Clinical and laboratory study

ANTIBODIES TO THE 46 KD LA/SSB PROTEIN DETECTED BY IMMUNOBLOTTING: THEIR CLINICAL RELEVANCE IN PATIENTS WITH CONNECTIVE TISSUE DISEASES

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ABSTRACT

The clinical relevance of antibodies against La/SSB was investigated in patients with systemic lupus erythematosus (SLE), subacute cutaneous lupus erythematosus (SCLE), discoid lupus erythematosus (DLE), mixed connective tissue disease (MCTD) and systemic scleroderma (PSS). La/SSB antibodies were detected by two methods: immunodiffusion against soluble rabbit thymus extract, and immunoblotting using a cytoplasmic HeLa cell extract as antigen source.

Both methods had an equal sensitivity, however by immunoblotting it was possible to differentiate the autoimmune response against different polypeptides.

By immunoblotting La/SSB positive sera were shown to react with a 46 kD protein and an additional 43 kD degradation fragment. Antibodies to these La/SSB proteins were found in the sera of 11 of 37 patients with SLE (30%), 9 of 23 patients with SCLE (39%), 1 of 12 patients with MCTD (85), 1 of 56 patients with PSS (2%), but in no serum of patients with DLE (0/14).

The clinical comparison of patients with or without antibody against the 46 kD La/SSB protein indicates that in SLE patients the presence of anti-La/SSB is associated with a more benign course of the disease. At the present state of investigation, the La/SSB antibody system showed no correlation with specific clinical findings of the other diseases studies, including SCLE.

KEY WORDS:
anti-La/SSB antibodies, connective tissue diseases, immunoblotting

INTRODUCTION

Autoantibodies reacting with different cell components are characteristic features of autoimmune connective tissue disorders (1). Many of these antibodies are directed against soluble RNA protein conjugates such as the nuclear antigens U1 RNP, Sm and La/SSB and the cytoplasmic Ro/SSA-antigen (2). Sm antibodies are mostly associated with antibodies to U1 RNP; similarly, antibodies to La/SSB are in most cases found together with Ro/SSA antibodies (3).

In recent years, great progress has been achieved in defining the specificities of antinuclear antibodies and in establishing the molecular identity of some of the antigens. Immunoblotting, immunoprecipitation and other techniques were Introduced to Identify the specificity of some of these antigens by determining the molecular weight of their protein components, by cDNA cloning, by autoepitope mapping and

by defining the nature of the nucleic acids bound to these proteins (4,5,6).

The correlation of the Ro/SSA antibody-system with specific clinical criteria has been investigated extensively; on the other hand, only few studies have been performed to elucidate the clinical significance of the La/SSB antibody system. for this reason, we have examined a large group of sera patients with connective tissue diseases (CTD) by a sensitive Immunoblotting technique for the presence of La/SSB antibodies in order to assess specific clinical manifestations associated with anti-La/SSB activity.

PATIENTS AND METHODS

Sera from 142 patients seen at the Department of Dermatology, Ludwig-Maximillians-Universität München, FRG, were collected for this Investigation. Patients Included 121 females and 21 males with ages ranging from 13 to 82 years (mean age of 52 years).

According to the modified classification of the American Rheumatism Association (ARA) (7), 37 patients fulfilled the diagnostic criteria of systemic lupus erythematosus (SLE). 23 patients had subacute cutaneous lupus erythematosus (SCLE); this subtype of LE was diagnosed on the basis of light sensitivity and widespread papulosquamous or annular skin lesions associated with less than 4 of the ARA criteria. The 14 patients with discoid lupus erythematosus (DLE) had typical scarring lesions limited to face and scalp. 12 patients were diagnosed to have mixed connective tissue disease (MCTD) as defined by Sharp (8). Systemic scleroderma (PSS) was diagnosed in 56 patients following the classification of Barnett modified by the "Arbeitsgruppe Sklerodermie" within the ADF (Arbeitsgemeinschaft Dermatologische Forschung 1986) (9).

Normal human sera (n=40) were obtained from age-and sex-matched apparently healthy individuals. Prototype sera for anti-La/SSB were obtained from the CDC (Center for Disease Control), Atlanta, GA.

CELLS AND EXTRACTS

Monolayer HeLa cells were cultured in RPMI 1640 supplemented with 10 % fetal calf serum, 2,5 µg/ml gentamicin sulfate, non-essential amino acids, 0,11 mg/ml sodium pyruvate, 2mM L-glutamine (all tissue culture reagents were from Gibco, Eggenstein, FRG) Collection of subconfluent cells and preparation of the cell extract was carried out at 4 °C. Cells on a 165 cm^2 Petri dish were lysed with 1 ml of ice-cold lysing buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 0.5% NP-40) harvested by scraping with a rubber policeman and freed of cell nuclei by centrifugation at 12.000 g for 15 min. The supernatant was added to the same volume of Laemmli's buffer (2 x conc.). The protein content of the cell fraction was determined by the method of Lowry (10). Extracts were stored in aliquots at -80°C and boiled for 2 min before use.

