

SS-B/La antigen purification, and ELISA detection of anti-SS-B/La antibodies in sera from patients with inflammatory connective tissue diseases

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SS-B/La antigen was purified by immunoadsorbent columns with immunoglobulin from a patient with primary Sjögren's syndrome. Monitoring of the purification was facilitated by the fused rocket-immunoelectrophoresis technique. Technical ELISA variables for the detection of serum antibodies against the SS-B/La antigen were evaluated, and a recommended procedure is described. Prospective investigation of anti-SS-B/La antibodies in 103 blood donors and 131 patients with chronic inflammatory connective tissue diseases, including 43 patients with primary Sjögren's syndrome was performed. Anti-SS-B/La antibody concentrations were above normal in 65% of the patients with primary Sjögren's syndrome (verified by the strictly objective Copenhagen criteria for keratoconjunctivitis sicca and xerostomia) and 9% of patients with other chronic connective tissue diseases. The predictive value for primary Sjögren's syndrome among patients with increased levels of the anti-SS-B/La antibodies attending a rheumatology clinic was 78%.

Key words: Sjögren's syndrome; connective tissue diseases; antinuclear factors; ELISA; SS-B/La antigen.

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CLINICAL ASPECTS

Antibodies against the soluble nuclear antigen SS-B/La seem to be a characteristic finding in patients with primary Sjögren's syndrome. There is a need for a simple serological screening test for this common chronic inflammatory connective tissue disease, which requires objective tests for evaluation of xerostomia as well as for keratoconjunctivitis sicca. This paper describes a prospective investigation of solid phase ELISA for the detection of serum IgG anti-SS-B/La antibodies.

Primary Sjögren's syndrome is a common inflammatory connective tissue disease, of which the cardinal symptoms are keratoconjunctivitis sicca and xerostomia (12). A number of cytoplasmic and nuclear antigen-antibody systems which are relatively specific for primary Sjögren's syndrome have been described independently by several investigators (1, 4, 13). By interlaboratory exchanges of reference sera and antigen preparations, it has become clear that the cytoplasmic RNA antigen, La, is immuno-

logically identical with the nuclear non-histone antigens, SS-B and Ha, as is the cytoplasmic antigen, Ro, with the nuclear antigen, SS-A (3). Of the two immunologically non-identical systems, Ha/SS-B/La and Ro/SS-A, the former seems to be the more specific with regard to primary Sjögren's syndrome (19).

Several methods for the detection of anti-SS-B/La antibodies, including counterimmune electrophoresis (10, 20), double immunodiffusion and enzyme linked immunosorbent assay

(ELISA) (9, 21), have been described. However, the reported sensitivity of these techniques range from 23% (2) to 87% (9), and even specificity varies to some degree. These discrepancies may reflect differences in detection methods, and/or differences in criteria for diagnosis of primary Sjögren's syndrome.

The aim of the present investigation was to purify the SS-B/La antigen, using immobilised immunoglobulin from a patient with primary Sjögren's syndrome, on an immunosorbent column and to use the purified antigen in a sensitive ELISA technique. A recommended experimental procedure is described, and technical variables in the ELISA procedure have been evaluated and optimised. In addition, the sensitivity, specificity and predictive value of anti-SS-B/La antibodies have been established among patients with primary Sjögren's syndrome and related connective tissue diseases.

MATERIAL AND METHODS

Preparation of immunoabsorbent from patient's serum

Serum from a patient with primary Sjögren's syndrome containing high anti-SS-B/La antibody activity, detected by Ouchterlony double immunodiffusion technique, was selected. Salting out was performed by adding 25% w/v $(\text{NH}_4)_2\text{SO}_4$ (1.7 M) to the patient's serum followed by overnight incubation at room temperature. The precipitated proteins containing the immunoglobulins were separated by centrifugation ($4000 \times g$) for 30 min and washed twice by resuspension in 50% w/v 1.7 M $(\text{NH}_4)_2\text{SO}_4$ followed by centrifugation.

