Molecular Properties of the Ro/SSA Antigen and Enzyme-linked Immunosorbent Assay for Quantitation of Antibody

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Abstract. Antibodies to the Ro/SSA antigen occur in patients with systemic lupus erythematosus and Sjögren's syndrome. An immunoaffinity method for the preparation of electrophoretically homogeneous Ro/SSA antigen is described. Several molecular properties of the antigen have been determined. The native RNA protein particle has a molecular weight of ~100,000 D determined by gel filtration. Sodium dodecyl sulfate-analysis of the purified Ro/SSA antigen and analysis by staining of bands with silver and Coomassie Blue, Western blotting, and RNAase treatment leads to a hypothesis for the structure of the particle in which an antigenic 60,000 protein is bound to 24,000–27,000 RNA molecules which are not antigenic. An enzyme-linked immunoabsorbent assay method for assay of anti-Ro/SSA is also described which sensitively measures antigen binding at dilutions of sera containing anti-Ro/SSA precipitins up to 10^7 fold. Normal sera on average have 10^3 less binding activity.

Introduction

Autoantibodies to the Ro/SSA antigen have been defined by precipitation in agar gels (Ouchterlony) and the disease specificity of this antibody has been shown to include systemic lupus erythematosus (SLE)[1] and Sjögren's syndrome by several groups (1–3). The antigenic epitopes for autoantibodies to Ro/SSA have recently been shown to reside on small cytoplasmic RNA protein particles (4). The presence of anti-Ro/SSA strongly correlates with distinguishable subsets of SLE and Sjögren's syndrome. Antibody to the Ro/SSA antigen occurs in 62% of patients with "ANA negative" SLE (5), 63% of patients with subacute cutaneous lupus erythematosus (6), 75% of the homozygous C2-deficient patients with an SLE-like picture, and in 80% of patients with Sjögren's syndrome who have vasculitis (7). Anti-Ro/SSA occurs in virtually all children with the neonatal lupus syndrome (8–12), which constitutes the strongest evidence to date that the antibody can be directly responsible for clinical aspects of SLE. Until now the only reliable method for the detection of anti-Ro/SSA has been the relatively insensitive and nonquantitative agar diffusion method of Ouchterlony. The molecular properties of the protein moiety of the RNA-protein complex have not been delineated.

In this report, purification and characterization of the Ro/SSA antigen by an immunoaffinity method is described. In addition, direct evidence for the size of the antigenically reactive protein moiety and a sensitive quantitative solid phase assay for the detection of anti-Ro/SSA antibodies are presented.

Methods

Tissue extract. Tissue extracts were prepared by homogenizing and extracting calf thymus gland or human spleen in an equal amount (wt/vol) of phosphate-buffered saline (PBS, pH 7.2) with 2 mM dithiothreitol at 4°C. After centrifugation at 10,000 *g* for 1 h, the supernatant was fractionated using ammonium sulfate precipitation. The precipitate at 30% saturation was discarded, and the precipitate at 60% saturation was dissolved in PBS and dialyzed against PBS for 48 h. The final protein concentration of this calf thymus extract (CTE) or human spleen extract was ~40 mg/ml.

Immunoadsorbent columns. The immunoadsorbent column used for purification of Ro/SSA antigen was prepared by coupling IgG from a patient (H.I.) who showed only anti-Ro/SSA precipitins in immunodiffusion to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) by the method suggested by the manufacturer. IgG was prepared by DEAE-cellulose chromatography (13), and dialyzed against the coupling buffer (0.05 M NaCl, 0.1 M $NaHCO_3$, pH 8.3). The protein concentration was adjusted to 30 mg/ml. 15 g of CNBr-activated Sepharose were washed with 1 liter of 1.0 mM HCl solution and reacted with 5 ml of IgG solution on a rotating turn table for 2 h at room temperature. Unbound protein was removed by washing with coupling buffer and the residual active sites on the Sepharose beads were blocked by reaction with 1.0 M ethanolamine, pH 8.0, for 1 h at

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1. *Abbreviations used in this paper:* CTE, calf thymus extract; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; SLE, systemic lupus erythematosus.

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room temperature. An anti-La/SSB immunoadsorbent column was prepared by the same method using IgG from a patient (M.U.) whose serum had only antibody to the La/SSB antibody by immunodiffusion.

Gel filtration. Pooled eluates from the anti-Ro/SSA immunoadsorbent column were further fractionated on a calibrated Bio-Gel A-0.5 m (Bio-Rad Laboratories, Richmond, CA) column (1.5 × 165 cm). Fractions containing Ro/SSA antigen activity detected by microcomplement fixation (14) or enzyme-linked immunosorbent assay (ELISA) were collected, concentrated and stored at −70°C.