RO/SSA ANTIGEN

Ro/SSA antigen was extracted from fresh human spleen tissue obtained within 24 hr post mortem according to the method described by Clark (11). The Ro/SSA fraction was further purified by affinity chromatography as described by Yamagata (12).

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

For 1-dimensional protein analysis 15% SDS-polyacrylamide gels were used as described by Laemmli (13). Protein bands were detected by staining with 0,1% Coomassie brilliant blue R-250. Molecular weight standards were obtained from BioRad Lab.,Munich, FRG.

WESTERN BLOTTING

Electrophoretic blotting was performed using the modified method of Towbin (14). Proteins were separated by SDS-PAGE and electrotransferred to an Immobilon PVDF sheet (Millipore, Eschborn,FRG) for 2.5 h at a constant voltage of 60 V at 4 °C. After transfer nonspecific protein-binding sites were blocked by overnight application of 3% nonfat milk in phosphate buffered saline (PBS) p H 7.4 at 4 °C. 5mm-Immobilon strips were incubated with a 1:100 dilution of sera, washed with PBS containing 0,05 % Tween 20 (Sigma, Deisenhofen, FRG), incubated with a 1:1000 dilution of biotin conjugated goat anti-human IgG (Tago, Burlingame, USA), washed with PBS-Tween and then incubated with a 1:1000 dilution of streptavidin-peroxidase (Amersham RPN. 1231) in 0.1M Tris pH 7.4. Ortho-dianisidine and sodium nitroprusside were used to detect bound streptavidin-peroxidase.

STANDARD TECHNIQUES

IMMUNOFLUORESCENCE

Antinuclear antibodies (ANAs) were detected by immunofluorescence technique using HEp2-cells (Kallestad, Freiburg, FRG) as antigen substrate. Patients' sera were diluted in phosphate buffered saline (PBS) pH 7.4 starting at 1:40 and then evaluated by indirect immunofluorescence IIF). Crithidia luciliale monolayers (Kallestad,Freiburg,FRG) were used as substrate for the detection of antibodies to doublestranded DNA. FITC-conjugated goat anti-human IgG (Tago, Burlingame, USA) diluted 1:20 in PBS was used as detecting agent.

IMMUNODIFFUSIONS

Antibodies to R0/SSA were detected by double immunodiffusion in 0.6% agarose using a partially purified human spleen extract (11) or by a newly developed ELISA

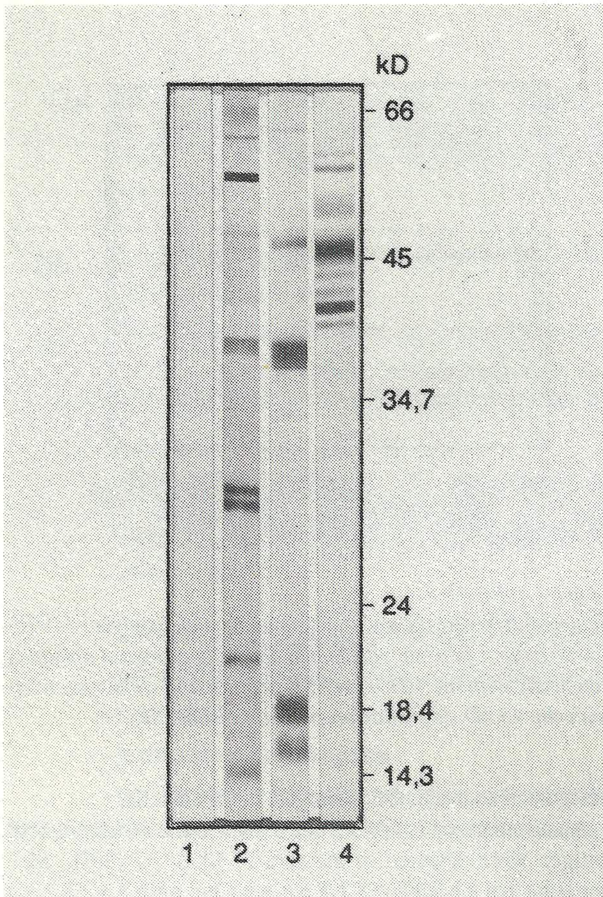


Figure 1:
Identification of antinuclear and anti cytoplasmic antibodies by Westernblot analysis using a cytoplasmic HeLa cell extract as antigen source:
Lane 1: normal human serum.
Lane 2: serum from patient with SLE and renal disease showing reactivity to 60 kD Ro/SSA, 38 kD rRNP, B/B and D protein of Sm, and C protein of U1-RNP
Lane 3: serum from patient with SLE and CNS involvement showing reactivity with 38, 19 and 17 kD proteins of rRNP
Lane 4: serum from patient with SLE without renal disease showing reactivity with 60 and 52 Ro/SSA and 46 and 43 kD La/SSB antigens.

