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USE OF HUMAN PANCREATIC RIBONUCLEASE ANTIBODIES TO DELINEATE PANCREAS RELATED RIBONUCLEASES IN HUMAN SERUM

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SUMMARY: Antibodies to the purified polycytidylate hydrolysing human pancreatic RNase were raised in rabbits. The partially purified antibody preparation is free from RNase. The polycytidylate hydrolysing human serum RNase is inhibited by the pancreatic RNase antibodies. Experiments have been carried out to establish optimal conditions for this inhibition. The serum RNase forms an inactive soluble complex with the antibody. Treatment of serum RNase-antibody complex with glycine-HCL buffer of pH 2.0 containing polyethylene glycol releases RNase activity. Goat antirabbit serum immunoglobulin precipitates serum RNase-antibody complex. In order to determine the pancreas related RNase in human serum, the serum RNase activity was measured with poly (C) as a substrate in the absence and presence of antibody. According to this assay about 98% of total polycytidylate hydrolysing human serum RNase activity is related to human pancreatic RNase. The main advantages of this assay over radio-immunoassay are its simplicity, economy of time and capital costs.

INTRODUCTION

Human serum contains RNases that hydrolyse poly (C) and poly (U). The poly (C) hydrolysing activity is most predominant and constitutes about 98% of total serum RNase activity (1). Human granulocytes and platelets contribute to the poly (U) hydrolysing activity, which is considerably enhanced in certain pathological conditions such as agranulocytosis and thrombocytopenia (2,3). Human liver and spleen were reported to contain RNase which does not hydrolyse homopolyribonucleotides but hydrolyses yeast RNA to yield oligonucleotides having purines at their 5'-terminii (4). This type of RNase acitivity was not observed in human serum by us nor was reported by any other investigators. The poly (C) hydrolysing RNase is present in the pancreas and also in the other organs of the body. The pancreas is the richest source of this RNase and contains about 100 times more than the other organs of the body (4). It is secreted by the exocrine cells of the pancreas and finds its way into the duodenum

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(5) and blood stream (1). It is biochemically related to the poly (C) hydrolsing RNase of human serum (1). The evidence presented in this communication further shows that the pancreatic RNase and poly (C) hydrolysing RNase of human serum are antigenically related.

This paper deals with the production of antibodies to the purified human pancreatic RNase, establishment of optimal conditions for the formation of serum RNase-antibody complex and the assay of the serum RNase activity antigenically related to human pancreatic RNase. This is part of an ongoing project, the objective of which is to find in the serum a specific biochemical marker for the pancreas, which might be helpful in the diagnosis of pancreatic ailments.

MATERIALS

Human Serum: The venous blood drawn from volunteer laboratory workers was allowed to clot at room temperature for 1 hr. and centrifuged at 750g for 15 min. at room temperature. The serum was removed with a capillary pipette and assayed immediately or stored at -20°C until used.

Goat AntiRabbit Immunoglobulin: The goat antirabbit immunoglobulin, isolated from serum by affinity chromatography was purchased from Boehringer Manheim Biochemicals, Indianapolis, IN.

Reagents: Polycytidylic acid was purchased from Miles Laboratory Inc., Elkhart, IN. Sephadex G-100 was purchased from Pharmacia Fine Chemicals Inc. Cellulose phosphate P11 was purchased from Whatman Biochemicals Ltd. polyethylene glycol (PEG 6000) was purchased from Fisher Scientific Co. All other chemicals used in this investigation were of reagent grade.

METHODS

Ribonuclease Assay: Because RNase of human serum, human pancreas and its secretions is highly specific to the secondary phosphate esters of cytidine-3'-phosphate, its assay with poly (C) as a substrate is more sensitive than with RNA which is hydrolysed only partially. The details of RNase assay were given in the previous publications (1,5).

Protein Determination: Protein was determined according to the colorimetric procedure of Lowry et al (6).

Polycytidylate Hydrolysing human pancreatic RNase: The polycytidylate hydrolysing RNase of human pancreas was purified according to the procedure described earlier. Briefly stated, the purification procedure consists of (i) extraction of pancreas with 3.5% $HClO_4$, (ii) fractionation of the extract with $(NH_4)_2SO_4$ (iii) filtration of the active fraction through Sephadex G-100 and (iv) chromatography on cellulose phosphate P11. This procedure resulted in the purification of RNase by about 1000-fold. The preparation was free from phosphatase, phosphodiesterase and deoxyribonuclease. However, the purified RNase is comprised of three variants, which differ only in their isoclectric points but are similar in all the other properties (7). The purified RNase preparation was used to immunize rabbits.