Polyacrylamide gel electrophoresis (PAGE). Electrophoresis of antigen preparations for detecting protein bands were carried out on 10% polyacrylamide slab gels with or without 0.1% sodium dodecyl sulfate (SDS) (15, 16). Electrophoresis was performed under nonreducing conditions and reducing conditions in which samples were boiled in the presence of 2-mercaptoethanol and SDS. Electrophoresed gels were stained with silver (17) or Coomassie Blue.

Western blot. Proteins with Ro/SSA antigenic determinants were detected by a method modified from Towbin et al. (18). Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose sheets in a Transblot cell (Bio-Rad Laboratories) at 200 mA for 5 h at 4°C.

After soaking with 3% bovine serum albumin for 30 min at 40°C, the sheets were incubated with an appropriate dilution of serum containing anti-Ro/SSA for 3 h at room temperature. Incubation with dilutions of normal human serum did not give rise to staining of any bands. After washing with PBS for 30 min, the sheets were incubated with an IgG fraction of goat anti-human IgG conjugated to peroxidase (Pel-Freez Biologicals, Rogers, AR) for 2 h. Sheets were washed again and soaked in substrate solution (1% *o*-dianisidine).

RNAse treatment. Ro/SSA antigen was incubated with RNAase A (Worthington Biochemical Corp., Freehold, NJ) for 2 h at 37°C. Changes in the mobility of the protein and RNA bands were determined by SDS-PAGE and their antigenicity assessed by the Western blot method as described above.

RNA extraction, radiolabeling, and urea gel. RNA was extracted from purified Ro/SSA antigen by phenol and precipitated with ethanol. ^{32}P-labeled cytidine 3',5'-biphosphate (ICN, Irvine, CA) was added to the 3' end of the extracted Ro/SSA RNA with T_4 RNA ligase (E.C. 6.5.1.3) (P-L Biochemicals, Milwaukee, WI) by the method of England et al. (19). PhiX174 DNA digested with Hae III (New England Biolabs, Beverly, MA) were used as nucleic acid molecular weight standards. The restriction fragments were treated with calf intestine alkaline phosphatase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and then T_4 polynucleotide kinase (P-L Biochemicals) in the presence of γ^{32}P-ATP (Amersham Corp., Arlington Heights, IL) by standard methods (20, 21). Samples and standards were applied to a 10% polyacrylamide gel containing 7 M urea. After electrophoresis, the dried gel was autoradiographed. These standards correctly predicted (±3%) the number of bases of known tRNAs.

Patient selection. 14 sera were selected for study with antibody to Ro/SSA by immunodiffusion from patients with SLE. All the patients fulfilled four or more of the revised American Rheumatism Association criteria for SLE (22). 15 normal sera were collected from healthy donors.

ELISA. The solid phase assay was modified from the ELISA technique of Engvall and Perlmann (23). Immulon plates (Dynatec Laboratories, Alexandria, VA) were coated overnight with 100 μl of affinity purified antigen in a carbonate buffer, pH 9.2. Preliminary experiments showed that 1 μg/ml of the antigen was optimal. After coating, plates were then washed thrice with 0.05% Tween in PBS. Sera were diluted in 0.05% Tween, 0.1% BSA, and 1% bovine IgG (Cohn fraction II, Sigma Chemical Co., St. Louis, MO) in PBS and allowed to incubate on the plate for 4–24 h at 4°C. In some experiments, serum dilutions prepared as described were preincubated at 4°C with 10 μg/ml Ro/SSA before being added to the plate. After washing the plates, a saturating dilution of the IgG fraction of goat antihuman IgG conjugated to alkaline phosphatase (Sigma Chemical Co.) in 0.05% Tween and 0.1% BSA in PBS was added to the plate and allowed to incubate at 4°C overnight. The plates were washed and incubated with paranitrophenylphosphate. Chromophore development was followed with a MR 580 Microelisa Auto Reader (Dynatec Laboratories) at 405 nm. A unit of binding activity was defined as that contained in a 10^{-7} dilution of an arbitrarily chosen monospecific anti-Ro/SSA serum. This was the limit of sensitivity for the assay.

Protein concentration. Protein concentrations were measured by a dye-binding assay (24).

Results

Purification of Ro/SSA. Purification of the Ro/SSA antigen from CTE was achieved by application of the dissolved and dialyzed 30–60% saturated ammonium sulfate fraction to the anti-Ro/SSA immunoadsorbent column. The column was washed with PBS until the optical density of the eluate became <0.01 at 280 nm. The column was then eluted with 0.1 M carbonate buffer (pH 11.2). The eluate was titrated immediately to pH 7.0 with 1.0 N HCl and concentrated by membrane filtration. This eluate contained La/SSB antigen detected by immunodiffusion and the Western blot method (data not shown) and was, therefore, passed through an anti-La/SSB immunoadsorbent column to remove contaminating La/SSB antigen.

