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ISOLATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST RIBONUCLEASE INHIBITOR

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Mouse monoclonal antibodies were generated against human ribonuclease inhibitor, an intracellular regulatory protein. A total of four antibodies were isolated, all of which were of the immunoglobulin G1 subtype. Western blot analysis of the antibodies suggested monospecificity. Based on immunoradiometric competition assays two of the antibodies were determined to be directed against the same antigenic epitope, while the other two were against a second and possibly third epitope. None of the antibodies appeared to be directed against the ribonuclease binding site of the antigen. Data is presented suggesting that ribonuclease inhibitor is present in normal human serum. The potential significance of these findings is discussed.

Ribonuclease inhibitor (RI) is a 48,000 dalton protein which was first identified in mammalian liver cytosol (1). It has since been found in a wide variety of tissues (2-4), and has been purified to homogeneity from human placenta (5). The protein binds stochiometrically and with high affinity to alkaline (pancreatic-type) ribonuclease (RNase), resulting in the non-competitive inihibition of nuclease activity (6). An increase in RI levels has been shown to precede bulk RNA accumulation in actively dividing cells (7-9) and to result in the stabilization of polysome structure (10,11). Conversely, reduction in RI activity (with concommitant increase in RNase

ABBREVIATIONS USED

RI, Ribonuclease inhibitor; RNase, Ribonuclease; IgG, immunoglobulin G; BSA, bovine serum albumin.

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activity) occurs in cells during periods of catabolism (8,9,12,13).

RI has also been shown to inhibit the RNase activity of "angiogenin", a protein isolated from conditioned medium of a human colon adenocarcinoma which may play an important role in blood vessel formation (14,15). Angiogenin shares sequence homology with RNase A, and has a discrete RNase activity which cleaves 28S and 18S ribosomal RNA to fragments of 100-500 nucleotides in length (16,17). The ribonucleolytic activity of angiogenin appears to be essential for its biological activity (18-20).

In this report we describe the production and characterization of monoclonal antibodies against RI and present data suggesting that RI might be present in human serum.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum were obtained from Gibco Laboratories. Hypoxanthine, aminopterin, RNase A, thymidine, polyethylene glycol, protein A-sepharose, and 2, 2'-azinobis (3-ethylbenzthiazoline sulfonic acid) (ABTS) were obtained from Sigma Chemical Co. Micro-titer plates (Immulon 2) were purchased through Fisher Scientific. Anti-mouse IgG-Peroxidase and anti-rabbit IgG were from ICN Biomedicals Inc. All chemicals used were of reagent grade.

Preparation of RI

RI was purified from human placenta essentially as described by Blackburn (5) or purchased from Sigma Chem Co.

Immunization of Mice

Balb/c mice (4-6 weeks old) were injected subcutaneously in the abdominal region with 0.2 ml of an emulsion containing equal volumes of RI (0.1 mg/ml saline) and complete Freund's adjuvant. After 3-4 weeks the animals were bled to determine whether antibodies to RI were detectable in the sera (see below), and if positive were given a boost of RI intraperitoneally (10 ug in 0.2 ml saline). Three days later animals were sacrificed and their spleens removed for production of hybridomas.

Production of Monoclonal Antibodies

Spleens were removed aseptically from positive mice and put in GKN buffer (8g/l NaCl, 0.4g/l KCl, 1.77 g/l $Na_2HPO_4.2H_2O$, 0.69 g/l $NaH_2PO_4.H_2O$, 2 g/l glucose, 0.01 g/l phenol red) (10 ml/spleen). A cell suspension was prepared by teasing the tissue with a forceps, and the released cells collected by centrifugation. Cells were resuspended in GKN and counted on a hemocytometer. The cells were recentrifuged and mixed with NS-1 mouse myeloma cells at an approximately equal ratio of lymphocytes to NS-1 cells. The cells were pelleted and fused using the polyethylene glycol fusion technique described by Lane (21). About 10^8 cells were mixed with 1.2 ml fusing medium (5 g

