Isolation and Characterization of Nuclear Ribonucleoprotein Complexes Using Human Anti-nuclear Ribonucleoprotein Antibodies* 

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We have investigated the feasibility of using human autoimmune antibodies to isolate and characterize specific nuclear ribonucleoprotein (nRNP) complexes. High titers of anti-nRNP antibodies occur in a syndrome called mixed connective tissue disease. IgG antibodies from two mixed connective tissue disease patients were used to construct affinity columns to isolate the antigenic complexes from rat liver nuclei. A maximum of 2.3% of the nuclear RNA and 0.7% of the protein bound to anti-nRNP but not to control IgG columns. A fraction of the bound antigen, comprising less than 0.15% of the total nuclear protein, was isolated in antigenically active form. The protein moiety of this fraction consisted of two quantitatively major polypeptides of molecular weights 30,000 (P30) and 13,000 (P13). Antigens isolated from both anti-nRNP columns possessed essentially these same two polypeptides. Immunological tests of crude and purified antigens against anti-nRNP sera from a total of four patients provided additional evidence that antibodies from different individuals are directed against the same nRNP antigen. There were no polypeptides in the isolated antigen which corresponded in molecular weight to the core proteins of heterogeneous nuclear ribonucleoprotein (hnRNP) particles described by other investigators.

The binding of both RNA and protein to anti-nRNP columns was greatly reduced by treating the crude antigen with pancreatic RNase A before chromatography. In particular, the binding of P13 was reduced to a fraction of the pre-RNase control. None of the four sera in this study contained antibodies to DNA, histones, RNA, DNA-histone complexes, or nonhistone chromosomal proteins.

Antibodies to specific cellular macromolecules can be raised in experimental animals. Such antibodies have been used to facilitate biochemical isolation of subcellular components (Duerre, 1967; Allen and Terrence, 1968; Holme et al., 1971) and to study their in situ localization in fixed cytological preparations (Lazarides and Weber, 1974; Silver and Elgin, 1976; Alfageme et al., 1976; Jamrich et al., 1977). There are certain human diseases in which antibodies directed against nuclear macromolecules appear spontaneously in the blood (Tan and Kunkel, 1966; Stollar, 1971; Tan and Lerner, 1972). These so-called systemic rheumatic diseases include systemic lupus erythematosus and mixed connective tissue disease. Antibodies to a variety of nuclear macromolecules including DNA (Tan and Natali, 1970), histones (Stollar, 1971), RNA-protein complexes (Notman et al., 1975; Kurata and Tan, 1976), and nonhistone proteins (Tan and Kunkel, 1996) can be found in autoimmune sera. These sera are potential sources of immunological tools which can aid in understanding the organization and function of the nucleus. However, the autoimmune antibodies have not been widely used for this purpose, primarily because immunological data which indicate that the sera may be good sources of specific antibodies have not yet been corroborated by biochemical studies. The possibility, therefore, has existed that individual sera may contain mixtures of antinuclear antibodies which are too complex to be useful for the study of one particular antigen.

The subject of this investigation is the MCTD1 syndrome which gives rise to high titers of antibodies to nRNP complexes (Northway and Tan, 1972; Sharp et al., 1972; Kurata and Tan, 1976; Alarcon-Segovia et al., 1978). The antigenic nRNP complexes have not previously been isolated or characterized chemically. A few of their properties are known from immunological studies. They are detectable by indirect immunofluorescence in the interphase nuclei of a variety of cell types including calf thymus, rabbit thymus, mouse kidney, and a human lymphocyte cell line W14 (Mattioi and Reichlin, 1971; Tan and Lerner, 1972; Notman et al., 1975; Kurata and Tan, 1976). The fluorescence patterns show that the antigenic complexes are not in the nucleoli, nor do the human anti-nRNP antibodies react with isolated nucleolar preparations in immunodiffusion tests (Pinnas et al., 1973). The antigenic complexes have not been sized accurately. However, the crude antigen migrates faster than 7 S markers in sucrose density gradients (Northway and Tan, 1972) and is thus larger than tRNA precursors which have been isolated from eukaryotic cells (Burdon and Clason, 1969; Mowshowitz, 1970; Blatt and Feldman, 1973; Weinmann and Roeder, 1974; Siddiqui and Chen, 1975). While in situ immunofluorescence data suggest that the antigenic nRNP complexes may be of ubiquitous occurrence in mammalian nuclei in vivo, other approaches, including biochemical isolation of the complexes and their component RNA and protein moieties, are needed to elucidate their function.

One of the objectives of this investigation was to determine...
by biochemical and immunological methods whether the human anti-nRNP antibodies react with what is quantitatively and qualitatively a specific subset of nuclear RNP complexes. This problem was approached by constructing affinity columns for isolating the antigenic ribonucleoprotein which could then be subjected to quantitative and chemical analyses. The second objective was to test available anti-nRNP sera for the presence of antibodies to other nuclear macromolecules which might interfere with isolation and characterization of nRNP complexes. Immunological tests conducted by medical investigators who provided the sera, including counterimmunoelectrophoresis, radioimmunossays, and indirect immunofluorescence, suggested that a high proportion of the anti-nRNP sera are free of antibodies to DNA, histones, and non-RNA-associated nonhistone proteins (see "Experimental Procedures" and Northway and Tan, 1972; Notman et al., 1975; Kurata and Tan, 1976). We screened six anti-nRNP sera of which four showed a single precipitin reaction against sonicated rat liver nuclei, a reaction which was sensitive to RNase A and trypsin. We used RNase A sensitivity and other procedures to test the four anti-nRNP sera for the presence of antibodies to other nuclear macromolecules. In addition to these four sera, a number of normal human sera were used as immunoglobulin sources in this investigation.

