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REPORTS

Molecular Characteristics of SS-B/La and SS-A/Ro Cellular Antigens*

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Anti-SS-B/La and anti-SS-A/Ro antibodies coexist in certain patients with connective tissue diseases such as systemic lupus erythematosus or Sjögren's syndrome. The respective antigenic structures with which these autoantibodies bind have not been fully characterized. The present study was conducted to better define these two different cellular antigens. WiL2 cell extracts were used to obtain partially purified SS-B/La and SS-A/Ro antigens. Both were found to be present in most fractions obtained after sequential purification with ammonium sulfate salt precipitation, G-200 gel filtration, DE-52 ion exchange chromatography, and preparative slab gel electrophoresis. However, SS-B/La antigenic activity was also found to be present in some fractions that did not contain detectable SS-A/Ro activity. These findings suggested the existence of two different forms of SS-B/La antigen: one containing the SS-B/La antigen only and the other containing both the SS-B/La and SS-A/Ro antigens. The RNA and protein components of these two ribonuclear protein particles were further defined by immunoprecipitation experiments using ^{32}P-labeled WiL2 cell extract. The SS-B/La antigen was found to be associated with several RNAs while the SS-A/Ro antigen was associated with several other distinct RNAs. Both antibodies precipitated a common 43K molecular weight phosphoprotein. The antigenic peptides of these 2 antibodies were analyzed using an immunoblot system. The SS-B/La antigen was present on a 43K peptide which was unstable and could be degraded to several peptides of lower molecular weight (40K, 38K, 30K), while the SS-A/Ro antigen occurred on a peptide having a molecular weight of about 60K.

Autoantibodies, especially antinuclear antibodies, are present in patients with various connective tissue diseases. Some of these antibodies are highly characteristic for certain diseases. For example, anti-nDNA and anti-Sm antibodies are characteristic of systemic lupus erythematosus (SLE), while anticentromeric antibodies are highly specific for the CREST syndrome. Many of these autoantibodies, however, are not disease-specific and can be present in patients in different clinical settings. Anti-nRNP antibodies, for example, can be seen in patients with SLE, rheumatoid arthritis, Sjögren's syndrome, and mixed connective tissue disease. Though such autoantibodies are not disease-specific, they can be important laboratory tools for studying cell structure and events related to cellular metabolism. Previously anticentromeric antibodies have been used in this way to localize the centromere in nuclei of cells during the resting phase of the cell cycle [1]. Antibodies against nonhistone nuclear components such as Sm, nRNP, and SS-B/La have also been used to gain a better understanding of the cellular distribution of their respective antigens. For example, our earlier studies have shown that SS-B/La antigen is present in the nucleoplasm of cells in the resting phase and in the nucleoplasm and nucleolus of cells in the late G_1 to early S phase of the cell cycle [2].

These autoantibodies have also been used to study the small cellular RNAs. In 1979, Lerner and Steitz reported that anti-Sm and anti-nRNP containing sera specifically precipitated the uridine-enriched nuclear RNAs called U1, U2, U4, U5, and U6 [3]. Subsequently Lerner et al reported that other unidentified cellular RNAs were precipitated by anti-La/SS-B and anti-Ro/SS-A sera [4]. In addition, anti-SS-B/La sera also precipitated adenovirus-associated RNA (VA-RNA) [4] and Epstein-Barr virus-associated RNAs (EBER) [5]. Further characterization of the SS-B/La and SS-A/Ro ribonucleoproteins showed that the small RNAs are mainly products of RNA polymerase III [6,7]. Recently, experiments have indicated that the SS-B/La antigen is involved in the maturation of RNA polymerase III transcripts and also binds to precursors of 5S ribosomal RNA [6,8,9].

SS-B/La and SS-A/Ro antigens are thought to be very soluble cellular proteins and sensitive to trypsin [10–14]. Molecular weight of the SS-B/La antigen-bearing protein has been variously estimated as 30K, 40–45K, 50K, and 67K [8,10,13,15–19]. Recent data did suggest that SS-B/La antigen was a peptide of around 45K [16,17,19] and also a phosphoprotein [15]. Molecular weight of SS-A/Ro was estimated to be 48K, 150K, or 60K [14,19,20]. There still is much confusion concerning the molecular characteristics of SS-B/La and SS-A/Ro antigens.

