LUPUS AUTOANTIBODIES TARGET RIBOSOMAL PROTEINS

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Although antibodies against nuclear antigens are considered the hallmark of systemic lupus erythematosus (SLE), antibodies against cytoplasmic constituents have also been identified. Despite their recognition more than 25 years ago, the prevalence, antigenic specificities, and disease associations of antiribosomal antibodies (ARA) are all controversial. Since these problems may relate to detection of different ribosomal or ribosome-associated antigens, we attempted to define more precisely the identity of the ribosomal protein antigens. The results of this study indicate that ARA reactive with ribosomal proteins occur in ~5–10% of SLE patients, but are highly specific, binding to epitopes on 3 out of a total of ~80 proteins.

Materials and Methods

Antibody Screening Procedures. Cellular localization of antigens was determined by indirect immunofluorescence using Hep-2 cells as substrate. The effect of the enzymes RNase (50 µg/ml), trypsin (5 µg/ml), and proteinase K (0.5 µg/ml) on the intensity of immunofluorescence was quantitatively analyzed using a FLAX fluorometer, kindly loaned by Dr. L. J. Kagen (The Hospital for Special Surgery). Reactivity with saline-soluble extracts of human spleen and rabbit thymus was determined by counterimmunoelectrophoresis (CIE) using standard reference serum to Ro, La, Sm, and ribonucleoprotein (RNP). An anti-Jo-1 reference serum was kindly provided by C. C. Bunn and G. R. V. Hughes, Hammersmith Hospital, London, Great Britain.

Isolation of Ribosomes. Ribosomes were isolated from dog, rat, and chicken livers essentially by the method of Fairhurst et al. (13). Briefly, livers were homogenized in 0.25 M sucrose in TK2-M buffer (20 mM Tris-HCl, 25 mM KCl, 5 mM MgCl2, pH 7.5). The postmitochondrial supernatant was adjusted to 1.0% deoxycholate and centrifuged through a discontinuous sucrose gradient using an SW40 rotor. In some cases, the KCl concentration in the sucrose was increased to 500 mM. The ribosomal pellet was washed and resuspended in distilled water or an appropriate buffer.

Analysis of Ribosomal Constituents. The isolated ribosomes had an optical density 260/280 ratio of ~1.6. The RNA composition was analyzed by sucrose density ultracentrifugation on a 5–20% linear gradient using a SW60 rotor, as well as by a 2% composite gel in a vertical gel apparatus. The ribosome was separated into large and small subunits either by incubation in 20 mM EDTA for 10 min at room temperature, or by

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Abbreviations used in this paper: ARA, antiribosomal antibodies; CIE, counterimmunoelectrophoresis; CM-cellulose, carboxymethylcellulose; NEPHGE, nonequilibrium pH gel electrophoresis; RNP, ribonucleoprotein; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SLE, systemic lupus erythematosus; TCM, Tris-potassium-magnesium buffer; TM, Tris-magnesium buffer; TPE, Tris-phosphate-EDTA buffer.

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exposure to 0.1 mM puromycin, as described by Blobel and Sabatini (16). The subunits were fractionated by sucrose density ultracentrifugation using a 15–30% gradient and SW27 rotor. The buffers used were either TKsoM (TKM buffer with 50 mM KCl), TKs00M (500 mM KCl), or 20 mM Tris HCl, 100 mM NaCl, 10 mM EDTA, pH 7.5. For further analysis of sucrose density fractions, A990 peaks were pooled and concentrated by pelleting at 200,000 g in a SW 60 rotor. Ribosomes were also analyzed on a 2% composite gel using either 25 mM Tris HCl, 1 mM MgCl2, pH 8.0, (TM) (whole ribosome) or 36 mM Tris HCl, 30 mM NaH2PO4, 10 mM EDTA, pH 7.8, (TPE) (subunits) as buffers. RNA was stained by ethidium bromide, and proteins were stained by Coomassie blue. For ultracentrifugation and composite gel studies, reference 16 and 23 S. E. coli ribosomal RNA (Boehringer Mannheim, Mannheim, Federal Republic of Germany) were used as markers.

