Anti-Endothelial Cell Antibody, Thrombomodulin, and von Willebrand Factor in Idiopathic Inflammatory Myopathies

KONSTANTIN V. SALOJIN,‡ ANNE BORDRON,† EVGENI L. NASSONOV,† VLADIMIR Z. SHTUTMAN,† NATALIA G. GUSEVA,‡ ANDREI A. BARANOV,† IRA N. TARGOFF,§ AND PIERRE YOUINOU†*

Laboratory of Immunology, Brest University Medical School, Brest, France†; University of Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma§

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Sera from 19 patients with idiopathic inflammatory myopathy (IIM) were examined for the presence of anti-endothelial cell antibodies (AECA) by an immunoglobulin G-specific cellular enzyme-linked immunosorbent assay. The mean binding index of AECA was found to be 37.7% ± 26.5% for the patients, compared with a mean of 7.2% ± 2.7% for normal controls (P < 0.04). Levels of thrombomodulin, von Willebrand factor antigen, and serum creatine kinase were also shown to be augmented. Interestingly, positive correlations between AECA on the one hand and Raynaud’s phenomenon and interstitial lung disease on the other were demonstrated. Given that the pathogenesis of IIM remains uncertain, these findings may be of importance.

Idiopathic inflammatory myopathies (IIM) are a group of autoimmune disorders, encompassing polymyositis (PM), dermatomyositis (DM) and a number of myositis disorders associated with connective-tissue diseases (5), such as systemic lupus erythematosus (SLE), systemic sclerosis (SS), and mixed connective-tissue disease (MCTD). Arthritis, Raynaud’s phenomenon, and interstitial lung fibrosis are prominent features of all these conditions (17). Patients with IIM have been shown to make antibodies that recognize ribonuclear phosphoproteins, e.g., PM/Scl, signal recognition particle (SRP), Mi-2, and Jo-1 (21).

Despite the availability of these myositis-specific autoantibodies (MSA) for classifying the patients (13), it is notoriously difficult to predict the outcome of the disease. One of the reasons is that the pathogenesis of these heterogeneous conditions remains uncertain. Though these disorders are believed to be mediated by cellular immune mechanisms, several investigators have identified immunoglobulin and complement in the muscles (10, 22) and focused on vascular deposition of autoantibodies in relation to pathogenesis. Relevant to this issue is the report of anti-endothelial cell antibodies (AECA) in PM/DM patients (6). The likelihood of such autoactivity was first suggested by indirect immunofluorescence analysis with mouse and rat kidney and liver as substrates (12, 20) and subsequently established by using purified immunoglobulin G (IgG) and F(ab’), fragments (7). AECA have since been detected in a variety of clinical conditions associated with vasculitis (15), showing promise as a sensitive indicator of endothelial cell (EC) damage.

Sera from a group of patients with IIM were, therefore, examined for the presence of AECA. The results were correlated to the serum titers of MSA, the concentration of serum creatine kinase (CK), as well as the levels of von Willebrand factor antigen (vWfAg) and thrombomodulin (TM) in plasma, which reflect EC injury (3, 4). Increased levels of AECA appeared to be associated with visceral complications in these patients with IIM.

MATERIALS AND METHODS

Source of sera. Nineteen Caucasian patients were recruited by faculty members in the Cardiology Research Center at the University of Moscow, Russia, all fulfilling the criteria of Bohan and Peter for adult-onset PM/DM (5). On the basis of these criteria, five and three patients were classified as having PM and DM, respectively. The 11 remaining patients suffered from connective-tissue disease-associated myositis, of whom 7 met the American College of Rheumatology criteria for SS (14) and 4 presented with clinical and serologic characteristics of MCTD (1). There were 13 women and 6 men, aged 22 to 72 years, and the mean duration of disease was 22.0 ± 11.2 months. Overall, 13 patients demonstrated nonerosive arthritis, 13 patients demonstrated Raynaud’s phenomenon, and 13 had clinical and radiological evidence for lung fibrosis. Global clinical assessment was based on a careful physical examination, including testing of manual muscle strength (neuromuscular score) and evaluation of daily activities.

Control sera were taken from 27 to 58 asymptomatic volunteers (27 serum samples for the AECA test, 40 plasma samples for the measurement of TM, 58 plasma samples for the detection of vWfAg, and 30 serum samples for that of CK). These were healthy medical students, members of the laboratory staff, and residents of a home for the aged located on the grounds of Brest University Hospital. Since levels of markers, notably vWfAg, have been shown to correlate with age, normal controls were matched by sex and age to the patients.

AECA test. Since we were aware of a need for standardization of the AECA test (23), the EA.hy926 hybrid cells (a generous gift of Cora-Jean S. Edgell, Chapel Hill, N.C.) were used as the substrate (9). Given that these cells express a variety of molecules, of which only a proportion are specific for EC (18), all the sera were absorbed with AS49/8 cells, the epithelial parent of the hybrid cell line (also kindly provided by Cora-Jean S. Edgell), before evaluation. Briefly, 400 μl of control or patient serum, diluted 1/800 in phosphate-buffered saline (PBS), was incubated with 5 × 10⁵ AS49/8 cells for 1 h at 37°C and centrifuged at 500 × g for 10 min. Sera were collected, not diluted further, and incubated again with another 5 × 10⁵ cells overnight at 4°C. Supernatants were concentrated back to 1/50 (Vivapore concentrators; Vivascience, Binbrook, United Kingdom) and used in the AECA test.

