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Cross reactivity and enzyme sensitivity of immunoaffinity purified Sm/RNP antigens

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Abstract. Immunoaffinity purified Sm/RNP antigens from buffalo and goat liver were studied to determine the role of RNA and proteins towards the antigenicity of Sm and RNP antigens. A more direct approach using enzyme-linked immunosorbent assay on nylon beads has been utilized to look into the problem. The effect of enzyme treatment and the role of RNA and protein fractions in influencing antigenicity have been described. RNA seems to be involved in the maintenance of RNP specific polypeptides in suitable conformation so as to keep them in solution. Removal of RNA leads to insolubilization of RNP specific polypeptides. Antibodies to Sm and RNP antigens have been shown to cross react with poly A containing heterogeneous nuclear ribonucleoprotein with no cross reactivity with thymus RNA or DNA.

Keywords. Sm/RNP; hnRNP; autoantibodies; enzyme-linked immunosorbent assay; enzyme sensitivity.

Introduction

Autoantibodies produced by patients with autoimmune diseases have been found to be useful tool for the characterization of small nuclear ribonucleoprotein (snRNP) complexes (Lerner and Steitz, 1979). These highly conserved RNA-protein complexes, which are targets of autoimmunity have been classified as Sm and RNP antigens on the basis of differential sensitivity to RNase (Mattioli and Reichlin, 1971; Northway and Tan, 1972; Ishaq and Ali, 1983a). Recently, the two antigens have been differentiated and characterized on the basis of the type and number of their antigenic polypeptides (Hinterberger *et al.*, 1983; Ishaq and Ali, 1983b; Steitz *et al.*, 1983). Role of RNA and the exact number and size of polypeptides responsible for the antigenic activity however, remains controversial. Further, the specificity and cross reactivity of the antibodies reactive with Sm and RNP antigens remains to be examined.

Abbreviations used: RNP, Ribonucleoprotein; snRNP, small nuclear RNP; hnRNP, heterogeneous nuclear RNP; SLE, systemic lupus erythematosus; DEAE, diethylaminoethyl; PMSF, phenylmethylsulphonyl fluoride; IgG, immunoglobulin-G; PBS, phosphate buffer saline; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; UV, ultraviolet.

The present study describes the isolation and characterization of Sm/RNP antigens from buffalo and goat liver. The immunoaffinity purified antigenic particles were subjected to characterization with an attempt to check the cross reactivity of antibodies with other antigens including hnRNP particles which are RNA-protein complexes residing with Sm/RNP particles in the same nuclear envelope.

Materials and methods

Isolation and purification of Sm/RNP antigen

Sm/RNP antigenic particles were isolated from fresh buffalo and goat liver as described earlier (Ishaq and Ali, 1983b). Nuclei were isolated (Douvas *et al.,* 1979) and extracted with STM buffer pH 8·0 (10mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride (PMSF), 100 μg/ml polyvinyl sulphate). The insoluble material was removed by centrifugation and the supernatant passed successively through Sepharose CL-2B and diethylaminoethyl (DEAE) cellulose columns (Ishaq and Ali, 1984). During purification, only those fractions containing RNA and protein and Sm/RNP activity were pooled and processed further. Ion exchange purified material was applied to an affinity column of immunoglobulin-G (IgG) of a Sm/RNP positive systemic lupus erythematosus (SLE) serum linked to CNBr-activated Sepharose 4B. The eluate after extensive dialysis was passed through another affinity column prepared by linking normal human IgG to Sepharose 4B. The column was washed with phosphate buffer saline (PBS) till no protein was eluted. Eluate from normal IgG-Sepharose column was used as purified Sm/RNP antigen.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) for Sm/RNP antigen was carried out with affinity purified antigen using nylon beads as described earlier (Ishaq and Ali, 1983a). ELISA for antibodies against RNA and DNA was performed as already reported (Kumar and Ali, 1984a; Ali and Ali, 1986). Sera tested for anti-RNA antibodies were pretreated with aurintricarboxylic acid polymer to overcome serum RNase activity (Kumar and Ali, 1984b).

Treatment of antigen with immobilized RNase and trypsin

RNase A and trypsin (Sigma Chemical Co., USA) were linked to CNBr-activated Sepharose 4B as per instructions of the manufacturer. Immunoaffinity purified RNP was digested for different time intervals individually with immobilized RNase and trypsin. At the end of incubation, the immobilized enzymes were separated by centrifugation in cold. The resulting soluble antigen was coated on nylon beads and processed for ELISA using Sm and RNP reference sera.

