Possible Sites of Origin of Human Plasma Ribonucleases as Evidenced by Isolation and Partial Characterization of Ribonucleases from Several Human Tissues

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ABSTRACT

The ribonucleases (RNases) present in a number of human tissues, including heart, brain, lung, and kidney, were purified, partially characterized, and compared in their properties to the previously described RNases from human liver, spleen, pancreas, and serum. The enzymes appeared to fall into two major classes: liver-spleen type RNase and plasma-type RNase. These two types of enzymes were present in varying proportions in all tissues examined. The extent to which the tissues studied possibly contribute to serum RNase levels is discussed.

INTRODUCTION

Results in this laboratory and elsewhere (1, 3, 6) have established the existence of 2 distinct classes of human RNases that differ in substrate specificity, pH optima, antigenic properties, and behavior toward metal ions (1, 3, 6, 10, 23, 25, 31, 33). The first type of RNase has been isolated from the pancreas (3, 7, 33), duodenal contents (3), plasma (1, 31), and urine (3, 6). The second type of RNase has been found in the liver and spleen (10, 23), urine (3, 6), and granulocytes (32). The first type of RNase (hereafter referred to as plasma-type RNase) exerts its maximal activity against the synthetic substrate poly(C)2 at pH 6.5 to 8.0, depending upon the buffer used. The second type of RNase (hereafter called the liver-spleen type RNase) also exhibits a buffer dependence but is maximally active in the range of 6.0 to 7.0 with yeast RNA as substrate. These 2 types of enzymes are present in varying proportions within different tissues.

Since elevated levels of RNase activity in the serum and urine have been reported for a wide variety of human cancers (2, 5, 7–9, 11, 13, 15, 17, 19, 20–22, 28, 29, 34), including multiple myeloma (9, 12, 15), chronic granulocytic leukemia (2, 20–22), and pancreatic carcinoma (28), some investigators feel that RNase activity may be a useful biomarker for these clinical conditions. However, the tissue or tissues of origin of normal serum and urine RNase activities are as yet unknown. It was of interest, therefore, to obtain a profile of distribution of the 2 classes of RNase in various tissues. This was accomplished by the isolation and partial characterization of RNases present in a number of human tissues. These studies revealed that in all the tissues examined both types of RNase could be detected and that one type or the other was usually predominant.

MATERIALS AND METHODS

Samples of human tissues were obtained from autopsy specimens supplied by the office of the Baltimore City Medical Examiner. The sources of yeast RNA and synthetic polynucleotides, as well as the methods used in their purification, are described elsewhere (31).

The following materials were obtained from commercial sources: spermidine (Calbiochem, San Diego, Calif.), Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N. J.), and poly(G)-agarose (P-L Biochemicals, Inc., Milwaukee, Wis.).

Assay of Human RNases. The standard assay system for the plasma-type enzyme (31) contained 1.5 μmoles of poly(C), 100 μmoles of Tris-HCl buffer (pH 7.5), 0.5 mg of bovine serum albumin, and enzyme in 1 ml. After incubation for 15 min at 25°C, the reaction was stopped by the addition of 1 ml of cold 2 n perchloric acid, and the reaction mixture was placed in an ice bath for 10 min. The cloudy solution was then clarified by centrifugation, and the absorbance of the acid-soluble nucleotides was measured at 260 nm (14). An enzyme unit is defined as that amount of enzyme that renders 1 nmole of substrate acid soluble in 1 min under the conditions of the assay.

The standard assay system for the liver-spleen type enzyme (10, 23) differed in that the reaction mixture contained 0.25 mg of yeast RNA, 100 μmoles of phosphate buffer (pH 6.0), 0.5 mg of bovine serum albumin, and enzyme in 1 ml. After incubation at 25°C for 15 min, enzyme activity was determined as above.