method with purified Ro/SSA protein as antigen (15).

Precipitating antibodies against nuclear U1-RNP and Sm were identified by double immunodiffusion using the soluble fraction (ENA; Pelfreeze, Rogers, AR) of rabbit thymus nuclei as antigen substrate (2).

HLA-B and-DR specifities were determined in selected patients using standardized microcytotoxicity assays (Prof. Albert, National Reference Laboratory for Tissue Typing, Children's Hospital, Universität München).

Clinical data were analysed using Chi-square test for statistical evaluation. P values less 0.05 were considered to be significant.

RESULTS

142 sera from patients with CT diseases were tested by immunodiffusion, ELISA and immunoblotting as described above.

118 of these sera were positive for anti-Ro/SSA antibodies by immunodiffusion and ELISA techniques (11.15), with immunodiffusion and immunoblotting, antibodies to La/SSB could be detected in 22 patients (15.5 %). By immunoblotting it was shown that all of anti-La/SSB positive patients specifically reacted with a 46 kD protein, which was recently identified as the major antigen of the undegraded La/SSB complex in human HeLa cells (4). 19 sera showed an additional reactivity with the 43 kD La/SSB degradation product. Sera of 3 patients (2 with SLE and 1 with SCLE) contained only antibodies against the 46 kD protein. In control experiments, we examined 40 sera from apparently healthy individuals: only 1 control serum (2.5 %) was found to react with the 46 kD protein.

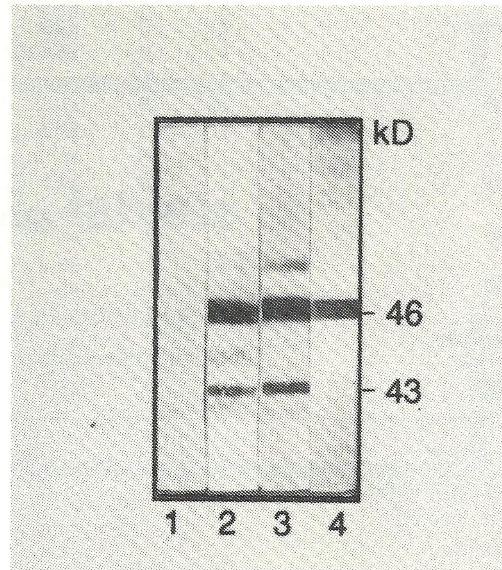


Figure 2:
Identification of antibodies to La/SSB by Westernblot analysis using a cytoplasmic HeLa cell extract as antigen source:
Lane 1: normal human serum.
Lane 2: CDC reference for La/SSB.
Lane 3,4: sera obtained from two patients with SLE.
Reference serum and patients sera showed reactivity to the 46 kD La/SSB protein. In lane 2 (CDC reference) and lane 3 there was additional reactivity to the 43 kD degradation product of La/SSB.

Table I Incidence of La/SSB antibodies in patients with autoimmune disorders using the immunoblotting method

	La/SSB-positive		antibodies to 43/46kD	antibodies to 46kD
SLE	11/37	(30%)	9/11	2/11
SCLE	9/23	(39%)	8/9	1/9
DLE	-/14	(0%)	-	-
MCTD	1/12	(8%)	1/12	-
PSS	1/56	(2%)	1/56	-
Healthy controls	1/40	(3%)	0/40	1/40

