IgG antibodies that recognize epitope Gly40-Arg43 in domain I of β₂-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis

Bas de Laat, Ronald H. W. M. Derksen, Rolf T. Urbanus, and Philip G. de Groot

Anti–β₂–glycoprotein I antibodies are known to have a heterogeneous reactivity against β₂–glycoprotein I. We performed this study to characterize the epitope on β₂–glycoprotein I to which pathologic anti–β₂–glycoprotein I antibodies are directed. Plasma samples from 198 patients with various systemic autoimmune diseases were tested for the presence of lupus anticoagulant and anti–β₂–glycoprotein I immunoglobulin G (IgG) antibodies. The reactivity of the anti–β₂–glycoprotein I–positive samples was further tested by coating recombinant full-length β₂–glycoprotein I and 8 deletion mutants of β₂–glycoprotein I onto hydrophilic and hydrophobic enzyme-linked immunosorbent assay (ELISA) plates. Full-length β₂–glycoprotein I with point mutations in domain I at positions 8, 40, and 43 were used in inhibition experiments. Fifty-two patients with anti–β₂–glycoprotein I IgG antibodies could be divided into 2 patterns. Type A antibodies only recognize domain I when coated onto hydrophobic plates; they do not recognize domain I coated onto hydrophilic plates. Type B antibodies have heterogeneous reactivity for all domains. Type A antibodies recognize the epitope around amino acids Gly40-Arg43 and cause lupus anticoagulant activity. In contrast to type B antibodies, those of type A strongly correlated with thrombosis. In conclusion, antibodies directed at domain I (epitope comprising Gly40 and Arg43) have lupus anticoagulant activity and strongly associate with thrombosis. (Blood. 2005;105:1540-1545)

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Introduction

The antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of circulating antiphospholipid antibodies (aPLs), notably anticardiolipin antibodies (aCLs) and antibodies that prolong phospholipid coagulation tests (lupus anticoagulant [LAC]) in patients with thrombosis and/or well-defined pregnancy morbidity. Initially it was thought that aPLs bind directly to negatively charged phospholipids. However, it is now generally accepted that the clinically relevant aPLs bind to proteins with affinity for phospholipids. Probably the most important epitope of APS-related aPL resides on β₂–glycoprotein I (β₂GPI). Most authorities do agree that anti-β₂GPI antibodies of the immunoglobulin G (IgG) class are more related to clinical symptoms like thrombosis than IgM-class antibodies.

β₂GPI is a 50-kDa glycoprotein that is present in plasma at a concentration of approximately 200 μg/mL. The protein is a member of the complement control proteins (CCPs). β₂GPI consists of 5 CCP repeats in which the first 4 domains are regular repeats consisting of 60 amino acids. The fifth domain is different from the other 4 domains as it consists of 82 amino acids, 4 conserved hydrophobic amino acids (313-3136), 3 disulfide bonds instead of 2, and a cluster of positively charged amino acids (282-287), which is responsible for binding of β₂GPI to phospholipids.

To understand the pathophysiology of APS, it is necessary to know the domains involved in anti-β₂GPI antibody binding and to know whether pathologic antibodies are all directed toward the same epitope. There is substantial controversy on the specificity of anti-β₂GPI antibodies. Some groups claim that the epitope for binding of anti-β₂GPI antibodies is located on domain IV. Other studies showed that binding of anti-β₂GPI antibodies to β₂GPI was abrogated when there was a clip at 316-317 in domain V, suggesting that domain V is involved. Arvieux et al found that anti-β₂GPI antibodies inhibit the binding of β₂GPI to cardiolipin, suggesting again that domain V harbors the epitope for binding of anti-β₂GPI antibodies. Blank et al found that anti-β₂GPI antibodies react with peptides that cover sequences present in domains I, II, III, and IV. Most of these groups used murine monoclonal antibodies or a restricted number of affinity-purified patient anti-β₂GPI antibodies for their studies. Interestingly, studies using only anti-β₂GPI antibodies purified from patients concluded that domain I is the major domain of β₂GPI involved in the binding of anti-β₂GPI antibodies.

