Anti-Prothrombin Antibodies and the Lupus Anticoagulant

By Rebecca A. Fleck, Samuel I. Rapaport, and L. Vijaya Mohan Rao

The investigators have evaluated the frequency and manifestations of anti-prothrombin antibodies in patients with the lupus anticoagulant. Thirty-one of 42 patients with lupus anticoagulants associated with a variety of underlying conditions (74%) had evidence on crossed immunoelectrophoresis of anti-prothrombin antibodies. Twenty-four of 25 patients with an activated partial thromboplastin time exceeding 50 seconds and 14 of 15 patients with a prothrombin time exceeding control by more than two seconds had demonstrable anti-prothrombin antibodies. Three of the 31 patients with anti-prothrombin antibodies had essentially no measurable plasma prothrombin, a presumed result of accelerated clearance of prothrombin/prothrombin antibody complexes. Each of these patients had bled abnormally. The remaining patients with anti-prothrombin antibodies had neither substantial hypoprothrombinemia nor hemorrhagic manifestations, which confirms the non-neutralizing property of anti-prothrombin antibodies associated with the lupus anticoagulant. Since lupus anticoagulant immunoglobulins are known to react with phospholipids, the high prevalence of antibodies binding prothrombin led us to test the hypothesis of antibody polyreactivity. Adsorption of three lupus anticoagulant plasmas with insolubilized prothrombin markedly diminished evidence of both prothrombin/prothrombin antibody complexes and anticoagulant activity. Eluates of the insolubilized prothrombin contained IgG that not only bound prothrombin but possessed lupus anticoagulant activity.

The Lupus Anticoagulant is a common laboratory abnormality found not only in patients with systemic lupus erythematosus but also in patients with a variety of disorders. It results from plasma immunoglobulins that impair the activity of anionic phospholipids in in vitro clotting tests, as in the activated partial thromboplastin time (APTT). Despite its prolonging clotting test times, the lupus anticoagulant rarely causes bleeding. When it does, the patient virtually always has evidence of a second plasma defect—a depletion of plasma prothrombin.

Although an association of the lupus anticoagulant with hypoprothrombinemia was first recognized many years ago, the pathogenesis of the hypoprothrombinemia remained obscure until recently. Material neutralizing prothrombin's activity could not be demonstrated in lupus anticoagulant plasma. However, in 1983, Bajaj et al demonstrated that the plasma of two patients with the lupus anticoagulant-acquired hypoprothrombinemia syndrome contained antibodies that bound prothrombin without neutralizing its coagulant activity. They postulated and later provided some indirect evidence that the hypoprothrombinemia could stem from a rapid clearance of prothrombin/prothrombin antibody complexes in vivo.

Because patients with the lupus anticoagulant-acquired severe hypoprothrombinemia syndrome are uncommon, it was thought that the coexistence of antibodies reacting with anionic phospholipids and antibodies reacting with prothrombin, although real, was infrequent. However, in 1984, Edson et al reported that anti-prothrombin antibodies could be demonstrated by crossed immunoelectrophoresis in the plasma of 14 of 21 patients with the lupus anticoagulant, many of whom had normal levels of plasma prothrombin.

This observation stimulated the investigators to obtain further data on the prevalence and manifestations of anti-prothrombin antibodies in a large group of patients with the lupus anticoagulant. After confirming that anti-prothrombin antibodies are indeed demonstrable in the majority of patients with the lupus anticoagulant, the investigators then carried out experiments to test the hypothesis that antibody polyreactivity accounts for the association.

MATERIALS AND METHODS

Specimens. Blood was collected in one tenth volume of either a buffered citrate anticoagulant (0.06 mol/L sodium citrate plus 0.04 mol/L citric acid) or 3.2% sodium citrate. Platelet-poor plasma was obtained by centrifugation at 2000 g for 15 minutes at room temperature and studied either immediately or after storage at −70°C.

