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PURIFICATION AND IDENTIFICATION OF ANTIGENIC POLYPEPTIDES OF Sm AND RNP ANTIGENS OF GOAT LIVER

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SUMMARY: A ribonucleoprotein complex containing Sm and RNP antigenic activity was isolated from goat liver. The sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the complex to contain four major polypeptides of 80,000, 70,000, 30,000 and 14,000 molecular weights. When the gels were probed for antigenic polypeptides by an enzyme-linked immunosorbent assay and fluorescent antibody method, anti-Sm sera were found to react with polypeptides of 14,000 and 30,000 molecular weights whereas anti-RNP sera recognized 70,000 and 80,000 molecular weight polypeptides with minor reactivity in the polypeptide of 14,000 molecular weight. Sera containing antibodies to both Sm and RNP reacted with all the four polypeptides.

Two nuclear antigens Sm and RNP have generated much interest because of the presence of circulating antibodies against them in systemic lupus erythematosus and mixed connective tissue disease and in view of the possible role of these antigens in the processing of nuclear RNA (1). A number of workers have attempted to purify and characterize the two antigens (2-9). Considerable controversies exist regarding the type and number of polypeptides recognized by the anti-Sm and anti-RNP sera and the role of RNA in the antigenicity of the two antigens. Here we report the purification of Sm and RNP antigens of goat liver and identify the type and number of polypeptides recognized by their respective antisera.

MATERIALS AND METHODS

Isolation and Purification of Sm/RNP Antigens. Sm and RNP antigens were isolated from goat liver nuclei. Nuclei were isolated (2) and extracted with STM buffer, pH 8.0 (10 mM tris-HCl/100 mM NaCl/1 mM $MgCl_2$ containing 1 mM phenylmethylsulfonyl fluoride and 10 µg/ml polyvinyl sulfate). The insoluble material was separated by centrifugation and the supernate designated as nuclear extract was used as the source of Sm/RNP antigens. The nuclear extract was

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filtered through a Sepharose CL-2B (Pharmacia) column (70 cm x 2 cm) equilibriated with STM buffer. Elution was carried out with the same buffer and fractions containing RNA and protein and Sm/RNP activity were pooled and dialysed against 10 mM Pi/50 mM NaCl, pH 7.3. The dialysed antigen was loaded onto a column of DEAE cellulose (6 cm x 1.5 cm) equilibriated with the dialysis buffer. The Sm/RNP antigens were eluted with 10 mM Pi/300 mM NaCl, pH 7.3. The eluate was dialysed against phosphate buffered saline (PBS) and concentrated. Ion exchange purified material was applied to an affinity column (10 cm x 2 cm) constructed by linking IgG fraction of a high titer Sm/RNP positive SLE serum to CNBr-activated Sepharose-4B (Pharmacia). The column was washed with a copious volume of 10 mM Pi/500 mM NaCl, pH 7.3 and the bound antigens were eluted by 6 M urea in PBS. The eluate was immediately dialysed against PBS to remove urea and concentrated. The concentrated antigen was passed through an affinity column (6 cm x 2 cm) prepared by linking normal human IgG to CNBr-activated Sepharose-4B. The column was washed with PBS till no protein was eluted. The eluate was used as purified Sm/RNP antigens.

Immunological Techniques. Immunodiffusion and counterimmunoelectrophoresis were used for monitoring the Sm/RNP activity during isolation. IgG was isolated as described (10). Sera were collected as reported earlier (11). Sm and RNP reference sera, provided by Dr. Eng.M.Tan, were a gift from Dr. A.N. Malaviya, All India Institute of Medical Sciences, New Delhi.

Sodium Dodecyl Sulfate Gel Electrophoresis. Samples for electrophoresis were solubilized in 10 mM tris-HCl pH 8.8 containing 1% SDS and 5% 2-mercaptoethanol and heated for 3 minutes at 90°C. 200 µg protein was applied to 10% polyacrylamide gels (0.6 cm x 18 cms) containing 0.1% SDS. Electrophoresis was performed as described by Laemmli (12). The proteins used as molecular weight markers were bovine serum albumin (68,000), ovalbumin (43,000), ∝-chymotrypsinogen (25,700), ribonuclease A (13,700) and cytochrome C (11,700).

Enzyme-Linked Immunosorbent Assay (ELISA). Immediately after the electrophoresis, the gels were sliced into 5 mm pieces. Each piece was crushed in 1 ml McIlvaine's buffer (20 mM Na_2HPO_4/150 mM NaCl, pH 5.0 adjusted with 100 mM citric acid) and the contents filtered through a millipore filter. An ELISA recently described by us (13), utilizing nylon as solid phase, was used for the determination of antigenicity in the filtrates. 0.5 ml of the gel filtrate was used for antigen coating of five activated nylon beads and ELISA was performed using a 100 fold dilution of anti-Sm, anti-RNP, anti-Sm/RNP and normal human sera.