Recently it was suggested that the positively charged epitope R39-R43 on domain I is important in binding of anti-β₂GPI antibodies. Here we further investigate that suggestion by testing the specificity of anti-β₂GPI antibodies in patients with systemic lupus erythematosus, lupuslike disease, and primary antiphospholipid syndrome by using deletion mutants of β₂GPI, recombinant full-length β₂GPI, point-mutated recombinant full-length β₂GPI, and both hydrophilic and hydrophobic enzyme-linked immunosorbent assay (ELISA)

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plates. Findings were related to the presence or absence of a history of thrombosis and anti-β₂GPI-dependent LAC activity.

Patients, materials, and methods

Patients

Included in this study was 198 patients with a diagnosis of systemic lupus erythematosus (SLE; n = 176), lupuslike disease (LLD; n = 16), and primary antiphospholipid syndrome (PAPS; n = 6) that were seen consecutively at the lupus clinic of the University Medical Center, Utrecht, the Netherlands. Patients with SLE meet at least 4 American College of Rheumatology (ACR) criteria for the classification of SLE, and patients with LLD meet at least 1 to 3 of these criteria. Patients with PAPS have aPLs and a history of thrombosis in absence of other signs for a systemic autoimmune disease. By chart review, the number of objectively verified thromboembolic events was recorded for each patient. Computed tomographic scanning or magnetic resonance imaging were used to diagnose a patient having thrombosis of intracerebral vessels. Typical electrocardiographic features and an elevated-fraction creatinine kinase muscle and brain (MB) diagnosed myocardial infarction. Periperal arterial thrombosis and thrombosis of the distal aorta was diagnosed by arteriography or thrombectomy at surgery. Retinal thrombosis was documented by fundoscopy and fluorescence angiography. Deep vein thrombosis was diagnosed by ultrasonography or venography, pulmonary embolism by radionuclide lung scanning, and portal vein thrombosis by angiography. The Institutional Review Board of the University Medical Center Utrecht approved this study, and informed consent was obtained from all patients.

Blood samples

Blood samples were taken at an arbitrary visit of the patients to the outpatient department and were collected by venipuncture using plastic tubes containing 3.8% trisodium citrate (0.129 M) as the anticoagulant (9:1; vol/vol). To obtain platelet-poor plasma, the samples were centrifuged twice at 2000 g for 10 minutes and subsequently stored at −50°C until use.

Serologic assays to measure antiphospholipid antibodies

All patients were measured with 2 LAC assays: an activated partial thromboplastin time (APTT)—like assay (PTT-LA) and a dilute Russell’s Viper Venom time (dRVVT). The APTT (PTT-LA; Diagnostica Stago, Gennevilliers, France) was performed as follows: 50 μL patient plasma was diluted 1:1 with normal pool plasma of 40 healthy volunteers and incubated with 50 μL APTT reagent (Diagnostica Stago). Coagulation was initiated by the addition of 50 μL CaCl₂ (25 mM). As a control, samples were tested in an APTT with actin-FS before.23 Nine IgG/IgM calibrators were used to report aCL levels as GPL positive if 1 of the 2 LAC assays was positive.

LAC screen/LAC confirm was greater than 1.20. A patient was considered LAC positive when the ratio APTT-LA/APTT-FS was greater than 1.20. 

Mutants of β₂GPI. By a generous gift of Dr Iverson of La Jolla Pharmaceutical Company, we obtained recombinant full-length β₂GPI (DI-V); 8 deletion mutants of β₂GPI (comprising domain I [DI]; domains I and II [DI-II]; domains I, II, and III [DI-III]; domains I, II, III, and IV [DI-IV]; domains II, III, IV, and V [DI-V]; domains III, IV, and V [III-V]; domains IV and V [DIV-V]; and domain V [DV]); and 3 recombinant β₂GPI molecules with different point mutations in domain I (aspartate-alanine mutation at position 8 [D8A], glycine-glutamate mutation at position 40 [G40E], and an arginine-glutamine mutation at position 43 [R43G]).21

