ARTERIAL DISEASE IN LUPUS AND SECONDARY ANTIPHOSPHOLIPID SYNDROME: ASSOCIATION WITH ANTI-BETA2-GLYCOPEPTIDE I ANTIBODIES BUT NOT WITH ANTIBODIES AGAINST OXIDIZED LOW-DENSITY LIPOPROTEIN

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SUMMARY

The prevalence and clinical significance of antibodies against β2-glycoprotein I (anti-β2-GPI) and antibodies against oxidized low-density lipoprotein (anti-ox-LDL) were evaluated as potential indicators of arterial disease in patients with systemic lupus erythematosus (SLE) and SLE with secondary antiphospholipid syndrome (APS). IgG anti-β2-GPI and IgG anti-ox-LDL were measured by enzyme-linked immunosorbent assay (ELISA) in serum samples from 118 patients with SLE, including 40 with secondary APS. IgG anti-β2-GPI were positive in 17% (20/118) of SLE patients. The presence and titres of IgG anti-β2-GPI were strongly associated with a history of arterial thrombosis. Haemolytic anaemia was also significantly associated with the presence of IgG anti-β2-GPI. The prevalence of IgG anti-ox-LDL was 53% (63/118), but there was no association with arterial thrombosis. The correlation between the values of anti-ox-LDL and those of anti-β2-GPI was found. These results suggest that IgG anti-β2-GPI could be a marker for arterial thrombosis in SLE patients, while IgG anti-ox-LDL were not associated with arterial disease in this group of lupus patients.

KEY WORDS: Atherosclerosis, Thrombosis, Anticardiolipin antibody, Antiphospholipid antibody, Haemolytic anaemia.
a lipid-binding protein, apolipoprotein B [20]. It had been suggested that anti-ox-LDL might be directed against β₂GPI, which in turn obtains increased antigenicity by binding to ox-LDL [24]. Matsuda et al. [25], however, recently suggested that anti-ox-LDL is an antibody to ox-LDL itself, and it is not an antibody to cryptic or neoantigenic β₂GPI.

Antibodies against β₂GPI have been found in patients with SLE and APS, and are associated with thrombosis [26–31]. Recently, we have demonstrated that anti-β₂GPI, using human β₂GPI as an antigen in the absence of cardiolipin on irradiated plates, is a more specific marker for the APS than aCL detected by the conventional aCL assay [32]. We have also shown that the titres of anti-ox-LDL in APS patients with arterial thrombosis are higher than those without such events [33]. In this study, we have investigated the prevalence of those antibodies in patients with SLE and SLE with secondary APS, and their correlation with arterial diseases.

PATIENTS AND METHODS

Patients

A total of 118 patients attending the Lupus Clinic at St Thomas’ Hospital were included in this study (114 females and four males; mean age 40 yr (range 10–71]). The average follow-up was 10.9 yr (range 1–41). All patients fulfilled the revised ARA criteria for the classification of SLE [34]. Of the 118, 40 patients were diagnosed as having secondary APS according to the proposed criteria [35]. Patients’ records were carefully reviewed, including the presence of risk factors for arterial disease. Hypercholesterolemia was defined as a total plasma cholesterol level >5 mmol/l. A history of hypertension was considered when the patient was under antihypertensive therapy or had in the supine position a diastolic blood pressure of >90 mmHg or a systolic blood pressure of >160 mmHg on three measurements in the hospital. Most of the patients included in our study (109/118, 92%) had been on steroids for an average of 6.3 yr. A patient was considered to be on a high dosage of steroids when receiving >20 mg/day of prednisolone or equivalent any time. Diagnostic techniques for arterial thrombosis included magnetic resonance imaging (MRI) and/or computed tomography (CT) scans of the head, biochemistry, electrocardiogram, myocardial perfusion scans, coronary angiography, renal scintigraphy and Doppler ultrasound scanning and venography in cases of venous thrombosis. One hundred and four sex- and age-matched healthy controls were also included.

