Relationship of Antiphospholipid Antibodies to Pregnancy Loss in Patients With Systemic Lupus Erythematosus: A Cross-Sectional Study

By Jeffrey S. Ginsberg, Patrick Brill-Edwards, Marilyn Johnston, Judah A. Denburg, Maureen Andrew, Robert F. Burrows, William Bensen, Alfred Cividino, and Aidan A. Long

To determine whether an association exists between the presence of antiphospholipid antibodies and pregnancy loss, a cross-sectional study was performed. Consecutive women who were referred to three outpatient rheumatology clinics and who had systemic lupus erythematosus (SLE) and a history of one or more pregnancies were evaluated. Patients were interviewed to determine outcomes of all previous pregnancies. Blood was taken on two separate occasions at least 3 months apart to test for the presence of the lupus anticoagulant and anticardiolipin antibodies; on both occasions, five tests of the lupus anticoagulant, with well-defined normal ranges, and an enzyme-linked immunosorbent assay to measure IgG anticardiolipin antibodies were performed. Patients were considered to be positive for the lupus anticoagulant if one or more tests was abnormal on both occasions and positive for anticardiolipin antibodies if the test was abnormal on both occasions. Forty-two women were studied. Statistically significant associations were shown between lupus anticoagulant positivity and previous pregnancy loss (odds ratio [OR], 4.8; 95% confidence intervals [CI], 1.0 to 23.6; \( P = .05 \)) and between anticardiolipin antibody positivity and previous pregnancy loss (OR, 20.0; 95% CI, 1.3 to 97.0; \( P = .01 \)). All seven women with multiple episodes of pregnancy loss were lupus anticoagulant positive and four of these were also anticardiolipin antibody positive. If patients who are transiently positive for lupus anticoagulant and/or anticardiolipin antibodies are considered to be test positive, the associations with pregnancy loss are no longer statistically significant. Within the group of lupus anticoagulant-positive patients, we observed stronger associations between the presence of six or more positive tests and pregnancy loss than between the presence of two to five positive tests and pregnancy loss. No single test for the lupus anticoagulant provides a statistically significant association with pregnancy loss. The results of our study show that by performing multiple lupus anticoagulant tests and by repeating testing for lupus anticoagulant and anticardiolipin antibodies on more than one occasion, significant associations between the presence of antiphospholipid antibodies and previous pregnancy loss can be shown in patients with SLE.

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ANTIPHOSPHOLIPID antibodies bind to charged phospholipids and inhibit phospholipid-dependent coagulation tests in vitro.\(^1\)\(^2\) In recent years, several studies have reported on the possible association between the presence of antiphospholipid antibodies and pregnancy wastage.\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\)\(^13\)\(^14\)\(^15\)\(^16\) However, a recent study critically reviewed the studies that have reported on this possible association and concluded that an association is suggested but not firmly supported.\(^17\) Six retrospective studies of patients with systemic lupus erythematosus (SLE) were reviewed; two reported no association between antiphospholipid antibodies and pregnancy loss and four reported nonsignificant trends.\(^11\)\(^13\)\(^15\)\(^17\) A limited number of patients has been studied prospectively and, to date, it has not been possible to conclusively establish the presence of antiphospholipid antibodies as an independent risk factor for adverse pregnancy outcome. Despite the lack of convincing evidence for an association between the presence of antiphospholipid antibodies and pregnancy loss, many experts believe such an association exists.\(^20\)\(^21\) Further, therapeutic interventions aimed at preventing pregnancy loss with agents such as acetylsalicylic acid (ASA), prednisone, and gammaglobulin have been evaluated in women with antiphospholipid antibodies and a history of pregnancy loss.\(^22\)\(^23\)\(^24\)\(^25\) There is a lack of agreement among experts concerning both the optimum laboratory assays to detect antiphospholipid antibodies and the optimum pattern of testing.\(^26\)\(^27\)\(^28\)\(^29\) The presence of these antibodies is usually inferred by performing one or more coagulation assays for the lupus anticoagulant and/or by performing an enzyme-linked immunosorbent assay (ELISA) to detect anticardiolipin antibodies. Many in vitro tests of the lupus anticoagulant have been described but the test, or combination of tests, that correlates best with clinical complications, including fetal loss and thromboembolic complications, has not been clearly established.

