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Identification of the immunogenically active components of the Sm and RNP antigens

(ribonucleoprotein/autoimmunity/affinity chromatography/limited proteolysis)

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ABSTRACT **The spectrum of cellular targets in the autoimmune diseases is both large and varied and includes among the nuclear components the so-called Sm and RNP antigens associated with systemic lupus erythematosus. The use of immunoaffinity chromatography with dual specificity for the Sm and RNP antigens has allowed for their substantial purification from rabbit thymus in parallel and in quantity. In lieu of a functional assay, the use of a counterimmunoelectrophoresis assay provided a sensitive and rapid means of monitoring the distribution of the two antigens during purification and ensured the isolation of complexes containing the components required for antigenicity. The resulting purified complex consisted of nine polypeptides having molecular weights of approximately 9000 to 44,000 and two small RNAs of similar size. However, limited proteolysis of the isolated complex suggested that most of these polypeptides were not actually required for antigenic activity. Unlike Sm in crude thymus extracts, purified Sm was RNase sensitive. Thus, one of the major diagnostic criteria used to distinguish Sm and RNP antigens in crude extracts was shown to be invalid for purified material, suggesting that both antigens from rabbit thymus are actually ribonucleoprotein complexes.**

Autoimmune diseases are characterized by the presence of circulating antibodies that react against components of the affected individual's own tissues (1–3). Isolation of the antigens associated with specific autoimmune diseases would facilitate the diagnosis of these diseases and, perhaps, would help in understanding the etiology of these diseases. Among the highly conserved cell components that can be targets of the autoimmune reaction are the so-called Sm and RNP (ribonucleoprotein) antigens. The presence of antibodies to Sm is diagnostic of the disease systemic lupus erythematosus; anti-RNP antibodies are found in patients suffering from lupus and, more specifically, mixed connective-tissue disease (4, 5). Sm and RNP are both located in the nucleus, giving similar speckled immunofluorescent staining patterns (5). They have been reported to have similar molecular weights (6, 7), to copurify during ion-exchange chromatography (7, 8), and to show partial antigenic identity (6, 9). In practice, they have been distinguished by differential sensitivity to RNase (4, 10).

There have been several reports in recent years on attempts to purify Sm and RNP (8, 11–14). Most recently Lerner and associates (9, 15), using the technique of immunoprecipitation, identified both Sm and RNP as ribonucleoprotein complexes. We report here a large-scale purification of the Sm and RNP antigens in quantities sufficient for physical and chemical characterization. Moreover, the isolation protocol allowed for continuous monitoring of antigenicity, thus ensuring that we were working with antigenically active material. The isolated complexes contained both protein and RNA moieties; and each moiety was composed of a subset of polypeptides and oligoribonucleotides. By using the technique of limited proteolysis, the protein immunogenic component could be identified with a limited number of these polypeptides. Both Sm and RNP required an RNA component for antigenicity; contrary to previous reports, Sm became increasingly sensitive to RNase as purification proceeded.

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MATERIALS AND METHODS

Isolation of Sm/RNP. All operations were carried out at 4°C and all buffers contained 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. Rabbit thymus acetone powder was obtained from Pel-Freez or Irvine Scientific (Santa Ana, CA).

Step 1: Extraction. Forty grams of rabbit thymus powder was suspended in phosphate-buffered saline (P_i/NaCl) at a concentration of 60 mg of powder per ml of buffer. The suspension was stirred gently for 4 hr and then centrifuged for 20 min at 16,300 × *g*. Solid enzyme-grade ammonium sulfate (144 g/liter) was slowly added to the supernatant to 25% saturation. The solution was stirred for 30 min and then centrifuged for 30 min at 16,300 × *g*. Ammonium sulfate (215 g/liter) was added slowly to the supernatant to 58% saturation. After 30 min, the solution was centrifuged as before. The precipitate was suspended in 40 ml of 10 mM sodium phosphate, pH 7.3/50 mM NaCl and dialyzed overnight against 50 vol of this buffer.