Anti-β₂GPI ELISA

Anti-β₂GPI were measured as previously described [32]. Briefly, irradiated microtitre plates, Type C (Sumilon Bakelite, Tokyo, Japan) were coated with 50 μl of 4 μg/ml of purified human β₂GPI in phosphate-buffered saline (PBS) and incubated overnight at 4°C. After washing with PBS, plates were blocked with 3% gelatine for 1 h at 37°C. After three washes with PBS containing 0.05% Tween 20 (PBS–Tweem), 50 μl of serum samples diluted in PBS containing 1% bovine serum albumin (BSA) (Sigma Chemical Co., St Louis, MO, USA) were added in duplicate and incubated for 1 h at room temperature, followed by 50 μl/well of a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-human IgG (Sigma). One hundred microlitres per well of 1 mg/ml p-nitrophenylphosphate disodium (Sigma) in 1 m diethanolamine buffer (pH 9.8) were added. Optical density was measured at 405 nm (OD 405) by a Titertek Multiskan MC apparatus (Flow Laboratories, Herts.). The anti-β₂GPI titre of each sample was derived from the standard curve according to the dilutions of the positive control.

Anti-ox-LDL ELISA

Antibodies against ox-LDL were detected as previously reported [33]. Native LDL was isolated by sequential ultracentrifugation from pooled plasma of healthy fasting adults and dialysed for 30 h against PBS. Malondialdehyde (MDA) was freshly generated from malonaldehyde bis dimethylacetal by acid hydrolysis as described by Palinsky et al. [36]. MDA–LDL was prepared by incubating LDL with MDA and, after conjugation, MDA–LDL was dialysed extensively against PBS.

For the ELISA, half of a microtitre plate (Immulon 4) (Dynatech Laboratories Inc., Virginia, USA) was coated with LDL and the other half with MDA–LDL, both at 5 μg/ml in PBS containing 2 mm ethylenediaminetetraacetic acid tetrasodium salt (EDTA) and 20 μm butylated hydroxytoluene (Sigma), incubated at 37°C for 2 h and then overnight at 4°C. After washing four times with PBS–Tweem, wells were blocked with 150 μl of 0.5% gelatine for 1 h at 37°C and washed again. Fifty microlitres per well of serum samples diluted 1:100 in PBS–Tweem containing 1% BSA were added in duplicate and incubated for 2 h at room temperature. Plates were washed and alkaline phosphatase-conjugated goat anti-human IgG (Sigma) in the sample diluent was added for 1 h at room temperature. After the addition of substrate, the colour was read at a wavelength of 405 nm. Results were expressed as OD values, and binding to ox-LDL was calculated by subtracting the binding of antibody to native LDL from that to MDA–LDL.

Statistical analysis

All statistical analysis was performed by Statview II (Apple Macintosh software). Categorical analysis was by χ² test and comparisons between two groups were determined by the Mann–Whitney non-parametric test.

RESULTS

Clinical features

Thirty-three patients (28%) had a history of arterial thrombosis, symptomatic cerebrovascular disease being the most frequent finding (Table I). Twenty-seven of the patients among those with arterial thrombosis (82%) were diagnosed as having secondary APS. Twenty-eight patients (24%) developed venous throm-
bosis, and 35 of the 111 women with available obstetric data (31%) had pregnancy loss, although only 15% (17/111) had two or more losses.

Fifty-nine per cent of our patients had at least one risk factor for arterial thrombosis. Hypercholesterolaemia was recorded in 19% (22/114) of our SLE patients, 32% (34/105) smoked regularly and 28% (33/118) had a history of hypertension. Only one patient had diabetes mellitus defined by American Diabetes Association criteria [37]. However, when analysed independently, only hypertension was significantly associated with arterial thrombosis (15/33, 45% vs 21%, 18/85; \( \chi^2 = 5.8, P < 0.01 \)) while there were no differences in the presence of hypercholesterolaemia or smoking habit between both groups. A total of 58% of the patients (62/106) who received corticosteroids were on high dosage during the follow-up. No association was found between a history of steroid treatment and the presence of arterial thrombosis (\( \chi^2 = 2.34, P = n.s. \)). No association was found with high dosages of steroids (\( \chi^2 = 0.003, P = n.s. \)).