Unlike antibodies to defined antigens which are raised in experimental animals, the human anti-nRNP antibodies arise spontaneously under the influence of disease mechanisms which are poorly understood. Therefore, a third objective was to determine whether different individuals affected with MCTD give rise to antibodies with the same antigen specificity. The availability of sera from four different individuals made this possible. Anti-nRNP antibodies are immunoglobulins of the IgG type (Tan and Vaughn, 1973). IgG was isolated from two of the sera and used to construct affinity columns for isolating and biochemically comparing purified antigens. The immunological specificities of all four sera were compared by double diffusion tests against crude antigen and affinity column fractions.

**EXPERIMENTAL PROCEDURES**

**Nuclear Isolation and Fractionation**—Nuclei were isolated from frozen rat livers (Pel-Freeze) with all procedures carried out at 0–4°C. In a typical isolation, six livers (approximately 100 g) were thawed for ½ h in 300 ml of homogenizing medium (HM) containing 0.25 M sucrose, 5 mM magnesium acetate, 5 mM 2-mercaptoethanol, 0.1 M ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride (a protease inhibitor from Calbiochem). Stock solutions of 50 mM phenylmethylsulfonyl fluoride were made up in isopropyl alcohol and diluted in HM immediately before use. After thawing, two livers at a time were blotted on paper, cut into 1-cm pieces, and homogenized in 30 ml of fresh HM using a motor-driven Potter-Elvehjem homogenizer (four strokes, 60 V). The homogenate was allowed to develop at 4°C for 1 h and designated A1. A2, the immunologically active fraction, was stored at 0°C for less than 24 h before chromatography or was frozen at −20°C for longer storage.

**Quantitative Assays**—Protein determinations were performed by the methods of Lowry et al. (1961) and Svednik and Grossberg (1977) for serum albumin as a standard. Nucleic acids were quantitated by a modification of the Schmidt-Tannhäuser procedure (TeY and Sato, 1959) and by the orcinol method for RNA (Dische and Schwarz, 1937), using yeast RNA (Sigma, Type III) as a standard.

**DNA, RNA, Histones, Nucleosomes, and Nonhistone Proteins**—

Rat liver chromatin was isolated by the method of Bonner et al. (1968) as a source of histones and nonhistones. The chromatin was extracted with 4.0 M NaCl, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, to dissociate chromosomal proteins (Sevall et al., 1975). Histones were separated from nonhistones by chromatography over Bio-Rex 70 (Dovas et al., 1977). Nucleosomes, donated by Thomas Sargent (Division of Biology, California Institute of Technology), were isolated from rat liver nuclei by digestion with staphylococcal nuclease as described elsewhere (Clark and Felsenfeld, 1971). They were stored at 0°C in 0.25 M disodium ethylenediaminetetraacetate, 25 mM Tris-HCl, pH 7.4, at 0°C. Calf thymus DNA (Worthington Biochemicals) and yeast RNA (Sigma, Type III) were dissolved in NaCl/0.1% sodium azide buffer, pH 7.4, to a concentration of 1 mg/ml.

**Selection and Testing of Human Sera**—Sera from MCTD patients were obtained from the Division of Rheumatic Diseases, University of Colorado Medical Center. MCTD sera were screened for anti-nRNP antibodies through a series of tests conducted in the clinical laboratory. These tests include comparisons with anti-nRNP reference sera and testing the sensitivity of the reactions to RNase A and trypsin (Northway and Tan, 1972). Other analyses to which these sera are subjected include radioactive immunodiffusion for the presence of antibodies to DNA, indirect immunofluorescence on mouse kidney sections for the presence of antibodies to histones, and hemagglutination for the presence of antibodies to nonhistone nuclear proteins (Tan and Ler, 1972; Robitaille and Tan, 1973, Tan and Peebles, 1976). We obtained six prescreened MCTD sera and tested them by double diffusion against sonicated rat liver nuclei. Four of the sera from the patients MP, MK, MM, and BN showed a single precipitin line against the nuclear sonicate and were used as antibody sources in this investigation. Six normal human sera for use as controls were obtained from laboratory personnel.

Double diffusion assays were conducted in plates of 0.4% agarose, 0.1% sodium azide in NaCl/Pi, pH 7.5. Freshly prepared sera were allowed to develop at 4°C for 18 to 42 h. The plates were then washed with 5% sodium citrate (48 h) followed by distilled water (24 h) and then photographed.

