Complexing Rauscher Leukemia Virus Reverse Transcriptase with Human Plasma Ribonuclease from Hodgkin’s Disease Patients*

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Human ribonucleases were purified from the sera of Hodgkin’s disease patients by sequential column chromatography. The purified enzyme interacted with reverse transcriptase of Rauscher leukemia virus and formed an additive complex of $M_r = 130,000$. RNase and oligo(dG)-directed reverse transcriptase activities were diminished in the complex. The complex could be dissociated with the subsequent restoration of both activities in the presence of spermidine. The molecular weight of the complex suggest that the 2 RNase molecules bind to a single reverse transcriptase molecule.

It is well established that reverse transcriptase is a marker of RNA tumor viruses (1, 2). These viruses, commonly found in the lower animals, have been demonstrated to be major etiologic agents in the development of cancer within the species studied (3-6). Studies in man have been performed to determine whether such viruses play an analogous etiologic role in the development of human neoplasia but with little success. The presence of reverse transcriptase and tumor virus-specific RNA in human cancer has been described (7, 8), but the expression of other RNA tumor virus-specific proteins (p30, gp71) within human tumors or established human tumor cell lines has not been found (9). Recent work in this laboratory on both animal and human models (10, 11) suggests that RNase activity may be altered in the pre-cancer or cancer states. Long standing evidence indicating that elevation of serum nucleolytic activity is found in the human patient with neoplasia (10, 11).

To determine whether a more definitive relationship might exist between the change in RNase activity in the cancer state and the presence of oncornaviral information, we have elected to compare the activities of purified human ribonucleases obtained from six individuals with and without Hodgkin’s disease (four Hodgkin’s patients and two normals). Since the enzyme reverse transcriptase utilizes RNA as a template, RNases might have a potential controlling role relative to oncornaviral-induced DNA synthesis. An attempt was made to visualize this in determining if reverse transcriptase could in any way bind to serum RNase. In this connection, Temin reported the difficulties in separating out trace amounts of RNase activity during the purification of reverse transcriptase from avian myeloblastosis virus (12). On the other hand, reverse transcriptase from Rauscher leukemia virus could be readily freed of RNase activity by the use of affinity chromatography (13).

It is demonstrated in this communication that six RNase preparations obtained from human sera possess similar degradative properties relative to synthetic substrates or purified RNA. Of significance, however, is the specific binding of four of these purified RNase preparations to reverse transcriptase.

EXPERIMENTAL PROCEDURES

The “Experimental Procedures” are presented in the miniprint supplement following this paper.¹

RESULTS

Purification and Characteristics of RNase—RNase was purified 2000- to 3000-fold with respect to the original activity from each of six human sera. When subjected to Sephadex G-100 column chromatography or glycerol gradient centrifugation, the enzymes yielded a single peak of activity without further purification. Furthermore, when examined for DNase, DNA polymerase, and RNA polymerase activity, the preparations were found to be free of them. The purified enzymes when stored in a solution of 0.02% bovine serum albumin at −20°C were stable for about 1 month.

Molecular Weight of RNase—The molecular weight was estimated by Sephadex G-100 column chromatography using various molecular weight markers, e.g. blue dextran ($M_r = 2 \times 10^6$), bovine serum albumin ($M_r = 70,000$), ovalbumin ($M_r = 44,000$), pancreatic DNase ($M_r = 33,000$), pancreatic RNase ($M_r = 13,000$). A single peak of activity of human serum RNase was eluted from the column having a molecular weight

¹ “Experimental Procedures,” Figs. 1 and 4 and Table I are presented in miniprint immediately following this paper. (Figs. 1 and 4 and Table I were cited in the main paper.) Full size photocopies are available from the Journal of Biological Chemistry, 9905 Rockville Pike, Bethesda, Md. 20014. Request Document No. 77m-836, cite author(s), and include a check or money order for $1.00 per set of photocopies.
of 30,000 confirming the previously published data of Schmutzler et al. (15).

The molecular weight determination by sedimentation analyses of the RNases in a glycerol gradient (5 to 20%) previously calibrated with various molecular weight protein markers (e.g., bovine serum albumin, ovalbumin, pancreatic DNAse, and pancreatic RNase) yielded the same result. The molecular weight of RNases either from normal sera or from Hodgkin's patients were the same.

