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Research Article

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Antibodies from Patients with Connective Tissue Diseases Bind Specific Subsets of Cellular RNA-Protein Particles

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ABSTRACT We characterized the RNA-containing antigens precipitated by sera from 260 patients with positive antinuclear antibodies. 49 individuals, most of whom had systemic lupus erythematosus or Sjögren's syndrome, possessed antibodies that precipitated the previously identified RNP, Sm, Ro, and La antigens either singly or in combinations. These antigens, which are located on discrete sets of small nuclear or cytoplasmic RNA-protein particles, exhibited a number of antigenic interrelationships. One patient's serum recognized a new particle containing a small RNA which we have called Th; it also precipitated the Ro complexes. Other patients with systemic lupus erythematosus, hepatitis B virus infection, juvenile rheumatoid arthritis, myositis, and rheumatoid arthritis had antibodies that precipitated specific subsets of ribosomal RNA and transfer RNA. One patient's serum contained a monoclonal immunoglobulin G that precipitated ribosomes. Most of these antibodies identified antigenic determinants constituted at least in part of protein. The specificity of the proteins bound to particular cellular RNA, probably explains the exquisite precision with which antibodies from rheumatic disease patients discriminate among RNA subsets. Such sera should be useful probes for investigating specific roles that different RNA and RNA-protein complexes play in cellular metabolism.

INTRODUCTION

The antigen-antibody systems that characterize systemic lupus erythematosus (SLE)[1] are important from a variety of viewpoints. For example, knowledge about the structure and normal biological function of "lupus" antigens could provide clinicians with insight into one of the central questions about the pathogenesis of this disease—why are nucleic acids, which are usually poor immunogens, selected so regularly as targets for seemingly spontaneous immune responses in these patients? Conversely, "lupus" antibodies could provide biochemists with probes for studying the metabolic function of molecules to which animal antisera cannot be induced conveniently.

Recently we developed methods for isolating ribonucleoprotein antigens and analyzing them electrophoretically (1). This new approach shows clearly that the RNP, Sm, Ro, and La antigens of SLE reside on discrete, small ribonucleoprotein particles composed of a single small nuclear or cytoplasmic RNA molecule complexed with protein (1, 2). For convenience, we refer to these particles as snRNPs (for small nuclear RNA-protein particles) or scRNPs (for small cytoplasmic RNA-protein particles). Immunofluorescent staining with specific antisera has shown that the RNP and Sm antigens are found within nuclei (3, 4)—hence on snRNP—while Ro is found within the cytoplasm (5)—hence on scRNP. Although the La antigen (also called Ha and SS-B) (6) has been thought to reside in the nucleus (7) or the cytoplasm (8, 9), our efforts indicate that it is largely located within the nucleus but readily leaks out during preparation of cell fractions (10). Consequently we refer to particles that bear this antigen as La RNPs.

Antibodies from patients with SLE are helping to clarify the function of snRNPs and scRNPs. For ex-

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[1] *Abbreviations used in this paper*: ANA, antinuclear antibodies; ENA, extractable nuclear antigen; scRNP, small cytoplasmic RNA-protein particle; SLE, systemic lupus erythematosus; snRNP, small nuclear RNA-protein particle.