Routine coagulation tests. APTT was performed with Automated APTT reagent (General Diagnostics, Morris Plains, New Jersey) and the prothrombin time with Thromboplastin C (American Dade, Aguada, Puerto Rico). Normal ranges are, for the APTT, 22 to 30 seconds; for the prothrombin time, 11 to 13 seconds.

Specific prothrombin coagulant assay. Prothrombin coagulant activity was measured in a one-stage assay based upon the ability of a dilution of test plasma to shorten the clotting time with tissue factor of a ready-prepared prothrombin deficient plasma. Thromboplastin C was used as the tissue factor reagent. The normal range is 60% to 150% of a pooled plasma reference standard.

Russell's viper venom (RVV) test system for assay of lupus anticoagulant activity. Lupus anticoagulant activity was assayed as activity in a test sample prolonging the clotting time with diluted RVV of a normal pooled plasma substrate. A stock solution of RVV (Wellcome Reagents Ltd. Beckenham, England) was reconstituted as suggested by the manufacturer and diluted one to 1,000 in 0.15 mol/L NaCl before use. A phospholipid reagent (Coagachek, Hyland Laboratories, Malvern, PA), reconstituted as the manufacturer suggested, was diluted 1:100 in 0.15 mol/L NaCl before use. The assay was performed by incubating 100 μL of normal pooled
plasma, 50 µL of test plasma, and 50 µL of dilute phospholipid for three minutes at 37°C. Then, 50 µL of RVV were added, followed exactly 30 seconds later by 100 µL of 25 mmol/L CaCl₂ warmed to 37°C. Normal control times, ie, clotting times obtained with isotonic saline or with non-IgG containing eluate fractions (see Results) as test material, varied between 62 and 70 seconds.

Prothrombin antigen assay. Prothrombin antigen was measured by electroimmunoassay essentially as described by Laurell⁸ using 150 µL of polyclonal rabbit anti-prothrombin antibody (Diagnostica Stago, Asnieres, France) added to 25 mL of 1% agarose (Bio-Rad Laboratories, Richmond, CA) for the electrophoresis plate and 5 µL of test or control plasma in sample wells. Rocket height of a test plasma was converted to percent prothrombin antigen from a reference curve constructed from the heights of dilutions of normal pooled plasma electrophoresed on the same plate.

Prothrombin crossed immunoelectrophoresis (CIE). Electrophoresis in the first dimension was carried out in 1% agarose in buffer on a 5 x 7.5 cm glass plate. Two sample wells, appropriately separated, were cut in the agar; the top well was filled with 5 µL of test material and the bottom well with 5 µL of normal pooled control plasma. Six milliamperes were applied across 7.5 cm for three hours. After the first electrophoresis, agarose strips approximately 7 mm wide along which the proteins had migrated, were left on the plates. Five milliliters of 1% agarose in buffer containing 40 µL of anti-prothrombin antibodies (Diagnostica Stago) were poured around the strips. Electrophoresis in the second dimension was carried out with 3 mA applied across 7.5 cm for 16 hours. The plate was then washed in 0.15 mol/L NaCl and distilled water, dried, and stained with Coomassie blue. The buffer used to dissolve the agarose was barbital buffer, pH 8.6, containing EDTA (15.47 g sodium barbital, 2.71 g barbital acid, and 2.60 g Na₂EDTA in 3.5 L distilled water). The same buffer without EDTA was used in the electrophoresis wells.

Adsorption with Staphylococcus aureus cell suspension. Five hundred microliters of a 30% suspension in Tris-buffered saline of a Staphylococcus aureus cell suspension (Pansorben, Calbiochem Behring Diagnostics, La Jolla, CA) was added to 500 µL of plasma. After incubation at room temperature for 15 minutes, the mixture was centrifuged in an Eppendorf centrifuge (Brinkmann Instruments Co., Westbury, NY) and the clear supernatant saved. Preparation of insolubilized prothrombin. Purified human prothrombin, prepared as described earlier,¹⁰ was coupled to Affi-Gel-15 (Bio-Rad Laboratories) at a concentration of 20 mg prothrombin/mL of beads. The purified prothrombin gave a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and contained no contaminating phospholipid measurable as phosphate in a 5 mg/mL sample as determined by the procedure of Ames.¹¹ The coupling reaction was carried out in 0.1 mol/L HEPES buffer, pH 7.5, at room temperature for four hours; unreacted resin sites were then blocked with glycine ethyl ester, 0.1 mol/L final concentration. Control beads were prepared by incubating Affi-Gel-15 with 0.1 mol/L glycine ethyl ester.

