Antiphospholipid Antibodies From Antiphospholipid Syndrome Patients Activate Endothelial Cells In Vitro and In Vivo

Silvia S. Pierangeli, PhD; Margaret Colden-Stanfield, PhD; Xiaowei Liu, MD; John H. Barker, MD; Gary L. Anderson, PhD; E. Nigel Harris, MD

Background—Antiphospholipid (aPL) antibodies are associated with thrombosis in patients diagnosed with antiphospholipid syndrome (APS) and enhance thrombus formation in vivo in mice, but the mechanism of thrombosis by aPL is not completely understood. Although aPL antibodies have been shown to inhibit protein C activation and activate endothelial cells (ECs) in vitro, no study has examined whether these antibodies activate ECs in vivo. Therefore, human affinity-purified aPL (ap aPL) antibodies from APS patients were tested in a mouse model of microcirculation using the cremaster muscle that allows direct microscopic examination of thrombus formation and adhesion of white blood cells (WBCs) to ECs as an indication of EC activation in vivo. Adhesion molecule expression on human umbilical vein endothelial cells (HUVECs) after aPL exposure was performed to confirm EC activation in vitro.

Methods and Results—All 6 ap aPL antibodies significantly increased the expression of VCAM-1 (2.3- to 4.4-fold), with one of the antibodies also increasing the expression of E-selectin (1.6-fold) on HUVECs in vitro. In the in vivo experiments, each ap aPL antibody except for 1 preparation increased WBC sticking (mean number of WBCs ranged from 22.7 to 50.6) compared with control (14.4), which correlated with enhanced thrombus formation (mean thrombus size ranged from 1098 to 6476 versus 594 μm² for control).

Conclusions—Activation of ECs by aPL antibodies in vivo may create a prothrombotic state on ECs, which may be the first pathophysiological event of thrombosis in APS. (Circulation. 1999;99:1997-2002.)

Key Words: antiphospholipid antibodies ■ thrombosis ■ endothelial cells ■ cell adhesion molecules

Antiphospholipid (aPL) antibodies are a heterogeneous group of antibodies detected in patients with antiphospholipid syndrome (APS), which is associated with thrombosis, pregnancy losses, and thrombocytopenia. 1–3 aPL antibodies isolated from patients with APS have been shown to enhance thrombus formation in mice,4–7 but the mechanism by which this occurs is not clearly understood. Investigators speculate that a prothrombotic state may be induced by aPL antibodies activating platelets8–10 or endothelial cells (ECs) or by inhibition of protein C activation.11–21 aPL antibodies may bind phospholipids or β₂-glycoprotein 1 (β₂GP1) in the membranes of ECs or platelets, resulting in their activation.8–10,16,22 EC activation by aPL antibodies has been demonstrated in vitro in several ways, including enhanced adhesion molecule expression and monocyte adherence22,23; however, no in vivo studies have shown such activation.

The present study used a unique microcirculation model in mice24,25 to demonstrate the thrombogenicity of aPL antibodies by activating ECs in vivo and correlated these findings with in vitro expression of adhesion molecules on the surface of aPL-exposed human umbilical vein EC (HUVEC) monolayers. These data provide evidence that EC activation may contribute to the hypercoagulable state in APS patients, thus predisposing these individuals to recurrent thrombosis.

Methods

Patients
Serum and plasma from 6 patients with various manifestations of APS1–3 were selected to participate in the study (Table 1). The patients signed a consent form approved by the Institutional Review Board Committee at the University of Louisville before donating blood specimens for the study. Anticardiolipin (aCL), anti-β₂GP1, and lupus anticoagulant (LA) activities were determined as previously described to confirm diagnosis of APS.26,27 Serum from normal, healthy individuals was pooled and used as controls.