**Serological results**

IgG anti-\( \beta_2 \)-GPI were found in 17% (20/118) of our patients, 18 of them (90%) diagnosed as secondary APS, but in only 2% (2/104) of healthy controls (\( \chi^2 = 12.34, P < 0.01 \)). The titre of anti-\( \beta_2 \)-GPI correlated strongly with that of aCL (correlation coefficient \( r = 0.781, P = 0.0001 \)). In the group of patients with IgG anti-\( \beta_2 \)-GPI, a history of arterial thrombosis was found more frequently than in anti-\( \beta_2 \)-GPI-negative patients (14/20, 70% vs 19%, 19/98; \( \chi^2 = 21.1, P < 0.01 \)). Titres of IgG anti-\( \beta_2 \)-GPI were significantly higher in patients with a history of arterial thrombosis than in those without (Fig.1). On the other hand, IgG anti-ox-LDL were found in 53% (63/118) of SLE patients, but in 6% (6/104) of healthy controls (\( \chi^2 = 56.3, P < 0.01 \)). There was no association between the presence of anti-ox-LDL and a history of arterial thrombosis, and the titres of these antibodies were not different between patients with and without this complication (Fig. 2). The association of other clinical manifestations with anti-\( \beta_2 \)-GPI and anti-ox-LDL is shown in Table II. These data were analysed further by comparing SLE patients with and without secondary APS. Only anti-\( \beta_2 \)-GPI were more frequent in the group of patients with secondary APS (\( \chi^2 = 30.8, P = 0.0001 \)), while the presence of anti-ox-LDL was not statistically different between both groups (\( \chi^2 = 3.58, P = n.s. \)).

**DISCUSSION**

The present study shows that IgG anti-\( \beta_2 \)-GPI are associated with a history of arterial thrombosis. Despite a high prevalence of anti-ox-LDL, we could not find any association between the presence of these antibodies and arterial thrombosis.

SLE is an autoimmune disorder with a wide range of clinical and laboratory manifestations, and arterial disease is an important complication. Patients with SLE have many risk factors for arterial disease, including hypertension and abnormalities in the lipid profile. In our population, only hypertension was associated with arterial thrombosis, while a history of treatment with a high dosage of steroids did not seem to play a role in acute events. Several studies have shown that over one-third of SLE patients had a positive aCL titre [38, 39], but only high titres of aCL are specific for the diagnosis of the APS. It has been reported that anti-\( \beta_2 \)-GPI represent a more specific marker for the clinical features of the APS than aCL detected by the standard assay [26–32]. In fact, anti-\( \beta_2 \)-GPI might be able to suppress the natural anticoagulant function of \( \beta_2 \)-GPI, facilitating thrombotic episodes. APS, unlike other thrombophilia states, is associated not only with venous thrombosis, but also with arterial complications. Thus, it has been suggested that additional factors could be involved in the pathophysiology of arterial thrombosis in APS, over and above this procoagulant state. It has been shown that in the presence of anti-\( \beta_2 \)-GPI, ox-LDL uptake by macrophages is markedly increased [19]. This indicates that anti-\( \beta_2 \)-GPI may favour the atherogenic process [19] and subsequent arterial thrombosis. In this study, we found a strong association between the presence and titres of anti-\( \beta_2 \)-GPI and a history of arterial thrombosis in SLE patients. Although there are many mechanisms implicated in the development of arterial complications in SLE, our data suggest that secondary APS could represent an important cause of arterial thrombosis in SLE. On the other hand, 53% of our patients were positive for anti-ox-LDL. This high prevalence in SLE
Fig. 1.—Titres of IgG anti-β2GPI in 118 patients with (n = 33) and without (n = 85) arterial thrombosis. SLE patients are represented by open circles and SLE with secondary APS by closed circles. Anti-β2GPI were determined by ELISA. SLE patients with arterial thrombosis showed higher titres of anti-β2GPI than those without (Mann–Whitney, P = 0.0004). The continuous line represents the cut-off value for the anti-β2GPI assay.