polyethylene glycol + 5 ml GKN) over a period of 45-60 seconds under agitation. Fusion was halted by dilution of cells with HAT medium (10^{-4}M hypoxanthine, 4 x 10^{-7}M aminopterin, 1.6 x 10^{-5}M thymidine in DMEM-medium containing 10% fetal bovine serum). Cells were ultimately suspended in HAT medium at a concentration of about 3 x 10^6 cells/ml. 0.1 ml of the suspension was added to 96-well tissue culture plates along with 0.1 ml of normal spleen feeder cells (about 1-3 x 10^6 cells/ml) and plates put in a 37°C incubator in an atomosphere of 94% air 6% CO_2. After about 2 weeks of undisturbed growth, clones of hybridoma cells became apparent in almost every well and supernatants were assayed for anti-RI activity (see below). Cells from positive wells were transferred to 24-well plates containing 1 ml HT medium (HAT lacking aminopterin) along with feeder cells. After cells became confluent they were recloned in 96-well plates by limited dilution (22).

Assay for Anti-RI Antibodies

RI was diluted to 0.25 ug/ml in 0.05M carbonate buffer (pH 9.6) and 0.1 ml aliquots applied to 96-well micro-titer plates. After incubation overnight at 4°C, the wells were washed with phosphate-buffered saline contained 0.05% tween 80 (pbs/tween) and additonal sites coated with a solution of bovine serum albumin (BSA) in carbonate buffer (1% BSA). After further washings with pbs/tween to remove excess BSA, serial dilutions of serum from control and immunized mice or hybridoma supernatants were then added and incubation continued for 2 hours at room temperature. The wells were then washed 3 times with pbs/tween followed by addition of a 1:100 dilution of rabbit anti-mouse immunoglobulin G (IgG) conjugated to peroxidase. Incubation was allowed to proceed for 2 hours, after which the wells were washed an additional 3 times, followed by addition of enzyme substrate (0.001 M ABTS in 0.1 M citrate pH 4.2 containing 1 ul 30% H_2O_2/ml). Color development (blue-green) was monitored by eye, and positive sera or supernatants ascertained subjectively relative to color in control wells.

Purification of Monoclonal Antibodies

Positive hybridoma clones were grown in tissue culture, and the spent media collected for antibodies to RI. The media were mixed with equal volumes of a saturated solution of ammonium sulfate (pH 7.5), to precipitate out the immunoglobulins. The precipitates were then washed once with 50% ammonium sulfate, and then resolubilized and dialyzed into .05 M phosphate buffer (pH 8.0) containing 0.15M NaCl. The dialyzed material was then passed over columns of anti-mouse IgG sepharose or protein A-sepharose and the adsorbed material eluted by sequential decreases in buffer pH (22). Alternatively, hybridomas were innoculated into balb/c mice and antibodies collected from ascites fluid (23).

Iodination of Antibodies

Purified anti-RI monoclonal antibodies were iodinated by the chloramine-T procedure (24) using 100-200 uCi $Na^{125}I$ (Amersham Corp.) per 10 ug antibody. Incorporation of iodine varied between 40 and 60%,

Immunoradiometric Competition Assays

RI (0.25 ug/ml) was coated onto micro-titer plates as described above. After blocking the wells with BSA, serial dilutions of monoclonal antibodies were added to the wells and

incubation continued for 2 hours at room temperature. The wells were then thoroughly washed and an ^{125}I-labelled monoclonal antibody added. Incubation was allowed to proceed for an additional 2 hours, after which the wells were washed 4 times with pbs/tween. 0.1 ml of 1M NaOH was added to each well, and after 15 minutes the extracted radioactivity measured in a gamma counter. Competition between the two antibodies for binding to RI was evidenced by a decrease in radioactivity bound to the well and would indicate activity against the same or a proximal epitope of the antigen; no competition would indicate activity against different epitopes.

A variation in this technique was used to assay for RI. Basically, serial dilutions of an RI standard or serum sample were incubated at 4°C overnight with an ^{125}I-labelled monoclonal antibody. The incubates were then added to micro-titer plate wells which had been coated with RI and blocked with BSA as described above. After 2 hours incubation at room temperature the wells were washed with pbs/tween and bound radioactivity extracted with 1M NaOH. Soluble RI in the sample effectively competed with bound RI for the antibody.