The washed precipitate was dialysed against 0.05 M tris/acetate buffer, pH 7.7 (20°C) and applied on DEAE® (diethyl-aminoethyl A 50 Sephadex anion-exchange chromatography column (Pharmacia Fine Chem.) equilibrated in dialysis buffer. The column was eluted in 3 column-volumes of 0.05 M sodium acetate buffer, pH 5.0. The effluent and eluate containing the major part of the immunoglobulins (90%) G and A were collected, concentrated and dialysed against 0.1M NaCl, 15 mM NaN_3 (7).

This protein fraction was then coupled to CNBr sepharose (Pharmacia Fine Chem.) at a concentration of 20 mg/ml.

Preparation of SS-B/La antigen

5 g of rabbit thymus acetone powder (Pelfreeze Biologicals, Rogers) was extracted by shaking for 15 min with 75 ml extraction buffer (0.1 M tris/acetate pH 7.7, 0.35 M NaCl, 200 kallikrein IE/ml aprotinin (Sigma), 0.2 mM phenyl-methylsulphonylfluoride, 15 mM NaN_3) in a bottle containing glass beads (3 mm). The suspension was centrifuged at 15 000 g for 20 min. The clarified supernatant was then applied to a 100 ml column of CNBr Sepharose coupled to the serum Ig fraction which had previously been equilibrated in extraction buffer (10).

After washing the immunosorbent column with 4–6 column volumes of extraction buffer elution of antigen was performed by adding one column volume of extraction buffer containing 6 mol UREA. Fractions were continuously collected using a Pharmacia FRAC 300 fraction collector and the elution profile was recorded using a Pharmacia UV-1 monitor measuring protein absorbance at 280 nm. The fractions obtained by immunosorbent chromatography were analysed by fused rocket immunoelectrophoresis.

Fused rocket immunoelectrophoresis (Fig. 1)

Gels for fused rocket immunoelectrophoresis (8) were cast on 10 × 10 cm glass plates or Gelbond® film.

Aliquots (5–10 ml) of fractions eluted from the immunosorbent column were loaded into wells punched in the previously casted antibody-free agarose gel. The content of the wells in the agarose thus represents the continuously collected fractions from the immunosorbent-chromatography.

The antibody-containing gels were prepared in two steps:

- 1) An intermediate gel, containing purified immunoglobulin (150 $\mu\text{g}/\text{cm}^2$) from a patient with primary Sjögren's syndrome, enabling identification of SS-B/La antigen.

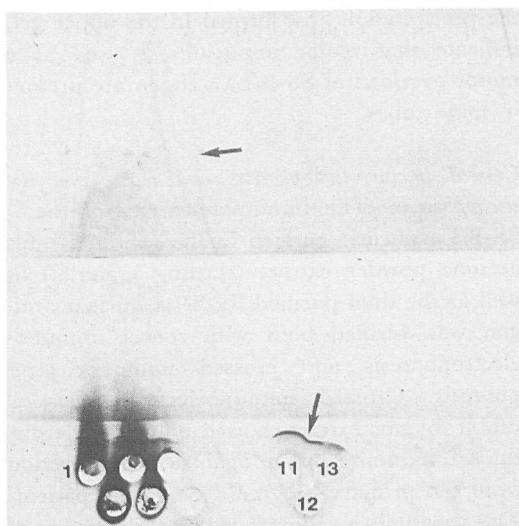


Fig. 1. Fused rocket immunoelectrophoresis. The upper arrow shows the precipitation arcs formed in the upper gel due to content of rabbit serum proteins. The lower arrow indicates the precipitation arcs formed in the intermediate gel due to SS-B/La in tubes 11-13.

2) An upper gel containing swine anti-rabbit serum proteins, 11 $\mu\text{l}/\text{cm}^2$, (Dakopatts A/S - code Z 195), enabling identification of rabbit serum proteins.