Fluorescent Antibody Labelling of SDS Gels. After the electrophoresis, the gels were fixed in 10% acetic acid/25% isopropanol for 12 hours, washed with PBS to remove the acid and treated for 12 hours with 25 fold diluted anti-Sm, anti-RNP, anti-Sm/RNP and normal human sera. The excess antibodies were washed with PBS and the gels were incubated for 12 hours with 50 fold diluted fluorescein isothiocyanate-labelled rabbit anti-human IgG. The gels were extensively washed with water for 2 days to remove the unreacted conjugate and cut into 5 mm pieces. Each piece was crushed in 1 ml of 50 mM carbonate-bicarbonate buffer, pH 9.5 and filtered through a millipore filter. The fluorescence intensity of the filtrate was recorded using Aminco Bowman spectrophotofluorometer (λ exc = 494 nm, λ em = 526 nm).

RESULTS

Goat liver was chosen as the source of Sm and RNP antigens in view of easy availability in bulk besides being a rich source of the

two antigens. Purification was affected in three steps. Bulk of the soluble chromatin and high molecular weight proteins in the nuclear extract were removed by chromatography on Sepharose CL-2B. DEAE cellulose chromatography removed most of the nonantigenic proteins and free nucleic acids. Final purification and the removal of normal human IgG binding material were performed by affinity chromatography. The material thus obtained was free of any detectable DNA and contained protein and RNA in the ratio of 5:1 by weight indicating the ribonucleoprotein nature of Sm/RNP antigens. It reacted with anti-Sm and anti-RNP reference sera and sera containing antibodies to both Sm and RNP antigens. No reactivity was observed with normal human sera and sera containing antibodies to DNA, RNA and SS-B antigens.

Figure 1 shows the electrophoretic pattern of purified Sm/RNP polypeptides. Four major polypeptide species of molecular weights 14,000, 30,000, 70,000 and 80,000 were consistently obtained with the purified antigen.

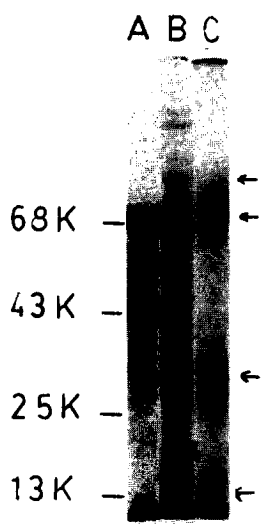

Figure 1. SDS-PAGE of polypeptides of Sm/RNP antigens. A: molecular weight markers; B: Sepharose CL-2B separated antigen and C: affinity purified antigen (arrows indicate the position of major Sm/RNP polypeptides).

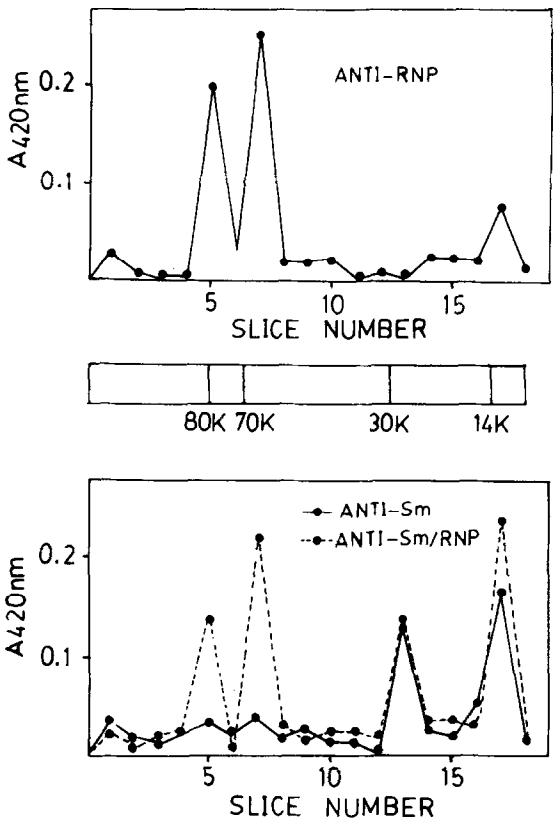

Figure 2. Enzyme-linked immunosorbent assay of polypeptides of SDS-PAGE fractionated purified Sm/RNP antigens. The gels were sliced and processed for ELISA. Upper panel - reaction with anti-RNP serum; lower panel - anti-Sm and anti-Sm/RNP sera. The small peak (extreme left) seen in both the panels was also obtained with normal human sera.