Preparation of Immobilized Antisera. Antisera to human liver (10) RNase and to human pancreatic RNase (25) were prepared by injecting independently each purified enzyme directly into the popliteal lymph nodes of New Zealand White rabbits (26). In each case, the presence of antibody was verified by precipitation of immune complexes with a 33% saturated, cold (NH4)2SO4 solution (24). The respective antisera, as well as preimmunization rabbit sera, were conjugated independently to cyanogen bromide-activated Sepharose 4B as described previously (26).

Binding of RNases to Immobilized Antisera. The standard reaction mixture for the measurement of enzyme binding to antibody consisted of 4.5 units of enzyme, 80 μmoles of Tris-HCl buffer (pH 7.5), and 0.5 mg of bovine serum albumin in a volume of 0.7 ml. To this was added 0.3 ml of a mixture consisting of equal volumes of either pre- or postimmunization serum conjugated to Sepharose 4B and 0.005 M Tris-HCl buffer, pH 7.5. After incubation at...
RESULTS

Crude Homogenates of Various Human Organs

For determination of the potential sources of plasma RNase, a number of human organs were homogenized (liver, spleen, lung, heart, kidney, and brain), and the crude extracts were examined for RNase activity. Previous work with human RNases had indicated there are at least 2 distinct species of human RNases: 1 isolated initially from liver (10) and spleen (23), and the other isolated from plasma (31). Included in this second class is a pancreatic RNase (25) which, although antigenically similar to the plasma enzyme, has not been well defined with respect to its attack on various internucleotide linkages. The 2 classes of enzymes have quite different properties, particularly with respect to their pH optima and to their hydrolytic activity against a variety of substrates. These differences can serve as a basis for differentiation between the 2 enzyme types (Table 1). The purified plasma enzyme is far more active against the synthetic substrate, poly(C), at pH 7.5 in Tris-HCl buffer, than is the purified liver enzyme. In contrast, the liver enzyme exhibits greater activity against yeast RNA at pH 6.0 in phosphate buffer than does the purified plasma enzyme. Thus utilization of different substrates and buffers can help in the determination of the relative concentrations of the 2 enzymes in a given preparation.

As to the concentration of the 2 types of RNase in various tissues (Table 2), both liver-spleen type and plasma-type RNases were found in all tissues examined, although their proportions were variable. In the spleen, liver, kidney, and lung, the concentrations of the 2 types of RNase are comparable. In the heart and brain, however, the plasma-type RNase predominates by as much as 10- to 20-fold over the liver-spleen type RNase. In the pancreas, the plasma-type RNase is present in a 100-fold greater concentration than is the liver-spleen type enzyme. In none of our studies, in which a variety of substrates, assay conditions, and fractionation techniques were used, was there any evidence for a third type of RNase.

For confirmation of the distribution of tissue RNases described above, immunological studies of the RNases present in these tissues were carried out with the use of antisera specific for either the liver-spleen type or the plasma-type RNase. As can be seen in Table 3, no cross-reaction between the purified plasma RNase and the purified liver RNase was detected when these antisera were used. When crude plasma was reacted with immobilized antiserum to liver-spleen RNase as well as to immobilized antiserum to the plasma RNase, 98.5% of the RNase activity in plasma bound to the antiplasma RNase immunoadsorbent, and only negligible amounts bound to the anti-liver-spleen RNase immunoadsorbent. It was concluded, therefore, that the liver-spleen type RNase is present in plasma to a very small extent, if at all, and that most, if not all, of the activity in plasma is from a plasma-type enzyme(s). Similarly, when the RNases present in the other 6 tissues were exposed to the same immobilized antiserum, the results (Table 3) correlated with the identity and distribution of the RNases outlined above, based on data in Tables 1 and 2.

Purification of Human Kidney RNase.

The purification and yield at each step are summarized in Table 4.

Step 1. Human kidney (20 g) was homogenized in 40 ml of 0.05 M Tris-HCl buffer, pH 7.5, for 1 min in a Sorvall Omnimixer, and cellular debris was removed by centrifugation at 20,000 x g for 20 min.

