Mechanism and Effects of the Binding of Lupus Anticoagulant IgG and Prothrombin to Surface Phospholipid
By L. Vijaya Mohan Rao, An D. Hoang, and Samuel I. Rapaport

We report here experiments on how lupus anticoagulant antibodies (LA IgG) that react with prothrombin bind to surface phospholipid and affect prothrombin’s affinity for surface phospholipid and activation to thrombin. LA IgG was purified by protein A chromatography from the plasma of 16 patients of whom four had associated hypoprothrombinaemia and 10 had experienced thrombosis. Many LA IgG bound, in the absence of phospholipid and calcium, not only to immobilized prothrombin but to both prethrombin 1 and fragment 1, which established at least an oligoclonal origin of LA IgG. No LA IgG bound to thrombin. Although prothrombin and Ca²⁺ were required to support binding of LA IgG to immobilized phosphatidylserine (PS), prothrombin at higher concentrations inhibited binding, presumably by competing with prothrombin/LA IgG complexes for PS binding sites. Prothrombin 1, which cannot bind to PS, also inhibited binding of many LA IgG to PS, presumably by forming competing soluble prothrombin 1/LA IgG complexes. Despite their ability to react with prothrombin independent of phospholipid, LA IgG enhanced binding of prothrombin to immobilized phospholipid and to cultured human umbilical vein endothelial cells. Prothrombin bound with LA IgG to the surface of endothelial cell monolayers could be activated to thrombin after supernatant prothrombin and LA IgG were washed away. The relation is discussed of these observations to a hypothesis that LA IgG mediated concentration of prothrombin on cell surface phospholipid represents a mechanism by which LA IgG could increase thrombotic risk.

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MATERIALS AND METHODS

Materials. Materials were obtained from the following sources: purified bovine phospholipids, phosphatidylcholine (PC), PS, and phosphatidylethanolamine (PE) (Avanti Polar Lipids, Alabaster, AL); Chromozym TH (Boehringer Mannheim, Indianapolis, IN); bovine serum albumin (BSA) (fatty acid free) (ICN Pharmaceuticals, Cleveland, OH); 96-well microtiter plates (Immulon 4) for ELISA (Dynatech, Chantilly, VA); reagents for cell culture (BioWhittaker, Walkersville, MD); plasticware for cell culture (Costar, Cambridge, MA); chemicals for gel electrophoresis (Bio-Rad, Richmond, CA). Other chemicals, reagent grade or better, were from Fisher Scientific (Springfield, NJ) or Sigma (St Louis, MO).

Purified proteins. Human prothrombin and factor X were purified as previously described. Factor X was activated with Russell’s viper venom and factor Xa was separated as described earlier. Factor Va was purchased from Haematological Technologies (Essex Junction, VT). Thrombin, protein C, and protein S were purchased from either Enzyme Research laboratories (South bend, IN) or from Haematological Technologies. Activation products of prothrombin were prepared with thrombinase as described in an earlier report. Prothrombin fragment 1 and prothrombin 1 were prepared by incubating prothrombin (2 mg/mL) with thrombin (2 U/mL) for 3 hours at 37°C and then subjecting the sample to DEAE Sephadex chromatography to purify fragment 1 and prothrombin 1.

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**Cell culture.** Human umbilical vein endothelial cells were obtained from Clonetics (San Diego, CA) and were cultured to confluency in 96-well plates as described in the product technical bulletin. In the present work, HUVEC passages between three and five were used. Before the experiment, confluent monolayers were washed three times with Ham's F12 media containing 5 mmol/L calcium ions.

**Plasma specimens.** Platelet-poor Plasma (PPP) from patients and normal subjects was prepared at UCSD Medical Center from venous blood collected in 1/10th volume of buffered citrate anticoagulant and centrifuged either one or two times at 4,000g for 10 minutes. PPP in buffered citrate anticoagulant from two patients was received from other institutions. The criteria used to establish the presence of lupus anticoagulant activity in plasma have been described earlier.6 Buffers. Tris-buffered saline (TBS) was 50 mmol/L Tris-HCl and 0.15 mol/L NaCl, pH 7.5. TBS/BSA (TBS containing 1 mg/mL BSA) was used for dilution of the reagents unless otherwise specified. Calcium containing buffer denotes the TBS/BSA supplemented with 5 mmol/L CaCl₂. For experiments with HUVEC monolayers Ham F12 culture medium was used in place of TBS.

