Increased Immunoglobulin Binding to Cerebral Endothelium in Patients With Antiphospholipid Antibodies

David C. Hess, MD; James C. Sheppard; Robert J. Adams, MD

Background and Purpose: There is a strong link between antiphospholipid antibodies and stroke. The mechanism of action of antiphospholipid antibodies is unknown. Most theories of pathogenesis center around platelet or endothelial cell dysfunction. Our aim was to determine if there were immunoglobulins in the sera of patients with antiphospholipid antibodies that bind human brain microvascular endothelial cells.

Methods: We studied sera from three groups of subjects: patients with antiphospholipid antibodies and stroke (group 1), healthy control subjects (group 2), and patients with stroke but without antiphospholipid antibodies (group 3). We isolated human brain microvascular endothelial cells from temporal lobectomy specimens and used a cellular enzyme-linked immunosorbent assay (ELISA) to measure immunoglobulin binding to endothelial cells derived from human brain and from human umbilical vein. We used a chromium release assay to measure cytotoxicity.

Results: Patients with antiphospholipid antibodies and stroke had significantly higher immunoglobulin binding to human brain microvascular endothelial cells than subjects in the other groups (ELISA index + standard deviation: 63±37 [group 1] versus 7±7 [group 2] versus 7±7 [group 3], P<.001). There was, however, poor correlation between binding to brain endothelial cells and binding to cardiolipin. The binding to brain microvascular cells was not specific to brain endothelium, as similar results were found in an ELISA using human umbilical vein cells. There was no evidence of complement-mediated brain endothelial cell cytotoxicity.

Conclusions: Patients with stroke and antiphospholipid antibodies frequently have human brain microvascular endothelial-reactive antibodies in their serum. These antibodies are distinct from those to cardiolipin. We found no evidence that these antibodies are cytotoxic. (Stroke 1993;24:994-999)

Key Words • anticoagulants, antiphospholipid antibodies • endothelium

There is a growing body of evidence that suggests that immunological mechanisms play a role in cerebral thrombosis and stroke. Antiphospholipid antibodies (aPL) are linked clinically with thromboembolism and ischemic cerebrovascular disease including transient ischemic attack, amaurosis fugax, acute ischemic encephalopathy, multi-infarct dementia, and stroke. It has not been established whether aPL are directly pathogenic and mediators of disease or merely serological epiphenomena, markers of a more fundamental disturbance in coagulation. Pathological studies in patients with aPL and stroke demonstrate a noninflammatory thrombotic occlusion of cerebral vessels. Theories of pathogenesis center around either platelet or endothelial cell dysfunction. There are suggestions that immunoglobulins in the sera of patients with aPL disrupt the thromboresistant properties of the endothelial cell, triggering intra-vascular coagulation. To date, no studies have addressed the interaction of aPL with human brain endothelium. We undertook this study to determine if there are immunoglobulins in the sera of patients with aPL and stroke that bind to human brain microvascular endothelial cells.

Subjects and Methods

Subjects

Sera from three groups of subjects were included. Group 1 included patients with a history of ischemic cerebrovascular disease who were found on diagnostic evaluation to have aPL: either elevated immunoglobulin G (IgG) anticardiolipin antibodies by enzyme-linked immunosorbent assay (ELISA) or the presence of a lupus anticoagulant. These patients were detected by an ongoing prospective screening for aPL in patients presenting with stroke at the Medical College of Georgia Hospital and its affiliated Veterans Affairs Medical Center or from referrals of patients to the outpatient clinics with known aPL and stroke. This group was further subdivided into patients with systemic lupus erythematosus (SLE) according to the criteria of the American Rheumatism Association criteria and those without a defined collagen vascular disorder.
Group 2 included 62 healthy control subjects: donors at a local community blood bank who were age and sex matched for subjects in group 1. Group 3 included patients with acute ischemic stroke without aPL or SLE. These were consecutive patients screened as above and found not to have aPL.

All patients in groups 1 and 3 were examined by a neurologist (one of the authors). Blood was drawn by venipuncture 3 days to 14 months after the index event from patients in group 1 and within 10 days of the ischemic stroke in group 3.

The criteria for aPL positivity was either elevation of IgG anticardiolipin antibody (aCL) by solid-phase ELISA (see below) or the presence of a lupus anticoagulant. Detection of a lupus anticoagulant was by (1) prolongation of the activated partial thromboplastin time, (2) failure to correct with a 1:1 dilution with normal plasma, and (3) positive tissue thromboplastin inhibition test.