Affinity Purification of aPL Antibodies
aPL antibodies from APS patients (ap IgG-APS) were affinity-purified by use of cardiolipin liposomes, elution with 1.5 mol/L NaI, and protein G Sepharose chromatography as previously de-
scribed\textsuperscript{28,29} to isolate the IgG fractions with confirmed aCL, anti-β₂GP1, and LA activities.\textsuperscript{30–32} IgG from normal, healthy individuals (IgG-NHS) was purified by protein G Sepharose. The sterile-filtered IgG fractions were determined to be free of endotoxin contamination by the limulus amoebocyte lysate assay (E-Toxate, Sigma Chemical Co.).\textsuperscript{33}

In Vitro Exposure of ECs to aPL Antibodies

Confluent monolayers of HUVECs (10⁴ cells/well) seeded in collagen-coated 96-well plates were incubated with complete MCDB107 culture medium, normal IgG (IgG-NHS; 100 μg/mL), or ap IgG-APS antibody (100 μg/mL) in Gey’s balanced salt solution for 4 hours at 37°C. As a positive control, some HUVEC monolayers were treated with lipopolysaccharide (LPS, 3 μg/mL) in complete MCDB107 for 4 hours to increase the surface expression of E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1). After paraformaldehyde fixation, adhesion molecule expression was assessed with a colorimetric ELISA described previously.\textsuperscript{34,35} Color development was stopped at 3 mol/L H₂SO₄ at 20 minutes, and the optical density was read at 492-nm wavelength on a SpectraMax 250 ELISA plate reader (Molecular Devices). The degree of specific antigen expression was calculated by subtracting nonspecific binding of the secondary antibody from all test values.

In another series of experiments to evaluate dose-dependence of the response, adhesion molecule expression was determined in HUVEC monolayers exposed to 2-fold serial dilutions of ap IgG-APS 6 (37.5 to 500 μg/mL) for 4 hours at 37°C.

In Vivo Exposure to aPL Antibodies

The ability of aPL antibodies to activate ECs in vivo and enhance thrombus formation was studied by examination of white blood cell (WBC) adhesion to endothelium in exposed cremaster muscle\textsuperscript{24,25} and study of the dynamics of thrombus formation in exposed femoral vein in the same mouse preparation.\textsuperscript{4–7} Briefly, CD1 mice (Charles River Breeding Laboratories; weight, 25 to 30 g) in groups of 9 were treated by intraperitoneal injection of ap IgG-APS preparation at time 0 and a second injection 48 hours later (500 μg/mL antibody per injection). Mice in a control group were treated by intraperitoneal injection of the same quantity of pooled normal IgG-NHS. aPL antibody levels were measured 72 hours after the first injection.\textsuperscript{4–7} In all cases, mice injected with ap IgG-APS preparations had levels >50 GPL units of aPL antibodies (data not shown). Animals were housed in the Center for Animal Resources at Morehouse School of Medicine, an approved facility, under the supervision of veterinarians and trained technicians.

For direct visualization of WBC adhesion in the microcirculation of the cremaster muscle, mice were anesthetized 72 hours after the first injection and the right cremaster muscle was exposed by a microsurgical technique as shown in Figure 1A.\textsuperscript{24,25,36} Briefly, animals were placed in the dorsal position on a specially designed Plexiglas observation platform with the hind legs straddled across a glass microscope slide. The scrotum was incised so that the cremaster muscle and testicle could be exposed. The sac-shaped cremaster muscle was splayed out and secured with 5-0 silk sutures onto the microscope slide for transillumination viewing. After a stabilization period of 30 minutes, the number of WBCs remaining stationary for a period of ≥30 seconds (“sticking”) within 5 different venules (diameter, 25 to 35 μm) was measured.

In another series of experiments to determine dose-dependence of the response, WBC sticking to ECs was determined in mice injected with different concentrations (100, 250, or 500 μg/mL) of ap IgG-APS from patient 6 at times 0 and 48 hours later. The surgical procedure was performed as described above at 72 hours after the first injection.

TABLE 1. Summary of Clinical Features of Patients Studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age, y</th>
<th>Thrombosis</th>
<th>Pregnancy Losses</th>
<th>Diagnosis</th>
<th>aCL, GPL U</th>
<th>Anti-β₂GP1, SGU</th>
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<tr>
<td>1</td>
<td>Male</td>
<td>42</td>
<td>Yes</td>
<td>N/A</td>
<td>APS</td>
<td>405.0</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>43</td>
<td>Yes</td>
<td>N/A</td>
<td>APS</td>
<td>723.6</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>38</td>
<td>No</td>
<td>Yes</td>
<td>APS</td>
<td>73.4</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>40</td>
<td>Yes</td>
<td>No</td>
<td>APS</td>
<td>286.1</td>
<td>+</td>
</tr>
<tr>
<td>5*</td>
<td>Male</td>
<td>39</td>
<td>No</td>
<td>Yes</td>
<td>APS</td>
<td>69.8</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>36</td>
<td>Yes</td>
<td>Yes</td>
<td>APS</td>
<td>83.3</td>
<td>+</td>
</tr>
</tbody>
</table>

N/A indicates not applicable; SGU, standard G units.

