Differential Effects of Anti–β2-Glycoprotein I Antibodies on Endothelial Cells and on the Manifestations of Experimental Antiphospholipid Syndrome

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Background—The antiphospholipid syndrome (APS) entails a prothrombotic state associated with the presence of antiphospholipid antibodies (aCL). aCL were shown to promote endothelial cell and platelet activation and to induce an APS-like syndrome in mice when administered intravenously. Recent data suggest that aCL target the plasma cofactor β2-glycoprotein I (β2GPI) rather than negatively charged phospholipids. However, it has not been determined whether different epitope-specific anti-β2GPI antibodies obtained from one patient possess pathogenic properties.

Methods and Results—Three β2GPI-binding IgM monoclonal antibodies (mAbs) (ILA-1, ILA-3, and ILA-4) were cloned from a patient with APS. The three antibodies were shown to bind β2GPI immobilized on irradiated plates, yet only ILA-1 bound β2GPI coated onto nonirradiated plates. Furthermore, when using the anti-β2GPI enzyme-linked immunosorbent assay, ILA-1 was the only mAb inhibited by fluid phase β2GPI. ILA-1 and ILA-3, but not ILA-4, induced adherence of U937 cells to endothelial cells in vitro (reflecting activation of endothelial cells). mAbs ILA-1 and ILA-3 as opposed to ILA-4 induced significant expression of adhesion molecules when preincubated with human umbilical vein endothelial cells. Passive administration of ILA-1 and ILA-3 to pregnant BALB/c mice induced clinical findings consistent with APS (increased fetal resorptions, reduced platelet counts, and prolonged activated partial thromboplastin time), whereas both ILA-4 and the control human IgM did not produce similar effects.

Conclusions—The results of the study demonstrate the differential effects of various populations of anti-β2GPI antibodies on endothelial cell activation and on experimental APS. (Circulation. 1998;97:900-906.)

Key Words: endothelium • adhesion molecules • antibodies • immune system

The APS is characterized by a combination of clinical findings consisting of thromboembolic events, thrombocytopenia, and recurrent fetal loss in association with aCL.1 The syndrome can accompany additional disorders defining it as secondary or exist as an isolated primary entity.

The clinical manifestations of APS have been considered to result from the occurrence of antibodies against negatively charged PLs. However, various hypotheses have been raised as to the true target of these antibodies. It has been suggested that aCL target either negatively charged PLs, various plasma cofactors, or, alternatively, an epitope exposed on either these molecules (ie, PLs or plasma cofactors) on their mutual interaction.2–5 One of the conceivable targets of aCL appears to be a 50-kD plasma cofactor termed β2GPI.6 This glycoprotein, initially described by Schulze et al in 1961,7 is composed of five respective consensus ("sushi") repeats.6 β2GPI binds negatively charged PLs through a lysine-rich locus in the fifth domain9,10 and possesses several in vitro properties that define it as an anticoagulant (ie, inhibition of prothrombinase activity, ADP-induced platelet aggregation, or platelet factor IX production).6

Several recent studies suggest that β2GPI alters its density11 or exposes a cryptic epitope on its binding to PLs, which allows subsequent aCL binding.12,13 Emerging clinical studies14,15 reinforce these observations, thus confirming the specificity of "autoimmune" aCL to β2GPI to the point that some consider them to be anti-β2GPI antibodies.

The role of aCL in conferring the prothrombotic predisposition in APS has been strengthened by in vitro studies showing their platelet-16,17 and EC-18,19 activating properties. Additionally, it has been demonstrated that passive transfer20 or active immunization21 with aCL of naive mice led to the clinical manifestations of APS. aCL have also been shown to possess thrombogenic properties in mice.22,23 However, it has not been established whether antibodies with different epitope specificities (ie, targeting different loci of the β2GPI) are capable of promoting activation of ECs and induction of an APS-equivalent mouse model.