**Anticardiolipin Assay**

aCL determination was made by solid-phase ELISA after the method of Harris. Ninety-six-well microtiter plates (Falcon) were coated with 30 μL per well of 50 μg/mL cardiolipin (Sigma) in ethanol. The ethanol was allowed to evaporate overnight at 4°C. The following day, the plates were washed twice with phosphate-buffered saline (PBS) and then blocked with 100 μL per well of 10% adult bovine serum in PBS for 1 hour at room temperature. After washing with PBS, test samples (sera) and standardized sera (obtained from the Antiphospholipid Standardization Laboratory) diluted 1:50 were added in triplicate and incubated for 2 hours at room temperature. After three washes with PBS, alkaline phosphatase-conjugated affinity-purified goat anti-human IgG or immunoglobulin M (IgM) diluted 1:1000 was added for 90 minutes. After three washes, 50 μL of 1 mg/mL p-nitrophenyl phosphate in diethanolamine buffer was added and the reaction stopped by 3N NaOH when the highest standard had an optical density (OD) of 1.0 at 405 nm in a microtiter plate reader (Flow Laboratories). A log-log plot of OD and anticardiolipin units using standardized samples was constructed, and test samples were assigned an anticardiolipin value by reference to the standard curve. Samples >10 GPL (IgG phospholipid units) or >10 MPL (IgM units) were considered positive.

**Endothelial Cells**

*Human brain microvascular endothelial cells.* These were isolated from temporal lobectomy specimens in patients undergoing ablative surgery for partial complex seizures or from autopsy material (traumatic death) by a method adapted from Bowman et al. Specimens in which malignancy or tumor were found were excluded. The tissue was washed in M199 with penicillin (50 U/mL) and streptomycin (50 μg/mL), then minced into pieces approximately 1 mm³ with a scalpel blade. The tissue then was homogenized with 10 to 20 strokes of a dounce. The homogenate then was mixed with a volume of 27.3% dextran to a final concentration of 15%, then centrifuged at 5800g at 4°C in a swinging bucket rotor to separate the microvessels from the neuropil. The microvessels then were resuspended in M199 and successively passed through 210- and 149-μm nylon meshes and subsequently trapped on a 40-μm nylon mesh. The microvessels then were treated with a mixture of 1 mg/mL collagenase and dispase (Boehringer-Mannheim) at 37°C overnight. The following morning, the treatment was terminated by washing with M199 and pelleting at 800g and resuspending in M199 with 10% fetal bovine serum (FBS). To isolate endothelial cells, the suspension was layered over a Percoll gradient. Fractions from the gradients were collected into sterile dishes and examined with inverted-phase contrast microscopy. Those dishes containing viable cells were diluted in M199 and centrifuged to remove Percoll. Isolated clumps of endothelial cells then were seeded in 32-mm plates and maintained in M199 with 10% FBS and penicillin (100 μg/mL) and streptomycin (100 μg/mL). The medium was changed every 2 days. The endothelial cells attached to the bottom of the plate, and 5 to 7 days later, colonies were evident (Fig 1). The endothelial cells were maintained in 20% fetal calf serum (FCS) in M199 with penicillin and streptomycin) at 37°C in 5% CO₂. Contaminating cell types were removed by mechanical wedging with a sterile 25-gauge needle. Cells were passaged every 5 to 7 days with pancreatin-EDTA and used in the first four passages.

Human umbilical vein endothelial cells were isolated according to the methods of Jaffe et al. and used in the first four passages. They were maintained in media consisting of 10% FBS in M199 with 15 U/mL heparin
FIG 2. Second-passage human brain microvascular endothelial cells demonstrating positive perinuclear granular immunofluorescence for von Willebrand factor.

and 20 U/mL endothelial cell growth factor (Upstate Biotechnology).

Endothelial cells were identified by (1) angiotension converting enzyme (ACE) activity and (2) von Willebrand factor immunofluorescence (Fig 2). ACE activity was measured as previously described26 and was 1.21±0.16 min⁻¹ per milligram of protein (mean±SEM of triplicate measurements in five wells) in the human brain microvascular endothelial cells.

von Willebrand factor immunofluorescence. Endothelial cells were grown to subconfluence on glass coverslips and then washed once with PBS. The cells then were fixed for 10 minutes in methanol at −20°C. After three washes with PBS, the cells were overlaid with rabbit anti-human von Willebrand factor (Sigma) at a 1:50 dilution for 30 minutes at 37°C. After three washes with PBS, goat anti-rabbit IgG (Sigma) at a 1:40 dilution was added for 30 minutes. After three washes with PBS, the slides were read. Controls consisted of omitting the first antibody or substituting diluted normal rabbit serum. In addition, smooth muscle from bovine aorta was run in parallel.