Antibodies to SS-B/La and SS-A/Ro have been found to coexist frequently in patients with SLE or Sjögren's syndrome [10]. Their mutual occurrence is similar to what has been observed for anti-Sm and anti-nRNP antibodies in certain SLE patients. Recent studies have shown that both Sm and nRNP antigenic particles share one common RNA—U1 RNA [3]. It has also been shown that there exist two forms of Sm antigen. One is free and the other consists of a complex of Sm and nRNP. These observations suggest that a similar kind of molecular interaction between SS-B/La and SS-A/Ro might also exist. Hendrick et al have shown that the SS-A/Ro particle carries the SS-B/La as well as the SS-A/Ro determinant [6].

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Abbreviations:
EBER: Epstein-Barr virus-associated RNAs
HRP: horseradish peroxidase
MEM: minimal essential medium
RNP: ribonuclear protein
SAS: saturated ammonium sulfate
SLE: systemic lupus erythematosus
VA-RNA: adenovirus-associated RNA

Their study suggested that there are two forms of SS-B/La antigen, one containing only the SS-B/La determinant and the other containing both SS-B/La and SS-A/Ro determinants.

A further clarification of these issues is required in order to gain a better understanding of the normal physiologic role played by these small RNP particles and any pathophysiologic alteration that antibodies to these particles might produce. Here, we report further biochemical characterization of SS-B/La and SS-A/Ro antigenic molecules extracted from WiL2 cells.

MATERIALS AND METHODS

Sera

Sera containing monospecific antibodies were obtained from patients with SLE, subacute cutaneous lupus erythematosus, mixed connective tissue disease, or primary Sjögren's syndrome. The monospecificity of these sera was based on the fact that each serum produced only one precipitin line on double immunodiffusion and counterimmunoelectrophoresis using rabbit thymus extract and WiL2 cell extract as the antigenic sources. The monospecificity of the sera containing only anti-SS-A/Ro antibodies was also substantiated by the fact that they gave a negative fluorescent antinuclear antibody assay on a mouse kidney section substrate, but a particulate nuclear staining pattern on human Hep-2 cell substrate. Sera from normal humans were used as a control.

Cells

The cells studied were WiL2, an Epstein-Barr virus-transformed cell line of continuously growing human diploid B lymphocytes originally obtained from the spleen of a patient with hereditary spherocytosis [21]. The cells were maintained in suspension culture with minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, MEM-nonessential amino acids, MEM-vitamins, sodium pyruvate, and 2 mM glutamine and antibiotics (Gibco, Buffalo, New York). The cells were subcultured at a concentration of 2×10^5/ml every 3 days.

Cell Extract

An equal amount of phosphate-buffered saline was added to packed WiL2 cell pellets plus 1 mM phenylmethyl sulfonylfluoride. The whole suspension was then subjected to ultrasonication to disrupt the cells. The supernatant, after clarification with centrifugation (100,000 g × 30 min), was then used as the crude antigenic material for further partial purification and double immunodiffusion analysis.

Procedures for Partial Purification

The WiL2 cell extract was subjected to salt fractionation with differential sequential saturated ammonium sulfate solution. Saturated ammonium sulfate (SAS) solution was added gradually to the cell extract to yield a 30% saturated solution. The precipitate (30% SAS ppt fraction) was collected after centrifugation at 10,000 g for 30 min and more SAS solution was added to produce 50% saturation. The precipitate (50% SAS ppt fraction) was collected and more SAS solution added to yield 66% saturation. Similarly this 66% SAS ppt fraction was collected and finally a 100% SAS ppt fraction was also obtained. The different ppt fractions were then dissolved in small amounts of phosphate-buffered saline which were then assayed in double immunodiffusion and counterimmunoelectrophoresis for the presence of SS-B/La or SS-A/Ro antigenic activities. The 50–66% SAS ppt fraction, which contained SS-B/La and SS-A/Ro antigens, was then applied onto a G-200 gel filtration column (diameter 2.6 cm, length 60 cm). After counterimmunoelectrophoresis for analyzing the antigenic activity of SS-B/La and SS-A/Ro, the second peak containing both antigenic activities was collected, concentrated, and applied onto a DE-52 ion exchange chromatography column using phosphate buffer 0.01 M, pH 8.0. The bound substances were eluted from the DE-52 column using NaCl gradients. The fractions containing the antigenic activities for SS-B/La and SS-A/Ro from 0.3 M NaCl gradient eluate were pooled, concentrated, and applied onto a 10% polyacrylamide gel electrophoresis under native condition relative to protein. The gel was then sliced into 5 mm-wide pieces and extracted in Tris-HCl buffer pH 7.6. SS-B/La and SS-A/Ro antigenic activity in these eluates was monitored by counterimmunoelectrophoresis. One part of the gel was stained with Coomassie Brilliant Blue and the protein profile was scanned using a densitometer.

