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Molecular Relationships between Two Nuclear Antigens, Ribonucleoprotein and Sm: Purification of Active Antigens and Their Biochemical Characterization†

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ABSTRACT: Two nuclear antigens, nuclear ribonucleoprotein (RNP) and Sm, which react with the serum antibodies of patients with mixed connective tissue disease and systemic lupus erythematosus were purified with maintenance of their immunological activities and characterized to elucidate their molecular relationships. Purifications from calf thymus nuclei were accomplished, in part, by affinity chromatography of the antigens on column resins covalently bound to purified human antibodies specific for RNP and Sm. Purified RNP and Sm had molecular weights of 160 000 and 70 000, respectively. A biochemical relationship between the two antigens was indicated by purified RNP exhibiting Sm activity which could not be separated from the former by RNP antibody affinity chromatography. Immunodiffusion lines of RNP against RNP and Sm antibodies were completely identical. Ribonuclease-treated RNP was immunologically inactive as RNP antigen and not retained on RNP antibody affinity columns, but did maintain immunological activity as Sm antigen. Purified Sm not containing RNP activity was retained only by Sm antibody columns. RNP and Sm were found by sodium dodecyl sulfate–polyacrylamide gel electrophoresis to contain the same four polypeptides of molecular weights approximating 13 000. RNP had four additional peptides, one approximating a molecular weight of 13 000, two approximating a molecular weight of 30 000, and one of molecular weight 65 000. Urea–polyacrylamide gel electrophoresis showed that RNP contained an RNA molecule similar in size to tRNA and that this RNA was not present in Sm or ribonuclease-treated RNP. The whole macromolecular complex has two antigenic sites. RNP antibody reacts with the portion of the molecule containing RNA and the polypeptides possibly having 65 000 and 30 000 molecular weights; Sm antibody reacts with polypeptides with a molecular weight of approximately 13 000. Sm having no RNP antigenic activity is composed of the 13 000 molecular weight polypeptides only.

Normally occurring macromolecular complexes of mammalian cell nuclei have been found to be antigens to which antibodies are produced in the autoimmune diseases, systemic lupus erythematosus (SLE),[1] and mixed connective tissue disease (MCTD) (Sharp et al., 1972, 1976; Parker, 1973; Notman et al., 1975; Bresnihan et al., 1977; Grennan et al., 1977; Koffler, 1980). High titers of circulating antibody to nuclear ribonucleoprotein (RNP) antigen are typical of MCTD (Sharp et al., 1972, 1976), while antibodies to RNP and Sm occur together in the serum of some patients with SLE (Notman et al., 1975; Sharp et al., 1976). RNP is an RNA–protein complex (Mattioli & Reichlin, 1971; Northway & Tan, 1972; Sharp et al., 1972; Douvas et al., 1979a; Lerner & Steitz, 1979; Lerner et al., 1980; Takano et al., 1980), and the immunological activity of the antigen is dependent on the integrity of RNA which can be destroyed by ribonuclease treatment of the antigen and protein which is sensitive to trypsin (Mattioli & Reichlin, 1971; Sharp et al., 1972; Douvas et al., 1979a; Takano et al., 1980). In contrast, Sm antigen is resistant to digestion with ribonuclease and trypsin (Tan & Kunkel, 1966; Sharp et al., 1972).

Isolation and biochemical characterization of RNP have been initiated by a number of investigators (Douvas et al., 1979a; Lerner & Steitz, 1979; Lerner et al., 1980, 1981; Takano et al., 1980), and the antigen has been found to be a highly conserved macromolecular complex in nuclei (Lerner et al., 1980). The biological roles of RNP and Sm are un-

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[1] Abbreviations used: SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease; RNP, nuclear ribonucleoprotein antigen; Sm, an RNase-resistant nuclear acidic protein antigen; CTE, calf thymus nuclear extract; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; NaSCN, sodium thiocyanate; RNase, ribonuclease A; DNase, deoxyribonuclease.

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known; however, the hypothesis has been presented that these RNA-containing antigens are involved in processing precursor RNA to mature RNA (Lerner et al., 1980) such as the RNA-requiring ribonuclease P (Kole & Altman, 1979).

A molecular relationship between RNP and Sm has been inferred from previous work showing (a) lines of partial identity in Ouchterlony tests with crude nuclear extracts or partially purified antigen and patient sera (Mattioli & Reichlin, 1973) and (b) prevalent expression of antibodies to both RNP and Sm by patients with SLE (Reichlin & Mattioli, 1974; Notman et al., 1975; Sharp et al., 1976). In this paper, we present the complete methodology for purification of immunologically active antigens and, through biochemical characterization of the pure antigens, describe the molecular relationships between RNP and Sm.

Experimental Procedures

Preparation of Crude and Partially Purified RNP Antigen. The initial extraction of nuclear antigens, RNP and Sm, used for this study was performed according to a modification of a published method (Northway & Tan, 1972). Briefly, calf thymus glands obtained from the slaughterhouse on sucrose ice cubes were trimmed of fat and connective tissues. All procedures were done at 4 °C. Thymus glands were cut into small pieces and placed into a Waring blender with 0.25 M sucrose solution containing 4 mM $CaCl_2$. Tissue in this solution was blended for 4 min with a rheostat setting of 55. The mixture was filtered through two layers of gauze and passed through white flannel. The filtrates were centrifuged at 600*g* for 5 min. Supernatant fluid was removed from the pellets, and 0.25 M sucrose solution with 4 mM $CaCl_2$ was added to resuspend the pellets by using a Vortex mixer. This suspension was passed through another layer of the flannel. The filtrate was then centrifuged at 600*g* for 5 min, and the resulting pellet was resuspended in 0.1 ionic strength phosphate buffer, pH 7.1, followed by stirring in a Sorvall homogenizer at high speed for 45 s. This homogenate was then left at 4 °C overnight. The solution was filtered through a single layer of gauze and ultracentrifuged at 106000*g* for 2 h. The resulting supernatant fluid was salted out with 30% saturated ammonium sulfate. The solution was centrifuged at 10000*g* for 60 min and the pellet discarded. The supernatant material was salted out with addition of ammonium sulfate to make a final concentration of 60% saturation. This solution was centrifuged at 10000*g* for 60 min, and this time the supernatant material was discarded. The pellet was dissolved in the smallest volume of 0.01 M PBS, pH 7.4, containing 0.15 M NaCl and dialyzed against the same buffer for 48 h. The final protein concentration was between 15 and 20 mg/mL. Agarose gel chromatography was used to partially purify the RNP antigen from crude calf thymus extract as previously described (Takano et al., 1980). Further purification was accomplished by antibody affinity chromatography.

