus anticoagulant-containing IgG fractions

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Anti-PT IgG</th>
<th>Anti-PT IgM</th>
<th>Anti-PE IgG</th>
<th>Anti-PE IgM</th>
<th>LA-containing IgG -PT</th>
<th>LA-containing IgG +PT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum/lupus control</td>
<td>0.15</td>
<td>0.28</td>
<td>0.22</td>
<td>0.46</td>
<td>0.46</td>
<td>0.43</td>
</tr>
<tr>
<td>1</td>
<td>0.14</td>
<td>0.07</td>
<td>1.28</td>
<td>1.10</td>
<td>0.66</td>
<td>2.00</td>
</tr>
<tr>
<td>2</td>
<td>0.23</td>
<td>0.26</td>
<td>1.16</td>
<td>&gt;2.65</td>
<td>0.62</td>
<td>&gt;2.65</td>
</tr>
<tr>
<td>3</td>
<td>0.21</td>
<td>0.11</td>
<td>0.30</td>
<td>0.27</td>
<td>0.41</td>
<td>&gt;2.65</td>
</tr>
<tr>
<td>4</td>
<td>0.20</td>
<td>0.28</td>
<td>0.59</td>
<td>1.80</td>
<td>0.46</td>
<td>0.52</td>
</tr>
<tr>
<td>5</td>
<td>0.83</td>
<td>0.12</td>
<td>0.24</td>
<td>0.42</td>
<td>0.35</td>
<td>0.31</td>
</tr>
<tr>
<td>6</td>
<td>0.05</td>
<td>0.23</td>
<td>0.18</td>
<td>0.91</td>
<td>0.24</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Values for sera indicate the binding (OD405) of prothrombin alone (anti-PT) or PE (anti-PE) in the presence of prothrombin. Values for IgG fractions indicate the binding (OD405) to PE (anti-PE) in the absence (+CaCl₂) or presence (-CaCl₂) of prothrombin. The sera sample for patient 2 was obtained five months prior to the plasma used for IgG isolation. The OD₄₀₅ value of 0.3 is considered to fall within the normal range. The positive values are shown in bold.

All of the preceding experiments were done in the presence of 1.25 mM CaCl₂ because of the calcium-dependent binding of prothrombin and annexin V to phospholipids. To determine whether the inhibition of lupus anticoagulant activity by PE and prothrombin was also calcium-dependent, incubations with this mixture were done in the absence and presence of 1.25 mM CaCl₂. Fig. 1 demonstrates that for all six lupus anticoagulant-containing IgG fractions, inhibition of the prolonged APTT by PE and prothrombin was at least, in part, dependent on calcium. In some cases, a small degree of inhibition was observed with PE and prothrombin, in the absence of calcium, compared to prothrombin only. However, in all cases, significantly more inhibition was observed in the presence of 1.25 mM CaCl₂.

**Antibodies to Prothrombin in Sera and Lupus Anticoagulant-Containing IgG Fractions**

The experiments described above demonstrate that the lupus anticoagulant activity of the IgG fractions is inhibited by the combination of PE and prothrombin in the presence of calcium, suggesting that the lupus anticoagulant antibodies in these fractions recognize prothrombin bound to PE. To demonstrate this reactivity by independent means, antibodies reactive with human prothrombin were assessed in the sera and isolated IgG fractions from the six SLE patients by ELISA (Table 4). For sera, reactivity was evaluated both on high-binding ELISA plates coated with prothrombin and on plates coated with PE to which prothrombin was bound. Interestingly, only one serum (patient 5) was positive in the ELISA on prothrombin-coated plates and this was for IgG antibodies only. In contrast, sera from three patients (#1, #2, and #4) contained IgG antibodies reactive with prothrombin bound to PE-coated plates. These sera also demonstrated IgM reactivity in this assay and one additional serum (patient 6) contained IgM antibodies only. Reactivity of the lupus anticoagulant-containing IgG fractions with PE-bound prothrombin was consistent with that found in the serum in all but one patient (#3). The positivity of the IgG fraction from patient 3 may be explained by the fact that the serum sample was obtained five months prior to the plasma used for IgG isolation. From the results shown in Table 4, it is clear that the IgG in these fractions reacted with prothrombin bound to PE and not with PE alone. No significant binding was observed on PE-coated wells incubated with buffer, while elevated binding was found for four of the six IgG fractions on PE-coated wells incubated with prothrombin. In contrast, incubation of PE-coated wells with β₂-gpI did not increase reactivity with PE (data not shown).