Adsorption of plasma with insolubilized prothrombin. Five hundred microliters of plasma were mixed with 200 µL of insolubilized prothrombin and incubated in an Eppendorf tube for either two hours at room temperature or 24 hours at 4°C. The mixture was centrifuged in an Eppendorf centrifuge, and the supernatant was adsorbed two more times.

Preparation of eluates from insolubilized prothrombin. Five milliliters of plasma were mixed with 1 mL of insolubilized prothrombin for three hours at room temperature. The slurry was then transferred to a small column and washed extensively with 20 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 0.02% Na azide, pH 7.5. Antibodies were eluted with 0.1 mol/L glycine and 0.15 mol/L NaCl buffer, pH 2.4. Eluate fractions of 900 µL were collected into tubes containing 100 µL of 1 mol/L Tris to adjust the pH to 7.6. The fractions were dialyzed in Tris buffer overnight at 4°C before testing.

Adsorption studies with barium citrate. Barium adsorbed plasma and barium eluates were prepared as described earlier.¹²

RESULTS

Patient characteristics. Plasma samples containing the lupus anticoagulant were available for study from 42 patients. Plasma was considered to contain the lupus anticoagulant if it fulfilled the following criteria: 1) an APTT that exceeded the upper limit of the normal range for the laboratory by at least five seconds; 2) an APTT of a 1:1 mixture of patient’s plasma and normal plasma that was at least five seconds longer than the APTT of the normal plasma alone; 3) less than 50% of normal activity of at least two of those factors that are measured in one stage APTT assays, specifically factors XII, XI, IX, and VIII; 4) evidence that the measured activity of one or more of the above mentioned clotting factors increased with increasing dilution of the patient’s plasma in the assay; and 5) no evidence of another cause for the above clotting abnormalities. Clinical information was available for all but three of the patients. The lupus anticoagulant was found to be associated with a variety of underlying disorders as summarized in Table 1.

Recognition of anti-prothrombin antibodies. An abnormal prothrombin arc with reduced electrophoretic mobility on crossed immunoelectrophoresis (CIE) was accepted as evidence of plasma prothrombin/prothrombin antibody complexes.⁵ In three patients with severe hypoprothrombinemia, the investigators first had to add normal plasma, a source of prothrombin, to the patient’s plasma in order to demonstrate an abnormal arc (Fig 1).

Adding prothrombin was unnecessary in the other patients whose plasma prothrombin level was normal or only moder-

| Table 1. Primary Diagnoses of Patients With Demonstrable Anti-Prothrombin Antibody |
|---------------------------------|--------|--------|--------|
| Diagnosis                        | No. of Patients | Prothrombin Antibody | Present | Absent |
| SLE                              | 7       | 7      | 0      |
| Fetal wastage                    | 4       | 2      | 2*     |
| Drug induced†                    | 10      | 7      | 3      |
| Infection associated‡            | 8       | 5      | 3      |
| Miscellaneous§                   | 10      | 8      | 2      |
| No history                       | 3       | 2      | 1      |
| Total                            | 42      | 31     | 11     |