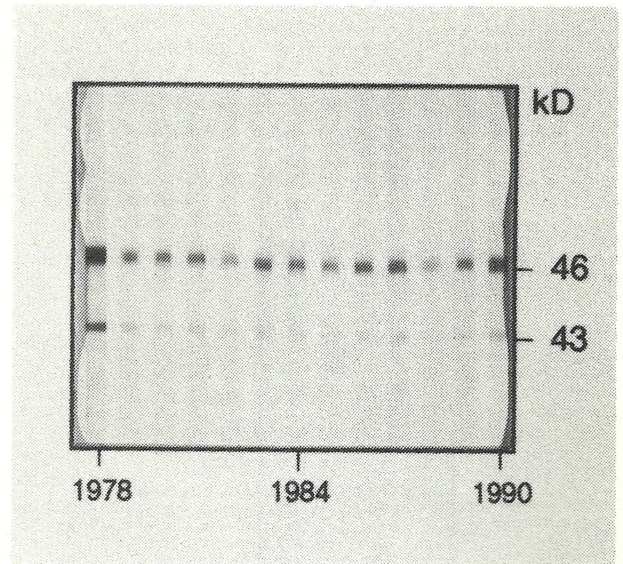
Table II Clinical and laboratory data of SLE patients with or without antibodies to La/SSB.

Clinical and laboratory features	La/SSB-positive (n = 11)	La/SSB-negative (n = 26)	Chi²-test
Butterfly rash	2	15	p≤0.05
Raynaud's phenomenon	2	17	p≤0.05
Vasculitis	6	13	n.s.
Sicca syndrome	2	2	n.s.
Arthralgia/arthritis	10	20	n.s.
Polyserositis	1	11	p≤0.05
CNS-involvement	2	10	n.s.
Renal involvement	2	20	p≤0.5
Cytopenia	3	14	n.s.
Rheumatoid factor	7	9	n.s.
Sm antibodies	1	6	n.s.
dsDNA antibodies	3	14	n.s.
Low C3 or C4	2	6	n.s.
Elevated gamma globulins	5	9	n.s.
Positive ANA ≥1 : 160	11	26	n.s.
Ro/SSA antibodies	11	21	n.s.

Legend to table II:
Chi2-test: critical value = 3.84
a = 0.05
DF = 1

Figure 1 demonstrates the spectrum of antinuclear and anti cytoplasmic antibodies detected by immunoblotting using a cytoplasmic HeLa cell extract.

The La/SSB-specific immunoblotting patterns are demonstrated figure 2: Lane 1 shows no specific reactivity using a healthy control serum sample, lane 2 shows the CDC La/SSB reference reacting with polypeptides of 46 and 43 kD. Lane 3 shows the reaction of a patient's serum with both the 46 kD La/SSB antigen and its degradation of 43 kD, whereas lane 4 shows a single band of 46 kD, which is designated as the La/SSB antigen in HeLa cells.



Legend to figure 3:
Sequential Westernblot analysis of serum, samples (1978-1990) from a patient with SCLE showing constant antibody reactivity with the 46 kD La/SSB protein and weak reactivity with the 43 kD degradation product of La/SSB.

As summarized in table I, antibodies to the 46 kD La/SSB antigen were detected in the sera of 11 of 37 (30%) SLE patients, 9 of 23 (39%) SCLE patients, but only 1 of 56 (2%) patients with PSS and in 1 of 12 (8%) MCTD patients. All 14 patients with DLE were anti-La/SSB negative.

Fig 3 presents a sequential Westernblot analysis of 12 different serum samples collected from one patient with SLE over a period of more than 10 years.

Antibodies to the 46 kD protein and an inconsistent weak reaction with the 43 kD protein could be detected in all serum samples. There was no change in the immunoblotting pattern, although the patient experienced several acute exacerbations with typical skin lesions and arthralgia, followed by long periods of remission. Levels of reactivity with the 46 kD protein did not correlate with disease activity.

When clinical and laboratory findings of SLE patients with or without the La/SSB antibody were compared, there were significant differences between the two groups with respect to butterfly rash, Raynaud's phenomenon, polyserositis and renal involvement. These findings were seen more frequently in patients without La/SSB antibodies. The differences in CNS involvement, cytopenia and presence of antibodies to DNA and Sm between the two groups did not reach statistical significance at the the $p \leq 0.05$ level (table II).

In contrast to SLE, no clinical or serological differences between SCLE patients with or without antibodies to La/SSB could be detected. Table 3 shows the pattern of clinical and

Table III Clinical and laboratory data of SCLE patients with or without antibodies to La/SSB.

Clinical and laboratory features	La/SSB-positive (n = 11)	La/SSB-negative (n = 26)	Chi²-test
Sicca syndrome	4	3	n.s.
Raynaud–s phenomenon	1	3	n.s.
Arthritis/arthralgia	5	7	n.s.
Polyserositis	-	-	-
CNS-involvement	-	2	n.s.
Renal involvement	-	1	n.s.
Cytopenia	1	1	n.s.
Sm antibodies	1	-	n.s.
dsDNA antibodies	2	1	n.s.
Rheumatoid factor	5	2	n.s.
Photosensitivity	6	11	n.s.
Positive ANA ≥1 : 160	8	8	n.s.
Ro/SSA	8	18	n.s.