The electrophoresis was run at 1.5 volt/cm for 20 h. Pressing, washing, drying and staining of the fused rocket immunoelectrophoresis were performed as previously described (8).

Material from relevant tubes containing SS-B/La (Fig. 1, fractions 11-13) was pooled and dialysed against dialysis buffer (0.05 M tris/acetate pH 7.7, 0.05 M NaCl, 15 mM NaN_3 , 0.2 mM paramethylsulphonylfluoride and 200 KIE aprotinin/ml Sigma). Protein concentration was estimated spectrophotometrically assuming specific absorbance of 10 for a 1% protein solution at 258 nm.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE in sodium dodecyl sulphate (SDS) was performed according to Laemmli (11).

Standard ELISA procedure

Buffer solution. A standard 0.01 M PBS buffer, pH 7.2, was used (NaH_2PO_4 , H_2O 3.45 g, Na_2HPO_4 , 2 H_2O 13.31 g, NaCl 85.21 g dissolved in 10 l distilled water).

Washing solution. 1 ml of Tween 20 was added to 1 l of PBS buffer.

Assay solution. 10 g of bovine serum albumin (BSA) was added (essential fatty acid-free, code No. A-7030 Sigma) to 1 l washing solution.

Substrate buffer. Citrate/phosphate buffer, pH 5.0, was prepared by dissolving citric acid (5.160 g) and Na_2PO_4 , H_2O (9.149 g) in 1 l distilled water.

Conjugate. Peroxidase-conjugated rabbit immunoglobulins against human IgG (gamma chains) (code No. P 214, Dakopatts A/S, Copenhagen).

Conditions for the standard assay. Microtitre plates (Nunc-Immuno-plates I 96) were coated with 100 μl antigen diluted in buffer solution, washed 3 times with washing solution and then incubated with 1% BSA in buffer solution (150 μl) for at least 12 h at 4°C followed by a further 3 washes.

Serum samples were diluted in assay solution and 100 μl aliquots incubated. After 3 washes peroxidase conjugate (100 μl) diluted in assay solution was added. After further 3 washes orthophenyldiamine 16 mg in substrate buffer 30 ml with 10 μl of H_2O_2 (30%) 100 $\mu\text{l}/\text{well}$ was added. The enzymatic colour reaction was stopped after 10 min with 150 μl H_2SO_4 1 N and read at 492 nm in Titertek® multiscan spectrophotometer. Each dilution was done in quadruplicate and a blank (containing only assay solution) as well as a negative and a positive reference serum were included on each plate.

Reference sera

Reference human sera containing antibodies against SS-A/Ro and SS-B/La antigen were obtained from the Arthritis Foundation Center for Disease Control, Atlanta, Georgia, USA, and anti-RNP, anti-Jo-1 and anti-Scl-70 reference sera from Biolab® Limal, Belgium.

Patients and normal material

131 patient sera were tested over a period of 6 months. All patients suffered from rheumatologic diseases and were diagnosed according to internationally accepted criteria. Definite or classical rheumatoid arthritis was diagnosed using the American Rheumatism Association (ARA) criteria (14). Primary Sjögren's syndrome was diagnosed in patients with at least two abnormal objective tests for keratoconjunctivitis sicca as well as for xerostomia (Copenhagen criteria) in the absence of another chronic inflammatory connective tissue disease (12). For diagnosis of systemic lupus erythematosus, the 1982 ARA diagnostic criteria were used (18). The diagnosis of mixed connective tissue disease (MCTD) was based on combined features of: systemic lupus erythematosus, Sjögren's syndrome, polymyositis and/or progressive systemic sclerosis together with a high serum anti-RNP antibody titre (15). "Collagenosis NUD" refers to diagnoses in patients who did not meet internationally accepted criteria for other connective tissue diseases, and where two or more organs (e.g. joints, muscles, skin) are affected. Other diseases were likewise diagnosed, using generally accepted clinical and paraclinical criteria. The patients referred to our department represent a non-selected population of rheumatological disorders.