Partial Purification of Sm. Crude Sm was obtained from calf thymus nuclei through modification of a published method (Winn et al., 1979). Briefly, calf thymus nuclei were homogenized in a Sorvall high-speed homogenizer at highest power for 45 s with 0.1 ionic strength phosphate buffer, pH 7.1. After overnight incubation at 4 °C, the homogenate was centrifuged at 10000*g* for 60 min, and the supernatant fraction was used for RNP purification. The pellet was homogenized with an equal volume of 0.1 M phosphate buffer, pH 6.7, containing 5 mM $MgCl_2$ in a Waring blender for 4 min. This homogenate was incubated with DNase at a final concentration of 100 μg/mL at 37 °C for 60 min followed by centrifugation at 12000*g* for 30 min. Pellets were discarded. Ammonium sulfate was added to the supernatant fraction in order to prepare a 30% saturated ammonium sulfate soluble, 60% saturated ammonium sulfate insoluble fraction. This fraction was shown to contain a large amount of Sm relative to that of RNP. For elimination of a RNP activity, this fraction was digested with RNase at the concentration of 0.5 mg/mL at 37 °C for 60 min. Sm was further purified by Sephadex G 150 column chromatography. Active Sm antigen fractions from the chromatography were pooled and concentrated by precipitation with 60% saturated ammonium sulfate. The protein concentration of this antigen was 20 mg/mL.

Purification of Antibody to RNP. RNP antibody was purified from serum monospecific for antibody to RNP as previously described (Takano et al., 1980). Only light and heavy immunoglobulin chains were found in the purified antibody preparation upon $NaDodSO_4$–polyacrylamide gel electrophoresis.

Purification of Antibody to Sm. Purified Sm antibody was prepared from an IgG fraction of an SLE patient serum containing high titers of Sm antibody. An affinity chromatography column in which Sepharose 4B was covalently coupled with partially purified Sm was employed. IgG was prepared from the serum by the methods previously described (Fahey & Horbett, 1959). The affinity column for purification of Sm antibody was constructed as follows: CNBr-activated Sepharose 4B (2 g) was swollen in 1 mM HCl and then washed with 0.1 M $NaHCO_3$ containing 0.5 M NaCl. Partially purified Sm (8 mL, 20 mg/mL protein) was mixed with Sepharose 4B and stirred gently at 4 °C for 18 h. Unbound protein was washed from the resin with 0.1 M $NaHCO_3$ buffer and reacted with 1 M ethanolamine at room temperature for 60 min. The resin was washed with (a) $NaHCO_3$ buffer, (b) 0.1 M acetate buffer, pH 4.0, containing 1 M NaCl, (c) 0.1 M borate buffer, pH 8.0, containing 1 M NaCl, and (d) 3 M sodium thiocyanate (NaSCN), poured into a column of 0.9 cm in diameter by 15 cm in length, and equilibrated with PBS. To this column was applied 10 mL of IgG fraction (20 mg/mL). After the column was extensively washed with PBS containing 0.5 M NaCl in order to remove unbound protein, highly purified Sm antibody was eluted with 3 M NaSCN. The specific antibody activity of this eluate was shown to be 169 times higher than that of the original serum. $NaDodSO_4$–polyacrylamide gel electrophoresis of purified antibody demonstrated the presence of only light and heavy immunoglobulin chains. Final concentration of purified Sm antibody was 0.82 mg/mL.

Affinity Chromatography for Purification of RNP and Sm. Affinity chromatography used for purification of RNP and Sm employed purified RNP and Sm antibodies coupled with CNBr-activated Sepharose 4B, respectively. In the case of the RNP antibody affinity column, 0.5 g of Sepharose 4B was coupled with 5.2 mg of purified RNP antibody by the methods suggested by the manufacturer. The coupling reaction of Sepharose 4B with RNP antibody was done at 4 °C for 18 h by gentle stirring in 10 mL of 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl. After reaction with 1 M ethanolamine at pH 8.0 for 60 min, the gel was washed with $NaHCO_3$ buffer, acetate buffer, borate buffer, and 3 M NaSCN and finally equilibrated with 0.01 M PBS, pH 7.5, containing 0.5 M NaCl. The Sm antibody affinity column (0.5 g of Sepharose 4B reacted with 6.5 mg of purified Sm antibody) was constructed in the same manner as the RNP antibody affinity column. Chromatography for purification of RNP and Sm was accomplished according to methods previously described (Takano et al., 1980).

Sodium Dodecyl Sulfate- and Urea-Polyacrylamide Gel Electrophoresis. Electrophoresis of crude and purified antigens for detection of polypeptide bands and RNA was carried out as previously reported (Takano et al., 1980).