Identification of Ribosomal Constituents. Precipitating antibodies to ribosomal antigens were determined by CIE and immunoelectrophoresis using standard methods (17). In some experiments, ribosomes were preincubated with RNase (50 µg/ml), trypsin (100 µg/ml), or proteinase K (100 µg/ml) before analysis. SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) (18) and Western blotting (19) were performed as described in detail previously (12). Two-dimensional gel electrophoresis was performed by the method of O’Farrell, using either isoelectric focusing (20) or nonequilibrium pH gel electrophoresis (NEPHGE) in the first dimension (21). Pharmalyte ampholines, pH 5–8 and pH 3–10 were used in a 4:1 ratio for isoelectric focusing, or in a 1:4 ratio for NEPHGE. Isoelectric focusing in agarose was performed as described previously (22).

For some experiments, ribosomal proteins were labeled with 125I using the chloramine T method (23). Screening for ARA was performed by a solid-phase radioimmunoassay as described by Cavanagh (24).

Analysis for Acidic Ribosomal Antigens. To determine whether the protein antigens identified had similar properties to any of the acidic ribosomal proteins described previously (25, 26), isolated ribosomes were incubated with 100 µg/ml RNase A (Worthington Biochemical Corp., Freehold, NJ) for 40 min at 37°C. The ribosomal proteins were then dialyzed against buffer E (6 M urea, 20 mM Na acetate, 0.5 mM dithiothreitol, 0.1% methylamine, pH 4.2) and applied to a carboxymethyl (CM)-cellulose column (Whatman Inc., Clifton NJ) equilibrated in the same buffer. Unbound proteins were eluted with buffer E, concentrated, and the protein antigens were detected by SDS-PAGE and Western blotting. Freshly isolated aliquots of ribosomes were also treated with either 1.0 M or 0.4 M NH4Cl and 50% ethanol (vol/vol) at 0°C as described by Sanchez-Madrid et al. (26). The supernatants and precipitates were analyzed by SDS-PAGE and Western blotting. Total proteins were detected by the ultrasensitive silver stain (27). To determine whether the antigens were phosphorylated, the isolated ribosome in 100 mM Tris HCl, 0.1 mM ZnCl2, 1 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, pH 8.0 was incubated with 5 U intestinal alkaline phosphatase (type VII; Sigma Chemical Co., St. Louis, MO) for 2 h at 37°C. The enzyme-treated preparation and buffer control (also incubated at 37°C for 2 h in the same buffer) were analyzed by two-dimensional electrophoresis and Western blotting as described above.

Results

Characterization of Antisera. Five sera with high-titer ARA were used for all results obtained in this study. Subsequently, during screening (see below), an additional four sera with ARA were identified. All of these patients had SLE. By immunofluorescence, three patients had virtually exclusive cytoplasmic staining, and two patients had both nuclear and cytoplasmic fluorescence. Prominent nucleolar staining was produced by two sera. By CIE, two sera had anti-Ro, and one serum contained anti-Sm-RNP antibodies, as well as ARA. None of these sera contained Jo-1 antibodies detected by CIE.

Ribosome Analysis. As shown in Fig. 1, ribosome-associated RNA had different
**Figure 1.** Localization of the ribosomal protein antigens. Ribosomes were resolved by composite gel electrophoresis in either magnesium- (TM) or EDTA- (TPE) containing buffers (A and B, respectively). The composite gel strips were layered over SDS-polyacrylamide gels, and the protein antigens detected by SDS-PAGE and immunoblotting (vertical dimension). The localization of the protein antigens was established by identifying the ribosome (lane r in A) or its subunits (lane r in B) with ethidium bromide staining of duplicate composite gel strips (shown in the horizontal dimension). Lane m in the composite gel strips contained the 16 and 23 S markers. Molecular masses (kD) of the protein antigens are shown on the left.

Electrophoretic mobilities when resolved on composite gels in TM or TPE buffers. In the TM buffer, both protein and RNA (polyribosome) were concentrated in the top 1 cm of the gel (Fig. 1A). Ribosomes exposed to EDTA were resolved into subunits, which migrated anomalously (14) on either side of the prokaryotic 23 S marker (Fig. 1B). Pretreatment of ribosomal subunits with SDS released two major RNA bands of estimated mol wt $7.4 \times 10^5$ and $1.6 \times 10^6$ (close to the reported [28] values for 18 and 28 S RNA). Analysis of ribosomal RNA by ultracentrifugation revealed two major peaks at 18 and 28 S (not shown). After exposure to puromycin (see Fig. 4) or EDTA, sedimentation values of ~60 and 40 S (puromycin), and 50 and 32 S (EDTA) were obtained for ribosomal subunits.