Step 2: Protamine Sulfate Fractionation. To 20 ml of the supernatant solution, 1 mg of protamine sulfate (2% solution; pH 7.5) per 20 mg protein was added slowly with stirring. After standing for 20 min, the cloudy mixture was clarified by centrifugation at 50,000 x g for 20 min, and the supernatant solution was retained.

Step 3: Immunoadsorption. Twenty ml of the enzyme obtained in the previous step were dialyzed against 2 changes (4 liters each) of 0.05 M phosphate buffer (pH 7.5) in 0.85% NaCl solution. The enzyme was then added to a centrifuge tube containing 5 ml of antiplasma-type serum
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Table 3

**Immunological differentiation and identification of liver-spleen and plasma-type RNases in crude tissue homogenates**

The crude extracts were prepared as described under "Enzyme Purification," and the purified liver and plasma enzymes were prepared as described elsewhere (10, 31). The binding of the RNases present in the crude extracts to either antiliver-spleen or antiplasma postimmunization sera conjugated to Sepharose 4B was carried out as described in "Materials and Methods." Control mixtures containing preimmunization serum conjugated to Sepharose 4B were also prepared. Unbound enzyme activity was then measured under 2 sets of conditions (Table 1) designed to detect liver-spleen activity (Condition 1) and plasma-type activity (Condition 2). The volume of each crude extract used was chosen to give an activity of about 1.0 absorbance unit in the control tubes that contained the immobilized preimmunization serum. For comparative purposes, purified plasma and liver RNases were also evaluated. The values listed below are the means of duplicate determinations and are representative of values obtained from the tissues of 3 trauma victims and from the plasma of 10 normal volunteers.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Amount of enzyme used (mg protein)</th>
<th>Condition 1</th>
<th>Condition 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antiliver-spleen RNase serum</td>
<td>Preimmunization serum</td>
<td>Antiplasma RNase serum</td>
</tr>
<tr>
<td>Crude extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>2.65</td>
<td>19.5</td>
<td>19.8</td>
</tr>
<tr>
<td>Brain</td>
<td>0.65</td>
<td>9.3</td>
<td>9.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.6</td>
<td>0.7</td>
<td>8.1</td>
</tr>
<tr>
<td>Heart</td>
<td>0.92</td>
<td>6.2</td>
<td>9.2</td>
</tr>
<tr>
<td>Lung</td>
<td>3.13</td>
<td>12.3</td>
<td>15.9</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.03</td>
<td>1.6</td>
<td>13.7</td>
</tr>
<tr>
<td>Liver</td>
<td>0.67</td>
<td>4.6</td>
<td>18.5</td>
</tr>
</tbody>
</table>

Purified enzymes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Amount of enzyme used (µg protein)</th>
<th>Condition 1</th>
<th>Condition 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antiliver-spleen RNase serum</td>
<td>Preimmunization serum</td>
<td>Antiplasma RNase serum</td>
</tr>
<tr>
<td>Liver</td>
<td>0.13</td>
<td>0.4</td>
<td>10.7</td>
</tr>
<tr>
<td>Plasma</td>
<td>5.7</td>
<td>15.0</td>
<td>13.7</td>
</tr>
</tbody>
</table>

* These values were obtained by the assay procedure for the human liver-spleen type RNase, i.e., yeast RNA as the substrate in 0.1 M Tris-HCl buffer, pH 7.5 (see "Materials and Methods"), since purified liver RNase hydrolyzes poly(C) so poorly.