For further purification, the pooled eluates were fractionated on a gel filtration column. The elution profile had two peaks in addition to the peak at the void volume (Fig. 1). Ro/SSA antigenicity was found in the void volume and in the second peak by ELISA but only peak II reacted with antibody in complement fixation reaction and by immunodiffusion. Due to the limited antigenicity of the void volume and because on SDS-PAGE under nonreducing conditions the void volume fractions did not show any sharp bands, the void volume was considered to contain aggregated Ro/SSA antigen. Fractions of peak II were collected and used as Ro/SSA antigen for all subsequent studies.

The relative antigenic activity of preparations were shown in Table I. As can be seen, the concentrated material from pooled peak II in immunodiffusion was purified 1672 times over the crude extract and therefore, the Ro/SSA antigen represented 0.06% of the soluble protein in the CTE if the final product is chemically homogeneous, which is suggested by the PAGE analysis in Fig. 2.

Characterization of Ro/SSA antigen. Affinity purified Ro/SSA antigen was shown to be active and antigenically identical to the Ro/SSA in crude CTE by agar diffusion (Fig. 3). Purified Ro/SSA in the well on the right fuses forming a line of identity with CTE in the well on the left, showing antigenic identity between purified Ro/SSA and CTE as identified by a monospecific anti-Ro/SSA serum. Normal human serum does not precipitate with either CTE or the affinity purified antigen. The

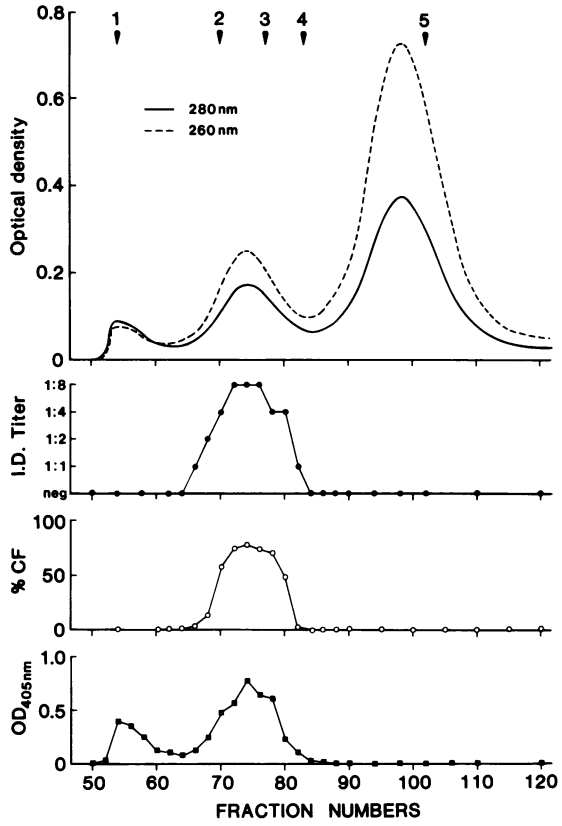

Figure 1. Fractionation of the antigen preparation eluted from anti-Ro/SSA immunoadsorbent column on Bio-Gel A-0.5-m column. Absorbance of fractions was measured at 280 nm (——) and 260 nm (-----). Arrows indicate elution volumes of (*a*) Blue dextran, (*b*) IgG: immunoglobulin G, (*c*) BSA (*d*) OVA: ovalbumin and (*e*) Mb: Myglobin. Immunological activity of the fractions was determined using anti-Ro/SSA reference serum by immunodiffusion (second level with I.D. titer as ordinate), microcomplement fixation (third level with % CF on ordinate) and ELISA (fourth level with OD_{405} nm on the ordinate). The dilutions of fractions used for micro CF and ELISA were 1:200 and 1:50, respectively.

elution behavior of the affinity purified Ro/SSA antigen on a Bio-Gel A-0.5-m column is seen in Fig. 1. As illustrated, only the second of the three peaks is antigenically active by complement fixation and immunodiffusion. Peaks I and II can both coat plates for an ELISA and are roughly similar on a weight basis. Its apparent molecular weight from the elution profile is ~100,000 which is a value similar to that previously determined with partially purified material (1, 25). The 260/280 ratios of the three peaks were found to be 0.83, 1.11 and 1.93, respectively, reflecting the protein rich character of the material in the void volume, the nucleic acid-enriched third peak, and the protein nucleic acid character of peak II, the active antigen. Purified calf liver RNA has a 260/280 ratio of 1.93, being very similar in its spectral properties to peak III.

Table I. Purification of Ro/SSA Antigen

	Protein concentration	Ro/SSA precipitin titer*	Relative specific purity‡
CTE	41 mg/ml	1:4	1
Eluate from immunoadsorbent column	125 μg/ml	1:8	654
Purified Ro/SSA from peak II	197 μg/ml	1:32	1672

* The highest titer at which a precipitin was detected by immunodiffusion.