Immunological Methods and Tests of RNP and Sm Purity. Immunodiffusion, hemagglutination, and hemagglutination inhibition methods were described in an earlier report (Takano et al., 1980). Immunological analysis of highly purified RNP and Sm using monospecific reagent antisera did not reveal the presence of any other nuclear protein antigens including PM-1 (Wolfe et al., 1977), Scl-70 (Tan & Rodnan, 1975; Douvas et al., 1979b), SS-A, or SS-B (Alspaugh & Tan, 1975).

Identification of RNP and Sm Antigenic Determinant Sites with Solid-Phase Radioimmunoassay. The RNP and Sm antigenic determinant sites in the purified antigens were investigated by using a solid-phase radioimmunoassay. Purified antigen was applied to a $NaDodSO_4$–polyacrylamide gel for electrophoretic separation of peptides. The gel was cut into 1-mm slices, and each gel slice was homogenized with 200 μL of PBS by using a Dounce glass homogenizer followed by centrifugation at 1000*g* for 5 min. A 30-μL sample of the eluate from each homogenate was added to a well of a flat-bottom polyvinyl microtiter plate (Dynatech Laboratories Inc., Alexandria, VA) and incubated at 4 °C overnight. All fluid was then removed from wells by aspiration, and the wells were filled with borate-buffered saline (0.05 M borate and 0.15 M NaCl, pH 8.0) containing 2% bovine serum albumin (BBS–BSA). After an overnight incubation at 4 °C, BBS–BSA was removed, and 30 μL of serum diluted 1:1000 in this buffer was added. Reference sera included sera specific for RNP antibody, Sm antibody, and various controls. After 3 h of incubation at room temperature, wells were washed 5 times with BBS–BSA, and then 30 μL of ^{125}I-labeled rabbit anti-human IgG (2.2×10^6 cpm/μg of protein, 6×10^4 cpm/well) was added to each well. After a 3-h incubation at room temperature, wells were washed 5 times with BBS–BSA, and the radioactivity of each was detected by γ scintillation counting.

RNP–RNA Isolation. RNA was isolated from purified RNP and Sm by extracting twice with a 1:1 mixture of phenol and isolation buffer, modified from methods previously described (Preisler et al., 1973) [46 mM NaCl, 20 mM sodium acetate, 10 mM $MgCl_2$, 2 mM EDTA (pH 5.2), 0.5% $NaDodSO_4$, 0.2% washed bentonite, and 1 μL/mL pyrocarbonate]. Alternately, RNP and Sm were boiled 10 min to dissociate the RNA–protein complex just prior to electrophoresis (Takano et al., 1980).

Molecular Weight Determination of Purified RNP and Sm. Purified RNP and Sm were subjected to chromatography on Sephadex G 150. Blue dextran, 7S IgG, and bovine serum albumin were used for molecular weight standards.

Nucleoside Composition Analysis. The nucleoside composition of RNP–RNA was determined by high-performance liquid chromatography of enzymatic digests (Davis et al., 1979).

Results

Purification of RNP by RNP Antibody Affinity Chromatography. RNP was purified from calf thymus nuclear extract (CTE) with maintenance of antigenic activity. Details of the purification methods are found under Experimental Procedures and in a previous publication (Takano et al., 1980). Double immunodiffusion in 0.4% agarose gel and hemagglutination inhibition assays were used to detect the antigenic activity of crude and purified antigens. The final purification step employed affinity chromatography with Sepharose 4B bound

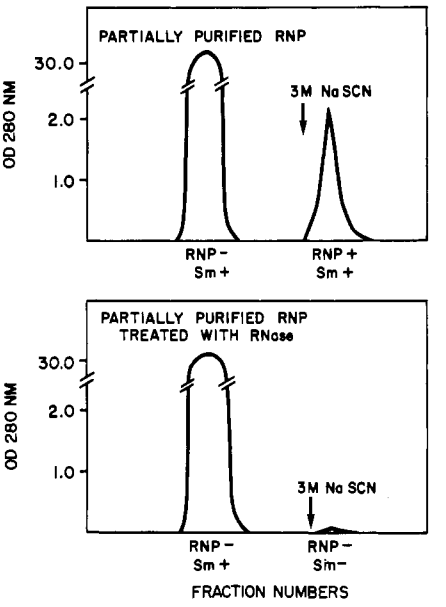

FIGURE 1: RNP antibody affinity column chromatography for purification of RNP antigen. Partially purified RNP antigen which was obtained from CTE through Sepharose 6B column chromatography was applied to the affinity column (shown in the upper panel). The first peak representing the pass-through of portions not specifically bound to the affinity column contained Sm but not RNP antigen. RNP antigen, which also contained Sm antigenic activity, was then eluted from the column by using 3 M NaSCN as the eluting solution. When partially purified RNP antigen which had been treated with RNase was applied to the column (shown in lower panel), the 3 M NaSCN eluate contained neither RNP nor Sm activities.

purified RNP antibody. This chromatography required prior purification of RNP-specific antibody from the serum of a patient with MCTD. Examples of the chromatography results are shown in Figure 1. The specific antigen was retained by the column and eluted with 3 M NaSCN. The specific activity of purified RNP antigen was 488 times higher than that of CTE (Takano et al., 1980). Purified RNP always contained Sm antigenic activity as well (Figure 1) but did not exhibit antigenic activity for other nuclear protein antigens such as PM-1, Scl-70, SS-A, or SS-B.

Purification of Sm by Sm Antibody Affinity Chromatography. Since Sm antibody is found in close association with RNP antibody in SLE sera, production of an Sm antibody affinity column for purification of Sm antigen first required separation of the two antibodies. This was achieved by applying the IgG fraction from a serum positive for Sm antibody to an affinity column composed of partially purified Sm antigen covalently linked to Sepharose 4B. As described under Experimental Procedures, this partially purified Sm antigen was treated with RNase in order to eliminate RNP before being bound to the resin. Sm antibody specifically eluted from this affinity column, free of RNP antibody, was then employed for production of the Sm antibody affinity column used for final purification of Sm.