Anti-endothelial Cell Assay

Human cerebral microvascular endothelial cells or human umbilical vein cells from passages 1 to 4 were plated and grown to confluence in 96-well microtiter plates (Costar) coated with 0.1% gelatin (Sigma). The wells were washed twice with PBS, then fixed for 20 minutes with 2% paraformaldehyde. The wells then were washed with buffer (20 mM Tris, 0.5 M NaCl, 0.2% Tween 20). To avoid nonspecific binding, the wells were blocked with 100 µL of 2% bovine serum albumin (BSA) in PBS for 1 hour at room temperature and then washed twice with buffer. Test samples (50 µL) diluted 1:1000 in buffer were added in triplicate and incubated for 2 hours at room temperature. After three washes with buffer, 100 µL of affinity-purified peroxidase-conjugated goat anti-human IgG (Sigma) diluted 1:1500 was added to each well and incubated for 90 minutes. Substrate (3,3,5,5 tetramethylbenzidine [10 mg/mL in DMSO] diluted 1:100 in citrate buffer [pH 5.0] with 1.3 mM H₂O₂) was added, and the reaction was terminated in 15 to 30 minutes with 25 µL of 3N H₂SO₄ and read at 450 nm in a microtiter plate reader. To ensure that antibodies were not binding directly to the plate, a plate coated with gelatin but without endothelial cells was run with every assay. The OD of antigen-free wells was subtracted from the OD of the antigen wells.

A known positive and a known negative sample (pooled human sera) was run with every plate. Each test sample was assigned a value in reference to these samples. This value, the ELISA index (EI), was derived from the formula 100×(U−N)/(P−N), where U=OD of unknown sample, P=OD of known high positive sample, and N=OD of known negative sample. The intra-assay and interassay coefficients of variation were <10% when the assay was performed with endothelial cells from the same donor.

Absorption With Cardiolipin Liposomes

Liposomes were prepared according to the methods of Pengo et al.27 A mixture of cardiolipin, cholesterol, and dicetyl phosphate (molar ratio 10:15:2) in chloroform was dried in a stream of nitrogen. The lipids were resuspended in 1 mL of 0.15 M NaCl by agitation using a vortex mixer; 500 µL of patient serum was incubated for 1 hour at 37°C and then overnight at 4°C. After centrifugation, the supernatants were kept as absorbed serum.

Cytotoxicity

Cytotoxicity was measured by a chromium release assay. Human brain endothelial cells were plated and grown to confluence in 24-well Costar plates. After aspiration of the medium and one wash with M199, the cells were incubated overnight with 1 µCi Na²⁴Cr per well in 10% FCS in M199. The following morning, the wells were again aspirated and washed three times with M199 to remove unincorporated chromium. The wells were then overlaid with 10% patient or control serum in M199 and incubated for 4 to 24 hours in a 5% CO₂ incubator at 37°C. All samples were done in triplicate. In addition, three wells were overlaid with 10% FBS in M199; 100 µL of pooled human serum was added to each well as a further source of complement. At the designated times, the medium was aspirated and the monolayers were washed three times with M199. The initial aspiration and subsequent washes were combined as the supernatant. The monolayers were then lysed with 1% Triton, and the lysate was collected. The radioactivity then was measured in a Beckman gamma counter. Percent cytotoxicity was calculated as cpm supernatant/(cpm supernatant+cpm lysate)×100. Per-
cent specific cytotoxicity was calculated by percent cytotoxicity of sample minus percent cytotoxicity with 10% FBS in M199.

**Statistical Analysis**

The mean ELISA index from the groups was compared by ANOVA. Each patient group was compared with the control group (group 3) by unpaired t test. Individual samples >3 SD above the control mean (group 3) were considered positive. Nonparametric data were compared by the Mann-Whitney U test.

**Results**

The group of patients with ischemic cerebrovascular disease and aPL (group 1) had significantly higher IgG binding to human brain endothelial cells than both the healthy control subjects and stroke patients without aPL (group 1 EI+SD: 63±37 versus group 2 EI: 7±7 versus group 3 EI: 7±7; P<.001). Of the patients in group 1 without SLE (n=12), the mean EI was 48±36, significantly higher than both control groups (P<.001). Individually, 16 of 20 (80%) patients from group 1 had IgG binding >3 SD above the control mean compared with 1 of 62 controls in group 2 (P=.0001, Mann-Whitney U test) and 1 of 40 stroke patients in group 3 (P=.0001, Mann-Whitney U test) (Fig 3). Of the patients in group 1, 8 of 8 patients with SLE and 8 of 12 without SLE had elevated IgG binding, with both subgroups significantly different than both control groups (group 1 SLE versus groups 2 and 3, P=.0001, Mann-Whitney U test; group 1 non-SLE versus group 2, P=.0004 and versus group 3, P=.0008).

