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MECHANISM OF THE ACTION OF THE INHIBITOR OF REVERSE TRANSCRIPTASE

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An inhibitor of reverse transcriptase of Moloney leukemia virus was reported previously in the cytoplasm of the cultured cells (1). In this report, the mechanism of the inhibition was examined. The inhibitory activity was completely abrogated by treatment at 55°C for 20 min. Destruction of the reaction products of reverse transcriptase was not observed. Strong inhibition was observed by preincubation of poly rA oligo dT used as a template-primer, together with the inhibitor, but was not by incubation of poly rA or oligo dT with the inhibitor separately. It is suggested that the inhibitor reacts with poly rA oligo dT complex and interferes with attachment of the reverse transcriptase to the template-primer complex.

Reverse transcriptase (E.C. 2.7.7.7) is an enzyme capable of transcribing RNA into DNA (2, 3). It was first detected in the virus particles of RNA tumor viruses after disruption of the virions by treatment with non-ionic detergent. It plays an important role at the early stage of infection of RNA tumor viruses. DNA synthesis by reverse transcriptase is influenced by factors which are as yet poorly defined. We have previously reported the presence of an inhibitor of the reverse transcriptase of Moloney leukemia virus in the cytoplasm of cultured cells (1).

It had a molecular weight of 85,000, based on its rate of sedimentation in glycerol gradients. Because the inhibition is inactivated by treatment with proteases, it is a protein in nature. It is shown by pretreatment of the inhibitor with reverse transcriptase that the inhibitor does not inactivate the enzyme directly. In this report, a further examination of the behavior of the inhibitor is described.

MATERIALS AND METHODS

Cells. HeLa and 78A-1 (4) cells were propagated in roller bottles, which were rotated at 0.5-1.0 rpm. Eagle's minimum essential medium enriched with two-fold the normal concentration of aminoacids and vitamins was used for tissue culture with 10 % heat inactivated calf serum. The

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cultured cells were harvested by trypsin treatment and washed 3 times with phosphate buffered saline without Mg++ and Ca++.

Cytoplasmic fractions. The cultured cells ($2-4 \times 10^9$ cells in 4-6 roller bottles) were harvested by trypsinization, washed with phosphate buffered saline, and suspended in 10 volumes of 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5 % NP-40 and 10 mM 2-mercaptoethanol. The cells were kept for 20 min. in an ice bath, broken with a Dounce homogenizer and centrifuged at 1,000 xg for 10 min. The supernatant was collected and stored at -80°C until use.

Reverse transcriptase. Purification of reverse transcriptase of the virions of Moloney leukemia virus was described previously (5).

Glycerol gradient centrifugation of the inhibitor. Glycerol gradient centrifugation of the inhibitor was performed as described below. 5-20 % (V/V) glycerol gradients were prepared in 10 mM Tris-HCl pH 7.4, 0.1 M KCl, 10 mM 2-mercaptoethanol and 0.2 % NP-40. Glycerol gradient columns were centrifuged at 40,000 rpm for 17 hours in a Beckman SW 50.1 rotor. After fractionation of the gradient columns (200ul/fraction), 10-20 units of reverse transcriptase or E. coli DNA polymerase I were added to each fraction. After incubation at 37°C for 30 min., the activity of the reverse transcriptase was assayed.

Assay of inhibitory activity of the inhibitor. The inhibitor and the purified reverse transcriptase were mixed, incubated for 30 min. at 37°C, and the activity of the reverse transcriptase was assayed as described before (5). Briefly, in a final volume of 100ul, the reaction mixture for reverse transcriptase assay contained 50 mM Tris-HCl pH 8.1, 1 mM manganase acetate, 60 mM KCl, 5 mM dithiothreitol, 0.02% NP-40, 5ug/ml $(dT_{12-18})(rA)_n$ (P-L Biochemicals, Inc.) and 25 uC/ml [^{3}H] dTTP (specific activity 53.7 C/mmol). Incubation was carried out at 37°C for 30 min. and the radioactivity incorporated into the acid insoluble form was assayed.

RESULTS AND DISCUSSION

Heat inactivation of the inhibitor. The inhibitor was incubated at 55°C and 60°C. Fig. 1 shows that the inhibitor was completely inactivated by treatment at 55°C for 20 min. or at 60°C for 10 min. In order to

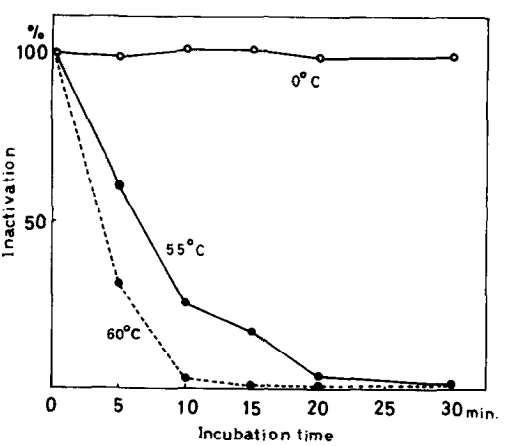

Fig. 1. Heat inactivation of the inhibitor. 10 units of the inhibitor were used in each reaction. The numbers on the ordinate indicate the percent of inactivation.
(o——o) 0°C, (●——●) 55°C, (●·····●) 60°C

inactivate the inhibitor, it is usually treated at 55°C for 20 min.

