Immunoblotting profiles in 55 systemic lupus erythematosus sera lacking precipitating antibodies to extractable nuclear antigens

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SUMMARY Serum samples from 55 patients with systemic lupus erythematosus (SLE) were selected for the absence of anti-extractable nuclear antigen antibodies after routine immunodiffusion tests. These sera were immunoblotted for anti-Sm and anti-RNP antibodies on a HeLa cell nuclear extract. Ten (18%) were negative and 45 (82%) produced complex patterns: 10 (18%) suggestive of anti-Sm, three (5%) anti-RNP, and 32 (58%) a combination of anti-Sm and anti-RNP antibodies. These data were very similar to those obtained from sera from a control group of 28 SLE sera selected for positivity of anti-Sm and anti-RNP precipitins with the immunodiffusion test. IgM isotype antibodies to the D peptide were significantly more prevalent than IgG isotype antibodies, whereas antibodies to the 68 kD polypeptide were of both IgM and IgG isotypes. Sera with an anti-Sm/RNP immunoblotting pattern stemmed from a group of patients with SLE with a higher titre of anti-dsDNA antibodies. Among clinical symptoms, the incidence of haemolytic anaemia was higher in the group of patients with the anti-Sm immunoblotting profile. Patients with an anti-RNP immunoblotting profile showed a higher incidence of cutaneous symptoms. It is concluded that immunoblotting for anti-Sm or anti-RNP antibody determination is a very sensitive diagnostic tool in patients with SLE.

Key words: antinuclear antibodies, Western blotting, anti-Sm/RNP antibodies.

Patients with systemic lupus erythematosus (SLE) produce antibodies which react with various nuclear components: DNA, histones, proteins, and RNA-protein complexes such as Sm and U1RNP antigens.1 The anti-Sm antibodies are considered to be a specific marker of SLE and constitute a major serological American Rheumatism Association criterion for diagnosis.2 Anti-U1RNP antibodies have a strong association with mixed connective tissue disease but are also present in SLE, frequently associated with anti-Sm antibodies 3-5 Anti-Sm antibodies, however, are less frequently present in SLE sera and are found in only 10–30% when immunodiffusion and counterimmunoelectrophoresis are used for their detection.1

Sera containing anti-Sm and anti-RNP react with a family of small nuclear RNA-protein complexes.67 Immunoblotting studies have shown that anti-Sm antibodies react preferentially with a set of at least two proteins of the RNP-protein complexes: B (25 kD) and D (14 kD) peptides,6 whereas anti-RNP sera react with the 68 kD, A (31 kD), and C (19 kD) peptides.6

The purpose of this study was to analyse the immunoblotting pattern in 55 patients with SLE lacking anti-Sm or anti-RNP precipitins on immunodiffusion and to investigate whether there were correlations between the blot pattern and distinct clinical and serological findings.
Patients and methods

PATIENTS AND SERA
Serum samples used in this study were obtained from 83 patients who fulfilled the revised American Rheumatism Association criteria for SLE.2 Fifty-five sera were found to be devoid of anti-Sm or anti-RNP precipitating antibodies were drawn from 55 patients with SLE (47 female, eight male, 51 white, four black; mean (SD) age: F 34 (12) years, M 31.5 (13.3) years) with a mean (SD) duration of disease of 7.2 (5.8) years.

Twenty-eight control sera, selected for a positive anti-Sm (23 sera) or anti-Sm and anti-RNP (five sera) precipitation test, were drawn from 28 patients with SLE (23 female, five male; 19 white, seven black, two Oriental) matched for the same mean duration of disease as the 55 patients with SLE without anti-Sm or RNP antibodies. All sera were stored at -20°C and complement inactivated at 56°C for 30 minutes before use.

IMMUNOBLOTTING FOR THE DETECTION OF ANTINUCLEAR ANTIBODIES

The technique described by Habets et al was used with only slight modifications.8 HeLa cell nuclear extract was dissolved in sodium dodecyl sulphate gel sample buffer and then subjected to 12% sodium dodecyl sulphate—polyacrylamide gel electrophoresis according to Laemmli9 for 18 hours at 50 V/0.3 A. After separation the nuclear proteins were transferred to transblot nitrocellulose sheets by electrophoretic blotting according to Towbin et al10 for four hours at 70 V/0.4 A. To probe for the presence of antigenic nuclear peptides the nitrocellulose strips were incubated with patient sera diluted 1:100 in phosphate buffered saline containing 0.1% Tween. Binding of the antibodies was detected by horseradish peroxidase conjugated rabbit anti-human IgG or IgM antibodies (Cappel, Fllobio France, 3201-0121) with dianimobenzidine as the substrate. Standard sera were applied on each blot as positive controls and as reference samples to allow identification of anti-RNP (68 kD band, A band, C band) and anti-Sm (B/B' doublet and D band) specificities.5