Dissociation of Sm/RNP particles

Affinity purified antigen was dissociated into protein and RNA fractions by a modified procedure of White *et al.* (1982). Antigen was dialysed against PBS containing 8 M urea,

2 mM 2-mercaptoethanol and 0.1 mM PMSF. The dialysed material was passed through the DEAE cellulose column equilibrated with the same buffer. Proteins eluted in the wash volume were collected and dialysed against PBS. RNA bound to the column was eluted with 10 mM P_i, pH 7·2 containing 3 M NaCl. The isolated RNA was extensively dialysed against PBS.

Spectral studies

Ultraviolet (UV) spectra of Sm/RNP particles, dissociated RNA and protein fractions were recorded in PBS, pH 7·2 using Spectronic-21 spectrophotometer.

Carbohydrate estimation

Carbohydrate content of affinity purified antigen was estimated by the method of Winzler (1955).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Polypeptide analysis of Sm/RNP antigens was performed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). Samples for electrophoresis were solubilized in 10 mM Tris-HCl, pH 8·8 containing 1 % SDS and 5 % 2-mercaptoethanol and heated for 5 min at 90°C. Fifty μg of protein was applied to 10 % polyacrylamide gel slab containing 0·1% SDS and electrophoresed at 10 mA and 50 volts for 20 h as described by Laemmli (1970). Phosphorylase B (97,400 d), BSA (68,000d), ovalbumin (43,000d), carbonic anhydrase (25,000d) and cytochrome C (11,700d) were used as molecular weight markers. At the end of electrophoresis, the slab was fixed for 1 h in 25 % isopropanol and 10% acetic acid. Staining was carried out for 20 min in 0·2% Coomassie brilliant blue R-250 in 50 % methanol and 10 % acetic acid. The slab was destained by 25% methanol plus 10% acetic acid.

Isolation of poly A-hnRNP particles by oligo-dT cellulose column chromatography

Oligo-dT cellulose (Sigma Chemical Co., USA) was treated with 0·1 M NaOH for 10 min, extensively washed with distilled water and equilibrated with binding buffer (10 mM P_i, pH 7·0,0·25 M NaCl). Nuclear extract was dialysed against binding buffer and loaded onto the oligo-dT cellulose column. The column was washed with copious volumes of buffer to remove unbound material. The bound material was eluted with 10 mM P_i, pH 7·0 having 50 % deionized formamide. Poly A-hnRNP thus eluted was dialysed against PBS and hybridized with an excess of poly U in PBS pH 7·2 and rechromatographed on oligo-dT cellulose. Ninety five per cent of the loaded material remained unbound and was eluted in wash.

Thymus RNA and DNA

RNA from buffalo thymus was isolated as described elsewhere (Kumar *et al.*, 1983). DNA obtained from Sigma Chemical Co., St. Louis, Missouri, USA, was purified by hydroxyapatite column chromatography (Ali and Ali, 1984). Double-stranded DNA was heat denatured to get single stranded DNA. Normal human sera were obtained

from healthy subjects. Sera of patients with SLE were collected as described earlier (Ishaq *et al.,* 1982). Serum IgG was isolated by ion-exchange chromatography using DEAE cellulose (Ali *et al.,* 1983).

Results

Isolation of Sm/RNP antigen was carried out under RNase free conditions. The RNA:protein ratio of the affinity purified material was 5·2:1 by weight with 27% carbohydrate content. The 280/260 ratio of the glycoprotein RNP complex was 1·10. The UV spectra of the Sm/RNP complex is depicted in figure 1. The affinity purified material was antigenically active when tested by ELISA and showed strong antibody activity with Sm, RNP and Sm/RNP reference sera.

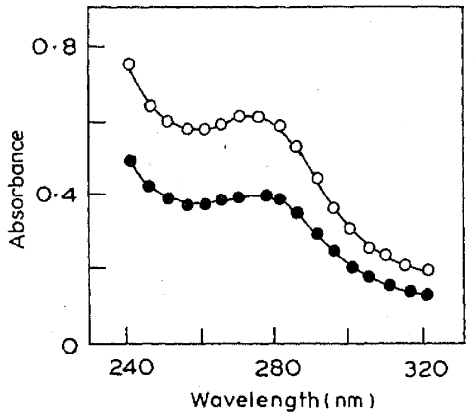

Figure 1. UV spectra of purified Sm/RNP antigen (O) and protein (●) fraction.

The polypeptide analysis of the antigen from buffalo and goat liver at different stages of purification was carried out by SDS-PAGE (data not given). Exactly similar profiles were observed with antigens isolated from two different sources. Five major polypeptides of molecular weight 80K, 70K, 29K, 13K and 12K were obtained from affinity purified antigen.

After dissociation and separation of RNA and protein parts of Sm/RNP antigens and upon removal of urea, a substantial precipitation occurs in the protein fraction. The UV spectral characteristics of the soluble protein fraction is given in figure 1. The soluble fraction was tested for Sm and RNP antigenicity using Sm and RNP reference

sera. Ninety per cent of activity was consistently obtained as Sm-activity in the soluble fraction with Sm reference sera as compared to 8–10 % of RNP activity (figure 2). No significant antibody activity was present in the RNA fraction with RNP, Sm and Sm/RNP reference sera (data not shown).