The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum plus 2 μM glutamine, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (Sigma Chemical Co., St. Louis, Mo.). They were harvested by using 0.25% trypsin-EDTA (Sigma), before evaluation. Briefly, 400 μl of control or patient serum, diluted 1/800 in phosphate-buffered saline (PBS), was incubated with 5 × 10⁵ AS49/8 cells for 1 h at 37°C and centrifuged at 500 × g for 10 min. The cells were collected, not diluted further, and incubated again with another 5 × 10⁵ cells overnight at 4°C. Supernatants were concentrated back to 1/50 (Vivapore concentrators; Vivascience, Bimbrook, United Kingdom) and used in the AECA test.

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The results were correlated to the serum titers of MSA, the concentration of serum creatine kinase (CK), as well as the levels of von Willebrand factor antigen (vWfAg) and thrombomodulin (TM) in plasma, which reflect EC injury (3, 4). Increased levels of AECA appeared to be associated with visceral complications in these patients with IIM.
and a member of the staff, respectively. After another three washes with PBS-BSA, horseradish peroxidase-conjugated goat F(ab')2 anti-human IgG (Jackson Immunoresearch, West Grove, Pa.) was added and the samples were incubated for 1 h at 37°C. After a further wash, the plates were developed with 1.2-phenylenediamine, the reaction was stopped with sulfuric acid, and optical density was read in a Titertek Multiskan microplate reader (Flow Laboratories, McLean, Va.). The intraplate and interplate coefficients of variation were 4% (0.746 ± 0.060 [range, 0.777 to 0.857]) and 8% (0.814 ± 0.053 [range, 0.654 to 0.831]), respectively. The standard curve was constructed using normal reference samples. The results were expressed as AECA binding indices (BI) equal to 100 × (S − A)/(B − A), where S is the optical density of the test serum and A and B are those of a negative control (0.089 ± 0.013 [range, 0.084 to 0.107]) and a positive control (0.968 ± 0.086 [range, 0.856 to 1.110]), respectively. Samples were recorded as positive if the BI was greater than the mean of the normal group plus 3 standard deviations (SD), i.e., above 15%.

**RESULTS**

As shown in Table 1, the mean IgG AECA BI was found to be 37.7% ± 26.5% in patients with IIM, compared with a mean of 7.2% ± 2.7% in normal controls (P < 0.04). Five sera positive for AECA in the ELISA were also examined by IF and gave tissue staining on rat kidney sections (remiscient of the autoantibodies described originally). Raised levels of TM (61.9 ± 34.6 versus 23.3 ± 8.5 ng/ml [P < 0.03]), vWF Ag (2.0 ± 1.8 versus 0.8 ± 0.5 IU/ml [P < 0.05]), and CK (492.9 ± 487.1 versus 19.3% [P < 0.03]) were also demonstrated in these patients. With the cutoff level of AECA set at 15%, 14 of the 19 patients showed EC-binding activity at 3 SD above the mean of healthy controls. There was a trend for more of the connective-tissue disease-associated myositis patients (four of four MCTD patients and six of seven myositis-SS overlap patients) than of the PM (two of five) and DM (two of three) patients to be positive by the AECA test, and indeed, the BI was significantly higher (P < 0.02) in the former group of patients (49.9% ± 24.2%) than in the latter (21.0% ± 19.3%).

U-1-RNP antibody was detected in the four patients with MCTD; PM-Scl antibody was detected in two of the three myositis-SS patients tested. Jo-1, RP, Ro, and Mi-2 antibodies were found in one PM/DM patient each. There was, however, no relationship between raised levels of AECA and these autoantibodies (including MSA).

In contrast, a positive association was found between EC-binding antibody and Raynaud’s phenomenon, as well as interstitial lung disease (Table 2). Whereas high concentrations of TM and vWF were not reflected in the presence of these complications, there was a significant correlation between the levels of TM and those of CK (r = 0.58 [P < 0.05]). None of these EC damage markers was significantly associated with the neuromuscular and daily activity scores.