The plasma (often pooled plasma obtained on different dates) used for IgG isolation and IgG fractions were also tested for antibodies reactive with cardiolipin (in the presence of fetal bovine serum) and human β₂-gpI. All six plasma pools and IgG fractions were negative for anti-cardiolipin and anti-β₂-gpI antibodies (data not shown), although the sera of several of these patients were positive for anti-cardiolipin antibodies in the clinical laboratory test. This discrepancy is likely due to the fact that plasma was pooled on the basis of lupus anticoagulant, and not anti-cardiolipin, activity. Therefore, the plasma pools often contained both anti-cardiolipin positive and negative samples. Furthermore, there are likely differences in sensitivity and specificity between the clinical laboratory and research laboratory tests.

**Discussion**

We have demonstrated that inhibition of IgG lupus anticoagulant activity by hexagonal (II) phase PE requires the presence of plasma. To identify the plasma component(s) involved in this inhibition, three different purified human plasma proteins (prothrombin, β₂-gpI, and annexin V) were evaluated for their ability to replace plasma in this interaction. Using lupus anticoagulant-containing IgG fractions isolated from six SLE patients, we demonstrate that prolongation of the APTT was inhibited by hexagonal (II) phase PE in the presence of human prothrombin, but not human β₂-gpI or annexin V. These data indicate that inhibition of some lupus anticoagulant antibodies by hexagonal (II) phase PE is dependent on prothrombin, suggesting that these antibodies recognize a complex of PE and prothrombin.

Although hexagonal (II) phase egg PE in the presence of human prothrombin showed significant inhibition of all six lupus anticoagulant-containing IgG fractions, neither egg PE nor prothrombin alone caused significant inhibition. In contrast, lamellar phase PE in the presence of prothrombin had no significant effect on lupus anticoagulant activity. Neither physiological concentrations of β₂-gpI or annexin V, alone or in combination with hexagonal (II) phase PE, had a significant effect on the lupus anticoagulant activity of the IgG fractions. These data
indicate that the lupus anticoagulant antibodies specifically recognize hexagonal (II), but not lamellar, PE in combination with prothrombin and are consistent with our previous findings (11, 14). For all six IgG fractions, inhibition of lupus anticoagulant activity by PE and prothrombin was dependent on or significantly enhanced by CaCl₂. This is consistent with the known calcium-dependent interaction of lupus anticoagulant antibodies with prothrombin and phosphatidylserine (PS)/phosphatidylincholine (PC) vesicles (4). All six IgG fractions were negative for anti-CL and anti-β₂-gpI antibody reactivity (data not shown), suggesting that these fractions contained β₂-gpI-independent lupus anticoagulant antibodies similar to those described by Galli et al. (17). The reactivity of β₂-gpI-dependent lupus anticoagulant antibodies with PE in the presence of prothrombin, β₂-gpI, and annexin V needs to be evaluated and these studies are currently ongoing in our laboratory.