Anti-β₂GPI IgG ELISA with β₂GPI deletion mutants. To determine the IgG reactivity against β₂GPI deletion mutants we used both 96-well hydrophilic high-binding ELISA plates (9102; Costar) and hydrophobic ELISA plates (2595; Costar). The plates were coated with deletion mutants in a concentration (10 μg/mL) that allows the use of the maximum binding capacity of the wells (1 hour at 37°C). The plates were washed 4 times with TBS/0.1% Tween and subsequently blocked with 4% BSA/TBS solution. Patient plasma (diluted 1:50 in blocking solution) was added to the wells (50 μL/well) and incubated for 1 hour at 37°C. The plates were washed 4 times (0.1% Tween/TBS) and incubated with alkaline-phosphatase–labeled goat antihuman IgG antibodies (Abs; diluted 1:1000; Biosource) for 1 hour at 37°C. Staining was performed by using PnPP (Sigma, St Louis, MO) at a concentration of 0.6 mg/mL diluted in diethynolamine (DEA) buffer. Adding 2.4 M/L NaOH to the wells after 10 minutes at room temperature stopped the reaction. Absorption was measured at 405 nm.

Extinctions obtained with normal pooled plasma were subtracted from those with patient plasma.

Inhibition experiments with point-mutated β₂GPI coupled to Sepharose beads. Recombinant β₂GPI and 3 different point-mutated forms of β₂GPI were covalently coupled to chelating Sepharose beads by the following procedure. Chelating Sepharose beads (Pharmacia, Uppsala, Sweden) were centrifuged for 15 seconds at 10 000g followed by removal of ethanol and washing of the beads with 4 volumes 50 mM EDTA (ethylenediaminetetraacetic acid)/H₂O. All washing procedures were done at room temperature by spinning down the beads at 57g. Subsequently beads were washed with 4 volumes H₂O, 4 volumes 50 mM CoCl₂/H₂O₂, 4 volumes H₂O₄, and 4 volumes PBS, followed by incubation for 15 minutes with point-mutated β₂GPI or full-length recombinant β₂GPI (2 volumes, 20 μM/mL) at room temperature during rotation. Then the beads were washed (with 6 volumes PBS) and incubated with 2 volumes 0.03% H₂O₂/PBS for 2 hours at room temperature during rotation, followed by washing with 4 volumes PBS, 1 volume 50 mM EDTA, 1 volume PBS, 1 volume 0.5 M imidazole/PBS, and 1 volume PBS. Blocking of aspecific binding places was done by incubation of beads with 4% BSA/PBS for 1 hour at room temperature during rotation.

Purified patient IgG was incubated for 1 hour at room temperature during rotation with Sepharose beads alone and Sepharose beads to which recombinant β₂GPI or mutated β₂GPI–Sepharose beads was coupled. Then the mixture was centrifuged for 1 minute at 57g. The supernatant was added to ELISA plates (Costar; high absorbing plates) coated with full-length recombinant β₂GPI and incubated for 1 hour at 37°C. After washing with 0.1% Tween-20/TBS five times, the plates were incubated with 50 μL/well alkaline-phosphatase–labeled goat antihuman IgG diluted 1:1000 in 4% BSA/PBS for 1 hour at 37°C. Then plates were washed 5 times with 0.1% Tween/TBS, and color development was performed by adding 100 μL/well PNPP at a concentration of 6 mg/10 mL DEA buffer. After 20 minutes, addition of 50 μL/well 2.4 M/L NaOH stopped the coloring reaction. Absorption was measured at 405 nm. From extinctions with purified patient IgGs, extinctions obtained with normal pool IgG were subtracted.
Table 1. Example of a β2 GPI-dependent LAC and a β2 GPI-independent LAC

