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Inhibition of Murine Leukemia Virus Replication by Poly(vinyluracil) and Poly(vinyladenine)

(vesicular stomatitis virus/Sindbis virus/RNA-dependent DNA polymerase/viral chemotherapy)

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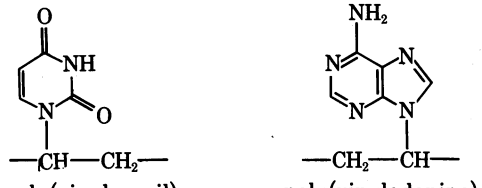
ABSTRACT **Poly(1-vinyluracil) and poly(9-vinyladenine), as well as the corresponding polynucleotides poly(uridylate) and poly(adenylate), inhibit acute murine leukemia virus infection in mouse-embryo cells, but they do not significantly inhibit the replication of Sindbis and vesicular stomatitis viruses. The polymers were most effective as inhibitors when added during an early stage of virus replication. Effects of vinyl polymers on the RNA-dependent DNA polymerase from the virions of murine leukemia virus were also observed.**

The replication of some RNA viruses, including oncogenic viruses, can be inhibited *in vitro* by polymers that are—to some degree—similar to the viral genome (1, 2). This observation suggests that it may be possible to design macromolecules that would inhibit viral replication without interfering significantly with host-cell metabolism, since the viral replicases have not been found in host cells. In addition, macromolecules are taken into cells through pinocytotic vesicles, rather than by diffusion through the cell membrane as are substances of low molecular weight. Since high pinocytotic activity appears to be characteristic of many tumor cells, macromolecules could be expected to be taken up preferentially by some types of neoplastic cells (3, 4).

In this work, we report that even some very distant analogs of polynucleotides can interfere effectively with virus replication in tissue culture and with the activity of viral RNA-directed DNA polymerase *in vitro*. The active compounds studied are vinyl analogs of polynucleotides, poly(1-vinyluracil) (5) and poly(9-vinyladenine) (6) (Scheme I). These



poly(vinyluracil) poly(vinyladenine)

SCHEME I

polymers (molecular weight more than 10^5) are electrically neutral, stable to chemical and enzymatic hydrolysis, and form complexes with complementary polynucleotides. These complexes do not have the simple stoichiometry found in nucleic acids; however, they resemble strongly distorted helical complexes of nucleic acids when examined by electron microscopy (7). The structural differences between vinyl polymers and polynucleotides, and the lack of sugar moieties and phosphate groups in the vinyl polymers, are probably the reasons why such polymers cannot serve as templates for transcription or translation. However, this lack of template activity does not mean that vinyl polymers are without any effect on the template-catalyzed enzymatic reactions. By complexing with template, they can interfere with its function *in vitro* (ref. 8 and manuscript in preparation). In this report, we show that selective inhibition of viral replication can be achieved with polynucleotides and vinyl polymers both *in vitro* and *in vivo*, probably by similar mechanisms.

Abbreviation: MLV, murine leukemia virus.

MATERIALS AND METHODS

Polymer Samples. Preparation of poly(vinyladenine) and poly(vinyluracil) was described (5–7). Polyuridylic acid [poly(U)] and polyadenylic acid [poly(A)] were commercial preparations (Miles Laboratories, Inc.); concentrations are expressed on a monomer or nucleotide basis, as determined spectrophotometrically.

Inhibition of DNA Polymerase Activity. Moloney mouse leukemia virus (MLV) was purchased from Electro Nucleonics Laboratories, Bethesda, Md. It had been purified by two cycles of banding in a sucrose gradient and contained 10^{12} particles per ml. Glycerine was added to the virus suspension to a final concentration of 8%, and the suspension was kept at 4°. The polymerase activity was stable for several months. [^{3}H]TTP (in 50% ethanol), Schwarz Mann Lot XR-2334, had a specific activity of 40 Ci/mmol and a concentration of 0.5 mCi/ml. Incubation temperature was 37°, and acid-precipitable radioactivity was measured by the filter-paper disc technique (9). Background counts were less than 75 cpm, with a counting efficiency of 6% for [^{3}H]TTP and 32% for [^{3}H]DNA; the methods are given in ref. 10.

Cells and Viruses. Mouse-embryo cells, NIH 3T3 cells, human-embryo lung cells WI-38 (passage 30), baby-hamster kidney cells (BHK), L cells, and the secondary chick-embryo cells were grown in Eagle's minimal medium supplemented with 10% fetal-calf serum.