Step 2: DEAE-Cellulose Chromatography. The dialysate was applied to a Whatman DE-52 column (5 × 20 cm). The column was washed with dialysis buffer until no further protein was removed. The column was then washed with P_i/NaCl until no protein was removed. The bulk of the antigens of interest was eluted with 10 mM sodium phosphate, pH 7.3/500 mM NaCl. This eluate was concentrated by the addition of ammonium sulfate to 60% saturation. The pellet was suspended in 20 ml of P_i/NaCl and dialyzed overnight against 60 vol of P_i/NaCl.

Step 3: Nonimmunoaffinity and Immunoaffinity Columns. The dialysate was applied to a 100-ml column (2.5 × 21 cm) of normal human IgG coupled to Sepharose-4B equilibrated in P_i/NaCl. The column was washed with P_i/NaCl until the A_{280} was less than 1. The wash fraction was then passed over a similar 100-ml column (2.5 × 21 cm) of IgG containing anti-Sm and anti-RNP specificity. The column was washed extensively with P_i/NaCl before the bound antigens were eluted with P_i/NaCl containing 6 M urea (enzyme-grade). The urea was removed from the eluate either by passage over a Sephadex G-25 column (1.6 × 66 cm) which had been equilibrated in P_i/NaCl containing 1 mM 2-mercaptoethanol and 15% (vol/vol) glycerol or by extensive dialysis against the same buffer.

Abbreviations: RNP, ribonucleoprotein; P_i/NaCl, phosphate-buffered saline; snRNA, small nuclear RNA; CIE, counterimmunoelectrophoresis.

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Protein Determination. Protein was measured by the method of Lowry *et al.* (16) with bovine serum albumin as a standard or by the Bio-Rad assay with human IgG as a standard.

Affinity Chromatography. Plasma obtained from the San Diego Blood Bank was the source of nonimmune IgG. Pleural effusion was the source of IgG with anti-Sm and anti-RNP specificity as determined by the Scripps Clinic Immunology Reference Laboratory. The IgG fraction from each was obtained by QAE-Sephadex chromatography and half-saturation ammonium sulfate precipitation. The isolated IgG was dissolved at 3 mg/ml in 0.1 M $NaHCO_3$ (pH 8.0). The IgG was coupled to cyanogen bromide-activated Sepharose by the procedure of Wilchek *et al.* (17). Sepharose-4B was suspended in distilled water at a concentration of 333 mg/ml and the pH was adjusted to 11–11.5 with NaOH. Solid cyanogen bromide (33 mg/ml) was added to the suspension with stirring and the pH was maintained at 10.5–11.5. The activated Sepharose was filtered and washed with an excess of cold water. The Sepharose and IgG were mixed (30 mg of Sepharose per mg of IgG) and the suspension was stirred overnight at 4°C. Greater than 95% of the IgG was bound to the Sepharose.

Electrophoresis. $NaDodSO_4$/polyacrylamide slab gel electrophoresis was carried out by using the system of Laemmli (18); the gels were stained by using the procedure of Fairbanks *et al.* (19). The following proteins were used as standards to calibrate the gels: ovalbumin (43,000), carbonic anhydrase (30,000), chymotrypsinogen A (25,700), RNase (13,700), and cytochrome *c* (11,700).

Phenol-extracted RNA was electrophoresed on 10% polyacrylamide gels in Tris/borate buffer containing 7 M urea (9); xylene cyanole FF (Eastman) was used as a dye marker. Gels were stained with Stains-all (Eastman) by the method of Dahlberg *et al.* (20). Rat liver nuclear RNA standards were prepared as follows. Nuclei were prepared from male S/A retired breeders (Simonsen) by the method of Blobel and Potter (21) and washed with RNA extraction buffer (40 mM EDTA/20 mM Tris/0.2 M NaCl, pH 7.4) containing 25 mM KCl, 5 mM $MgCl_2$, and 0.8 M sucrose. The washed nuclei were homogenized in RNA extraction buffer and extracted twice at room temperature with distilled phenol saturated with RNA extraction buffer. The aqueous phase was dialyzed against distilled water, divided into portions, and lyophilized. The dry portions were stored at −70°C until used. The individual bands were identified from their mobilities relative to 4S RNA as reported (9). Methyl mercury/10% polyacrylamide slab gel electrophoresis was carried out by the system of Chandler *et al.* (22). The rat liver RNA contained small nuclear (snRNA) species of defined length as follows: U2, U1, 5S, and 4.5S RNA representing 196, 171, 120, and 96 nucleotides per molecule (23, 24).