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Coagulation times</th>
<th>Ratios of coagulation time of patient to normal pool plasma</th>
<th>Differences in ratio between 25 μM CL and 0 μM CL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μM CL</td>
<td>25 μM CL</td>
<td>0 μM CL</td>
</tr>
<tr>
<td>Normal pool plasma</td>
<td>40.0</td>
<td>41.7</td>
<td>NA</td>
</tr>
<tr>
<td>Patient with β2 GPI-dependent LAC</td>
<td>106.3</td>
<td>88.8</td>
<td>2.66</td>
</tr>
<tr>
<td>Patient with β2 GPI-independent LAC</td>
<td>69.7</td>
<td>73.5</td>
<td>1.74</td>
</tr>
</tbody>
</table>

To discriminate between a β2 GPI-dependent LAC and a β2 GPI-independent LAC we added 25 μL PTT-LA reagent, 50 μL patient plasma (mixed 1:1 with normal pool plasma), and 25 μL of different concentrations cardiolipin (0, 25, 50, 100, 200 μM) to a KC-10 microcoagulometer (Amelung, Lemgo, Germany). After 3 minutes of incubation at 37°C, coagulation was initiated by the addition of 50 μL CaCl2 and clotting time was measured. A LAC was considered β2 GPI dependent when the ratio of coagulation times of patient plasma and normal pool plasma was decreased by at least 0.05 with addition of cardiolipin vesicles at a concentration of 25 μM cardiolipin vesicles. CL indicates cardiolipin; and NA, not applicable.

Anti-β2 GPI–specific LAC assay

To discriminate between a β2 GPI-dependent LAC and a β2 GPI-independent LAC we made use of an apTT-based clotting test (PTT-LA; Diagnostica Stago), as described before.25 In short, 25 μL PTT-LA reagent, 50 μL patient plasma that was mixed 1:1 with normal pool plasma (of 40 healthy volunteers), and 25 μL of different concentrations cardiolipin (0, 25, 50, 100, 200 μM) of cardiolipin vesicles diluted in TBS were mixed and added to a KC-10 microcoagulometer (Amelung, Lemgo, Germany). The cardiolipin vesicles were made as described before.26 After 3 minutes of incubation at 37°C, coagulation was initiated by the addition of 50 μL CaCl2 and clotting time was measured. A LAC was considered C1–C4 dependent when the ratio of coagulation times of patient plasma and normal pool plasma was decreased by at least 0.05 with addition of cardiolipin vesicles at a concentration of 25 μM cardiolipin vesicles.27 An example of a patient with a β2 GPI-dependent LAC and a patient with a β2 GPI-independent LAC is given in Table 1.

LAC assay with point-mutated β2 GPI

First, β2 GPI-deficient plasma was obtained by adding normal pool plasma (mixture of 40 healthy volunteers) to a Sepharose column coupled with 21B2 (a monoclonal murine anti-β2 GPI antibody; generous gift of Prof J. Arnout). After this procedure, β2 GPI could not be demonstrated anymore by chromatography. The β2 GPI-deficient plasma was reconstituted with either recombinant β2 GPI or mutated β2 GPI (R43G). Of this mixture, 75 μL was added to the KC-10 (Amelung) and incubated at 37°C. After 3 minutes of incubation, a dRVVT solution (Gradipore, Frenchs Forrest, Australia) was added and the coagulation time was measured.

Statistical analysis

Chi-square statistics was used to compare the prevalence of thrombosis with serologic findings. Odds ratios and 95% confidence intervals were calculated by binary logistic regression. Student t test or Mann-Whitney test was used to calculate differences between 2 groups. For these calculations we used SPSS (Chicago, IL). P values less than .05 were considered significant.

Table 2. Presence or absence of anticardiolipin antibodies (aCLs), lupus anticoagulant (LAC), and IgG class anti-β2 GPI antibodies in patients of the study population

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Total, n</th>
<th>Present, n</th>
<th>Absent, n</th>
<th>Present, n</th>
<th>Absent, n</th>
<th>Present, n</th>
<th>Absent, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>176</td>
<td>93</td>
<td>83</td>
<td>48</td>
<td>130</td>
<td>36</td>
<td>140</td>
</tr>
<tr>
<td>LLD</td>
<td>16</td>
<td>13</td>
<td>3</td>
<td>12</td>
<td>4</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>PAPS</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>198</td>
<td>112</td>
<td>86</td>
<td>63</td>
<td>135</td>
<td>52</td>
<td>146</td>
</tr>
</tbody>
</table>

SLE indicates systemic lupus erythematosus; LLD, lupuslike disease; and PAPS, primary antiphospholipid syndrome.