ASSAY FOR PRECIPITATING ANTIBODIES AGAINST EXTRACTABLE NUCLEAR ANTIGENS

Extractable nuclear antigens were prepared by the method of Mattioli and Reichlin11 from fresh calf thymus, or using an extract of rabbit thymus powder (Pel Freez, Rogers, Arkansas). The extractable nuclear antigen preparations were used at a final concentration of 40 mg/ml for calf thymus and 100 mg/ml for rabbit thymus in phosphate buffered saline pH 7.4. Antibodies were detected by immunoprecipitation with 0.8% agarose in phosphate buffered saline. Patients' sera were tested undiluted, and diffusion was carried out at room temperature in a humidified chamber for 48 hours.

Precipitins were identified by comparison with reference anti-RNP and anti-Sm sera from the Center For Disease Control, Atlanta, Georgia, USA12 and by searching for lines of complete identity with the patient's precipitin(s). Antibodies detected against Ro (SSA) antigen were also tested by immunodiffusion with a human splenic soluble extract13 and identified with a reference anti-SSA (Ro) serum.12

OTHER LABORATORY PROCEDURES

The following laboratory procedures were performed routinely: assay for antinuclear antibodies by indirect immunofluorescence on rat liver sections,14 and for anti-dsDNA antibodies by indirect immunofluorescence on Crithidia luciliae as described by Aarden et al.15

STATISTICS

Statistical comparisons between groups were made by the χ² method, with Yates's continuity correction, or Fisher's exact test if the number of patients was low. A value of p<0.05 was considered significant.

Results

Sera from the 55 patients with SLE without anti-Sm or anti-RNP precipitins showed a variety of immunoblot patterns of peptide reactivity (Table 1). Thirty-three sera (60%) reacted against the D peptide and 28 (51%) against the B/B' doublet. Twenty sera (36%) reacted against the 68 kD polypeptide; 14 (25%) and 17 (31%) sera reacted against the A and C peptide respectively. Ten sera

Table 1 Immunoblot results of sera from 55 patients with systemic lupus erythematosus without Sm or Smi/RNP precipitins. Values show the number of sera binding to specific polypeptides and detected with antisera specific for immunoglobulin isotype

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG and IgM</th>
<th>IgG or IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>68 kD</td>
<td>5</td>
<td>3</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>A (31 kD)</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>B/B' (25 kD)</td>
<td>6</td>
<td>7</td>
<td>15</td>
<td>28</td>
</tr>
<tr>
<td>C (19 kD)</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>D (14 kD)</td>
<td>3</td>
<td>10</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td></td>
<td>10</td>
<td>18</td>
</tr>
</tbody>
</table>

No %
Both antibody reactivities with the polypeptide in (18%) showed a negative Sm or RNP immunoblot reactivity on two different runs.

Table 1 also shows the patterns of IgG and IgM reactivities with the U1RNP and Sm polypeptides. Both antibody isotypes were present against each polypeptide in approximately 50% of the sera.

IgM was the dominant isotype of anti-D (14 kD) polypeptide positive sera ($\chi^2=4.7$; $p<0.05$), whereas IgG was the dominant isotype of anti-68 kD polypeptide positive sera.

Most sera produced a complex pattern suggestive of anti-Sm, anti-RNP, or anti-Sm and anti-RNP antibodies (Table 2). Ten sera (18%) recognised either the B/B' doublet or the D protein and were identified as anti-Sm sera. Three sera (5%) recognised the 68 kD polypeptide, alone or in conjunction with the A or C peptide, or both, and were called anti-RNP positive sera. Finally, 32 sera (58%) recognised simultaneously the 68 kD, B/B' doublet, and D peptides as well as either the A or C peptides and were identified as anti-Sm/RNP positive sera (Fig. 1). The control group of 28 SLE sera with Sm or Sm/RNP precipitins produced very similar immunoblot patterns of peptide reactivity (Table 2).