Counterimmunoelectrophoresis (CIE) was carried out on projector slides layered with 1.75 ml of 1% agarose (Marine Colloids) in 25 mM barbital buffer (pH 8.4), followed by 13 ml of 0.6% agarose in the same buffer. The electrophoresis was carried out in 50 mM barbital buffer (pH 8.4) at 12 mA for 30 min at room temperature. An ammonium sulfate concentrated extract of rabbit thymus (10 mg/ml) was used as the control. RNase sensitivity was assayed as follows. A 30-μl sample to be tested and 10 μl of RNase (300 μg/ml) were incubated at 37°C for 30 min prior to CIE. Reference antisera for Sm and RNP were obtained from the Scripps Clinic Immunology Reference Laboratory and were used at the following dilutions: anti-Sm, 1:200; anti-RNP, 1:20; anti-Sm/RNP, 1:20. Sm reactivity was verified with anti-Sm antiserum supplied by Morris Reichlin.

Limited Proteolysis. An aliquot of the immune eluate was dialyzed overnight against 100 vol of P_i/NaCl (pH 7.5) containing 10% (vol/vol) glycerol. Trypsin free of chymotrypsin activity (Worthington, type TPCK) was prepared at a concentration of 1 mg/ml in P_i/NaCl. The Sm/RNP dialysate was made 0.4% in trypsin (weight of trypsin/weight of total protein) and incubated at 37°C. At appropriate times, 50-μl aliquots were removed and mixed with 1.6 μl of phenylmethylsulfonyl fluoride (100 mM in isopropyl alcohol) to inhibit further trypsin activity. Samples to be analyzed by $NaDodSO_4$/15% polyacrylamide slab gel electrophoresis were immediately lyophilized. Samples were assayed for activity by CIE.

RESULTS

Isolation of Sm/RNP Antigens. Because of the reported lability of the Sm antigen under various conditions, it was essential to determine first which conditions could be varied without loss of antigenicity during the purification, particularly relative to the affinity columns. By using parallel aliquots of a crude thymus extract, it was determined that both Sm and RNP were sensitive to low pH (0.1 M glycine·HCl, pH 2.6); RNP was resistant to 3.5 M NaSCN but Sm was not; both were resistant to relatively high salt [3 M $(NH_4)_2SO_4$]; both were resistant to 6 M urea. Thus, the immunoaffinity columns were routinely eluted with 6 M urea. Urea was then quickly removed in the presence of buffer containing 1 mM 2-mercaptoethanol. No cellular role has been definitely assigned to Sm and RNP, precluding a functional assay to monitor these two antigens during purification. However, the CIE assay provided a sensitive and rapid means of monitoring the distribution of the two antigens, thus ensuring that we were isolating antigenically active complexes. Beginning with the immunoaffinity columns the protein profiles were also followed by using $NaDodSO_4$/polyacrylamide slab gel electrophoresis.

We have arrived at a substantial purification of these two autoimmune-associated antigens from rabbit thymus in essentially three steps: ammonium sulfate fractionation, ion exchange chromatography, and immunoaffinity chromatography. The isolation protocol was deliberately designed on a scale sufficiently large to allow for the recovery of the antigens in quantities necessary for physical analyses. The use of an immunoabsorbant column with dual specificity for the Sm and RNP antigens also allowed for the parallel isolation of these antigens. Thus, to this stage of purification there was no need to consider potential problems of an Sm–RNP complex when trying to isolate just one of the two antigens. Less than 1% of the protein starting material was retained in the urea eluate of the immunoaffinity column. A summary of this isolation is shown in Table 1. Throughout the purification, all fractions were tested for antigenic activity by using CIE. Thus, this isolation protocol did not result in complete recovery of activity. Rather, side fractions containing activity were generally recycled separately through the immune column. At this stage of the Sm/RNP characterization, we chose not to maximize recovery yields in order to obtain more easily an antigenically active preparation with a limited number of components.