We have previously shown the importance of using a comprehensive array of assays on at least two separate occasions when looking for clinical associations of antiphospholipid antibodies.\(^30\) We observed that reliance on a single measurement at a single time point, which may yield a transiently positive result, has the potential to obscure the association between the presence of antiphospholipid antibodies and thromboembolic complications. Although it has been recognized that titers of anticardiolipin antibodies may fluctuate in a given patient,\(^17\) the clinical relevance of transiently versus repeatedly abnormal tests with respect to fetal loss is unknown. In the studies mentioned above, addressing the possible association between antiphospholipid antibodies and pregnancy loss, the investigators did not perform antiphospholipid antibody assays on more than a single occasion and did not use multiple assays for the lupus anticoagulant.

To determine whether an association between pregnancy...
loss and antiphospholipid antibodies exists in patients with SLE, we have performed a cross-sectional study in consecutive women with SLE and a history of one or more pregnancies. These women were evaluated using a combination of five tests for the lupus anticoagulant and an ELISA to detect IgG anticardiolipin antibodies and the tests were performed on two separate occasions at least 3 months apart.

MATERIALS AND METHODS

Study Population

The study population consisted of consecutive female patients with SLE and a history of at least one pregnancy referred to three rheumatology clinics between March 1, 1987 and April 1, 1988 in Hamilton, Ontario, Canada. All patients fulfilled the 1982 revised American Rheumatism Association (ARA) criteria for SLE and patients with lupus-like disorders who failed to fulfill these criteria were excluded. The patients were referred because of their SLE and were not selected because they had antiphospholipid antibodies or a history of pregnancy loss.

Intervention

Eligible patients were interviewed by investigators, blinded to the results of testing for antiphospholipid antibodies, to determine details of the outcomes of all previous pregnancies. The outcomes were corroborated in all cases by review of medical charts, which were available for all patients. An abortion was defined as a pregnancy loss occurring before week 20 of gestation and a stillbirth as a pregnancy loss occurring after week 20 of gestation.

All patients had blood drawn at their initial visit and again on a separate occasion at a follow-up visit at least 3 months after the initial visit (mean, 4 months; range, 3 to 6 months). Patients who had blood drawn on only one occasion were excluded from the study (nine patients).

Laboratory Methods

Lupus anticoagulant. Blood was drawn into a vacutainer tube (Becton Dickinson no. 6-416; Becton Dickinson, Mississauga, Canada) containing 0.102 mol/L buffered citrate. Plasma was immediately separated from cellular elements by centrifugation at 1,700g for 15 minutes at room temperature, with maximal removal of platelets being assured by the use of Sure-sep (Organon Teknika, Scarborough, Canada), a silicone based gel, during centrifugation. Plasma was subsequently aliquotted and frozen until batch assays were performed.

The assays used to determine lupus anticoagulant activity were:

1. Xa clotting time (XaT) was performed using purified human Xa and human phospholipid prepared by the method of Bell and Alton. In brief, 50 µL of patient plasma was added to 100 µL of the Xa/phospholipid mixture, incubated for 60 seconds at 37°C, followed by the addition of 100 µL of 0.025 mol/L CaCl₂. Results of greater than 72 seconds (2 standard deviations above the mean of 39 normal volunteers) were considered abnormal.

2. The Kaolin Cephalin activated partial thromboplastin time (KCaPTT) was performed as previously described, using a reagent prepared at McMaster University using a human brain phospholipid preparation and 4% kaolin in saline. Results of greater than 75 seconds (2 standard deviations above the mean of 39 volunteers) were considered abnormal.

3. The activated partial thromboplastin time (aPTT), was performed using a commercially available reagent, automated aPTT (Organon Teknika), as previously described. Results of greater than 32 seconds (2 standard deviations above the mean of 39 normal volunteers) were considered abnormal. (4) Dilute Russell viper venom time (RVVT) was performed as previously described. If the observed clotting time was greater than the upper limit of normal for our laboratory (44 seconds, based on 2 standard deviations above the mean of 39 normal volunteers), ionophore-treated platelets were substituted for the bovine phospholipid and the test was repeated. Results of less than 33 seconds (2 standard deviations above the mean of 38 normal volunteers) were considered abnormal. (5) Dilute one-stage prothrombin time (DPT) was performed using a 1/500 dilution of rabbit brain thromboplastin (Dade Thromboplastin C; Baxter Diagnostics, Mississauga, Canada) in a saline/CaCl₂ mixture. Results of greater than 61 seconds (2 standard deviations above the mean of 4 normal volunteers) were considered abnormal.