Sepharose 6B fractionated CTE was applied to the Sm antibody affinity column. The 3 M NaSCN eluate from this column was shown to contain Sm antigen with a specific activity 149 times higher than that of crude CTE by using hemagglutination inhibition. In addition, the eluate also contained RNP antigenic activity as shown by immunodiffusion (Figure 2).

In order to obtain purified Sm antigen free from RNP activity, two different partially purified antigens were applied to an Sm antibody affinity column. In the first case, partially purified Sm antigen was prepared by using RNase treatment

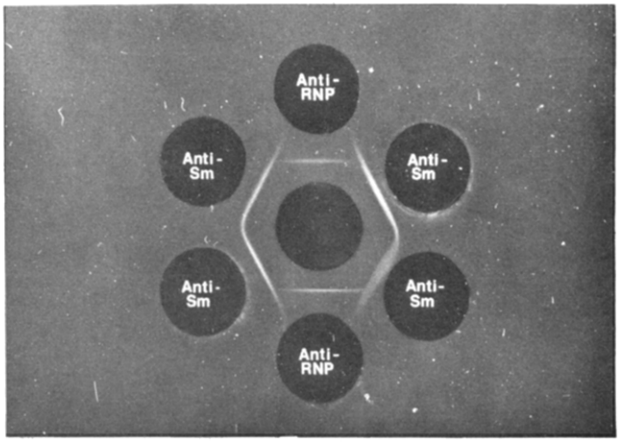

FIGURE 2: Demonstration of RNP antigenic activity in Sm antigen which was purified by an Sm antibody affinity column. The center well contained the 3 M NaSCN eluate from the Sm antibody affinity column to which Sepharose 6B fractionated CTE had been applied. This eluate gave a precipitin line against Sm antibody and also gave a line against RNP antibody. These precipitin lines were shown to be partially identical.

as explained under Experimental Procedures. The second partially purified Sm antigen was prepared by chromatography of Sepharose 6B fractionated CTE on an RNP antibody affinity column. Material having Sm antigenic activity, but no RNP, was not retained by the column and eluted in the pass-through fraction as shown in Figure 1. This material was again subjected to RNP antibody affinity chromatography, and the Sm not being retained by the column was collected. Therefore, this latter procedure yielded Sm lacking RNP without requiring RNase treatment. These two partially purified antigens were applied to Sm antibody affinity columns, and purified Sm, now being retained by the resin, was eluted with 3 M NaSCN. Purified Sm exhibited neither RNP antigenic activity nor that of other nuclear protein antigens, such as PM-1, Scl-70, SS-A, or SS-B, and was 270-fold higher in specific activity than crude CTE determined by the hemagglutination inhibition assay.

Analysis of Purified RNP and Sm. The approximate molecular weights of purified RNP and Sm were determined by gel filtration as shown in Figure 3. Purified RNP was excluded from the column at a position similar to that of 7S IgG, thus exhibiting an approximate molecular weight of 160 000, identical with that of RNP in crude extracts (Takano et al., 1980). The approximate molecular weight of purified Sm without RNP activity, determined in a similar manner, was 70 000, as compared to 70 000–160 000 found for Sm in crude extracts (Northway & Tan, 1972).

The protein contents of CTE, purified RNP, and purified Sm were compared by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4). Purified RNP (lane B) exhibited eight polypeptide bands when subjected to gel electrophoresis: five peptides migrated, in comparison to standards, in the molecular weight range of 12 000–13 000; two peptides migrated with an approximate molecular weight of 30 000; one migrated with a molecular weight of 65 000. Sm (lane C) which was purified from Sepharose 6B fractionated CTE by Sm antibody affinity chromatography exhibited very similar polypeptide bands. All eight bands seen in lane B were also observed in lane C. This result suggests that we obtained the same molecule from Sepharose 6B fractionated CTE with use of either an RNP antibody affinity column or an Sm antibody affinity column. However, when Sm antigen devoid of RNP was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (lane B in Figure 5), only four polypeptide bands were observed in

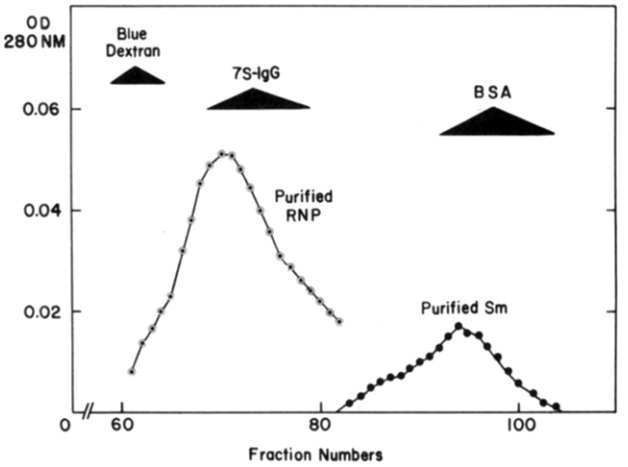

FIGURE 3: Sephadex G 150 column chromatography of purified RNP and purified Sm antigens. Bovine serum albumin (M_r 67 000) and 7S IgG (M_r 160 000) were used as molecular weight standards. Purified RNP antigen was excluded from the column in approximately the same fractions as 7S IgG; therefore, the molecular weight of this antigen was estimated to be approximately 160 000. Purified Sm having no RNP activity was excluded from the column in fractions corresponding to the exclusion of bovine serum albumin, and its molecular weight was estimated to be approximately 70 000.