The protein could be further fractionated by the use of phosphocellulose chromatography (Fig. 1). The polypeptide pattern was very similar to that found by Lerner and Steitz (9) using the technique of immunoprecipitation, with the addition of two higher molecular weight bands at 44,000 and 38,000. However, the results of other such columns suggested that all these protein species were not necessarily required for antigenic activity.

In using the CIE, fractions to be tested were assayed in the presence and absence of RNase with the intent to differentiate the Sm and RNP reactivities. As the purification proceeded, the antigen detected by the anti-Sm antiserum became progressively more RNase sensitive (Table 1). This progressive RNase

Table 1. Isolation of RNP and Sm antigens by affinity chromatography

Step	Protein mg/ml	Protein mg	Protein %	RNP*	Sm
Rabbit thymus extract	11.3	6203	100	+	+
25–58% sat. ammonium sulfate precipitate	58.6	3986	64	+	+
DEAE-cellulose conc. 500 mM NaCl eluate	23.4	808	13	+	+†
Nonimmune IgG affinity chromatography, wash	4.5	642	10	+	+†
Immune IgG affinity chromatography, 6 M urea eluate	0.27–1.31	33.7	0.5	+	+*

* RNase sensitive.
† Partially RNase sensitive.

sensitivity was highly reproducible and was not dependent on the particular anti-Sm antiserum used in the CIE or on the particular purification procedure used to isolate the antigen. In a separate experiment under RNase-free conditions, Sm purified from a crude extract by ammonium sulfate fractionation and gel filtration on Sepharose-6B was as RNase-sensitive as the RNP. Thus, the Sm antigen being isolated did not meet the accepted classical criteria for this antigen—namely, RNase resistance.

As is obvious from the purification protocol, the isolation was not normally performed under RNase-free conditions. Although the product of the immune column was antigenically active, the RNA profile upon gel electrophoresis did not allow for the assignment of definite RNA species (Fig. 2). However, when diethylpyrocarbonate was added directly to the rabbit thymus powder during extraction and to all subsequent buffers, a more distinct profile was seen. The RNA bands present in the immune eluate but not in the immune wash were a doublet of the mobility of the U1 species of snRNA. A corresponding protein profile was not available because the crosslinking engendered by the diethylpyrocarbonate precluded much of the protein from entering the $NaDodSO_4$ gels.

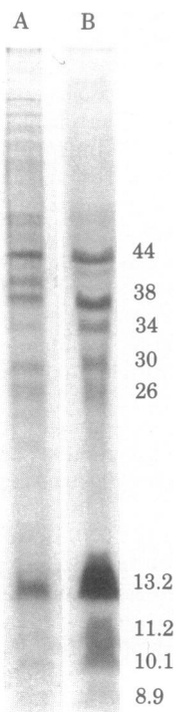

FIG. 1. $NaDodSO_4$/12.5% polyacrylamide slab gel electrophoresis of Sm/RNP-associated protein. Lanes: A, urea eluate (25 μg) of immunoaffinity column (anti-Sm/RNP); B, urea eluate (49 μg) of phosphocellulose column loaded with sample A. The phosphocellulose column was equilibrated in 10 mM sodium phosphate, pH 7.3/50 mM NaCl/1 mM EDTA/1 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride/15% (vol/vol) glycerol. The column was eluted in succession with buffer containing 600 mM NaCl and 6 M urea. Molecular weights are shown $\times 10^{-3}$.