Fig. 2.—Titres of IgG anti-ox-LDL in 118 SLE patients with (n = 33) and without (n = 85) arterial thrombosis. SLE patients are represented by open circles and SLE with secondary APS by closed circles. There was no difference in the titres between the two groups. The continuous line represents the cut-off value for the anti-ox-LDL assay.

has been reported by others [24]. We could not find any association between the presence of these antibodies and AT, which is also consistent with the Finnish population [24]. Long-term and prospective studies are necessary to evaluate whether anti-ox-LDL might have a clinical significance in the pathogenesis of the arterial complications in SLE. In this study, anti-β2GPI were associated with a history of haemolytic anaemia. Some authors have suggested that autoimmune haemolytic anaemia should be considered as a manifestation of APS in SLE since aPL might induce erythrocyte destruction, possibly by direct binding and complement-mediated damage or by receptor-mediated entrapment by the reticuloendothelial system. Previous studies have suggested an association of IgM aCL with haemolytic anaemia [40–42]. In our series, we confirmed this finding. Whether haemolytic anaemia associates with anti-β2GPI or relates primarily with aCL remains unknown. aPL might recognize phospholipids in the erythrocyte membrane together or in close association with membrane proteins [43].
In conclusion, this study recognizes that the co-existence of atherosclerosis and aPL does not necessarily indicate a causal relationship. However, we suggest that certain autoantibodies, including anti-β₂GPI, may play a direct role in this accelerated arterial disease process.

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**REFERENCES**

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### TABLE II

Association of clinical or laboratory manifestations with anti-β₂GPI and anti-ox-LDL

<table>
<thead>
<tr>
<th>Manifestations</th>
<th>IgG anti-β₂GPI (n = 118)</th>
<th>IgG anti-ox-LDL (n = 20)</th>
<th>IgG anti-ox-LDL (n = 63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malar rash</td>
<td>60 (51%)</td>
<td>9</td>
<td>31</td>
</tr>
<tr>
<td>Discoid rash</td>
<td>18 (15%)</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Photosensitivity</td>
<td>48 (41%)</td>
<td>9</td>
<td>26</td>
</tr>
<tr>
<td>Oral ulcers</td>
<td>36 (31%)</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Arthritis</td>
<td>80 (68%)</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Pleuritis</td>
<td>56 (47%)</td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td>Pericarditis</td>
<td>15 (13%)</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>34 (29%)</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>Casts</td>
<td>6 (5%)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Convolutions</td>
<td>10 (8%)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Psychosis</td>
<td>9 (8%)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Haemolytic anaemia</td>
<td>8 (7%)</td>
<td>4*</td>
<td>5</td>
</tr>
<tr>
<td>Leucopenia</td>
<td>21 (18%)</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Lymphopenia</td>
<td>85 (72%)</td>
<td>14</td>
<td>49</td>
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<tr>
<td>Thrombocytopenia</td>
<td>17 (14%)</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Venous thrombosis</td>
<td>28 (24%)</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Pregnancy loss</td>
<td>35/111 (31%)</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>Recurrent pregnancy loss</td>
<td>17/111 (15%)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Anti-dsDNA antibodies</td>
<td>77/117 (66%)</td>
<td>13</td>
<td>48*</td>
</tr>
<tr>
<td>Anti-Sm antibodies</td>
<td>18/116 (15%)</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Venereal Disease Research Laboratory (VDRL)</td>
<td>2/117 (2%)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Anticardiolipin antibodies</td>
<td>45/38%</td>
<td>20</td>
<td>28</td>
</tr>
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<td>Lupus anticoagulant</td>
<td>30/95 (32%)</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Antinuclear antibodies</td>
<td>112/117 (96%)</td>
<td>19</td>
<td>59</td>
</tr>
</tbody>
</table>

*S*² test: *P* < 0.05.