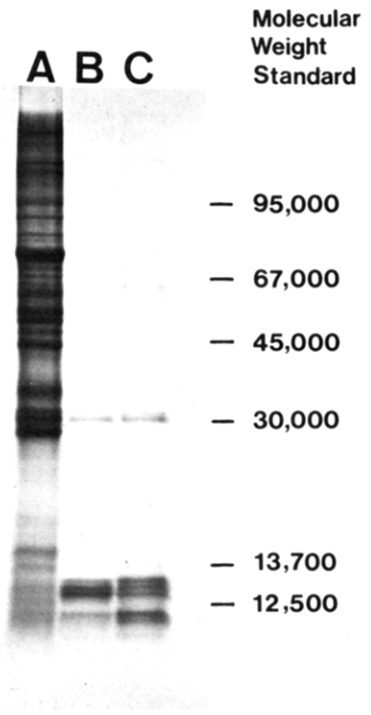

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of Sepharose 6B fractionated CTE (A), purified RNP (B), and purified Sm containing RNP activity (C). The gel was formed as 1.5 × 120 × 106 mm slabs which was prepared by the methods described previously (Takano et al., 1980). Phosphorylase *b* (M_r 95 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 30 000), RNase (M_r 13 700), and cytochrome *c* (M_r 12 500) were used as molecular weight standards. The gel was stained by 0.04% Coomassie brilliant blue in 25% isopropyl alcohol and 10% acetic acid solution overnight. In lane B, purified RNP antigen gave eight polypeptide bands: one polypeptide with approximately 65 000 molecular weight, two bands with molecular weights of approximately 30 000, and five polypeptides with molecular weights of 12 000–13 000. Although several additional faint polypeptide bands could be seen in lane C, all eight polypeptides in lane B were also found in lane C.

contrast to the eight peptides demonstrated for RNP (lane A). These four bands migrated in the 12 000–13 000 molecular weight region. Thus, the three larger molecular weight po-

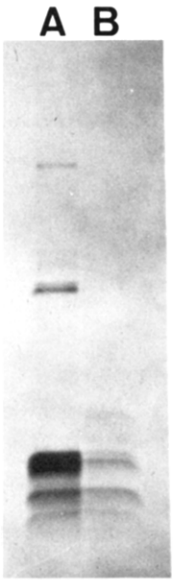

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of Sm antigen without RNP antigenic activity. In lane A, purified RNP antigen gave eight polypeptide bands. In lane B, Sm without RNP activity showed only four polypeptide bands which migrated to the 12 000–13 000 molecular weight region. Three larger polypeptides and one polypeptide of approximately 13 000 molecular weight noted in the case of RNP antigen were totally missing in the case of Sm antigen.

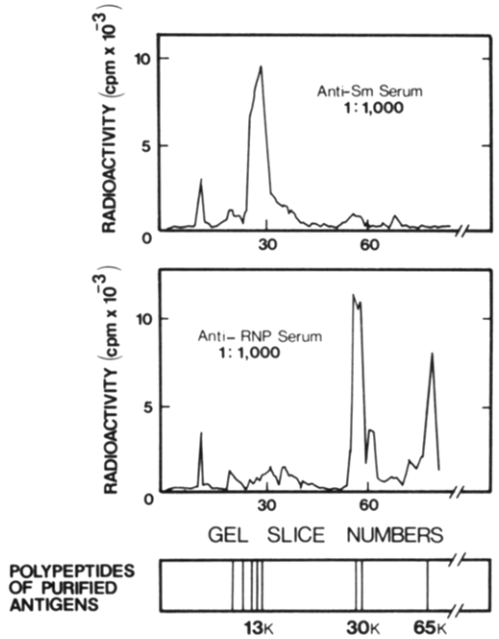

FIGURE 6: Detection of Sm and RNP antigenic activity in eluates from gel slices with a solid-phase radioimmunoassay. A purified antigen containing both RNP and Sm antigenic activity, 20 μg of protein per lane, was applied to a $NaDodSO_4$–polyacrylamide gel and electrophoretically separated. Methods of electrophoresis, PBS elution from the gel, and radioimmunoassay are described under Experimental Procedures. The upper and middle panels represent the radioactivity of wells which had been coated with samples eluted from gel slices, reacted first with antibodies to Sm and RNP, respectively, and then with ^{125}I-labeled rabbit anti-human IgG antibody. The bottom panel shows the diagrammatic representation of polypeptides seen on Na-$DodSO_4$–polyacrylamide gel electrophoresis of the purified RNP–Sm antigens. One small radioactive peak seen in the low molecular weight area (slice number 13) in the upper and middle panels was also seen in experiments using control sera.

lypeptides and one polypeptide with a molecular weight of approximately 13 000 present in both purified RNP (lane A) and purified Sm containing RNP activity (Figure 4, lane C)