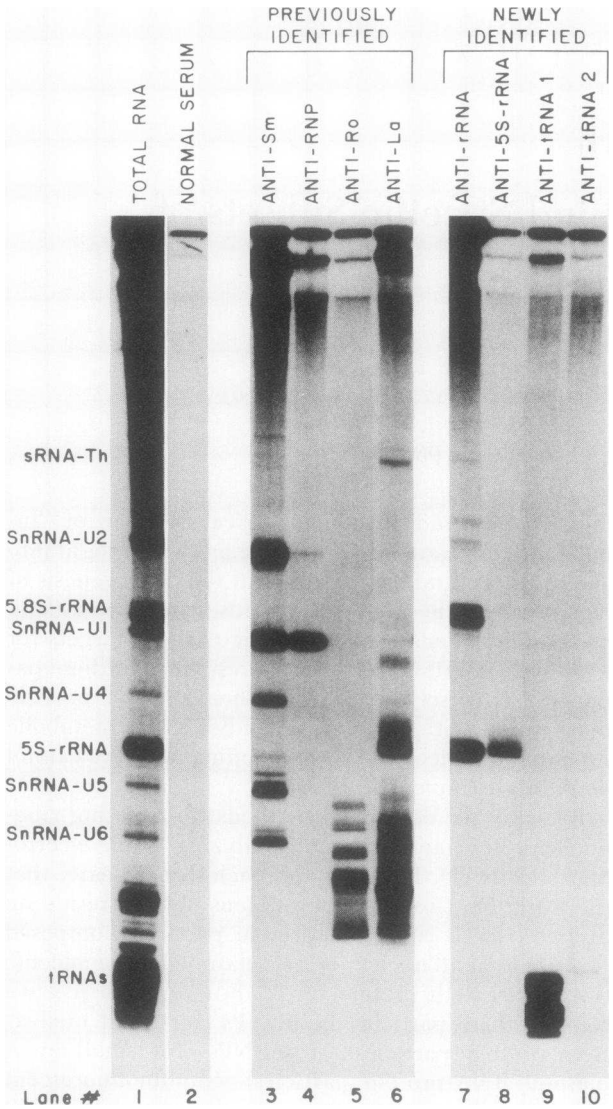

FIGURE 1 Specific subsets of small RNA are recognized by patients' sera. The most prominent small RNA components in total cellular RNA (lane 1) along with a newly recognized RNA called Th (lane 6) are identified. Anti-Sm (lane 3), anti-RNP (lane 4), anti-rRNA (lane 7), anti-5SrRNA (lane 8), anti-tRNA1 (lane 9) and anti-tRNA2 (lane 10) precipitate highly selected subsets of these prominent species. Normal serum (lane 2) precipitates no small RNAs. Other sera such as anti-Ro (lane 5) and anti-La (lane 6) precipitate RNA components so low in abundance that they are not even visible in total cellular RNA; much larger amounts of sample were loaded to detect the RNA species shown in these lanes. It can be seen that the Ro scRNPs migrate in close approximation with a subset of the La RNPs. RNA fingerprint analysis and reconstitution experiments prove that the RNPs precipitated by anti-Ro are a subset of those precipitated by anti-La (10). The large amounts of material at the origin in some lanes is attributed to the presence of antibodies that react with DNA because DNAase digestion of the radiolabeled substrate results in its disappearance.

ample, the RNP antigen isolated with anti-RNP antibodies, consists of the small nuclear RNA U1 and seven proteins (1). The U1-snRNP appears to regulate the posttranscriptional processing of messenger RNA by ensuring the precise excision of nonsensical intron segments from newly transcribed heterogeneous nuclear RNA (11). In cell-free systems, this processing activity is blocked by the anti-RNP antibody (12).

Our earlier studies suggested that antibodies from SLE patients are precise in their ability to discriminate among various RNA-protein complexes (1, 2). Consequently we have sought to determine how many different RNA or RNA-protein particles become targets of immune responses in human diseases. We have found that small nuclear and cytoplasmic RNA, ribosomal RNA, and transfer RNA can be divided into discrete antigenic families on the basis of their reactivity with sera from rheumatic disease patients.

METHODS

Patients. All sera submitted over a 3-mo period to the Clinical Immunology Laboratory of the Yale-New Haven Hospital for routine antinuclear antibody (ANA) testing were studied, provided that an ANA titer ≥ 1:8 was detected by immunofluorescence using human peripheral blood cells as substrate. When antibodies to RNA or RNA-protein were detected in our assay, the patients' physicians were interviewed and hospital records were examined to establish a diagnosis. Patients were categorized as having SLE, scleroderma or rheumatoid arthritis if they satisfied the American Rheumatism Association's criteria for these diseases (13–15). Patients with an incomplete clinical picture of SLE were classified as probable SLE. Myositis was diagnosed if persistent elevations of creatine phosphokinase and/or muscle biopsy were indicative of inflammatory muscle disease. If data were insufficient to permit a diagnosis to be assigned with confidence, patients were classified as diagnosis not available (four cases).