With the exception of the RVVT, all patient samples were run neat and in a 1:1 mix of normal pooled plasma and patient plasma. The normal pooled plasma consisted of 20 normal hospital personnel processed to assure minimal platelet activation. The pooled plasma was frozen in small aliquots at −70°C. Values for the 1:1 mix above the defined upper limit of normal for each test (except the RVVT) were considered positive for the lupus anticoagulant. All lot numbers of reagents were constant for the duration of the study.

Patients were considered to be repeatedly positive for lupus anticoagulant if one or more of the tests was positive on both occasions. Thus, patients with one test positive on the first occasion and another test positive on the second occasion were considered to be repeatedly positive. On the other hand, patients were considered to be transiently positive for lupus anticoagulant if one or more of the tests was positive on one occasion and all tests were negative on the other. Finally, patients were considered lupus anticoagulant negative if all five tests were normal on both occasions.

Anticardiolipin antibodies. The detection and quantitation of IgG anticardiolipin antibodies was performed using an ELISA technique similar to that described by Loizou et al with minor modifications. Briefly, 25 µL of 100 mg/ml cardiolipin (Sigma Chemicals, St Louis, MO) in ethanol was coated on polystyrene microtitre wells (Immulon I; Dynatech Laboratories, Fisher Scientific, Unionville, Canada) and evaporated under current of air. After washing in phosphate-buffered saline (PBS), the plates were blocked for 2 hours with 10% adult bovine serum in PBS (ABS-PBS) and washed three times with PBS. Fifty-microliter aliquots of each serum sample diluted 1/50 in ABS-PBS were added to duplicate wells on the plates and incubated for 1 hour at room temperature. After washing five times with PBS, alkaline phosphatase-conjugated affinity-purified rabbit antihuman IgG (diluted 1/350 in ABS-PBS) was added and incubated for 1 hour at room temperature, with the addition of alkaline phosphatase substrate (1.5 µg/mL in diethanolamine buffer pH 9.8) after five further washes with PBS. After 30 minutes of incubation in the dark at room temperature, absorbance was measured at 405 nm. Mean values of the duplicates greater than four standard deviations above the mean of 55 normal sera (obtained from the blood bank at the Canadian Red Cross, Hamilton, Ontario, Canada) were considered raised (namely, optical density at 405 nm of greater than 0.58).

Known positive and negative controls were included on each ELISA plate as references.

Patients were considered to be repeatedly positive for anticardiolipin antibodies if the assays were positive on both occasions measured, whereas they were considered to be transiently positive if the tests were positive on one occasion and negative on the other.

Patients were considered negative for anticardiolipin antibodies if testing was normal on both occasions.
Statistics

The primary analyses were to compare (1) pregnancy outcomes in patients who were repeatedly lupus anticoagulant positive with those who were transiently positive or negative for lupus anticoagulant and (2) pregnancy outcomes in patients who were repeatedly anticardiolipin antibody positive with those who were transiently positive or negative for anticardiolipin antibodies.

Odds ratios and their corresponding 95% confidence intervals (CI) were calculated where indicated. An odds ratio (OR) was considered statistically significant when the lower limit of the 95% CI was ≥ 1.0. The χ² tests and Fisher exact tests were used where indicated. A P value of less than .05 was considered to be statistically significant.

RESULTS

Forty-two women were studied, of whom five had one previous pregnancy, 19 had two previous pregnancies, nine had three previous pregnancies, three had four previous pregnancies, two had five previous pregnancies, three had six previous pregnancies, and one had 12 previous pregnancies. There were 11 women with one pregnancy loss; one had one previous pregnancy, four had two previous pregnancies, four had three previous pregnancies, and two had five previous pregnancies. There were three women who had lost two of two pregnancies, one had lost all three pregnancies, two had lost three of six pregnancies, and one had lost 6 of 12 pregnancies. Of the 122 pregnancies, 30 had resulted in spontaneous abortions and two had resulted in stillbirths, yielding an overall pregnancy loss rate of 26.2%. Of the 42 women, 11 had a history of one previous pregnancy loss and seven had a history of two or more episodes of pregnancy loss.

