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Preparation and Characterization of Monoclonal Antibodies Specific for Ribonuclease Inhibitor from Rat Liver

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Monoclonal antibodies specific for ribonuclease inhibitor (RNasin) were obtained by the method of production of hybridomas from spleen cells and myeloma cells. The specificity of monoclonal antibodies obtained by cloning was investigated by Western blot analysis. The addition of ribonuclease (RNase) A to RNasin resulted in the inhibition of antibody binding to the antigen. However, the addition of RNase A to the antigen-antibody complex did not cause dissociation of the complex.

Keywords——rat ribonuclease inhibitor (RNasin); ribonuclease A; monoclonal antibody; enzyme linked immunosorbent assay

Numerous mammalian tissues are known to contain ribonuclease inhibitor (RNasin), which is thought to be involved in the catabolism of RNAs, that is, RNasin regulates ribonucleic acid (RNA) biosynthesis and hence protein synthesis. There have been reports that RNasin activity is increased in tissues where protein synthesis is active but ribonuclease (RNase) activity is decreased.[1-3] Scheele and Blackburn found that the addition of RNasin to a cell free system of protein synthesis increased the amount of translation product.[4] This would suggest that the stimulation of protein synthesis is due to the inactivation of potential RNase activity by RNasin and, therefore, stabilization of messenger ribonucleic acids (mRNAs).

Bartholeyns and Baudhuin have determined the activity ratio of RNasin and RNase in various rat tissues. The result showed a proportional relationship between the RNasin and RNase activities.[5] However, the biological role of RNasin *in vivo* remains unknown. Therefore, detailed studies on RNasin are required in order to evaluate its biological roles *in vivo*. In the current studies on RNasin, the quantitative analysis of RNasin in tissue extracts has been performed by measuring the inhibition of RNase activity instead of measurement of the absolute amount of RNasin protein. In this paper, we describe the preparation of monoclonal antibodies specific for rat liver RNasin and we report some properties of anti-RNasin.

Materials and Methods

Materials——Pancreatic ribonuclease A (RNase A) was purchased from Sigma Chemicals Co. Yeast RNA as a substrate for RNase A was prepared by the reported[4] method.

RNasin Activity Assay——A 2% RNA solution was added to a mixture of RNase A (10 ng) and an appropriate amount of RNasin solution (50 μl) in 100 mM Tris–HCl (pH 7.9)–8 mM 2-mercaptoethanol–2 mM ethylenediaminetetraacetic acid (EDTA) under ice cooling. After incubation at 37 °C for 30 min, ice-cold 1 N HCl–75% EtOH (1 ml) was added and the mixture was kept for 30 min in an ice-bath. Then the mixture was centrifuged at 3000 rpm for 20 min at 4 °C and the ultraviolet (UV) absorbance of the supernatant at 260 nm was measured. The calculation of inhibition percentages was based on the optical densities obtained from the reaction mixture without RNasin and

without RNase A, as 0% and 100% respectively. The unit of RNasin activity was defined to be one unit when the RNase activity of 5 ng of RNase A was inhibited by 50%.

Purification of RNasin from Rat Liver——Isolation and purification of RNasin were performed principally by the method of Blackburn *et al.*[6] The lysate obtained from 200 g of rat liver (Wistar strain) was subjected to $(NH_4)_2SO_4$ fractionation (30—60%). The precipitate was dissolved in 200 ml of 20 mM Tris–HCl (pH 7.5)–1 mM EDTA–20 mM 2-mercaptoethanol (buffer A) and dialyzed against 2 l of buffer A followed by centrifugation at 32000 rpm for 1 h. The supernatant was loaded onto a diethylaminoethyl (DEAE)-Sephacel column (3.7 × 25 cm) which was equilibrated with buffer A and the elution was carried out using a linear gradient of NaCl (0—0.5 M, 1 l + 1 l). Fractions containing RNasin were combined and dialyzed against buffer A. Then RNasin was purified by affinity chromatography using RNase coupled Sepharose.[6] The yield was 2.3 mg (175000 units).

Preparation of Monoclonal Antibodies——Immunization of RNasin was accomplished by the method of Goding.[7] An emulsion of 75 μg RNasin in Freund's adjuvant was injected twice into BALB/c mice at intervals of 2 weeks and, 3 d before the fusion, the mice were given intravenously a final boost of 75 μg of RNasin in phosphate–saline buffer. Then the spleens were removed and a single cell suspension was prepared.