Table 4

**Purification of plasma-type RNases from human kidney**

Values are representative of the values obtained from 3 different purifications of the kidney enzyme.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein* (mg)</th>
<th>Plasma-type activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>20</td>
<td>1200</td>
<td>38,900</td>
<td>32.4</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>20</td>
<td>595</td>
<td>35,900</td>
<td>51.6</td>
<td>92</td>
</tr>
<tr>
<td>3.</td>
<td>6</td>
<td>0.066</td>
<td>24,100</td>
<td>365,000</td>
<td>62</td>
</tr>
<tr>
<td>4.</td>
<td>7</td>
<td>0.018</td>
<td>18,100</td>
<td>1,010,000</td>
<td>46</td>
</tr>
</tbody>
</table>

* In Steps 1 and 2, protein was determined by the biuret method (16). In Steps 3 and 4, protein was determined by the fluorescamine assay (4).

conjugated to Sepharose 4B (see "Materials and Methods") and 10 ml of the dialyzing buffer. This mixture, shaken mechanically for 30 min, was centrifuged at 500 x g for 10 min, and the supernatant solution was discarded. The imunoabsorbent to which enzyme was now bound was resuspended in 0.05 M phosphate buffer (pH 7.5) containing 0.8% NaCl. The process of alternate washing and centrifugation was repeated 5 additional times. After the final wash, the supernatant solution was carefully aspirated and discarded. To the immobilized antisera to the plasma-type RNase, 5 ml of 0.25 n H₂SO₄ were added to dissociate the enzyme from the antisera, and after 30 min of shaking the supernatant enzyme solution was clarified by centrifugation at 500 x g for 10 min, and the elutant was collected. This solution was then neutralized with 1 M NaOH and used in the next step.

**Step 4: Poly(G) Affinity Chromatography.** Six ml of the enzyme solution were dialyzed against 2 changes (4 liters each) of 0.05 M sodium phosphate buffer (pH 6.2). The enzyme was then applied to a column (0.5 x 14 cm) containing 4 g of CNBr-activated Sepharose 4B to which 22 µmoles of poly(G) have been covalently bound and which had been equilibrated with 0.05 M sodium phosphate buffer (pH 6.2) (31). After the column was washed with 65 ml of equilibrating buffer to remove all unbound protein, the enzyme was eluted with 1 M KCl in 0.05 M phosphate buffer (pH 6.2). Fractions, 1 ml in volume, were collected at a flow rate of 15 ml/hr, and those fractions with the
highest specific activity were combined. Unless otherwise noted, this preparation was used for all subsequent studies.

**Purification of Human Lung, Brain, and Heart RNases**

The procedure followed for the lung RNase was as described above, except that the specific activity and yield of the lung enzyme was slightly less than that of the kidney RNase. Nonetheless, a 10,000-fold purification was attained.

With respect to the brain and heart RNases, the preparation of the crude extract was altered because of the presence of a protein inhibitor. Both tissues were homogenized in 0.25 N H$_2$SO$_4$, which eliminated the need for a protamine sulfate precipitation (Step 2). The remaining steps in the purification were as described, yielding, in each case, enzyme preparations purified approximately 2600-fold.

**Properties of Plasma-type RNases**

**Hydrolysis of Synthetic Polyribonucleotides.** Each of the purified plasma-type RNases was compared to the plasma enzyme in its ability to hydrolyze a variety of synthetic polyribonucleotides (Table 5). The results suggest that the hydrolysis of each synthetic substrate was quite comparable to that found for the original plasma enzyme (31). Those copolymers, for example, that contained cytidylic acid residues were hydrolyzed most rapidly and, of the homopolymers poly(C), poly(A), poly(G), and polyuridylicate, only poly(C) was hydrolyzed at a significant rate.

**pH Optima.** All the plasma-type enzymes isolated from human brain, heart, lung, and kidney had pH optima similar to those reported for the plasma RNase (31). A pH curve for the brain enzyme, which was representative of all the enzymes studied, is presented in Chart 1. At pH 6.0 in

![Chart 1. pH curve for plasma-type RNases purified from human brain. The hydrolysis of poly(C) was measured in Tris-HCl (□) and sodium phosphate buffers (Δ) in the standard assay system, except that incubation was for 7.5 min and 12 units of enzyme were used. Similarly, the hydrolysis of RNA was assayed in Tris-HCl (△) and sodium phosphate buffers (○) with the use of the standard assay system with 12 units of enzyme. Buffer concentration was 0.1 M.](image)

