HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

Procoagulant Effect of Anti–β2-Glycoprotein I Antibodies With Lupus Anticoagulant Activity

By V. Pengo, T. Brocco, A. Biasiol, P. Rampazzo, P. Carraro, and R. Zamarchi

Prothrombin time (PT) is routinely used to monitor oral anticoagulant treatment in patients with the antiphospholipid antibody syndrome (APS). The fact that PT is a phospholipid (PL)-dependent coagulation test raises the possibility that lupus anticoagulant (LA) might interfere with this test, thus complicating the control of anticoagulant treatment. The effect of 6 affinity-purified preparations of anti-β2-glycoprotein I (β2-GPI) antibodies with LA activity on PT was tested. Instead of prolonging PT as expected, the β2-GPI antibodies reduced the PT of both normal plasma and anticoagulated plasma by a mean of 2.4 seconds and 5.6 seconds, respectively. This effect was also observed using other 5 commercially available preparations of thromboplastin. The β2-GPI-mediated reduction in PT was dose-dependent and was lost upon removal of β2-GPI. The failure of β2-GPI antibodies to express LA activity in PT was found to depend on the fact that calcium ions were added together with PL at the beginning of the assay. In fact, modification of the standard diluted Russell viper venom time (dRVVT) test by adding calcium ions together with PL resulted in a loss of β2-GPI antibody anticoagulant activity. The procoagulant effect was not as evident in an assay that used stimulated monocytes as a source of thromboplastin. These results show that β2-GPI antibodies exhibit an ‘in vitro’ procoagulant effect in PT and an anticoagulant effect in dRVVT only when the interaction with their antigen and PL occurs in the absence of calcium ions.

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fied aβ2-GPI antibodies and control IgG were tested after a 1:4 dilution in aβ2-GPI IgG ELISA as previously described.22

Preparation of β2-GPI–depleted plasma. Pooled normal plasma was passed through a CL-ocyt1 Sepharose column as previously reported.22 To estimate the quantity of residual β2-GPI in the flow through, 24-µL samples (diluted 1:2 in 20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4 [TBS]) were applied to 4.0-mm wells of a 1% agarose gel containing 6% vol/vol polyclonal aβ2-GPI rabbit antisera, and subjected to single radial immunodiffusion. Purified β2-GPI was serially diluted with TBS, pH 7.4, and analyzed in parallel to provide a calibration curve. Results of this analysis showed that β2-GPI was no longer detectable in the depleted plasma.

Coagulation studies. Pooled plasma from 5 healthy subjects and pooled plasma from 5 patients on stable oral anticoagulant treatment were used as coagulation substrates. Clotting times were recorded using an automated fibrometer (Mechrolab Clot-timer Model 202A; Heller Laboratories, Santa Rosa, CA). LA activity of purified aβ2-GPI IgG preparations was evaluated by dRVVT as previously described.22

Standard PT was assayed by preincubating 100 µL of plasma at 37°C for 2 minutes, followed by the addition of 200 µL of calcium thromboplastin and measurement of clotting time. To introduce 100 µL of aβ2-GPI IgG into the test without altering the proportions between reagents, tissue calcium thromboplastin was reconstituted with one-half volume of the appropriate diluting agent. Distilled water was used for the thromboplastin preparations Innovin (Dade, Miami, FL), Thromborel S (Istituto Behring, L’Aquila, Italy), Thromboplastin IS (Baxter Diagnost-ic Division, Milan, Italy), and IL-LS (Instrumentation Laboratories, Milan, Italy). Recombiplastin (Ortho Clinical Diagnostics, Milan, Italy) was reconstituted with 30 mmol/L CaCl2. The final reaction mixture contained 100 µL of pooled plasma, 100 µL of either TBS or normal pooled or aβ2GPI IgG in Tris buffer, and 100 µL of concentrated calcium thromboplastin. In experiments performed to test the importance of the sequence of reagent addition, tissue thromboplastin was incubated with aβ2-GPI antibodies in the presence or absence of calcium ions for 2 minutes before addition of anticoagulated plasma. In some experiments, the reconstituted Recombiplastin was further diluted with 30 mmol/L CaCl2.

