Enzyme Immunoassay of Antibodies to Sjögren’s Syndrome B Antigen

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I describe a solid-phase enzyme immunoassay of IgG-, IgA-, and IgM-type antibodies to Sjögren’s syndrome B antigen. Polystyrene tubes are coated with the purified antigen. The antibodies are allowed to bind with their antigens, and then detected with alkaline-phosphatase-conjugated anti-human IgG, IgA, or IgM sera. The amount of alkaline phosphatase fixed to the tubes is determined in pH 10.0 diethanolamine buffer at 37 °C with p-nitrophenyl phosphate as substrate. The absorbance of the p-nitrophenolate ion liberated in 1 h at 37 °C is measured at 406 nm. When rheumatoid factor is present, the values obtained for IgG antibodies are too low, and those for IgM antibodies are too high.

Additional Keyphrases: antibodies to nuclear antigens rheumatoid factor

In Sjögren’s syndrome (SS), there is some evidence that the etiology and prognosis of the disease depend on the concentration of antibodies to SS-B antigen, changes in the serum concentration of these antibodies correlating with disease activity (1). Further studies of this relationship have been hampered by lack of a method for quantitative determination. The presence of SS-B antibodies in serum has been demonstrated earlier by double diffusion (1–4). However, the method is insensitive and nonspecific, and gives only a rough estimate of the concentration of these antibodies.

I present an enzyme immunoassay of SS-B antibodies. Enzyme immunoassay was selected because of its sensitivity, the small amounts of purified antigen needed, the ease of automation, and the possibility of separating the antibodies into the immunoglobulin classes.

SS-B antibodies have been found most frequently in sera of patients with Sjögren’s syndrome (1–8). Because these patients often have high rheumatoid factor activity, the effect of the presence of rheumatoid factor on the values obtained by enzyme immunoassay was also studied.

Materials and Methods

Blood samples. The material consisted of 157 sera, classified as follows:

1. SS-B-antibody-positive group: 43 sera that showed antibodies to SS-B antigen in double diffusion.
2. SS-B-antibody-negative group: 68 sera that revealed no SS-B antibodies in double diffusion but had antibodies against other extractable nuclear antigens. Antibodies to ribonucleoprotein were found in eight sera, to Sjögren’s syndrome A antigen in six, to Smith antigen in 24, to both ribonucleoprotein and Smith antigen in 10, to scleroderma-70 antigen in two, and to DNA in 18.

3. Controls: 46 sera from healthy blood donors (Finnish Red Cross).

SS-B antigen was purified from rabbit thymus acetone powder (Pel-Freez Biologicals, Rogers, AR 72756) by precipitation with ammonium sulfate, followed by gel filtration with Blue Sepharose CL-6B (Pharmacia, Uppsala, Sweden), and preparative electrophoresis on agarose gel (9). Pure antigen (2 g/L) in phosphate-buffered isotonic saline, pH 7.4, was stored at −20 °C.

Alkaline-phosphatase-conjugated anti-human immunoglobulin sera were obtained from Orion Diagnostica, Espoo, Finland. The conjugates were prepared from purified alkaline phosphatase (Type VII, EC 3.1.3.1) from calf intestine, and pure, heavy-chain-specific, high-avidity antibodies were isolated from swine antisera with use of immunosorbents. Unconjugated antibodies and unconjugated enzyme were removed by gel filtration. Conjugated antibodies were stored at −20 °C in Tris-HCl buffer solution (50 mmol/L), pH 8.0, containing 10 g of albumin and 1 g of NaNO₃ per liter.

Rheumatoid factor. IgM-type rheumatoid factor was isolated from the IgG–IgM type cryoglobulin by gel filtration at 37 °C on Sephadex G 200 in sodium acetate buffer (0.1 mol/L, pH 5.0) (10). The protein concentration of the rheumatoid factor solution was 5.2 g/L, and the latex titer was 5000.