Production of antibody to polycytidylate hydrolysing human pancreatic RNase: Two male white New Zealand rabbits were used. One mg of purified pancreatic RNase in 0.5 ml of 0.9% NaCl was mixed with an equal volume of Freund's adjuant and injected subcutaneously into several sites on the back of the rabbit. Subsequent injections of each containing 0.5 mg of RNase was given 14, 21, and 28 days after the first injection. The first blood samples were drawn a week after the last injection. The blood was allowed to clot for 3 hr. at room temperature and stored overnight at 4°C. The serum was then separated by centrifugation and stored at -20°C until used. The serum was similarly obtained from the blood samples drawn from the rabbits prior to immunization and stored until used.

The antiserum was fractionated with $(NH_4)_2SO_4$ to yield gammaglobulin fraction. To 20 ml of antiserum were added while stirring 10 ml of saturated $(NH_4)_2SO_4$ solution. The mixture was held at 0° for 30 min and centrifuged in the cold at 6040g for 20 min. The supernatant was discarded. The precipitate was dissolved in 10 ml of 0.9% NaCl and the precipitation with $(NH_4)_2SO_4$ was repeated as above four more times. The resulting precipitate was dissolved in 10 ml of 0.9% NaCl and dialysed at 4°C with gentle stirring against 2 changes of 2L of 0.9% NaCl over a 24 hr. period. A white precipitate that was formed in the dialysis bag was removed by centrifugation in the cold at 12,100g for 30 min. The supernatant was free from the polycytidylate hydrolysing RNase activity and contained 6.25 mg of protein per ml. This was distributed in 0.2 ml and 0.5 ml aliquots and stored at -20°C.

The nonimmune rabbit serum was fractionated with $(NH_4)_2SO_4$ as above. The final preparation contained 2.13 mg of protein per ml. This was distributed in 0.5 ml aliquots and stored at -20°C.

RESULTS

The following experiments were done to determine the optimal conditions for the interaction of pancreatic RNase antibody with human serum RNase.

Effect of Antibody Concentration: The effect of pancreatic RNase antibody on serum RNase activity was tested. The results presented in Fig. 1 show that nonimmune rabbit serum fraction has no effect on human serum RNase activity, while the pancreatic RNase antibody is a potent inhibitor of serum RNase. The inhibition is dependent on the antibody concentration. Thus, 10µg, 20µg, and 30µg of antibody respectively brought about 24%, 48%, and 72% reduction in the serum RNase activity. However, the inhibition was not linear when the antibody concentration was increased above 30µg, but progressed steadily until it reached a plateau. Sixty or 80µg of antibody inhibited about 95% of serum RNase activity.

Effect of Time: The results presented in Fig. 2 show that nonimmune rabbit serum fraction has no effect on serum RNase activity even after a prolonged in-

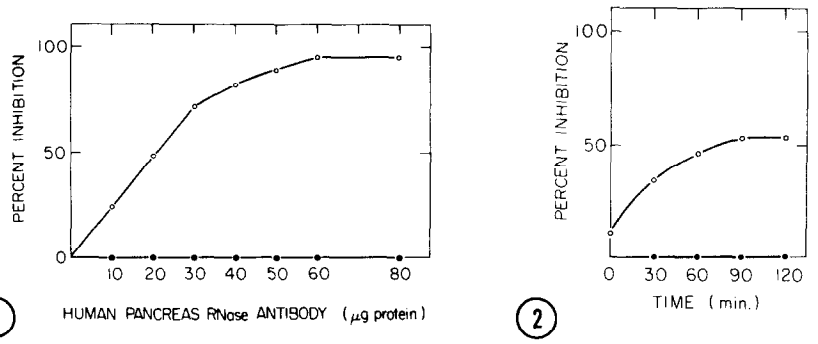


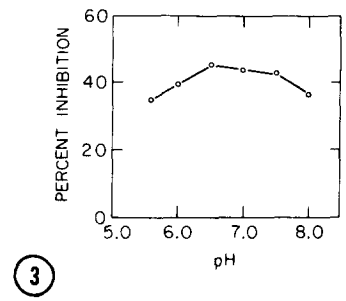
Fig. 1. Effect of pancreatic RNase antibody on serum RNase activity. Incubation mixtures consisting of 0.05 ml of dilute human serum (5-fold diluted with 0.9% NaCl), 0.05 ml of 0.1 M EDTA, 0.1 ml of 0.25 M sodium phosphate buffer of pH 7.0 and 0.05 ml of antibody or nonimmune rabbit serum fraction, the concentrations of which are given in Fig. 1, were held at 4°C for 2 hr. They were then diluted to 1.0 ml with H_2O and their RNase activities were determined. Reaction mixtures consisting of 0.05 ml of poly (C) (100μg), 0.05 ml of 0.25 M sodium phosphate buffer of pH 6.5, 0.1 ml of H_2O and 0.05 ml of test solutions, were incubated at 37°C for 15 min. and then transferred to an ice bath. To each tube was added with mixing 0.25 ml of cold 12% $HClO_4$ containing 0.02 M lathanum nitrate. After 20 min at 0°C, the precipitates were removed by centrifugation in the cold at 12,100g for 30 min. The supernatants were diluted with H_2O and measured at 278nm (1,5).(●) antibody to pancreatic RNase; (0) nonimmune rabbit serum fraction.