Radiolabeling of Cells and Preparations of Cell Extract for Immunoprecipitation

Two- to three-day-old WiL2 cells were used in the immunoprecipitation experiments. Cells were labeled with [^{32}P]phosphoric acid at a concentration of 2×10^7 cells/l mCi for 15 h (50 μCi/ml). The labeling was carried out in a medium of phosphate-free MEM, (Flow Laboratory, California) and supplemented with 2 mM glutamine, antibiotics, and 10% heat-inactivated fetal calf serum. After labeling, the cells were washed twice with 50 mM Tris, 150 mM NaCl, pH 7.4, and resuspended into the same buffered solution at the concentration of 2×10^7/ml. The cells were then disrupted extensively by ultrasonication. The cell supernatant after clarification by centrifugation at 12,000 g for 20 min, was collected as the source of radiolabeled antigen.

Immunoprecipitation of Protein-RNA Complexes

The ^{32}P-labeled WiL2 cell extract was used in the immunoprecipitation, and the RNA and phosphoprotein components of the immune complexes were analyzed using the methods described earlier by Pizer et al [15].

Immunoblot Analysis

Either the crude WiL2 cell extract or the partially purified materials were subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were blotted onto nitrocellulose paper according to the method described by Burnett [22]. Thereafter, the nitrocellulose paper was soaked in a monospecific serum dilution containing either anti-SS-B/La or SS-A/Ro antibodies followed by a horseradish peroxidase (HRP)-labeled goat antihuman IgG solution (Cappel Company). After incubation, the nitrocellulose paper was washed extensively in Tris-HCl-NaCl solution, pH 7.4, and the coloring reagent (substrate solution) of hydrogen peroxide and 4-chloro-1-naphthol was added. The colored band indicated the reactive peptide location. Four different sera containing SS-B/La or SS-A/Ro precipitin antibodies were used in this assay.

RESULTS

Coexistence of SS-B/La and SS-A/Ro Antigenic Activities

Sixty milliliters of WiL2 cell extract was subjected to differential biochemical purification procedures in order to get partially purified antigenic preparations. As shown in Table I, the bulk of SS-B/La antigenic materials was recovered from a wide range of ammonium sulfate-precipitated fractions. SS-A/Ro antigenic activity, however, was recovered only from the fractions precipitated by 30–50% and 50–66% SAS solutions. Both antigenic materials were present in the 30–66% SAS-precipitated fractions, while SS-B/La was also present in the fraction that did not contain detectable SS-A/Ro activity (66–100%). The fraction precipitated by 30–50% SAS solution also contained Sm and nRNP antigenic activity and therefore was not used for further purification. the 50–66% SAS solution-precipitated fraction (total protein 150 mg) was then applied onto G-200 gel filtration columns and the SS-B/La and SS-A/Ro antigenic activities were recovered in the fractions present in second peak as demonstrated in Fig 1. Fractions from the second peak were collected, pooled, concentrated (total protein 30.5 mg), and applied onto a DE-52 ion exchange column using phosphate buffer 0.01 M, pH 8.0. The bound materials were eluted with NaCl gradients and the elution profiles are illustrated on Fig 2. SS-B/La antigenic activity can be detected in fractions eluted with 0.1, 0.2, 0.3, and 0.5 M NaCl gradients

TABLE I. *Antigenic activities in ammonium sulfate-precipitated fractions*

Antigen	% Saturated ammonium sulfate solution fractions			
	0–30	30–50	50–66	66–100
SS-B/La	–	+	++	+
SS-A/Ro	–	++	++	–
Total protein	125 mg	210 mg	150 mg	115 mg

while SS-A/Ro can be detected only in the fractions eluted with 0.2 and 0.3 M NaCl gradients. Since the 0.3 M NaCl fraction contained a lower protein concentration and was relatively enriched for SS-A/Ro and SS-B/La activities, this fraction (total protein 3 mg) was further fractionated by 10% acrylamide gel electrophoresis under native conditions (i.e., without SDS). The electrophoresed protein profile is shown in Fig 3. The right-hand side was the dye front and the left-hand side was the stacking gel. In this native gel condition, the separation of protein was based on the molecular weight and charge of the protein molecules. After electrophoresis, SS-B/La and SS-A/Ro were found in the same fraction ranges, as indicated in Fig 3, though SS-B/La was also present in some fractions not having detectable activity of SS-A/Ro.