At the time of sampling 47 patients (85%) were positive in the indirect immunofluorescent test on Crithidia luciliae for anti-dsDNA antibodies: 21 patients had an anti-dsDNA antibody titre over 40. Among these 21 patients, 14 (67%) produced an anti-Sm/RNP pattern on immunoblots. One third of sera with an anti-RNP or anti-Sm pattern and 20% of sera with a negative reactivity produced a similarly high titre of anti-dsDNA antibody ($p=NS$). Sera from patients with anti-Sm/RNP antibodies had a low anti-dsDNA antibody titre less frequently than all the other groups of sera ($p<0.03$) (Table 3).

Table 2
Comparison of the immunoblot results of SLE sera without Sm or Sm/RNP precipitins and 28 SLE sera with Sm or Sm/RNP precipitins

<table>
<thead>
<tr>
<th>Immunoblot antibody specificity</th>
<th>Ouchterlony result for anti-Sm or Sm/RNP precipitins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>Sm*</td>
<td>10</td>
</tr>
<tr>
<td>RNP†</td>
<td>3</td>
</tr>
<tr>
<td>Sm/RNP‡</td>
<td>32</td>
</tr>
<tr>
<td>Blank</td>
<td>10</td>
</tr>
</tbody>
</table>

*Sm*=B/B'+D or D alone or B/B' alone.
†RNP=68 kD, alone or associated with either A or C polypeptides.
‡Sm/RNP=any combination of RNP polypeptides with Sm polypeptides.

Table 3
Relation between anti-dsDNA antibody titres and immunoblot antibody specificities of 55 systemic lupus erythematosus sera without Sm or Sm/RNP precipitins

<table>
<thead>
<tr>
<th>Immunoblot antibody specificity</th>
<th>Sm (n=10)</th>
<th>Sm/RNP (n=32)</th>
<th>RNP (n=3)</th>
<th>blank (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-dsDNA titre</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\leq 10$ (n=17)</td>
<td>6</td>
<td>6*</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>$&gt; 20$ (n=38)</td>
<td>4</td>
<td>26</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

*$\chi^2=5.3$; $p<0.03$.

To investigate a possible relation between immunoblotting results and clinicoserological findings in SLE the 55 patients were divided into four groups according to their immunoblotting patterns and...
listed according to the symptoms they displayed during the course of the disease (Table 4). Patients with anti-Sm/RNP antibodies showed a decreased incidence of Raynaud’s syndrome (p<0.05); patients with anti-Sm antibodies had an increased incidence of haemolytic anaemia (p<0.05). Patients with anti-RNP antibodies showed a higher incidence of cutaneous involvement (p<0.05). The incidence of other American Rheumatism Association criteria, such as renal or central nervous system involvement as well as vasculitis, was not statistically different in the anti-Sm group as compared with the anti-Sm/RNP and anti-RNP antibody positive groups.

Discussion

The aim of this study was to investigate the sensitivity of the immunoblotting technique for the detection of anti-Sm and anti-RNP nuclear antibodies in sera from patients with SLE lacking precipitins against these nuclear antigens. For this purpose we selected a group of 55 patients with SLE, comprising 35 from the rheumatology unit and 20 from the nephrology unit. All sera were repeatedly negative for anti-Sm and anti-RNP precipitins with the double diffusion technique against rabbit and calf soluble nuclear extracts. Several studies have shown that Sm and RNP antigens contain a series of small nuclear RNA species associated with at least eight polypeptides, which consist of a 68 kD polypeptide and others of lower molecular weight designated A to G, according to Lerner and Steitz nomenclature. The RNP antigenic determinants are present mainly on the 68 kD, A (31 kD), and C (19 kD) proteins; Sm determinants are found mainly on polypeptides B/B’ (25 kD) and D (14 kD).

The B/B’ polypeptides are sometimes faintly recognised by anti-RNP positive sera, however. In line with these previously published data we decided to designate anti-Sm as those antibodies which recognised either of the B/B’ and D polypeptides, anti-RNP as those antibodies which recognised the 68 kD polypeptide or either of the A and C polypeptides, and anti-Sm/RNP as those antibodies which recognised simultaneously any of the RNP polypeptides and either of the D or B/B’ polypeptides. According to Pettersson et al. and Van Venrooij it seems that anti-RNP specificities in patients with SLE are primarily composed of reactions with A and C polypeptides.