Immunoblotting

5-15% gradient SDS PAGE was performed as described by Laemmli(25). Gels were stained with Coomassie Brilliant Blue. Proteins were electrophoretically transferred to nitrocellulose using a transfer apparatus (Bio-Rad Labs.) and the procedure described by the manufacturer. Nitrocellulose filters were fixed, then saturated with 0.1% BSA and 0.05% Tween 20 in TBS, pH 7.5. Filters were incubated at 4°C overnight with primary antibodies suspended in TBS, pH 7.5, containing 0.1% BSA and 0.05% Tween 20. Filters were washed three times with TBS containing 0.05% Tween, then incubated 1 hour at 4°C with peroxidase-conjugated goat anti-mouse IgG diluted in TBS containing 0.1% BSA and 0.05% Tween 20. After three washes with TBS containing 0.05% Tween 20 and one wash with 20 mM Tris, 0.5M NaCl, pH 7.5, reactive bands were visualized with 4-chloro-1-naphthol and 0.015% hydrogen peroxide.

RESULTS

Figure 1 shows a Western blot analysis on four monoclonal antibodies generated against RI. Monoclonal antibodies 2-H-10, 1-F-9, and 12-2-F reacted against a purified placental RI standard but did not appear to interact with any other proteins in a placental extract. Monoclonal antibody 2-A-12 did not exhibit antigen-specific reactivity in a Western blot assay. Also shown are results obtained using a rabbit polyclonal antibody against purified human RI. Unlike the monoclonal antibodies this antibody recognized several antigens in the placental extract. All four monoclonals were shown to be of the IgG1 sub-type using a mouse monoclonal typing kit (Bioproducts for Science, Inc.).

To determine whether the antibodies were directed against different epitopes of RI we employed an immunoradiometric