Standard sera. Sera containing anti-Sm, anti-RNP, anti-Ro, and anti-La antibodies were gifts of Drs. Morris Reichlin and Eng Tan (8, 16). These sera were monospecific when tested in gel diffusion against saline extracts of cell nuclei and they precipitated the snRNPs and scRNPs reported previously (1, 2). Specifically, when extracts from mouse Ehrlich ascites cells were used in the assay, the anti-Sm serum precipitated particles containing the U1a, U1b, U2, U4, U5, and U6 small nuclear RNAs. Similarly, the anti-RNP serum precipitated snRNPs containing only the U1a and U1b molecules. The anti-Ro serum precipitated the small cytoplasmic RNAs Y1 and Y2, and the anti-La serum precipitated a distinctive highly banded pattern of small RNAs.

In the present study, extracts were prepared from human HeLa cells. The small RNA profile in these cells is slightly different from that of mouse cells; U1 is a single molecular species so that the closely related doublet of U1a and U1b is not observed and the small cytoplasmic RNA in scRNPs precipitated by anti-Ro antiserum are more numerous, consisting of at least five different molecules.

Analysis of RNAs precipitated by patient sera. Extracts of ^{32}P-labeled HeLa cells were prepared as described previously (2). Approximately 3×10^7 cells were suspended in

TABLE I

*ANA Positive Patients**

	SLE	Prob SLE	RA	DM	SJ	SCL	JRA	CA	HBV	NA	Hepatitis B pts *n* = 20	Lyme disease pts *n* = 20	Normal donors *n* = 30
Anti-Sm	9	1	—	—	—	—	—	—	—	—	—	—	—
Anti-RNP	4	—	1	—	—	1	—	1	—	4	—	—	—
Anti-Ro	8	1	—	—	1	—	—	—	—	—	—	—	—
Anti-La	2	2	—	—	2	—	—	—	—	—	—	—	—
Anti-SM + Anti-RNP	6	2	—	—	—	—	—	—	—	—	—	—	—
Anti-RNP + Anti-Ro	2	1	—	—	—	—	—	—	—	—	—	—	—
Anti-Ro + Anti-Sm	1	—	—	—	—	—	—	—	—	—	—	—	—
Anti-Th	1	—	—	—	—	—	—	—	—	—	—	—	—
Anti-rRNA	3	—	1	—	—	—	—	—	—	—	—	—	—
Anti-5S RNA	—	—	—	—	—	—	—	—	1	—	—	—	—
Anti-tRNA1	1	—	—	—	—	—	1	—	—	—	—	—	1
Anti-tRNA2	1	—	—	1	—	—	—	—	—	—	—	—	—

* 260 patients studied.

SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; DM, dermatomyositis; SJ, Sjögren's syndrome; SCL, scleroderma; JRA, juvenile rheumatoid arthritis; CA, lung cancer; NA, not available; HBV, hepatitis B-virus infection; pts, patients.

5 ml of Tris buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and sonicated three times at 0°C for 15 s with a Branson sonifier (Branson Sonic Power Co., Danbury, CT) at setting 2. The sonicate was centrifuged at 15,000 *g* for 10 min to remove cellular debris.

Before assay, test sera were precipitated with 40% ammonium sulfate and IgG was isolated with DEAE cellulose (17). The isolated IgG was resuspended in phosphate-buffered saline (130 mM NaCl, 20 mM $NaPO_4$, pH 7.3) at a volume equal to one-half that of the starting serum.

In the assay, 10 μl of IgG solution were combined with 500 μl of radiolabeled cell extract. The mixture was incubated on ice for 15 min, then 100 μl of Pansorbin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) was added for an additional 15 min. Immune precipitates were sedimented at 8,000 *g*, washed five times with Tris buffer containing 0.5% NP40 and resuspended in 400 μl of the same buffer. This suspension was extracted with phenol/chloroform/isoamyl alcohol (50:50:1), containing 0.1% hydroxyquinone, to remove protein. The isolated RNA were precipitated with ethanol and fractionated on polyacrylamide gels. The gels were autoradiographed and RNA species were identified from their electrophoretic mobility as previously assigned by fingerprint analysis (1, 2, 10).