*Patient 5 was the son of a patient diagnosed with APS with no clinical symptoms of APS at the time of the study.

Figure 1. Cremaster muscle mouse model of microcirculation.
A, Close view under microscope of exposed cremaster muscle; B, photograph of WBC (1) adhesion to ECs (2) the fiber of mouse cremaster muscle (3) the lumen of a postcapillary venule (18-μm diameter).
Analysis of thrombus dynamics in a mouse model has been described previously. In brief, mice subjected to the previously described treatment were anesthetized 72 hours after the first anti-IgG-APS or IgG-NHS injection, and the right femoral vein was exposed and pinched with a pressure of 1500 g/mm² to induce the formation of a thrombus. Clot formation and dissolution in the transilluminated vein were visualized with a microscope equipped with a closed-circuit video system. Thrombus size (in mm²) was measured after the pinch injury by freezing the digitized image and tracing the outer margin of the thrombus; the times (in minutes) of formation (from appearance to maximum size) and disappearance (from maximum size to disappearance) of the thrombus were measured.

Data Analysis
An unpaired Student's t test was used to compare the means of thrombus sizes and times (formation, disappearance) and WBC adhesion numbers between groups. Statistical significance was achieved at P<0.05. Statistically significant differences on surface antigen expression of endothelial adhesion molecules on IgG-NHS– or ap IgG-APS–exposed HUVEC monolayers were evaluated by unpaired Student’s t test. Statistical significance was achieved at P≤0.05.

Results
Characterization of Patient Sera and Plasma
As indicated in Table 1, all 6 APS patient plasma samples had aCL and LA activities and were positive for anti-β₂GP1 antibodies.

Characterization of ap IgG-APS Fractions
All 6 ap IgG-APS preparations were positive for aCL activity (range, 69.7 to 289.1 GPL units) and anti-β₂GP1 activity (range, 35.2 to 68.5 SGU) and were positive for LA activity (Table 2).

Enhanced In Vitro Expression of EC Adhesion Molecules After aPL Antibody Exposure
Under unstimulated conditions, surface antigen expression of VCAM-1 and E-selectin on HUVEC monolayers was low (Figure 2A and 2B), whereas ICAM-1 was constitutively expressed (Figure 2C). LPS treatment of HUVEC monolayers for 4 hours increased expression of E-selectin (7.1-fold), VCAM-1 (3.6-fold), and ICAM-1 (3.5-fold). Although normal IgG-NHS did not alter the surface expression of the 3 adhesion molecules, incubation of the cells with ap IgG APS 1 to 6 for 4 hours increased VCAM-1 expression 2.3- to 4.4-fold (Figure 2A) in all but 1 patient (ap IgG-APS 4). The induction of VCAM-1 by ap IgG-APS antibodies ranged from 88% (ap IgG-APS 2) to 169% (ap IgG-APS 1) of the LPS-stimulated VCAM-1 expression. E-selectin expression was enhanced 3.8-fold by ap IgG-APS 6 (Figure 2B). No ap IgG-APS sample increased ICAM-1 expression over its constitutive expression (Figure 2C).

To assess whether this enhancing effect on ECs by aPL was dose-dependent, HUVEC monolayers were incubated with 2-fold serial dilutions of ap IgG-APS 6 (31.25 to 500 μg/mL), the preparation that increased both VCAM-1 and E-selectin expression. Exposure to ap IgG-APS 6 dose-dependently increased VCAM-1 and E-selectin, with concentrations of >125 μg/mL producing a >10-fold increase in

| TABLE 2. Characterization of the ap IgG-APS Preparations |
|-----------------|-----------------|-----------------|
| IgG-NHS         | aCL IgG, GPL U  | Anti-β₂GP1 IgG, SGU | LA |
| ap IgG-APS 1    | 98.5            | 54.6            | +  |
| ap IgG-APS 2    | 75.6            | 48.9            | +  |
| ap IgG-APS 3    | 73.3            | 25.6            | +  |
| ap IgG-APS 4    | 289.1           | 68.5            | +  |
| ap IgG-APS 5    | 69.7            | 45.8            | +  |
| ap IgG-APS 6    | 86.3            | 35.2            | +  |

