Anti-Scl-70 antibodies detected by immunoblotting in progressive systemic sclerosis: specificity and clinical correlations

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SUMMARY One hundred and forty five serum samples from patients with a connective tissue disease and 30 serum samples from healthy blood donors were analysed by immunoblotting. The presence of anti-Scl-70, which seems to discriminate between progressive systemic sclerosis (PSS) and the CREST (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, telangiectasia) syndrome, was found in 31/64 (48%) patients with PSS, in 6/55 (11%) patients with systemic lupus erythematosus, in 2/26 (8%) patients with mixed connective tissue disease, and in none of 30 healthy blood donors. These data resulted in a specificity of 93% for this antibody in systemic sclerosis. For patients with PSS the duration of disease was significantly shorter in those with anti-Scl-70 antibodies than in those without, whereas the presence of anti-Scl-70 did not correlate with severity of disease. An 82% prevalence of anticientromere antibodies in patients with the CREST variant compared with a 4% prevalence in patients with PSS or with overlap syndrome confirms the high diagnostic value of this autoantibody for the CREST variant of PSS.

Progressive systemic sclerosis (PSS) has been defined as a generalised disorder of small arteries, microvessels, and the diffuse connective tissue, characterised by scarring (fibrosis) and vascular obliteration in the skin, gastrointestinal tract, lungs, heart, and kidneys, in which hidebound skin is the clinical hallmark, and organ compromise the prognostic keystone. Various autoantibodies reacting with nuclear components may be present in the sera of patients with scleroderma. Antibodies that precipitate a soluble 70 000 dalton nuclear protein called Scl-70, identified recently as topoisomerase I, and antibodies directed against the centromere region of chromosomes have been reported to characterise respectively diffuse systemic sclerosis and the CREST variant of systemic sclerosis. Antibodies to Scl-70, which are considered almost specific for PSS and are also called Scl-86, are found in only up to 20% of patients with diffuse PSS and therefore are of limited diagnostic value using standard techniques. Anticientromere antibodies, however, are present in 44–98% of patients with the CREST syndrome as opposed to only 6–12% in PSS. These data were obtained in studies using routine techniques such as counterimmunoelectrophoresis or immunodiffusion. In the past decade detection of antinuclear antibodies by immunoblotting was introduced.

The purpose of our study was to analyse the different antinuclear antibodies in a group of 64 patients with PSS from a single centre, to compare the results obtained with different methods, such as immunodiffusion and immunoblotting, for antinuclear antibody determination, and to define the specificity and sensitivity of these autoantibodies and their correlation with the clinical features.

Patients and methods

The study was performed retrospectively. We considered 64 patients with PSS who attended our...
centre between 1985 and 1987 and for whom serum samples were available. They were classified into three types according to Barnett and Coventry. Type I comprised patients with sclerodactyly (n=15), type II those with skin changes involving hands, forearms, face, and neck (n=13), and type III those in whom the skin changes were diffuse and usually most marked on the trunk (n=7). Forty nine of 63 (78%) patients fulfilled the recent criteria of the American Rheumatism Association (ARA) for diagnosis of PSS.12

Patients with the CREST variant of the disease were identified in a separate group (n=11) as were patients with PSS with features of overlap syndrome (n=18). Our CREST population was characterised by the prominence of calcinosis, Raynaud’s phenomenon, sclerodactyly, telangiectasia, associated in two patients with oesophageal dysfunction.

The case reports were reviewed for sex, age, clinical manifestations, available laboratory and immunological data, histological and radiological documents, treatment, and outcome.

The control population consisted of 55 patients with systemic lupus erythematosus (SLE), who fulfilled at least four of the ARA 1982 revised criteria for the classification of SLE, 26 patients with mixed connective tissue disease (MCTD) according to the Alarcon-Segovia classification, and 30 healthy blood donors.

STANDARD TECHNIQUES
Serum samples were collected at the time of clinical examination, stored at -20°C until use, and analysed without knowledge of the clinical data, together with the serum from control groups and healthy blood donors.

Immunofluorescence
Antinuclear antibodies were detected by indirect immunofluorescence on two different substrates: unfixed rat liver sections and acetone treated HEP-2 cell preparations. In each case the conjugate used was a fluorescein labelled polyvalent antihuman immunoglobulin raised in goats.