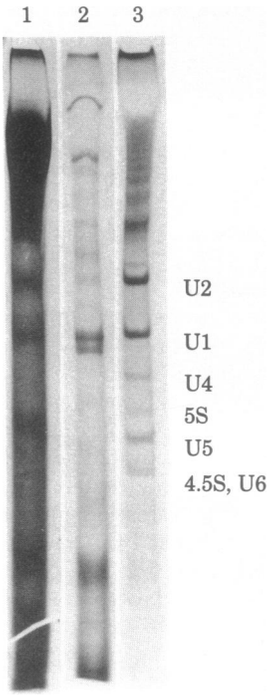

FIG. 2. Urea/polyacrylamide slab gel electrophoresis of Sm/RNP-associated RNA. The gel concentration was 10%. Lanes: 1, urea eluate of immunoaffinity column (anti-Sm/RNP); 2, urea eluate of immunoaffinity column under RNase-free conditions; 3, rat liver nuclear RNA standards.

In an early protocol for the isolation of RNP containing the metabolically stable RNA molecules referred to as snRNAs, nuclei from Novikoff ascites cells were solubilized in 10 mM Tris·HCl (pH 8.0) and passed over a Sepharose-6B column (25). Using rabbit thymus powder as the starting material, we repeated this experiment as an alternative method of monitoring the probable association of U1 RNA with the Sm/RNP activity. The Sm and RNP activities overlapped, and the intensity of this activity paralleled the fractions containing the U1 RNA doublet, in particular the slower migrating of the two species (Fig. 3). Although in this experiment a faster migrating doublet also coincided with Sm/RNP activity, the U1-size doublet was the only one consistently found associated with the antigens (e.g., see Fig. 2). These two RNAs were examined on a methyl mercury/10% polyacrylamide gel (22). The two bands were calculated to have 170 and 159 nucleotides per molecule relative to the rat liver snRNA standards including the U1 band of 171 nucleotide length.

Limited Proteolysis of Sm/RNP. Protein from the urea eluate of the immunoaffinity column was subjected to limited proteolysis with trypsin to determine if certain polypeptides could be selectively digested to leave a protein composite similar to that seen after phosphocellulose chromatography. The time course of a mild proteolytic digestion of Sm/RNP by trypsin is shown in Fig. 4. At each time point the samples were examined as to their protein profile and were assayed for activity by using the CIE. At each point a significant amount of both Sm and RNP activities was detectable. The protein profile indicated