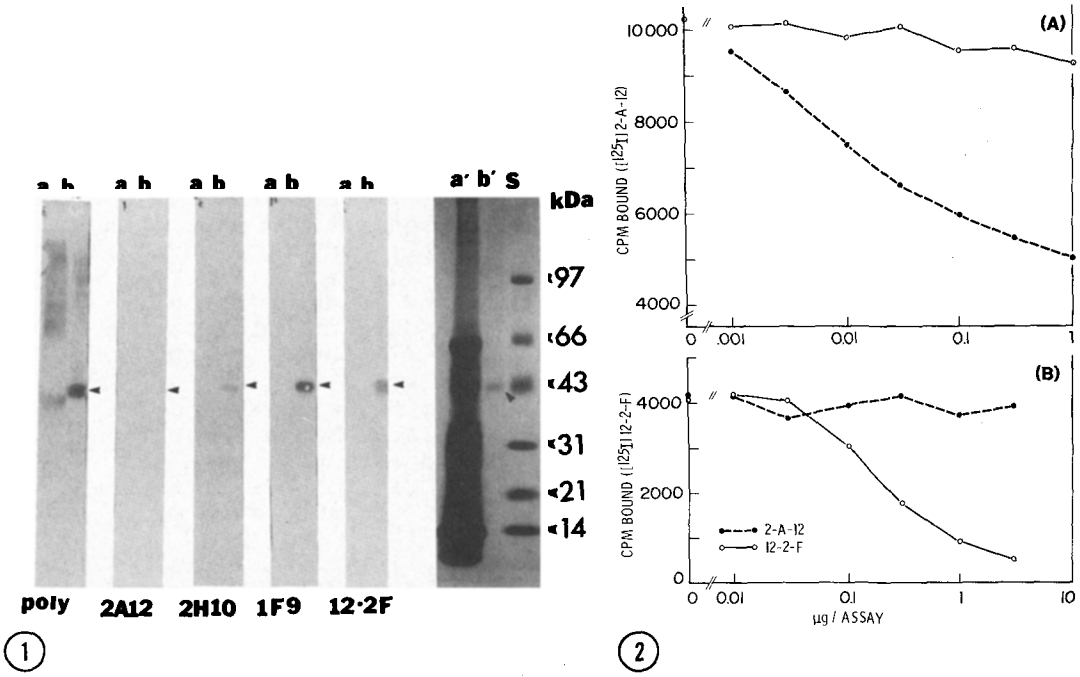


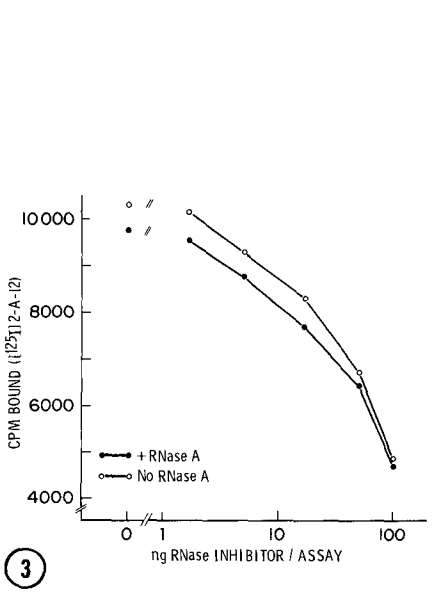
Figure 1. Western blot analysis of anti-RI monoclonal antibodies. Placental extracts (a lanes) or purified RI (b lanes) were resolved by SDS PAGE and either transferred to nitrocellulose (a,b lanes) or stained with Coomassie blue (a',b' lanes). Samples transferred to nitrocellulose were Western blotted with the indicated monoclonal antibodies, or polyclonal rabbit anti-RI (poly).

Figure 2. Detection of antibodies against different epitopes of RI. Immunoradiometric competition assays were done as described in the text. Dilutions of 2-A-12 and 12-2-F were competed with either [^{125}I]2-A-12 (Panel A) or [^{125}I]12-2-F (Panel B).

competition assay. Data on two of the antibodies (12-2-F and 2-A-12) are shown in Figure 2. It can be seen that 0.25 ug non-labelled 12-2-F caused a 50% inhibition in binding of [^{125}I] 12-2-F whereas 3 ug 2-A-12 was totally ineffective in this regard (panel B). Conversely, as little as 0.003 ug non-labelled 2-A-12 could compete with [^{125}I]2-A-12 whereas 1 ug 12-2-F was virtually without effect (panel A). These data indicate that 12-2-F and 2-A-12 are directed against different epitopes. Using this assay we have found that antibody 2-H-10 is directed against the same epitope as 2-A-12 and that 1-F-9 shows partial competition with 12-2-F (but not 2-H-10 or 2-A-12) suggesting a third epitope which might overlap with the 12-2-F epitope (data not shown). None of the antibodies appeared to be directed against the RNase binding site of RI. Thus as shown in figure 3 (using antibody 2-A-12), preincubation of RI standards

with an excess of RNase A (10 ug/ml) did not effect the slope of the RI standard curve. However, it is interesting to note, that the presence of RNase did cause a slight reduction in the absolute amount of antibody bound at each concentration of RI, suggesting that binding to RNase might cause some degree of non-competitive interference or steric hindrance. Similar results were obtained with the other antibodies.

Using the immunoradiometric competition assay, we were also able to set up standard curves for quantitation of RI (figure 4); with [^{125}I]2-A-12 we could detect down to 10 ng RI (panel A), while [^{125}I]12-2-F allowed us to detect as little as 5 ng RI (panel B). To determine whether RI is present in serum we made serial dilutions of pooled normal male sera and ran these samples along with the RI standards. As shown in figure 4 the curves are parallel suggesting that RI might be present in serum at a concentration of about 2-3 ug/ml. Further evidence that RI is present in serum is demonstrated by the fact that addition of