Enzyme immunoassay procedure. Polystyrene tubes (10 × 50 mm; Kone Oy, Espoo, Finland) were coated with SS-B as follows: 1 mL of SS-B solution (3 mg/L) in phosphate buffer (50 mmol/L, pH 7.3, plus 0.5 g of NaNO₃ per L) was added to each tube. The tubes were stored at 4 °C until used (within three weeks). Before use, the tubes were washed three times with distilled water, and 1 mL of a solution of the patient’s serum, diluted 100-fold in phosphate-buffered saline containing 5 mL of Tween 20 surfactant per liter, was added to each tube. The tubes were stored overnight at 4 °C, to allow the antibodies to react with the surface-absorbed antigen. The tubes were then washed with phosphate-buffered saline containing Tween 20. The alkaline-phosphatase-labeled antibody to human IgG, IgA, or IgM (1 mL of 250- or 500-fold dilution) was added, and incubated at room temperature for 3 h. The amount of alkaline phosphatase fixed to the tubes was determined in diethanolamine (1.0 mol/L)-magnesium chloride (0.5 mol/L) buffer, pH 10.0, at 37 °C, with p-nitrophenyl phosphate as substrate (11). The enzyme reaction (at 37 °C for 1 h) was stopped by adding 0.1 mL of 1 mol/L NaOH, and the absorbance of the p-nitrophenolate liberated was measured at 406 nm. Results are given as changes in absorbance (ΔA), i.e., absorbance of the test sample minus that of the blank. In the blank tubes phosphate-buffered saline was used instead of serum.

Other methods. SS-B antigen was labeled with 125I (Radiochemical Centre, Amersham, U.K.) by the lactoperoxidase method (12) in the presence of mercaptoethanol (9). Rheumatoid factor activity was determined by the rheumatoid factor–latex agglutination test (RF-latex test; Behring,

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Received Feb. 19, 1981; accepted April 6, 1981.
Frankfurt, F.R.G.) (13). Rheumatoid factor was eliminated by treatment with dithiothreitol (0.6 g/L) in glycine-EDTA buffer (pH 7.0, 100 mol/L) at 37°C for 2 h (14).

**Results**

**Assay Conditions**

125I-labeled SS-B antigen was used to determine the optimum coating concentration. Because adherence was similar in alkaline and neutral buffers (Figure 1), and because SS-B antigen is unstable at acid and alkaline pH (4), neutral (pH 7.3) phosphate buffer was used in further experiments. With increasing concentration of the coating solution the amount of antigen adhering to the tubes increased linearly up to 2 mg/L, after which the curve leveled off (Figure 2). Accordingly, any concentration ≥3 mg/L would be appropriate for coating.

To find the optimum concentration of the coating solution, I diluted sera of SS-B-antibody-positive patients 10-, 100-, and 1000-fold and added some to the tubes coated with different concentrations of SS-B antigen. The antibodies bound to the antigen were detected with alkaline-phosphatase-conjugated anti-IgG serum. Alkaline phosphatase activity was measured at 406 nm. Three curves are for serum diluted 10-, 100-, 1000-fold.

**Fig. 1. Effect of buffer on the adherence of SS-B antigen**

Binding of 125I-labeled SS-B to polystyrene tubes with different concentrations of the coating solution. The radioactivity remaining in the tubes after washings was measured.

**Fig. 2. Dependence of the antigen binding on the concentration of the coating solution**

Binding of 125I-labeled SS-B to polystyrene tubes with different concentrations of coating solution.

**Fig. 3. Dependence of the antibody binding on the concentration of coating solution**

The antibodies bound to antigen were detected with alkaline-phosphatase-conjugated anti-IgG serum. Alkaline phosphatase activity was measured at 406 nm. Three curves are for serum diluted 10-, 100-, 1000-fold.