‡ Values calculated by dividing the protein concentration by the Ro/SSA precipitin titer for each stage of the preparation and then normalizing by the same calculation for CTE. In effect, this calculation reflects the minimum protein concentration required for a precipitin reaction.

The ability of these three peaks to coat plates for a solid phase assay to detect anti-Ro/SSA is illustrated in the lowest panel of Fig. 1. Although the relative quantitative yields in each of the three peaks vary between preparations, the relative position of the peaks in the gel filtration column has been identical. As seen, both peaks I and II are active in coating plates for an anti-Ro/SSA ELISA but peak II has greater specific antigenicity. A 1/50 dilution of each fraction was used to coat the plate and then a dilution of anti-Ro/SSA serum was applied. After suitable washing and addition of the conjugate, color development was read at 405 nm on the Dynatec reader. The OD_{405}/OD_{280} ratio calculated for peak I at the maximum was 210 while the maximum values for peak II was 222, which is very similar. The alteration in the antigenicity of peak I is apparent by its failure to either precipitate or fix complement with anti-Ro/SSA serum. It is well known that both the precipitin and complement fixation reaction not only require multivalent antigens of appropriate epitope density, but are very sensitive to conformational change in complex macromolecular antigens. The ability of peak I to efficiently coat plates for an ELISA likely indicates that only a primary binding reaction is required for reactivity in the ELISA assay. Peak III had no measurable antigenicity for coating plates at the 1/50 dilution as illustrated.

Fig. 2 shows the PAGE pattern of the native peak II material. Only a single band is observed in contrast with the large number of bands seen in crude CTE.

The subunit structure of the Ro/SSA antigen under nonreducing and reducing conditions in the presence and absence of pretreatment with RNAase was analyzed by SDS-PAGE. The affinity-purified and gel-filtered Ro/SSA from peak II was electrophoresed in SDS-PAGE under reducing and nonreducing conditions and stained with Coomassie Blue and silver, respectively (Fig. 4). These experiments were performed with a single preparation from the peak of peak II without further

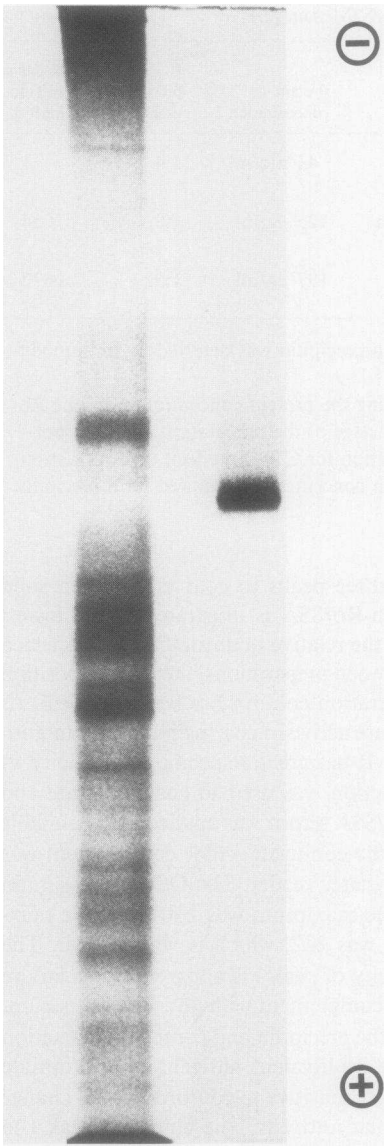

Figure 2. Photograph of a native 10% polyacrylamide gel with crude CTE (200 μg protein) on the left and affinity purified gel filtered Ro/SSA antigen (1 μg protein) on the right.

concentration. Only staining with the silver stain is presented in the figure. In lanes 1 and 2 are seen the antigen under non-reducing conditions while in lanes 3 and 4 the antigen is seen under reducing conditions. Lanes 1 and 3 are not preincubated with RNAase while lanes 2 and 4 are preincubated with RNAase. Lane 1 shows one strong band and two weak ones with apparent molecular weight of 40,000, 60,000 and 16,000, respectively. RNAase treatment leads to virtual loss of the 40,000 band, complete loss of the 16,000 band, and some increased staining of the 60,000 band (lane 2). Lane 3 shows that under reducing