Fig. 2. Effect of time on the inhibition of human serum RNase activity by pancreatic RNase antibody. Incubation mixtures consisting of 0.2 ml of dilute human serum (5-fold diluted with 0.9% to NaCl), 0.2 ml of 0.1 M EDTA, 0.4 ml of 0.25 M sodium phosphate buffer of pH 7.0 and 0.2 ml of pancreatic RNase antibody (80μg of protein) or 0.2 ml of nonimmune rabbit serum fraction (80μg of protein) were held at 4°C. At intervals of time, 0.1 ml aliquots were withdrawn and diluted to 0.4 ml with H_2O and their RNase activities were determined according to the procedure given in the legend to Fig. 1. (●) antibody to pancreatic RNase; (0) nonimmune rabbit serum fraction.

cubation. The inhibition of serum RNase by pancreatic RNase antibody progressed with time and reached optimal within 90 min.

Effect of pH: The results presented in Fig. 3 show that the inhibition of serum RNase by the antibody is dependent on pH. In 0.05 M sodium phosphate buffer of pH 6.5, maximum inhibition of RNase was produced. In all the experiments described below, the incubation mixtures contained 0.05 M sodium phosphate buffer of pH 6.5.

Dissociation of Serum RNase-Antibody Complex: The results presented in Table 1 show that RNase activity can be recovered from serum RNase-antibody complex. The treatment of the complex with 0.05 M glycine-HCl buffer of pH 2.0 released about 72% of RNase activity. The presence of 2.5% PEG 6000 in 0.05 M glycine-HCl buffer released about 91% of the original RNase activity. Thus, the inhibition of serum RNase by the pancreatic RNase antibody is reversible.



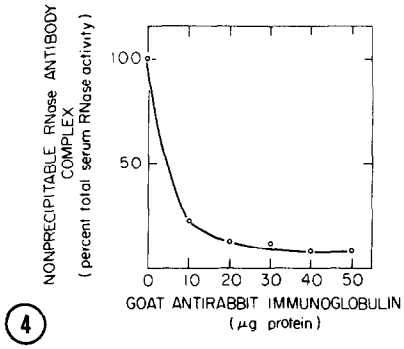


Fig. 3. Effect of pH on the inhibition of human serum RNase activity by pancreatic RNase antibody. The pH effect was studied with 0.25 M sodium phosphate buffers ranging in pH from 5.6 to 8.0. The rest of the experimental conditions were those given in the legend to Fig. 1.

Fig. 4. Precipitation of serum RNase and pancreatic RNase antibody complex with goat antirabbit immunoglobulin. Incubation mixtures consisting of 0.05 ml of serum (5-fold diluted), 0.05 ml of 0.1 M EDTA, 0.1 ml of 0.25 M sodium phosphate buffer of pH 6.5, 0.05 ml of pancreatic RNase antibody (60μg of protein) or 0.05 ml of 0.9% NaCl were incubated at 4°C for 2 hr. To each incubation mixture were added 0.1 ml of 10% PEG 6000 and 0.05 ml of goat antirabbit immunoglobulin, concentration of which is given in the figure and the incubation was continued for 24 hr. at 4°C. They were then centrifuged in the cold at 12,100g for 30 min. The nonprecipitable serum RNase-antibody complex present in the supernatants was dissociated by treating with glycine-HCl buffer of pH 2.0 containing PEG 6000 as described in the legend to Table 1 and their RNase activities were determined according to the procedures given in the legend to Fig. 1.

Precipitation of Serum RNase-Antibody complex: Since the pancreatic RNase antibody preparation used in this study is not pure, it is possible that the inhibition of serum RNase may not be related to the immunoglobulin fraction, but it could be due to some contaminant in the preparation. To test this

Table 1. Dissociation of serum RNase - pancreatic RNase antibody complex.