Results

Population

The median age of the 198 patients (180 female) was 33 years. In 60 (30%) of 198 patients there was a history of objectively verified thrombosis. Thirty-two patients had a history of arterial thrombosis (stroke, n = 19; myocardial infarction, n = 5; peripheral artery, n = 7; distal aorta, n = 2; retinal artery, n = 3) and 38 patients had a history of venous thrombosis (deep venous thrombosis, n = 30; pulmonary embolism, n = 19; thrombophlebitis, n = 7; portal vein thrombosis, n = 1). Anticardiolipin antibodies were present in 112 (57%) of 198 patients, LAC in 63 (32%) of 198 patients, and anti–β2 GPI IgG antibodies in 52 (26%) of 198 patients (Table 2). Table 3 shows the interassay variability as an indication for the quality of the assays used in this study.

Domain specificity of anti–β2 GPI IgG antibodies

To investigate the domain specificity of the anti–β2 GPI IgG antibodies that was found in 52 patients, separate wells of hydrophilic and hydrophobic ELISA plates were coated with 8 domain-deleted mutants of β2 GPI and full-length recombinant β2 GPI. It appeared that the 52 samples with anti–β2 GPI IgG antibodies could be divided into 2 types (designated A and B) based on the reactivity that these had (Figure 1). On a hydrophilic high-binding plate the samples of type A (n = 30) only recognized full-length recombinant β2 GPI, whereas those of type B (n = 22) recognized full-length recombinant β2 GPI, domains I and V, and also domains I and V extended with additional domains. When a hydrophobic plate was used, samples of type A recognized not only full-length recombinant β2 GPI (albeit the OD was lower) but also domain I. On a hydrophobic plate the reactivity of samples from type B was similar to that found with hydrophilic ELISA plates,
Table 3. Interassay variability of all assays used in this study

<table>
<thead>
<tr>
<th>Assay</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Coefficient of variation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>dRVVT, coagulation time, s</td>
<td>66.1</td>
<td>2.1</td>
<td>3.2</td>
</tr>
<tr>
<td>PTT-LA, coagulation time, s</td>
<td>55.1</td>
<td>1.1</td>
<td>2.0</td>
</tr>
<tr>
<td>(\beta_2)GPI-dependent LAC, difference in ratio between 25 (\mu)M CL and 0 (\mu)M CL*</td>
<td>0.28</td>
<td>0.03</td>
<td>10.7</td>
</tr>
<tr>
<td>aCL ELISA, GPL units</td>
<td>40.9</td>
<td>2.8</td>
<td>6.9</td>
</tr>
<tr>
<td>Anti-(\beta_2)GPI IgG ELISA, OD</td>
<td>0.220</td>
<td>0.025</td>
<td>11.5</td>
</tr>
<tr>
<td>Anti-domain I ELISA, OD</td>
<td>0.315</td>
<td>0.012</td>
<td>3.8</td>
</tr>
</tbody>
</table>

To ensure the quality of the assays used in this study, we determined the interassay variability. For all assays, we performed 10 runs on duplicate samples on different days. The interassay coefficient of variation (CV) is then calculated as follows: (standard deviation of the means of all 10 duplicates/mean of all 10 duplicates) \(\times 100 = \%\) CV.

*See Table 1.

albeit the ODs were lower. A typical example of the reactivity of samples of types A and B is presented in Figure 1.