Using the immunoblotting technique in conjunction with this nomenclature, we found that 45 (82%) of the 55 SLE sera contained non-precipitating antibodies against the polypeptide components of the Sm and RNP particles: Sm polypeptides were recognised by 10 (18%) of the sera, RNP polypeptides by 3 (5%), and Sm plus RNP polypeptides by 32 (58%) of the sera.
These data are very similar to those obtained with our control group of 28 SLE sera selected for positive immunodiffusion precipitins against Sm or Sm/RNP antigens.

Our results suggest either that immunoblotting is a highly sensitive technique for detection of anti-Sm and anti-RNP antibodies or that the sensitivity of double immunodiffusion, in our hands, is poor. Recent data reported from our unit estimated the prevalence of anti-Sm precipitins in patients with SLE as 6% in 40 European Caucasians, 22% in 20 Arab North African patients, and 13% in 20 Caribbean black patients.16 The prevalence of anti-Ro antibodies was estimated to be 27% in a group of patients with SLE.17 These data are very similar to those published concerning Caucasian patients with SLE.16 18 Very few published reports have compared immunodiffusion and immunoblotting for the detection of anti-Sm/RNP antibodies in SLE sera: Williams et al reported that four of the 13 anti-Sm sera detected by immunoblotting were not detected by counterimmunoelectrophoresis and immunodiffusion.19 20 These sera were all IgM antibodies. They also observed four anti-RNP sera that were not detected by counterimmunoelectrophoresis and immunodiffusion. These sera contained specific antibodies of the IgG and IgM classes. Our data on antibody isotypes are in good agreement with published work.20 21 Only one study has previously reported differential positivity of counterimmunoelectrophoresis and immunoblotting.22 Of 66 SLE sera without anti-RNP precipitins as indicated by counterimmunoelectrophoresis, none was positive for anti-U1RNP polypeptide antibodies on immunoblotting, whereas 20 out of 71 sera without anti-Sm precipitins as indicated by counterimmunoelectrophoresis were positive for anti-Sm polypeptide antibodies on immunoblotting. The prevalence of anti-Sm and anti-RNP which we found by immunoblotting is rather high compared with these studies. Moreover, discrepancies between immunoblotting and immunodiffusion appear to occur in both directions as we found 5/28 sera in the control group with anti-Sm precipitins on immunodiffusion who had a negative immunoblot pattern. Such discrepancies have been reported previously20 22 and can be explained partly by the different species origin of the nuclear extract (calf and rabbit thymus for immunodiffusion, human HeLa cells for immunoblotting) or by the antibody isotype not being detected by our immunoblotting technique. As reported by previous studies an enzyme linked immunosorbent assay (ELISA) is more sensitive than counterimmunoelectrophoresis for the detection of anti-Sm and anti-RNP antibodies,23 24 but there is no published comparison of ELISAs and immunoblotting assays. Evans et al, however, were able to detect anti-U1RNP/Sm antibodies in some sera from patients with SLE that were negative for precipitin and ELISA assays. These data suggest but do not prove that immunoblotting may have a higher sensitivity than ELISA for the detection of anti-Sm and anti-U1RNP antibodies.

Studies concerning the clinical and serological significance of anti-Sm and anti-RNP antibodies detected by immunoblotting have shown different correlations: patients with SLE with anti-Sm/RNP antibodies have a higher titre of anti-dsDNA antibodies, a low incidence of Raynaud’s syndrome, and a high incidence of haemolytic anaemia; patients with SLE with anti-RNP antibodies have a higher incidence of cutaneous manifestations. In contrast with previously published reports of the clinical significance of anti-Sm and anti-RNP precipitating antibodies in SLE,25–30 we found no correlation between anti-Sm antibodies and vasculitis, renal disease, or central nervous system symptoms. Recent data using immunoblot suggested that patients with anti-Sm antibodies might have a higher incidence of renal disease.31 Our findings do not confirm this claim. Patients with anti-RNP antibodies had the same incidence of renal disease as patients with or without anti-Sm antibodies.

The reason for such discrepancies is still unclear, and further studies with more patients will be necessary to understand the prognostic value of anti-Sm and anti-RNP antibodies detected by immunoblotting.

Our present data suggest, however, that immunoblotting is a very sensitive method for the detection of anti-Sm and anti-RNP antibodies in SLE and could be very useful for diagnosis of forms of SLE with little symptomatology.

References
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Immunoblotting profiles in systemic lupus erythematosus sera