**IgG Isolation and Affinity Column Procedures**—IgG was isolated from two anti-nRNP sera (MP and MK) using 2 ml of each serum for a yield of 20 mg of IgG. A pool of normal human serum was used for isolation of control IgG. The IgG was purified essentially as described by Weir (1967). Each serum aliquot was heated in a 56°C waterbath for 30 min to inactivate complement and then centrifuged at 484 × g for 15 min at 4°C and the pellet was discarded. All subsequent procedures were at 4°C. The supernatant was centrifuged at 20,000 × g for 15 min, and fat pellets which formed on top were discarded. Two of these supernatants were dialyzed against 0.015 M NaCl/Pi to one-half the original volume (1 ml) and dialyzed for 24 h. The suspension was then dialyzed for 24 h against 0.015 M sodium phosphate buffer, pH 7.4, resulting in the formation of a precipitate which was removed by centrifugation at 10,000 × g for 30 min. The supernatant was chromatographed on DE52 (Whatman) as follows: A column (1 × 24 cm) was packed and equilibrated with 0.015 M phosphate buffer, pH 7.4. The serum was applied at a flow rate of 15 ml/h, and the IgG emerged in the effluent. The effluent was pooled and concentrated by addition of ammonium sulfate to 40% (v/v), and the resulting precipitate was collected by centrifugation at 10,000 × g for 30 min. The supernatant was dialyzed against 0.015 M sodium phosphate buffer, pH 7.4, and then dialyzed against NaCl/Pi, pH 7.5. The IgG was tested for purity by gel electrophoresis in SDS (as described below) and stored at −20°C.
Antibodies to Nuclear Ribonucleoprotein

For affinity column construction, 0.5 g/column of CNBr-activated Sepharose 4B (Pharmacia) was swollen and washed to remove stabilizers as prescribed by the supplier. The gel, in 10 ml of 0.05 M NaCl, 0.01 M phosphate buffer, pH 7.5, was combined with 1 ml of IgG (from patients MP, MK, MM, and BN) at a concentration of 20 mg/ml, placed in a stopped glass cylinder, and mixed for 15 h at 4°C by inversion on a motor-driven wheel. Essentially all (>96%) of the IgG bound to the gel. The IgG-Sepharose gel was poured into a 7-mm-diameter column, washed for three cycles with 1 M NaCl, 0.1 M acetic acid, pH 4.0, followed by 1 M NaCl, 0.05 M phosphate buffer, pH 7.5 (a total of six washes) and then equilibrated with NaCl/P, pH 7.5.

Crude nuclear antigen (A2) was dialyzed extensively in NaCl/P, pH 7.5, before application to IgG columns. In all affinity column experiments described here, 24 mg of nuclear fraction A2 protein in 3 ml of NaCl/P, were applied to each column. The flow rate was controlled by a Gilson Minipuls pump at a setting of 50 for loading the antigen and washing with NaCl/P. When the absorbance at 280 nm reached zero, the column was developed with two-step elutions to remove bound antigen. The first elution, 1 M NaCl, 0.01 M phosphate buffer, pH 7.5, was intended to remove nucleic acid and protein adventitiously bound to the antigen. The second elution, 0.15 M NaCl was intended to dissociate antigen-antibody complexes for recovery of pure antigen. Both step elutions were run at a pump speed of 175 (approximately 30 ml/h). The recovered material was pooled and stored at −20°C until use. To determine how much A2 was needed to saturate the most avid anti-nRNP column (MP), increasing loads of A2 (at a protein concentration of 7.5 mg/ml) up to a total of 45 mg of protein were applied. With each load, the NaCl/P, rinse buffer was collected and tested for immunoprecipitating activity against all anti-nRNP sera by double diffusion. Loads of 30 and 38 mg were not saturating. Immunoprecipitating activity against all sera was observed in the NaCl/P, runoff from the 45-mg load. With increasing A2 loads, a corresponding increase in the amount of protein bound to the column was observed up to 45 mg.

RNase A Digestion—Crude nuclear antigen (A2) was digested with RNase A, preboiled to inactivate DNase, under the following conditions: A2 (7.5 mg/ml) was incubated with a final concentration of 0.3 mg/ml of RNase A (Worthington, RAF grade) in an incubation buffer of 10 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol for 1 h at 37°C. Control aliquots were incubated at 37°C without RNase A. Reactions were terminated by transferring the samples to 0°C.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate—To dissociate protein-nucleic acid complexes, samples were dialyzed at room temperature for a minimum of 18 h against 2% SDS, 55 mM Tris-HCl (pH 6.8), 2.5% mercaptoethanol was added to 5% (v/v) and the samples were boiled 2 min. Glycerol (to 10% v/v) and bromophenol blue (1%) were added, and electrophoresis was performed according to King and Laemmli (1971) as modified by Weinstaeb et al. (1976) for 15% acrylamide, in gel tubes (8 mm x 13 cm). At an altitude of 5280 feet, it was necessary to reduce the amount of ammonium persulfate by 50% to prevent air bubble formation. The gels were allowed to polymerize overnight. Samples were applied and then stacked at 1 mA/gel for 1 h at 4°C. Gels were fixed and stained with Coomassie brilliant blue as described elsewhere (Garrard and Bonner, 1974). The gels were scanned at a wavelength of 600 nm using a Varian 634 spectrophotometer equipped with scanning attachment.