In order to confirm the inhibition results of the APTT assays by more direct and independent means, the lupus anticoagulant-containing IgG fractions were assessed for binding to PE-bound prothrombin by ELISA. Four of the six IgG fractions demonstrated binding to PE in the presence, but not the absence, of prothrombin. This reactivity was also observed in sera of three patients from whom IgG had been isolated. The reason for the lack of reactivity of two of the IgG fractions is not clear, but may be due to differences in specificity or binding affinity. Clearly, the APTT assay appears to be more sensitive for detecting antibodies with this specificity than the ELISA. Furthermore, it is likely that structures presented by hexagonal (II) phase PE and prothrombin in suspension are not represented by prothrombin bound to PE on a solid phase. Lupus anticoagulant antibodies, which do not react with PE-bound prothrombin in the ELISA, may be directed against epitopes found on these structures. Nevertheless, four of the six SLE patients had serum IgG and/or IgM antibodies reactive with PE-bound prothrombin by ELISA, while only one patient had antibodies reactive with prothrombin alone. These findings are consistent with those of previous studies. Rao et al. (9) studied the binding of lupus anticoagulant-containing IgG fractions to prothrombin alone and prothrombin bound to PS or PE. Binding to phospholipid-bound prothrombin was significantly higher than binding to prothrombin alone for many lupus anticoagulant fractions. The patterns of reactivity on PS and PE were similar, but binding of the antibodies to PE-bound prothrombin appeared to be somewhat lower, possibly due to subtraction of an apparently high background binding on PE. It is noteworthy that neither protein C nor protein S supported the binding of the same lupus anticoagulant antibodies on PE or PS coated plates. More recently, Galli et al. (10) compared the binding of plasma antibodies from patients with lupus anticoagulant activity to prothrombin bound to PS or prothrombin alone, and found a much higher prevalence of antibody binding to PS-bound prothrombin (90%) than to prothrombin alone (58%). Although these investigators were unable to link lupus anticoagulant activity to reactivity in a particular assay, they demonstrated that some IgG fractions bound in one assay and not the other. Puurunen et al. (16) have demonstrated that antibody binding to prothrombin coated to high-binding plates can be enhanced by Tween 20, which is known to contain fatty acids. Taken together, these data highlight the differences between these assays and the likelihood that new epitopes are exposed on prothrombin bound to PE or PS. Evidence that human prothrombin undergoes phospholipid-specific conformational changes upon binding to phospholipid-containing model membranes strongly supports this possibility (18).

The finding that prothrombin interacts with hexagonal (II) phase PE to form an antigen recognized by some lupus anticoagulant antibodies was somewhat unexpected. Lupus anticoagulant antibodies inhibit phospholipid-dependent coagulation reactions, possibly at the level of prothrombin conversion to thrombin by the prothrombinase complex (factor Va, factor V a cofactor, Ca²⁺, and a phospholipid membrane) (19-21). Negatively charged phospholipids, in particular PS, can provide the appropriate surface for prothrombin activation (22, 23) and are believed to be essential constituents of the membrane that support the catalytic conversion of prothrombin to thrombin during blood coagulation (24, 25). Although there has been little investigation into the interaction of prothrombin with zwitterionic phospholipids, such as PE, Gerads et al. (26) demonstrated that prothrombin activation occurred on model membranes containing 20 mol % PE. PE has also been reported to support the anticoagulant activity of activated protein C (27) and antibodies reactive with PE in lupus anticoagulant-containing plasmas demonstrated inhibition of activated protein C activity (28). Furthermore, a number of studies have shown the presence of antibodies to PE in patients with thrombosis (29-31). In the latter studies, the phospholipid-binding proteins relevant to PE binding in ELISA appear to be kininogens and kininogen-binding proteins (32, 33). Our data indicate that inhibition of lupus anticoagulant activity by hexagonal (II) phase PE is dependent on prothrombin for some lupus anticoagulant antibodies. In most cases, reactivity with PE-bound prothrombin could also be demonstrated by direct binding ELISA. These findings suggest that certain lupus anticoagulant antibodies found in patients with SLE recognize a complex of PE and prothrombin. Furthermore, this recognition is responsible for the inhibition of lupus anticoagulant activity by PE in vitro. The relevance of these findings to the pathogenesis of thrombosis associated with lupus anticoagulant antibodies remains to be determined.

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