Group 1 was further divided into those patients who had their sera drawn within 10 days of the index ischemic event and those later than 10 days (see Table). The mean EI±SD was 75±38 in those drawn within 10 days and 49±31 for those drawn later (P=.11). In the subgroup of group 1 without SLE, the EI was 56±39 in those drawn early (n=6) and 39±32 in those drawn late (n=6) (P=.44).

To ensure that the increased binding was not specific to any individual endothelial cell line, at least 10 sera from each group were tested on human brain microvascular endothelial cells derived from at least three different donors. In all instances, positive sera (>3 SD above control mean) remained positive and negative sera remained negative.

To determine if the increased binding was specific to brain endothelial cells, we performed the ELISA with

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**Data for Individual Patients, Including Anticardiolipin Titer, Lupus Anticoagulant Status, and ELISA Index**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y)</th>
<th>aCL</th>
<th>LA</th>
<th>EI</th>
<th>Sample</th>
<th>Comment</th>
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<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>15</td>
<td>+</td>
<td>110</td>
<td>&lt;10 d</td>
<td>Multi-infarct dementia</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>60 (15)</td>
<td>+</td>
<td>100</td>
<td>&lt;10 d</td>
<td>SLE</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>15</td>
<td>+</td>
<td>96</td>
<td>&lt;10 d</td>
<td>SLE</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>32</td>
<td>+</td>
<td>30</td>
<td>&lt;10 d</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>32 (22)</td>
<td>+</td>
<td>21</td>
<td>14 mo</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>21</td>
<td>+</td>
<td>35</td>
<td>9 mo</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>34</td>
<td>106 (12)</td>
<td>+</td>
<td>78</td>
<td>&lt;10 d</td>
<td>DVT, PE, miscarriages</td>
</tr>
<tr>
<td>8</td>
<td>36</td>
<td>18</td>
<td>+</td>
<td>34</td>
<td>2 mo</td>
<td>SLE</td>
</tr>
<tr>
<td>9</td>
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<td>34</td>
<td>+</td>
<td>48</td>
<td>12 mo</td>
<td>SLE</td>
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<tr>
<td>10</td>
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<td>12</td>
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<td>26</td>
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<tr>
<td>11</td>
<td>54</td>
<td>126</td>
<td>+</td>
<td>104</td>
<td>2 mo</td>
<td>DVT</td>
</tr>
<tr>
<td>12</td>
<td>35</td>
<td>23 (18)</td>
<td>+</td>
<td>65</td>
<td>3 mo</td>
<td>SLE</td>
</tr>
<tr>
<td>13</td>
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<td>110</td>
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<td>33</td>
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<td>Livedo reticularis</td>
</tr>
<tr>
<td>14</td>
<td>29</td>
<td>15</td>
<td>+</td>
<td>12</td>
<td>1 mo</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>35</td>
<td>21</td>
<td>+</td>
<td>88</td>
<td>&lt;10 d</td>
<td>SLE</td>
</tr>
<tr>
<td>16</td>
<td>29</td>
<td>92</td>
<td>+</td>
<td>104</td>
<td>&lt;10 d</td>
<td>SLE</td>
</tr>
<tr>
<td>17</td>
<td>51</td>
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<td>+</td>
<td>82</td>
<td>&lt;10 d</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>29</td>
<td>42</td>
<td>+</td>
<td>21</td>
<td>&lt;10 d</td>
<td>Chorea</td>
</tr>
<tr>
<td>19</td>
<td>30</td>
<td>30 (12)</td>
<td>+</td>
<td>65</td>
<td>8 mo</td>
<td>SLE, MI</td>
</tr>
<tr>
<td>20</td>
<td>51</td>
<td>14</td>
<td>+</td>
<td>126</td>
<td>&lt;10 d</td>
<td>SLE</td>
</tr>
</tbody>
</table>

aCL, anticardiolipin titer; LA, lupus anticoagulant status; EI, ELISA (enzyme-linked immunosorbent assay) index; Sample, time from ischemic event to time sample was drawn; SLE, systemic lupus erythematosus; DVT, deep venous thrombosis; PE, pulmonary embolus; MI, myocardial infarction. Under aCL, the first number represents immunoglobulin titer in GPL units; number in parenthesis indicates immunoglobulin titer in MPL units. If no number is in parentheses, the value was <10 MPL.
endothelial cells derived from human umbilical vein. Twelve sera that were positive and four that were negative in the ELISA using human brain cerebral endothelial cells from group 1 were tested. In all instances, positive sera remained positive (defined by 3 SD above control group [n=20]) and negative sera remained negative in the ELISA using human umbilical vein cells.