Substrate Specificity – RNases were tested with different kinds of substrates. Human serum RNase have high specificity for poly(C) but can hydrolyze both poly(U) and RNA with lower efficiency. Spermidine, putrescine, and spermine amplify the poly(C)-hydrolizing activity of human serum RNases to the same extent as reported earlier and no significant increase in activity was noted in the presence of Ca²⁺ or Mg²⁺ (15). Human serum RNases are quite stable in the presence of 1 mM dithiothreitol but 70 to 80% loss of activity was observed at 5 mM dithiothreitol. In the presence of dithiothreitol, spermidine was unable to restore the activity.

Synthetic polynucleotides like poly(G) or poly(X) inhibit the serum RNase activity to the same extent as reported earlier (15). The metal chelating agents such as phenol comcarbazide, o-phenanthroline, and ammonium thiocyanate had no effect on serum RNase activity.

Interaction of Purified Viral Reverse Transcriptase and Serum RNase – Preliminary studies with RNase from each individual serum indicated that when enzymatic activity was measured in the presence of differing concentrations of reverse transcriptase, a consistent decrease in RNase activity from 10 to 20% could be seen (Fig. 1, see miniprint supplement). If reverse transcriptase activity was examined in the presence of each RNase enzyme, a similar decrease in reverse transcriptase activity (20 to 25%) occurred, but only if the enzymatic activity was measured with oligo(dG)·poly(rC) for [3H]dGMP incorporation. When oligo(dT)·poly(rA) was used as a template for [3H]TMP incorporation, no difference in reverse transcriptase activity was apparent. Although the reduction in the respective activities of both enzymes was small, it was consistent, and suggested that formation of some type of complex between the two enzymes. In an attempt to visualize the formation of this complex more clearly, the two enzymes were subjected to glycerol gradient centrifugation either separately or as a mixture of the two. Shown in Fig. 2 are results of such a study. Reverse transcriptase when measured by either [3H]TMP incorporation or [3H]dGMP incorporation, is sedimented as a single entity of activity in Fractions 14 to 20 (Fig. 2). When, however, the mixture of both enzymes (i.e. RNase and reverse transcriptase) is centrifuged, two distinct peaks of reverse transcriptase activity can be seen (Fig. 2); a lighter component (Fractions 14 to 20) sedimenting in the gradient in the same position as before and an apparently heavier one sedimenting in Fractions 8 to 12. Of particular interest is that the [3H]dGMP incorporation in the heavier sedimenting fractions (8 to 12) is only about 10% of [3H]TMP incorporation. In the lighter fractions (14 to 20), [3H]dGMP incorporation is only about one-third of the other activity in accord with results reported previously for purified reverse transcriptase (13, 14, 22).

If these fractions were re-examined for RNase activity, a small percentage (3 to 10%) of the total activity was found to sediment in Fractions 8 to 12. However, most of the RNase activity could be found as a lighter component sedimenting in Fractions 20 to 28. In the absence of reverse transcriptase enzyme (i.e. when RNase alone was subjected to glycerol gradient centrifugation), all of the RNase activity was found as a lighter component in Fractions 20 to 28.

Effect of Polyamine on the Glycerol Gradient Fractions – Although the polyamine spermidine is known to stimulate RNase activity (15), the compound when tested under a variety of conditions showed no significant effect on reverse transcriptase activity.

In accord with this, as shown in Fig. 2, no significant stimulation of reverse transcriptase could be seen if the enzyme was sedimented alone in the glycerol gradient. Similarly, no significant stimulation of reverse transcriptase activity could be found in lighter fractions (14 to 20) or in heavier fractions (8 to 12) of the enzyme when assayed by [3H]TMP incorporation (Fig. 2). If the same fractions were examined for reverse transcriptase activity using [3H]dGMP incorporation, a 3- to 4-fold increase in enzyme activity was found in the heavier fractions, however, no change was apparent in the lighter ones. RNase activity, on the contrary, was stimulated considerably in the presence of spermidine, both in Fractions 20 to 28 and in the heavier fractions, 8 to 12, consisting of the complex between the reverse transcriptase and RNase. The RNase enzyme sedimenting alone also showed an increase in enzyme activity.

