Effects of Poly(1-vinyluracil) and Poly(9-vinyladenine) on Viral RNA-directed DNA Polymerase

Josef Pitha

SUMMARY

The effects of poly(1-vinyluracil) [poly(vU)] and poly(9-vinyladenine) [poly(vA)] on the RNA-dependent DNA polymerase activity of murine leukemia virus (Moloney strain) were studied. Vinyl polymers themselves cannot act as templates for the polymerase. However, if a vinyl polymer is added to a polymerase reaction mixture in which a complementary polynucleotide serves as the template, the reaction is inhibited: thus with polyribocytidylic acid as template and oligodeoxyguanylic acid as primer, neither poly(vU) nor poly(vA) had a significant effect; when polyribouridylic acid was used as template and oligodeoxyadenylic acid as primer, poly(vA) inhibited polymerase activity while poly(vU) had little effect; when polyriboadenylic acid was a template and oligodeoxy thymidyllic acid was a primer, poly(vU) was an inhibitor. Complex effects were noted with the latter system and poly(vA); either stimulation or inhibition of the reaction was observed, depending on the concentration of poly(vA). The stimulation brings about a decrease in the amount of lower-molecular-weight materials in the product and is caused by the interaction of poly(vA) with the template-primer. Thus vinyl polymers differ from polynucleotides in their mechanism of inhibition of viral polymerase, since the latter inhibit the enzyme by binding to it.

INTRODUCTION

Viral replication can be specifically inhibited by only a few low-molecular-weight compounds. The mechanisms of their antiviral actions are difficult to elucidate fully, and no easy approach to the design of more effective derivatives can be expected. Consequently, the possibility of blocking viral replication by nucleic acids is an attractive alternative. Compounds with more specific action can eventually be developed; the design of such compounds may be based on the considerable amount of knowledge being accumulated in the field of viral nucleic acids. Even polynucleotides and their analogs exert some influence on viral replication. Poly(rU) and poly(rA) alone, without their corresponding primer, interfere with RNA-dependent DNA polymerase (reverse transcriptase), an enzyme contained in the virions of Rauscher leukemia virus (19). Poly(rA) also inhibits the replication of MLV in cell culture (18). In previous work (14), we found that the replication of MLV in cell culture is inhibited not only by both of these easily degradable polynucleotides, but also by their nondegradable analogs, poly(vU) and poly(vA). The data indicate that the virus is blocked in some of the early stages of its replication cycle, possibly those which involve reverse transcriptase, while the later stages of replication are not affected by the polymeric inhibitors (13, 17). To gain further insight into this phenomenon, we have studied the effects of vinyl polymers on reverse transcriptase in detail and have found that the mechanism of action of the vinyl polymers is different from that of the polynucleotides.

MATERIALS AND METHODS

Samples of vinyl polymers and polynucleotides were of the same origin as previously described; all compounds have average molecular weights greater than 10^6 (14). Oligodeoxynucleotides (n = 14 to 18) were purchased from Collaborative Research, Inc., Waltham, Mass. The molarities of all polymers or oligomers are calculated per base residue. The suspension of MLV was purchased from Electronucleons Laboratories, Inc., Bethesda, Md., and contained 0.3 mg protein per ml. Radioactive deoxyribonucleoside triphosphates ([Me^3H]dATP, 17.4 Ci/mmole; [8-^3H]dGTP, 10.2 Ci/mmole) were purchased from Schwarz/Mann, Orangeburg, N. Y.

For immobilization of poly(vA), a washed sample of 15 ml of Sepharose 4B200 (Sigma Chemical Co., St. Louis, Mo.) activated by BrCN (1), was stirred with a solution of 50 mg poly(vA) in 30 ml of 0.1 M borate buffer (pH 9.5) overnight at 4°C. The beads, after exhaustive washing, gave a deep orange color in the test with trinitrobenzenesulfonic acid (6), and 10 µl of beads had the capacity to adsorb 130 nmoles of poly(rU) at room temperature. The immobilization was com-
The reaction mixture (100 µl) was prepared in the following way. A solution of the primer oligodeoxynucleotide and vinyl polymer was added to an aqueous solution of the polyribonucleotide template. Then 5-µl portions of stock reagents were added, forming the following final concentrations: 50 mM Tris-HCl (stock solution had pH 8.3 at 37°), 60 mM NaCl, 20 mM DTT, 0.05% Triton X-100, 1 mM MnCl₂, and 5 µl of the MLV suspension. This mixture was incubated for 10 min at 37°, and the reactions were started by the addition of deoxynucleoside triphosphate solution (free of ethanol). Incubations were performed at 37°; samples of 10 µl were taken at 0, 15, 30, 45, and 60 min, spotted on Whatman No. 3MM paper discs and processed by the technique developed by Bollum as described previously (14). The initial rate of reaction was determined by the tangent method from the plot of cpm versus time. With every individual experimental run, a standard assay (as specified) was included and used for the normalization of the other results. Under conditions of the standard assay of Table 1 and Chart 1, the following cpm values were obtained in 5 independent experiments: 0 min, 57 cpm (S.D., 8); 5 min, 525 cpm (S.D., 21); 30 min, 1000 cpm (S.D., 26); 45 min, 1475 cpm (S.D., 30); 60 min, 1925 cpm (S.D., 33). Results in Table 1, Experiment 1 indicate that all reaction components are near saturation or optimal concentration, and the rate of reaction is governed by the amount of lysed virus. Furthermore, the system is not affected in any critical way by degradative processes: the addition of fluoride ions and ATP, chemicals that are known to protect the template primer against hydrolysis and regenerate the hydrolyzed deoxynucleoside triphosphate (15), is without effect (Table 1, Experiment 2). Similarly, in the system no critical radiation damage occurs, as the addition of a protective agent, catalase (9), is without any effect (Table 1, Experiment 2).