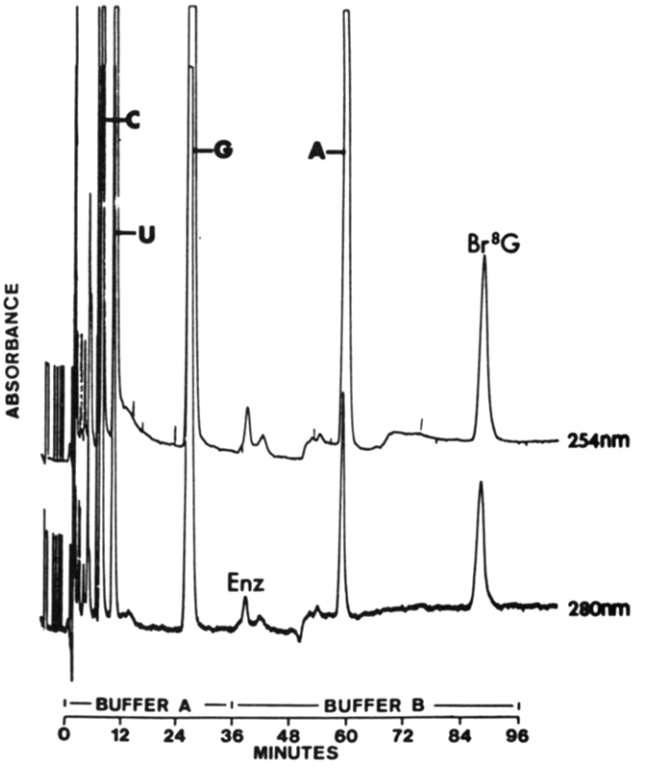

FIGURE 7: Reverse-phase high-performance liquid chromatographic separation of RNA from purified RNP antigen. RNA, 5.4 μg, was enzymatically digested and applied to the column (μBondack C18, 4 × 600 mm). Chromatography procedures were performed according to the methods described previously (Davis et al., 1979). Buffer A: 2.5% methanol in 0.01 M $NH_4H_2PO_4$, pH 5.3. Buffer B: 8.0% methanol in 0.01 M $NH_4H_2PO_4$, pH 5.1. The modified nucleoside, 8-bromoguanosine (Br^8G), was added as an internal standard. Note that only four nucleosides, cytidine (C), uridine (U), guanosine (G), and adenosine (A), were found.

were totally missing in this preparation of Sm.

The RNP and Sm antigenic determinant sites of electrophoretically separated components of the purified antigens were examined by using a solid-phase radioimmunoassay. The results are depicted in Figure 6. When Sm antibody reference serum was used in this assay, the major radioactive peak occurred in fractions corresponding to the 13 000 molecular weight polypeptide bands. In contrast, when RNP antibody reference serum was used, the two major peaks occurred in fractions corresponding to the 30 000 and 65 000 molecular weight polypeptide bands. These radioimmunoassay results were reproducible and specific for RNP and Sm antibody sera. When sera specific for SS-A, SS-B, PM-1, and DNA antibody were tested by using the same radioimmunoassay system, no specific radioactive peak was found.

The RNA component of purified RNP was found to be required for antigenicity as has been reported for crude or partially purified preparations of RNP (Sharp et al., 1972; Douvas et al., 1979a; Takano et al., 1980). However, purified RNP–RNA alone did not exhibit antigenic activity in hemagglutination inhibition assays in which the amount of RNA far exceeded that found in the purified protein–nucleic acid complex which demonstrated high immunological activity.

RNA isolated from purified RNP was analyzed for its nucleoside composition. High-performance liquid chromatography of enzymatically digested RNA and dual-wavelength UV microcell detection capable of discerning one modified nucleoside per thousand showed that only A, G, C, and U were present (Figure 7). These results are in contrast to work by other authors (Lerner & Steitz, 1979) who reported the

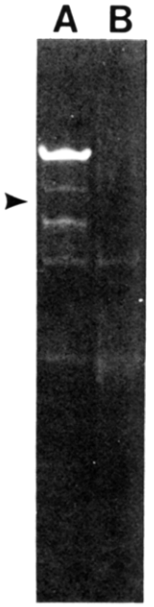

FIGURE 8: Urea–polyacrylamide gel electrophoresis of RNA from RNP antigen (A) and RNA from Sm antigen without RNP activity (B). Slab gels (1.5 × 165 × 165 mm) consisting of 10% acrylamide and 7 M urea were run at 15-mA constant current. RNA was detected by staining with ethidium bromide (0.1 mg in 200 mL of H_2O). The arrow indicates phenylalanine transfer RNA as a molecular weight standard of 76 nucleosides. In lane A, one prominent polynucleotide with approximately 90 nucleosides and four faint polynucleotide bands were seen. In lane B, only three faint polynucleotides were observed, and two polynucleotide bands seen in lane A (first and third bands from the top of the gel) were totally absent.

presence of modified nucleosides in RNA obtained from immunoprecipitated RNP.

The polynucleotide compositions of RNA from purified RNP and Sm antigens were analyzed by using urea–polyacrylamide gel electrophoresis. The arrow in Figure 8 indicates the migration of *Escherichia coli* phenylalanine transfer RNA as a molecular standard of 76 nucleosides. Lane A, which contained RNA from purified RNP, shows one very prominent and four other polynucleotide bands of much lesser amounts. Lane B contains RNA from purified Sm antigen that had no RNP activity but was purified without RNase treatment. Only three faint bands are seen, and two polynucleotide bands that were present in RNP, band 1 and band 3 from the top of the gel in lane A, were totally absent.

Discussion

Relationship between RNP and Sm. A relationship between RNP and Sm has been inferred from previous immunodiffusion observations using crude nuclear extracts or partially fractionated materials and patient sera (Mattioli & Reichlin, 1973) and attempts at chromatographic separation (Peltier et al., 1977). This molecular association was supported recently by results from our laboratory (Takano et al., 1980) and by other authors (Lerner & Steitz, 1979; Lerner et al., 1980). Other publications have emphasized the immunological distinctness of these two antigens (Northway & Tan, 1972; Douvas et al., 1979a). The purpose of this study was to purify and characterize the two antigens and to determine what, if any, molecular relationship existed between them.