**Detection of Ribosomal Antigens and Effect of Enzyme Treatment.** Preincubation of ARA, but not anti-Sm-RNP serum, with isolated ribosomes completely abolished cytoplasmic immunofluorescence staining of Hep-2 cells. Four of the five ARA sera produced precipitin line(s) of identity with human spleen extract as seen in CIE. The serum with a negative result had the lowest ARA titer by radioimmunoassay. Although several experiments suggested that some ARA without Ro or Sm-RNP reactivity produced two lines to spleen extract or isolated ribosomes, clear separation of these lines was not obtained, and in no case were lines of nonidentity observed (Fig. 2A).

CIE and immunoelectrophoresis showed that both patient and normal serum precipitated with the isolated ribosome regardless of whether Mg$^{2+}$ (5 mM) or
Characterization and Localization of Ribosomal Antigens. Western blot analysis of the isolated ribosome revealed a major antigenic protein of 38 kilodaltons (kD), and two less strongly reactive antigens at 17/19 kD for all five sera (Fig. 3). These antigens were present in similar intensity, regardless of whether the ribosomes were isolated by centrifugation through sucrose containing 25 or 500 mM KCl. Monospecific high-titer antisera against Ro, La, Sm-RNP, Jo-1, and microsomal antigens failed to react with any of the ribosomal proteins transferred to nitrocellulose paper. Of the four ARA detected by screening, three reacted with the 38, 17, and 19 kD proteins, and one reacted with additional protein of 27 kD.

To detect the protein antigens in polyribosomes and in the ribosomal subunits, ribosomes were electrophoresed on composite gels in either TM or TPE buffers.
After completion of the run, gel slices were removed, soaked in equilibration buffer (20), and electrophoresed in the second dimension on a SDS-PAGE gel. Proteins were subsequently blotted to nitrocellulose and probed with patient serum as described above. The positions of polyribosomes, and small and large subunits were determined by ethidium bromide staining of duplicate tracks of ribosome run on the same composite gel. In the presence of magnesium (Fig. 1A), the 38 and 17/19 kD protein antigens were located in the same position as the polyribosomes. When resolved in the presence of EDTA, both the 38 and 17/19 kD proteins migrated with the slower of the two major RNA bands (Fig. 1B), presumably indicating an origin from the large ribosomal subunit. This conclusion was supported by Western blot studies performed after ultracentrifugation of ribosomal subunits in TK500M buffer, as described above. Western blots of untreated ribosome (Fig. 4a) and puromycin-treated ribosome (b) are compared. In the untreated ribosome, antigens were concentrated in the pellet of the 10–30% gradient (not shown), whereas maximal antigen activity was recovered from the 60 S subunit region of the puromycin-treated ribosome (Fig. 4d).

Immunoblots of antigens detected by patient sera after two-dimensional gel electrophoresis are shown in Fig. 5. In the NEPHGE gel system, the 38 kD antigen (three discrete dots) and the 17 kD antigen showed a similar charge distribution at the anodal end of the gel, whereas the majority of the radiolabeled ribosomal proteins were found at the cathodal region of the first dimension of the gel (not shown). In Fig. 5A, the Western blot performed after isoelectric focusing and SDS-PAGE of the ribosome indicated that the 38 kD protein was more basic than the 17 kD antigen, which in turn was more basic than the 19 kD antigen. Following exposure to alkaline phosphatase, the lower molecular mass antigens shifted to the more basic region of the first dimension gel,
suggestion loss of acidic phosphate residues (Fig. 5B). The first- and second-dimension gels were run together in Fig. 5A and B. After RNase treatment, the pI of the ribosomal antigens determined by agarose isoelectric focusing was 5.6–6.5.

Identity of Ribosomal Antigens. To determine whether the 38 kD and 17/19 kD antigens contained cross-reactive epitopes, an antibody affinity-purification experiment was performed. After transfer of ribosomal antigens to the nitrocellulose paper in the standard Western blot, the 38 and 17/19 kD strips, and a <15 kD (control) strip were cut out and blocked with 3% bovine serum albumin and 1% goat serum. After applying ARA to each strip, the strips were washed and then eluted with 0.1 M glycine HCl, 500 mM NaCl, pH 2.8. The eluted antibodies were neutralized and used to probe the high and low molecular mass antigens. As shown in Fig. 6, antibodies eluted from the 38 kD strip reacted equally well with the 17/19 kD antigens, and vice versa. Proteins eluted from the control strip failed to bind to either set of antigens. Both the 38 kD and 17/19 antigens were detected in Western blots of fresh whole cells (human lymphocytes), isolated nuclei (rat hepatocytes), and wheat germ and rabbit reticulocyte extracts (not shown).