**Fine specificity of anti-\(\beta_2\)GPI IgG antibodies**

We hypothesized that patients of group A recognize a positive epitope on domain I that is shielded off from recognition when coated to hydrophilic plates. To demonstrate the involvement of this positively charged epitope on domain I in recognition by type A anti-\(\beta_2\)GPI antibodies, we performed inhibition experiments. We coated Sepharose beads with native full-length recombinant \(\beta_2\)GPI or 1 of 3 point mutations (D8A, G40E, or R43G) of full-length recombinant \(\beta_2\)GPI, blocked the beads with BSA, and incubated them with IgG fractions isolated from plasma from 3 patients of type A. The supernatants were added to a hydrophilic plate that was coated with full-length recombinant \(\beta_2\)GPI. We found that in all 3 samples with type A anti-\(\beta_2\)GPI antibodies, the anti-\(\beta_2\)GPI activity could be fully absorbed by full-length recombinant \(\beta_2\)GPI and with D8A and only partially with G40E or R43G (Figure 2).

When we tested the affinity for \(\beta_2\)GPI in an anti-\(\beta_2\)GPI IgG-class ELISA in the presence of 1 M NaCl, 26 of 30 patients from group A recognized an epitope in the presence of 1 M NaCl (data not shown), indicating the recognition of a specific epitope rather than charge. Only 8 of 22 of the IgG of group B recognized \(\beta_2\)GPI in the presence of 1 M NaCl, indicating that the binding of those antibodies was of lower affinity (data not shown).

**Specific anti-\(\beta_2\)GPI reactivity and LAC**

Out of the 30 samples from type A, 28 (93%) were LAC positive. In 23 of 28 samples the LAC activity was dependent on \(\beta_2\)GPI (Table 4).

To give support to the association between anti-\(\beta_2\)GPI reactivity and \(\beta_2\)GPI-dependent LAC, we added anti-\(\beta_2\)GPI IgG isolated from plasma of 3 patients with type A reactivity to \(\beta_2\)GPI-depleted plasma that was reconstituted with either recombinant full-length \(\beta_2\)GPI or point-mutated \(\beta_2\)GPI (R43G). We observed an increase in coagulation time from 104.0 ± 3.4 (mean ± SEM, n = 4) seconds to 175.5 ± 32.4 (mean ± SEM, n = 4) seconds when plasma was reconstituted with 150 \(\mu\)g/mL recombinant \(\beta_2\)GPI and 100 \(\mu\)g/mL type A anti-\(\beta_2\)GPI IgG antibodies. However, little prolongation (104.0 ± 3.4 → 122.8 ± 17.3, mean ± SEM, n = 4) was seen when plasma was reconstituted with 150 \(\mu\)g/mL R43G and 100 \(\mu\)g/mL type A anti-\(\beta_2\)GPI IgG antibodies. This experiment is representative of 4 experiments performed in the same way.

**Correlation between thrombosis and presence of type A or type B anti-\(\beta_2\)GPI antibodies**

A history of thrombosis was present in 32 of 52 patients with IgG anti-\(\beta_2\)GPI antibodies, in 25 (83%) of 30 patients IgG anti-\(\beta_2\)GPI antibodies with type A reactivity, and in 7 (32%) of 22 patients with type B reactivity (Table 4). The odds ratios for thrombosis were 6.7

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**Figure 1. Recognition of domain-deleted mutants by anti-\(\beta_2\)GPI antibodies coated to different plates.** Typical recognition of domain-deleted mutants of \(\beta_2\)GPI and full-length \(\beta_2\)GPI coated onto hydrophilic or hydrophobic ELISA plates by a sample from a patient with type A (left) and type B (right) reactivity. ODs are displayed after subtraction of the OD obtained by pooled normal plasma. Error bars represent mean ± SEM of triplicate points.