Figure 9 shows immunodiffusion reactions of purified RNP and antibodies to Sm and RNP. At the top of the figure, purified RNP antigen gave an immunodiffusion line with Sm antibody which was completely identical with the line formed with RNP antibody. On the other hand, a line between crude CTE and Sm antibody was only partially identical with a line

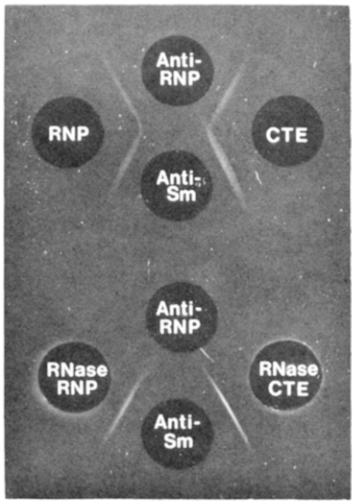

FIGURE 9: Immunodiffusion reaction of purified RNP and crude CTE antigens against antibodies to RNP and Sm. At the top of the figure, purified RNP antigen gave an immunodiffusion line with Sm antibody which was completely identical with the line formed with the RNP antibody. A line between crude CTE antigen and Sm antibody was partially identical with a line between CTE and RNP antibody. At the bottom, when the antigens were treated with RNase, only the lines with RNP antibody were eliminated.

between unfractionated CTE and antibody to RNP. As shown at the bottom of the figure, when the antigens were treated with RNase, only lines with RNP antibody were eliminated. In addition, Figure 2 shows that Sm purified by Sm antibody affinity chromatography of Sepharose 6B fractionated CTE exhibited a precipitin line in immunodiffusion with Sm antibody that was partially identical with one with RNP antibody. From these immunodiffusion results, we hypothesized that RNP and Sm are distinct antigenic determinant sites on the same nuclear macromolecular complex.

The lines of partial identity seen in immunodiffusion experiments with CTE can be explained by the heterogeneous nature of this crude extract. The RNP antigen is known to be thermally labile and is easily destroyed by digestion with RNase and trypsin. Thus, CTE would be heterogeneous with respect to macromolecular complexes containing RNP and Sm determinant sites; some complexes would contain both antigenic sites whereas the lability of the RNP antigenic site in comparison to that of the Sm would produce many complexes which have only the Sm determinant sites. The two types of antigenic molecules, RNP plus Sm antigenicity and Sm antigenicity alone, would be separable by RNP antibody affinity chromatography. This is indeed the case as seen in Figure 1 and, as explained earlier, allowed us to purify Sm without the use of RNase. Sm purified by Sm antibody affinity chromatography of Sepharose 6B fractionated CTE is similarly heterogeneous with respect to molecules containing Sm sites alone, and those with Sm and RNP determinants. In contrast, purified RNP produced completely identical precipitin lines against RNP and Sm antibodies because it is a homogeneous population of macromolecular complexes containing both RNP and Sm antigenic determinant sites.

This interpretation is supported by the inability to separate Sm from immunologically active RNP by affinity chromatography. When partially purified RNP antigen was applied to either an RNP antibody or an Sm antibody affinity column, the material retained by the column and eluted with 3 M NaSCN contained both RNP and Sm antigenic activities. On the other hand, when partially purified RNP pretreated with RNase was applied to an RNP antibody affinity column, the 3 M NaSCN eluate contained neither RNP nor Sm antigenic

activity. Sm antigenic activity alone, always present in material not retained by the RNP antibody affinity column, was retained by an Sm antibody affinity column. Sm has been shown to have an affinity for single-stranded DNA and to be retained by such columns (Reyes & Tan, 1979). We have found that both purified Sm and purified RNP are retained by single-stranded DNA resins.

RNP and Sm Antigenic Determinant Sites. The RNP–Sm molecular complex whether isolated by RNP antibody or Sm antibody affinity chromatography was found to contain the same eight polypeptides (Figure 4). However, when Sm devoid of RNP was analyzed, only four polypeptide bands migrating in the molecular weight range of 12000–13000 were observed. Therefore, the 30000 and 65000 molecular weight peptides and one peptide with a molecular weight approximating 13000 appear to be related to the RNP antigenic determinant site. The remaining four 12000–13000 molecular weight peptides appear to be related to the Sm antigenic determinant site. This hypothesis of the location of the RNP and Sm antigenic determinant sites in the whole RNP–Sm molecular complex was further supported by the results from a solid-phase radioimmunoassay. Using this assay, we found that Sm antibody specifically bound to samples eluted from the 13000 molecular weight polypeptide region, and RNP antibody specifically bound to samples eluted from the 30000 and 65000 molecular weight polypeptide regions. These results strongly suggest that the RNP antigenic determinant site is associated with the 30000 and 65000 molecular weight polypeptides, and the Sm antigenic determinant site is associated with the 13000 molecular weight polypeptide. Our present results are different from those of Douvas et al. (1979a) and our previous results (Takano et al., 1980). Although the reason for this difference is not clear, these radioimmunoassay results were very reproducible and are consistent with other results from the protein analysis of the purified RNP and Sm antigens.

RNA from the RNP–Sm complex is required only for RNP and not for Sm antigenicity. A comparatively large amount of a single polynucleotide of approximately 90 nucleosides was present in RNP–RNA. This RNA and a smaller polynucleotide were absent from RNA obtained from purified Sm, indicating a close relationship of these polynucleotides to the RNP antigenic determinant site. Preliminary sequence data suggest that the smaller polynucleotide is a fragment of the more prominent larger polynucleotide.

The role played by the RNA in specifying RNP antigenicity in conjunction with the protein is not known. RNA could be recognized by the RNP antibody or could be responsible for affecting the polypeptide structure that is recognized, or both. This aspect of the problem is under study.

Since modified nucleotides have been shown to be antigenic determinants (Rainen & Stollar, 1978), the nucleoside composition of RNP–RNA was investigated. No modified nucleosides or messenger RNA-like CAP structures were found by high-performance liquid chromatography analysis. A structure such as CAP, containing *N*-7-methylguanosine, or a small oligonucleotide terminus may have been lost during purification of RNP, and the resulting RNA component would then be smaller than that reported by Steitz and co-workers (Lerner & Steitz, 1979). The RNA we analyzed was extracted from antigenically active purified RNP, and therefore, it retained its important and necessary contribution to the antigenic determinant for RNP antibody whether or not any modified nucleoside was originally present. Preliminary sequence data indicate that the RNA is similar to one of the small nuclear RNAs, Ula, reported by others (Lerner et al., 1980, 1981) to be present in immune precipitates of nuclear extracts.

