A
tiphospholipid antibodies are a heterogeneous family of antibodies that react to negatively charged phospholipids or phospholipid-protein complexes. Currently, they are detected by either ELISA, with anionic phospholipids used as the antigens (eg, anticardiolipin antibodies, antiphosphatidylserine antibodies), or by the ability to prolong coagulation time in a phospholipid-dependent assay (lupus coagulant). Antiphospholipid (aPL) antibodies are an element of the antiphospholipid syndrome, a condition characterized by arterial and venous thrombosis, recurrent fetal loss, and thrombocytopenia in the presence of aPL antibodies. There also appears to be an association between aPL antibodies and ischemic events even in the absence of the other features of the syndrome. Progress has been made in the last decade in describing mechanisms of action of aPL antibodies. However, the pathogenesis of aPL antibodies and the precise mechanisms by which they promote thrombosis remain unknown. The association between aPL antibodies and autoimmune disease has long been recognized, but more recently it has also been suggested that infection may trigger the generation of aPL antibodies by exposure of antigenic phospholipids, which induce immunologic responses. However, it has been suggested that infection-induced aPL may not be thrombogenic. Because many infections tend to follow a seasonal pattern, we decided to investigate the seasonal distribution of aPL antibodies among patients with acute stroke and community-based control subjects, reasoning that the risk associated with these antibodies may also demonstrate seasonal variability.

Conclusion
Our data suggest some aPL antibodies arise from different origins in patients and control subjects. The seasonality observed in the apparently normal population may be related to antibodies of infectious origin and is consistent with the reported lack of association with thrombosis of infection-related antibodies. (Stroke. 2001;32:1707-1711.)

Key Words: antibodies, anticardiolipin • antibodies, antiphospholipid • phosphatidyl serines • stroke • seasons
ucts, Westminster Co). The assays were performed as follows: Sera were diluted 1:50 with sample diluent, which contained a standardized concentration of β2-glycoprotein I (β2-GPI). Then 100 μL of the diluted sera was distributed to each well of microtiter plates coated with cardiolipin (or phosphatidylserine) in duplicate. After a 15-minute incubation at room temperature, the wells were washed 4 times with PBS, pH 7.4, to remove the unbound serum proteins. Then, 100 μL of solutions containing goat antibodies specific for human IgG or IgM labeled with horseradish peroxidase were added to the wells. After another 15 minutes of incubation at room temperature and washing 4 times with PBS, 100 μL of substrate solution containing tetramethylbenzidine and hydrogen peroxide as the chromogenic substrate were distributed to the well. After a 10-minute incubation at room temperature, 100 μL of stopping solution (0.36N sulfuric acid) was added to the wells. Within 30 minutes after the addition of stopping solution, the optical density was read in a microplate reader (Molecular Devices kinetic microplate reader) with a dual beam at 450-nm and 650-nm wavelength.

The results of IgG and IgM anticardiolipin antibodies (aCL IgG, aCL IgM) were expressed in GPL or MPL units. One GPL unit is equivalent to 1 μg/mL and 1 MPL unit is equivalent to 1 μg/mL of affinity-purified IgG and IgM sera, respectively. In the same concentration of purified sera, the values of antiphosphatidylserine antibodies (aPS IgG, aPS IgM) were expressed in GPS or MPS units. The results were defined as positive if they were >23 GPL or >11 MPL for aCL antibodies and >16 GPS or >22 MPS for aPS antibodies, according to the instructions of the manufacturer.

Statistical Methods

The χ² test was used to compare the difference in prevalence of stroke risk factors between patients and control subjects. The differences between cases and control subjects in geometric mean aPL titers were assessed by using a t test (Satterhwaite method for unequal variances) on log-transformed data. The effect of season and case-control status and the interaction of these variables on the log transformation of the geometric mean titers of each aPL antibody were assessed with a 2-way ANOVA. The χ² test developed by Walter and Elwood12 was used to evaluate the seasonal trends of aPL antibody variation. Odds ratios with 95% confidence intervals were calculated by logistic regression analysis, with adjustment for age, race, sex, current cigarette smoking, and history of hypertension, diabetes mellitus, atrial fibrillation, and coronary artery disease (defined as previous myocardial infarction, angina, or a coronary artery revascularization procedure). The Breslow-Day Test for Homogeneity was used to evaluate the difference in odds ratios.