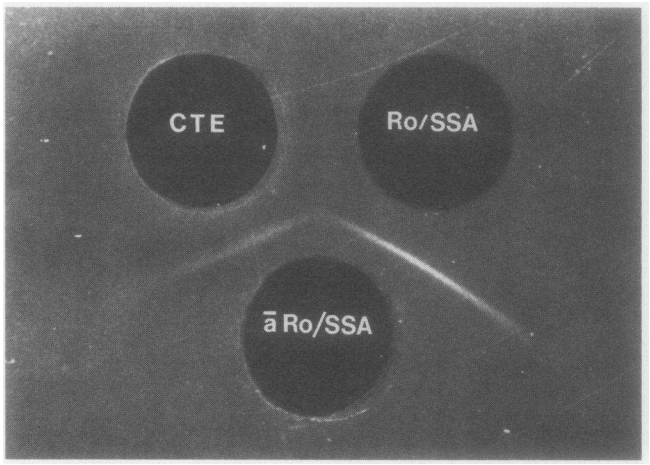

Figure 3. Antigenicity of crude CTE (40 mg protein/ml) in the upper left well compared with affinity-purified, gel-filtered Ro/SSA (60 μg protein/ml) in the upper right well in reaction with monospecific human anti-Ro/SSA serum. Note the reaction of complete antigenic identity.

conditions, in the absence of RNAase, there is a strong 60,000 band and a weak 16,000 band. RNAase treatment of the reduced material leads to loss of the 16,000 band and little change in the 60,000 band. In lanes 2 and 4, the new band at 18,000 is RNAase itself. Coomassie staining of these gels reveals that the 16,000 band never stains with Coomassie Blue (even if the antigen is concentrated threefold over the concentration used in these experiments) but that the 40,000 and 60,000 bands invariably stain with Coomassie Blue albeit with somewhat lesser intensity than with the silver stain.

Experiments in which the Ro/SSA antigen is boiled without mercaptoethanol and then subjected to SDS-PAGE also show that the 40,000 band disappears and the 60,000 band increases in intensity. This result suggests that reduction is not solely responsible nor is necessarily required for the loss of the 40,000 band. Conversion of the 40,000 band to the 60,000 band is suggested by the greater staining intensity of the 60,000 band in lane 2 as compared with lane 1 (Fig. 4). Two-dimensional gels (26) in which nonreduced antigen is electrophoresed in one dimension and reduced heated material[2] is electrophoresed in the second dimension have directly proven that the 40,000 band material is converted to the 60,000 band (experiments not shown). Thus, both the 40,000 and 16,000 bands appear to contain RNA (Fig. 4) and there is no evidence that protein is associated with the 16,000 band. Other experiments with twice the concentration of RNAase used here also show diminished intensity but persistent staining of the 60,000 band after RNAase treatment with the silver stain.

2. In the case of the two-dimensional gels, the first gel was heated to 60°C in reducing agent.

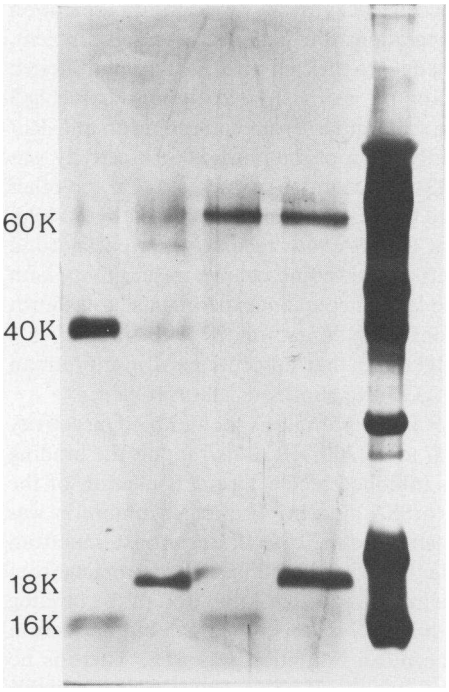

Figure 4. 2 μg of Ro/SSA were applied to each lane on SDS-PAGE with or without pretreatment of RNAase (0.6 μg). Two lanes on the left were run under nonreducing conditions (lane 1 without and lane 2 with RNAase). The two lanes on the right were run under reducing conditions (boiled in the presence of 2-mercatopethanol). Lane 3 was not pretreated with RNAase while lane 4 was pretreated with RNAase. Marker proteins are on the far right in lane 5.

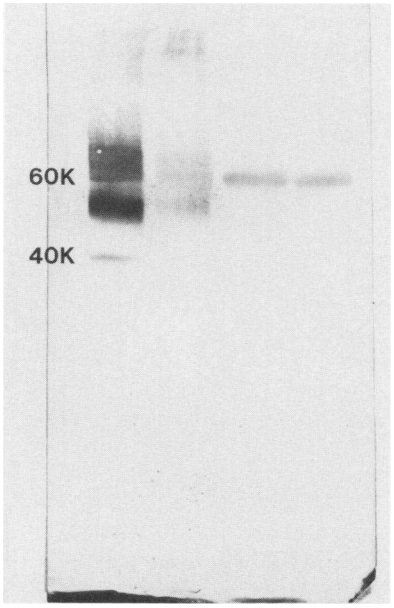

Figure 5. Western blotting of Ro/SSA preparation subjected to SDS-PAGE. Lane 1 on the left is Ro/SSA under nonreducing conditions. Lane 2 is Ro/SSA treated with 2.4 μg RNAase. Lane 3 is Ro/SSA treated with RNAase (2.4 μg enzyme) and reducing conditions. Lane 4 is Ro/SSA with reducing conditions alone.