Dissociation of Reverse Transcriptase·RNase Complex –

![Fig. 2. Reverse transcriptase and RNase interaction. Seventy to one hundred nanograms of reverse transcriptase were mixed with 10 μg of RNase in the presence of 1 mM MnCl₂ at pH 6.2. The reaction mixture was incubated for 10 min at 37°C and then layered over a 4.4-ml glycerol gradient (5 to 20%) followed by centrifugation in a Beckman L2 65B ultracentrifuge in SW 65 rotor at 44,000 rpm for 16 h at 4°C. Equal (200 μl) aliquots were collected by puncturing the bottom of the tube. RNase activity was measured by using poly(C) as a substrate (15) and reverse transcriptase activity was determined either by [3H]TMP incorporation into (dT)₅·(rA)₅ or [3H]dGMP incorporation into (dG)₅·(rC)₅, as described under "Experimental Procedures." Each activity was assayed by using 20-μl aliquots from the gradient fractions. The arrows at the top of the panel indicate the peak of enzyme activity for either reverse transcriptase (RT) or RNase when each was run separately in the gradient. In panel A, [3H]TMP incorporation is shown, in panel B, [3H]dGMP incorporation, and in panel C, RNase activity. O—O, enzyme activity without spermidine; •—•, activity in presence of spermidine.
The polyamine, spermidine, has been shown to dissociate the aggregate consisting of protein bound to either polynucleotides or other proteins (23, 24). To determine if the polyamine was acting in a similar manner on the reverse transcriptase-RNase complex, the complex isolated as described above was incubated with spermidine at room temperature for 15 min. The mixture after being subjected to glycerol gradient centrifugation sedimented as a peak of RNase activity in Fractions 20 to 28 and as a peak of reverse transcriptase activity in Fractions 14 to 20. It should be noted that the sedimentation pattern for each enzyme was similar to the patterns obtained when each enzyme was subjected to glycerol gradient centrifugation alone (Fig. 3).

Molecular Weight of Reverse Transcriptase-RNase Complex—When glycerol gradient centrifugation was used to measure the molecular weight of the reverse transcriptase-RNase complex, the molecular weight was estimated to be 130,000.

Conditions Influencing Formation of Reverse Transcriptase-RNase Complex—A number of cations were examined for their effect on either the formation or the inhibition of the formation of reverse transcriptase-RNase complex. When reverse transcriptase activity in Fractions 8 to 12 is used as a measure of the formation of the complex, it can be seen that Mn"+ at 1 mm contributes greatly to its formation (Table I, see miniprint supplement).

Denaturation of reverse transcriptase by boiling for 5 min apparently prevented the formation of the complex since under a variety of conditions studied, no binding of the enzyme with serum RNase could be seen. Similar denaturation of the RNase protein also resulted in an inability to find evidence for binding between the two enzymes. From this it follows that there are probably specific conformational requirements for each enzyme to maintain for the complex to form.

Specificity of Interaction between Reverse Transcriptase and Serum RNase—The binding between serum RNase and Rauscher leukemia virus reverse transcriptase appeared to be limited to RNase obtained from sera of Hodgkin's disease patients (Table II). Although several attempts were made to bind serum RNase obtained from normal volunteers to reverse transcriptase, no evidence for complexing of any kind could be seen. To examine if the binding between the two enzymes was nonspecific, an attempt was made to bind the serum RNase from Hodgkin's or from normal sera to a variety of other proteins. Of interest is the that major viral specific protein, p30, did not bind to RNase obtained from either source (Table II), nor was there any immunological or physical evidence for the existence of a complex (e.g. the migration of a complex in the heavier region of the gradient) between p30 and human serum RNase (Fig. 4). Similarly, nonviral proteins also failed to show any binding to the serum RNase (Table II). Reverse transcriptase, on the other hand, did not bind to bovine pancreatic RNase.

DISCUSSION

Impetus for the present study arose because of suggestive evidence accumulating over the last 20 years relating alteration of nucleolytic activity with the presence of malignancy. More recent work has demonstrated that elevation of RNase activity exists not only within the cancer state but also within the pre-cancer state (25). Hodgkin's disease sera were selected

![Fig. 3. Dissociation of reverse transcriptase-RNase complex. The reverse transcriptase-RNase complex was treated with 2 x 10^-4 M spermidine at room temperature for 15 min and then layered over a glycerol gradient (10 to 30%) and processed as described in Fig. 2. Each activity (RNase or reverse transcriptase) was measured using 20-μl aliquots from the gradient fractions. The position in the gradient of the peak of activities of reverse transcriptase-RNase complex (Peak I) and each enzyme when run separately is shown by an arrow in the top part of the figure. a, reverse transcriptase activity after dissociation of the complex; b, RNase activity.

| Table II Specificity of reverse transcriptase-RNase interaction |
|---------------------------------------------------------------|
| Components | Reverse transcriptase-RNase complex |
| Hodgkin's disease patient RNase* + Reverse transcriptase | + |
| Hodgkin's disease patient RNase* + Bovine serum albumin | - |
| Hodgkin's disease patient RNase* + DNA polymerase α* | - |
| Hodgkin's disease patient RNase* + DNA polymerase β* | - |
| Hodgkin's disease patient RNase* + p30* | - |
| Normal human serum RNase* + Reverse transcriptase | - |
| Normal human serum RNase* + Bovine serum albumin | - |
| Normal human serum RNase* + DNA polymerase α* | - |
| Normal human serum RNase* + DNA polymerase β* | - |
| Normal human serum RNase* + p30* | - |
| Bovine pancreatic RNase + Reverse transcriptase* | - |

* + indicates the isolation of reverse transcriptase-RNase complex from glycerol gradient. - indicates no detection of such a complex in the glycerol gradient.