**Fig. 4. Determination of optimum serum dilution for SS-B antibody determination**

Tubes are coated with SS-B antigen, 3 mg/L. Sera 1, 2, 3 are SS-B-antibody-positive, and sera 4 and 5 are negative, as shown by double diffusion.
Methods. The difference between the absorbances of the SS-B-antibody-positive (1, 2, and 3 in Figure 4) and -negative (4 and 5 in Figure 4) sera was greatest at 100-fold dilutions, and this serum dilution was used in further experiments. The optimum dilutions of the conjugates (alkaline-phosphatase-conjugated anti-human immunoglobulins) were determined by measuring the maximum ΔA at different conjugate concentrations, with a coating concentration of 3 mg/L and a

100-fold dilutions of pooled patients' serum. The greatest dilution that resulted in a high ΔA value was selected for further use (Figure 5).

Effect of Rheumatoid Factor

Addition of rheumatoid factor. Different amounts of purified rheumatoid factor were added to an rheumatoid factor-negative serum that had both IgG- and IgM-type antibodies to SS-B antigen. With increasing concentrations of rheumatoid factor the ΔA values of IgM-type SS-B antibodies increased, whereas those of IgG-type antibodies decreased markedly (Figure 6).

Elimination of rheumatoid factor. The 34 rheumatoid factor-positive sera, which had either IgG- or IgM-type SS-B antibodies, were treated with dithiothreitol to eliminate rheumatoid factor. After dithiothreitol treatment the latex titer of all samples was 0, whereas the treatment with dithiothreitol did not change the IgG or IgM concentration of the sera, as measured nephelometrically (15). Elimination of rheumatoid factor increased the ΔA value of IgG-type SS-B antibodies in every serum, whereas the reverse was true with IgM-type antibodies (Figure 7); in fact, all IgM antibody values were near zero.

In contrast, when rheumatoid factor-negative, SS-B-antibody-positive serum was treated with dithiothreitol, the IgM-type SS-B antibodies were eliminated as above, but the ΔA value of IgG-type antibodies decreased only slightly (Table 1).

Precision

Twenty-nine separate assays run within four months from

<table>
<thead>
<tr>
<th>Sample</th>
<th>ΔA IgG</th>
<th>ΔA IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>2.06</td>
<td>0.97</td>
</tr>
<tr>
<td>DTT, treated for 30 min</td>
<td>1.96</td>
<td>0.16</td>
</tr>
<tr>
<td>DTT, treated for 2 h</td>
<td>1.89</td>
<td>0.03</td>
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Table 1. Effect of Dithiothreitol (DTT) Treatment on SS-B Antibodies in Rheumatoid Factor-Negative Serum

Fig. 5. Determination of optimum dilutions of conjugated antisera to human IgG, IgA, and IgM
Tubes are coated with SS-B antigen, 3 mg/L, and the sample of pooled SS-B-positive sera diluted 100-fold. Arrows indicate the optimum dilutions of conjugated antisera

Fig. 6. Effect of purified rheumatoid factor on ΔA values of IgG- and IgM-type SS-B antibodies
Different amounts of purified IgM-type rheumatoid factor (RF) was added to a rheumatoid factor-negative serum, which had both IgG- and IgM-type antibodies to SS-B antigen. The arrows indicate the corresponding rheumatoid factor latex titers of the serum

Fig. 7. Determination of IgG- and IgM-type SS-B antibodies before and after elimination of rheumatoid factor (RF) with dithiothreitol
The latex titer of the rheumatoid factor-positive sera before dithiothreitol treatment (mean ± SD) was 120 ± 186, and after treatment 0
one serum, which had a ΔA of 1.10, gave a coefficient of variation of 9.0%. Forty-eight parallel determinations of sera with ΔA values of 0.3 and 1.1 resulted in within-batch coefficients of variation of 6.3 and 4.8%, respectively.