A reference range of values was obtained in studying samples from 103 blood donors, 19-74 years old, female/male ratio 1:1. All patient and control sera were stored at -20°C.

RESULTS

Methodology

Fused rocket immunoelectrophoresis (Fig. 1). The tubes (11-13) containing the specific SS-B/La activity are easily identified by the precipitation arcs formed in the gel opposite the wells. By including swine anti-rabbit serum proteins in the upper gel, the tubes containing major impurities (rabbit serum proteins) are identified. The heavy staining above wells 1-5, and

the precipitation arcs formed in the upper gel, indicate that major impurities as well as a minor overload of SS-B/La antigen are present in these tubes.

Crossed immunoelectrophoresis and rocket immunoelectrophoresis. The immunoreactivity of the SS-B/La nuclear antigen in the rabbit thymus acetone powder extract (starting material) as well as the final purified SS-B/La nuclear antigen was verified both with rocket immunoelectrophoresis and crossed immunoelectrophoresis. Crossed immunoelectrophoresis of rabbit thymus extract showed only a single distinct immunoprecipitate against the Ig-fraction from the primary Sjögren's syndrome patient. The sensitivity was improved by the addition of 2% w/v polyethyleneglycol MW 6000 to the agarose gels. A minor change was observed in the form of the precipitation arc of purified SS-B/La as compared with that of the starting material.

Polycrylamide gel electrophoresis and immunoblotting. Polycrylamide gel electrophoresis (PAGE) in sodium dodecyl sulphate (SDS) of the purified SS-B/La antigen showed a 50 KD band and two degeneration products (Fig. 2). This finding was subsequently confirmed by Dr. S.O. Hoch at the Agouron Institute, California, by a Western blotting technique using iodinated protein A.

Optimization of conditions for the ELISA technique

A chessboard titration with dilutions of the local positive and negative reference sera in one dimension (interval 1/100-1/1600) and antigen concentration in the other was performed. A serum dilution of 1:200 and a SS-B/La concentration of 20 µg/ml gave the best discrimination between the positive and negative standards, and these were used in all subsequent experiments. Fig. 3 indicates extinction as function of antigen concentration at the optimal serum dilution.

Antigen coating with carbonate buffer pH 9.6 (1.59 g Na₂CO₃ in 2.93 g NaHCO₃ dissolved in 1 l distilled water) gave lower extinction values than coating at neutral pH.