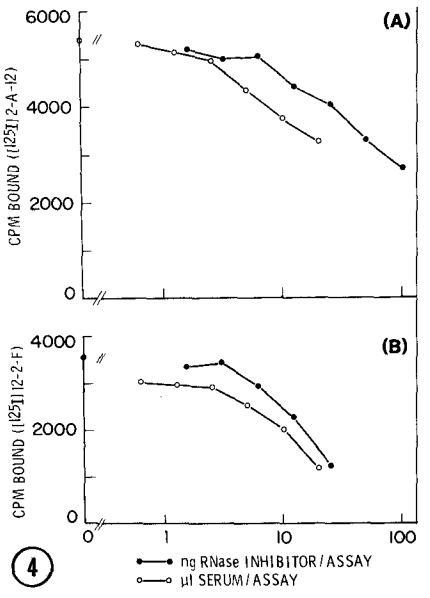


Figure 3. Effect of RNase on antibody binding to RI. Serial dilutions of an RI standard were incubated with [^{125}I]2-A-12 in the presence or absence of 10 ug/ml RNase A. The incubates were used in an immunoradiometric competition assay as described in the text.

Figure 4. Demonstration of RI in serum. Serial dilutions of an RI standard or pooled human serum were incubated with [^{125}I]2-A-12 (Panel A) or [^{125}I]12-2-F (Panel B). The incubates were used in an immunoradiometric competition assay as described in the text.

exogenous RI to the serum samples gave values equal to the sum of the exogenous and endogenous RI levels (data not shown).

DISCUSSION

We have generated four monoclonal antibodies against human RI, all of which are of the IgG1 sub-type. Three of the antibodies recognize purified RI run on Western blots but do not appear to recognize any proteins from placental extracts, suggesting mono-specificity. In contrast, a rabbit polyclonal antisera against human RI recognizes a number of different proteins.

Using an immunoradiometric competition assay we have determined that two of the monoclonals (2-H-10 and 2-A-12) appear to be directed against the same epitope of RI; however, these monoclonal antibodies are probably not idiotypically identical because only 2-H-10 is effective in Western blotting. Monoclonals 12-2-F and 1-F-9 appear to be directed against a second and possibly third epitope, since they do not compete with the other two monoclonals and only partially compete with each other. None of the epitopes appear to involve the RNase binding domain of RI, since addition of an excess of RNase A to the competition assay could not effectively inhibit the binding of any of the antibodies to RI. Future studies with these antibodies might thus prove useful in determining the topography and antigenic domains of the RI molecule.

The data presented in this paper also suggest the RI is present in normal human serum. This is based on immunoradiometric competition curves using monoclonal antibodies against two different epitopes. However, actual proof that this is the case must await the purification of RI from serum, so that at present the evidence is only circumstantial. Since most studies to date have centered on RI as an intracellular mediator of ribonuclease activity (26-28), the possibility of it being in serum raises the question of whether RI might have additional functions as a secretory protein. It has recently been shown that the protein angiogenin, an inducer of neovascularization which has RNase-like activity is present in normal serum at a concentration of about 0.4 ug/ml (20,29). The serum concentration of RI in our study is about 2-3 ug/ml. Since the molecular weight of angiogenin is only 14,000 daltons, the molar concentrations of the two proteins would be in the same range.

Since our antibodies are not directed against the RNase binding site of RI, it is impossible to say whether the RI we are measuring is circulating free or complexed to angiogenin.

Like RI, angiogenin is found in a wide variety of tissues and is most prominant in adult liver (30). Surprisingly, the angiogenin mRNA content in different rat tissues during development does not appear to correlate with vascular growth (30). This suggests that if angiogenin is indeed a mediator of blood vessel formation its activity must be regulated post-transcriptionally. Moreover, unlike other angiogenic peptides such as basic and acidic fibroblast growth factors (31,32), angiogenin does not appear to be mitogenic for endothelial cells (33,34) and has not been demonstrated to interact with specific membrane receptors. Regardless of the actual in vivo role of angiogenin, its high affinity for RI as well as its tissue distribution make it likely that RI could function physiologically as a mediator of angiogenin (19,20). The utilization of monoclonal antibodies against RI could thus prove useful in helping to answer some of the questions surrounding this interesting peptide.

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