Immunodiffusion
Antibodies to extractable nuclear antigen were assayed by double immunodiffusion in agarose using the Ouchterlony technique against saline soluble antigens either from fresh calf thymus extract (U1RNP, Sm, SSB/La) or from human spleen extract (SSA/Ro). The extract of rabbit thymus powder (Pel-Freez, Rogers Arkansas, USA) used as the source of Scl-70 was made fresh as the Scl-70 antigen is labile. The precipitating reaction was read after a three day incubation at 6°C in a humid atmosphere and compared with an anti-Scl-70 reference serum.

Immunoblotting
For blotting analyses a total nuclear protein fraction from HeLa cells was prepared. Protein samples were dissolved in 2% sodium dodecyl sulphate, 10% glycerol, 5% β-mercaptoethanol, 0·1 M TRIS HCl pH 6·8. Samples were heated for three minutes at 100°C followed by electrophoresis on 12% polyacrylamide gels. Proteins were loaded over the entire width of the gel and separated according to their molecular weight. After an 18 hour run at 50 mA per gel (50 V/cm) replicas of the gels were made in nitrocellulose by transferring the proteins electrophoretically using a Bio-Rad trans blot system. Transfer was performed for four hours at 40 mA (80 V/cm) and at 25°C in 192 mM glycercol, 25 mM TRIS pH 8·3, and 20% methanol. The protein blots were cut into strips of about 6 mm and treated with preincubation buffer (3% bovine serum albumin, 10 mM NaCl, TRIS HCl pH 7·6, 0·5 mM phenylmethylsulphonyl fluoride (PMSF)) for two hours at room temperature under constant agitation to saturate protein binding sites on the nitrocellulose.

Incubation with diluted patient’s serum (1/20) was performed for two hours in the following buffer: 0·3% bovine serum albumin, 150 mM NaCl, 10 mM TRIS HCl pH 7·6, 0·1 mM PMSF, 1% Triton-X 100, 0·5% deoxycholic acid, and 0·1% sodium dodecyl sulphate. After extensive washing with this buffer (5×5 minutes) IgG and IgM immune complexes were detected by incubating the blots for one hour with a horseradish peroxidase conjugated IgG fraction goat antihuman γ chain specific or μ chain specific serum (Cappel Labs, Cochranville, PA), diluted 1:500 or 1:200 in 0·5% bovine serum albumin phosphate buffered saline, 0·5% Triton-X 100. After five additional washes the immunostain reaction was visualised with diaminobenzidine and H2O2 as the substrate. A kit of seven coloured protein standards (Amersham-Rainbow marker) was used to determine molecular weights ranging from 200 kilodaltons (myosin) to 14·3 kilodaltons (lysozyme). Anti-Scl-70 antibody was identified as an immunostain reaction at 92 kilodaltons and compared with an anti-Scl-70 reference serum.

STATISTICAL ANALYSIS
χ² Tests, with or without Yates’s correction for small groups, Student’s t test for paired groups, and Fisher’s exact test were performed.

Results
Table 1 shows the demographic characteristics of the
patients with CREST, PSS, and overlap syndrome. Ten of 64 (16%) patients were male. The patients with CREST were significantly older than those with overlap syndrome (p<0.05). The mean duration of disease was significantly longer in patients with CREST than in patients with PSS type III (p<0.02) and patients with overlap syndrome (p<0.05).

Antinuclear antibodies as determined by indirect immunofluorescence on rat liver sections were present in the sera of 44/64 (69%) patients. The most common pattern was homogeneous 23/44 (52%), followed by a speckled 15/44 (34%) and a nucleolar pattern 6/44 (14%).

With HEp2 cells as substrate antinuclear antibodies were detected in 47/64 (73%) patients' sera. Eight of 11 serum samples from patients with CREST were negative on liver sections, seven of these nine showed a speckled pattern on HEp2 cells characteristic of anticentromere antibodies (Table 2). A comparison of antinuclear antibodies detected on rat liver sections and on HEp2 cells showed a similar pattern in 31/64 (48%) cases. Antinuclear antibodies were detected in eight cases exclusively on liver section, in seven cases exclusively on HEp2 cells, and in either one or the other test in 55/64 (86%) patients.