A modified dRVVT was performed by changing the sequence of added reagents. In the dRVVT used to measure the LA activity of purified antibodies, the addition sequence was (1) normal plasma; (2) TBS, normal IgG, or affinity-purified aβ2-GPI IgG; (3) viper venom; (4) PL, followed by incubation at 37°C for 30 seconds; and (5) CaCl2, followed by measurement of clotting time. In a modified test the sequence was (1), (2), and (3), followed by the addition of CaCl2 and PL and measurement of clotting time. A second modified dRVVT was performed by incubating (2), (3), (4), and without (5) (calcium ions) for 30 seconds followed by initiation of the clotting reaction by adding (1) (normal pooled plasma). Some experiments were performed by using a range of CaCl2 concentrations (3 mmol/L to 7 mmol/L).

Induction of tissue factor–like activity in monocytes. Peripheral blood mononuclear cells (PBMCs) were obtained as described elsewhere.23 Briefly, blood was collected in heparin from a healthy staff volunteer. PBMCs were isolated by Ficoll-Hypaque gradient centrifugation (Pharmacia LKB Biotechnology Inc, Piscataway, NJ) and washed twice with RPMI. PBMCs were then resuspended in RPMI-15% fetal calf serum (FCS) and incubated in a tissue-culture flask (Falcon) for 1 hour at 37°C in a humidified, 5% CO2 atmosphere. Nonadherent cells were then discarded, and adherent cells were collected by scraping and incubated overnight in RPMI-10% FCS. This method resulted in a 3-fold enrichment in CD14+ mononuclear cells, as evaluated by cytfluorimetry after staining with an anti-CD14 antibody. The cells were resuspended at a concentration of 1 × 107 cells/mL, incubated for 6 hours with 10 µg/mL lipopolysaccharide (LPS) to stimulate mono-ocytes, and then centrifuged and resuspended in TBS containing 30 mmol/L CaCl2. PT was measured by adding 50 µL of the stimulated monocyte/CaCl2 preparation to 50 µL of normal pooled plasma that had been preincubated with 50 µL of TBS or aβ2-GPI IgG for 2 minutes at 37°C.

Patients. Six patients (2 males and 4 females, 40 to 58 years of age) with APS were studied; 4 patients had primary APS, and 2 patients had the syndrome associated with systemic lupus erythematosus. All patients tested positive for anticardiolipin IgG and LA activity. Two patients also had low titers of anticardiolipin IgM. Qualifying thrombotic events were venous thromboembolism in 3 patients and arterial thromboembolism in the remaining 3 cases (1 had acute myocardial infarction and 2 had acute arterial insufficiency in the lower limbs). All patients tested positive for aβ2-GPI IgG.

RESULTS

Affinity-purified preparations of aβ2-GPI IgG antibodies were obtained from plasma samples of 6 patients with APS. All 6 preparations showed LA activity in dRVVT, with prolongation of clotting time ranging from 3.7 to 8.3 seconds over control IgG. All 6 preparations also showed marked positivity in aβ2-GPI IgG ELISA, with the OD405 ranging from 2.587 to 3.320 for patient no. 5 to 3.492 for patient no. 1 (Table 1). These preparations were then tested by PT (see Materials and Methods) with the thromboplastin routinely used in our laboratory (Recomiplastin) and pooled normal plasma as a substrate. Comparison of results obtained with normal IgG showed that all of the aβ2-GPI antibody preparations slightly shortened the PT, with the reduction ranging from 1.1 seconds in patient no. 5 to 2.9 seconds in patient no. 2 (mean, 2.4 seconds) (Fig 1). As shown in Fig 2, this procoagulant effect was also observed in assays performed using pooled plasma from 5 patients on stable long-term anticoagulant treatment (International Normalized Ratio [INR] of pooled plasma was 2.28). Compared with normal IgG, the aβ2-GPI antibody preparations shortened the PT of the anticoagulated pool by a mean of 5.6 seconds (from a minimum of 4.5 seconds in patient no. 5 to a maximum of 6.7 seconds in patient no. 6). Results were comparable when using different pools of normal or anticoagulated plasma (data not shown).

