Lymphocytotoxic autoantibodies in progressive systemic sclerosis

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Summary. In 53 patients with progressive systemic sclerosis (PSS) the lymphocytotoxic activity of their serum was measured in a microlymphocytotoxicity assay. In 21 of the 53 patients the test reacted distinctly positively in the heterologous system, and in 9 of these 21 also in the autologous system. After preparation of the immunoglobulins from these positive sera, whole cytotoxic activity was detected only in the IgM fraction but not in the IgG fraction. When using prepared T lymphocytes as target cells in the microlymphocytotoxicity test, the cytotoxic activity of the positive PSS sera showed itself to be directed against this lymphocyte population. Further analysis using the Western-blot technique showed that the IgM autoantibody in PSS sera reacted with the cell surface of CD4+ lymphocytes. The cross reactivity with extractable nuclear antigens was rather improbable. These results suggest that lymphocytotoxic autoantibodies may play a role in immunological disturbances in PSS.

Key words: Progressive systemic sclerosis — Microlymphocytotoxicity test — Lymphocytotoxic autoantibodies — Antinuclear antibodies — T-helper lymphocytes — Western-blot technique

Progressive systemic sclerosis (PSS) is a generalized disease with well-known abnormalities in the connective tissue metabolism [10]. The etiology is poorly understood; however, it is generally accepted that immunological disturbances play an important role in the triggering or maintenance of the disease [10]. This pathogenetic conception is further supported by the association of PSS with other autoimmune diseases [4, 26]. Autoantibodies that recognize a wide range of membrane, cytoplasmatic, and nuclear determinants have been detected in human sera. In particular these autoantibodies are diagnostically helpful, e.g., DNA antibodies are viewed as specific serologic markers of systemic lupus erythematosus (SLE). In other diseases they may be responsible for the pathogenesis, e.g., in bullous pemphigoid and pemphigus vulgaris.

The presence of a wide range of autoantibodies reacting with nuclear and structural proteins [15, 19, 21] and abnormalities in the cell-mediated immunity are well-described phenomena in PSS [3, 6]. Several previous reports have demonstrated reduced proportions of T lymphocytes [9] and their subpopulations [12] due to an increase in the T-helper cell function. These data, however, are partially in conflict with a number of studies demonstrating normal proportions of T lymphocytes and their subsets [1]. As a consequence of such immunological disturbances, immune cells may overproduce soluble mediators (lymphokines, monokines) which can stimulate the fibroblast metabolism leading to the typical histological and clinical findings in PSS [8, 28].

In recent studies we have also found a decrease in the absolute numbers of circulating T-lymphocytes using monoclonal antibodies and a distinct depression of their proclivity for rosette formation using sheep red blood cells (SRBCs) in patients with PSS [20].

On the other hand, the presence of lymphocytotoxic autoantibodies is a well-known laboratory phenomenon associated with SLE and to a lesser extent with other connective tissue diseases [18]. For this reason, we wanted to answer the question whether the diminished numbers of circulating T-lymphocytes are a result of the occurrence of lymphocytotoxic autoantibodies in PSS.

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Table 1. Antinuclear autoantibodies (ANAs) in the sera from patients with progressive systemic sclerosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Cases (n)</th>
<th>Antinuclear autoantibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IF⁺⁺⁺⁺ (HEp-2) positive</td>
<td>IF⁺⁺⁺⁺ (rat liver) positive</td>
</tr>
<tr>
<td></td>
<td>(n/%)</td>
<td>(n/%)</td>
</tr>
<tr>
<td>Acrosclerosis</td>
<td>12</td>
<td>7/58</td>
</tr>
<tr>
<td>CREST syndrome</td>
<td>19</td>
<td>14/74</td>
</tr>
<tr>
<td>Diffuse sclerosis</td>
<td>22</td>
<td>20/91</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>41/77</td>
</tr>
</tbody>
</table>

* IF, immunofluorescence method
b Sel-70: anti-Sel-70 antibody

**Material and methods**

**Patients**

The patient group consisted of 53 individuals, 42 women and 11 men, with a mean age of 62 years (range, 13–78 years). Patients were classified according to the ARA criteria [16]. The 53 scleroderma patients consisted of 12 patients with the acral form of the disease, 19 patients with the typical CREST syndrome (having at least 4 of 5 features such as calcinosis, Raynaud’s phenomenon, esophageal involvement, sclerodactyly, and telangiectasia), and 22 patients with a generalized diffuse form. In the majority of patients (46) the esophagus was involved, in some of them the lung (34), intestinum (12), heart (6), and kidney (4), as well. Forty-one patients exhibited antinuclear antibodies (Table 1).