Legend to table III:
Chi2 test: critical value = 3.84
a = 0,05
DF = 1

laboratory features.

Data of HLA-phenotyping was available for a limited number of patients. A comparison of the specific HLA markers in the La/SSB-positive group and the La/SSB-negative group revealed a difference in the frequency of HLA B8 and DR3 antigens, which were found more often in the La/SSB positive patients: 5 of 6 La/SSB positive patients had HLA-DR3 compared with only 2 of 9 patients without the La/SSB antibody. HLA-B8 was found in 5 of 6 La/SSB positive patients but in only 5 of 9 patients without anti-La/SSB antibodies.

DISCUSSION

La/SSB antibodies recognize a cellular phosphoprotein that is nuclear in location and has been shown to be transiently associated with RNA polymerase III transcripts (4,5,6,16). The undegraded human La/SSB protein in HeLa cells is a molecule of 46 to 48 kD. An additional 43 kD polypeptide can be present as a proteolytic degradation product of La/SSB; different molecular weights of La/SSB have been reported in other species (4).

Antibodies to La/SSB were first detected by agar diffusion according to the method of Ouchterlony using extractable nuclear antigens from animal organs or human lymphoblastoid cells as antigen source (3). In addition to double immunodiffusion La/SSB antibodies have been detected by different techniques, including enzymelinked immunosorbent assay (ELISA), radioimmunoassay (RIA) and immunoblotting procedures. By immunofluorescence, the localization of the La/SSB antigen is primarily nuclear. However, most of the phosphoproteins precipitable by anti-La/SSB antibodies leak into the cytoplasmic fraction during aqueous fractionation procedures, which we used for this study (17).

In most cases antibodies to La/SSB are associated with antibodies to Ro/SSA (18) which are more frequent and are often found in the absence of La/SSB antibodies in patients with rheumatic diseases. In human cells, the Ro/SSA antigen has ben characterized as polypeptides of 60 kD and 52 kD (19), which are bound to nonantigenic small cytoplasmic ribonucleoprotein particles (scRNPs) (20).

In this study 118 of 142 CTD-patients (83%) were anti-Ro/SSA positive, whereas only 22 of these 142 patients (15,5 %) had Ro/SSA as well as La/SSB antibodies. La/SSB antibodies were comparatively frequent in patients with SLE (30 %) and SCLE (39 %), but were rarely found in PSS (2 %) and MCTD (8 %).

In order to find out if La/SSB antibodies are linked to certain disease manifestation, not yet known to be associated with the Ro/SSA antibody system (21), patients with SLE or SCLE were divided into a La/SSB positive and a La/SSB negative group. The analysis of sera and clinical diagnosis of 37 SCLE patients did not allow for the detection of a significant difference between the different subsets of patients.

However in patients with SLE the presence of La/SSB antibodies indicates a more benign course of the disease, since renal involvement and polyserositis were significantly less frequent in La/SSB positive SLE patients (p=0,05).

Similar to our findings, Maddison et al. (22) and Harley et al. (23) have reported that antibodies to La/SSB occur in a subgroup of patients with SLE with a later disease onset and a lower frequency of renal disease and anti-DNA antibodies. Wasicek et al. (24) have studied SLE patients with antibodies to Ro/SSA alone or with antibodies to Ro/SSA and to La/SSB. This group gained similar results to ours, demonstrating in anti-La/SSB positive SLE patients a low incidence of renal involvement and antibodies to DNA.

The humoral response to the La/SSB antigen seems to be heterogenous since some sera react with the 46 kD protein but not with its 43 kD degradation product. This finding corroborates the results of Rauh et al. (25), who identified at least three B-cell epitopes on the La/SSB protein using La/SSB fusion proteins. Further studies should clarify whether there are differences between the subgroups of patients reacting with the 46 kD protein with or without its 43 kD degradation product. In addition, we could demonstrate in one patient with SLE that the humoral response to the antigens of a HeLa cell cytoplasmic extract is rather constant (figure 3) and does not correlate with disease activity.

Although the number of patients available for HLA typing

was too small for statistical analysis, our results are consistent with the reported association of La/SSB antibodies with the HLA B8, DR3 haplotype (23,26). It has been suggested that class II restriction of autoantibody production in SLE patients may determine the serological abnormalities and clinical manifestation in these patients (27). This study shows, that the identification of La/SSB antibodies by a highly sensitive immunoblotting technique is helpful for the diagnosis of connective tissue diseases and provides distinct information concerning prognosis in individual patients with SLE.

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