A Western blot of proteins eluted from the CM-cellulose column revealed that the 17/19 kD proteins were present in the fall-through fraction (Fig. 7A). Similarly, only two proteins (of molecular mass ~17/19 kD, as determined by silver stain) were extracted with NH₄Cl-ethanol at 0°C, and both these proteins contained antigenic determinants for ARA (Fig. 7B). The 38 kD antigen, in contrast, was not extracted by either 400 or 1,000 mM NH₄Cl, and remained with the ribosomal core.

The identity of the antigens as the P ribosomal proteins was confirmed by one-
FIGURE 5. Two-dimensional gel electrophoretic analysis of ribosomal antigens using isoelectric focusing in the first dimension, and Western blotting. Ribosomes were treated either with buffer alone (A) or alkaline phosphatase (B) for 2 h at 37°C immediately before analysis. The anode (+), as well as the directions of charge (horizontal arrow) and size (vertical arrow) separation are indicated.

and two-dimensional Western blots using a mouse monoclonal anti-P (kindly provided by Dr. H. Towbin, Ciba/Geigy Ltd., Basel, Switzerland) (35) and a rabbit anti-L44/45 (kindly provided by Dr. J. G. P. Ballesta, Centro de Biologia Molecular, Madrid, Spain) (38). Two-dimensional analysis of the human autoantibody (Fig. 8A) and mouse monoclonal antibody (B) indicated binding to the same three proteins in chicken ribosomes. The human autoantibody and rabbit anti-L44/45, but not normal human serum, bound to a highly purified P protein (kindly provided by Drs. I. Wool and A. Lin, University of Chicago, IL), as shown in Fig. 8C.
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Figure 6. Crossreactive epitopes. Antibodies eluted from the immobilized 38 kD (a), 17/19 (b) or <15 kD (c) ribosomal proteins were used to probe separate strips of nitrocellulose containing high- and low-molecular mass ribosomal proteins (see Results). Antibodies eluted from either the 38 or 17/19 kD antigen-containing strips (but not the <15 kD strip) reacted with both the 38 and 17/19 kD proteins.

Frequency of ARA. Considerable variation was noted in the binding of normal serum to solid-phase-adsorbed ribosomes. Five of seven ARA had elevated binding levels, while two sera had binding within the normal range. Only 2 of 43 (5%) of randomly selected SLE sera had positive results by radioimmunoassay. Of 20 SLE patients screened for ARA by Western blotting, 2 (10%) gave positive results.

Since completion of this study, five further ARA have been identified that react with the same 38, 19, and 17 kD proteins.

Discussion

Recent application of cell labeling with immunoprecipitation as well as Western blotting methods have enabled the precise specificities of lupus autoantibodies to be determined (reviewed in 29). By combining subcellular and protein fractionation techniques with immunoblotting, we have identified the major protein antigens targeted by antiribosomal antibodies in lupus sera. These proteins are almost certainly part of the ribosome, rather than loosely bound contaminating
proteins, for the following reasons: (a) the isolated ribosome appeared homogeneous when analyzed both by ultracentrifugation and composite gel electrophoresis, and contained none of the other well-characterized lupus antigens; (b) the localization of the antigens was predictably influenced by exposure of the ribosome to puromycin, EDTA, and SDS; and (c) when ultracentrifuged through a sucrose gradient containing a high concentration of KCl (500 mM), the protein antigens could be localized to the position of the large ribosomal subunit (60 S). Loosely associated cytoplasmic proteins are dissociated from the ribosome subunits under these conditions (30). The ribosomal antigens identified in this report
were readily distinguishable from the two-dimensional fingerprints of the other major intracellular targets of lupus autoantibodies (31).