RESULTS

We characterized the RNAs precipitated from extracts of labeled HeLa cells by sera from 260 patients who were selected because of a previously positive test for ANA. Four standard sera known to contain either anti-RNP, anti-Sm, anti-Ro, or anti-La antibodies served as positive controls. These four antibodies precipitate characteristic spectra of RNAs by reacting with specific snRNPs or scRNPs as shown in Fig. 1 (lanes 3–6). These known RNA patterns were matched exactly by 37 of the patients' sera (Table I).

As might be expected, some sera appeared to contain multiple antibodies (Table I). In eight cases, the U1 band observed in the assay was disproportionately prominent relative to the U2, U4, U5, and U6 bands. It seems fair to assume that these samples contain a combination of anti-RNP (precipitating only the U1-snRNP) and anti-Sm (precipitating the U1, U2, U4, U5, and U6-snRNPs). Similarly, three sera precipitated the U1 snRNPs and the Ro scRNPs as if they contained a combination of anti-RNP and anti-Ro. One serum appeared to contain both the anti-Sm and the anti-Ro antibodies. In such examples, it remains possible that a single antibody that reacts with an antigen not restricted to the previously defined RNP subsets are responsible for these results. This point is most vividly illustrated by a serum from patient Th that precipitated a single member of the La RNP spectrum and all of the Ro scRNPs (not shown in Fig. 1; the gel was identical to the lower half of lane 5 combined with the upper half of lane 6). This serum must contain a new antibody, anti-Th, but it is not possible to know if it binds an antigenic protein that complexes both with the Th RNA and the Ro RNA or if anti-Th is present along with a conventional anti-Ro antibody.

Five sera exhibited specificity for constituents of ribosomes. Mammalian ribosomes contain the RNA species—5S, 5.8S, 18S, and 28S—as well as a group of specific proteins. Only the first two of these ribosomal RNAs (rRNAs) are small enough to penetrate 10% ac-

rylamide gels. Fig. 1 (lane 7) shows an example of a serum that precipitated equal amounts of 5.8S and 5S rRNAs, apparently because it reacts with an antigenic determinant on intact ribosomes. In contrast, the serum studied in lane 8 precipitated only 5S rRNA, presumably by binding to a 5S-RNA-protein complex at a stage when it is not included in the intact ribosomal structure.

Five sera specifically precipitated transfer RNAs (tRNAs). RNAs of this class range in molecular weight from ~23,000 to 30,000. As shown in Fig. 1, they are the most rapidly migrating components of total cellular RNA. Antibodies directed against specific subsets of these molecules were identified. Two patient sera and one control serum precipitated the tRNA1 subset shown in lane 9. In contrast, two other patient sera precipitated a different tRNA subset called tRNA2, shown in lane 10.

It should be noted that the Ro scRNPs as well as rRNAs and tRNAs are found in cytoplasm. Obviously, the antibodies that react with these structures do not cause positive results in conventional assays for ANA. Consequently it is likely that ANA positive sera that bind these antigens contain a variety of different antibodies.

Table I summarizes the distribution of the various antibody specificities found in our ANA-positive patients and in control groups. Antibodies that precipitated the Sm and RNP snRNPs or the Ro scRNPs singly or in combinations were the most prevalent. The majority of these sera as well as the one that contained the anti-Th antibody came from patients with SLE or probable SLE. Six sera precipitated the La snRNPs. They were from patients with SLE or probable SLE in four cases and Sjögren's syndrome in two others. Among the patients with antiribosomal antibodies, three had SLE while a fourth had rheumatoid arthritis and multiple myeloma. The serum that precipitated only 5S rRNA came from an individual with hepatitis B virus infection. The anti-tRNA1 antibodies were found in patients with juvenile rheumatoid arthritis in one case and SLE complicated by moysitis in another, as well as in 1 of 30 normal controls. One of the patients with anti-tRNA2 antibodies had dermatomyositis; the other, SLE.

The observation that the antibody precipitating 5S rRNA alone came from a patient with hepatitis B virus infection raised the possibility that this infection might regularly induce these antibodies. Consequently we screened 20 patients with this diagnosis but none of their sera precipitated RNA molecules.