Vesicular stomatitis virus, New Jersey serotype, was propagated in DEAE-dextran (10 μg/ml)-treated (mouse) L cells infected at low multiplicity and harvested 24 hr later, when the titer was 10^9 plaque-forming units (PFU)/ml. Sindbis

virus was propagated in DEAE-dextran-treated BHK cells, harvested at 24 hr, at a titer of 10^8 PFU/ml. Murine leukemia virus (Moloney strain) was prepared from lysates of NIH Swiss mouse-embryo cells grown in tissue culture. The virus preparations were passed through a 0.22-μm membrane filter (Millipore Co.) immediately before use, and titrated on NIH 3T3 cells.

One-step growth curves were obtained by infection of 3T3 cells with vesicular stomatitis virus and WI-38 cells with Sindbis virus at high multiplicity [multiplicity of infection (MOI) = 100]. Virus titers were assayed under standard conditions with L cells for formation of vesicular stomatitis virus plaques and secondary chick-embryo cells for Sindbis virus.

MLV was replicated in NIH Swiss mouse-embryo cells, which were treated for 1 hr with DEAE-dextran (25 μg/ml) 4 hr before inoculation with virus; virus titer was determined 5 days later by use of the XC test (11).

RESULTS

Effects on cells in tissue cultures

For the purpose of comparison, the effects of poly(A) and poly(U) were investigated in parallel with those of poly(vinyladenine) and poly(vinyluracil). Since even small changes in the growth and metabolism of the host cell have significant effects on the replication of oncogenic RNA viruses (12), we determined the relative cellular toxicity of the polymers. Poly(vinyladenine) and both polynucleotides, even at a high concentration (1 mM), do not show any detectable effect on the growth rate of mouse fibroblasts (mouse-embryo cells and 3T3 cells). However, 1 mM poly(vinyluracil) shows a selective toxicity on rapidly growing cells, while its effect on the viability of nongrowing cells is negligible (Fig. 1); 10–100 μM poly(vinyluracil) did not influence the growth rate of the cells. Similar results were obtained when the effects of polynucleotides and vinyl analogs on DNA synthesis in these cells (measured by incorporation of [^{3}H]thymidine) were studied. All four polymers are without inhibitory effect at 0.1 mM concentration, and only poly(vinyluracil) is inhibitory at 1 mM.

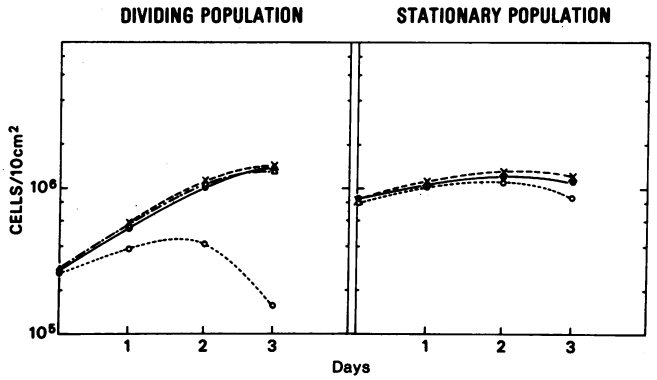


FIG. 1. Effect of poly(vinyluracil) on 3T3 cells. Growth curves were measured on NIH 3T3 cells inoculated at 1×10^5 cells per petri dish, 24 hr before addition of poly(vinyluracil) in growth medium [10% fetal-calf serum]; for measurements of the effect on nondividing populations of cells, different concentrations of poly(vinyluracil) in growth medium [10% fetal-calf serum] were added on confluent 3T3 monolayers (1 day). The ability of the cells to exclude trypan blue was used as a criterion of viability (27); only viable cells were counted. ×——×, control; △——△, 10 μM; ●——●, 100 μM; O- - -O, 1 mM.