*Both of these patients were receiving adrenocorticosteroids at the time of study.
†Tricyclic antidepressants, two patients; procainamide, six patients; hydralazine, two patients.
‡Infections included: osteomyelitis, two patients; pharyngitis, two patients; disseminated gonococcal infection, one patient; pneumonia, one patient; sepsis associated with leukemia, one patient; multiple infections complicating AIDs, one patient.
§Diagnoses included: hip fractures; osteoporosis with multiple fractures; hyperthyroidism; hypothyroidism; Whipple’s disease; peripheral vascular disease; autoimmune hemolytic anemia; coronary artery disease; and well differentiated lymphocytic lymphoma.
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Fig 1. Measurement of prothrombin antigen by CIE of plasma from a patient with severe acquired hypoprothrombinemia (Patient C, Table 3). (A) Absence of prothrombin antigen rocket on electroimmunoassay. (B) Absence of prothrombin arc on CIE of patient’s plasma (top well); a prothrombin arc of normal pooled plasma (bottom well). On this and subsequent CIE figures the control plasma arc shown was obtained by electrophoresis performed simultaneously with the patient’s plasma. (C) The broad, low prothrombin arc, with a peak persisting at the origin, seen on CIE of a 1:1 mixture of patient C’s plasma and control plasma (top well); control plasma (bottom well).

ately reduced. The abnormalities seen on CIE in patients with anti-prothrombin antibodies varied. Whereas a broad low arc, with a peak persisting at the origin, was found in some patients (Fig 2), most patients had a less striking abnormality consisting of a trailing shoulder of the prothrombin arc (Fig 3). When plasma from a patient containing high titer prothrombin antibodies and no residual prothrombin antigen was diluted in increasing amounts of normal plasma, the resulting prothrombin arcs on CIE were converted from a broad low arc to a progressively more subtle trailing shoulder (Fig 4). When purified prothrombin was added to the plasma shown in Fig 3 to approximately double its prothrombin concentration, the trailing shoulder of the prothrombin arc increased substantially in prominence (data not shown).

As reported by Edson et al,8 evidence that prothrombin/prothrombin antibody complexes were responsible for abnormal arcs could be obtained by demonstrating that an abnormal arc reverted nearly to normal after plasma was adsorbed with Staphylococcal protein A (Fig 3). Moreover, adsorbing plasma with an excess of prothrombin antigen, insolubilized on agarose beads, caused abnormal arcs to revert nearly to normal (Fig 5).

Prevalence of anti-prothrombin antibodies. Thirty-one of the 42 patients in this series (74%) had evidence of anti-prothrombin antibodies (Table 1). These included all seven of the patients with systemic lupus erythematosus and seven of the ten patients in whom the lupus anticoagulant appeared related to the use of a drug. All but one of 25 patients whose APTT exceeded 50 seconds had demonstrable prothrombin/prothrombin antibody complexes (Table 2).

Relations between prothrombin time test results, measurements of prothrombin activity, antigen and antibodies, and evidence of clinical bleeding. The prothrombin time test is normal or only minimally prolonged in most patients with the lupus anticoagulant;1 in 27 of our 42 patients the test result was within two seconds of a control value. In these 27 patients, levels of prothrombin antigen and specific prothrombin coagulant activity exceeded 55% of our pooled plasma reference standard. Nevertheless, 17 of the 27 patients had a trailing shoulder of the prothrombin arc on CIE.

The prothrombin times of the remaining 15 patients exceeded a control value by more than two seconds. An abnormal prothrombin arc was found in all but a single patient. In ten of the patients the prothrombin time was only moderately prolonged (<16.5 seconds). All ten had levels of prothrombin antigen and activity above 40%, and none had bled abnormally because of impaired systemic hemostasis.

Data from five patients whose prothrombin times exceeded 16.5 seconds are summarized in Table 3. In three patients, the long prothrombin time was associated with severe hypoprothrombinemia, with essentially unmeasurable plasma prothrombin antigen (Fig 1) and minimal prothrombin coagulant activity. In the other two patients, prothrombin antigen levels were normal (Fig 2) despite the long prothrombin time. Values for prothrombin coagulant activity were low when the test was carried out at the 1:5 or 1:10
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Fig 3. An example (patient Hu) of the more subtle "trailing shoulder" abnormality (△) of the prothrombin arc seen on CIE of plasma from many patients with the lupus anticoagulant. Its disappearance after adsorption of the plasma with Staphylococcal protein A is also shown. (A) Patient's plasma before adsorption (top well); control plasma (bottom well). (B) Patient's plasma after adsorption (top well); control plasma (bottom well). The reduced height of the arc of the patient's plasma after adsorption reflects both loss of prothrombin/prothrombin antibody complexes and dilution of prothrombin by the buffer of the Staphylococcal protein A suspension.