Fig. 5 contains the Western blot analysis of Ro/SSA antigen after several treatments and analysis by SDS-PAGE. Lane 1 contains Ro/SSA under nonreducing conditions, and as can be seen, there is heterogenous activity in the 60,000 D region, a sharp 40,000 band, and some intermediate heterogenous material of about 50,000 D. Lane 2 contains RNAase-treated material subjected to SDS-PAGE under nonreducing conditions. One sees disappearance of the 40,000 band and diminution of intensity with the 60,000 band and the intermediate heterogenous material. Both lanes 3 and 4 contain Ro/SSA under reducing conditions but the lane 3 material was also pretreated with RNAase. They both reveal antigenic activity restricted to the 60,000 band.

These observations lead to the conclusion that there are two forms of the same polypeptide in the particle and that it is primarily 40,000 under nonreducing conditions on SDS gels and 60,000 under reducing conditions. This interesting behavior has been seen with several other polypeptides as will be discussed. It may also be the RNA which accounts for the paradoxical behavior in SDS since antigenicity by Western blotting is demonstrated in the 40,000 position before RNAase treatment but not afterwards. While this could also conceivably represent antigenicity dependent on RNA, this is unlikely since the same RNAase treatment has no demonstrable effect on the antigenicity of the Ro/SSA antigen in either precipitin or complement fixation reactions. In summary, the major evidence for believing that the 60,000 band is protein is the strong staining with Coomassie blue after all treatments including boiling, reduction, and high concentration of RNAase.

In addition, there is a band with the mobility in SDS-PAGE of a 16,000 protein which appears to be RNA as evidenced by its staining with silver, failure to stain with Coomassie Blue, and disappearance with RNAase (27). That this material was nucleic acid was established by phenol extracting the purified Ro/SSA antigen and repeating the SDS-PAGE. Only the 16,000 band remained; neither the 60,000 nor the 40,000 band were present. The true molecular weight of the RNA was established in a nucleic acid denaturing gel. The phenol extract of purified Ro/SSA was radiolabeled with T4 RNA ligase and compared with known standards (Fig. 6). The 3′ end labeled RNA formed three major bands and two minor bands which ranged in molecular weight from 24,000–27,000 or from 74 to 83 bases.

Finally, Ro/SSA antigen prepared from human spleen was compared with the calf thymus antigen in SDS-PAGE. The detailed subunit structure of material from both species revealed indistinguishable bands, at 60,000, 40,000 and 16,000 under nonreducing conditions and at 60,000 and 16,000 under reducing conditions.

ELISA. The solid phase assay method was applied to the quantitation of anti-Ro/SSA in sera with anti-Ro/SSA precipitins and in normal individuals.

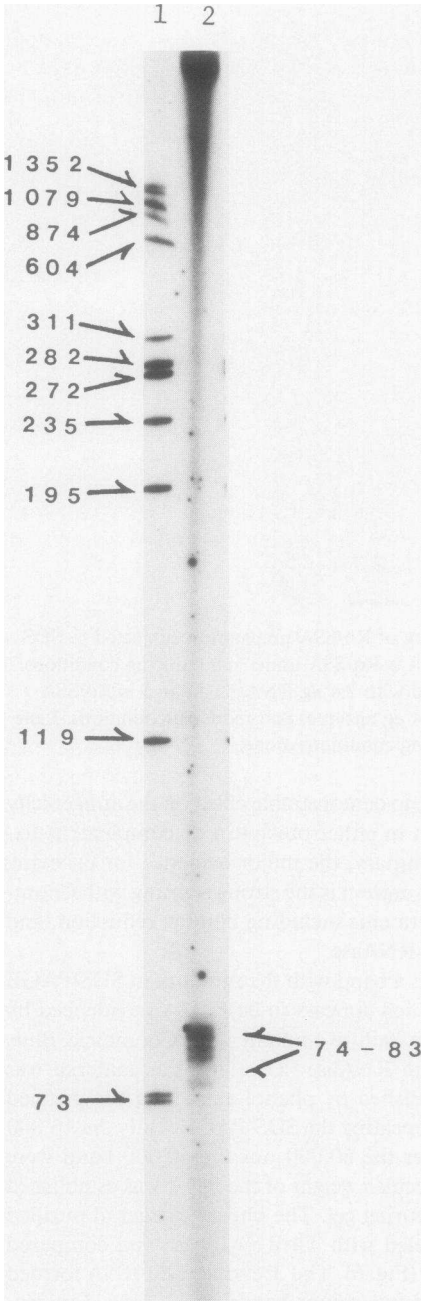

Figure 6. Autoradiography of urea gel from Ro/SSA RNA. Lane 1 shows the number of bases in the nucleic acid standards (Hae III restriction fragments of ϕX174). Lane 2 shows the phenol extract from purified Ro/SSA RNA containing five bands between 74 to 83 bases. Both standards and Ro/SSA RNA were end-labeled with a ^{32}P-containing base (see Methods), and therefore, are one base larger than before the labeling procedure was performed.