RNA Profiles Associated with the Respective Nuclear Antigens

Sequential immunoprecipitation using ^{32}P-labeled cell extract and monospecific sera was performed. The respective antigen-associated RNA profiles are shown in Fig 4. Lanes 1 and 2 represent SS-B/La and SS-A/Ro, respectively. SS-B/La antibodies could bring down 7 RNAs. Two of them were similar to EBER electrophoretically; two others were similar to 5S RNA and 4.5S RNA. Anti-SS-A/Ro antibodies were associated with 4 RNAs as shown in lane 2 in Fig 4. Lanes 3 and 4 show the RNA profiles for Sm and nRNP, respectively. Sm was found associated with at least 5 different RNAs and the most abundant were U1 and U2 RNAs, while nRNP was associated with U1 RNA primarily. Lane 5 was the normal human serum control which did not bring down specific RNA.

Distinct Phosphoproteins Associated with the SS-B/La and SS-A/Ro Antigens

Fig 5 shows the SS-B/La and SS-A/Ro antigen-associated phosphoprotein profiles. Lanes 1 and 2 show that anti-SS-B/La antibodies are associated with a distinct 43K peptide containing phosphoamino groups. Similarly, anti-SS-A/Ro antibodies can also bind to a 43K phosphoprotein as shown in lanes 3 and 4. This 43K peptide was not bound by normal human serum (lane 5), anti-Sm (lane 6), nor anti-nRNP (lane 7). Anti-Sm and anti-nRNP antibodies bound commonly to 3 phosphoproteins, while anti-Sm also bound to a distinct phosphoprotein of 38K peptide.

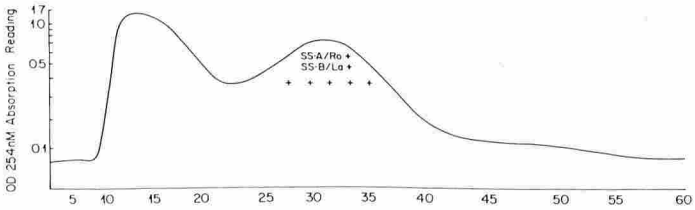

FIG 1. G-200 gel filtration protein elution profile. The original material was from the fractions precipitated with 50–65% SAS solution. The SS-A/Ro and SS-B/La were present in the second peak fractions, based on counterimmunoelectrophoresis. Fraction number is indicated on abscissa.

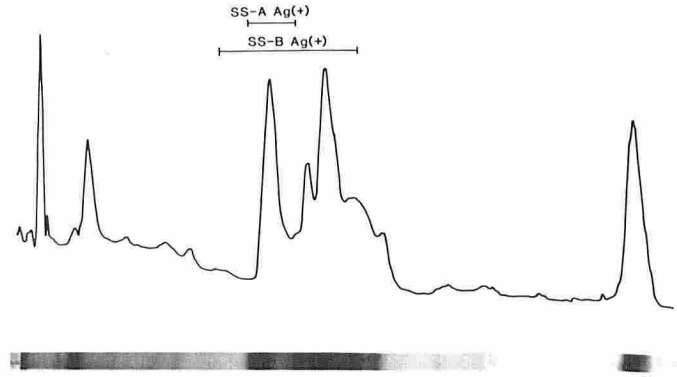

FIG 3. Protein scanning profile of preparative 10% polyacrylamide gel electrophoresis. The starting material was obtained from fractions eluted with 0.3 M NaCl gradient in DE-52 ion exchange chromatography.