Treatment of antigen-antibody complex	Serum RNase activity recovered (% of total serum RNase activity)
None	2.5
0.05 M glycine - HCl, pH 2.0	72.3
0.05 M glycine - HCl, pH 2.0 Containing 2.5% PEG 6000	90.7

Incubation mixtures consisting of 0.05 ml serum (5-fold diluted with 0.9% NaCl), 0.05 ml of 0.1 M EDTA, 0.1 ml of 0.25 M sodium phosphate buffer of pH 6.5 and 0.05 ml of antibody (60μg of protein) or 0.05 ml of 0.9% NaCl (controls) were held at 4°C for 2 hr. At the end of incubation time, to tube one was added 0.25 ml of 0.9% NaCl; tube two, 0.25 ml of 0.1 M glycine-HCl, pH 2.0; tube three 0.25 ml of 0.1 M glycine-HCl, pH 2.0 containing 5% PEG, and similar additions were made to the control tubes. They were held at 0°C for 2 hr., appropriately diluted with H_2O and their RNase activities were determined according to the assay procedure given in the legend to Fig. 1.

possibility, the goat antirabbit immunoglobulin was added to serum RNase-antibody complex. The results presented in Fig. 4 show that the antigen-antibody complex is precipitated by goat antirabbit immunoglobulin, suggesting that the inhibition of serum RNase is due to the immunoglobulin fraction of the pancreatic RNase antibody preparation. Fifty µg of goat antirabbit immunoglobulin precipitated about 92% of serum RNase-antibody complex. The immunoglobulin concentrations above 50µg did not bring about 100% precipitation of the complex. About 8% of the complex remained in a soluble state under these experimental conditions.

Determination of human serum RNase antigenically related to human pancreatic RNase: Since human serum contains RNases derived from sources other than pancreas, an attempt was made to delineate the polycytidylate hydrolysing RNase of serum related to the pancreas. In order to achieve this objective, the serum was assayed in the presence and absence of pancreatic RNase antibody. The results presented in Table 2 show that the serum RNase levels of 25 apparently healthy persons ranged from 14.2 to 37.7 units per ml of serum. When the RNase assays were performed in the presence of a large excess of pancreatic RNase antibody, the serum RNase levels ranged from 0 to 2.7 units per ml of serum. The pancreas related serum RNase thus constitutes on the average about 98.0% of the total polycytidylate hydrolysing serum RNase activity.

DISCUSSION

It is evident from the results presented in this paper, that the antibodies to human pancreatic RNase inhibit the polycytidylate hydrolysing RNase activity of human serum. The inhibition is dependent on the pH of the incubation medium, duration of the contact of serum RNase with the antibody and the antibody concentration. The serum RNase activity can be recovered from serum RNase-antibody complex by treating the complex with 0.05 M glycine-HCl buffer of pH 2.0 containing 2.5% PEG 6000, suggesting that the inhibition of serum RNase is reversible. The serum RNase-antibody complex can be precipitated with goat antirabbit immunoglobulin thereby establishing that the inhibition of serum RNase is antibody specific.

Table 2. RNase of normal human serum as determined in the presence and absence of pancreas RNase antibody.

Donor No.	Units of RNase/ml Serum - Antibody	+ Antibody	Pancreas related Serum RNase (% of total serum RNase)
1	37.7	2.4	93.6
2	15.7	0	100.0
3	18.7	0	100.0
4	27.6	0.4	98.6
5	19.2	0	100.0
6	25.7	0.7	97.3
7	14.2	0	100.0
8	21.3	0	100.0
9	26.7	0	100.0
10	30.2	0.5	98.3
11	20.9	0	100.0
12	32.3	1.5	95.4
13	20.3	0	100.0
14	38.7	2.7	93.0
15	26.8	0	100.0
16	18.8	0	100.0
17	18.3	0	100.0
18	25.3	0.4	98.4
19	16.2	0	100.0
20	34.9	2.0	94.3
21	20.8	0.8	96.2
22	32.3	1.7	94.7
23	25.9	0.4	98.5
24	21.5	1.0	95.4
25	28.6	0.8	97.2
Average	24.7 ± 6.85	0.6	98.0 ± 2.38

Incubation mixtures consisting of 0.05 ml serum (5-fold diluted with 0.9% NaCl); 0.05 ml of 0.1 M EDTA, 0.1 ml of 0.25 M sodium phosphate buffer of pH 6.5 and 0.05 ml of 0.9% NaCl or antibody to pancreatic RNase (100µg of protein) were held at 4^0C for 2 hr. They were then diluted 4-fold with cold H_2O and their RNase activities were determined according to the procedure described in the legend to Fig. 1.

Since the inhibition of polycytidylate hydrolysing serum RNase activity by the antibody to the polycytidylate hydrolysing pancreatic RNase is highly specific, the assay of serum RNase with poly (C) as a substrate in the presence and absence of antibody helps in measuring the pancreatic type RNase in the serum. This assay has several advantages over the radioimmunoassay which involves expensive equipment, expensive unstable reagents and long hours of incubation. The inhibition procedure herein used in assaying the pancreatic type RNase in the sera derived from 25 apparently normal persons, is simple and specific involving stable inexpensive reagents, short periods of incubation and a spectrophotometer.

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