**Separation of IgG from plasma.** IgG from patients' plasma and control plasma was purified by affinity chromatography on a protein A column (Pierce, Rockford, IL). In an earlier study it was found that such purified IgGs are apparently homogenous, devoid of phospholipid, and contain insignificant amounts of β2GPI.

**Sodium dodecyl sulfate (SDS) gel electrophoresis and immunoblotting.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli in 10% polyacrylamide gels. Proteins were either stained with Coomassie blue or transferred for immunoblotting onto a PVDF membrane. Immunoblotting was carried out with lupus anticoagulant IgG and normal IgG preparations as described earlier.6

**Radiolabeling of prothrombin.** Prothrombin was labeled with [125I] using the Iodogen method according to the manufacturer's technical bulletin and free radioactivity was removed by dialysis against TBS.

**ELISA for binding of IgG to immobilized prothrombin, prothrombin activation products, protein C, and protein S.** Microtiter wells were coated overnight at 4°C with 100 μL of 50 μg/mL of one of the following proteins in 0.1 mol/L sodium bicarbonate buffer, pH 9.6: prothrombin, prethrombin 1, fragment 1, thrombin, protein C, or protein S. Then the wells were blocked with 0.3% gelatin and 1% BSA in TBS at room temperature for 3 hours. Further steps of the assay to measure binding of IgG to the immobilized proteins were as described earlier.6

**ELISA for binding of IgG to PS or PE.** Microtiter wells were coated with 40 μL of PS or PE in ethanol, 50 μg/mL, or with ethanol alone. The wells were blocked with TBS containing 0.3% gelatin and 1% BSA for overnight at 4°C. Fifty microliters of test sample (50 μg/mL LA IgG or control IgG) was added to each well in gelatin/BSA buffer containing 5 mmol/L Ca²⁺ with 50 μL of 10 μg/mL of one of the following proteins as a cofactor: prothrombin, fragment 1, prethrombin 1, protein C, or protein S. Further steps of the assay were as described earlier.6

**RESULTS**

**Patient Data.**

Clinical and laboratory findings of seven of the 16 patients providing LA IgG used in the present study (Cr, Ga, Hu, Ka, Lo, Ta, and Te) have been summarized in an earlier report.6 Two of the seven patients had a lupus anticoagulant associated with acquired hypoprothrombinemia (prothrombin levels: Cr, 4%; Lo, 10%). Two of the nine additional LA IgG used in the present study were from patients who also had an associated hypoprothrombinemia (prothrombin levels: Ca, 15%; Ma, 3%). Thus, 4 of the 16 LA IgG were from plasmas containing antibodies reacting towards prothrombin with an affinity and/or tier sufficient to deplete plasma prothrombin as a consequence of accelerated clearance of prothrombin/antiprothrombin immune complex.
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Fig 1. Binding on Western blots of lupus anticoagulant IgG to purified human prothrombin and its activation products. Purified prothrombin and its activation products (300 μg per gel) were subjected to SDS-PAGE and transblotted onto a PVDF membrane at 45 V for 3 hours. The membrane was blocked with 5% milk in TBS overnight at 4°C. After washing the membrane once with TBS, the membrane was placed in a multiscreen apparatus (Multiscreen II, Bio-Rad). Lanes were probed with LA IgG from different patients (200 μg IgG in 750 μL of TBS containing 5% milk). After 3 hours, the lanes were washed four times with 1 mL of TBS containing 0.05% Tween-20 (TTBS). Then, the membrane was removed from the Multiscreen, washed 2 more times with TTBS, and probed with 125I-labeled protein A (200,000 cpm/mL) for 90 minutes. After this, the membrane was washed 6 times with TTBS and exposed overnight to x-ray film at -80°C. The farthest lane labeled “C” depicts the binding observed with normal control IgG. Arrow marks indicate (from top to bottom) the position of prothrombin, prethrombin 1, thrombin/fragment 1-2, fragment 1, and fragment 2. Immunoblotting with Ma’s LA IgG was performed separately because the plasma from this patient was obtained at later date.