RESULTS

Fractionation of Rat Liver Nuclei and Testing of Anti-nRNP Sera—A fractionation procedure was devised with the objective of isolating a nuclear subfraction enriched in the antigenic nRNP complexes. The procedure which proved to be the most suitable for this purpose is outlined in Fig. 1. Of the four resulting fractions, A3 contained most of the nuclear protein (57%) and more than 90% of the DNA and histones. Fraction A2, comprising 10% of the nuclear protein, was an insoluble residue and was not tested further. The greatest enrichment in RNA relative to protein was seen in A2 which contained 38% of the nuclear RNA, 1.5% of the DNA, and 11% of the protein. The mass ratio of RNA to protein in this fraction was 0.5:1. A1 contained 12.3% of the nuclear protein. Fractions A1, A2, and A3 were tested for the presence of antigenic complexes by double diffusion against anti-nRNP sera.

Anti-nRNP sera, obtained from a serum bank, had been prescreened by clinical laboratory tests for the presence of anti-RNP antibodies as described under “Experimental Procedures.” Four of these sera from the patients MP, MK, MM, and BN were used as antibody sources in this investigation. In addition, IgG was isolated from the two sera with the highest titers of anti-RNP antibodies (MP and MK). The four sera and two IgGs were tested against the nuclear fractions A1, A2, and A3 as shown in Fig. 2. Optimal antibody concentrations were determined by testing several dilutions of each serum and IgG against each antigen. Of the three nuclear fractions shown in Panels A, B, and C, A2 clearly contained the highest antigen concentration. A trace of activity was observed in fraction A1. As A1 was the least concentrated, A2 was diluted to the concentration of A1 and retested. Panel D shows that diluted A2 was more antigenic than A1 at the same protein concentration. When A2 was tested against six normal human sera, no reaction was observed (Panel E). Panel F shows the reactions of MP serum against whole nuclear sonicate, the low speed supernatant from which A2 is derived, and A2. The lines of identity observed with all three nuclear fractions indicate that all of the antigenic determinants present in unfractonated nuclei are also present in A2. The data show only one precipitin reaction between anti-nRNP antibodies and nuclear fractions.

Previous studies demonstrated that the immunoprecipitating activity of the nRNP antigen is destroyed by treatment with either ribonucleases or proteases (Mattioli and Reichlin, 1971; Northway and Tan, 1972). We re-examined the question of RNase A sensitivity and extended it to interactions between antigen and antibodies affixed to Sepharose 4B. The effects of RNase A and trypsin on the immunoprecipitating activity of A2 are shown in Fig. 3. Panel A shows the reaction of the buffer-incubated control antigen against anti-nRNP sera and

![Fig. 1](http://www.jbc.org/)

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Fig. 1. Fractionation of rat liver nuclei. Nuclei were isolated and then sonicated in 0.01 M phosphate buffer, pH 7.5, 0.15 M NaCl (NaCl/P, PBS, pH 7.5) at a protein concentration of 20 mg/ml. As described under Experimental Procedures. All centrifugations were performed at 4°C. The fractionation procedure outlined here yielded two fractions, A1 and A3, in NaCl/P, pH 7.5, and one fraction, A2, in 10 mM Tris-HCl, 5 mM 2-mercaptoethanol, pH 8.0. The protein concentrations of A1 and A3 averaged 2 and 8 mg/ml, respectively. The protein concentration of A2 ranged from 7.1 to 7.7 mg/ml and averaged 7.5 mg/ml. Nuclear isolation and fractionation took approximately 10 h to complete.
FIG. 2. Immunological reactions of nuclear fractions against anti-nRNP and normal sera. Nuclear fractions: all fractions were in NaCl/Pi, pH 7.5. The center wells of Panels A through E, respectively, contained A1, 2.0 mg/ml of protein; A2, 7.5 mg/ml; A3, 7.7 mg/ml; A4, 2.0 mg/ml, A5, 7.5 mg/ml. In Panel F, WNS = whole nuclear sonicate, 19 mg/ml of protein; LSS = low speed supernatant, 6.0 mg/ml; and A6, 7.5 mg/ml. Anti-nRNP sera: wells 1 to 6 of Panels A to D contained the following antibodies, diluted, if indicated, in NaCl/Pi, pH 7.5: 1, MP serum, diluted 1/20; 2, MK serum, diluted 1/3; 3, MM serum, undiluted; 4, BN serum, undiluted; 5, MP IgG, 1 mg/ml; 6, MK IgG, 2 mg/ml. These concentrations were found to produce optimal reactions against the nuclear fraction A2 at a concentration of 7.5 mg/ml. Panel F, center well, contains MP serum at a dilution of 1/20. Normal sera: the undiluted sera of laboratory personnel BB, LM, MW, MS, PR, and CP were tested against A2 (7.5 mg/ml in NaCl/Pi, pH 7.5) in Panel E. Reaction conditions: agarose plates (0.4%) were used at room temperature. The wells held 150 μl of solution. Plates were allowed to develop 18 to 48 h and were then washed with 5% sodium citrate followed by distilled H2O.

FIG. 3. Effects of RNase A and trypsin on the antigenic activity of A2. Enzyme digestions: bovine pancreatic RNase A (Worthington RAF) at a concentration of 2 mg/ml was boiled for 1 h and then dialyzed into 10 mM Tris-HCl, 5 mM 2-mercaptoethanol, pH 7.5. 50 μl of RNase were added to 300 μl of A2 (7.1 mg/ml of protein) in the same buffer. Twenty-five microliters of a 3 mg/ml solution of trypsin (Worthington) were added to 300 μl of A2 in the Tris buffer. The enzyme-containing aliquots of A2 and a control aliquot were incubated at 37°C for 1 h and then transferred to 0°C to stop the reactions. Reaction conditions: wells 1 to 6, Panels A to C, contained the anti-nRNP sera MP, MK, MM, BN, and MP and MK IgG, respectively, at concentrations given in Fig. 2. The center wells of Panels A, B, and C contained control-incubated A2 (A2c), RNase A-treated A2 (A2r) and trypsin-treated A2 (A2t), respectively. The plates were incubated and processed as described in the legend to Fig. 2.