Anticentromere antibodies were found in nine (82%) of the 11 patients with CREST and in two of

Table 1  Demographic characteristics of the patients with systemic sclerosis (n=64)

<table>
<thead>
<tr>
<th></th>
<th>CREST (n=11)</th>
<th>PSS*</th>
<th>Overlap syndrome (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I (n=15)</td>
<td>II (n=13)</td>
<td>III (n=7)</td>
</tr>
<tr>
<td>Sex ratio (M/F)</td>
<td>0/11</td>
<td>3/12</td>
<td>1/12</td>
</tr>
<tr>
<td>Mean (SD) age (years)</td>
<td>66-4 (10)</td>
<td>59-4 (15-3)</td>
<td>54-2 (11-3)</td>
</tr>
<tr>
<td>(Range)</td>
<td>(36-75)</td>
<td>(15-75)</td>
<td>(22-65)</td>
</tr>
<tr>
<td>Mean (SD) disease duration (years)</td>
<td>25-9 (16-9)</td>
<td>15-3 (16-1)</td>
<td>11-0 (7-4)</td>
</tr>
<tr>
<td>(Range)</td>
<td>(1-52)</td>
<td>(1-49)</td>
<td>(1-31)</td>
</tr>
</tbody>
</table>

*PSS=progressive systemic sclerosis.

Mean age: CREST v overlap syndrome: p<0.05.
Mean disease duration: CREST v type III PSS: p<0.02; CREST v overlap syndrome: p<0.05.

Table 2  Prevalence of antinuclear antibodies on rat liver sections and HEp2 cells and of anticentromere antibodies in various subtypes of systemic sclerosis. Values show number (%) of patients

<table>
<thead>
<tr>
<th></th>
<th>CREST (n=11)</th>
<th>PSS*</th>
<th>Overlap syndrome (n=18)</th>
<th>Total (n=64)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type I (n=15)</td>
<td>Type II (n=13)</td>
<td>Type III (n=7)</td>
<td></td>
</tr>
<tr>
<td>ANA (liver section)</td>
<td>3 (27)</td>
<td>10 (77)</td>
<td>7 (100)</td>
<td>15 (83)</td>
</tr>
<tr>
<td>ANA (HEp2 cells)</td>
<td>9 (82)</td>
<td>10 (67)</td>
<td>1 (8)</td>
<td>1 (6)</td>
</tr>
</tbody>
</table>

*p<0.000001 (x²=26) CREST v PSS.

†PSS=progressive systemic sclerosis; ANA=antinuclear antibodies.
Table 3 Prevalence of serum anti-Scl-70 antibody (anti-Scl-70) detected with immunodiffusion and immunoblotting in 64 patients with systemic sclerosis. Values show number (%) of patients

<table>
<thead>
<tr>
<th></th>
<th>CREST (n=11)</th>
<th>PSS* (types I-III) (n=35)</th>
<th>Overlap syndrome (n=18)</th>
<th>Total (n=64)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Scl-70 detected with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunodiffusion</td>
<td>0</td>
<td>6 (17)</td>
<td>0</td>
<td>6 (9)</td>
</tr>
<tr>
<td>Immunoblotting</td>
<td>2 (18)</td>
<td>22 (63)</td>
<td>7 (39)</td>
<td>31 (48)</td>
</tr>
</tbody>
</table>

*PSS=progressive systemic sclerosis.

the remaining patients, one with PSS type II and one with overlap syndrome (PSS associated with rheumatoid arthritis). With immunoblotting all the patients with anticentromere antibodies also showed the presence of autoantibodies against a 17 kilodalton antigen (Fig. 1).

With immunodiffusion, anti-Scl-70 antibodies were detected in 6/64 (9%) PSS sera. None of the controls (SLE, MCTD, healthy blood donors) was positive. Anti-Scl-70 antibodies were not detected in sera from patients with the CREST variant or with the overlap syndrome. All patients with anti-Scl-70 antibodies detected by immunodiffusion were also positive for anti-Scl-70 antibodies on immunoblotting.