The fusion of spleen cells with myeloma cells (X63-Ag 8, 6, 5, 3) was carried out using the polyethyleneglycol fusion procedure reported by Galfre *et al.*[8] The selection of hybridoma cells was accomplished by culture in hypoxanthine–aminopterin thymidine containing Dulbecco's modified essential medium (DMEM). Hybridoma cells producing an antibody specific for RNasin were screened by enzyme linked immunosorbent assay (ELISA) and then cloned using the limiting dilution method. The large-scale production of the antibody was carried out by propagation *in vivo*, by injecting hybridomas i.p. into pristene-primed BALB/C mice. The ascitic fluid was centrifuged and the supernatant was subjected to 40% ammonium sulfate precipitation. Then the precipitate was dissolved in phosphate–saline buffer (PBS), and after dialysis against the same buffer, the antibody solution was obtained.

ELISA Method——The immunoassay performed in this study was essentially the same as the general ELISA method.[9] The antigen was immobilized on the wells of a 96 well microtiter plate and subsequent reaction of antibody conjugated to alkaline phosphatase with the antigen was carried out. Then, the amount of the antibodyantigen complex bound to the ELISA plate was analyzed by color reaction of *p*-nitrophenyl phosphate as a substrate and alkaline phosphatase linked to the antibody.

Western Blot Analysis——Proteins on polyacrylamide gel were electrophoretically transferred to nitrocellulose paper. Then an antigen bound to nitrocellulose paper was reacted with the anti-RNasin after blocking of the nitrocellulose paper with ovalbumin.

The detection of the antibody bound to the antigen was performed by reaction of anti-mouse immunoglobulin G plus M (IgG + IgM) conjugated with peroxidase and subsequent color reaction of peroxidase with H_2O_2 and diaminobenzidine.

Effect of RNase A on the Complex Formation between RNasin and Its Antibody——One unit of RNasin was immobilized on well plates and various amounts (0—50 ng) of RNase A were added to the wells. After a 2 h incubation at room temperature, the wells were rinsed with PBS. Then, an excess amount of the antibody was added to the wells and the mixture was worked up as in the ELISA assay. The effect of RNase A on the immunoreaction of RNasin and its antibody was evaluated from the amount of antibody bound.

Results and Discussion

Purification of RNasin

The purification of RNasin from rat liver was carried out according to the method reported by Blackburn *et al.*[6] The results of the purification are summarized in Table I. From 200 g of rat liver, 175000 units (2.3 mg) of RNasin was obtained. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis revealed a single band with a molecular mass of 50 kilo daltons (kDa) (Fig. 1A).

Preparation of Monoclonal Antibodies Specific for Rat RNasin

Preparation of monoclonal anti-RNasins was performed by production of hybridomas from spleen cells and mouse myeloma cells according to the method established by Kohler and Milstein.[10] After immunization of mice with RNasin, spleen cells were obtained and fused with myeloma cells to produce hybridomas. Then hybridomas producing anti-RNasin were screened by the ELISA method. Higher anti-RNasin activity was shown by 12 out of 120 hybridomas producing specific antibodies. The cloning of hybridomas was performed using the limiting dilution method. Cloned hybridomas termed as A443, B335, D5810, C296

TABLE I. Summary of Purification of RNase Inhibitor

	Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Recovery (%)
1	Crude extract	26000	500000	18.9	100
2	30—60% sat. $(NH_4)_2SO_4$ ppt	11000	490000	44.5	99
3	DEAE-Sephacel	320	370000	1156	76
4	RNase A-Sepharose	2.3	175000	75000	36