We next performed additional PT assays to test the influence of the tissue thromboplastin preparations and the sequence of reagent addition on the observed reduction in clotting time. To limit experimental variability, these assays were performed

### Table 1. Anticoagulant and ELISA Activities of Affinity-Purified Anti-β2-GPI Antibody Preparations

<table>
<thead>
<tr>
<th>Protein concentration (µg/mL)</th>
<th>Normal IgG</th>
<th>No. 1</th>
<th>No. 2</th>
<th>No. 3</th>
<th>No. 4</th>
<th>No. 5</th>
<th>No. 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 5</td>
<td>100</td>
<td>46</td>
<td>41</td>
<td>51</td>
<td>30</td>
<td>54</td>
<td>50</td>
</tr>
<tr>
<td>dRVVT (seconds prolongation over buffer)</td>
<td>0.0</td>
<td>6.7</td>
<td>3.7</td>
<td>4.2</td>
<td>3.9</td>
<td>8.3</td>
<td>4.9</td>
</tr>
<tr>
<td>Anti-β2-GPI IgG ELISA (OD405)</td>
<td>0.035</td>
<td>3.492</td>
<td>3.056</td>
<td>3.026</td>
<td>3.121</td>
<td>3.287</td>
<td>3.320</td>
</tr>
</tbody>
</table>
Table 2 summarizes results of PT performed using 4 additional preparations of tissue thromboplastin (ie, Innovin, Thromborel S, Thromboplastin IS, and IL-HS) and either α2-GPI preparation no. 1A (52 µg/mL) or normal IgG (100 µg/mL). The α2-GPI IgG antibodies shortened the PT regardless of the thromboplastin preparation used, yielding a mean reduction of 2.1 and 10.1 seconds using normal and anticoagulated plasma, respectively. The reduction in PT was particularly evident in tests performed using anticoagulated plasma and either Thromborel S or Thromboplastin IS (9.8 and 19.3 seconds, respectively).

In the standard PT assay, plasma was preincubated at 37°C for 2 minutes before the addition of calcium-thromboplastin and measurement of clotting time. Results of modified assays in which α2-GPI preparation (no. 1B) and thromboplastin were incubated in the absence (−Ca) or presence (+Ca) of calcium ions for 2 minutes before the addition of anticoagulated plasma showed that the sequence of reagent addition did not influence the procoagulating effects of the antibodies with PT reduced by 9.2 seconds in standard assay, and by 7.6 and 10.1 seconds in the −Ca and +Ca assays, respectively.

Results of PT assays performed using a pool of anticoagulated plasma (INR = 3.0) and increasing amounts of a α2-GPI preparation no. 1B showed that the reduction in PT was dose-dependent (Fig 3). This IgG preparation shorted PT by 3.2, 6.7, 7.5, 9 seconds when added at a concentration of 5.8, 11.6, 23.2, and 46.5 µg/mL, respectively. The undiluted preparation (93 µg/mL) shortened the PT by 9.2 seconds (ie, from 47.7 seconds using normal IgG to 38.5 seconds).

To ascertain the role of the target antigen, β2-GPI, in α2-GPI–mediated PT shortening, PT assays were performed using plasma that had been depleted of β2-GPI by affinity chromatography (see Materials and Methods). Results showed that β2-GPI–depleted plasma exhibited a substantial loss in the ability to shorten PT (ie, from a 9.2-second reduction to a 1.5-second reduction), indicating that the procoagulant effect of α2-GPI antibodies is dependent on the presence of their antigen. Moreover, in PT performed by incubating pooled anticoagulated plasma with PL and recombinant-tissue factor (r-TF) for 2 minutes before adding calcium ions, α2-GPI IgG did not exhibit procoagulant effect (ie, 45.8 seconds for α2-GPI preparation no. 1B v 47 seconds for normal IgG). Therefore, procoagulant activity of α2-GPI IgG is not detectable in PT when plasma is incubated with PL in the absence of calcium ions.

Previous results had shown that the use of diluted tissue
thromboplastin in TTI yielded comparable PTs in the presence or absence of αβ2-GPI IgG with LA activity. This prompted us to test whether diluting thromboplastin would affect the procoagulant activity of αβ2-GPI IgG. As shown in Fig 4, dilution of Recombiplastin progressively abolished the αβ2-GPI–mediated reduction in PT of both normal and anticoagulated plasma. Therefore, the observed procoagulant effect also depend on the PL/r-TF concentration.