Thus, RNP and Sm are distinct antigenic determinant sites on the same nuclear molecular complex. The whole complex containing both RNP and Sm activities consists of RNA and eight polypeptides. The Sm molecule without RNP activity is composed of only four polypeptides. One polynucleotide of approximately 90 nucleosides and three polypeptides of approximately 65000 and 30000 molecular weight appear to be associated with the RNP antigenic determinant. The Sm antigenic determinant appears to be associated with the 12000–13000 molecular weight polypeptides.

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Isolation and Sequence Analysis of the Intramembranous Hydrophobic Segment of the H-2K^b Murine Histocompatibility Antigen†

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ABSTRACT: The primary structure of the intramembranous segment (TC-1) of the mouse transplantation antigen, H-2K^b, has been determined. The segment contains a stretch of 31 uncharged amino acid residues and is localized between the NH_2-terminal and the COOH-terminal hydrophilic regions of the molecule. The amino acid sequence of TC-1 is Trp-Asp-Glu-Pro-Pro-Ser-Thr-Val-Ser-Asn-Met-Ala-Thr-Val-Ala-Val-Leu-Val-Val-Leu-Gly-Ala-Ala-Ile-Val-Thr-Gly-Ala-Val-Val-Ala-Phe-Val-Met-Lys-Met-Arg-(Arg)-(Arg).

The classical murine H-2 major histocompatibility antigens (H-2 antigens) are integral membrane glycoproteins which, although found on all cells, are more densely concentrated on lymphocytes. The molecules are composed of two noncovalently associated polypeptide chains, a highly polymorphic 45 000 molecular weight polypeptide coded for by genes within the major histocompatibility complex (MHC)[1] and a non-MHC-encoded 11 500 molecular weight polypeptide, β_2-microglobulin (β_2m) (Snell et al., 1976; Vitetta & Capra, 1979; Nathenson et al., 1981). Structural studies on the 45 000 molecular weight H-2K^b polypeptide have been pursued for the last 4 years by using radiochemical methodology (Nathenson et al., 1981; Coligan et al., 1981). The H-2K^b polypeptide was initially cleaved by CNBr, and three large fragments (>50 residues) and six small fragments (<35 residues) were isolated (Ewenstein et al., 1978). The amino acid sequence and alignment have been determined for the three large fragments and two of the smaller fragments, which comprise the NH_2-terminal 284 residues (Coligan et al., 1978; Uehara et al., 1980a,b; Martinko et al., 1980). As described in the following paper in this issue (Uehara et al., 1981), the four remaining CNBr fragments are found to constitute the COOH-terminal portion of the molecule (residues 308-346). However, the nine CNBr fragments which were recovered did not contain an extensive hydrophobic stretch which might interact with the lipid bilayer of the membrane. The presence of such a hydrophobic peptide would be expected, since it has been found in a number of integral membrane proteins such as cytochrome b_5 (Ozols & Gerard, 1977; Fleming et al., 1978; Takagaki et al., 1980), glycophorin A (Furthmayr et al., 1978), and membrane-bound IgM (Rogers et al., 1980). In addition, the total number of amino acid residues included in these CNBr fragments was smaller than the number predicted from the apparent molecular weight of the intact molecule. Thus, attempts were made to isolate a peptide containing the putative membrane-associated portion of the H-2K^b molecule employing procedures other than CNBr cleavage. In this paper, we report the isolation and amino acid sequence analysis of a tryptic peptide which contains the putative, intramembranous hydrophobic region of the H-2K^b molecule.

Materials and Methods

Radiolabeling, Cell Extraction, and Alloantisera. Incorporation of radiolabeled amino acids into EL-4.BrdU cells and cell extraction with the nonionic detergent Nonidet P-40 were performed as described previously (Uehara et al., 1980a; Nairn et al., 1980). Alloantisera against H-2K^b (H-2.33) were prepared as described (Ewenstein et al., 1978; Uehara et al., 1980a).

Isolation of H-2K$^b_{trp}$ (a Fragment Obtained by Limited Trypsin Digestion of the Intact H-2K^b). The glycoprotein fraction purified by lentil lectin affinity column chromatography of NP-40 cell lysates (Brown et al., 1974) was digested by trypsin (trypsin-TPCK, Worthington Biochemical Corp., Freehold, NJ) at 37 °C for 10 min in the presence of bovine serum albumin (usually 0.2 mg/mL) at trypsin concentration of 60-80 μg/mL. The digestion was halted by the addition of an equal amount of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), and after 30 min at room temperature, H-2K$^b_{trp}$ was isolated by immunoprecipitation (Brown et al., 1974). After reduction and alkylation in 9% $NaDodSO_4$ as described before (Brown et al., 1974), the reaction solution was diluted about 10 times with distilled water and precipitated by the addition of Cl_3CCO_2H at a final concentration of 15%. The Cl_3CCO_2H precipitate was washed successively with 5% Cl_3CCO_2H (2 times), ethanol/ether (1:1 v/v), and ether, after which it was dried at room temperature.

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[1] Abbreviations used: MHC, major histocompatibility complex; H-2K$^b_{trp}$, H-2K^b glycoprotein derived by trypsin cleavage of the NP-40 solubilized H-2K^b molecule; β_2m, β-2-microglobulin; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; IgM, immunoglobulin M; V8, *Staphylococcus aureus* V8 protease; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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