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DEFINITION OF THE ANTIGENIC POLYPEPTIDES IN THE Sm AND RNP RIBONUCLEOPROTEIN COMPLEXES

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SUMMARY: The antigenically-active polypeptides of the Sm and RNP autoimmune ribonucleoprotein complexes from rabbit thymus were distinguished using protein blots. The complexes were fractionated electrophoretically by SDS gel electrophoresis, transferred to nitrocellulose and probed with individual autoimmune sera. Anti-Sm sera recognized a 13,000 molecular weight protein almost exclusively. Anti-RNP sera consistently recognized proteins of 70,000 (a doublet) and 40,000 molecular weight. Reactivities of the immobilized proteins were not dependent on RNA. RNA was necessary for activity when assayed by counterimmunoelectrophoresis, as demonstrated by RNase sensitivity, suggesting a role for RNA in mediating a precipitin reaction of the two antigens.

INTRODUCTION: Antinuclear antibodies are a hallmark of autoimmune disease. Clinical use of these antinuclear antibodies as a diagnostic tool remains empirical because of our ignorance of the biochemical nature of the nuclear antigens recognized by these antibodies. Two prime examples are the Sm and RNP antigens associated with the diseases systemic lupus erythematosus and mixed connective tissue disease, respectively (1,2). Recent reports have shown that Sm and RNP antigenic activity is present in complexes which may be immunoprecipitated (3,4) or immunoaffinity purified (5,6) and consist of a limited number of polypeptides and small RNAs. Some evidence was obtained that the lower molecular weight proteins were antigenically active (5-7). In none of these studies were the components carrying specific Sm and RNP antigenic sites distinguished. We report here that by fractionating Sm/RNP polypeptides by SDS/polyacrylamide gel electrophoresis and transferring the protein bands to nitrocellulose filters, the antigenicity of individual bands can be probed. The predominant RNA-independent sites recognized by anti-Sm and anti-RNP sera reside on different polypeptides.

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MATERIALS AND METHODS: Isolation of Sm and RNP. Sm and RNP antigens were isolated from rabbit thymus acetone powder (Pel-Freez) by a modification of the method reported previously (6). Use of a high titer anti-Sm/RNP immunoaffinity column allowed the elimination of several of the purification steps. RNase activity was minimized by pretreating all buffers and glassware, and by including heparin sulfate (0.5 mg/ml) in the extraction buffer (10 mM sodium phosphate, pH 7.3/150 mM NaCl (PBS)/1 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride). Rabbit thymus powder was extracted and concentrated by the addition of ammonium sulfate to 58% saturation. The precipitate was suspended in extraction buffer containing 10 mM vanadyl ribonucleoside complex, dialyzed overnight and clarified by centrifugation. The sample was applied directly to an immunoaffinity column. The Sm/RNP antigenically active material eluted from the immunoaffinity column by urea is subsequently termed the immune eluate. Samples to be assayed for antigenic activity were dialyzed into extraction buffer containing 10% (v/v) glycerol. Material for gel electrophoresis was dialyzed against 50 mM NH_4HCO_3 and lyophilized.

For extraction of Sm/RNP from calf thymus, fresh-frozen calf thymus (Irvine Scientific) was homogenized in a Waring blender in 2 volumes (v/w) extraction buffer containing 0.2 mM phenylmethylsulfonyl fluoride. The homogenate was filtered through cheesecloth, clarified by centrifugation, and processed in the same way as the rabbit thymus powder extract.

Electrophoresis. Counterimmunoelectrophoresis (CIE) was performed as described (6). SDS/polyacrylamide gels were prepared as described by Laemmli (9), using linear gradients of 12-18% acrylamide solutions containing only 0.025% (v/v) tetramethylethylenediamine and 0.01% (w/v) ammonium persulfate. Proteins used as molecular weight markers were: ovalbumin, 43,000; carbonic anhydrase, 30,000; chymotrypsinogen, 25,700; RNase A, 13,700; cytochrome c, 11,700; trypsin inhibitor, 6,500. In one experiment the gel standards were radioiodinated by the method of Bolton and Hunter (10).

Gel transfer and antigen identification. All steps were carried out at room temperature. Electrophoretic transfer of protein bands from the SDS/polyacrylamide gels to nitrocellulose filters (Schleicher and Schuell) was carried out as described by Towbin et al. (11). Since the gels were larger than the electrophoretic transfer apparatus they were cut into sections which were transferred separately. After transfer, the filters were saturated for 1 hr with PBS containing 10% (w/v) each bovine serum albumin and ovalbumin, 10% (v/v) normal rabbit serum, and 0.2% (w/v) NaN_3. The filters were rinsed with PBS and incubated 1 hr in human serum (normal, anti-Sm, or anti-RNP) diluted in PBS containing 1% each bovine serum albumin and ovalbumin (incubation buffer). The filters were washed 30 min in 5 L PBS then incubated 1 hr in incubation buffer containing 2.5×10^5 cpm/ml ^{125}I-labeled rabbit IgG with anti-human IgG specificity. The filters were again washed 30 min in 5 L PBS, dried, and autoradiographed using Kodak XRP or XAR film. Anti-Sm sera were obtained from three separate laboratories and anti-RNP sera were obtained from the Scripps-Miles Immunology Reference Laboratory.