There was poor correlation between endothelial cell binding and binding to cardiolipin ($r=23$, $r^2=56$) (Fig 4). Moreover, in inhibition experiments with sera from two patients with high endothelial binding and high cardiolipin binding (patients 2 and 7 from Table 1), incubation of the sera with cardiolipin liposomes had no significant effect on endothelial binding (<20%), whereas cardiolipin binding was reduced by $>80%$.

Sera from group 1 patients with elevated IgG binding to brain endothelial cells had no significant cytotoxic effect on human brain microvascular endothelial cells as measured by chromium release at both 4 and 24 hours (mean specific cytotoxicity at 4 hours: 1.19% [aPL, n=12] versus 1.22% [control subjects, n=15], NS; mean specific cytotoxicity at 24 hours: 2.4% [aPL] versus 2.6% [control subjects], NS). Moreover, no individual sera from group 1 exhibited cytotoxicity as defined by $>2$ SD above control mean.

**Discussion**

We have demonstrated for the first time that patients with stroke and aPL have human brain microvascular endothelial cell-reactive antibodies. These antibodies were detected both in patients without an associated collagen vascular disease and in those with SLE. This binding, however, was not specific to brain endothelium, as it was also demonstrated with endothelial cells derived from human umbilical vein. In addition, the endothelial cell-reactive antibodies and those binding to cardiolipin appeared to be distinct.

Our findings reinforce those of McCrae et al,28 who, using human umbilical vein endothelial cells, found endothelial cell-reactive antibodies in 47 of 76 patients with aPL. Moreover, when they specifically studied the subset of aPL patients with a history of thrombosis, 17 of 19 had endothelial cell-reactive antibodies. They also determined that immunoglobulins binding to cardiolipin and those binding to endothelial cells were distinct populations. Similar results have been found by other investigators in SLE populations,29,30

We were unable to demonstrate a cytotoxic effect of these sera despite the use of a sensitive assay. In our system, however, we specifically assayed for complement-mediated cytotoxicity. Because there were no inflammatory cells, a neutrophil- or mononuclear cell-mediated cytotoxicity cannot be excluded. The vascular pathology of this disorder does not involve inflammatory cells but rather a bland thrombosis. It is quite possible that these immunoglobulins may disturb endothelial cells in more subtle ways by disrupting the delicate balance between their anticoagulant and procoagulant functions.

Many of our patients with aPL fulfilled criteria for the primary antiphospholipid syndrome. The antiphospholipid syndrome is a multisystem disease consisting of arterial and venous thrombotic events, recurrent spontaneous miscarriages, and a high frequency of neurological events.31,32 The cause for the high incidence of neurological complications in patients with the antiphospholipid syndrome remains unknown. There are at least two possibilities. In a generalized hypercoagulable state, thrombotic occlusion of vessels in the brain may be more likely to be clinically manifest than thrombotic occlusions in other organs. To date, there are too few pathological studies to determine the frequency of vascular occlusions in various organs. An alternative explanation is that cerebral endothelium may be selectively targeted or may have a selective vulnerability. There are significant differences between endothelial cells derived from different organs.33-37 This heterogeneity includes differences in prostaglandin synthesis,34 expression of adhesion molecules35,36 and cell surface epitopes,37 antigen presentation,38 and thrombogenicity.39 Brain endothelium is regarded as the most atypical. Besides well-documented differences in barrier function,40 even coagulation may be regulated differently in the brain than in other organs.41 Further studies are ongoing to determine if immunoglobulins in the sera of patients with aPL disturb brain endothelial cell anticoagulant functions in vitro and to define the precise epitopes to which these immunoglobulins bind.

Our finding and that of others of a population of endothelial cell-reactive antibodies distinct from those to cardiolipin highlight another avenue of investigation in the pathogenesis of antiphospholipid-related complications. There is now evidence that anticardiolipin antibodies are directed against a complex of $\beta_2$-glycoprotein I and phospholipid42,43 and the lupus anticoagulant against a complex of phospholipid and human prothrombin.44 Endothelial cell damage or perturbation could lead to increased exposure of procoagulant surfaces (ie, phospholipids) with the binding of $\beta_2$-glycoprotein I or human prothrombin and the subsequent development of antiphospholipid antibodies. Endothelial cell-reactive antibodies may reflect the initial or primary insult in this disorder.

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