plexes. Three of these four patients presented with abnormal bleeding or bruising. The fourth patient (Ca) was discovered to have hypoprothrombinemia when tested for lupus anticoagulant activity because of a 20-week miscarriage and a history of two past miscarriages. An additional patient (Ga) had an associated hypoprothrombinemia of 20% in plasma used to prepare LA IgG for the earlier study cited but a prothrombin level of 50% in the plasma sample used to prepare the LA IgG for the present study. This 36-year-old patient died of a myocardial infarction several weeks after the second plasma sample was obtained.

Ten of the 16 patients experienced thrombotic events (Br, Es, Ga, He, Hu, Je, Ka, Su, Te, and To), which in three patients (Ga, Te and To) were arterial. One patient (Ka) had both a venous thrombosis and at least two cerebral ischemic episodes. As already mentioned, one patient (Ca) experienced multiple miscarriages; a second patient (Hu) experienced a reproductive loss associated with almost total infarction of the placental bed; and two patients (Es and Ta) experienced severe preeclampsia.

Binding of LA IgG to Immobilized Prothrombin, Prothrombin Activation Products, Protein C, and Protein S

The ability of 16 LA IgG to bind on Western blots to human prothrombin and to human prothrombin activation products was determined (Fig 1). As noted earlier, most LA IgG (11 of 15 preparations) bound to prothrombin in the absence of Ca2+ and phospholipid. Three LA IgG that failed to give a signal on Western blots possessed weak lupus anticoagulant activity only minimally prolonging the dRVVT of normal plasma (clotting times on adding 2 mg/mL of the LA IgG, 44 to 48 seconds; clotting time on adding 2 mg/mL of control IgG, 39 seconds). Eight of the 15 LA IgG also bound to prethrombin 1, in two instances with an intensity of signal equal to or exceeding that obtained for binding to prothrombin. In contrast, no LA IgG bound on Western blots to fragment 1, fragment 2, or thrombin.

Fourteen LA IgG were also tested in an ELISA for their ability to bind to prothrombin, prethrombin 1, fragment 1, or thrombin (Fig 2). Eleven LA IgG could be demonstrated to bind to wells coated with prothrombin and eight LA IgG could be demonstrated to bind to wells coated with prethrombin 1. In contrast to their inability to give a signal on Western blots, 8 of the 14 LA IgG could also be demonstrated to bind to a varying extent to wells coated with fragment 1. No LA IgG bound to a well coated with thrombin in excess of its binding to a well-coated with normal control IgG (data not shown).

The ability of LA IgG to bind in an ELISA to immobilized protein C and to immobilized protein S was also examined (Fig 3). No LA IgG bound to protein C in excess of its binding to a control well blocked with BSA. Although most of the LA IgG failed to bind to protein S, one LA IgG (He) definitely bound to protein S (P = .02) and two additional LA IgG had questionably significant (Cr, P = .05 and Je, P = .11) excess binding to protein S than to control BSA. Whereas the LA IgG of patient Cr bound to a much greater
extent to prothrombin than to protein S, the LA IgG of both patient He and patient Je appeared to bind to a greater extent to protein S than to prothrombin.

Requirements for the Binding of LA IgG to Immobilized PS and to Immobilized PE

The ability of LA IgG to bind to immobilized PS in the presence of prothrombin, prethrombin 1, or fragment 1 was compared. Prothrombin consistently supported the binding of LA IgG to immobilized PS, whereas prethrombin 1, which cannot bind to anionic phospholipid, failed to support the binding of any LA IgG to immobilized PS. Fragment 1 supported binding to PS of 5 of the 11 LA IgG tested but, in each instance, to a lesser extent than that observed with prothrombin (eg, binding of patient Ca LA IgG to PS supported by prothrombin, 1.65 OD, supported by fragment 1, 0.56 OD; binding of control IgG to PS supported by either prothrombin or fragment 1, 0.08 OD).