TABLE 1. *Inhibition of acute MLV infection by poly(vinyladenine) and poly(A)*

Polymer	Hours of treatment	Plaques per plate	% Inhibition
—	—	106, 126	
Poly(vinyladenine)	−2→ 0	66, 36	60
(1 mM)	0→ 6	20, 26	80
	6→ 12	46, 46	60
	12→ 18	63, 72	40
	18→ 24	90, 80	30
	0→ 12	16, 28	80
	0→ 24	31, 27	80
Poly(A)	−2→ 0	4, 3	100
(1.3 mM)	0→ 6	1, 0	100
	6→ 12	74, 48	50
	12→ 18	74, 90	30
	18→ 24	79, 93	30
	0→ 12	1, 0	100
	0→ 24	2, 0	100

Effects on virus replication *in vivo*

We next investigated the effects of the polymers on the replication of MLV in tissue culture and found that, at subtoxic concentrations (on cell viability), all four polymers inhibit acute infection. When the two ribopolymers are compared with each other (Tables 1 and 2), it can be seen that poly(A) is a stronger inhibitor; it is able to completely inhibit virus replication at concentrations as low as 30 μM; poly(U), which is a very effective inhibitor *in vitro* (ref 2, and see below) seems to be less effective against virus in tissue culture. The extent of inhibition of MLV replication by poly(A) is in agreement with the recent observation of Tennant *et al.* (13). However, in their system poly(U) was inactive; this difference in activity may be due to the difference in poly(U) preparations used. Factors such as their molecular weight are of critical importance for the activity of polynucleotides, as the extent of cellular uptake of macromolecules seems to be directly proportional to their molecular weight (14).

Table 1 compares the effects of treatment with poly(A) and poly(vinyladenine), either before or after inoculation

TABLE 2. *Inhibition of acute MLV infection by poly(vinyluracil) and poly(U)*

Polymer	Hours of treatment	Plaques per plate	% Inhibition
—	—	22, 23	–
Poly(vinyluracil)	−2→ 0	11, 9	60
(0.1 mM)	0→ 6	24, 12	20
	6→ 12	11, 18	40
	12→ 18	8, 18	40
	18→ 24	11, 19	30
Poly(U)	−2→ 0	6, 6	70
(0.1 mM)	0→ 6	8, 8	60
	6→ 12	14, 19	30
	12→ 18	20, 21	10
	18→ 24	22, 23	0

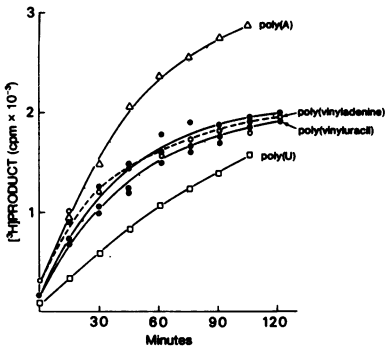


FIG. 2. Inhibition of DNA polymerase activity of MLV by polymers at a low concentration of detergent. Concentration of polymers was 1 mM, except for poly(A) where 0.1 mM was used to prevent precipitation after addition of $MnCl_2$. The reaction mixtures (500 μl) were assembled as follows, with final concentrations being: 50 mM Tris·HCl (pH 7.6), 60 mM NaCl, 2 mM dithiothreitol, 0.015% Triton X, 5 mM $MnCl_2$, 0.8 mM dATP, dCTP, dGTP (each). 50 μl of MLV suspension was then added, and the mixture was incubated for 5 min at 37°; the reaction was started by the addition of 50 μl of [³H]TTP. 50-μl Aliquots were taken. *Multiple points* on the curves show the variation observed in experiments performed over several months; ●——● represents experiments in which no polymer was added.