dilution of patient's plasma usually used in the assay. However, values were normal when the dilution of the patient's plasma used in the assay was increased from 1 to 20, which eliminated error due to carry over of lupus anticoagulant in the patient's plasma into the final reaction mixture of the assay. Thus, in these two patients a prolonged prothrombin time was not associated with evidence of hypoprothrombinaemia but appeared to stem from an effect of a potent lupus anticoagulant upon the prothrombin time test.

It is important to note that all three patients in whom a markedly prolonged prothrombin time was associated with true severe hypoprothrombinaemia had bled abnormally. In contrast, the two patients in whom a markedly prolonged prothrombin time reflected an effect of the lupus anticoagulant upon the prothrombin time test but whose plasma prothrombin levels were normal, had not bled abnormally.

Figure 6 is a log/log plot of percent prothrombin activity against percent prothrombin antigen in all 42 patients studied. No difference was apparent in the relation of prothrombin activity to antigen between those patients with and those patients without demonstrable anti-prothrombin antibodies. This provides added evidence that anti-prothrombin antibodies associated with the lupus anticoagulant are non-neutralizing antibodies.

Evidence of antibody polyspecificity. The prolonged clotting time of lupus anticoagulant plasma in various clotting tests, including a dilute RVV test, stems from antibodies that react with anionic phospholipids. When it became clear that plasma from most patients with the lupus anticoagulant contained antibodies that bind prothrombin, the investigators asked the question: can the antibodies binding prothrombin also lengthen the clotting time of a dilute RVV test system?

Plasma samples from three patients were adsorbed three times with insolubilized prothrombin. Since the plasma of one patient (patient G of Table 3) had no measurable prothrombin antigen, the plasma was mixed with an equal volume of normal plasma before study. The markedly abnormal prothrombin arc of this mixture is shown in Fig 5 A. The log-log plot of the dilute RVV clotting time obtained when
increasing dilutions of the mixture were added to normal plasma is depicted in Fig 7. After three adsorptions, the mixture gave a nearly normal prothrombin arc on CIE (Fig 5B) and was less effective in prolonging the RVV clotting time than was a 1:64 dilution of the mixture before adsorption (Fig 7). Adsorption with control agarose beads coated with glycine or an unrelated protein such as factor X, did not reduce the ability of the mixture to prolong the RVV clotting time.

Adsorption with insolubilized prothrombin of plasma from the other two patients also markedly reduced both the abnormality on CIE and the ability of the plasma to lengthen the dilute RVV clotting time of normal substrate plasma. In one patient, the following RVV clotting times were obtained: before adsorption of the patient’s plasma, 130 seconds; after prothrombin adsorption, 88 seconds; after control adsorption, 145 seconds. In the second patient (patient Hu of Fig 3) the corresponding values were: before adsorption, 132 seconds; after prothrombin adsorption, 89 seconds; after control adsorption, 118 seconds.

It is important to note that plasmas were adsorbed three times to obtain the above results. When CIE was performed after each of the three adsorptions, the prothrombin arc shifted progressively towards normal following each adsorption. A single adsorption only partially removed lupus anticoagulant activity from the plasma. For example, patient Hu’s plasma prolonged the RVV time to 131 seconds before adsorption and to 117 seconds after a single adsorption.

Antibody was eluted from the prothrombin beads used to adsorb plasma from patient Hu. Eluate fractions containing IgG, as demonstrated by immunodiffusion against rabbit anti-human IgG, prolonged the dilute RVV clotting time of normal plasma (Table 4). These eluate fractions, when added to normal plasma, also caused a subtle but definite shoulder to appear on the prothrombin arc obtained by CIE. Eluates lacking measurable IgG neither prolonged the RVV clotting time nor gave rise to an abnormal prothrombin arc. Eluates from control experiments, in which patient Hu plasma was adsorbed with glycine-coated agarose beads and normal plasma was adsorbed with insolubilized prothrombin, contained no measurable IgG and failed to affect either the RVV clotting time or the prothrombin arc of normal plasma.