The ability of protein C and protein S to support the binding of LA IgG to PS was examined in an experiment in which prothrombin served as a positive control and buffer as a negative control. Neither protein C nor protein S could be demonstrated to support the binding of LA IgG to immobilized PS (Fig 4).

PE has recently been reported to support the anticoagulant activity of activated protein C" and some antiphospholipid autoantibodies have been reported to bind preferentially to PE." Therefore, the ability of prothrombin, protein C and protein S to support the binding of LA IgG to PE was also evaluated. Although prothrombin supported the binding of many LA IgG to immobilized PE, neither protein C nor protein S could support the binding of LA IgG to immobilized PE (Fig 5).
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Fig 4. The inability of protein C and protein S to support the binding of LA IgG to immobilized PS. LA IgG, 50 μg/mL, with protein C or protein S, 10 μg/mL, in calcium containing buffer were added to wells containing immobilized PS. After 1 hour at room temperature, the wells were washed with calcium containing buffer and LA IgG bound to immobilized PS was measured in an ELISA. LA IgG binding to PS in the presence of the control buffer or prothrombin (10 μg/mL) is also shown. The farthest left group of bars labeled "C" depict binding observed with normal control IgG.

The Effect of Excess Prothrombin, Prethrombin I, or Fragment I on Prothrombin-Mediated Binding of LA IgG to Immobilized PS

LA IgG, 10 μg/mL, and prothrombin, 5 μg/mL, were incubated with control buffer or with buffer containing a 100-fold excess (wt/wt) of fragment I, prethrombin I, or prothrombin. The mixtures were then added to wells containing immobilized PS. The 100-fold excess of fragment I prevented the binding of every LA IgG to immobilized PS (Fig 6). The 100-fold excess of prethrombin I diminished the binding of 6 of the 10 LA IgG tested to immobilized PS. This was particularly evident for the LA IgG from patients Ca, Cr, and Lo (Fig 6).

Increasing the prothrombin concentration 100-fold impaired the binding of some LA IgG to immobilized PS, had little or no effect on the binding of other LA IgG, and substantially increased the binding of one LA IgG (Cr) (Fig 6). In an additional experiment five LA IgG were incubated at a 10 μg/mL concentration with increasing concentrations of prothrombin before being added to wells coated with PS. Binding of each LA IgG to PS increased as the prothrombin concentration in incubation mixtures was increased from 1 μg/mL to between 10 and 100 μg/mL. However, when the prothrombin concentration was increased beyond 100 μg/mL, the binding of four of the LA IgG to PS decreased (Fig 7).

The Effect of LA IgG on Prothrombin Binding to Surface Phospholipid

Increasing concentrations of 125I-prothrombin, from 10 nmol/L to 4 μmol/L, were incubated with a fixed 2 mg/mL

Fig 5. Binding of LA IgG to immobilized PE in the presence of prothrombin, protein C, or protein S. LA IgG, 50 μg/mL, with prothrombin, protein C, or protein S, 10 μg/mL, in calcium containing buffer were added to wells containing immobilized PE. After 1 hour at room temperature, the wells were washed with calcium containing buffer and the binding of LA IgG to immobilized PE was measured in an ELISA. The values shown were obtained by subtracting the values obtained for the binding to PE of individual IgG preparations in the absence of added protein (background) from the values obtained for the binding to PE of individual IgG preparations in the presence of prothrombin, protein C or protein S. The farthest left group of bars labeled "C" depict binding observed with normal control IgG.
concentration of either control IgG or LA IgG in wells coated with a mixture of PS/PC (40/60). Four LA IgG preparations were used: two from patients with associated hypoprothrombinemia (Ma, 3% prothrombin and Lo, 10% prothrombin) and two from patients without associated hypoprothrombinemia (Hu and Te). The resultant binding isotherms could be fitted to a rectangular hyperbola (Fig 8). Kd values obtained by curve fitting and from Scatchard plots were similar. Each LA IgG increased the affinity of binding of prothrombin to immobilized PS/PC (Table 1). The most pronounced effect was observed with the LA IgG from patient Ma (Table 1).