The ap IgG-APS used in this study had aCL, anti-β₂GP1, and LA activity. IgG preparations tested at 100 μg/mL protein concentration. Cut-off value for anti-β₂GP1 is 20 SGU and for aCL, 10 GPL units.
TABLE 3. Dynamics of Thrombus Formation (Area and Time of Thrombus) in Mice Injected With ap IgG APS

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of Animals Tested†</th>
<th>Thrombus Area, μm² (mean±SD)</th>
<th>Thrombus Disappearance Time, min (mean±SD)</th>
<th>Total Time, min (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-NHS</td>
<td>9</td>
<td>594±370</td>
<td>2.5±0.5</td>
<td>3.9±0.7</td>
</tr>
<tr>
<td>ap IgG-APS (1)</td>
<td>9</td>
<td>1756±818*</td>
<td>4.9±1.1*</td>
<td>6.9±1.2*</td>
</tr>
<tr>
<td>ap IgG-APS (2)</td>
<td>8</td>
<td>2289±1542*</td>
<td>4.3±2.1*</td>
<td>6.4±2.7*</td>
</tr>
<tr>
<td>ap IgG-APS (3)</td>
<td>7</td>
<td>844±386</td>
<td>4.1±2.0*</td>
<td>5.8±1.9*</td>
</tr>
<tr>
<td>ap IgG-APS (4)</td>
<td>8</td>
<td>1098±261*</td>
<td>4.5±1.3*</td>
<td>6.3±1.5*</td>
</tr>
<tr>
<td>ap IgG-APS (5)</td>
<td>5</td>
<td>2405±625*</td>
<td>4.1±0.6*</td>
<td>5.7±0.6*</td>
</tr>
<tr>
<td>ap IgG-APS (6)</td>
<td>7</td>
<td>6476±597*</td>
<td>7.5±1.4*</td>
<td>9.1±1.5*</td>
</tr>
</tbody>
</table>

*Significantly different from IgG-NHS control group (P<0.05).
P values for thrombus sizes were 0.044, 0.01, 0.098, 0.02, 0.001, and 0.0001 for ap IgG-APS 1 to 6, respectively.
P values for thrombus disappearance were 0.03, 0.032, 0.04, 0.034, 0.025, and 0.001 for ap IgG-APS 1 to 6, respectively.
P values for total thrombus time were 0.025, 0.012, 0.016, 0.03, 0.04, and 0.002 for ap IgG-APS 1 to 6, respectively.
†Nine mice were included in each group. However, some mice died during surgical procedure.

VCAM-1 and E-selectin (data not shown). In addition, ICAM-1 expression on HUVEC monolayers after exposure to 500 μg/mL ap IgG-APS 6 was increased 4-fold.

Enhanced In Vivo Thrombus Formation After aPL Antibody Treatment
Five of the 6 ap IgG-APS preparations significantly enhanced thrombus size in mice compared with mice immunized with IgG-NHS (sample from patient 3 being the exception) (Table 3). All 6 ap APS samples delayed the disappearance of induced thrombi and total times compared with the IgG-NHS control group. It was noteworthy that ap IgG-APS purified from patient 5, who had no history of thrombosis, also enhanced thrombus size and delayed thrombus disappearance. Conversely, ap IgG-APS from patient 3, who had experienced a stroke, did not have enhanced thrombus formation (Table 3).

Enhanced In Vivo Leukocyte Adhesion After aPL Antibody Treatment
The 6 ap IgG-APS and IgG-NHS control samples were tested to determine whether WBC adhesion to ECs in the microcirculation was affected. As shown in Table 4, 5 of the 6 ap IgG-APS samples significantly increased WBC sticking to ECs compared with mice injected with IgG-NHS. It was noteworthy that sample 3, which was found not to enhance thrombus size in previous experiments, also did not enhance leukocyte sticking to endothelial cells (Figure 3).

When mice were injected with various concentrations of aPL antibodies from patient 6, a dose-dependent effect on WBC sticking to ECs was observed that correlated with an increase in the level of aCL antibodies present in the sera of the mice at the time of surgery (Figure 4) (high levels for group A: >80 GPL units; medium levels for group B: >20 and <80 GPL units; and low levels for group C: >10 and <20 GPL units).