The separation of radioactive products from radioactive substrate by acid precipitation in the presence of poly(vA) could give anomalous results: electroneutral poly(vA) is transformed by protonization into a polycation, presence of which might considerably change the efficiency of separation. To check for the possibility of such artifacts, in several experiments we used an alternative precipitation by cetyltrimethylammonium bromide. A recent modification of this procedure (16) gave high background counts, but after the appropriate corrections were made, values parallel to the acid-precipitated samples were obtained.

Study of reverse transcriptase binding to immobilized poly(vA) was carried out at 4° with a column 0.9 cm in diameter and containing 4 ml of beads. The suspension of MLV (30 to 90 µl) and 60 µl of column buffer (60 mM KCl; 10 mM K₂HPO₄, pH 7.1; 1 mM DTT; 0.1% Triton X-100) was incubated at 37° for 10 min, placed on the column, and eluted with the same buffer. From the collected fractions, 75-µl samples were taken and assayed for reverse transcriptase activity as described above and in Chart 1, with the exception that the concentration of nonradioactive dTTP was decreased to 50 µM, and the acid-insoluble radioactivity contained in the whole assay (100 µl) was measured after 60 min of reaction time.

For the rate zonal sedimentation analysis, the polymerase assay mixtures after a 45-min reaction were processed by bringing them to the following concentrations: 0.15 mM poly(vA), 1% sodium dodecyl sulfate, 0.4 mM NaOH, 0.2 M NaCl, 4 mM EDTA, and DNA, 40 µg/ml. The mixtures were then incubated at 80° for 1 hr (3). The resulting solution was layered over 4.8 ml of a 5 to 25% linear sucrose gradient containing 0.2 M NaOH, 2 mM EDTA, and DNA, 20 µg/ml. Gradients were centrifuged at 45,000 rpm for 135 min at 20° in a SW50.1 rotor and were processed as described previously (4).

**RESULTS**

Assay conditions were such that viral RNA is not used as template by viral polymerase (2, 14); vinyl polymers also cannot function as template even in the presence of the corresponding primers (14), and, as mentioned in “Materials and Methods,” all components of the assay mixture are at nearly optimal concentration and the degradative processes are without noticeable influence.

**Effects of Poly(vU) on the Polymerase Reaction.** Poly(rA)

### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Conditions as described in “Materials and Methods” and legend of Chart 1.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>Standard mixture</td>
<td>1.00</td>
</tr>
<tr>
<td>Poly(rA) 3 times increased</td>
<td>1.13</td>
</tr>
<tr>
<td>Oligo(dT) 4 times decreased</td>
<td>0.80</td>
</tr>
<tr>
<td>Oligo(dT) 3.5 times increased</td>
<td>1.24</td>
</tr>
<tr>
<td>Triton X-100 5 times decreased</td>
<td>0.32</td>
</tr>
<tr>
<td>Mn²⁺ 2 times decreased</td>
<td>0.48</td>
</tr>
<tr>
<td>Mn²⁺ 2 times increased</td>
<td>0.78</td>
</tr>
<tr>
<td>MLV 3 times increased</td>
<td>3.33</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>Standard mixture plus 15 mM NaF</td>
<td>1.00 plus 0.15 mM poly(vA) 1.74</td>
</tr>
<tr>
<td>NaCl concentration decreased to 45 mM plus catalase, 50 µg/ml</td>
<td>1.07 plus 0.15 mM poly(vA) 1.60</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
</tr>
<tr>
<td>Standard mixture plus bovine serum albumin, 1 mg/ml</td>
<td>1.00 plus 0.15 mM poly(vA) 1.59</td>
</tr>
</tbody>
</table>

CANCER RESEARCH VOL. 36
in the presence of a primer, oligo(dT), is a very efficient template for dTTP incorporation; poly(vU) inhibits this reaction. The concentration dependence of inhibition is shown in Chart 1. The complex of poly(vU) with poly(rA) has a 9:1 molar ratio of components (12), and full inhibition begins only when the entire template is complexed. The nature of the inhibition was further analyzed by variation of the template-primer concentration. The results (Chart 2) show that poly(vU) inhibition is prevented by a high concentration of template-primer and that maximum velocities areapproximately the same for both the inhibited and uninhibited reactions.