LA IgG from patient Ma was also found to enhance markedly the specific binding of $^{125}$I-prothrombin to the surface of monolayers of cultured HUVEC. The resultant binding isotherm (Fig 9) was similar to that observed for the binding of the LA IgG to immobilized PS/PC.

The Effect of LA IgG on HUVEC-Supported Activation of Prothrombin

Cold prothrombin, 100 μg/mL, was added with control IgG or LA IgG (2 mg/mL) to monolayers of cultured HUVEC. After 1 hour the wells were washed repeatedly with the medium containing 5 mmol/L Ca$^{2+}$ to remove unbound prothrombin and LA IgG before factors Xa (0.01 pg/mL) and Va (3 μg/mL) were added. The concentration of thrombin

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**Fig 6.** The effect of excess fragment 1, prothrombin 1, or prothrombin upon the binding of LA IgG to immobilized PS. LA IgG, 10 μg/mL, in calcium containing buffer were incubated with 5 μg/mL prothrombin alone (□) or 5 μg/mL prothrombin plus a 100-fold (wt/wt) excess of fragment 1 (●), prothrombin 1 (○), or prothrombin (●). After 1 hour at room temperature mixtures were added to microtiter wells containing immobilized PS. After a further 1 hour at room temperature, the wells were washed and binding of LA IgG to PS was measured in an ELISA (n = 4). The farthest left group of bars labeled "C" depict binding observed with normal control IgG.

**Fig 7.** The effect of increasing prothrombin concentration on the ability of a low concentration of five LA IgG to bind to immobilized PS. A fixed low concentration of LA IgG, 10 μg/mL, was incubated with increasing concentrations of prothrombin from 0.1 to 1,000 μg/mL in the presence of calcium containing buffer for 1 hour at room temperature. Then, 100 μL of mixtures was added to microtiter wells that had been coated with PS. The binding of IgG to immobilized PS was measured in an ELISA. The IgG preparations used in the experiment were from patients Br (●), Ca (□), Ka (■), Lo (○), and Te (▲).

**Fig 8.** The effect of LA IgG on the binding of $^{125}$I-prothrombin to PS/PC coated wells. PS/PC coated wells were incubated for 1 hour with increasing concentrations in calcium containing buffer of $^{125}$I-prothrombin in the presence of 2 mg/mL of LA IgG or control IgG. The wells were then washed 10 times with calcium containing buffer. Following this, the wells were detached from the plate and counted for radioactivity. The IgG preparations used in this experiment were from normal (■), Hu (○), Ma (●), Te (▲) plasma.
generated was measured after 1 hour with a chromogenic substrate. More thrombin was present in wells containing prothrombin that had come down onto the cell surface in the presence of each of the five LA IgG tested than in wells containing prothrombin that had come down onto the cell surface in the presence of control normal IgG (Table 2). Under these experimental conditions LA IgG bound with prothrombin onto the monolayer surface was unable to prevent the conversion of bound prothrombin to thrombin.

A further experiment was performed to compare the time course of thrombin generation from prothrombin brought down onto the cell surface in the presence of LA IgG from patient Ma and the time course of thrombin generation from prothrombin brought down onto the cell surface in the presence of control IgG. A mixture of prothrombin, 100 pg/mL, and either control IgG or LA IgG from patient Ma, 2 mg/mL, in medium containing calcium ions was added to a series of wells containing monolayers of cultured HUVEC. After 1 hour, the wells were washed and factor Xa and Va were added as above. At increasing times chromogenic substrate was added to the well to measure the amount of thrombin that had been generated. As shown in Fig 10, at 5 minutes a similar amount of thrombin had been generated from prothrombin brought down on the cell surface in the presence of LA IgG. However, additional thrombin continued to be slowly generated from prothrombin brought down in the presence of LA IgG over the next more than 1 hour.