Pregnancy Loss and Lupus Anticoagulant

A summary of the antiphospholipid antibody testing and the pregnancy outcomes is provided in Table 1. There were 15 women who showed repeat lupus anticoagulant positivity, four who showed transient lupus anticoagulant positivity, and 23 who showed lupus anticoagulant negativity. All seven of the women experiencing multiple pregnancy losses showed repeatedly positive testing for the lupus anticoagulant (OR, 47; 95% CI, 2.4 to 923.9; P = .003). Table 2 shows the significant relationship between repeat lupus anticoagulant positivity and history of pregnancy loss (OR, 4.8; 95% CI, 1.0 to 23.6; P = .05). If transiently lupus anticoagulant-positive patients are grouped with repeatedly positive patients, the OR decreases to 3.8 (95% CI, 0.8 to 17.4; P = .08), corroborating the hypothesis that repeat positivity is more strongly associated with fetal loss than transient positivity.

A history of pregnancy loss occurred in 24 of the 55 pregnancies in women with repeat lupus anticoagulant positivity compared with 8 of the 67 pregnancies in lupus anticoagulant-negative women (OR, 5.7; 95% CI, 2.3 to 13.9; P < .0001).

Pregnancy Loss and Anticardiolipin Antibodies

There were five women who were repeatedly positive for anticardiolipin antibodies, all of whom had one or more episodes of previous pregnancy loss, compared with 13 of the 37 antibody negative or transiently positive women who had one or more episodes of previous pregnancy loss (OR, 20.0; 95% CI, 1.3 to 97.0; P = .01; Table 2). There were five women who had transient antibody positivity, one of whom had previous pregnancy loss. If patients who are transiently positive for anticardiolipin antibodies are grouped with patients who are repeatedly positive, the OR decreases to 3.5 (95% CI, 0.5 to 22.3; P = .14), corroborating the hypothesis that repeat antibody positivity is more strongly associated with pregnancy loss than transient positivity.

Ten of the 14 pregnancies in the women who were repeatedly positive for anticardiolipin antibodies resulted in pregnancy loss compared with 22 of the 108 pregnancies in antibody-negative or transiently positive women (OR, 9.8; 95% CI, 3.0 to 32.4; P = .002). One of the 12 pregnancies in a woman who was transiently antibody positive resulted in pregnancy loss. Of note is that all women who were positive for anticardiolipin antibodies were also positive for lupus anticoagulant.

Of the five women who tested repeatedly positive for anticardiolipin antibodies, four had high titres (>6 SD above the mean) on both occasions and one had a high titre on one occasion and a low titre (4 SD above the mean) on the second occasion. Three of the four women with persistently high-titre antibodies had suffered multiple pregnancy losses and the fourth had suffered one pregnancy loss.

Table 1. Pregnancy Loss and Antiphospholipid Antibody Results

<table>
<thead>
<tr>
<th>APLA Test Results</th>
<th>No. of Episodes of Pregnancy Loss per Patient</th>
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<tbody>
<tr>
<td></td>
<td>≥ 1</td>
</tr>
<tr>
<td>LA+ ACLA+</td>
<td>4</td>
</tr>
<tr>
<td>LA+ ACLA−</td>
<td>3</td>
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<tr>
<td>LA− ACLA+</td>
<td>0</td>
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<tr>
<td>LA− ACLA−</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
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</tr>
</tbody>
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Abbreviations: APLA, antiphospholipid antibody; LA, lupus anticoagulant; ACLA, anticardiolipin antibody; +, repeatedly positive (see Materials and Methods); −, negative or transiently positive (see Materials and Methods).

Table 2. Relationship Between Antiphospholipid Antibodies and Pregnancy Loss

<table>
<thead>
<tr>
<th>APLA Test Results</th>
<th>No. of Episodes of Pregnancy Loss per Patient</th>
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<tbody>
<tr>
<td></td>
<td>≥ 1</td>
</tr>
<tr>
<td>LA+</td>
<td>10</td>
</tr>
<tr>
<td>−</td>
<td>8</td>
</tr>
<tr>
<td>ACLA+</td>
<td>5</td>
</tr>
<tr>
<td>−</td>
<td>13</td>
</tr>
</tbody>
</table>

Abbreviations: LA, lupus anticoagulant; ACLA, anticardiolipin antibodies; +, repeatedly positive; −, negative or transiently positive.

*χ² test.
†Fisher exact test.
whereas the one woman with mixed titres had suffered multiple pregnancy losses. The small number of study participants with anticardiolipin antibodies does not allow us to determine whether there is a relationship between the titre of anticardiolipin antibodies and the risk of pregnancy loss.

Patterns of Lupus Anticoagulant Results

Of the 15 women who repeat lupus anticoagulant positivity, five had identical patterns of testing at both times of testing, whereas 10 had different patterns of positivity. Details of the results of each of the tests in patients who were repeatedly positive are shown in Table 3. Based on the results we are unable to show that one test is clearly superior. Of those four women who exhibited transient positivity for lupus anticoagulant, two had a single abnormal test and two had two abnormal tests.