In the present study, we generated three IgM anti-β2GPI mAbs from a patient with primary APS. We provide a description of their binding characteristics and show that...
Selected Abbreviations and Acronyms

aCL  =  anti-cardiolipin antibodies  
APS  =  antiphospholipid syndrome  
apTT  =  activated partial thromboplastin time  
EC  =  endothelial cell  
ELISA  =  enzyme-linked immunosorbent assay  
β2GPI  =  β2-glycoprotein I  
HUVEC  =  human umbilical vein endothelial cell  
ICAM-1  =  intracellular adhesion molecule 1  
mAb  =  monoclonal antibody  
PL  =  phospholipid  
VCAM-1  =  vascular cell adhesion molecule 1

despite their various epitope specificities, two mAbs had pathogenic properties manifested by in vitro activation of cultured ECs. Infusion of these mAbs to naive mice resulted in the clinical manifestations of APS.

Methods

Patient
The patient was a 32-year-old woman with primary APS, diagnosed after the occurrence of a previous episode of deep vein thrombosis and two abortions. The patient’s serum was found to be positive for aCL (using the standard aCL ELISA) in high titers and for lupus anticoagulant.

Production of Human mAbs
Single-cell suspension of lymphocytes were derived from peripheral blood cells of a patient with primary APS. After a 5-day exposure to pokeweed mitogen (GIBCO Laboratory, Grand Island Biological Co), the lymphocytes were fused with the GM4672 human lymphoblastoid cell line according to a method described previously.24 After fusion, the cells were seeded onto 96-well tissue culture plates (Nunc–Immunol), with hypoxanthine aminopterin thymidine selection media for 4 to 5 weeks. The clones were screened for binding to β2GPI with the modified aCL ELISA. All clones were subjected four times to limiting-dilution cloning procedures in a regular medium. The hybridomas were grown in culture supernatant. The culture fluid was collected, and ammonium precipitation (50%) was performed. Dialyzed ammonium precipitate was loaded onto anti-HigM Sepharose columns (Pharmacia), and the immunoglobulins were eluted using 5 mol/L MgCl2 and dialyzed against PBS.

Detection of aCL
ELISA plates were coated with cardiolipin (Sigma Chemical) at a concentration of 50 μg/mL in ethanol. Plates (Nunc) were blocked with 1% gelatin in PBS, and serial dilutions of the mAbs were added for 2 hours. Bound antibodies were detected using 1:10,000 dilution of goat anti-human IgM conjugated to alkaline phosphatase (Jackson Immunoresearch) and the addition of its substrate p-nitrophenylphosphate. Absorbance was read at 405 nm in a Titertek ELISA reader (S.L.T. Laboratory Instruments).

Detection of mAb Reactivity by Modified aCL and Anti-β2GPI ELISA
β2GPI dependence of the binding of the mAbs to cardiolipin was assessed with a modified aCL ELISA. Cardiolipin coated onto ELISA plates were blocked with 1% gelatin in TBS and washed after 2 hours. Serial concentrations of β2GPI (0 to 100 μg/mL) with 10 μg/mL purified mAbs were added. The binding was detected as described above (aCL ELISA).

To detect reactivity of the mAbs with human mouse, or bovine sera (1% to 10% in TBS), followed by the addition of the mAbs (10 μg/mL), and the assay was performed as described above.

For the purpose of determining the binding to human β2GPI alone, high binding (γ-irradiated; Nunc Maxisorp) or nonirradiated (Nunc Polysorp) plates were coated with purified human β2GPI (10 μg/mL), the mAbs were added in different concentrations, and the ELISA was performed as described previously.14

Inhibition of Binding of Anti-β2GPI mAbs by β2GPI
The concentration was determined of mAbs giving 50% maximal binding to β2GPI-coated plates. Different concentrations of β2GPI were preincubated with each of the mAbs for 2 hours and placed on precoated high-binding β2GPI-coated plates (Nunc) overnight at 4°C. The assay was continued as described in the anti-β2GPI ELISA.