RESULTS: The Sm and RNP antigens are differentiated in crude extracts on the basis of their RNase sensitivity; RNP is RNase sensitive and Sm is not. We recently reported that after purification, Sm was also sensitive to RNase treatment when measuring antigenicity by CIE (6). However, we felt that failure to observe a positive CIE reaction with the isolated protein fraction implied only that RNA was necessary for maintenance of a precipitin reaction,

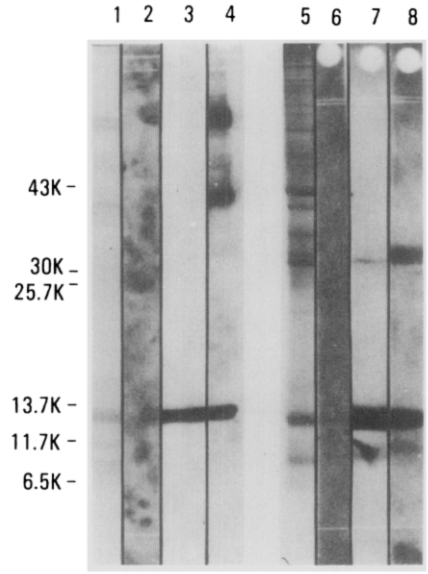

Figure 1. Protein blots of immobilized Sm/RNP polypeptides with anti-Sm and anti-RNP sera. Sm/RNP purified by immunoaffinity chromatography from rabbit and calf thymus, were electrophoresed on an SDS/polyacrylamide gradient gel, transfered to nitrocellulose filters which were probed by a double antibody procedure. Lanes 1-4 represent rabbit thymus material; lanes 5-8, the calf thymus material. Lanes 1 and 5 show the stained gel after transfer. The remaining lanes show autoradiographs of the filters probed with normal human serum (lanes 2 and 6), anti-Sm serum (lanes 3 and 7), and anti-RNP (lanes 4 and 8). The numbers on the left represent molecular weight standards.

particularly in view of a report that isolated RNP polypeptides were antigenically active in a hemagglutination inhibition assay (5). To test this, the antigenic activity of isolated polypeptides was investigated by the protein blot method (11) using rabbit and calf thymus immune eluates. The nitrocellulose filters were probed using normal human serum or anti-Sm or anti-RNP sera as sources of the first antibody and radiolabeled rabbit anti-human IgG as the second antibody. Radioactive bands were visualized by autoradiography. Figure 1 shows that the autoimmune sera did indeed recognize isolated polypeptides, and that furthermore, the Sm and RNP antisera showed striking differences with regard to the rabbit thymus polypeptides each recognized. The anti-Sm serum reacted only with a band of approximately 13,000 molecular weight, while the anti-RNP serum recognized a 13,000 band and three other bands: a doublet of approximately 70,000 and a single band of approximately 40,000 molecular weight. Since immunoaffinity-purified

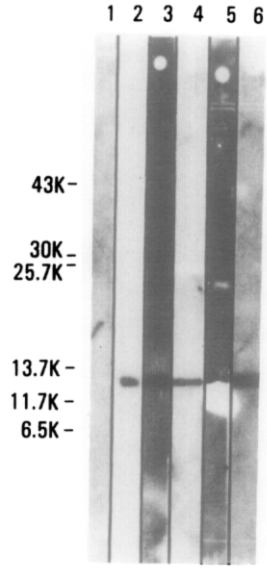

Figure 2. Anti-Sm serum screen. Immunoaffinity purified rabbit thymus Sm/RNP polypeptides were electrophoretically separated and transferred to a nitrocellulose filter. Lane 1 shows an autoradiograph of a filter strip probed with normal human serum. Lanes 2-6 represent filter strips reacted with anti-Sm sera obtained from five different patients. The numbers on the left represent molecular weight standards.

material might contain fewer antigenically-reactive polypeptides than unfractionated material, crude rabbit thymus extract was used in a separate experiment. The crude material revealed the same reactive bands with each serum as seen in Figure 1. With the calf thymus material, both sera recognized a 13,000 molecular weight band, while the anti-RNP serum recognized two proteins of approximately 30,000 molecular weight as well.