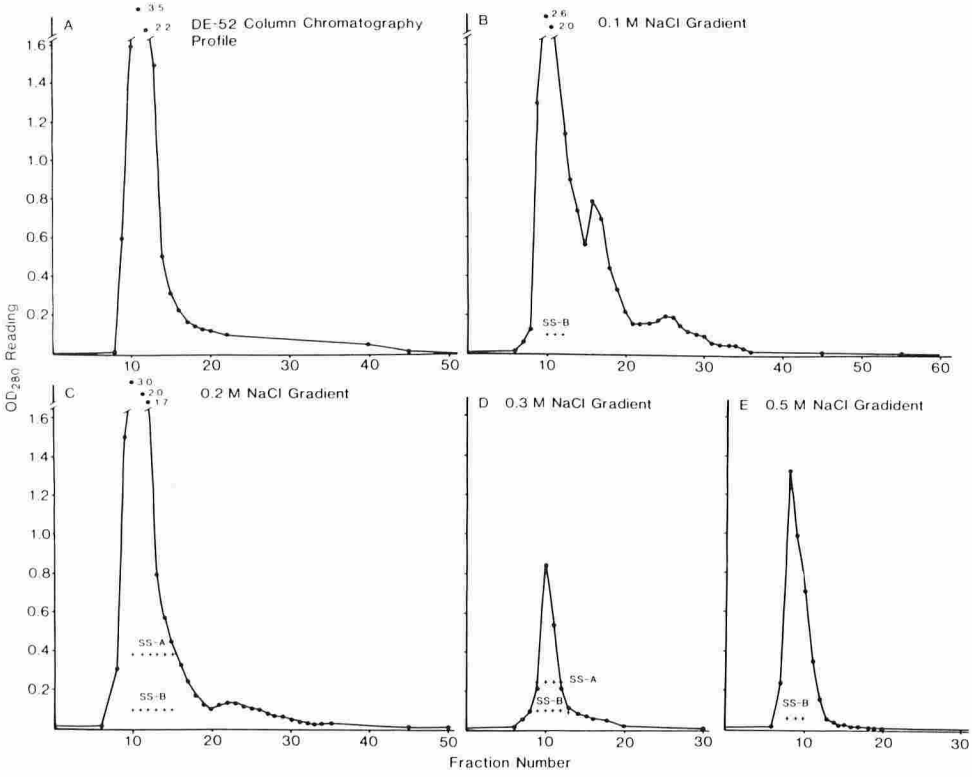

FIG 2. Protein elution profile from DE-52 ion exchange chromatography with phosphate buffer 0.01 M, pH 8.0. The starting material was obtained from the second peak fractions of G-200 gel filtration column eluate. Initially 500 ml phosphate buffer was used to wash out all the unbound substances (*A*), then the following were applied: 500 ml phosphate buffer with 0.1 M NaCl gradient (*B*), 500 ml phosphate buffer with 0.2 M NaCl gradient (*C*), 500 ml phosphate buffer with 0.3 M NaCl gradient (*D*), and finally 500 ml phosphate buffer with 0.5 M NaCl gradient (*E*), and used to elute out the bound substances.

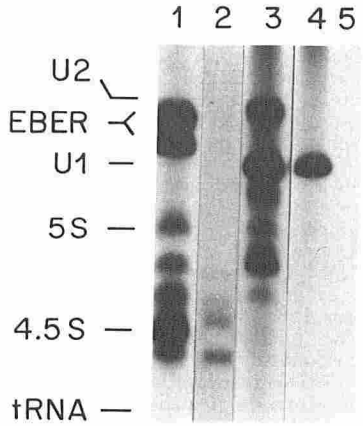

FIG 4. Cellular antigens associated RNA profiles. ^{32}P-labeled WiL2 cell extract was added to specific serum and then Pansorbin (*Staph. aureus*) added. The immune complexes were then eluted out from the *Staph. aureus* with SDS containing Tris-HCl buffer and then treated with proteinase K and subjected to 7 M cerea–10% acrylamide gel electrophoresis. *Lane 1,* SS-B/La-associated RNAs. *Lane 2,* SS-A/Ro-associated RNAs. *Lane 3,* Sm-associated RNAs. *Lane 4,* nRNP-associated RNA. *Lane 5,* Normal human serum control.