IgG. Panels B and C show the reactions of RNase A-treated and trypsin-treated A2, respectively. A complete loss of immunoprecipitating activity was observed upon treatment of A2 with either hydrolytic enzyme.

The presence of a single precipitin reaction between sera and either whole nuclear sonicate or A2 and the RNase sensitivity of the A2 reaction suggested that precipitating antibodies to other nuclear molecules (e.g. histones and DNA) were not present in the four anti-RNP sera. This was confirmed directly by double diffusion assays of anti-nRNP antibodies against DNA, rat liver histones, yeast RNA, rat liver nonhistone chromosomal proteins, and nucleosomes (= bodies). The reactions against all anti-nRNP antibody sources were negative and are not shown. (See “Experimental Procedures” for sources and preparation of these antigens.) Normal human sera were also tested and were similarly negative.

Isolation of the Antigen by Affinity Column Chromatography—Three affinity columns were constructed by linking anti-nRNP IgG from two MCTD patients and IgG from a pool of normal human serum (NHS) to CNBr-activated Sepharose 4B. Chromatography of A2 over anti-nRNP columns resulted in three fractions: a runoff in phosphate-buffered saline (NaCl/Pi), pH 7.5, and two fractions recovered by step elutions with 1 M NaCl, 0.01 M phosphate buffer, pH 7.5, followed by 10 mM HCl, 0.15 M NaCl, pH 2.2. Affinity column fractions were tested for immunological activity by double diffusion as shown in Fig. 4. The runoff fraction from the MP anti-nRNP column was devoid of immunoprecipitating activity against all sera (Panel A). The runoff from the MK anti-nRNP column was also inactive and is not shown. In contrast, the runoff from the control NHS column was fully active (Panel D). The amount of antigen applied to these columns was calculated to be nonsaturating with respect to anti-nRNP antibodies as described under “Experimental Procedures.” The NaCl eluate from the MP anti-nRNP column was inactive, but the HCl eluate was active against all antibody sources
Fig. 4. Immunological reactions of affinity column fractions. Column fractions. Panels A to D: 24-mg aliquots of A2 protein in 3-ml volumes were chromatographed over each of two columns, the anti-nRNP column MP and the NHS column. The A2 aliquots were dialyzed against NaCl/Pi, (PBS), pH 7.5, overnight before loading onto the columns. The center wells of Panels A, B, and C contain the following fractions from the MP column: 150 μl of NaCl/Pi, (PBS) runoff, 1.5 mg/ml of protein; 150 μl of NaCl eluate, 146 μg/ml; 150 μl of HCl eluate, 120 μg/ml, respectively. The center well of Panel D contains 150 μl of NaCl/Pi, (PBS) runoff from the NHS column at a protein concentration of 1.5 mg/ml. The NaCl and HCl eluates were tested either immediately upon recovery from the columns or were dialyzed into NaCl/Pi, pH 7.5, for 12 to 15 h and then tested. The NaCl eluate was inactive by either procedure. The HCl eluate was active under both conditions. However, its immunoprecipitating activity showed the greatest stability in the pH 2.2 elution buffer.

(Panel B and C, respectively). Panel E shows that the immunological relationship between the HCl eluate and the crude antigen A2 is one of partial identity. The HCl eluate is impoverished in RNA relative to A2 (10 ± 4% by mass compared to 33 ± 7%, respectively, based on data from five experiments). However, the RNA which is present in the HCl eluate appears to have an essential immunological role as treatment of this fraction with RNase A destroyed its immunoprecipitating activity as shown in Panel F.

Quantitative data on the affinity column experiments, including the two anti-nRNP and control NHS columns, are shown in Tables I and II. The distributions of protein in runoff, 1 mM NaCl, and HCl eluates are shown in Table I. The NHS IgG column bound 0.2% of the applied protein. The anti-nRNP columns MP and MK bound 6.7% and 3.9%, respectively. Most of the retained protein was eluted by 1 mM NaCl. The antigenic activity, however, was found in the 10 mM HCl eluate, as shown in Fig. 4. The reasons for the quantitative differences between the MP and MK column experiments are not known and may not prove to be statistically significant. Neither column was saturated with respect to antigen. Protein recovery from all column experiments averaged 98.5%. To determine the effect of RNase treatment on the binding of ribonucleoprotein to anti-nRNP antibodies, A2 was treated with RNase A. The antisera, Panels A to D, wells I to 6 of Panels A, B, and D contain 150 μl of the following sera diluted, if indicated, in NaCl/Pi, pH 7.5: 1, MP, 1/10 dilution; 2, MK, 1/25 dilution; 3, MM, undiluted; 4, BN, undiluted; 5, MP IgG, 1 mg/ml; 6, MK IgG, 2 mg/ml. Wells 1 to 6, Panel C, contain a 4-fold higher concentration of all antibodies with the exception of the undiluted sera. Panel F: left HCl well contains 150 μg/ml of HCl eluate protein from the MP column, incubated as a control for 1 h at 37°C following adjustment of the pH to 7.5. Right HCl well contains an aliquot of the same fraction incubated with 25 μg/ml of RNase A. MP serum, at the dilutions indicated, was reacted with the HCl eluates.