Since polypeptides alone have been shown to react with anti-Sm and anti-RNP sera in SDS gels (4,7) we also attempted to identify electrophoretically separated antigenic determinant sites in the Sm/RNP antigens. ELISA was used for the purpose and individual anti-Sm, anti-RNP and anti-Sm/RNP sera were used to probe the antigenic polypeptides. The results of these experiments are shown in figure 2. When anti-Sm sera were used to probe the gels, two polypeptides of molecular weights 14,000 and 30,000 strongly reacted with such sera. Anti-RNP antibodies recognized 70,000 and 80,000 molecular weight polypeptides almost exclusively with minor reactivity in 14,000 molecular weight region. Sera containing antibodies to both Sm and RNP antigens reacted with all the four polypeptides.

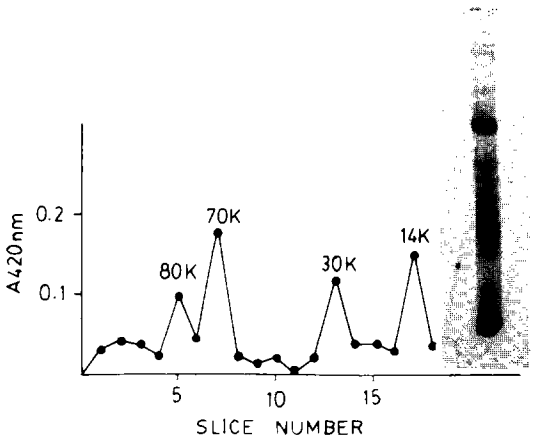

Figure 3. Enzyme-linked immunosorbent assay of polypeptides of partially purified Sm/RNP antigens. Sepharose CL-2B filtered Sm/RNP antigen was subjected to SDS-PAGE (Inset). The gels were sliced and processed for ELISA using an SLE serum containing antibodies to both Sm and RNP antigens.

Figure 3 shows that when partially purified Sm/RNP antigens obtained by Sepharose CL-2B chromatography of the nuclear extract, was electrophoresced and the gels probed for antigenic polypeptides, the pattern of polypeptides recognized by the antisera was similar to that obtained with affinity purified antigen. These results indicate that none of the antigenic polypeptides of the crude antigen was lost during purification.

When the results obtained by ELISA were compared with fluorescent antibody procedure, identical polypeptide profiles were obtained (data not shown). However, the ELISA was far more sensitive and less time consuming than fluorescent antibody assay.

DISCUSSION

Although considerable work has been done to elucidate the fine antigenic structure of Sm and RNP antigens, no clearcut picture has so far emerged. Only recently attempts have been made to identify the antigenic determinant polypeptides of these antigens (4,7). The results described here reveal that Sm and RNP antigens contain a limited number of polypeptides and the larger part of antigenicity

is present in these polypeptides. Employing a highly sensitive and specific ELISA (13) we report that the Sm and RNP antigenic determinant sites are located in different polypeptides and the two antigens can conveniently be differentiated on the basis of type of polypeptides recognized by anti-Sm and anti-RNP sera. The results show that major RNP activity is present in two high molecular weight polypeptides of 70,000 and 80,000 molecular weights with minor reactivity in 14,000 molecular weight polypeptide. Sm antigenicity was detected in 30,000 and 14,000 molecular weight polypeptides and sera containing antibodies to both Sm and RNP react with all the four polypeptides. Since the sera reacted with polypeptides alone without the presence of RNA it seems that RNA is not necessary for the antigenicity of Sm and RNP antigens as has been suggested by other workers (8). As the major RNP activity is associated with two high molecular weight polypeptides, the presence of low reactivity in 14,000 molecular weight polypeptide (a major Sm determinant) might represent the associated anti-Sm reactivity of apparently monospecific RNP sera. The presence of Sm reactivity in RNP antigen has been reported previously (3). The 14,000 molecular weight polypeptide could be a likely candidate for such associated Sm activity.

It seems that the polypeptides of small nuclear ribonucleoproteins are the main targets of autoimmunity. The identification and characterization of such polypeptides therefore forms an essential part of research in this area. ELISA provides an efficient means of screening patients sera for the type of polypeptide reactivity. The method is simple and highly sensitive and can provide alternative to the nitrocellulose transfer assays which involve costly radioactive chemicals and special technical skill.

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