Although the majority of ribosomal proteins are basic (32) a number of acidic ribosomal proteins have more recently been characterized (25, 26, 33–35). P₁ and P₂ are two acidic proteins from the large ribosomal subunit, with molecular masses of ~16 and 15 kD (25). P₁ and P₂ are the only large-subunit proteins not retained by CM-cellulose at pH 4.2 in 6 M urea (25). Towbin et al. (36) have identified a previously unrecognized 38 kD large-subunit acidic protein (P₀) that shares highly conserved antigenic determinants with the P₁/P₂ proteins. These three proteins were present in the nucleoli, suggesting that they are early-assembled proteins of the ribosome (36). The proteins identified in our study share all of the above properties with P₀, P₁, and P₂. Their identification in Western blots of whole cells (see Results) suggest that the 17/19 kD proteins are not breakdown products of the 38 kD protein, in keeping with the observations of Towbin et al. (36). Most importantly, the 17/19 kD antigens detected in this study were extracted under stringent conditions for the P₁/P₂-equivalent proteins (400 mM NH₄Cl/50% ethanol at 0°C [26]), whereas the 38 kD protein remained in the precipitate as anticipated (36). Although the pI values previously reported (35, 37, 38) for the P₁/P₂-equivalent proteins varied between 4.4 and 5.2, the higher pI values (5.6–6.5) for the antigens identified here are partly due to the near-neutral pI of the 38 kD antigen, as revealed by the two-dimensional studies. Differences in protein preparation and pI estimation in the absence of urea and SDS may also have influenced these findings. The charge heterogeneity observed for the 17/19 and 38 kD antigens, and basic shift after exposure to alkaline phosphatase is compatible with their identification as phosphoproteins (25, 35, 36). The co-identity of the proteins targeted by lupus autoantibodies in this study, and the P proteins (25) or their equivalent (L₄₄/₄₅) in yeast (38) was confirmed using reference antigen (25), as well as reference monoclonal (36) and polyclonal (38) antibodies. P₁/P₂ are regarded as the mammalian equivalent of the L₇/L₉ proteins in E. coli, and have also been shown (26) to influence elongation factor–dependent GTP hydrolysis.

The 80 S mammalian ribosome is composed of approximately equal amounts of protein and RNA (32). Previous studies (3, 4, 6–9) have provided inferential evidence, based on enzyme digestion and inhibition methods, for the existence of both protein and RNA, or an RNP antigen(s) reactive with lupus antibodies. In this study, we have shown that all lupus sera that produced specific precipitin lines with spleen extract in CIE reacted with ribosomal proteins in the absence of RNA in Western blots. This finding is similar to observations (39) made with other RNP target antigens in SLE and related diseases. However, the disappearance of precipitin lines from some patient sera after RNase treatment of the spleen extract or isolated ribosome suggests that ribosomal RNA may enhance the antigenicity of the ribosomal proteins, or serve as an additional antigen. Evidence for the coexistence of anti-ribosomal RNA antibodies is also provided by the persistence of precipitin lines after protease treatment of the ribosome, and a decrease in fluorescence intensity of ARA after exposure of Hep 2 cells to RNase. The results of these experiments should, however, be cautiously interpreted because of the possibilities of incomplete digestion and changes in protein
charge or solubility after enzyme treatment of an RNP complex. Reactivity with rRNA, RNP, or apparently nonspecific binding of normal serum to the isolated ribosome in some test systems (see Results) may explain the higher estimates (up to 70%) of the frequency of ARA in SLE. Screening of lupus sera in this study suggests a frequency of 5–10% for ARA binding to the 38 and 17/19 kD protein antigens.

Although the significance of ARA in SLE is uncertain, the ribosomal P proteins share similarities with the targets of other lupus autoantibodies (29). The proteins are part of a soluble protein–nucleic acid complex, and are highly conserved. Further studies are necessary to determine whether the chemical structure (e.g., phosphate groups), localization, or function of the P proteins is related to their antigenicity.

Summary

All nine SLE (systemic lupus erythematosus) sera with antiribosomal antibody activity targeted the same three ribosomal protein antigens, of molecular masses 38 and 17/19 kD when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting. One serum reacted with an additional protein of ~27 kD. Ribosomal subunit fractionation by composite gel electrophoresis and sucrose density ultracentrifugation showed that these proteins were part of the large subunit. Isoelectric focusing in agarose, and two-dimensional polyacrylamide gel electrophoresis revealed that the antigens had pI between 4.5 and 6.5, but that the 17/19 kD antigens were more acidic than the 38 kD antigen. Similarities in the molecular masses, charges, as well as the presence of highly conserved crossreactive epitopes, failure to bind to carboxymethylcellulose at pH 4.2, and extractability of the 17/19 kD proteins by 400 mM NH₄Cl-ethanol at 0°C indicated that these antigens were analogous to the proteins P₀ (38 kD) and P₁/P₂ (17/19 kD) described previously (25, 36). Co-identity was confirmed using reference antibodies and antigen. Although antibodies to these proteins were only found in 5–10% of more than 50 sera screened by radioimmunoassay or Western blotting, the selective production of antibodies to epitopes on three (out of a total of more than 80) ribosomal proteins may provide further clues to autoantibody induction of SLE.

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