As both dRVVT and PT depend on PL, we reasoned that the distinct abilities of these tests to disclose LA might be influenced by the sequence in which the reagents are mixed. One major difference between the PT and dRVVT protocols is that calcium ions are added together with PL in the former, while they are added after PL in the latter. In fact, modification of the dRVVT protocol by concomitant addition of calcium ions and anionic PL (as in PT) resulted in normal clotting times (Fig 5). The same effect was observed when dRVVT was performed by incubating all reagents for 30 seconds before adding normal pooled plasma. In fact, while affinity-purified αβ2-GPI antibody preparation no. 1B prolonged conventional dRVVT by 10.3 seconds, it showed no prolongation when normal pooled plasma was added last. The same behavior, ie, no prolongation, was observed when preparation no. 1B was used and calcium ions and normal pooled plasma were added after 30 seconds of incubation of the other reagents. Moreover, an increase in final concentration of calcium from 3 mmol/L to 7 mmol/L completely abolished the LA activity of αβ2-GPI antibodies (data not shown). These observations showed that detection of αβ2-GPI LA in dRVVT assay requires the incubation of plasma with PL in the absence of calcium ions.

Finally, we tested whether this procoagulant effect could be mediated by monocytes, cells known to express tissue factor activity on their surface when provided with specific stimuli. As shown in Table 3, compared with nonactivated monocytes, activated monocytes shortened the clotting time of normal plasma from 220 to 42.6 seconds in the presence of Tris buffer and from 217 to 43.3 seconds in the presence of normal IgG; αβ2-GPI IgG preparations nos. 4 and 6 exhibited a modest procoagulant effect (ie, reduction in clotting time by 3 seconds and 2.4 seconds, respectively) in the presence of activated monocytes.

**DISCUSSION**

LA is a strong risk factor for thrombosis, and its association with either arterial or venous thrombosis defines the APS syndrome. Most patients with this condition are treated with oral anticoagulants and undergo PT-INR monitoring, the internationally accepted method used to assess oral anticoagulant treatment. It has been reported that the prolongation of PT in patients with LA reflects both the effect of warfarin and the ‘in vitro’ anticoagulant effect of their autoantibodies; therefore, PT-INR values might not accurately reflect the true level of anticoagulation. The present study was undertaken to elucidate the effect of αβ2-GPI LA, a common LA in patients with APS syndrome, on PT. Results showed that, instead of prolonging PT, these LA accelerate coagulation of both normal plasma and plasma from patients on oral anticoagulant treat-

<table>
<thead>
<tr>
<th>PT (s) (normal plasma)</th>
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<tbody>
<tr>
<td>Buffer + nonactivated monocytes</td>
</tr>
<tr>
<td>Normal IgG + nonactivated monocytes</td>
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<tr>
<td>Buffer + activated monocytes</td>
</tr>
<tr>
<td>Normal IgG + activated monocytes</td>
</tr>
<tr>
<td>αβ2-GPI IgG no. 1</td>
</tr>
<tr>
<td>αβ2-GPI IgG no. 2</td>
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<tr>
<td>αβ2-GPI IgG no. 3</td>
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<tr>
<td>αβ2-GPI IgG no. 4</td>
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<tr>
<td>αβ2-GPI IgG no. 5</td>
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<tr>
<td>αβ2-GPI IgG no. 6</td>
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ment. This explains the observation that (1) PT is seldom influenced in the presence of LA; (2) PT with Simplastin is not affected by monoclonal antibodies against β2-GPI; and (3) the TTI is not influenced by αβ2-GPI LA.

The mechanism by which αβ2-GPI antibodies shorten PT (with possible implications between this phenomenon and the occurrence of thromboembolic events) is difficult to explain. Our data show that αβ2-GPI-mediated PT shortening exhibits the following characteristics: (1) it is dose-dependent; (2) it requires the presence of the plasma antigen, β2-GPI; (3) it is not dependent on the type of thromboplastin; (4) it disappears upon dilution of TF and the template PL through which these antibodies recognize their antigen; and (5) it is abolished when calcium ions are added last to initiate clotting. Therefore, αβ2-GPI, β2-GPI, and PL in the presence of calcium ions accelerate thrombin formation when the activator of coagulation is TF.

Some investigators suggest that αβ2-GPI antibodies determine a PL-dependent reduction in the activity of tissue factor pathway inhibitor (TFPI).

...The ‘in vitro’ anticoagulant effect of αβ2-GPI antibodies is in agreement with the observation that binding of αβ2-GPI/β2-GPI complexes to PL membranes severely impairs the absorption or hinders the lateral mobility and activation of clotting factors.

...It was recently shown that increasing ionic strength or hinders the lateral mobility and activation of clotting factors.

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