Detection of Anti–Endothelial Cell Antibodies
HUVECs were isolated as described previously and cultured under standard conditions.26 Cells were used at passage 1 to 2 and plated onto gelatin-coated 96–well plates. Cyto-ELISA was performed as detailed previously.26

Biotinylation of mAbs
The mAbs were dialyzed (100 μg/100 μL against 0.1 mol/L NaHCO3) overnight at 4°C. NHS (biotinylandacaprate n-hydroxy succinimide ester in 1 mL dimethylformamide; Sigma) biotin solution (2.5 μL/100 μL mAb) was added for 2 hours at room temperature and dialyzed against PBS.

Determination of β2GPI Epitopes Recognized by Different mAbs
To clarify whether the mAbs recognize similar epitopes on β2GPI, 50% of the binding of each of the mAbs onto β2GPI-coated plates was determined. Subsequently, each mAb and total immunoglobulins from the APS patient sera were biotinylated in a set of separate experiments. The purpose of the first experiment was to assess whether all the mAbs accounted for the total anti-β2GPI reactivity of the APS patient sera. Thus, a mixture of the nonbiotinylated mAbs (ILA-1 plus ILA-2 plus ILA-4; final concentration, 100 μg/mL) were used for competition with biotinylated total IgG. Subsequently, each of the nonbiotinylated mAbs (or control IgM) was used as competitors for binding in different concentrations (0 to 100 μg/mL) to the single biotinylated mAb (at concentration giving 50% maximal binding) in the anti-β2GPI ELISA. The binding was probed by streptavidin alkaline phosphatase (Jackson) and appropriate substrate. The percentage of inhibition was calculated as follows: % inhibition=absorbance control−absorbance with inhibitor/absorbance control×100.

Adherence of U937 Cells to Endothelial Cells in the Presence of mAbs
The assay was performed as described previously.27 Briefly, U937 (a monocyte/macrophage–like cell line) cells were pretreated with mouse anti-human anti–MHC class I mAb (PharMingen) for 30 minutes at 37°C (to block Fc receptor binding without affecting the activation of the ECs) and labeled with 0.5 μCi/mL [3H]thymidine (Amersham International) for 24 hours. Adhesion assays were performed on HUVEC monolayers that were preincubated with β2GPI, and mAbs were added overnight. The EC monolayers were washed extensively, and radiolabeled U937 cells were added to each well, in RPMI 1640 medium containing 0.2% BSA for 30 minutes at 37°C. The nonadherent cells were removed by washing, and the cells were lysed with formic acid. Radioactivity associated with adherence was quantified by β-scintillation spectroscopy. The results were expressed as percentage of added U937 cells that adhered and are presented as mean±SD from three to five replicate wells.
ELISA for Detecting Expression of Adhesion Molecule

HUVECs were grown onto 96-well plates, preincubated with mAbs (100 μg/mL), washed and fixed with 0.1% glutaraldehyde, and treated with PBS containing 0.2% Triton X-100 to permeabilize the cell membrane. Plates were blocked with 3% BSA and incubated with biotinylated mouse anti-human E-selectin, anti-human ICAM-1, or anti-human VCAM-1 (PharMingen) (1 μg/mL) for 1 hour. Cells were then exposed to streptavidin alkaline phosphatase (1:5000 dilution) and the appropriate substrate.

A separate set of experiments was designed to test the specificity of the effect of the mAbs on the expression of adhesion molecules. Accordingly, a cocktail of anti–ICAM-1, VCAM-1, and E-selectin mAbs (PharMingen) (20 μg/mL each) was added to the HUVECs after preincubation with each of the mAbs (25 μg/mL), and the assay was continued as described in the adhesion assays.

Induction of Murine Experimental APS

Mice were infused intravenously with 100 μg of each of the mAbs at day 0 (the day at which a vaginal plug was observed after mating) and day 7 of pregnancy. The mice were bled and killed on day 15 of pregnancy. Fetal resorptions, aPTT, and platelet counts (markers of the APS-equivalent in mice) were determined as described previously.

Statistical Analysis

In the solid phase assays and competition and inhibition experiments, the Student’s t test was used for comparison between the mAbs. The ANOVA test was used for comparison between the clinical parameters in the experimental groups.