Results

Of the 884 patients tested for aPL antibodies, 467 had aCL testing only, 402 were tested for both aCL antibodies and aPS antibodies, and 15 were tested only for aPS antibodies because of an insufficient amount of sera. In the control group, 1024 individuals were tested for aCL antibodies, and 653 of them (those recruited in the latter part of the study for whom serum samples were stored) were also tested for aPS antibodies.

The demographic and clinical characteristics of patients and control subjects are provided in Table 1. The control subjects were younger, and women represented 54% of the cases and 65% of the control subjects. Most risk factors for stroke, including hypertension, diabetes mellitus, coronary artery disease, and atrial fibrillation were more common in patients. However, we have previously demonstrated that adjusting for these factors does not alter the estimated relative risk associated with a positive aPL antibody titer in our population.13,14

Geometric mean aPL titers are given in Table 2. For each aPL subtype, cases had a higher mean titer. To assess the effect of seasonality on the difference in titer between cases and control subjects, we performed a 2-way ANOVA on the log transformations of the geometric means for summer and nonsummer months, including season, case-control status, and the interaction between these variables for each aPL isotype. The probability value for this interaction was <0.0001 for both aCL IgG and IgM, 0.017 for aPS IgM, and 0.34 for aPS IgG, confirming that season of blood draw has an important effect on the magnitude of the difference of aPL titers between stroke patients and nonstroke control subjects, although this interaction did not reach statistical significance for aPS IgG. The mean aPL values for cases and control subjects by month are given in Table 3. With few exceptions, the largest differences are observed during the warm weather months.

The distribution of any positive aPL (either aCL or aPS) titer by month of blood draw for control subjects and cases is depicted in Figure 1. Among the control group who had both aPS and aCL testing, there was a lower proportion of any TABLE 1. Age, Sex, and Clinical Characteristics of Stroke Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Cases (n=884)</th>
<th>Control Subjects (n=1024)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, y</td>
<td>70.5 ± 11.7</td>
<td>65.1 ± 10.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Men, %</td>
<td>471 (46%)</td>
<td>362 (35%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>596 (68%)</td>
<td>456 (45%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>296 (34%)</td>
<td>129 (13%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>241 (27%)</td>
<td>92 (9%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>95 (11%)</td>
<td>9 (1%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td>124 (14%)</td>
<td>157 (15%)</td>
<td>0.440</td>
</tr>
</tbody>
</table>

TABLE 2. Geometric Mean Titers for Cases and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Control Subjects</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCL IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>13.87</td>
<td>8.50</td>
<td>0.0001</td>
</tr>
<tr>
<td>Summer</td>
<td>15.19</td>
<td>7.58</td>
<td>0.0001</td>
</tr>
<tr>
<td>Nonsummer</td>
<td>13.35</td>
<td>9.01</td>
<td>0.0001</td>
</tr>
<tr>
<td>aCL IgM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>3.83</td>
<td>3.27</td>
<td>0.0001</td>
</tr>
<tr>
<td>Summer</td>
<td>4.20</td>
<td>2.96</td>
<td>0.0001</td>
</tr>
<tr>
<td>Nonsummer</td>
<td>3.69</td>
<td>3.44</td>
<td>0.11</td>
</tr>
<tr>
<td>aPS IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>5.84</td>
<td>5.16</td>
<td>0.0006</td>
</tr>
<tr>
<td>Summer</td>
<td>6.09</td>
<td>5.15</td>
<td>0.0073</td>
</tr>
<tr>
<td>Nonsummer</td>
<td>5.71</td>
<td>5.17</td>
<td>0.02</td>
</tr>
<tr>
<td>aPS IgM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>7.99</td>
<td>6.68</td>
<td>0.0001</td>
</tr>
<tr>
<td>Summer</td>
<td>8.40</td>
<td>6.05</td>
<td>0.0001</td>
</tr>
<tr>
<td>Nonsummer</td>
<td>7.80</td>
<td>7.01</td>
<td>0.059</td>
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</tbody>
</table>
positive aPL titer during the warm weather months. The probability value was <0.001 for this seasonal variation. There was no evidence of a seasonal pattern in the distribution of aPL antibodies in the corresponding patient group. A similar seasonality pattern was noted for each individual aPL isotype among the control subjects (Figure 2, A and B) for whom that aPL result was obtained. Seasonal trends for individual aPL isotypes were not noted among cases (Figure 3, A and B). The probability value for these seasonal trend analyses are given in Table 4.