When poly(vU) was added to the system where poly(rU) and oligodeoxyadenylic acid were template and primer, respectively, no significant effects on dATP incorporation were detected (Chart 3, upper graph). A similar situation was found for the polyribocytidylic acid templated reaction [Table 2 and independent observations (7)].

Effects of Poly(vA) on the Polymerase Reaction. When poly(vA) was added to the poly(rU)-oligodeoxyadenylic acid templated reaction, the incorporation was inhibited in a concentration-dependent fashion (Chart 3, lower graph).

Poly(vA) did not have significant effects on dGTP incorporation using polyribocytidylic acid as template and oligodeoxyguanylic acid as primer (Table 2).

Previously, we reported that the addition of a low concentration of poly(vA) to a polymerase reaction where poly(rA) and oligo(dT) were used as template and primer considerably increased the rate of dTTP incorporation (14). Upon more detailed investigation we have found this phenomenon rather complex; low concentrations of poly(vA) increase the incorporation while high concentrations decrease it (Chart 4). Components of the assay are at nearly optimal concentrations (Table 1); thus the observed increase cannot be due to a simple change in effective concentration of some reagent that is achieved through complexing of reagent with poly(vA). The dependence of poly(vA) effects on template-primer concentrations was further studied. When the results were plotted in a double reciprocal plot, it became apparent that the stimu-
anion-polymerase binding, irrespective of the possibility of base pairing between the inhibitor and template (19). The same mechanism was proposed for the inhibition of viral polymerase by a synthetic polyanion, pyran copolymer (10). In agreement with such a mechanism, the viral polymerase is strongly bound to oligo(dT) immobilized on cellulose (8) or pyran copolymer immobilized on Sepharose (5). Both polynucleotides and pyran copolymer have polyanionic character, and the inhibition of viral polymerase is probably dependent on that feature.

The effects of electroneutral poly(vU) and poly(vA) on viral polymerase are summarized in Table 3. The inhibition is strongly dependent on the template-primer used, which indicates that interaction between the inhibitors and poly-
nucleotides are of primary importance. The results on binding of polymerase to immobilized poly(vA) confirm this interpretation; the interaction between enzyme and inhibitor is nonspecific and occurs only in the absence of other proteins and thus is of very limited importance for in vivo situations. Thus, in contrast to polynucleotide inhibitors, vinyl polymers act on viral polymerase primarily through their interaction with template-primer.

In systems in which base pairing between the vinyl polymer and template occurs, the template is apparently inaccessible to the enzyme. The reaction templated by poly(rA) can form base pairs with the primer, oligo(dT), as well as a weaker complex with poly(vA) (probably by intercalation; cf. Ref. 11). This complexity of interactions is reflected in the complexity of the observed poly(vA) effects: stimulation at low concentration and inhibition at high concentration of poly(vA). A rigorous assessment of the relative importance of base pairing and intercalation for the observed effects can be made only after the specific mechanisms of initiation and elongation steps of the polymerase reaction are better understood.

The biological effects of poly(vA) are markedly selective; the replication of leukemia viruses is reduced while the induction of leukemia viruses, the growth of the host cells, and the replication of nononcogenic viruses are not affected (13, 14). The present results show that this selectivity is not achieved through a specificity of interaction of poly(vA) with viral polymerase.

A possible explanation of the observed selective action of poly(vA) lies in its polymeric character. Poly(vA), as other polymers, diffuses slowly and tends to stay localized; the resulting uneven intracellular distribution of poly(vA) may result in its selective antiviral effects. One can speculate that the specificity of other antiviral compounds could be increased in a similar way, i.e., by incorporating them into polymers.

ACKNOWLEDGMENTS

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REFERENCES


Table 3

Summary of the effects of vinyl polymers on the incorporation of deoxynucleoside triphosphates in the MLV system

<table>
<thead>
<tr>
<th>Vinyl polymer</th>
<th>Template</th>
<th>None</th>
<th>Poly(vV)</th>
<th>Poly(vA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>No incorporation</td>
<td>No incorporation</td>
<td>Not investigated</td>
<td>No incorporation</td>
</tr>
<tr>
<td>Poly(rA)</td>
<td>Incorporation</td>
<td>Inhibited</td>
<td>Inhibited</td>
<td>Bimodal</td>
</tr>
<tr>
<td>Poly(rU)</td>
<td>Incorporation</td>
<td>No effect</td>
<td>No effect</td>
<td>Inhibited</td>
</tr>
<tr>
<td>Poly(rC)*</td>
<td>Incorporation</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>

* Poly(rC), polyriboctydyl acid.
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