**DISCUSSION**

Given the limitations of TM and vWF as circulating markers for EC damage in connective-tissue diseases, e.g., vasculitis (18) and systemic sclerosis (19), a cellular ELISA was developed to detect AECA in patients with IIM. Fourteen of 19 patients (77%) were shown to have EC-binding activities 3 SD above the mean for healthy controls. We interpret the SD as being high, because this sample population represents a variety of patients. Yet, the main message emerging from the present study is that EC-specific IgG autoantibodies were more often encountered in MCTD and SS-associated myositis than in idiopathic PM and DM. Since most of the MSA are relatively uncommon in PM/DM (21), and certain antinuclear antibodies are not specific for a given connective-tissue disease, AECA may be another potential tool for characterizing these two groups of IIM. Importantly, no difference in the level of AECA

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**TABLE 1. Levels of AECA, TM, vWF Ag, and serum CK in patients with IIM compared with normal controls and in patients with PM/DM compared with patients with connective-tissue disease-associated myositis**

<table>
<thead>
<tr>
<th>Group</th>
<th>AECA (%) [n]</th>
<th>TM (ng/ml) [n]</th>
<th>vWF Ag (IU/ml) [n]</th>
<th>CK (IU/ml) [n]</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIM</td>
<td>37.7 ± 26.5% [19]</td>
<td>61.9 ± 34.6% [19]</td>
<td>2.0 ± 1.8% [17]</td>
<td>492.9 ± 487.1% [18]</td>
</tr>
<tr>
<td>Normal controls</td>
<td>7.2 ± 2.7% [27]</td>
<td>23.3 ± 8.5% [40]</td>
<td>0.8 ± 0.5% [58]</td>
<td>151.7 ± 109.0% [30]</td>
</tr>
<tr>
<td>Difference</td>
<td>P &lt; 0.04</td>
<td>P &lt; 0.03</td>
<td>P &lt; 0.03</td>
<td>P &lt; 0.02</td>
</tr>
<tr>
<td>PM/DM</td>
<td>21.0 ± 19.3% [8]</td>
<td>59.3 ± 44.8% [8]</td>
<td>2.5 ± 2.2% [8]</td>
<td>482.2 ± 592.6% [8]</td>
</tr>
<tr>
<td>Difference</td>
<td>P &lt; 0.02</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*The data are means ± SD. CTD, connective-tissue disease. NS, not significant.*

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**TABLE 2. AECA, TM, and vWF Ag in relation to visceral complications**

<table>
<thead>
<tr>
<th>Serologic finding</th>
<th>Arthritis</th>
<th>Raynaud’s phenomenon</th>
<th>Interstitial lung disease</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No. with</td>
<td>No. without</td>
<td>P</td>
</tr>
<tr>
<td>AECA</td>
<td>11/13</td>
<td>3/6</td>
<td>NS</td>
</tr>
<tr>
<td>TM increase</td>
<td>8/13</td>
<td>4/6</td>
<td>NS</td>
</tr>
<tr>
<td>vWF Ag increase</td>
<td>2/12</td>
<td>3/5</td>
<td>NS</td>
</tr>
</tbody>
</table>

*NS, not significant.*
was observed between patients positive and negative for MSA or antinuclear antibodies. Ro and La antibodies were never or rarely found, which is not surprising inasmuch as the former is associated with SLE and both are associated with Sjögren’s syndrome, rather than with IIM.

Noteworthy is the fact that the assay described here does not detect antibodies specific for structures on other blood cells; the autoantibodies we have detected are thus directed exclusively against EC. However, there was a clear relationship between high levels of AECA and the presence of Raynaud’s phenomenon as well as interstitial lung fibrosis. This is in line with our finding of AECA in SS (19) and the frequency of such autoantibodies in PM patients with lung complications (6). The antibody titer paralleled disease activity, as indicated by the correlation between the concentration of CK and the presence of AECA (r = 0.58 [p < 0.05]). Surprisingly, while AECA were more frequent in PM/DM patients than in connective-tissue disease-associated myositis patients, the levels of CK in serum were comparable in these two subgroups of patients. Therefore, a correlation between AECA titer and CK concentration could not be found in these small subgroups of patients.

Though vascular injury is not acknowledged as the hallmark of IIM (5), the levels of circulating TM and vWF were found to be increased (16). AECA may thus be pathogenetic, through complement activation (7), antibody-dependent cell cytotoxicity (15), or apoptosis of EC (8a). Serum TM originates mainly from damaged vascular EC, but also from platelets and monocytes. Furthermore, elevated levels of serum TM have been reported in patients with functional impairment of the kidney. Because of the discrepancies between the data currently available (8), serial measurements are still needed to clarify the diagnostic value of serum TM levels. With regard to vWF levels (8), the highest concentrations have been previously found in plasma of patients with necrotizing vasculitis, irrespective of the type of vasculitis. This lesion is not encountered in IIM.

In fact, the antigens recognized by the sera are still unknown. A subset of AECA may recognize negatively charged glucosaminoglycans (17a). Because the AECA from the above patients were not absorbed to the A549/8 cells, one may assume that the antiglucosaminoglycan subgroup of AECA does not represent all the EC activity in IIM.

Another attractive project aims at establishing EC clones in the hope that these would differentially express specific cell surface markers as potential targets for AECA. We have previously reported the successful establishment of several clones that appeared to be slightly different from each other (11). AECA from PM/DM and from overlap myositis might thus become distinguishable. No doubt the clones constitute valuable tools for analyzing different sets of AECA. Such an approach would be of importance in furthering the understanding of mechanisms involved in IIM.

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REFERENCES