Table I: The distribution of protein in anti-nRNP and control column fractions

<table>
<thead>
<tr>
<th>Column</th>
<th>% of recovered protein</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>NaCl/Pi; runoff*</td>
</tr>
<tr>
<td>MP anti-nRNP</td>
<td>93.3 ± 3</td>
</tr>
<tr>
<td>MK anti-nRNP</td>
<td>98.1</td>
</tr>
<tr>
<td>MP anti-nRNP (RNase A-treated antigen)</td>
<td>99.9</td>
</tr>
<tr>
<td>Normal</td>
<td>99.8</td>
</tr>
</tbody>
</table>

*Average of two to five experiments per column.
with RNase A in 10 mM Tris, pH 7.5, before chromatography on the MP anti-nRNP column. The RNase treatment caused a major reduction in the quantity of protein which bound, as shown in Table I. This material was eluted with 10 mM HCl, 0.15 M NaCl and was analyzed by gel electrophoresis.

The amounts of RNA bound by control and anti-nRNP columns are shown in Table II. Chromatography of A2 over the MP anti-nRNP column resulted in the binding of 7.7% of the applied RNA. The partitioning of bound RNA into NaCl and HCl eluates is shown in the table. Digestion of A2 in 10 mM Tris completely abolished RNA binding. The effects of RNase A on RNA binding to the column are consistent with its effects on the immunoprecipitating ability of crude antigen, as shown in Fig. 3. The NHS column retained no RNA.

RNase A digestions were performed in 10 mM Tris-HCl, pH 7.5, instead of NaCl/Pi, pH 7.5, because we have found that, in general, A2 is more soluble in Tris than in NaCl/Pi. RNase A digestions performed in NaCl/Pi, often do not go to completion, possibly as a result of inhibition by the inorganic phosphate in the buffer. Moreover, there is extensive precipitation in the course of RNase digestions performed in NaCl/Pi.

The absence of immunoprecipitin reactions between MCTD sera and isolated histones or r bodies (above) and the RNase A sensitivity of both crude and purified antigens (A2 and HCl eluate, Figs. 3 and 4, respectively) suggested that antihistone antibodies are not present in these sera. To further test for antihistone activity, we applied 1 mg of histones in 2.5 ml of NaCl/Pi, pH 7.5 (Case 1) or in 1 mM NaCl, 0.01 mM PB, pH 7.5 (Case 2), to the MP anti-nRNP column. The column was then eluted with either 1 mM NaCl, 0.01 mM PB, pH 7.5, followed by 10 mM HCl, 0.15 M NaCl (Case 1) or with the 10 mM HCl solution (Case 2). The absorbance of these eluates at 230, 260, and 280 nm was zero in both cases, indicating no histone binding. Also, there was quantitative recovery of applied protein in the runoffs.

**TABLE II**

The distribution of RNA in MP anti-nRNP and normal column fractions

<table>
<thead>
<tr>
<th>Column</th>
<th>NaCl/Pi, runoff*</th>
<th>1 mM NaCl eluate*</th>
<th>HCl eluate*</th>
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</thead>
<tbody>
<tr>
<td>MP anti-nRNP</td>
<td>92.3</td>
<td>7.3</td>
<td>0.4</td>
</tr>
<tr>
<td>MP anti-nRNP (RNase A-treated antigen)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Average of two experiments.

Fig. 5. SDS-polyacrylamide gel electrophoresis of anti-nRNP column fractions. The protein moieties of A2 and of anti-nRNP column fractions were electrophoresed by the method of King and Laemmli (1971) as modified by Weintraub et al. (1975) for 15% acrylamide. The molecular weights on the left were calculated from the mobilities of the indicated bands relative to the following molecular weight markers: bovine serum albumin, 68,000; pyruvate kinase, 57,000; human heavy chain IgG, 50,000; ovalbumin, 43,000; DNase I, 31,000; trypsin, 23,000. Gels were run, fixed, and stained as described under “Experimental Procedures.” Panels A to F contain: (A) 100 μg of A2 protein; (B) 100 μg of NaCl/Pi runoff protein following chromatography of A2 over the MP column; (C) 60 μg of 1 mM NaCl eluate, MP column; (D) 60 μg of 10 mM HCl eluate, MP column; (E) 60 μg of 10 mM HCl eluate, MK column; (F) 60 μg of rat liver histones.