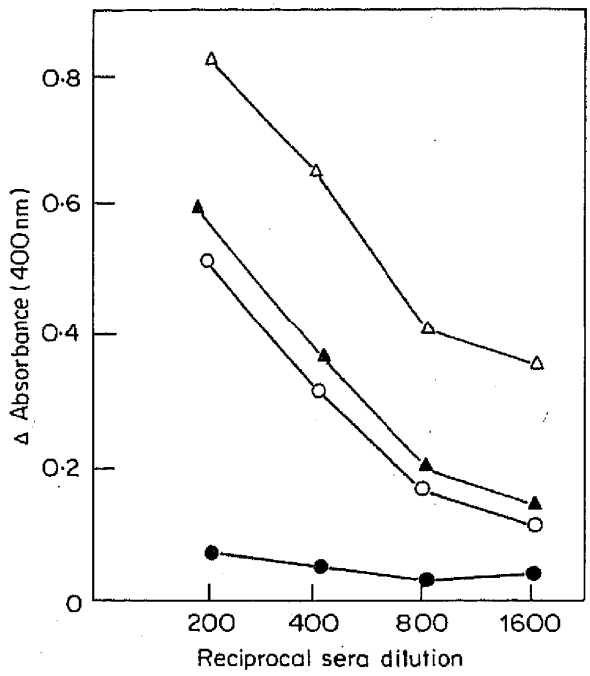

Figure 2. Antigenicity of the soluble protein fraction obtained by dissociation of RNP complex.

RNP complex was dissociated into RNA and protein fractions as described in 'materials and methods'. Soluble protein fraction (100 μg/ml) was coated on nylon beads and tested by ELISA using anti-Sm (O) and anti-RNP (•) reference sera. Undissociated Sm/RNP coated beads tested with Sm (▲) and RNP (Δ) reference sera.

RNP antigenicity was lost on incubation with the immobilized RNase when tested with RNP reference serum. The loss in antibody activity was dependent on the period of incubation with the enzyme. Sm activity, on the other hand, remained almost unaffected by RNase treatment. Trypsin treatment resulted in the loss of both Sm and RNP antigenic activity (figure 3). The specificity and cross reactivity of Sm/RNP antibodies were checked by ELISA using RNA, ssDNA, dsDNA and poly A containing hnRNP. No cross reactivity was observed when Sm/RNP reference sera were tested for reactivity with mammalian RNA and ds- or ssDNA (data not given). With poly A-hnRNP, around 22% reactivity was consistently observed with sera positive for antibodies against Sm/RNP antigens (figure 4).

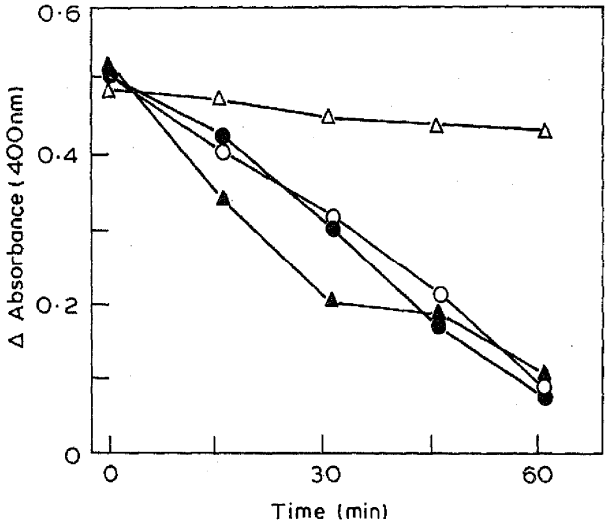

Figure 3. Enzyme sensitivity of Sm/RNP antigens.
Sm/RNP antigens were treated with immobilized trypsin and RNase for different time intervals. Trypsin treated antigen was coated on nylon beads and tested with anti-Sm (O) and anti-RNP (•) reference sera. Similarly, RNase treated antigen was coated on nylon beads and processed for ELISA using anti-Sm (Δ) and anti-RNP (▲) reference sera. Serum dilution was 1:200.