Results

Binding of Human mAbs to β2GPI and Cardiolipin

Three mAbs were generated (ILA-1, ILA-3, and ILA-4), all of which were of the IgM isotype. The three mAbs bound β2GPI added to CL in a dose-dependent manner, yet no binding was evident to cardiolipin alone (Fig 1A). Binding to CL in the presence of human β2GPI (10 μg/mL) was as follows: ILA-3 absorbance, 1.302±0.104; ILA-1 absorbance, 1.008±0.098; ILA-4 absorbance, 1.005±0.118; and HIgM absorbance, 0.145±0.113. Binding of mAbs to sample blanks (the assay devoid of CL) was negligible (mean absorbance: ILA-1, 0.091; ILA-3, 0.084; and ILA-4, 0.095).

Higher binding of the mAbs to CL was evident in the presence of increasing concentrations of human, mouse, and bovine serum (Fig 1B, 1C, and 1D, respectively). Binding of all three mAbs to β2GPI-coated high-binding plates was observed in a dose-dependent manner compared with control human IgM (Fig 2A). However, when the antibodies were assayed on nonirradiated plates, dose-dependent binding to β2GPI was noted only with ILA-1 (Fig 2B).

Inhibitory Effect of Fluid Phase β2GPI on Binding of mAbs in Anti-β2GPI ELISA

Dose-dependent inhibition of binding of ILA-1, but not of ILA-3, ILA-4, or IgM, by fluid phase human β2GPI was
evident in three separate experiment using high-binding plates (Fig 3). The $K_d$ value of ILA-1 was estimated as $2.2 \times 10^{-7}$.

**Epitope Recognition by mAbs and Total Immunoglobulins for Binding to $\beta_2$GPI/CL**

The mixture of the three mAbs obtained from the APS patient partially inhibited the binding of biotinylated total immunoglobulins from the APS patient through the use of the anti-$\beta_2$GPI ELISA (Fig 4).

To assess whether the obtained mAbs were directed against different epitopes on $\beta_2$GPI, we biotinylated each mAb and performed competition assays with either of the three nonbiotinylated mAbs (ILA-1, ILA-3, and ILA-4). Binding of ILA-1 to $\beta_2$GPI was inhibited only by ILA-1, whereas no competition was achieved with ILA-3 and ILA-4 (ILA-1: 89\%±7\% compared with 5\%±1\% for ILA-3 and 7\%±2\% for ILA-4, at ILA-1 concentrations of 30 \mu g/mL). Binding of biotinylated ILA-3 was inhibited only by nonbiotinylated ILA-3 and not by ILA-1 or ILA-4 (ILA-3: 79\%±6\%, 8\%±2\% for ILA-1 and 6\%±1\% for ILA-4, at biotinylated ILA-3 concentrations of 30 \mu g/mL), thus suggesting that the three mAbs target different non-cross-reactive epitopes on the $\beta_2$GPI.

**Anticoagulant Activity of mAbs**

The anticoagulant activity of each of the anti-$\beta_2$GPI mAbs was measured by the aPTT as described previously and is shown in Fig 5.

**Binding of mAbs to HUVECs by Cyto-ELISA**

All three mAbs bound HUVECs through the use of a cyto-ELISA (Fig 6). No significant differences were noted between the mAbs with respect to the binding to HUVECs. No binding to HUVECs was evident when the ECs were incubated in a serum-free medium (devoid of $\beta_2$GPI) (Fig 6).
Figure 5. Anticoagulant activity of anti-β2GPI mAbs. aPTT was measured (in seconds) when mAbs (5 μg/mL) were added to a normal plasma using a mixing test with an equivalent dose of cephalin and incubation for 2 minutes at 37°C. Each point represents the mean±SD of three separate experiments.