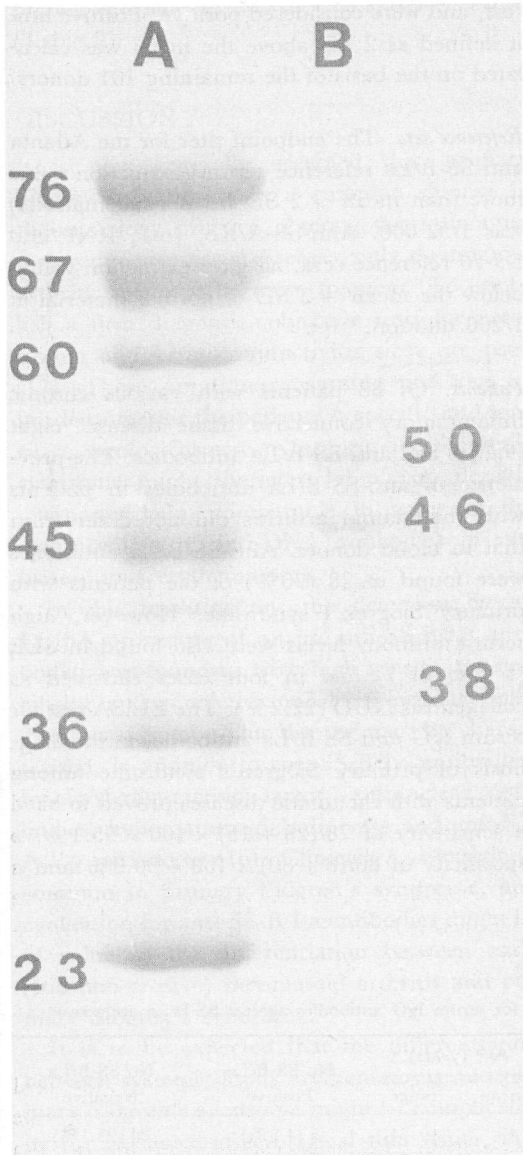


Fig. 2. Analysis by SDS-PAGE of (A) molecular weight standards and (B) purified SS-B/La antigen.

Antigen was incubated at 4°C and 24°C for different periods from 1 h to 8 days. Incubation at 4°C for 3 days was selected because of highest positive/negative extinction ratio.

Positive and negative reference sera were incubated at 4°C and 24°C for periods of between 5 min and 6 h (Fig. 4), and 2 h at

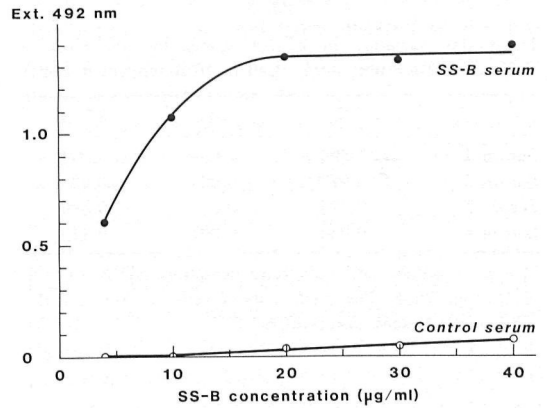


Fig. 3. Absorbance at 492 nm as a function of SS-B/La antigen concentration. Serum dilution, 1:200. Filled circles = anti-SS-B/La positive reference serum. Open circles = anti-SS-B/La negative reference serum.

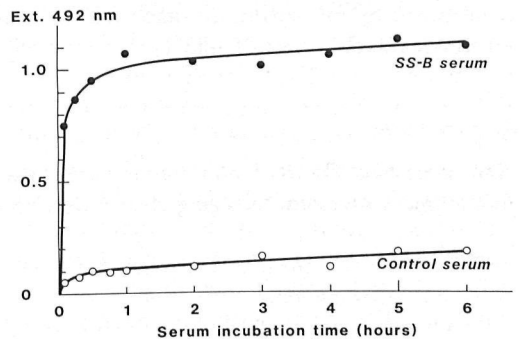


Fig. 4. Absorbance at 492 nm as a function of serum incubation time. Filled circles = anti-SS-B/La positive reference serum. Open circles = anti-SS-B/La negative reference serum. Temperature 24°C.

24°C was selected as optimal. Likewise, incubation times and temperatures were varied for the conjugate step and 2 h at 24°C selected. Addition of 3% polyethyleneglycol (6000 MW) to the assay buffer did not increase the sensitivity of the test.

Under these conditions, a 1:5000 dilution of the conjugate gave an average extinction of 1,000 at 492 nm when testing the local SS-B/La reference serum. All test sera were corrected using the formula:

Table 1

Day-to-day variation in ELISA values for anti-SS-B/La antibodies. Four sera were tested in 10 independent assays

	X	SD	SD/X
Serum 1	0.994	0.034	0.034
Serum 2	0.943	0.028	0.030
Serum 3	0.896	0.043	0.048
Serum 4	0.060	0.007	0.116

X = mean values, SD = standard deviation, SD/X = coefficient of variation. The coefficients of variation were calculated using corrected absorbances.

absorbance of sample
absorbance of positive reference (22), to minimise day-to-day variation.