As a result of the high density of protein in band P13 of Gels D and E, the intensity of staining with Coomassie blue was not linearly related to protein load. Therefore, the loads...
of P13 monomers, and >85% of the applied protein in a molecular weight range of 110,000 to 65,000. There was no binding of P30 is unaffected by the RNase treatment. An interpretation consistent with the quantitative effects of these treatments were presented in Tables I and II. With the same amount of protein applied to each gel, it is evident that the ratio of P13 to P30 decreased with RNase treatment. The HCl eluate was applied to a column of Sephadex G-150 M

on these gels appear to be lower than 60 μg of protein. Another problem we encountered was incomplete absorption of dye into the P13 band due both to the density of protein and to the density of acrylamide which is significantly higher at the bottom of the gels if they are allowed to polymerize overnight. This problem has been partially overcome by using 10% acrylamide instead of 15%.2

Fig. 6 shows a scan of a gel containing HCl eluate protein from the MP column (upper scan). From gel scans of five MP anti-nRNP column experiments, we estimate that P30 and P13 comprise more than 75% of the protein mass of HCl eluates. The effect of RNase A on the protein composition of the HCl eluate can be seen by comparing the upper and lower scans of Fig. 6. Shown in the lower scan is the HCl eluate from an experiment in which A2 was pre-treated with RNase A before chromatography on the MP column. The quantitative effects of these treatments were presented in Tables I and II. With the same amount of protein applied to each gel, it is evident that the ratio of P13 to P30 decreased with RNase treatment. An interpretation consistent with the quantitative data is that the binding of P13 is greatly reduced, but the binding of P30 is unaffected by the RNase treatment.

The HCl eluate was applied to a column of Sephadex G-150 in 10 mM HCl, 0.15 M NaCl to determine the approximate sizes of the macromolecules in the antigenically active fraction. At least three major components were present, all within a molecular weight range of 110,000 to 65,000. There was no evidence of P13 monomers, and >85% of the applied protein fell within the molecular weight range of 110,000 to 65,000.

**DISCUSSION**

One of the aims of this study was to determine how much of the nuclear ribonucleoprotein reacts with human anti-nRNP antibodies. Antiserum from the patient MP was the most potent of the four compared here both with respect to titer and antigen binding, as shown in Fig. 2 and Tables I and II. A2 was chromatographed over the MP column under conditions of antibody excess so that all of the applied antigen bound. As A2 represents 11% of the nuclear protein and 38% of the nuclear RNA, it follows from the data in Tables I and II that 0.7% of the total nuclear protein and 2.9% of the RNA bind to MP antibodies.

Some assumptions implicit in these calculations are: 1) that A2 contains the majority of the nuclear antigenic complexes; 2) that most of the antigen is not inactivated during nuclear isolation and fractionation; and 3) that adventitious sticking of nonantigenic ribonucleoprotein is not quantitatively significant. The data in Fig. 2 show that most of the antigenic activity is found in A2. These data also show that A2 is immunologically identical with whole nuclear sonicate. Thus, it is unlikely that the fractionation scheme outlined in Fig. 1 results in gross inactivation of the antigen. We found it necessary to sonicate nuclei to release the antigenic activity. It is possible that sonication has a deleterious effect. While the quantitative data may be affected to an unknown degree by these assumptions, they do illustrate that the antigenic complexes represent a small fraction of the nuclear ribonucleoprotein.

Most of the ribonucleoprotein which bound to the MP and MK columns was eluted with 1 M NaCl and had no immunological activity, as shown in Fig. 3, Panel B. It is evident from the reaction in Fig. 4, Panel D, that the HCl eluate, although active, lacks some of the antigenic determinants present in the crude antigen. It is likely that the NaCl eluate consists, in whole or part, of salt-labile antigenic complexes which together with the salt acid-resistant complexes of the HCl eluate make up the whole antigen. When nuclear extracts were reacted against anti-nRNP sera in nondissociating media such as phosphate-buffered saline, a single precipitin reaction was observed, both by counterimmunoelectrophoresis (Kurata and Tan, '76) and by immunodiffusion, as shown in Fig. 2. Thus, under nondissociating conditions, complexes which are sensitive and resistant to salt and acid may be found on the same RNP particle.

Four antisera were compared in this study in order to determine whether they are specific for the same antigen. The protein moieties of HCl eluates from two anti-nRNP columns (MP and MK) were compared in Fig. 5. The polypeptides P30 and P13 are the quantitatively major protein species in HCl eluates from both columns. With the exception of minor polypeptides, the protein composition of this fraction is very reproducible. The HCl eluate from the MP column reacted with antibodies from all four sera, and the reactions were immunologically identical, as shown in Fig. 4, Panel C. Reactions of the four sera against the crude antigen A2 were also identical. Moreover, fractions which were inactive against one serum were inactive against all four, e.g. the NaCl/Pi runoff from the MP column, shown in Fig. 4, Panel A. Thus, there were no detectable differences in the specificities of anti-nRNP antibodies from different individuals.

The HCl eluate consists of less than 0.15% of the nuclear protein based on data in Table I. We have no direct evidence that any of the antigenic determinants in this eluate are on polypeptides P30 and P13. However, they comprise most of the protein mass of this fraction and are the only reproducible protein components with the possible exception of a minor polypeptide of M, = 14,000. The binding of P13 to affinity columns is RNase A-sensitive, as are the immunoprecipitin activities of A2 and of the HCl eluate. Characterization of the separate nucleic acid and protein moieties of the HCl eluate is in progress. Isolation of a sufficient quantity for further characterization is now feasible. Chromatography of 24 mg of A2 protein over a 20-μg IgG column yields about 240 μg of protein in the HCl eluate. We have now constructed a 100-μg column and have chromatographed 75 mg of A2 with a yield

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of 700 µg of protein and about 70 µg of RNA in the HCl eluate. It should be possible to chromatograph 100 mg of A2 without saturating the column. Moreover, the column can be used several times; the original MP anti-nRNP column has been used 10 times without noticeable deterioration in quality.