Effect of mAbs on Adhesion of U937 Cells to HUVECs

The adhesion of monocytes to EC is considered a marker of EC activation. The percentage of adhesion expresses the portion of added U937 cells adhering the HUVECs (thus reflecting the percentage of EC coverage by the moncytic cells). The most significant adhesion of U937 cells to EC was accomplished by preincubation of the U937 cells with ILA-1 and then subjecting them to HUVECs. Adhesion to EC was dependent on the dose of the ILA-1 mAb achieving a maximal 79±6% (at ILA-1 concentration of 50 μg/mL) adhesion compared with control HlgM (3±2%) (P<.001) (Fig 7A). A similar, although less pronounced, dose-dependent effect on adhesion was evident by preincubation with ILA-3 (at a concentration of 50 μg/mL) reaching a 43±4% adhesion compared with control HlgM (3±2%) (P<.003). ILA-4 was not potent in affecting U937 adhesion, giving a negligible 13±2% compared with the control IgM inducing 3±2% adhesion (P<.5). Prior incubation with antibodies to ICAM-1, VCAM-1, and E-selectin using the same assays significantly decreased the effect of the ILA-3 but only partially that of ILA-1 on U937 adherence to ECs (Fig 7B).

Preincubation with heat-aggregated IgG to block Fc receptor interactions (expressed by U937 cells) ruled out the possibility that the effects on adhesion resulted from nonspecific Fc interactions of the mAbs with the ECs or leukocytes.

Effect of mAbs on Expression of Adhesion Molecules by HUVECs

The most pronounced effect on E-selectin expression by HUVECs was evident after their preincubation with ILA-3 (absorbance, 1.674±0.112) (Fig 8). Preincubation of the mAb ILA-1 with HUVECs also resulted in significant E-selectin expression (absorbance, 1.232±0.212), whereas ILA-4 and human IgM did not produce marked effects on E-selectin expression (absorbance, 0.457±0.078 for ILA-4 and 0.112±0.058 for human IgM; Fig 8). Expression of ICAM-1 was most impressive with ILA-3 (absorbance, 1.365±0.01) compared with ILA-1 (absorbance, 0.856±0.075), ILA-4 (absorbance, 0.507±0.075), and control IgM (absorbance, 0.111±0.068).

A similar pattern was noted with VCAM-1 expression, obtaining the most significant effect with ILA-3 (absorbance, 0.989±0.100) in comparison with ILA-1 (absorbance, 0.779±0.091), ILA-4 (0.377±0.068), and control IgM (0.092±0.012).

Induction of APS Manifestations in Naive Pregnant Mice by mAbs

Significant fetal loss was induced after passive intravenous administration of two of the three mAbs. The fetal resorption percentage was mostly pronounced with ILA-3 compared with ILA-1, ILA-4, and normal IgM (Table). Significantly reduced platelet counts were noted in the mice passively infused with ILA-1 and ILA-3 compared with ILA-4 and control human IgM (Table). ILA-1 and ILA-3 prolonged aPTT significantly compared with ILA-4 and human IgM.

Discussion

In the present study, we cloned three IgM mAbs that bound β2GPI attached to solid phase coated CL. The antibodies were obtained from a single primary APS patient with well-documented thrombotic episodes. The competition assays using the different mAbs show that a mixture of the IgM (containing all three mAbs) was responsible for only a fraction of anti-β2GPI activity in the serum of the patient. Thus, not all β2GPI-reactive antibodies from the patient serum were recovered. The three mAbs were found to target different domains on human β2GPI, evident by the competition assays. All mAbs bound β2GPI immobilized on high binding plates, yet only ILA-1 bound β2GPI coated onto nonirradiated plates. ILA-1 also was the only mAb inhibited by fluid phase human β2GPI. Thus, it appears that ILA-1 is directed to an epitope present on the native structure of β2GPI, whereas ILA-3 and ILA-4 recognize targets that are exposed on β2GPI after conformational changes (exposed by its binding to γ-irradiated plates).

Two of the three mAbs (ILA-1 and ILA-3) induced activation of HUVECs manifested by enhanced adhesion of U937 cells, which was associated with an elevated expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin). It should be mentioned in this respect that the activating effect on the EC was not directly correlated with the binding to HUVECs as measured by the ELISA, thus excluding the
possibility that mere binding of the mAbs to ECs is sufficient to elicit monocyte adhesion.