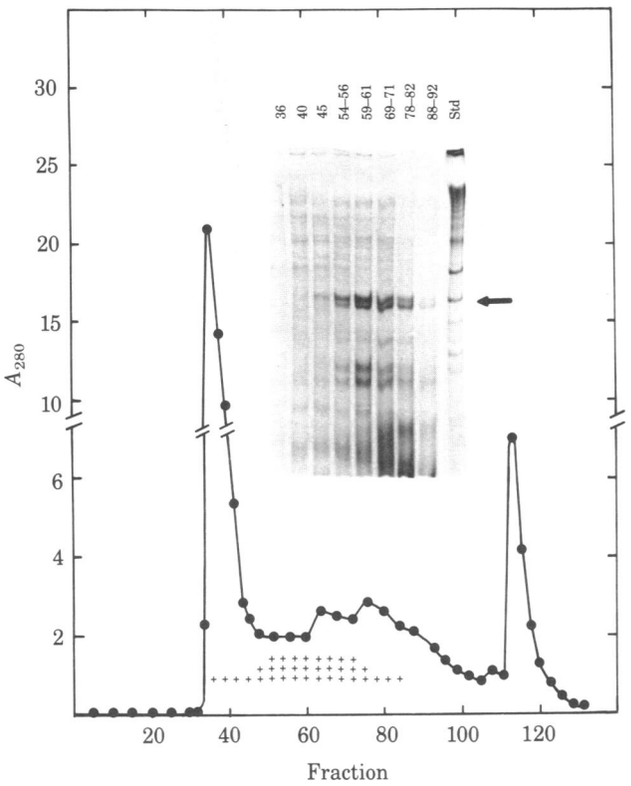

FIG. 3. Sepharose filtration of rabbit thymus extract. Rabbit thymus powder (15 g) was extracted with 225 ml of 10 mM Tris·HCl, pH 8.0/0.1 mM phenylmethylsulfonyl fluoride/heparin (0.5 mg/ml). The soluble extract was concentrated by ammonium sulfate precipitation and passed through a Sepharose-6B column (1.5 × 78 cm) equilibrated with 10 mM Tris·HCl, pH 8.0/140 mM NaCl/1 mM $MgCl_2$. Fractions containing Sm and RNP antigenic activity are indicated by +. (*Inset*) RNA (50 μg) was extracted from selected pooled fractions and displayed on a 7 M urea/10% polyacrylamide slab gel. Numbered lanes refer to Sepharose-6B fractions; Std, rat liver nuclear RNA. The arrow designates the RNA doublet consistently associated with the Sm/RNP antigens.

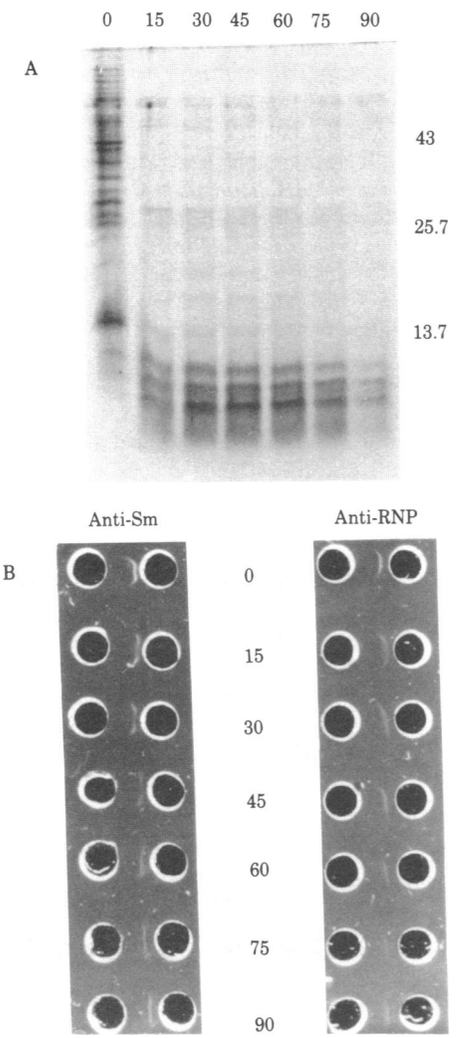

FIG. 4. Time course of limited trypsinization of Sm/RNP. (*A*) $NaDodSO_4$/15% polyacrylamide slab gel electrophoresis of samples at 0, 15, 30, 45, 60, 75, and 90 min (0 is time of introduction of the trypsin). (*B*) CIE of samples at the same times. (*Left*) Samples assayed with antisera with anti-Sm specificity; (*Right*) anti-RNP specificity.

an approximately 50% decrease in Coomassie blue-stainable material by the first point at 15 min as determined by a scanning densitometer. The profile would suggest that only the three low molecular weight species seen after phosphocellulose chromatography were required for antigenic activity for Sm/RNP. The weights of the three distinct low molecular weight species were calculated to be 10,800, 9800, and 8800.

The $NaDodSO_4$/polyacrylamide gel profile in Fig. 4, however, represented the total trypsin digest. To determine if all of these low molecular weight species were associated with Sm/RNP activity, a trypsin digest was subjected to gel filtration chromatography on Sephadex G-150. The Sm and RNP activity peaks partially overlapped in the region of the IgG marker (150,000). The nondenatured molecular weight ranges seen for the two antigens on the calibrated column were remarkably similar to those reported for the native antigens in crude extracts (6, 7). When aliquots from these column fractions were subjected to $NaDodSO_4$/polyacrylamide slab gel electrophoresis, a comparison could be made of the number and size of the polypeptides represented in the region containing both Sm and RNP activity and the region containing Sm activity alone. There was no obvious difference in the protein profiles. All three low molecular weight species were represented in these patterns and there was, in addition, a fourth band at molecular weight 7500 which was now distinguishable and was obviously a product of trypsin digestion. To characterize the antigenically active species further, a protein sample from the Sephadex G-150 column containing both Sm and RNP activity was passed back over an immunoaffinity column (anti-Sm/RNP). The protein profile in the adsorbed fraction did not change.