Initially, a very high background was found with normal sera which was not inhibited by purified Ro/SSA, and therefore, was thought to represent nonspecific binding. The difficulty caused by this nonspecific background varied from preparation to preparation. In preparations where this was a serious problem, the addition of bovine IgG to the sample at 10 mg/ml inhibited the nonspecific binding by nearly 20-fold. When bovine IgG was incubated with autoimmune serum containing an anti-Ro/SSA precipitin, the inhibition of the anti-Ro/SSA activity was decreased in the ELISA by as much as two-fold. On the other hand, the background found in normals decreased by 10-fold. Other preparations of Ro/SSA with relatively low background binding of normal serum showed no change in sensitivity with the addition of bovine IgG. Subsequent experiments have shown that some preparations were contaminated with minor (<5% wt/wt) amounts of IgG and that absorption with antihuman IgG and antibovine IgG have eliminated this problem.

14 sera with anti-Ro/SSA precipitins showed binding activity ranging from 545,000 to 18,200,000 units/ml and the binding of all these sera was inhibited >60% by preincubation of the sera with 10 μg of Ro/SSA antigen. The mean inhibition was 86%. These data are tabulated in Table II. In contrast, sera from normal donors (Table III) showed binding activity ranging from 1,582 to 18,445 units/ml and percent inhibition of the binding by preincubation with Ro/SSA was <44% in all but one serum (69% inhibition). The mean inhibition was 32%. There is no overlap in the level of autoanti-Ro/SSA between SLE patients with anti-Ro/SSA precipitins and normal individuals. Indeed, the highest normal value is almost 30-fold less than the lowest precipitin-positive SLE patient. The difference in the logarithmic average of the two groups was almost 1000-fold (Tables II and III).

Table II. Anti-Ro/SSA Binding in 14 SLE Sera with an Anti-Ro/SSA Precipitin

	Anti-Ro/SSA Binding (units)*		
SLE patient	Serum	Serum + 10 μg/ml Ro/SSA	Percent binding inhibition
1	13,100,000	1,210,000	91
2	1,480,000	302,000	80
5	1,080,000	113,000	90
9	8,110,000	420,000	95
10	13,200,000	1,710,000	87
12	18,200,000	986,000	95
13	866,000	331,000	62
14	2,320,000	836,000	64
15	545,000	124,000	77
16	1,350,000	23,100	98
17	3,810,000	109,000	97
18	6,970,000	988,000	86
19	1,380,000	206,000	85
21	9,650,000	495,000	95

* 1 unit of anti-Ro/SSA is the binding activity contained in a 10^{-7} dilution of an arbitrarily chosen anti-Ro/SSA monospecific serum. The log average of serum binding was 3.338×10^6 units.

Table III. Anti-Ro/SSA Binding in 15 Normal Sera

Normal donor	Anti-Ro/SSA binding (units)*		
	Serum	Serum + 10 μg/ml Ro/SSA	Percent binding inhibition
1	4,325	2,937	32
2	4,303	2,815	35
3	18,445	13,657	26
4	12,786	10,316	19
5	4,522	3,003	34
6	10,314	6,957	33
7	3,157	1,814	43
8	4,945	3,146	36
9	2,569	2,005	22
10	2,970	2,692	9
11	2,786	2,572	8
12	12,358	6,927	44
13	1,582	1,241	22
14	14,927	4,636	69
15	1,952	1,159	41

* 1 unit of anti-Ro/SSA is the binding activity contained in a 10^{-7} dilution of an arbitrarily chosen anti-Ro/SSA monospecific serum. The log average of serum binding was 3.388×10^3.

Discussion

A method is described for purifying Ro/SSA antigen from CTE and for a solid phase assay to detect antibody to the Ro/SSA antigen. The final preparation contains immunologically active Ro/SSA antigen with a specific antigenicity 1,672-fold higher than in the CTE starting material. The estimated molecular weight of the purified Ro/SSA antigen by gel filtration was ~100,000, which is similar to that reported previously (1, 25). The presence of a heavy protein material (peak I) and a lighter nucleic acid-enriched material (peak III) in Fig. 1 suggest some alteration of the native material induced by the alkaline buffers in the final elution step from the immunoaffinity column. As already mentioned, peak I is primarily aggregated protein while peak III is low molecular weight nucleic acid material; the former with some limited antigenicity and the latter with no measurable antigenicity.