**Figure 2. A mutation in domain I of \(\beta_2\)GPI at positions 43 and 40 abrogates binding of anti-\(\beta_2\)GPI antibodies.** A hydrophilic ELISA plate was coated with 10 \(\mu\)g/mL recombinant full-length \(\beta_2\)GPI. IgG (20 \(\mu\)g/mL) of 3 patients (A, B, C) was preincubated with Sepharose beads coated with full-length recombinant \(\beta_2\)GPI or 1 of 3 \(\beta_2\)GPI molecules with point mutations (D8A, G40E, or R43G) or only blocked with BSA. Supernatants were added to the plate and IgG binding was detected by an alkaline-phosphatase–labeled goat antihuman IgG antibody. The OD obtained with IgG incubated with BSA-blocked Sepharose beads was set at 100%. Binding of IgG anti-\(\beta_2\)GPI antibodies to \(\beta_2\)GPI coated onto a hydrophilic ELISA plate can be fully absorbed by full-length recombinant \(\beta_2\)GPI and D8A but only partially by R43G and G40E. Error bars represent mean ± SEM of triplicate points.
Table 4. Association between type A or type B anti-β2GPI antibodies and (β2GPI-dependent) LAC activity and thrombosis

<table>
<thead>
<tr>
<th></th>
<th>Total population, N = 198</th>
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<tbody>
<tr>
<td></td>
<td>Anti–β2GPI IgG present, n = 52</td>
</tr>
<tr>
<td>Type A, n = 30</td>
<td></td>
</tr>
<tr>
<td>Type B, n = 22</td>
<td></td>
</tr>
<tr>
<td>LAC, n = 63</td>
<td>28</td>
</tr>
<tr>
<td>β2GPI-dependent LAC, n = 25</td>
<td>23</td>
</tr>
<tr>
<td>Thrombosis, n = 60</td>
<td>25</td>
</tr>
<tr>
<td>Odds ratio for thrombosis</td>
<td>18.9</td>
</tr>
<tr>
<td>95% CI</td>
<td>5.3-6.8</td>
</tr>
</tbody>
</table>

Numbers are depicted as absolute number of patients. NA indicates not applicable.

(95% confidence interval [95% CI]: 13.5-3.4) for IgG anti-β2GPI antibodies, 18.9 (95% CI: 53.2-6.8) for type A reactivity, and 1.1 (95% CI: 2.8-0.4) for type B reactivity.

Anti–domain I ELISA for clinical practice

To enable differentiation between patients with IgG anti-β2GPI antibodies of type A and type B reactivity, one can coat domain I of β2GPI onto a hydrophilic and a hydrophobic ELISA plate. Plasma from patients with type A IgG anti-β2GPI antibodies do not recognize domain I on a hydrophilic plate but recognize domain I on a hydrophobic plate, whereas samples from patients with type B reactivity recognize domain I on both plates. A ratio of greater than 2 between the OD measured with the hydrophobic plate and the OD with the hydrophilic plate discriminates between type A and type B IgG anti-β2GPI antibodies. This ratio is an indication for the relative amount of IgG anti-β2GPI antibodies that recognize the positive charge on domain I. We determined a coefficient of variation of 3.7% (interassay variability) as an indication for the quality of this assay (Table 3). Figure 3 shows that within the group of patients with IgG anti-β2GPI antibodies with type A reactivity, the ratio in those with a thrombotic history is significantly higher than in patients without thrombosis ($P = 0.014$). Within the group of patients with IgG anti-β2GPI antibodies of type B reactivity, the ratios are low and there is no difference in ratio between patients with or without thrombosis.

Discussion

The identification of the domains of β2GPI that are involved in binding of anti-β2GPI antibodies has been the subject of many studies. The cumulative results show that each domain of β2GPI has been indicated as the location where anti-β2GPI antibodies may bind. A recent paper strongly suggests that for binding of anti-β2GPI antibodies purified from patient plasma, a positively charged epitope, spanning amino acids 40-43 on domain I of β2GPI, is very important. We tested this suggestion by studying the reactivity of 52 samples (52 patients) with anti-β2GPI IgG antibodies that were found within a group of 198 patients with SLE, LLD, or PAPS. The major conclusions from this study are as follows: (1) not all anti-β2GPI IgG antibodies have the same epitope specificity; (2) a substantial number of antibodies found in patients recognize an epitope around G40-R43 on domain I; (3) these latter antibodies seem to be the pathologic ones because their presence was highly correlated with a history of thrombosis; (4) the anti-β2GPI IgG antibodies against epitope G40-R43 in domain I are antibodies that cause LAC activity; and (5) anti–β2GPI IgG antibodies that do not recognize G40-R43 in domain I are not related to a history of thrombosis. For anti–β2GPI antibodies of the IgM class we did not find an increase in their association with thrombosis by using the anti–domain I ELISA. Based on these observations we have developed a simple assay to detect a subset of anti–β2GPI IgG antibodies that highly correlates with thrombosis. Although the proportion of patients with primary APS was very small in our population, previous studies found similar clinical and laboratory features for primary and secondary APS. It seems likely that this also accounts for this anti–domain I ELISA.