**Table 6**

**Comparison of hydrolysis of synthetic polyribonucleotides by purified human plasma-type RNases**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Enzyme activity (nmoles/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
</tr>
<tr>
<td>poly(C)</td>
<td>44.8</td>
</tr>
<tr>
<td>poly(A,C) *</td>
<td>52.3</td>
</tr>
<tr>
<td>poly(A,C,U)</td>
<td>22.4</td>
</tr>
<tr>
<td>poly(A,U)</td>
<td>6.9</td>
</tr>
<tr>
<td>poly(A,G,U)</td>
<td>2.7</td>
</tr>
<tr>
<td>poly(A,G)</td>
<td>0.9</td>
</tr>
<tr>
<td>poly(G,U)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* The abbreviations used are: poly(A,C), random copolymer of adenylic and cytidylic acids; poly(A,C,U), random copolymer of adenylic, cytidylic, and uridylic acids; poly(A,U), random copolymer of adenylic and uridylic acids; poly(A,G,U), random copolymer of adenylic, guanylic, and uridylic acids; poly(A,G), random copolymer of adenylic and guanylic acids; poly(G,U), random copolymer of guanylic and uridylic acids.

phosphate buffer, hydrolysis of yeast RNA is minimal, whereas under the same conditions, the liver-spleen type RNase activity has been shown to have optimal activity (10, 25). Such differences in pH optima and in the hydrolysis of substrates permitted the plasma-type RNase and the liver-spleen type RNase to be differentiated as illustrated in Table 1.

**Inhibition by Poly(A).** It was of interest also to examine the effects both of polyamines and of ordered polynucleotides on enzyme activity since the plasma enzyme is known
to be affected greatly by these agents (18). It is apparent (Table 6) that low concentrations of poly(A) can inhibit completely all the activity of the RNases. In all cases, too, spermidine enhanced enzyme activity considerably and reversed poly(A)-induced inhibition of RNase activity. These results are in good agreement with those found for the plasma enzyme under the same experimental conditions.

**DISCUSSION**

Levels of plasma RNase have been reported to be elevated in certain neoplastic conditions (2, 11, 29, 34). The pathophysiology of these changes, however, is unclear since the tissue(s) of origin of this enzyme has not been established. In this study, an attempt has been made to identify the possible site or sites of origin of the plasma enzyme with the use of normal tissues.

In earlier studies, in which RNases from human liver, spleen, pancreas, and plasma were purified, the human enzymes differed in their properties from the plasma enzyme and is supported by our results (13). Leukocytes have also been suggested to be the source of serum RNase (11), but the leukocyte enzyme appears to have little effect (11). It has been suggested that the kidney is the sole source of serum RNase, but the fact that chronic pancreatitis and pancreatectomy has little effect on the plasma-type RNase per g of tissue. Therefore, in this study, an attempt has been made to determine the site of synthesis of plasma RNase under the same experimental conditions.

Our results suggest that any of a number of tissues could be possible sources of serum RNase. Therefore, we feel that the attribution of serum RNase to any 1 specific source may be premature at present. Evaluation of the relative contributions of various tissues to serum RNase levels must await further biochemical characterization of the tissue enzymes and also kinetic data on synthesis and secretion of RNase from these tissues.

With respect to the number of different RNases present in normal tissues and plasma, we have found only 2 different types, a plasma-type (31) and a liver-spleen type (10, 23) enzyme. Recent results of Akagi et al. (1) suggest the presence of 5 RNases (differing in molecular weights) in normal serum, but this may be a result of the aggregation phenomenon observed by Schmukler et al. (31).

The low level of the liver-spleen type enzyme in plasma (Table 2) may not be the case for individuals with neoplastic disorders that involve tissues that contain the liver-spleen type enzyme. Emphasis should be placed on the fact that assay conditions usually used in clinical studies for the measurement of crude plasma RNase activity may be inadequate for detection of the presence of the liver-spleen RNase (13).

**REFERENCES**

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