Discussion
This study clearly demonstrates that 5 of the 6 ap IgG-APS antibodies from patients with diverse clinical features of APS increase adhesion of leukocytes to ECs in vivo, indicating activation of ECs. Furthermore, by use of an in vivo model of thrombosis, 5 of the 6 ap IgG-APS preparations enhanced thrombus formation, and all the 6 preparations delayed the time of thrombus disappearance significantly. This is in agreement with previously published work from our group that indicated that monoclonal and polyclonal aPL antibodies isolated from APS patients with thrombosis enhanced thrombus formation in vivo in a dose-dependent fashion.4–7,37,38 This study, using in vivo and vitro techniques, also provides evidence that the prothrombotic state induced in APS may be the result of aPL antibodies acting on the vascular endothelium.

Figure 3. Activation of endothelial cells in vivo by ap IgG-APS. In vivo leukocyte adhesion (sticking) to endothelial cells in microcirculation of mice injected with ap IgG-APS and in controls (IgG-NHS) was determined as described in Methods. *Significantly different from IgG-NHS control group (P<0.05). Nine mice were included in each group. However, some mice died during surgical procedure.

Figure 4. Activation of ECs in vivo by ap IgG-APS at different concentrations. ap IgG-APS from patient 6 was injected into mice as described in Methods in different doses to achieve different levels of aCL antibodies in animals (group A, high positive; group B, medium positive; and group C, low positive). Surgical procedure to determine number of WBCs adhering to endothelium was performed at 72 hours after first injection. Values are expressed as mean±SD. *Statistically significantly different from control (IgG-NHS) (P<0.05).
Several studies have investigated EC binding and activating properties of aPL antibodies. Del Papa et al.39,40 showed direct binding of aPL to ECs, a finding that could not be confirmed by McCrae and colleagues.40 These investigators concluded that binding to ECs may be mediated by β₂GP1.23 This study was not designed to determine how aPL antibodies interact with ECs but rather whether or not they activate ECs. Simantov et al.22 demonstrated that IgG fractions from APS patients activatedHUVECs, as reflected by the increased monocyte adherence to ECs and expression of adhesion molecules. Our studies confirm those of Simantov et al. and showed that aPL antibodies activate ECs, as evidenced by enhanced expression of adhesion molecule expression on HUVECs. This study also illustrates a varied enhanced expression of adhesion molecules by the 6 aPL preparations, suggesting a heterogeneity in the function of aPL antibodies. It is also possible that different aPL antibodies may activate various intracellular mechanisms that will lead to the upregulation of ≥1 adhesion molecules. In agreement with these findings, heterogeneity in function and specificity of aPL has been reported by other investigators.41

It is known that endotoxin (LPS) can induce EC activation by increasing cytokine production and adhesion molecule expression. The activation of ECs seen in this study could not be attributed to the presence of endotoxin, because the samples were tested to be free of endotoxin by the limulus amoebocyte lysate test.

The mechanism(s) by which aPL antibodies activate ECs is uncertain. Antibody–EC–mediated injury has been identified as 1 potential factor that may be involved in the pathogenesis of thrombosis in patients with APS. Vascular endothelium maintains the anticoagulant surface of blood vessels by constitutive expression of (1) thrombomodulin by activating protein C; (2) heparan sulfate by activating antithrombin III to accelerate thrombin inhibition; and (3) annexin V, which prevents binding of coagulation factors,42–43 as well as by (4) release of tissue factor pathway inhibitor, which blocks the activation of ECs by aPL observed in the in vitro experiments, because monocytes were absent in the tissue cultures. The interaction of aPL antibodies with ECs may occur via phospholipids, phospholipid-protein (β₂GP1) complexes, or other unknown protein receptors on the surface of ECs.42,39 Del Papa et al.39 showed that polyclonal anti-β₂GP1 antibodies from APS patients bound ECs through β₂GP1 to activate the cells, as evidenced by the increased expression of adhesion molecules, interleukin-6, and 6-keto-prostaglandin. The aPL antibody preparations selected for this study included antibodies to phospholipids and to anti-β₂GP1 antibodies.

In summary, this study provides the first evidence that aPL antibodies activate endothelium in vivo and that these effects correlate with thrombogenic effects in vitro. The data presented in this study suggest that aPL antibodies activate ECs to create a hypercoagulable state and that “thrombogenicity” of aPL antibodies may be directly related to this. In addition, this study provides new relevant information that may explain why APS patients are prone to recurrent thrombosis.

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**References**

2002

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