Of the women who were repeatedly positive for lupus anticoagulant, there were five episodes of pregnancy loss in 20 pregnancies in women who had a total of two to five positive tests compared with 19 episodes of pregnancy loss in 35 pregnancies in women who had a total of 6 to 10 positive tests (OR, 3.6; 95% CI, 1.1 to 11.5; P = .03). The results of our study show significant associations between antiphospholipid antibodies and thromboembolic disease,30 the performance of repeat testing is important because the association between repeat test positivity and pregnancy loss is stronger than the association between transient test positivity and pregnancy loss. The performance of several assays for lupus anticoagulant shows a much stronger association (greater sensitivity) for previous pregnancy loss than the performance of single assays. Furthermore, within the group of lupus anticoagulant-positive patients, the association is stronger when a greater number of different assays is abnormal as we observe stronger correlations between the presence of six or more positive tests and pregnancy loss than between the presence of two to five positive tests and pregnancy loss.

The study design chosen was cross-sectional rather than cohort because the cross-sectional study is more efficient. Because of the design, great care was taken to minimize bias in order to provide valid conclusions. Patient selection and referral bias were minimized by entering consecutive patients into the study from clinics that do not have specific selection criteria.
the investigators, the information obtained was corroborated in all cases by the review of medical records.

The greatest limitation of the cross-sectional design used in this study is the inability to establish a temporal relationship between antiphospholipid antibody testing and obstetrical complications. The study population was a group of women with a wide age range (25 to 61 years) when entered into the study. The patients had had the diagnosis of SLE made for between 1 month and greater than 20 years. Therefore, a small proportion of the women had not even been diagnosed with SLE during their pregnancies. Testing was performed from 30 years after the last pregnancy to immediately postpartum. Thus, we are unable to determine when the assays became abnormal in women who tested positive in this study and whether these tests became abnormal before or after their pregnancies. It is possible (and perhaps likely) that some women who were negative or transiently positive when tested in the study were abnormal when they were pregnant. Additionally, we cannot exclude the possibility that pregnancy events cause test positivity rather than vice versa, although such a relationship seems unlikely. The best study design to clearly establish the temporal relationship between the diagnosis of SLE, antiphospholipid antibodies, and obstetrical complications is prospective cohort.

The results of our study are weighed relatively heavily by the women with multiple pregnancy losses, all of whom were positive for both lupus anticoagulant and anticardiolipin antibodies or a lupus anticoagulant alone. Because the women in this study were not evaluated for other causes of habitual abortion, such as husband-wife immunologic incompatibilities, it is not possible to exclude these as causes of habitual abortions in this study. The results of our study show an association but not a cause and effect relationship between antiphospholipid antibodies and pregnancy loss; such as relationship could only be shown in large prospective cohort studies. Although our results are likely to be valid for patients with SLE, it cannot be concluded that there is an association between the presence of antiphospholipid antibodies and pregnancy loss in patients without SLE.

The aim of our study was not to determine the best test for lupus anticoagulant or combination of tests that correlate with clinical outcomes. Rather, it confirms our hypothesis that a combination of tests is superior to a single test and that testing on more than a single occasion increases the strength of the clinical associations. No single test provides significant superiority over any other test, although the relatively small number of patients evaluated does not allow us to exclude the possibility that one test is superior to another. The variability of test positivity between patients and within the same patient, over time, suggests variability in the titer of antibodies and heterogeneity of the antibodies being detected. Although we cannot exclude an effect on test results due to corticosteroid or immunosuppressive therapy, we do not believe that this biases the results of the study because there was no clear relationship between the use of these agents and patterns of anticardiolipin antibody testing. It is likely that the results of our study can be extrapolated to most populations of SLE patients because a proportion of patients in most SLE clinics will be taking corticosteroid and/or immunosuppressive therapy.

This study points to likely explanations for the lack of demonstration of an association between antiphospholipid antibody positivity and pregnancy loss in previous studies. By optimizing the pattern of testing, namely by performing multiple lupus anticoagulant tests and by repeating testing on more than one occasion, and by using rigorous laboratory methods and interpretation of the tests, the strength of the association between antiphospholipid antibody positivity and pregnancy loss can be shown.

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Relationship of antiphospholipid antibodies to pregnancy loss in patients with systemic lupus erythematosus: a cross-sectional study [see comments]

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