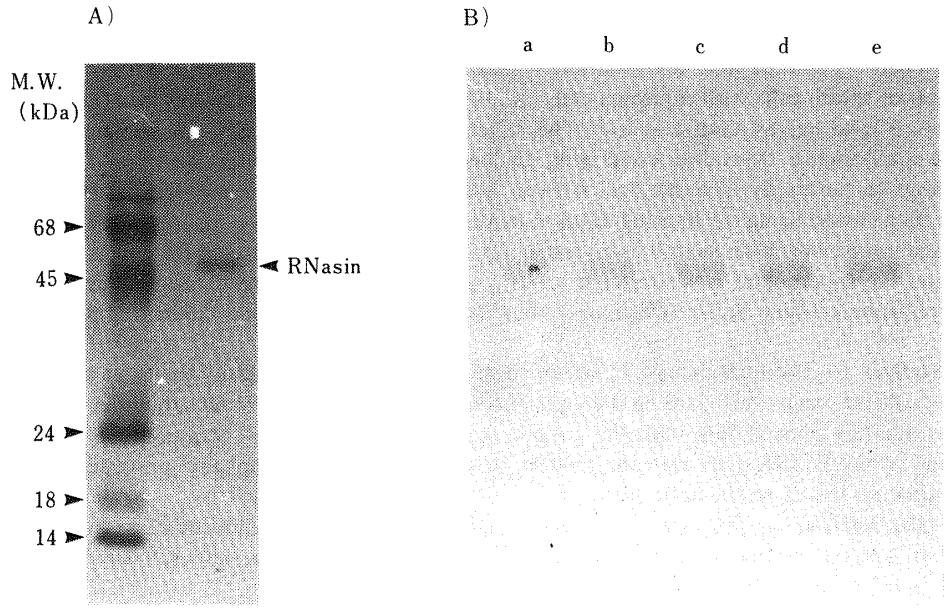

Fig. 1. Western Blot Analysis

A) SDS–15% polyacrylamide gel electrophoresis. Proteins were detected by staining with Coomassie Brillian Blue. M.W.: molecular weight (kDa). B) Western blotting using anti-RNasins (a, A443; b, B335; c, C296; d, D5810; e, E4116) after SDS–15% polyacrylamide gel electrophoresis.

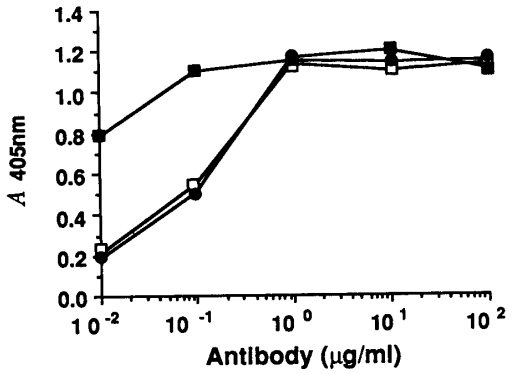

Fig. 2. Titration Curve of Monoclonal Antibodies to Rat Liver RNasin

RNasin (*ca.* 250 mg/well) was used as an antigen. □—□, A443; ●—●, B335; ■—■, D5810.

and E4116 producing specific antibodies were propagated in mice and characterized. In order to examine the antibody titer of cloned antibodies, the titration curve against RNasin adsorbed on ELISA plate was obtained as shown in Fig. 2. The result showed that monoclonal antibodies had higher titer. The specificity of the monoclonal antibodies against RNasin was examined by Western blot analysis using anti-mouse IgG conjugated with peroxidase.

As shown in Fig. 1, the protein peak of RNasin on SDS–polyacrylamide gel electrophoresis coincided with the position stained by reaction with diaminobenzidine and antibody-conjugated peroxidase. This confirmed that the monoclonal antibodies obtained by cloning were specific for RNasin.

Species Specificity of Rat Anti-RNasin

It has been shown that all RNasin from rat liver, mouse liver and human placenta inhibited bovine RNase A.[6,11,12] This suggested that structures of RNasins from different mammalian sources should be very similar to each other.

We examined whether or not the rat and human RNasins were similar to each other in terms of antigenic activity using monoclonal anti-rat RNasin. As shown in Fig. 3, antibody A443 reacted with both rat and human RNasins, but D5810 reacted with only rat RNasin. This shows that A443 and D5810 have distinct epitopes and the structures of rat and human RNasins show some differences.