The same positive reference sera as well as a negative control was included on each plate. Antigen-coated plates were stable after blocking with BSA in PBS for at least 3 months at 4°C. Interassay variation of ELISA method was determined by measuring the same four sera in 10 independent assays (Table 1).

Clinical studies

Normal material. Of 103 blood donors tested, two had extinction values diverging clearly from the

rest, and were considered positive. Positive limit defined as 2 SD above the mean was calculated on the basis of the remaining 101 donors.

Reference sera. The endpoint titer for the Atlanta anti-SS-B/La reference serum (extinction value more than mean + 2 SD for normal material) was 1/52 000. Anti-SS-A/Ro, Jo-1, RNP and Scl-70 reference sera, all gave extinction values below the mean + 2 SD of normal material at 1/200 dilution.

Patients. Of 88 patients with various chronic inflammatory connective tissue disease, eight (9.1 %) had anti-SS-B/La antibodies. The prevalence of anti-SS-B/La antibodies in patients with rheumatoid arthritis did not differ from that in blood donors. Anti-SS-B/La antibodies were found in 28 (70 %) of the patients with primary Sjögren's syndrome. However, high serum antibody levels were also found in SLE (1 case of 7) and in four cases classified as collagenosis NUD (22.2 %). The ELISA test for serum IgG anti-SS-B/La antibodies in the diagnosis of primary Sjögren's syndrome among patients with rheumatic diseases proved to have a sensitivity of $28/(28 + 15) \times 100 = 65.1 \%$, a specificity of $80/(8 + 80) \times 100 = 90.9 \%$ and a

Table 2

Patient and control sera analysed with ELISA technique for serum IgG antibodies against SS-B/La antigen

Diagnosis	No. patients	F	M	Age (years)		No SS-B/La Positive	No SS-B/La Negative
				mean	range		
Blood donors	103	51	52	40	19-74	5 (4.9 %)	98 (95.1 %)
Rheumatoid arthritis	53	43	10	63	23-84	3 (5.6 %)	50 (94.3 %)
Collagenosis NUD	18	15	3	45	25-76	4 (22.2 %)	14 (77.8 %)
SLE	7	7	0	51	25-86	1 (14.2 %)	6 (85.7 %)
Prog. syst. sclerosis	3	2	1	59	55-63	0	3
MCTD	2	1	1	65	56-72	0	2
Polymyalgia rheumatica	1	1		72		0	1
Mb Jaccoud	1	1		49		0	1
Dermatomyositis	1	1		33		0	1
CREST	1	1		68		0	1
Mb Reiter	1		1	48		0	1
Total	88	72	16	58	23-86	8 (9.1 %)	80 (90.9 %)
Primary Sjögren's syndrome	43	40	3	59	19-86	28 (65.1 %)	15 (34.9 %)

Sensitivity: 65.1 %. Specificity: 90.9 %. Predictive value: 77.7 %.

predictive value of $28/(28 + 8) \times 100 = 77.7\%$ (Table 2).

DISCUSSION

It is now generally accepted that primary Sjögren's syndrome is a common disease in rheumatology practice. Among the inflammatory connective tissue diseases, only rheumatoid arthritis seems to be more frequent. To establish a firm diagnosis, objective tests for xerostomia and keratoconjunctivitis sicca are used (12). These are time-consuming and can be burdensome for the patient. A specific and sensitive serological screen for primary Sjögren's syndrome might therefore be of considerable diagnostic help, comparable in value to the measurement of anti-DNA antibodies in systemic lupus erythematosus.

In this investigation, the detection by an ELISA procedure of serum anti-SS-B/La antibodies was found to have high sensitivity, specificity and predictive value in primary Sjögren's syndrome. This disease may be characterized, in addition to anti-SS-B/La antibodies, by IgM-rheumatoid factor, antinuclear antibodies, hypergammaglobulinemia and anti-SS-A/Ro antibodies (16). Clinically, arthralgia is common in primary Sjögren's syndrome, and evaluation for anti-SS-B/La antibodies might be of value in the differentiation between early (still non-erosive) rheumatoid arthritis and primary Sjögren's syndrome.