We found no evidence for the presence of antibodies to free protein or free nucleic acid in any of the four anti-nRNP sera in this study. Within the limits of sensitivity of chemical assays, there was no DNA in ribonucleoprotein fractions isolated by affinity chromatography. Nor did any of the sera react with DNA, histones, histone-DNA complexes, RNA, or nonhistone chromosomal proteins in immunodiffusion assays. As shown in Fig. 3, the antigenic activity of A2 is sensitive to either RNase A or trypsin, indicating that both RNA and protein moieties are essential for activity. Also, treatment of crude antigen with RNase A greatly reduced the quantity of protein which bound to the anti-nRNP column, as shown in Table I. These data indicate that the antigenic ribonucleoprotein is in the form of complexes rather than separate RNA and protein moieties. The smallest major component of the HCl eluate has a molecular weight of 65,000 based on Sephadex G-150 gel filtration, whereas the majority of the protein has a molecular weight of 13,000 in SDS gels (Fig. 5). Therefore, this antigenically active fraction is clearly composed of molecular aggregates. Due to the heterogeneity of these aggregates, we have not yet determined their nucleic acid to protein ratios. The size of the RNP antigen has been estimated previously by subjecting crude tissue extracts to gel exclusion chromatography (Mattioli and Reichlin, 1971) or to sucrose density sedimentation (Northway and Tan, 1972). These estimates indicate a size of 200,000 daltons or ≥7 S, respectively, for the crude antigen. As the HCl eluate is a partial antigen (Fig. 4) representing acid- and salt-resistant antigenic complexes, it is not surprising that it is composed of aggregates smaller than Mₐ = 200,000.

The rates of migration of polypeptides P30 and P13 on SDS gels are very similar but not identical with those of histones H1 and H2A, respectively. Preliminary data show that HCl eluate proteins do not migrate as rapidly as histones in acetic acid/urea gel systems which resolve basic proteins on the basis of charge and size. Moreover, a number of observations made in this study indicate that antiboistone antibodies are not present in the Mₐ< 10 S fraction. The majority of the histone mass in the HCl eluate is composed of histone-like proteins. At least two types of histone-like proteins have been found in mammalian nuclei or nucleoli. The so-called high mobility group nuclear proteins include a polypeptide of Mₐ = 11,000 which has a high content of basic amino acid residues and is partially homologous to histone T (Goodwin et al., 1975; Walker et al., 1976). A24, a protein found in nucleoli, is composed of histone H2A covalently linked to a nonhistone polypeptide (Olsen et al., 1976). Further characterization of the HCl eluate proteins should reveal any similarities between these proteins and histones.

For this study, we intentionally selected human sera which contained no precipitating antibodies to RNase-resistant nuclear antigens. Sera from patients with systemic lupus erythematosus occasionally contain antibodies to both the nRNP antigen and to a nuclear protein (or proteins) called Sm (Mattioli and Reichlin, 1971; Tan et al., 1973; Tan and Vaughan, 1973; Kurata and Tan, 1976). Using sera with this dual specificity, Mattioli and Reichlin (1971) reported that the Sm antigen appears to be physically associated with the nRNP antigen. We have tested prototype anti-Sm sera against nuclear Fractions A1 and A2, the NaCl/P, runoff from the anti-nRNP affinity columns, and the NaCl and HCl eluates. With the exception of the NaCl and HCl eluates, all of these reactions proceeded with anti-Sm sera in double diffusion tests. The absence of precipitin reactions between anti-Sm sera and the HCl eluate suggests that this antigen contains no physically associated Sm.

The very high prevalence of anti-nRNP antibodies in MCTD sera suggests that malfunctions in specific nuclear mechanisms, e.g., some aspect of nuclear RNA synthesis or metabolism, may be the stimulus for antibody production. To understand which part of the nuclear RNA synthesizing or processing machinery, or both, is reacting with the anti-nRNP antibodies from these sera, it will be necessary to characterize the RNA moiety of the isolated complexes. As we have not isolated the entire nRNP antigen in immunologically active form, it is not possible to draw extensive comparisons between this antigen and other nonnucleolar RNP particles which have been isolated from nuclei. However, some differences between the nRNP antigen and 30 to 55 S hnRNP particles (composed of hnRNA and protein) are evident. First, it is necessary to disrupt nuclei by sonication to dissociate the nRNP antigen. We attempted to extract the antigen from intact nuclei by the method of Martin et al. (1973) and found that the extract, which contained 30 S RNP particles, was immunologically inactive. Second, the 30 to 55 S hnRNP particles are composed of between two and seven distinct polypeptides ranging in molecular weight from 32,000 to 40,000 (see LeStourgeon et al., 1978 and Martin et al., 1978 for references to earlier work). The HCl eluate is devoid of quantitatively major polypeptides of Mₐ = > 30,000 (Fig. 5, Panels D and E). Also, the majority of the protein mass in the NaCl eluate corresponds to Mₐ ≤ 30,000 (Panel C). These observations do not exclude the possibility that the particles comprising the nRNP antigen represent a different stage of hnRNA processing than do the hnRNP particles. Using the human anti-nRNP antibodies, it should be possible to investigate the distribution of the antigenic particles in nuclei and determine the degree to which they are chromosome-associated.

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