These results raised the possibility that anti-Sm and anti-RNP sera may be reliably distinguished by the polypeptides recognized by each. Figures 2 and 3 show the results obtained with a series of anti-Sm and anti-RNP sera reacted with rabbit thymus immune eluates. The 13,000 molecular weight protein is the most prominent band recognized by the anti-Sm sera (Figure 2), and the anti-RNP sera consistently recognized the 70,000 molecular weight doublet and the 40,000 molecular weight band (Figure 3).

In a separate experiment, native or denatured rat liver nuclear RNA bound to nitrocellulose by the dot-blot method of Thomas (13) or native RNA

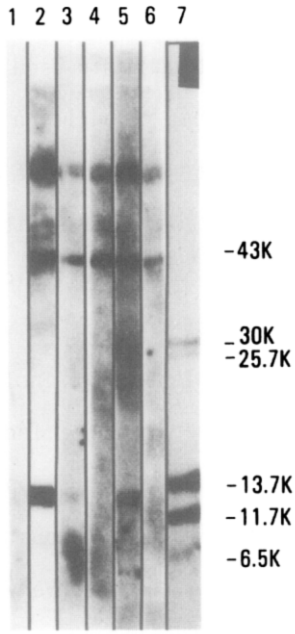

Figure 3. Anti-RNP serum screen. Immunoaffinity purified rabbit thymus Sm/RNP polypeptides were electrophoretically separated and transferred to a nitrocellulose filter. Lane 1 represents a probe with normal human serum; lanes 2-6 show probes with anti-RNP sera obtained from different patients. Lane 7 shows an autoradiograph of a filter containing radiolabeled protein standards of the indicated molecular weights.

dot-blotted onto Whatman DE81 DEAE-cellulose filters showed no reactivity with anti-Sm or anti-RNP sera. The rat liver RNA was chosen because it is known to reconstitute Sm and RNP activity with isolated immune eluate protein (White, unpublished).

DISCUSSION: Recently a technique has been described for immunological detection of proteins fractionated by polyacrylamide gel electrophoresis and transferred to nitrocellulose filters (11). The technique allows for the rapid detection of specific proteins in impure mixtures. We have used this technique to identify the antigenically active polypeptides d signated Sm and RNP. We have shown here that while an RNA component was necessary for detection of Sm and RNP antigenicity in a CIE assay, isolated Sm/RNP polypeptides were able to bind anti-Sm and anti-RNP specific immunogloublins in the absence of RNA. The proteins recognized by the two types of antisera were sufficiently different that the two specificities could be distinguished by

this criterion. In our screen, we chose to use material isolated from rabbit thymus as this is the source commonly used in the clinical diagnostic laboratory. The results suggest that recognition of the 13,000 molecular weight band to the exclusion or near exclusion of other reactivities may be used to define anti-Sm specificity, while recognition of the 70,000 and 40,000 molecular weight proteins is characteristic of anti-RNP specificity. Earlier reports of a large number of proteins in various combinations reputedly associated with the Sm and RNP complexes have included individual polypeptides of the molecular weights reported here (4-7). Indirect evidence was obtained for RNP-associated antigenicity of proteins of approximately 13,000 molecular weight (5,7), in agreement with our direct demonstration of antibody binding to a protein of this size. However, the discovery that the 70,000 and 40,000 molecular weight polypeptides are recognized by anti-RNP sera and a 13,000 molecular weight polypeptide by anti-Sm are new findings, made possible by the greater sensitivity and specificity of the protein blot assay compared to previously used methods.

The results reported here clearly show that at least some antigenic sites in both the Sm and RNP complexes are not dependent on RNA. Therefore, previous reports that both antigens are RNase-sensitive (6,14) raise questions as to the role of RNA in the detection of Sm and RNP antigenicity by some assays. There have been conflicting reports as to the antigenicity of the isolated RNA (3,5); in our attempts to resolve this question, we were unable to detect any binding of anti-Sm or anti-RNP immunoglobulins to rat liver nuclear RNA (known to be active in reconstitution experiments). Although we cannot rule out a role for RNA in the formation of some antigenic sites, it is also possible that in some assays, RNA is required for detection of antigenicity for relatively trivial reasons; e.g., to maintain antigenic polypeptides in solution, to provide sufficient negative charge to the complexes so that they migrate towards the anode in the CIE assay, or to prevent dis-

persal of antigenic sites into fragments with too few sites to form precipitating immune complexes.

The protein transfer procedure described here should have broad implications in determining the precise antigens associated with a variety of autoimmune syndromes. This technique provides a rapid, efficient means of screening a large number of antibody populations and does not require a precipitin reaction. The latter is of particular importance for the determination of diagnostically-important antigens that may be the target of non-precipitating antibodies or for the screening of hybridomas such as we are now producing from autoimmune mice.

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