By SDS-PAGE, Ro/SSA antigen was composed of a major 60,000 band, and a 16,000 band which lacked antigenicity and whose RNA nature is suggested by its complete disappearance with RNAase, failure to stain with Coomassie Blue, and its presence in the phenol extracted Ro/SSA antigen.

The true molecular weight of the apparent 16,000 material on SDS-PAGE is between 24,000 and 27,000 on nucleic acid-denaturing gel analysis. Indeed, the multiple bands of RNA found from the bovine source corresponds with the multiple bands and forms of Ro/SSA RNA found in mouse, rat, and man (4, 28, 29). In man, sequencing data show that the RNA species vary from 83 to 112 bases (29, 30), which closely corresponds to the ~73–82 base composition of the bovine Ro/SSA shown here. In addition to intrinsic differences in bovine Ro/SSA RNA, the heterogeneity presented in Fig. 6 may also be due to partial degradation and the larger part of each doublet could be explained by adding more than one base to the Ro/SSA RNA. How many truly different Ro/SSA RNA species are present in bovine tissue must await similar sequencing data from this species.

In gels studied under nonreducing conditions, three bands are regularly seen with the silver stain and a 40,000 band is prominent. This prominent band is not seen after boiling alone, boiling and mercaptoethanol, or RNAase treatment. The disappearance of the 40,000 band after these treatments is worthy of comment. Such behavior could be due to fragmentation of the molecule by the treatment or a change of mobility on SDS-PAGE. The latter interpretation seems likely since the 60,000 band clearly increases as the 40,000 band decreases in gels stained with Coomassie Blue, suggesting that the former derives from the latter. Direct evidence for the conversion of the 40,000 material to the 60,000 material has been obtained from 2 D gels stained with silver and/or subjected to the Western blotting technique. There are at least five additional proteins which behave this way, i.e., the reduced boiled protein has a slower mobility in SDS-PAGE than the nonreduced protein. These include the T_{10} antigen (31), the carboxyl peptide of procollagen IV (32), fetuin (33), plasminogen activator from human melanoma cells (34), and the ^{125}I-multiplication stimulating activity receptors labeled species of liver plasma membranes (35). The mechanism of this effect with the Ro/SSA antigen may reflect incomplete SDS binding because of the polyanionic nature of the RNA-protein complex. Heat or RNAase would appear to disrupt these remaining bonds and thus facilitate full binding of SDS, which increases the Stokes radius of the protein, and therefore, causes decreased mobility in SDS-PAGE.

In any case, it is reasonable to hypothesize that the native 100,000-D RNA-protein complex is composed of a single 60,000 protein molecule and one 24,000–27,000 RNA molecule. The error in determining molecular weights by gel filtration is at least 10%, which means that the particle could be 90–110 D and have one or two 24,000–27,000 D RNA molecules. Thus far, antigenicity has only been demonstrated on the isolated 60,000 protein band by the Western blotting technique. There may be antibodies directed against the RNA protein particle which are specific for the complex but other experiments are necessary for their demonstration.

A comment is in order about the coelution of La/SSB antigen from the anti-Ro/SSA affinity column. The particular serum used for the construction of this column had an anti-La/SSB titer in the normal range by a sensitive ELISA (36). We are confident that the La/SSB antigen was effectively removed as evidenced by the absence of its characteristic 40,00 and 30,000 peptides in Western blotting experiments in which anti-La/SSB serum was used to probe for the presence of these peptides in the Ro/SSA preparations and as also evidenced by the inability of the Ro/SSA preparation to inhibit anti-La/SSB activity in

the anti-La/SSB ELISA (data are not presented). Despite this, it is possible that this serum contained small amounts of specific anti-La/SSB, which accounted for the binding of the La/SSB antigen. Alternatively, it may be that there are molecules of Ro/SSA which are complexed to La/SSB molecules. This coelution behavior of a proportion of the Ro/SSA and La/SSB molecule has also been noted by Venables et al. (37) who also reported an antigenically active Ro/SSA human spleen antigen in denaturing gels of 55,000 molecular weight. This slight difference in molecular weight might represent the difference between the bovine and human species or more likely variation in experimental results. Upon direct comparison, the purified human and bovine Ro/SSA antigen cannot be distinguished by SDS-PAGE analysis in our laboratory.

Availability of a sensitive and quantitative assay for anti-Ro/SSA and the Ro/SSA antigen should provide new information on the role of this antigen-antibody reaction in disease as well as permit the exploration of the cellular mechanism for the production of this autoantibody.

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