With immunoblotting 31/64 (48%) sera were positive for an anti-Scl-70 protein antibody also recognised by the anti-Scl-70 reference serum. When only patients with PSS types I to III were considered anti-Scl-70 were detected with immunodiffusion in 6/35 (17%) sera and with immunoblotting in 22/35 (63%) sera (Table 3) ($\chi^2=15.2$, $p<0.0001$). Anti-Scl-70 antibodies were also found in 6/55 (11%) patients with SLE, in 2/26 (8%) patients with MCTD, and in none of 30 healthy blood donors (Table 4). As calculated from our data the anti-Scl-70 antibody detected with immunoblotting has a specificity of 93% for PSS.

As judged by the clinical features associated with anti-Scl-70 antibodies or anticientromere antibodies, the duration of the disease was significantly shorter in patients with anti-Scl-70 antibodies than in those without ($p<0.02$), the mean age was similar in both groups. The two groups did not differ from each other for any other clinical (Table 5) or biological features such as erythrocyte sedimentation rate, rheumatoid factor, serum immunoglobulin concentrations, anti-tissue antibodies.

Table 4 Prevalence of serum anti-Scl-70 antibody (anti-Scl-70) detected with immunodiffusion and immunoblotting in 145 sera from patients with connective tissue disease and in 30 sera from healthy blood donors. Values show number (%) of patients

<table>
<thead>
<tr>
<th></th>
<th>Systemic sclerosis (n=55)</th>
<th>MCTD* (n=26)</th>
<th>HBD* (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Scl-70 detected with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunodiffusion</td>
<td>6 (9)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Immunoblotting</td>
<td>31 (48)</td>
<td>6 (11)</td>
<td>2 (8)</td>
</tr>
</tbody>
</table>

*SLE=systemic lupus erythematosus; MCTD=mixed connective tissue disease; HBD=healthy blood donors.

Table 5 Characteristics of 31 anti-Scl-70 antibody positive patients and 33 anti-Scl-70 antibody negative patients with systemic sclerosis. Values show number (%) of patients

<table>
<thead>
<tr>
<th></th>
<th>Anti-Scl-70 present (n=31)</th>
<th>Anti-Scl-70 absent (n=33)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) age (years)</td>
<td>54.4 (12.6)</td>
<td>52.2 (15)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean (SD) disease duration (years)</td>
<td>11.5 (9.5)</td>
<td>17.6 (15.6)</td>
<td>$p&lt;0.02$</td>
</tr>
<tr>
<td>Calcium</td>
<td>7 (23)</td>
<td>15 (45)</td>
<td>$p&lt;0.05$</td>
</tr>
<tr>
<td>Telangiectasia</td>
<td>14 (45)</td>
<td>19 (58)</td>
<td>NS</td>
</tr>
<tr>
<td>Digital pitting ulcers</td>
<td>12 (39)</td>
<td>15 (45)</td>
<td>NS</td>
</tr>
<tr>
<td>Arthritis</td>
<td>16 (52)</td>
<td>10 (30)</td>
<td>NS</td>
</tr>
<tr>
<td>Myositis</td>
<td>10 (32)</td>
<td>7 (21)</td>
<td>NS</td>
</tr>
<tr>
<td>Lung fibrosis</td>
<td>18 (58)</td>
<td>16 (48)</td>
<td>NS</td>
</tr>
<tr>
<td>Cardiac involvement</td>
<td>5 (16)</td>
<td>5 (15)</td>
<td>NS</td>
</tr>
<tr>
<td>Oesophageal dysfunction</td>
<td>19 (61)</td>
<td>15 (45)</td>
<td>NS</td>
</tr>
<tr>
<td>Renal involvement</td>
<td>3 (10)</td>
<td>3 (9)</td>
<td>NS</td>
</tr>
</tbody>
</table>
Discussion

Detection of antinuclear antibodies in 44/64 (69%) of the cases using rat liver section and 47/64 (73%) using HEP2 cells as substrate is comparable with previously published data.\textsuperscript{19,20} Recently, Giordano et al reported that antinuclear antibodies with a diffusely grainy and nucleolar pattern associated with anti-Scl-70 antibodies were significantly more prevalent in a form of PSS they called 'diffuse cutaneous systemic sclerosis',\textsuperscript{21} which was characterised by poor prognosis. This finding has not been confirmed by other authors.\textsuperscript{22} In our series the different fluorescent nuclear patterns did not correlate with the severity of the disease.