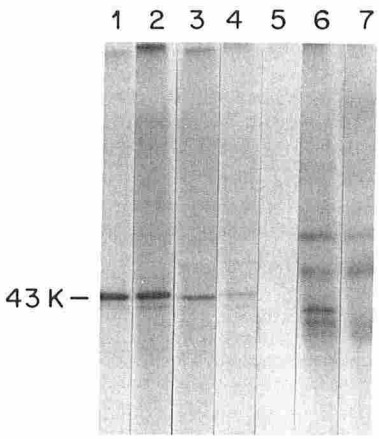

FIG 5. Cellular antigen-associated phosphoprotein profiles. ^{32}P-labeled WiL2 cell extract was added to specific serum and then Pansorbin (*Staph. aureus*) added. The immune complexes were then subsequently eluted out from the *Staph. aureus* with SDS containing Tris-HCl buffer and then treated with RNase subjected to 10% SDS-polyacrylamide gel electrophoresis. *Lanes 1 and 2,* SS-B/La-associated phosphoproteins. Primarily a 43K phosphoprotein was found associated with SS-B/La. *Lanes 3 and 4,* SS-A/Ro-associated phosphoprotein profiles. This 43K peptide was not bound by normal human serum (*lane 5*), anti-Sm (*lane 6*), nor anti-nRNP (*lane 7*).

Peptides Reacting Specifically with Anti-SS-B/La and Anti-SS-A/Ro Antibodies

When the WiL2 cell extract was analyzed by the immunoblot technique, as shown in Fig 6, lane 3, anti-SS-B/La antibodies were found to react with a specific peptide of molecular weight 43K. Anti-SS-A/Ro antibodies, however, reacted with a 60K peptide (Fig 6, lane 4). Four different sera containing SS-B/La and SS-A/Ro precipitin antibodies were used and all showed the same kind of reactivity. Lane 1 contains the molecular weight standards and lane 2 represents the protein profile of the crude WiL2 cell extract. When the partially purified materials were used in the immunoblot analysis, a 60K peptide was consistently observed and recognized by anti-SS-A/Ro antibodies (data not shown). However, when fractions from different steps of purification were used, anti-SS-B/La antibodies reacted with other peptides of lower molecular weight, as shown in Fig 7. Lane 1, obtained from crude WiL2 cell extract, showed that 43K peptide reacted with anti-SS-B/La antibodies. This indicated that the SS-B/La antigen is a peptide of molecular weight of 43K. As the cell extract was subjected to ammonium sulfate fractionation (lane 2) and DE-52 ion exchange chromatography (lane 3), the intensity of the 43K peptide band decreased and 3 or 4 lighter protein bands were detected. One band of 30K peptide gradually increased in intensity. When fractions from preparative 10% polyacrylamide gel electrophoresis were used, only the 30K peptide was recognized by the anti-SS-B/La antibodies (lane 4). Similarly, 30K peptides from partially purified rabbit thymus extract was recognized by the anti-SS-B/La antibodies (lane 5).

DISCUSSION

SS-B/La and SS-A/Ro cellular antigens have been found by others to be associated with distinct small cellular RNAs. We have found that SS-B/La is associated with 7 RNAs in our experimental system using cellular extract from WiL2 cells—an Epstein-Barr virus transformed cell line. As has been shown previously, SS-B/La is associated with at least 4 RNAs from cells not infected with adenovirus, or Epstein-Barr virus [4,15]. Two of the RNAs are 5S RNA and 4.5S RNA [6,8,9]. The other 2 RNAs are still unidentified and needed to be further defined. When adenovirus-infected cells have been used, VA-RNA have been found to be strongly associated with SS-B/La antigen [4,15]. In addition, other small RNAs synthe-

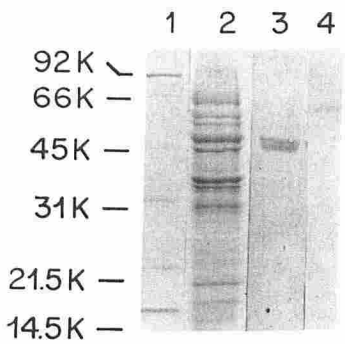

FIG 6. Protein profile of WiL2 cell extract and the immunoblot profiles for SS-B/La and SS-A/Ro. *Lane 1,* Molecular weight standard. *Lane 2,* Protein profile of WiL2 cell extract. WiL2 extract was subjected to 10% SDS-polyacrylamide gel electrophoresis and then blotted onto nitrocellulose paper (*lane 2*). After blotting some were reacted with anti-SS-B/La monospecific serum (*lane 3*) and some with anti-SS-A/Ro monospecific serum (*lane 4*) and then detected with HRP-coloring reagent.