It is to be expected that the differentiation between systemic lupus erythematosus and primary Sjögren's syndrome might be complicated by the existence of serological similarities, and in keeping with this, anti-SS-B/La antibodies were found in one of seven patients with SLE (giving a sensitivity of 14.2%). Although the number of patients is small, these results are in accordance with the findings of Tan (19). Further, the SLE patient who gave a positive result had skin lesions as the most prominent of her clinical manifestations with no nephritis or anti-DNA antibodies, and had the characteristic clinical finding of subacute cutaneous lupus erythematosus (SCLE), a subset of SLE patients with high levels of serum anti-SS-B/La anti-

bodies (17). She also had a pathological lower lip biopsy but normal unstimulated whole sialometry (6.4 ml/15 min) and no objective signs of keratoconjunctivitis sicca.

Four of 18 patients with unclassified connective tissue disease (collagenosis NUD) had increased serum anti-SS-B/La antibodies, but in only one was the extinction value more than 3 SD above the mean. This patient also had focal lymphocyte infiltrations in small salivary glands and an abnormal salivary gland scintigraphy, but no objective signs of keratoconjunctivitis sicca. This patient will be followed for the development of keratoconjunctivitis sicca.

For a number of years now, fused rocket immunoelectrophoresis has been a well-established method for observing the distribution of proteins obtained in fractionation experiments. In the present study, this technique proved to be a quick and easy method for detecting fractions containing the SS-B/La antigen, and thus facilitated the purification procedure.

The purified SS-B/La antigen was found to have a molecular size, estimated by SDS-PAGE, of 50 kD with two smaller breakdown products. This is in accordance with the findings of Elkon & Jankowski (6). Furthermore, immunoblotting of our purified SS-B/La antigen by Hoch et al. showed a 50 kD band in accordance with their findings (10). No 57 kD protein band, indicative of SS-A/Ro impurities (6), was found in the antigen preparation, and the homogeneity of the SS-B/La antigen was further shown by the absence of reactivity with the anti-SS-A/Ro, -Scl-70, -RNP and -JO-1 reference sera. The minor change in precipitation morphology of the purified SS-B/La antigen compared with the starting material could indicate a change in the structure of the SS-B/La molecule, probably due to proteolytic degradation occurring during processing of the antigen (5).

We found antigen concentration and serum dilution to be the two most important of the technical variables in the ELISA. The optimal antigen concentration of 2.0 µg/well found by us accords with the findings of Venables et al. (21). The more efficient antigen coating at neu-

tral rather than high pH might be because SS-B/La is an acidic protein, easily destroyed at high pH.

The indirect immunofluorescence techniques, often used as a screening test for antinuclear antibodies in rheumatological diseases, give a speckled nuclear pattern in sera with anti-SS-B antibodies. However, it requires exceptional skill to distinguish this pattern from the speckled pattern found in sera with antibodies to Sm, RNP and Scl-70. The sensitive ELISA technique has several advantages compared with Ouchterlony double diffusion and counter-immune electrophoresis. The ELISA technique is quantitative and less time-consuming, and allows the measurement of non-precipitating antibodies and the detection of antibody Ig-class. In this study we have focused solely on the IgG-class.

We conclude that among patients with chronic inflammatory connective tissue diseases, the measurement of anti-SS-B/La antibody by ELISA constitutes a sensitive, specific and highly predictive test for the diagnosis of primary Sjögren's syndrome, defined by objective tests for xerostomia as well as keratoconjunctivitis sicca (Copenhagen criteria) (12). Very little is known about the levels of anti-SS-B/La antibodies in patients with primary Sjögren's syndrome, followed for several years. However, this has not been our objective in the present study and must await further investigations.

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