Treatment of the reverse transcriptase reaction product with the inhibitor.

In order to examine whether the inhibitory activity of the inhibitor resulted from the destruction of the reaction products of reverse transcriptase, the inhibitor was incubated with these reaction products.

(a) The reverse transcriptase was incubated at 37°C in the complete reverse trascriptase reaction mixture for 10, 20 and 30 min. and heated at 55°C for 10 min. in order to inactivate it. After heat inactivation, the inhibitor was added to the reaction mixture and incubated at 37°C for 30 min.

(b) The reverse transcriptase was incubated in the same reaction mixture at 37°C for 30 min. and heated at 55°C for 10 min. The inhibitor was added to the reaction mixture and incubated at 37°C for 10, 20 and 30 min.

The results were shown in Fig. 2. No difference was observed between with and without the inhibitor. It is concluded that the destruction of the reaction products of the reverse transcriptase by treatment with the inhibitor was not the cause of the inhibition.

Incubation of the reaction mixture of the reverse transcriptase with the inhibitor. As reported previously (1), the inhibitor did not inactivate the reverse transcriptase molecules directly. In order to examine whether the inhibitor reacts with template, primer and/or substrates of

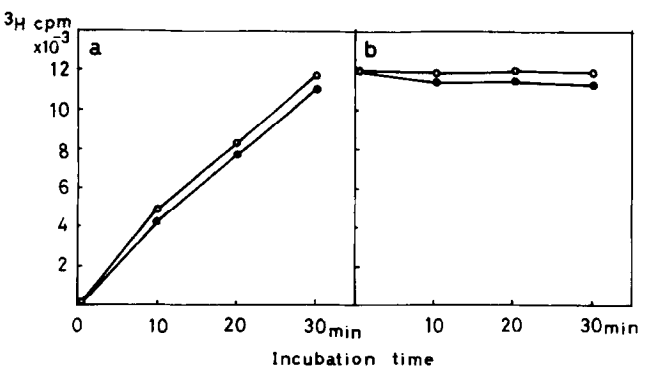

Fig. 2. Treatment of reverse transcriptase reaction product with the inhibitor. The numbers on the ordinate indicate the acid insoluble radioactivity.
(o——o) without the inhibitor
(●——●) with the inhibitor

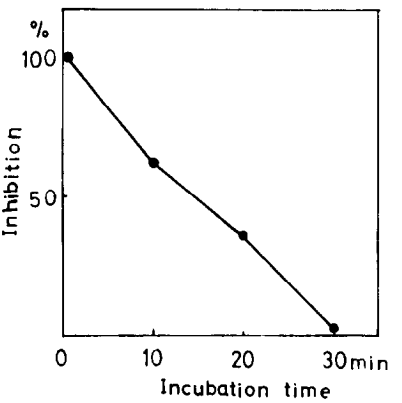

Fig. 3. Treatment of the reaction mixture with the inhibitor. The ordinate shows the percent inhibition calculated as follows:

$$\frac{\text{with the inhibitor}}{\text{without the inhibitor}} \times 100$$

DNA synthesis by reverse transcriptase, the reaction mixture for the assay of reverse transcriptase was preincubated with the inhibitor. The inhibitor was incubated with the reaction mixture at 37°C for 10, 20 and 30 min. and heated at 55°C for 20 min. The reverse transcriptase was added to the heated reaction mixture and incubated at 37°C for 30 min. Incubation of the reaction mixture without the inhibitor at 55°C for 20 min. did not affect DNA synthesis by the reverse transcriptase.

Fig. 3 shows that preincubation of the reaction mixture with the inhibitor inhibited the reverse transcriptase activity. It is suggested that the inhibitor reacts with template, primer or substrate of reverse transcriptase reaction.

Recovery of activity of reverse transcriptase inhibited by pretreatment of the reaction mixture with the inhibitor. In order to examine which component(s) of the reaction mixture was responsible for inhibition of reverse transcriptase by the inhibitor, the reaction mixture was incubated with the inhibitor at 37°C for 30 min. After treatment at 55°C for 20 min., poly rA, oligo dT, poly rA oligo dT or dTTP was added to the reaction mixture. After addition of reverse transcriptase, this mixture was incubated at 37°C for 30 min. and the activity of the reverse transcriptase was assayed.