Effect of RNase A on Immunoreactivity of Anti-RNasin

RNasin is present in tissue as the complex with RNases.[1-3] Therefore, it is necessarry to investigate whether RNases affect the immunoreactivity between anti-RNasin and RNasin to

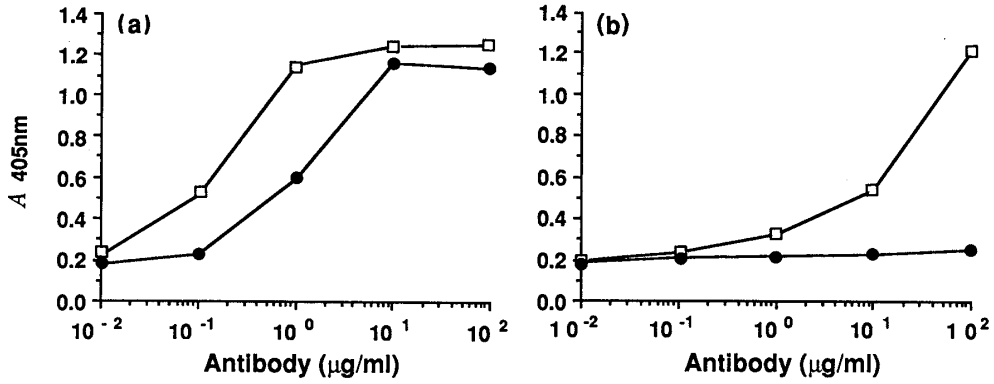

Fig. 3. Reaction of Monoclonal Antibodies with Rat Liver RNasin (a) and Human Placenta RNasin (b)

□—□, A443; ●—●, D5810.

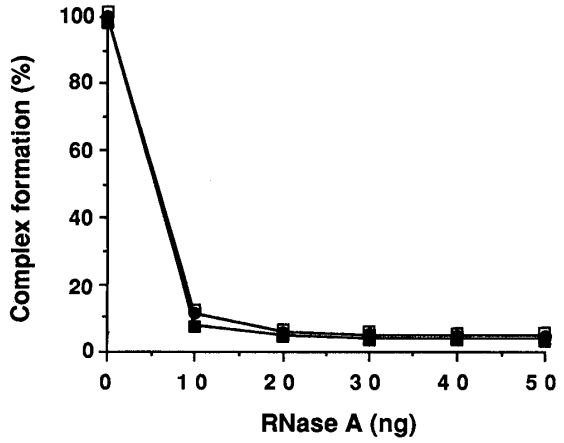

Fig. 4. Effect of RNase A on the Complex Formation between Antigen and Antibody

Antibodies: □—□, A443; ●—●, B335; ■—■, D5810.

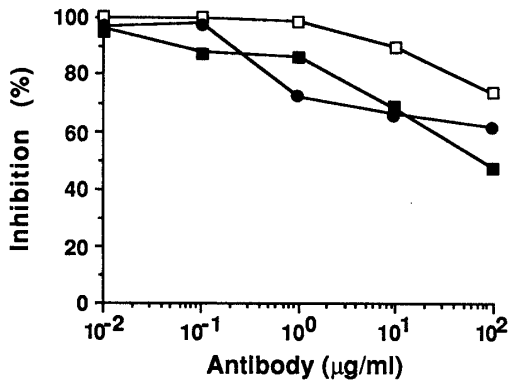

Fig. 5. Inhibitor Activity of RNasin Complexed with Antibodies

○—○, A443; ●—●, B335; ■—■, D5810.

allow the application of antibodies to quantitate RNasin in tissues. An assay was carried out by the ELISA method; RNase A was added to immobilized RNasin before addition of the antibody. As shown in Fig. 4, the reaction of the antibodies tested with RNasin was inhibited. This shows that anti-RNasins did not react well with RNasin complexed with RNase A, and the antigenic site in RNasin may be at or near the site forming the complex with RNase. Therefore, dissociation of the RNase–RNasin complex in the tissue extract is required in order to estimate the absolute amount of RNasin in the tissue.

Effect of Anti-RNasin on RNase Inhibitor Activity

We next examined whether the formation of the complex between RNasin and the antibody affected the RNasin activity. The assay was performed by quantitation of inhibition of RNase A activity. As shown in Fig. 5, the formation of the complex of RNasin with its antibody did not result in complete inhibition of RNasin activity although the extent of inhibition was slightly reduced when a large amount of antibody was added. This means that the active site of RNasin is not the site of antigen-antibody recognition. On the other hand, the addition of antibody to the complex of RNasin with RNase A did not regenerate RNase activity (data not shown). This means that the addition of antibody did not cause dissociation of the RNasin–RNase A complex.

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