DISCUSSION

We present here a large-scale procedure for isolation of the Sm and RNP antigens. The protocol consists of extraction of rabbit thymus acetone-insoluble residue with phosphate-buffered saline followed by ammonium sulfate fractionation, ion-exchange and immunoaffinity chromatography, and, finally, chromatography on phosphocellulose. The resulting purified complex consists of nine proteins having molecular weights of approximately 9000 to 44,000 and two small RNAs of similar size. We also observe that, unlike Sm in crude extracts (4, 10), the purified Sm antigen is RNase sensitive. Thus, one of the major criteria used to distinguish Sm and RNP in crude extracts is shown not to be valid for purified material.

There is substantial agreement between the work reported here and that of Lerner and associates (9, 15) as to the protein and RNA composition of the Sm and RNP antigens. Those work-

ers examined immunoprecipitates formed in reactions between Ehrlich ascites cell nuclear extracts and anti-Sm or anti-RNP antiserum. The precipitates consistently showed protein bands, on one-dimensional gels, whose molecular weights were similar to those of the seven smallest proteins in our phosphocellulose eluates (Fig. 1). It is impossible to say whether all of the proteins we observed were part of the Sm/RNP complex or whether they merely copurified with the antigenically active polypeptides. The fact that the six largest could be destroyed by trypsin without seriously affecting reactivity in the CIE assay (Fig. 4) or apparent molecular size suggests that they in fact are not part of the Sm/RNP antigenic complex. However, we cannot rule out the possibility that Sm and RNP are heterogeneous with respect to protein composition and that reactivity with other anti-Sm or anti-RNP antisera would require that some of these proteins remain intact. These results also do not rule out the possibility that these small antigenically active polypeptides were generated from a much larger protein during extraction and processing of the thymus source material. Regardless, these results suggest a limited number and defined size for the polypeptides actually required for Sm and RNP antigenic activity. Our immunoaffinity eluates contained two prominent small RNAs of similar size. Their mobilities were similar to those of the U1 snRNA. We did not see any other RNAs consistently associated with Sm/RNP activity but this may be dependent on the particular IgG used for the affinity column.

That both RNA and polypeptide material are required for Sm and RNP antigenicity is shown by the fact that either RNase or autodigested Pronase rapidly destroyed antigenic activity and also by the fact that isolated protein and RNA moieties were not antigenic (unpublished data). However, in preliminary experiments, both Sm and RNP antigenic activity have been reconstituted from the isolated moieties. Reconstitutions were successful only with preparations relatively enriched for the low molecular weight polypeptide species. Protein derived from an immune eluate, such as that shown in Fig. 1*A*, was not suitable; however, a trypsin-treated eluate was reconstituted. New results suggest the possibility of fractionating the protein moiety and recombining individual fractions with purified RNA to reconstitute antigenic activity, a procedure which would characterize more completely the components required for antigenicity.

The pathogenesis of autoimmune disease is undoubtedly complex, possibly involving a number of genetic, immunological, viral, and environmental factors (26). One critical aspect of any understanding of this chain of events is determination of the specific targets of the autoimmune response. One target area includes the nonhistone nuclear proteins, but it remains to be determined how many of these proteins are synonymous with autoimmune antigens, what property or properties of each of these proteins give it this unique role as an autoimmune antigen, and why each of these antigens is associated with a limited array of autoimmune diseases and not all. To answer these and other questions requires, first, the availability of antigens in a purified form. And having defined their basic structure, and possibly their cellular function, one can begin to extrapolate the role of these proteins in the sequence of events that is the autoimmune response. The target proteins appear to be functionally important for the most part as judged by their nuclear conservation. As such, there seems to be a natural correlation between the efforts of those studying the nuclear components as they relate to normal cell function and those studying the same components as they relate to the autoimmune phenomena.

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