In additional experiments with the LA IgG from patient Ma, prothrombin, 100 μg/mL, LA IgG, 2 mg/mL, or both were added back to the washed monolayers along with factors Xa and Va. LA IgG brought down onto the monolayer during the initial incubation with prothrombin did not substantially inhibit activation of prothrombin added back after

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<th>Table 1. Prothrombin Binding Constants to Immobilized PS/PC in the Presence of Lupus Anticoagulant IgG</th>
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<td>Source of IgG</td>
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</tr>
<tr>
<td>Control</td>
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<td>Hu</td>
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<th>Table 2. Activation of Prothrombin Bound to HUVEC in the Presence of Lupus Anticoagulant IgG (n = 4)</th>
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<td>Source of IgG</td>
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<tr>
<td>---------------</td>
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<tr>
<td>Control</td>
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Prothrombin, 100 μg/mL (1.4 μmol/L) and 2 mg/mL LA IgG in 100 μL of Ham F12/BSA media containing calcium ions (5 mmol/L) were added for 1 hour to monolayers of HUVEC cultured in a 96-well plate. Then, unbound prothrombin and LA IgG were removed and the wells were washed five times with the above media containing calcium ions. Following this, factor Xa, 0.01 μg/mL and factor Va, 3 μg/mL in 50 μL of the media containing calcium ions were added to the wells and the amount of thrombin generated in the well was measured after 1 hour by adding a chromogenic substrate.

Fig 10. Kinetics of activation of prothrombin bound to HUVEC in the presence of LA IgG. Prothrombin, 100 μg/mL (1.4 μmol/L) and 2 mg/mL LA IgG in 100 μL of Ham F12/BSA media containing calcium ions (5 mmol/L) were added to multiple wells of monolayers of HUVEC cultured in a 96-well plate. After 1 hour, unbound prothrombin was removed and the wells were washed five times with the media containing calcium. Following this, factor Xa, 0.01 μg/mL and factor Va, 3 μg/mL in 50 μL of the media containing calcium ions were added to the wells. At increasing times thereafter, 50 μL of chromogenic substrate was added to wells to measure the amount of thrombin generated. Values are mean ± SD (n = 3).
washed (mean thrombin generation supported by monolayers exposed in the initial incubation to LA IgG; 3.9 pmol/min/well; mean thrombin generation supported by monolayers exposed in the initial incubation mixture to control IgG, 4.5 pmol/min/well, n = 2). Adding back LA IgG without prothrombin failed to inhibit activation of the prothrombin bound to the surface in the initial incubation (thrombin activity measured after 1 hour in the presence of added back LA IgG, 1.38 pmol/well; in the presence of added back LA IgG, 1.52 pmol/well). However, when the LA IgG from patient Ma and prothrombin were added back together, the monolayer surface failed to support prothrombin activation (mean thrombin generation for prothrombin added back alone, 3.9 pmol/min/well; for prothrombin added back with LA IgG, 0.002 pmol/min/well, n = 2).

Similar results were obtained in experiments in which monolayer surfaces were incubated initially for 1 hour with 100 μg/mL prothrombin and 2 mg/mL LA IgG and factors Xa and Va were added without removing the supernatant prothrombin and LA IgG. Under these conditions all 5 LA IgG tested substantially impaired the ability of the monolayer to supply the activity needed for prothrombinase-catalyzed activation of prothrombin (Table 3).

**Table 3. Effect of Lupus Anticoagulant IgG on HUVEC Supported Activation of Prothrombin (n = 4)**

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<tr>
<th>Source of IgG</th>
<th>Thrombin Generated (pmol/10 min/well)</th>
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<tr>
<td>Control</td>
<td>37.1 ± 3.6</td>
</tr>
<tr>
<td>Hu</td>
<td>11.7 ± 1.2</td>
</tr>
<tr>
<td>Ka</td>
<td>10.2 ± 0.6</td>
</tr>
<tr>
<td>Lo</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Ma</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>Te</td>
<td>5.9 ± 2.6</td>
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Confluent monolayers were incubated in media containing calcium with prothrombin, 100 μg/mL (1.4 μmol/L) in the presence of control IgG or LA IgG; 2 mg/mL for 1 hour at 37°C. Then factor Xa, 0.01 μg/mL and factor Va, 3.0 μg/mL were added to the wells. An aliquot of supernatant was removed at 10 minutes, diluted 1/20 in TBS/BSA buffer containing 5 mmol/L EDTA and assayed for thrombin with a chromogenic substrate.