The patient with myeloma and antiribosomal antibodies was of special interest. Her serum contained an IgG kappa M component with cathodal electrophoretic mobility. 200 μl of her serum was fractionated electrophoretically (18) on a preparative agarose block. The M component and polyclonal immunoglobulins were eluted separately by mincing appropriate slices of this gel in phosphate-buffered saline. Equivalent amounts of each protein preparation were reacted with ^{32}P-labeled cell extract in our assay. Only the M component protein was active in precipitating ribosomal RNAs.

It seemed possible that some of the antibodies described here might react with antigenic determinants that are constituted solely by RNA rather than by associated proteins tightly bound to RNA molecules. Such antibodies have been reported previously (19, 20). To test this possibility, we prepared radiolabeled cell extract as usual. It was then further extracted with 10 vol of phenol-chloroform to free the radiolabeled RNA of associated proteins. These RNAs were then reacted in the assay with sera of all of the specificities shown in Fig. 1. Only the sera that reacted with intact ribosomes (as in lane 7) and with the tRNA1 subset (as in lane 8) were capable of precipitating their characteristic RNAs. The remainder precipitated no RNA at all. We conclude that the majority of the anibodies examined in this study recognize antigenic sites constituted at least in part of protein.

DISCUSSION

Approximately 15 yr ago, patient sera began to be tested for antibodies to saline soluble components of cell nuclei (extractable nuclear antigen, ENA), because these antibodies seemed to correlate with a favorable prognosis in SLE (4). Later it became clear that ENA contained, among other things, the RNAase-sensitive ribonucleoprotein (RNP) antigen and the RNAase-resistant Sm antigen (21). Although these antigens seemed to be physically associated (22), antibodies directed against them appeared to characterize mixed connective tissue disease and SLE, respectively (21). Subsequently, it has become evident that rheumatic disease patients make a variety of different antibodies to the many constituents of ENA (6, 23). In the present study, we demonstrate within ENA nine different antigens that characterize specific subsets of small RNAs, ribosomal RNAs, and transfer RNAs. Strictly speaking, ribosomes, tRNAs, and the Ro scRNPs are found in cytoplasm but because most nuclear preparations retain significant amounts of cytoplasmic material, it is not surprising that they could be present in nuclear extracts also.

Antibodies to RNA-associated antigens are not rare. They were found in 59 of 260 individuals (23%) selected only because of a positive ANA. The incidence

among patients with known connective tissue diseases may be substantially higher. On the other hand, since our Institution is a tertiary referral center for complex patients, this survey may have overestimated the probability that any given patient with a positive ANA will have one of these antibodies.

Our method has the advantages of exquisite specificity and sensitivity. In most cases, as little as 1 μl of serum contains enough antibody to precipitate readily detectable quantities of RNA. Yet there are several limitations that must be recognized. Large molecules, such as DNA and high molecular weight RNA, do not penetrate the gels used for electrophoresis. Instead, as seen in lanes 1, 3, and 7 of Fig. 1, these molecules remain near the point of sample application. We have begun to digest samples with DNAase and RNAase to characterize further the molecules in this portion of our gels. As currently performed, our method detects only antibodies that are bound by staphylococcal protein A, so specificities restricted to IgM or IgA classes would go undetected. Finally, our method gives little information about antibody titer.

It is now recognized that most RNA molecules in cells exist tightly associated with proteins and there are compelling reasons to believe that most of the antigenic sites detected in this study are constituted, at least in part, of protein. In an earlier study, we found that the Sm and RNP antigens reside on small RNA-protein complexes, each containing one RNA molecule and several different proteins (1). In the present study, we deproteinized whole cell extract with phenol and found that only two of the nine antibodies identified here—anti-rRNA and anti-tRNA1—were capable of precipitating their RNA antigens when freed from proteins. Indeed, this observation seems reasonable since it is difficult to imagine how the remarkable degree of antigenic specificity observed could occur with RNA molecules alone. RNA subclasses would have to be distinguished by their content of rare nucleotides (which are most plentiful in rRNAs and tRNAs), unique sequences, or unusual tertiary configurations. It must be pointed out however that if the antigens are proteins, their reactive configurations might nonetheless depend on a physical association with RNA.