Although the investigators’ prothrombin preparation contained no measurable phosphate, and was presumed not to be contaminated with phospholipid, they performed a further experiment in which plasma from two patients was adsorbed with either control insolubilized prothrombin or prothrombin that had been washed two times with ether to assure that it was lipid free before being insolubilized. Washing with ether did not prevent insolubilized prothrombin from binding IgG with lupus anticoagulant activity. For example, when eluate fractions from the ether-washed insolubilized prothrombin used to adsorb plasma from patient Hu were tested for lupus anticoagulant activity in the RVV clotting system, the following times were obtained: fraction preceding the IgG peak, 62 seconds; the IgG peak, 112 seconds; and fraction following the IgG peak, 63 seconds.

Additional experiments were carried out in which plasma from two patients (patient Hu and patient H of Table 3) was adsorbed with barium citrate, a material that depletes plasma of vitamin K-dependent clotting factors. It was reasoned that adsorption with barium citrate would remove only those antibodies from the plasma that were bound to prothrombin. No prothrombin antigen was detected by electroimmunoassay in the plasmas after barium citrate adsorption. Each plasma, when added undiluted to normal substrate plasma, gave the following coincidentally identical clotting times in the RVV test system before and after adsorption of the patient’s plasma with barium citrate, ie, 115 seconds. CIE of prothrombin, eluted from the barium plasma,
citrate pellets, gave abnormal prothrombin arcs similar to but less striking than the abnormal arcs of the plasmas before adsorption with barium citrate.

**DISCUSSION**

The abnormal prothrombin arcs on CIE of lupus anticoagulant plasmas described herein can be accepted as evidence of prothrombin/prothrombin antibody complexes in plasma for the following reasons: 1) When lupus anticoagulant plasma lacking prothrombin (and therefore having no prothrombin arc) was mixed with an equal part of normal plasma, CIE yielded a highly abnormal, broad, low prothrombin arc with a persisting peak at the origin, (Fig 4, A and B). As the concentration of patient's plasma in the mixture was reduced, the abnormality in the prothrombin arc became less prominent and was manifest as a more subtle trailing shoulder (Fig 4, C and D). 2) As first demonstrated by Edson et al, adsorption with Staphylococcal protein A caused an abnormal prothrombin arc to revert to an essentially normal arc (Fig 3B). This reflects removal of prothrombin/prothrombin antibody complexes by the binding of Fc sites on IgG antibodies in the complexes to Staphylococcal protein A. 3) Repeated adsorption with excess insolubilized prothrombin caused abnormal prothrombin arcs to revert nearly to normal (Fig 5). This presumably reflects dissociation of prothrombin/prothrombin antibody complexes in the presence of excess prothrombin antigen and binding of the antibodies to the insolubilized antigen.

Therefore, finding abnormal prothrombin arcs in most patients with the lupus anticoagulant, including 24 of 25 patients with an APTT of 50 seconds or longer, means that most patients have demonstrable circulating anti-prothrombin antibodies. The detection of anti-prothrombin antibodies in only seven of 17 patients with a less prolonged APTT could reflect insensitivity of the CIE technique to the presence of a low concentration of prothrombin/prothrombin antibody complexes in plasma with weaker lupus anticoagulant activity.

Our current understanding of the clinical significance of anti-prothrombin antibodies in patients with the lupus anticoagulant may be summarized as follows. Because they are non-neutralizing, anti-prothrombin antibodies, even when present in apparent high titer (eg, patient H of Fig 2 and Table 3), will not cause abnormal bleeding. Only when the plasma is depleted of prothrombin, presumably as a result of an accelerated clearance of prothrombin/prothrombin antibody complexes, will clinical bleeding be encountered. A markedly prolonged prothrombin time in a patient with the lupus anticoagulant alerts one to this possibility, but in occasional patients (eg, patients H and N of Table 3) the potent anti-phospholipid activity of a high titer lupus anticoagulant may lengthen the prothrombin time. Therefore, both prothrombin activity and antigen should be measured specifically in the patient with a long prothrombin time to establish the presence or absence of hypoprothrombinemia. If only