**DISCUSSION**

Although an association between lupus anticoagulant activity and acquired hypoprothrombinemia had been recognized many years earlier,23-24 it was not until the early 1980s that the plasma of a patient with the acquired hypoprothrombinemia-lupus anticoagulant syndrome was shown to contain non-neutralizing antiprothrombin antibodies capable of binding to prothrombin in solution.18 Soon thereafter it was reported20 and confirmed21 that abnormal prothrombin arcs indicative of prothrombin/antiprothrombin complexes were demonstrable by crossed immunoelectrophoresis of plasma from most patients with a lupus anticoagulant. In further experiments it was shown: that lupus anticoagulant activity could be adsorbed from plasma onto and eluted from insolubilized prothrombin22; that a number of LA IgG preparations could bind in the absence of phospholipid and calcium with a varying intensity of signal to prothrombin on Western blots; and that several LA IgG preparations could bind in the absence of phospholipid to human prothrombin in solution.3

We report here that many LA IgG can bind, in the absence of phospholipid and calcium ions, not only to the intact prothrombin molecule but to prothrombin 1 and fragment 1. Binding to prothrombin 1 could be demonstrated both by Western blotting (Fig 1) and by ELISA (Fig 2), whereas binding to fragment 1 could be demonstrated only by ELISA (Fig 2). In contrast, of 16 LA IgG tested none could be shown to bind to α-thrombin by either technique. This observation has implications for the consideration of mechanisms whereby LA IgG increase thrombotic risk. The finding that LA IgG often bind to both prothrombin 1 and fragment 1 provides evidence that LA IgG have immunologic specificity for more than a single epitope on prothrombin, ie, are at least of oligoclonal origin.

We also tested whether LA IgG could bind to protein C or protein S immobilized on microtiter wells. In agreement with an earlier report26 none of 14 LA IgG tested could be shown to bind to protein C. Also in agreement with earlier reports,26,27 most of the LA IgG failed to bind to immobilized protein S. However, one LA IgG definitely bound to immobilized protein S and two additional LA IgG exhibited questionably significant binding (Fig 3).

As reported earlier,2,5-7 prothrombin, in the presence of Ca2+, was again found to support the binding of LA IgG to immobilized PS. In contrast, neither protein C nor protein S could be shown to support the binding of LA IgG to immobilized PS (Fig 4). PE has been reported to be an important membrane component for supporting activated protein C anticoagulant activity.20 Prothrombin also supported the binding of most of the LA IgG tested to PE whereas neither protein C nor protein S could be shown to enhance the binding of LA IgG to immobilized PE (Fig 5). These data are of interest in that antibodies reacting with PE in lupus anticoagulant plasmas have been reported to inhibit activated protein C activity.28 They are also of interest in view of an unresolved issue of whether autoantibodies to protein S reported in patients with thrombosis have lupus anticoagulant activity or anticardiolipin reactivity.27,29

If LA IgG can bind to unaltered prothrombin, then the hypothesis is tenable that LA IgG comes down as a "passenger" when prothrombin, in the presence of Ca2+, binds to immobilized PS. Two types of evidence were obtained in support of this hypothesis. First, it was found that incubating LA IgG with prothrombin in the presence of a large excess of prothrombin 1 diminished the ability of the prothrombin to support the binding of many of the LA IgG to PE (Fig 6). Prethrombin 1 cannot bind to PE. Therefore, excess prethrombin 1 could impede prothrombin-supported binding of LA IgG to PS only by competing with prothrombin in solution for LA IgG and so diminishing the number of prothrombin/IgG complexes available to come down into immobilized PS.

Second, it was found that incubating a low, 10 μg/mL concentration of LA IgG with increasing concentrations of prothrombin initially increased the binding of five LA IgG to immobilized PS but then, at concentrations of prothrombin between 100 and 1,000 μg/mL, decreased the binding of LA IgG...
IgG to immobilized PS (Fig 7). At the higher prothrombin concentrations free prothrombin could compete with limited numbers of prothrombin/LA IgG complexes for binding sites on immobilized PS.