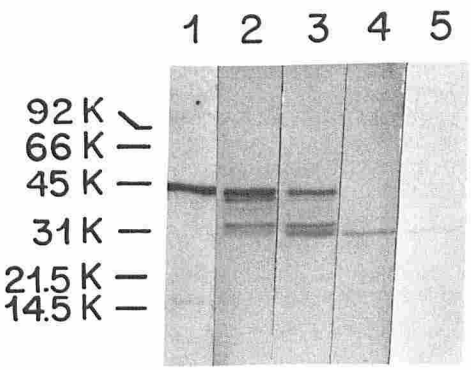

FIG 7. Immunoblot analysis for SS-B/La cellular antigen. Antigenic preparations from different preparations were subjected to 15% SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose paper, treated with anti-SS-B/La antiserum, and then HRP-labeled goat antihuman IgG. *Lane 1,* From crude WiL2 cell extract. *Lane 2,* From ammonium sulfate-precipitated fractions. *Lane 3,* From DEAE ion exchange chromatography fraction. *Lane 4,* From fractions eluted from 10% preparative acrylamide gel electrophoresis. *Lane 5,* From partially purified fractions from rabbit thymus extract.

sized by cells infected with adenovirus have also been found to be associated with SS-B/La antigen as confirmed by RNA-DNA hybridization [15]. Similarly, when Raji cells and WiL2 cells (both are transformed by Epstein-Barr virus) have been used, 2 additional RNAs have been found bound to SS-B/La antigen and have been designated EBER I and II [5,7]. Our studies have shown that the SS-B/La-associated RNAs are different from those associated with SS-A/Ro (Fig 4, lane 2), Sm (Fig 4, lane 3), or nRNP (Fig 4, lane 4). Some of the SS-B/La molecules seemed to be related to SS-A/Ro molecules [6]. There are possibly 2 forms of SS-B/La antigen. One is a free SS-B/La antigen which contains a 43K peptide and SS-B/La-associated RNAs. The second form of SS-B/La antigen contains SS-A/Ro peptide, SS-A/Ro-associated RNAs, and the 43K peptide. This might be related to the fact that SS-A/Ro is present in few copies per cell [4]. This hypothesis could be confirmed by using monoclonal antibodies against SS-B/La and SS-A/Ro antigen. The 43K peptide is the core molecule present in both forms of SS-B/La antigens. The 43K peptide is also the antigenic peptide for SS-B/La. This is similar to the results reported earlier [9,13,15–17,19] In the present report, SS-B/La was also found to be associated with other peptides when analyzed on immunoblot analysis, including a 30K peptide. We believe that the 43K peptide is unstable and is sensitive to spontaneous degradation or proteolytic digestion, as suggested by Venables et al [19]. As seen in Fig 7, lane 1, only the 43K peptide band is recognized by SS-B/La antibody. When WiL2 cell extract was subjected to ammonium sulfate precipitation, seen in Fig 7, lane 2, 4 more light bands were detected. This indicates that the 43K peptide is degraded gradually. When the preparation is further purified with DE-52 ion exchange chromatography, more of the 43K peptide is degraded and finally only the 30K peptide is detectable after preparative polyacrylamide gel electrophoresis. When a preparation from rabbit thymus extract was used, only the 30K peptide was found. This could explain the difference in molecular weights for SS-B/La that have been reported in the past several years [9,13,15–17].

Recent evidence has suggested that SS-B/La can also bind to some precursors of 5S RNA [8], which is a product of ribosomal RNA. The distribution of SS-B/La is strongly associated with the cell cycle [2]. SS-B/La is present in the nucleoplasm of cells in the resting phase. It is also present in the nucleolus of cells during the late G_1 to early S phases of the cell cycle, a phase in which rapid synthesis of ribosomal RNA occurs. These observations suggest SS-B/La may have a functional role related to ribosomal RNA synthesis.

The nature of SS-A/Ro is still unknown. As shown in Fig 6, lane 4, the molecular weight of SS-A/Ro peptide is around 60K. This is similar to that described recently by M. Reichlin et al (personal communication). Its presence in the cellular extract is frequently associated with SS-B/La from which separation is difficult. This kind of molecular association between SS-A/Ro and SS-B/La might give rise to the simultaneous coexistence of two autoantibodies—anti-SS-B/La and anti-SS-A/Ro—in certain patients with various connective tissue diseases.

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