Specificity

The SS-B antigen used in this study migrated as a single homogeneous band both in cellulose acetate and in sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 8). In double diffusion it gave a single precipitin line with anti-SS-B sera, but did not react with antisera against any of the following antigens: SS-A, ribonucleoprotein, DNA, scleroderma-70 antigen, or Smith antigen (9). ΔA values exceeding 0.21 for IgG, 0.12 for IgA, and 0.27 for IgM were found only in SS-B-antibody-positive sera (Table 2). There was no overlapping in IgG antibodies between SS-B-positive sera and the controls or SS-B-negative sera. The method was not as specific for IgA and IgM antibodies.

### Table 2. ΔA Values of SS-B Antibodies in Various Sera

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ΔA, range (mean)</th>
</tr>
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<tbody>
<tr>
<td>Controls</td>
<td>46</td>
<td>0.00–0.14</td>
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<tr>
<td></td>
<td></td>
<td>(0.09)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00–0.06</td>
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<tr>
<td></td>
<td></td>
<td>(0.03)</td>
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<tr>
<td></td>
<td></td>
<td>0.00–0.26</td>
</tr>
<tr>
<td>(right)</td>
<td></td>
<td>(0.13)</td>
</tr>
<tr>
<td>SS-B-positive a</td>
<td>43</td>
<td>0.27–1.52</td>
</tr>
<tr>
<td>(right)</td>
<td></td>
<td>(0.86)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00–0.66</td>
</tr>
<tr>
<td>(right)</td>
<td></td>
<td>(0.16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00–1.84</td>
</tr>
<tr>
<td>(right)</td>
<td></td>
<td>(0.40)</td>
</tr>
<tr>
<td>SS-B-negative b</td>
<td>68</td>
<td>0.00–0.21</td>
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<tr>
<td></td>
<td></td>
<td>(0.10)</td>
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<tr>
<td></td>
<td></td>
<td>0.00–0.12</td>
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<tr>
<td></td>
<td></td>
<td>(0.04)</td>
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<tr>
<td></td>
<td></td>
<td>0.00–0.27</td>
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<tr>
<td></td>
<td></td>
<td>(0.15)</td>
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</table>

* Sera positive for SS-B antibodies in double diffusion. b Sera negative for SS-B antibodies but positive for antibodies against other extractable nuclear antigens.

**Discussion**

Enzyme immunoassay for SS-B antibodies has the advantages of sensitivity and economy, and requires only small amounts of purified antigen.

In this assay, the optimum antigen density was obtained with a coating concentration of 3 mg/L. The specificity of the solid-phase enzyme assay depends on the purity of the antigen and the amount of nonspecific binding. The antigen used here was known to be pure (9). The SS-B-antibody-negative test sera may have contained antibodies to SS-B titers too low to be detectable in double diffusion, but they all had high titers of antibodies to other extractable nuclear antigens. Because these sera, as well as the control sera, had only low ΔA values, the assay may be considered specific for clinical purposes. In fact, an increased ΔA value of SS-B antibodies has been found only in patients with primary or secondary Sjögren’s syndrome (7, 8).

Rheumatoid factor, usually IgM, reacts with antigen-bound IgG. Hence, in the presence of rheumatoid factor the values of IgG-type SS-B antibodies should be too low and those of IgM antibodies too high. This was found to be the case. Accurate values for IgG- and IgM-type antibodies can be obtained only after elimination of rheumatoid factor. This can be achieved by treatment with dithiothreitol but, unfortunately, the ability of IgM-type SS-B antibodies to bind to their antigens is also destroyed. No methods for these antibodies are yet available in the presence of rheumatoid factor. For determination of IgG-type SS-B antibodies, prior elimination of rheumatoid factor with dithiothreitol treatment is recommended.

**References**

7. Manthorpe, R., Teppo, A.-M., Bendixen, G., and Wegelius, O., Antibodies to SS-B antigen in patients with primary and secondary Sjögren's syndrome. (Submitted for publication.)