Several studies investigated the EC binding and activating properties of aCL. In a very recent work, Simantov et al used purified IgG aCL from patients with APS and demonstrated that these antibodies activated cultured HUVECs, as reflected by enhanced monocyte adherence to ECs and expression of adhesion molecules. The activation properties were found to depend on the presence of $\beta_2$GPI in the medium. However, it was not possible to conclude whether the EC-activating effect is contributed by a single monospecific population of antibodies or, alternatively, induced by different subgroups of aCL. More recently, Del-Papa et al showed that anti-$\beta_2$GPI antibodies from APS patients activated ECs provided $\beta_2$GPI was present in the medium (assayed by the expression of adhesion molecules, IL-6, and 6-keto-prostaglandin). However, the correlation of these interesting in vitro findings with the procoagulant state induced in animal models of APS has not been investigated. In this study, we used three mAbs produced from the same patient that had different binding targets on $\beta_2$GPI. Despite the differences in epitope binding, mAbs ILA-1 and ILA-3 were potent in promoting EC activation when added to a culture containing $\beta_2$GPI. The EC-activating properties of aCL were previously presumed to result from attachment of the antibodies to $\beta_2$GPI deposited on the EC surface after its preactivation and resultant exposure of negatively charged phosphatidylserine. This mechanism, however, remains largely speculative in view of the scarce evidence on loss of membrane asymmetry in EC. Regardless of the mechanism, our observations also show that all mAbs bound cardiolipin in the presence of mouse and bovine sera (presumably native $\beta_2$GPI), a finding that could support the role of endogenous $\beta_2$GPI-mediated activation of the EC in vivo.

Previously, we have shown that passive administration of monoclonal aCL as well as active immunization of naive BALB/c

Clinical Parameters in Pregnant Mice Infused With Different mAbs

<table>
<thead>
<tr>
<th>Tested mAb</th>
<th>ILA-1 (n=14)</th>
<th>ILA-3 (n=9)</th>
<th>ILA-4 (n=16)</th>
<th>HlgM (n=17)</th>
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<tbody>
<tr>
<td>Platelet count, cells/mm$^3$$\times10^{-3}$</td>
<td>453±10</td>
<td>389±118</td>
<td>1007±212</td>
<td>1218±217</td>
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<tr>
<td>aPTT, s</td>
<td></td>
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<tr>
<td>$P&lt;.008^\dagger$</td>
<td>$P&lt;.05^\dagger$</td>
<td>$P&gt;.5^\dagger$</td>
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<tr>
<td>Resorptions, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P&lt;.09^\dagger$</td>
<td>$P&lt;.05^\dagger$</td>
<td>$P&gt;.5^\dagger$</td>
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*Percent resorptions equals (Live fetuses minus resorbed fetuses)/live fetuses.
†Statistical analyses were performed with ANOVA. The groups of mice infused with ILA1 were compared with the group of mice infused with HlgM.
with these antibodies was capable of inducing experimental APS.20,21 The aCL-infused mice were found to develop thrombocytopenia, prolonged aPTT, and increased fetal resorptions after mating. These clinical parameters aided in establishing the primary pathogenicity of the antibodies. Moreover, antibodies produced by immunization with human β2GPI were found to induce APS in naïve BALB/c mice,25 thus supporting an important role of aCL in the pathogenesis of the syndrome.

In the present study, we used these parameters to assess the pathogenic potential of the three mAbs to sort out whether it could be attributed to a monospecific antibody. Herein, we show that two of the three mAbs produced from the same patient harbored pathogenic in vivo characteristics evidenced by the induction of a syndrome resembling human APS. The correlation between the pathogenic effects in the passive transfer experiments and the in vitro adherence assays was not complete. These findings could be explained by the multiple effects of aCL on hemostasis, including activation of platelets,16 induction of tissue factor,25 and so on. Furthermore, the effect of the mAbs on the EC seems to be multifactorial and probably is not restricted merely to expression of the three adhesion molecules that were evaluated. This observation is supported by the partial inhibitory effect of prior incubation with antibodies to adhesion molecules on the ILA-1–mediated EC-activation compared with the complete inhibition of the ILA-3 effect.

The results of the present study show for the first time that different anti-β2GPI antibodies possess both EC-activating properties and are capable of inducing a murine APS.

Acknowledgments

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References

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