The control group consisted of 83 healthy volunteers, 64 women and 19 men, with a mean age of 58 years (range, 24–82 years).

Blood from the patients and controls was allowed to clot at room temperature for 60 min and to react at 4°C for 120 min. After separation, serum was aliquoted and stored at −20°C. To inactivate complement, each serum aliquot was heated at 56°C for 1 h immediately before use.

**Determination of antinuclear antibodies (ANA)**

The indirect immunofluorescence technique was used to determine the patterns and titers of ANA with HEp-2 cells or rat liver sections as substrate. A clear fluorescent staining with discernible nuclear pattern at serum dilution of 1:40 in phosphate buffered saline (PBS) was considered to be a positive result in the present study. The end-point dilutions were used as titers of ANA.

The Sel-70 antibody as a specific marker of PSS was detected by immunodiffusion according to the method of Tan et al. [22]. The Sel-70 antigen, the extractable nuclear antigen (ENA), and the positive control serum were kind gifts from Dr. Th. Krieg, Munich, FRG and from the Center for Disease Control, Atlanta, USA.

**Lymphocyte separation**

Peripheral blood lymphocytes (PBLs) were separated from heparinized venous blood of PSS patients using the Ficoll-Hypaque density gradient centrifugation technique according to the method described by Boyum [2]. The separated cells were washed twice in 0.15 mol/l PBS at pH 7.2, once in Eagle’s basal medium, and adjusted to a concentration of 5 × 10⁶/l. The cell suspension contained 93%–97% viable lymphocytes as determined by the trypan blue dye exclusion technique. The separation of T lymphocytes was carried out using a sheep erythrocyte suspension according to Wybran and Fudenberg [30]. Briefly, monocyte-depleted lymphocytes obtained from Ficoll-Hypaque gradients were mixed with 0.5% sheep erythrocyte suspension in Eagle’s medium and incubated at 4°C for 1 h followed by density-gradient sedimentation on Visotrasst-Dextran 70. The remaining erythrocytes were eliminated by hypotonic shock and afterwards the T-cell suspension was washed three times with Eagle’s medium. Using monoclonal antibodies, specific for T lymphocytes (CD 3, Ortho Diagnostics) the prepared T lymphocytes showed a positive immunofluorescence of approximately 96%.

**Microlymphocytotoxicity test**

Lymphocytotoxic antibodies were determined using lymphocytes from 20 normal donors (heterologous system) or lymphocytes from 53 PSS patients (autologous system) in accordance with the microcytotoxicity technique originally described by Terasaki et al. [23]. Using this procedure we have also tested in parallel T lymphocytes isolated by rosette formation with sheep erythrocytes. The extent of the cytotoxic reaction was defined as the percentage of killed cells measured using the trypan blue dye technique: grade 0, < 10%; grade 1, 11%–25%; grade 2, 26%–50%; grade 3, 51%–75%; grade 4, > 76%. Cytotoxicity with complement alone or with complement plus normal serum was 10%.

**SDS-polyacrylamide gel electrophoresis and immunoblotting**

We have used membrane preparations of CD4⁺ HIV-infected H9 cells and extractable nuclear antigens as proteins for the SDS electrophoresis. The proteins were boiled for 10 min in SDS sample buffer and were electrophoresed by using 10% polyacrylamide slab gels under reducing conditions according to the technique of Laemmli [15].

Protein standards (BIO-RAD) were used to calibrate the molecular weight. After gel electrophoresis, proteins were electrophoretically transferred to sheets of nitrocellulose at 60 V for 3 h in 25 mM Tris-HCl (pH 8.3) containing 0.195 M glycine and 20% methanol, using an LKB Transphor Vertical Electrophorizzare Apparatus. The nitrocellulose was then soaked in PBS and saturated in 3% (w. vol.) nonfat milk powder in PBS, pH 7.2. Nitrocellulose strips were then incubated with the PSS sera diluted 1:100 in PBS containing 3% nonfat milk powder, followed by washing three times with PBS containing 0.02% Tween 20. Strips were incubated for 1 h with appropriate peroxidase-conjugated anti-immunoglobulin reagents and were developed for color [25]. The protein concentration was measured according to the method of Lowry et al. [14].

**Results**

Table 1 shows the distribution of antinuclear antibodies (ANA) in patients with PSS corresponding to their clinical forms and extent of skin involvement, respectively. With HEp-2 cells as the substrate for indirect immunofluorescence, 41 patients (77%) had ANA and with rat liver sections as substrate 37