**Table 3. A Summary of Data From Five Patients With Markedly Prolonged Prothrombin Times**

<table>
<thead>
<tr>
<th>Patient</th>
<th>APTT (sec)</th>
<th>Prothrombin Time (sec)</th>
<th>Prothrombin Antigen (%)</th>
<th>Prothrombin Activity (%)</th>
<th>Bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>70/32*</td>
<td>16.5/10.5</td>
<td>&lt;5</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>G</td>
<td>75/24</td>
<td>29/13</td>
<td>&lt;5</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>89/27</td>
<td>23/12</td>
<td>&lt;5</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>H</td>
<td>66/26</td>
<td>28/12</td>
<td>94</td>
<td>8</td>
<td>80</td>
</tr>
<tr>
<td>N</td>
<td>89/26</td>
<td>23/12</td>
<td>94</td>
<td>15</td>
<td>96</td>
</tr>
</tbody>
</table>

* Numerator is patient result, denominator is control result.
† The dilution at which the patient's plasma was assayed.

![Fig 6](image_url) Fig 6. Log/log plot of prothrombin antigen vs prothrombin activity in 42 patients with the lupus anticoagulant. The symbol (●) identifies patients with an abnormal prothrombin arc on CIE. The symbol (○) identifies patients with a normal prothrombin arc on CIE. A larger symbol has been used for emphasis for three patients with markedly depressed prothrombin activity and antigen. Their antigen levels, which were less than 5% prothrombin antigen, have been arbitrarily plotted as 2%.

![Fig 7](image_url) Fig 7. A reference curve prepared by plotting log clotting time against log dilution of lupus anticoagulant plasma (patient C) added to the normal plasma substrate of a dilute RVV test system. Also shown are clotting times obtained on adding a 1:1 mixture of normal plasma and patient's plasma that had been adsorbed with either insolubilized prothrombin (△) or control agarose beads (▲). The initial percentages shown on the abscissa represent a 1:1 dilution of the lupus anticoagulant plasma (patient G. Table 3) in normal plasma for the 50% value and further dilutions of this 50% mixture in isotonic saline for the lower percentages.
specific prothrombin activity is measured, then finding a low level should be accepted as evidence of hypoprothrombinemia only after further dilution of the patient's plasma in the assay has ruled out inaccuracy due to carry over of anti-phospholipid activity (patients H and N in Table 3).

The investigators do not know why an occasional patient may appear to have large amounts of prothrombin/prothrombin antibody complexes in the plasma (patient H of Fig 2 and Table 3) yet fail to develop hypoprothrombinemia. The rapid rise of prothrombin levels in patients with acute hypoprothrombinemia after treatment with adrenal corticosteroids suggests that the functional activity of the mononuclear phagocytic system in clearing such complexes could be an important variable.

Lupus anticoagulants may have immunological specificity for several anionic phospholipids: phosphatidylserine, cardiolipin, phosphatidylinositol, and phosphatidic acid. The high prevalence, which we have herein confirmed, of associated anti-prothrombin antibodies suggests that polyreactivity extends beyond anionic phospholipids to include epitopes on prothrombin.