The odds ratio for any positive aPL titer during the warm weather months (June through September), adjusting for age, sex, race, and traditional stroke risk factors, was 12.3 (95% CI, 6.5 to 23.5). During the other months, the corresponding odds ratio was 2.9 (95% CI, 2.0 to 4.2). This 4-fold difference is statistically significant ($P<0.0003$).

**Discussion**

The presence of aPL antibodies in either primary or systemic lupus erythematosus–related antiphospholipid syndrome is associated with increased risk for thrombosis, but aPL antibodies have been found in up to 14% of normal subjects. Antiphospholipid antibodies have also been detected in malignancies, infections, and drug ingestion; in these conditions, aPL antibodies have not appeared to increase the risk for thrombosis. Drugs that have been reported to induce aPL antibody production include procainamide, phenothiazine, quinidine, and hydralazine. Antiphospholipid antibodies have been detected in sera from individuals with a variety of bacterial and viral infections: mycoplasma, chlamydia, human immunodeficiency virus, Lyme disease, rubella, chicken pox, hepatitis C, and syphilis. Antiphospholipid antibodies in sera from patients infected with Gram-negative bacteria and chlamydia were shown to react to lipopolysaccharide from a rough strain of salmonella. Because phospholipids are constituents of cell membranes, it has been proposed that aPL antibody generation might be an autoimmune response to human tissue damage or a result of cross-reactivity to similar antigens of human tissue and microorganism structures.

Several studies have shown that anionic phospholipids require a cofactor to form a phospholipid–protein complex that presents the antigenic epitope to aPL antibodies. The expression of epitope occurs as the result of the conformational changes of the antigen after the binding of a cofactor to phospholipid. β$_2$-glycoprotein I (β$_2$GPI), a major cofactor, has been extensively studied. Other proposed cofactors include prothrombin, protein C, protein S, and annexin-V. It has been suggested that aPL antibodies present in autoimmune diseases are thrombogenic and β2-GPI–dependent, as opposed to infection-related aPL antibodies, which are thought less likely to be thrombogenic and are β2-GPI–independent.

In the present study, the high frequency of aPL antibodies among control subjects occurring in the fall, winter, and
spring is similar to the trends of seasonal respiratory tract infections and resembles the epidemic curve of acute rheumatic fever. Interestingly, there was no evidence of a seasonal distribution of aPL antibodies among the stroke patients. Thus, some of the aPL antibodies of patients and control subjects appear to arise from different origins. Because we have no information regarding previous history or testing for infection in either group, the idea that infection was a contributing factor to aPL antibody production remains speculative. However, if this is the explanation for seasonal variability in antibody positivity in the control group and if aPL antibodies induced by infection are less likely to induce or be associated with a thrombogenetic state, we would expect to see the observed lack of seasonality in the aPL titers of the patient population because any seasonal variation would be obscured by the high proportion of antibodies unrelated to infection. We do not exclude the possibility that even thrombogenic aPL antibodies might arise in response to infection through a “molecular mimicry” mechanism analogous to acute rheumatic fever. Further studies on the prevalence of other autoantibodies, which would elucidate this point, are planned.

Our results suggest that in an apparently normal population, there is seasonal variability in aPL antibody titers. This could arise from certain seasonal infections, but this remains to be proven. The nonthrombogenicity of aPL antibodies related to infection and drug induction has not yet been established, but our data suggest that the apparent relative risk for stroke associated with aPL antibodies may be considerably higher in the summer months and conversely lower at

Figure 2. Distribution of proportion of positive aPL antibody titers for each subtype by month in control subjects.

Figure 3. Distribution of proportion of positive aPL antibody titers for each subtype by month in cases.
other times. If this seasonal variability is confirmed in other cohorts, it may be necessary to consider the implications for subsequent study design. It may also help to explain the apparent discrepancies in previous studies. If, for example, subjects were recruited exclusively during cold weather months, the prevalence in unselected (nonstroke) populations would tend to appear higher, but the association with stroke (and possibly other vascular events) would appear weaker, whereas a prospective cohort recruited primarily in warm weather months may demonstrate a lower prevalence of elevated aPL titers but stronger relation of those titers to stroke.

Acknowledgments

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References

Seasonal Distribution of Antiphospholipid Antibodies
Thanh-Ha Luong, Jacob H. Rand, Xiao-Xuan Wu, James H. Godbold, Mayra Gascon-Lema and Stanley Tuhrim

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