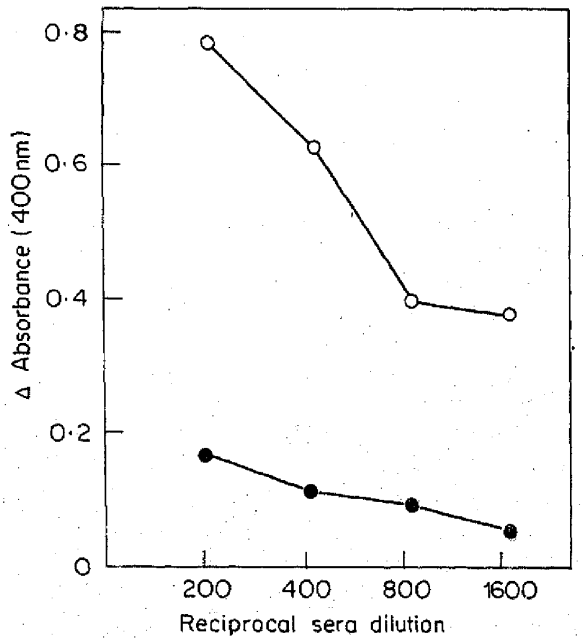

Figure 4. Cross reactivity of anti-Sm/RNP antibodies with poly A containing hnRNP. ELISA was performed using affinity purified Sm/RNP (O) and poly A containing hnRNP (•) coated nylon beads at various dilutions of anti-Sm/RNP positive serum.

Discussion

Sm/RNP ribonucleoprotein antigens were isolated from buffalo and goat liver by immunoaffinity chromatography. The polypeptide profiles from two different sources were identical revealing no species difference and reiterating the belief that these molecular complexes are highly conserved molecules. When the nuclear extract was directly loaded onto the immunoaffinity column, the yield of the purified antigen was considerably low although, no difference was observed in the polypeptide profile (data not shown). Thus the purification could be shortened considerably permitting rapid isolation of the antigen provided yield is of secondary importance. The UV spectra of RNP complex, proteins: RNA ratio and carbohydrate content of the preparation reveals that the antigen is a glycoprotein-RNA complex. There has been considerable variation in the reported molecular weight values of antigenic polypeptides associated with Sm and RNP antigens. The demonstration of 5 major polypeptides of molecular weight 80K, 70K, 29K, 13K and 12K in this study is in good agreement with those found by other investigators in preparations from other sources (White *et al.,* 1981; Lerner and Steitz, 1979; Billings *et al.,* 1982). A similar number of polypeptides was reported from this laboratory in an earlier study (Ishaq and Ali, 1983b).

The antigenicity of purified RNP requires an intact RNA for antibody activity. RNase treatment of antigen in crude or purified form, destroys the immunological determinant for RNP antibody whereas RNase-treated RNP does retain Sm reactivity (figure 3). It is generally believed that Sm determinant is defined by protein alone (Takano *et al*.,1981; White *et al.,* 1982; Lerner *et al.,* 1981). To look into the role of RNA in antigenicity, a non-conventional procedure was adopted. The purified antigen having both Sm and RNP activities was subjected to the action of immobilized RNase for different time intervals. The resulting antigenic preparations were coated on nylon beads and ELISA was carried out using Sm and RNP reference sera. RNP antigenicity was gradually lost and within an hour of incubation with RNase, almost complete loss in antigenicity was observed. No significant change in the Sm activity was noticed. To further explore the requirement of RNA in RNP antigenicity, Sm/RNP complex was separated into RNA and protein fractions. The isolated RNA, free of protein contamination, was tested for its reactivity with Sm, RNP and Sm/RNP reference sera by ELISA. No antibody activity was found to be associated with RNA fraction of Sm/RNP antigen thereby implying that on its own, RNA has got no activity in Sm/RNP antigenicity. In a recent study a small portion of the entire RNA sequence and few peptides have been implicated as the RNP antigenic determinant in calf thymus preparation (Agris *et al.,* 1984).

During the separation of RNA and protein from Sm/RNP complex, it was observed that a significant part of the protein fraction becomes insoluble upon removal of urea. The soluble portion was coated on nylon beads and processed for ELISA. The antigen on the solid support was fully active towards Sm antibody and showed insignificant activity towards anti-RNP antibody. The insolubilization of these polypeptides was successfully reversed when RNA fraction was presented in solution at the time of removal of urea. The RNP antigenicity was also restored. This led us to believe that RNA maintains RNP polypeptides in solution and somehow expose the antigenic determinant in these complexes. Upon removal or degradation of RNA, the antigenic

polypeptides are rendered insoluble and it is inferred that the RNP activity is lost as a result of RNase treatment.

The close association of RNP complexes with poly A-hnRNP in the nucleus and possible association of some of the antigenic polypeptides in these particles is likely to explain their cross reactivity (figure 4). Sera from patients with mixed connective tissue disease were found to contain antibodies that react with at least two species of RNP, snRNP and a high molecular weight hnRNP/RNA bound to the nuclear matrix (Ali and Tan, 1979; Fritzler *et al.,* 1984). In another study, human autoimmune sera were found to interact with hnRNP and these autoantibodies recognized common epitopes present on snRNP and hnRNP (Zouali and Eyquem, 1984).

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