Several interesting relationships exist among the antigen-antibody systems demonstrated here. First, the RNP antigen is indeed located on a particle that also possesses the Sm antigen—the U1 snRNP—yet the RNP determinant is sensitive to RNAase while the Sm determinant is not. The occurrence of both anti-Sm and anti-RNP in the same patient was the most prevalent form of multiple antigen-antibody systems that we encountered. One wonders if the physical association of the two antigens on the same particle in some way facilitates the simultaneous induction of both antibodies.

Similarly, the La determinant is also present on the Ro scRNPs since the latter particles are a subset of those precipitated by all anti-La serums (Fig. 1; reference 10). This observation may explain why all patients with anti-La antibodies have been thought also to have anti-Ro antibodies (8). The Ro and La interrelationship is now further complicated by the observation that one patient's serum (Th) precipitated the Ro ScRNP and a single La RNP—the one containing the relatively high molecular weight small RNA designated Th in Fig. 1. We do not yet know if this serum contains two separate antigen-antibody (Ro-anti-Ro, Th-anti-Th) systems or a single new system that involves an antigen common to the Ro and Th particles.

Previous studies of antibodies to purified RNAs have demonstrated their occurrence in patients with SLE. Little specificity for RNA subsets was found except that in general, reactivity seemed to favor double-stranded rather than single-stranded molecules (19, 20). It must be emphasized that these studies used deproteinized RNAs as antigens. One of the advantages of our assay method is its ability to exploit the fact that in cell extracts specific proteins are associated with specific RNAs. As noted above, the ability to categorize RNA subsets immunologically is likely to be much greater when the structural variability of these RNA-binding proteins can be utilized.

The availability of antibodies that recognize specific subsets of RNA-protein particles has opened new avenues to understanding the molecular biology of gene expression in mammalian cells. One example is the role played by the U1-containing SnRNP in messenger RNA biosynthesis. When a mammalian gene is transcribed into RNA, the molecule that is synthesized is much larger than the ultimate messenger RNA. This first approximation, called heterogeneous nuclear RNA (hnRNA, or hnRNP when complexed with proteins), includes large nonsensical segments. These segments, referred to as introns or intervening sequences, are subsequently excised to form the mature messenger molecule that codes for a specific protein. The mechanism for this precise splicing was hypothesized to involve the U1 snRNP, since a portion of the U1 sequence is complementary, by Watson-Crick base pairing, to the nucleotide segments that surround the splice junctions in hnRNA (11, 24). The U1 snRNP could potentially align these junctions before cleavage. Experimental evidence supporting this idea has been provided by Yang et al. (12) who showed that anti-RNP and anti-Sm antibodies, which bind the U1 snRNP, do in fact block the splicing of newly transcribed hnRNA in a cell-free system. Although biological roles for

other small RNA-protein particles have not yet been established, it is reasonable to expect that the other Sm snRNPs, which share an antigenic determinant with the U1 SnRNP, may participate in related events in RNA biogenesis. Thus, the lupus antibodies may be able to identify families of RNA-proteins in which individual members have common metabolic responsibilities.

It remains a mystery that nucleoproteins so often become targets for immune responses in rheumatic diseases. However, several properties of these macromolecular complexes seem to be important in determining what happens when they interact with the immune system. First, they are ubiquitous, highly conserved across species, and abundant in nearly all types of mammalian cells (10). Second, their biochemical stability suggests that they may be found within the circulation, as has been demonstrated for DNA and RNA (25, 26). Thus, the immune system may be constantly exposed to them. These factors would seem to favor immunologic tolerance. If spontaneous immune responses to these structures is to be explained by a basic immunologic defect, that lesion must be one associated with variable yet narrowly selected antigenic specificity since the repertoire of RNA-protein antibodies in any given patient is highly restricted. It remains possible that a more precise identification of the antigenic structures on RNA-protein complexes will explain how these antigens escape normal regulatory controls. Immunologic cross-reactivity between endogenous RNA-protein and exogenous RNA-protein—like the ability of anti-La antibodies to recognize RNPs containing adenovirus or Epstein-Barr virus encoded RNAs as well as the normal cellular La RNPs (2)—might prove to be a trigger that circumvents the normal state of tolerance in individuals with the appropriate immunogenetic background.

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