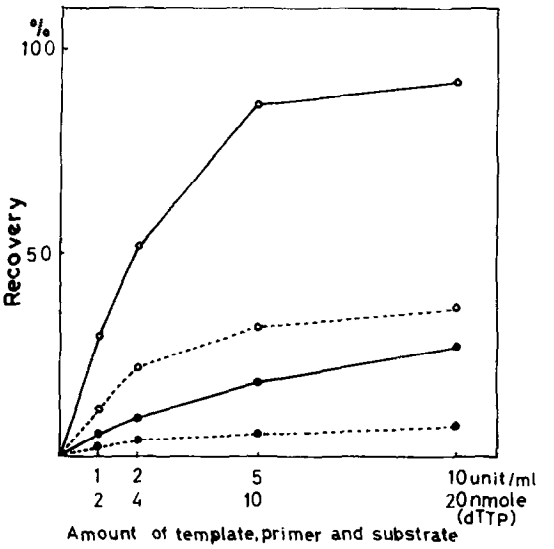

Fig. 4. Recovery of activity of reverse transcriptase inhibited by pretreatment of the reaction mixture with the inhibitor. The numbers on the ordinate indicate the percent of recovery calculated as follows:

$$\frac{\text{with the inhibitor}}{\text{without the inhibitor}} \times 100$$

(o——o) poly rA oligo dT, (o·····o) poly rA
(●——●) oligo dT, (●·····●) dTTP

Fig. 4 shows that when poly rA oligo dT was added to the reaction mixture, the recovery of activity of the reverse transcriptase was 92 %, but when only poly rA or oligo dT was added, the recovery was 36 % or 28 %, respectively. Addition of dTTP did not result in the recovery of the activity of the reverse transcriptase. It appears that the inhibitor reacts with the poly rA oligo dT complex but does not react with poly rA and oligo dT individually.

Preincubation of template, primer or substrate with the inhibitor.

To confirm the above observations, poly rA, oligo dT, poly rA oligo dT or dTTP was incubated with the inhibitor. After treatment at 55°C for 20 min., the matching reaction mixture, that is,with the already present template, primer, template-primer complex or substrate here omitted, was added, respectively.

After addition of the reverse transcriptase, the reaction mixture was incubated at 37°C for 30 min. and the activity of the enzyme was assayed.

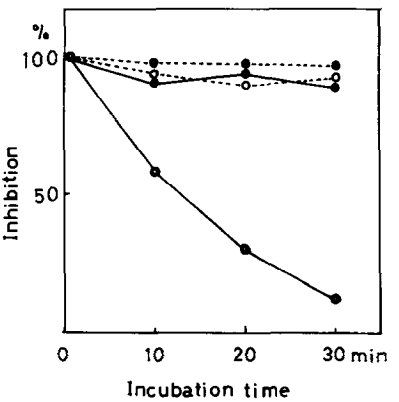

Fig. 5. Preincubation of template, primer or substrate with the inhibitor. The numbers on the ordinate indicate the percent of inhibition calculated as follows:

$$\frac{\text{with the inhibitor}}{\text{without the inhibitor}} \times 100$$

(o——o) poly rA oligo dT, (o·····o) poly rA
(●——●) oligo dT, (●·····●) dTTP

Fig. 5 shows that incubation of poly rA oligo dT complex with the inhibitor suppressed the activity of the enzyme. Preincubation of poly rA, oligo dT or dTTP with the inhibitor did not affect the activity of the enzyme. It was noted that the inhibitor reacted with the poly rA oligo dT complex but not with poly rA, oligo dT or dTTP.

The inhibitor does not inhibit E. coli DNA polymerase I (1), when poly dA olgo dT was used as template-primer. But when poly rA oligo dT was used, E. coli DNA polymerase I was inhibited by the inhibitor. These findings suggested that inhibition of reverse trascriptase activity by the inhibitor did not result in the destruction of template-primer and reaction products. Also, destruction of poly rA and oligo dT by the inhibitor was not observed (data not shown). Treatment of poly rA oligo dT with the inhibitor was effective after incubation at 55°C for 20 min., though the inhibitor was inactivated by incubation at 55°C for 20 min. It is suggested that the inhibition of the reverse transcriptase might be the result of an irreversible attachment of the inhibitor to the template-primer. Further, it is supposed that the inhibitor is able to distinguish between poly rA and poly dA in the presence of oligo dT.

A variety of agents were reported to inhibit reverse transcriptase activity. One general class of inhibitors covalently attaches to functional groups of the enzyme (6,7,8). Another general class of inhibitors binds templates (9,10). A third class of inhibitors competes either for the template or primer or for the substrate. The inhibitor reported here may very well attach to the template-primer complex specifically.

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