By coating 8 domain-deleted mutants of β2GPI and full-length recombinant β2GPI on both hydrophilic and hydrophobic ELISA plates we discriminated 2 different reactivity patterns (designated types A and B) for the anti-β2GPI antibodies. Samples with type A reactivity (present in 58% of anti-β2GPI antibody–positive samples) recognize full-length β2GPI but not deletion mutants when hydrophilic ELISA plates are used and a strong reactivity for domain I (and extensions of this domain) with use of hydrophobic ELISA plates (Figure 1). Samples designated type B do not seem to have a preferred binding site on β2GPI. With hydrophilic ELISA plates these antibodies react both with full-length β2GPI, domain I and V, and extensions of these. When hydrophobic ELISA plates are used, the pattern of reactivity against β2GPI and domain-deleted mutants is similar, albeit the ODs are much lower.

We hypothesized that our observation of the reactivity of type A antibodies is dependent on the type of ELISA plate used
and could indicate that type A anti-β2GPI antibodies are directed against the G40-R43 epitope in domain I because it is conceivable that interactions between the positive charge of this epitope and the negative charge of a hydrophilic ELISA plate may hamper interaction of antibodies with that epitope.21 We showed that the reactivity of type A anti-β2GPI antibodies against β2GPI can be fully absorbed by full-length recombinant β2GPI and full-length recombinant β2GPI mutated at position 8 (D8A). When we absorbed type A anti-β2GPI antibodies with full-length recombinant β2GPI that had point mutations at 1 amino acid within the epitope G40-R43, namely R40E or R43G, 35% to 40% of the reactivity against β2GPI remained (Figure 2). This strongly suggests that these amino acids are part of the epitope to which type A anti-β2GPI antibodies are directed. We previously reported that β2GPI-dependent LAC activity is a strong risk factor for thrombosis.22 We now demonstrated that 23 (92%) of 25 samples with β2GPI-dependent LAC activity contained type A anti-β2GPI antibodies and that presence of type A antibodies is a strong risk factor for thrombosis (Table 4). The finding that reconstitution of β2GPI-depleted plasma with full-length β2GPI and type A anti-β2GPI antibodies induces more prolongation of the clotting time than reconstitution with R43G and type A anti-β2GPI antibodies suggested that type A anti-β2GPI antibodies cause LAC activity. As a β2GPI-dependent LAC activity correlates very strongly with thrombosis (odds ratio, 42.3), it also supports the concept that pathologic antibodies are directed against the epitope G40-R43.

Collectively, type B antibodies directed to β2GPI are not correlated with a history of thrombotic complications. Whether this population contains clinical relevant subpopulations of antibodies needs further studies.

In conclusion, our study shows that anti-β2GPI antibodies react with different epitopes on β2GPI. However, a substantial number of patient samples with anti-β2GPI antibodies (in our series 58%) recognize an epitope on domain I, including positions 40 and 43 (type A antibodies). The strong correlation between such type A antibodies with histories of thrombosis and presence of β2GPI-dependent LAC activity strongly suggests that type A antibodies constitute a pathologic subset. Our observations form the basis for a simple assay (using domain I coated onto hydrophilic and hydrophobic ELISA plates) for the detection of type A anti-β2GPI antibodies.

References

IgG antibodies that recognize epitope Gly40-Arg43 in domain I of β2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis

Bas de Laat, Ronald H. W. M. Derksen, Rolf T. Urbanus and Philip G. de Groot