An 82% prevalence of anticentromere antibodies in patients with the CREST variant as compared with a 4% prevalence in patients with PSS or with overlap syndrome confirms the diagnostic value of this autoantibody for the CREST variant of PSS. The prevalence of anticentromere antibodies in patients with CREST has been estimated as between 55% and 96%.\textsuperscript{4,6,23}

Similar results were recently published by Rothfield et al using cloned autoantigen CENP-B for enzyme linked immunosorbent assay (ELISA) detection of anticentromere antibodies.\textsuperscript{24}

According to other authors, patients with the CREST variant are characterised by a significantly longer mean duration of disease and less involvement of internal organs.\textsuperscript{25,26} Weiner et al did not find that patients with scleroderma with anticentromere antibodies had a longer disease duration nor that these antibodies identify patients with less severe disease.\textsuperscript{27} When the mean age at diagnosis is considered the patients with anticentromere antibodies are significantly older than those without. We suggest that variability of serological and clinical characteristics is the consequence of differences in selection and classification of patients.\textsuperscript{1,5}

With immunoblotting anti-Scl-70 were found in 31/64 (48%) patients with PSS, in 6/55 (11%) patients with SLE, in 2/26 (8%) patients with MCTD, and in none of 30 healthy blood donors. The data resulted in a specificity of 93% of this antibody in systemic sclerosis. Anti-Scl-70 antibodies are therefore not absolutely specific for PSS as they are also found in SLE and MCTD. It is possible that reactions with unidentified intracellular peptides with the same molecular weight as Scl-70 have been identified and it is not certain that all the antibodies are truly against Scl-70. The sensitivity of anti-Scl-70 antibody detected by immunoblotting (48%) is significantly higher than that reported in previous studies using immunodiffusion as standard detection technique.\textsuperscript{3,5} In those studies anti-Scl-70 antibodies were detected in up to 20% of cases.

Our study showed that the specificity of anti-Scl-70 for PSS is lower by immunoblotting than immunodiffusion (93% v 100% in our series). The information contained in both tests is complementary as the source of antigen is not the same. Immunodiffusion is fast and reliable. With immunoblotting it is possible to obtain more precise information about the different antigenic components and, in addition, to detect several other specificities. The prevalence of anti-Scl-70 in the different subgroups was 18% in the CREST syndrome, 39% in scleroderma associated with overlap syndrome, and 63% in types I–III of diffuse scleroderma. These results re-emphasise the diagnostic value of this antibody, which seems able to discriminate between subsets of systemic sclerosis—for example, PSS and the CREST variant. This was first suggested by Tan et al.\textsuperscript{28} Comparable results have been published by Weiner et al, who found that anti-topoisomerase I was positive in 26% of patients who fulfilled the ARA criteria for systemic sclerosis and in 10% of patients with four clinical features of the CREST syndrome.\textsuperscript{23} With immunoblotting Van Venrooij et al detected the presence of autoantibodies against an 86 000 mol.wt marker antigen in 13/22 (59%) patients with diffuse scleroderma and in only 1/11 (9%) patients with limited scleroderma.\textsuperscript{7}

The presence of anti-Scl-70 antibody in patients with diffuse scleroderma according to the Barnett and Coventry classification does not correlate with the severity of the disease. Our data do not support the conclusion previously published that anti-Scl-70 antibodies are associated with skin ulcerations and cardiac\textsuperscript{23} or lung involvement.\textsuperscript{27,28} On the other hand, duration of disease was significantly shorter in patients with anti-Scl-70 antibodies than in those without. This suggests that screening for anti-Scl-70 antibody with immunoblotting is particularly useful in the early stages of the disease. Whether the presence of anti-Scl-70 antibody has any prognostic value so far as severity and progression of disease are concerned will have to be confirmed by prospective longitudinal studies.

We would like to thank the Swiss Rheumatology Society for financial support. The anti-Scl-70 reference serum was a gift from Dr Humbel, Centre Hospitalier. Luxembourg.

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doi: 10.1136/ard.48.12.992

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