* RNase, purified from four Hodgkin's patients.

* Reverse transcriptase and p30 were purified from Rauscher leukemia virus.

* DNA polymerase α and DNA polymerase β were purified from mouse bone marrow cells JLS-V9.

* RNase, purified from two normal individuals.
for a more definitive study because of the known elevation of nucleolytic activity found in these patients (73%) (25). Four patients selected in this study have about a 2-fold higher serum RNase activity than the two other normal individuals.

Purification and partial characterization of serum RNase from two normal individuals and the four Hodgkin's disease patients demonstrated no significant difference between RNase with respect to molecular weight, template specificity, and their elution profile when subjected to column chromatography. Serum RNase from Hodgkin's disease patients appear, however, to complex specifically with Rauscher leukemia virus reverse transcriptase when a variety of other viral and nonviral proteins do not. Formation of the RNase-reverse transcriptase complex can be studied by the addition of Mn$^{2+}$ but not by a number of other divalent cations.

Although the exact molar ratio of RNase and reverse transcriptase in the complex is not known, it is suggested because of a molecular weight of about 130,000, that 2 RNase molecules bind to a single reverse transcriptase molecule. This assumes a molecular weight of 30,000 for the RNase and one of 70,000 for reverse transcriptase (13, 15).

The present observation demonstrating the binding of Hodgkin's disease serum RNase with Rauscher leukemia virus reverse transcriptase leading to the specific inhibition of dGMP incorporation suggests a potential controlling role of the RNase in the synthesis of viral DNA. Further work utilizing a variety of other type C virus reverse transcriptases and RNases obtained from Hodgkin's disease patients as well as other patients with neoplasia is now underway and may add to our understanding of these observations.

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Supplemental Material

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Supplemental Material

Tissue Preparation

The purification of reverse transcriptase from human leukemic tissue was carried out on a column of agarose-agarose 4B. The tissue was homogenized in 10 mM Tris-HCl, pH 8.0, and 100 mM NaCl. The homogenate was centrifuged at 2000 g for 10 min. The supernatant was then dialyzed against 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM DTT. The dialyzed supernatant was concentrated to a volume of 10 ml, and then applied to a column of agarose-agarose 4B. The column was washed with 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM DTT. The reverse transcriptase activity was eluted from the column with 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 50% (v/v) glycerol. The purified enzyme was then dialyzed against 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM DTT. The purified enzyme was then stored at -80°C until use.

Reverse Transcriptase

The purified enzyme was used to transcribe 16S and 28S rRNA from human leukemic tissue. The transcribed rRNA was then purified by ethanol precipitation and analyzed by agarose gel electrophoresis.

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Figure 1

Conditions for the formation of reverse transcriptase-esterase complexes

Table 1

| Condition | Reverse Transcriptase-esterase Complex | Reverse Transcriptase-esterase Complex | Reverse Transcriptase-esterase Complex |
|-----------|----------------------------------------|----------------------------------------|----------------------------------------|
| None      | 25 ± 0.5                                | 10 ± 0.5                               | 5 ± 0.5                                |
| Reverse transcriptase | 10 ± 0.5                               | 5 ± 0.5                                | 2.5 ± 0.5                              |
| Esterase  | 25 ± 0.5                                | 10 ± 0.5                               | 5 ± 0.5                                |
| Reverse transcriptase + Esterase | 25 ± 0.5                               | 10 ± 0.5                               | 5 ± 0.5                                |

* About 50 μg of reverse transcriptase was mixed with 10 μg of Esterase in each case. The reaction mixture was incubated at 37°C for 20 min.
* Each reaction contained 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.5 mM DTT, and 0.5 mM spermine.
* The enzyme activities were determined fluorometrically as described in Materials and Methods.

Figure 2

RNAse Reverse Transcriptase Interaction

R.N. Reverse Transcriptase Interaction 7787

Figure 1

pH 7.5

pH 8.0

pH 8.5

pH 9.0

pH 9.5

pH 10.0
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