Two pieces of evidence support this hypothesis. The first was obtained from a comparison of the effect of adsorption with barium citrate and adsorption with insolubilized prothrombin upon the lupus anticoagulant activity of plasmas containing anti-prothrombin antibodies. Adsorption with barium citrate removed all prothrombin from the plasmas, including prothrombin complexed to anti-prothrombin antibodies. Yet the lupus anticoagulant activity of the plasmas, as measured by their ability to prolong the dilute RVV clotting time of a normal plasma substrate, was unaffected. The investigators conclude that the free lupus anticoagulant antibodies of lupus anticoagulant plasma, ie, the antibodies not complexed with prothrombin, can lengthen the clotting time of a dilute RVV assay system. In contrast to barium citrate adsorption, adsorption of plasmas with insolubilized prothrombin caused the near disappearance of both demonstrable prothrombin/prothrombin antibodies on CIE (Fig 5) and the ability of the plasmas to prolong the RVV clotting time (Fig 7). When interpreted with the results of the barium citrate experiment, this represents strong indirect evidence that excess insolubilized prothrombin can bind and remove from plasma free lupus anticoagulant antibodies that prolong the RVV clotting time. Although the prothrombin used for these experiments contained no measurable phospholipid, we also repeated the experiment with ether-washed prothrombin to assure that the insolubilized prothrombin added to lupus anticoagulant plasmas was lipid free. Nevertheless, one can not rule out the possibility that lipids reacted with the insolubilized prothrombin after it was added to the plasma to give rise to a prothrombin/lipid epitope.

The second piece of evidence was obtained from testing eluates of insolubilized prothrombin used to adsorb plasmas containing anti-prothrombin antibodies. Eluate fractions containing IgG prolonged the dilute RVV clotting time of normal plasma (Table 4). This is direct evidence that antibodies in lupus anticoagulant plasma that bind to prothrombin can also lengthen the dilute RVV clotting time.

One should note that in at least some patients, such as patient Hu, the binding affinity for prothrombin of the lupus anticoagulant antibodies must be low. One can conclude by comparing the appearance of the CIE arc before and after adsorption of the plasma of patient Hu with Staphylococcal protein A (Fig 3, A and B) that most of the plasma prothrombin was free prothrombin not bound to IgG. One can further conclude from the barium citrate adsorption study that the majority of the antibodies with lupus anticoagulant activity were not bound to prothrombin. Nevertheless, essentially all antibodies with lupus anticoagulant activity could be removed from the plasma by repeated adsorption with insolubilized prothrombin. When eluted from the prothrombin, these antibodies exhibited lupus anticoagulant activity. Taken as a whole, these findings suggest that the binding affinity of the lupus anticoagulant antibodies for prothrombin was low enough in patient Hu's plasma to permit the existence of both free antibodies and free prothrombin at the concentrations of prothrombin antigen and of lupus anticoagulant antibodies present in the plasma.

The epitopes on prothrombin to which the antibodies bind are unknown. In preliminary experiments the investigators were unable to demonstrate binding of antibodies in lupus anticoagulant plasma to purified prothrombin by a Western blotting technique. This suggests that denaturation of prothrombin by SDS disrupts essential discontinuous epitopes that are dependent upon the tertiary structure of the molecule. However, the intact prothrombin molecule is not required since Bajaj et al have shown that anti-prothrombin antibodies in patients with the acute hypoprothrombinemia-lupus anticoagulant syndrome may react with prothrombin cleavage products containing only the COOH-terminal segment of the molecule.

Although the data provide strong initial evidence that immunoglobulins responsible for the lupus anticoagulant phenomenon exhibit polyreactivity for epitopes on anionic phospholipids and prothrombin, further work is needed to confirm this conclusion. We must establish that antibodies eluted from prothrombin interfere with the coagulant function of anionic phospholipid in reaction mixtures independent of prothrombin, eg, in reaction mixtures in which anionic phospholipid functions as a cofactor for factor Xa-catalyzed activation of factor VII. Moreover, we must establish that antibodies eluted from prothrombin can bind to anionic phospholipids. Such studies are in progress.

### Table 4. Properties of Eluates From a Prothrombin Adsorption Study

<table>
<thead>
<tr>
<th>Elution Fraction No.</th>
<th>RVVT* (sec)</th>
<th>A_{597}</th>
<th>Presence of IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>76.0</td>
<td>0.028</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>123.0</td>
<td>0.750</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>108.2</td>
<td>0.700</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
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*Absorbance at 280 nm as a measure of protein concentration.
†Detected by immunodiffusion against rabbit anti-human lgG.
ACKNOWLEDGMENT

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REFERENCES

Anti-prothrombin antibodies and the lupus anticoagulant

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