The concept that prothrombin/LA IgG complexes forming independent of phospholipid come down onto anionic phospholipid because prothrombin binds to phospholipid does not preclude postulating that reactions between LA IgG and prothrombin enhance the affinity of prothrombin for anionic phospholipid. This hypothesis was tested in experiments in which increasing concentrations of 125I-prothrombin were exposed to surface phospholipid in the presence of Ca++ and a 2 mg/mL concentration of LA IgG. The binding isotherms obtained in the presence of this higher concentration of LA IgG provided evidence that LA IgG can increase the affinity of prothrombin both for binding to immobilized PS/PC (Fig 8 and Table 1) and for binding to the surface membrane of monolayers of HUVEC (Fig 9). It is presently unknown whether this stems from an LA IgG-induced alteration in prothrombin conformation enhancing prothrombin’s ability to bind to phospholipid or from reactions between LA IgG and new epitopes arising after prothrombin binds to anionic phospholipid that could tighten the binding. Regarding the latter possibility, it should be emphasized that our evidence for LA IgG coming down as formed prothrombin/LA IgG complexes onto anionic phospholipid in no way rules out the possible additional binding of LA IgG to new epitopes arising after the binding of prothrombin to anionic phospholipid. Indeed, in our earlier work it was observed that whereas several LA IgG could bind to soluble human prothrombin in the absence of PS/PC vesicles, adding PS/PC vesicles enhanced the extent of the binding.7

The reason or reasons why the presence of LA IgG in plasma is associated with an increased thrombotic risk are still not clear. After showing that 125I-prothrombin in the presence of LA IgG binds with enhanced affinity to immobilized phospholipid and to monolayers of cultured endothelial cells, we thought it important to determine whether prothrombin bound with LA IgG to the surface cell monolayers could be activated to thrombin. Therefore, cold prothrombin was bound in the presence of either LA IgG or control IgG to the surface of HUVEC monolayers and, after the supernatant prothrombin and IgG were washed away, the surface-bound prothrombin was exposed to factors Xa and Va. One hour after addition of the factors Xa and Va, more thrombin had been generated from prothrombin that had bound in the presence of LA IgG than from prothrombin that had bound in the presence of control IgG (Table 2). Thus, the binding of prothrombin to monolayers in the presence of LA IgG was not associated with an inherent loss of prothrombin’s activatability. However, when a time course experiment was performed, it was found that despite the increase in total amount of thrombin generated more than an hour of activation, binding in the presence of LA IgG appeared to slow the rate of generation of thrombin from that prothrombin which had bound in excess of the amount bound in the presence of control IgG (Fig 10). Moreover, if experimental conditions were changed and supernatant prothrombin and LA IgG were not removed before factors Xa and Va were added, then prothrombin activation was markedly impaired. Under these conditions the monolayers of HUVEC were unable to provide anionic phospholipid or an equivalent activity required for prothrombin activation (Table 3).

The data herein provide evidence: (1) that LA IgG bound to prothrombin in the absence of phospholipid can be brought down as a “passenger” when prothrombin binds to phospholipid; (2) that prothrombin binds to immobilized phospholipid or to cultured HUVEC monolayers with higher affinity in the presence of LA IgG than in the presence of control IgG; and (3) that prothrombin bound to cultured monolayers of HUVEC in the presence of LA IgG retains its inherent activatability to thrombin. Although the data of Table 3 raise doubt about the validity of an hypothesis that LA IgG increases thrombotic risk by concentrating activatable prothrombin on cell surface phospholipid, it may be premature to discard the hypothesis. It should be noted that patients Hu, Ka, and Te of Table 3, who did not have an associated hypoprothrombinemia, had not experienced abnormal bleeding and had each experienced one or more serious thrombotic events. Yet under the experimental conditions of Table 3, their LA IgG impaired substantially the ability of an HUVEC monolayer to support prothrombinase activity, ie, yielded data that at face value would fit best with an increased propensity for bleeding than for thrombosis. For these patients, the experimental conditions of Table 2 and Fig 10, in which only the conversion of surface bound prothrombin to thrombin was determined, yielded data concordant with the pathophysiologic effects of LA IgG. The design of additional ways to test the hypothesis that LA IgG increase thrombotic risk by enhancing the binding of substrate prothrombin to cell surfaces remains for us a challenge of the future.

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