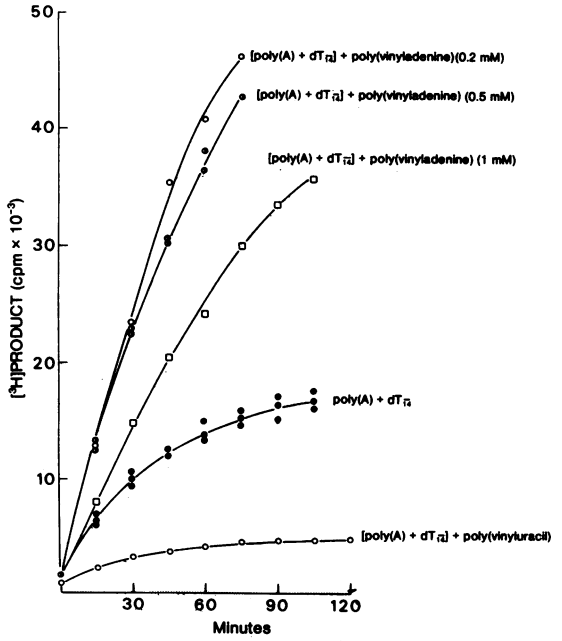


FIG. 3. Inhibition of DNA polymerase activity of MLV by polymers: The reaction mixture (200 μl) was assembled as follows: addition of polymer as specified in the figure–poly-(vinyluracil), 0.5 mM; poly(U), 0.5 mM; poly(vinyladenine) as given; poly(A), 40 μM; dT_{12}, 20 μM; then followed: 50 mM Tris·HCl (pH 8.3), 60 mM NaCl, 20 mM dithiothreitol, 0.05% Triton X, 1 mM $MnCl_2$, and 15 μl of MLV suspension. The mixture was incubated for 5 min at 37°, and the reaction was started by the addition of TTP to 0.8 mM and 20 μl of [³H]TTP solution; 20-μl aliquots were sampled. When specified, dCTP, dATP, and dGTP were present at 0.8 mM each. No incorporation into product was observed with (dCTP, dATP, and dGTP), no additions, [poly(A) + dT_{12}] and poly(U), poly(vinyladenine), or [poly(vinyladenine) + dT_{12}].

with virus. The maximum inhibitory effect of poly(A) was achieved with either 2 hr of treatment before infection or with treatment during the first 6 hr after virus inoculation. After the first 6 hr after infection, the inhibitory effect of poly(A) is much less profound. Poly(vinyladenine) was also able to inhibit MLV replication, although the inhibition was less than that observed with poly(A). However, the inhibitory effect of poly(vinyladenine) was observed not only during the early stages of the replicative cycle, but also when it was added as late as 12 hr after infection. While poly(A) at 0.1 mM inhibited MLV replication completely, poly(vinyladenine) at the same concentration was inactive.

A comparison of the effects of poly(U) and poly(vinyluracil) is presented in Table 2. Poly(U) inhibits virus replication most effectively when it is applied during the early stages of infection. Even at a low concentration of poly(U), there is more than 60% inhibition of virus replication. Poly(vinyluracil), which was tested at a concentration that does not cause cell toxicity, showed a maximum of 50% inhibition when added before virus inoculation. Once again, a small inhibitory effect was observed even when poly(vinyluracil) was added 12 hr after infection. Thus, both vinyl polymers seem to have a small, but definite, effects, even on the later events in MLV replication, while the effect of the corresponding polynucleotides is apparently confined to the early stages in viral replication. The difference in overall effectiveness of natural polynucleotides and vinyl polymers is probably not due to the different internal concentrations; the vinyl polymers are taken up by the mouse fibroblasts to a similar [poly(vinyladenine))], or greater [poly(vinyluracil)], extent than are the corresponding polynucleotides (Pitha *et al.*, manuscript in preparation).

To see if the observed inhibition was specific for MLV, or whether the replication of other RNA viruses would be affected in a similar way, we tested the effect of polynucleotides and their vinyl analogs on the replication of two lytic viruses, vesicular stomatitis and Sindbis, both of which are single-stranded RNA viruses. Vesicular stomatitis virus contains and RNA-dependent RNA polymerase in its virion; however, this enzyme seems to be involved in the formation of mRNA rather than in replication of the viral genome. On

TABLE 3. *Effects of polynucleotides and vinylpolymers on the yields of vesicular stomatitis virus and of Sindbis virus*

Polymer	Hours of treatment	PFU/ml (×10⁻⁷) Vesicular stomatitis	Sindbis
—	—	3.0	1.8
Poly(vinyladenine)	−2→0	2.0	1.7
	0→8	2.3	1.2
Poly(vinyluracil)	−2→0	4.0	1.0
	0→8	3.2	1.3
Poly(A)	−2→0	2.6	2.0
	0→8	3.1	1.5
Poly(U)	0→8	3.5	2.1

Experiments were done in duplicate; the mean values are given. Both viruses were harvested 15 hr after infection. Polymers were tested at 1 mM. Similarly no significant differences in yields were observed where viruses were harvested at 3, 4, 5, 6, 7, and 8 hr after injection.

the other hand, the RNA that is present in Sindbis virus acts directly as viral mRNA (15). Data in Table 3 show that neither the polynucleotides nor their vinyl analogs affect the replication of these two viruses. Furthermore, none of the tested polymers affected the adsorption and yield of vesicular stomatitis virus in 3T3 cells or of Sindbis virus in human fibroblast cells. Infection with each virus was performed in nongrowing cells, where cell division was stopped by contact inhibition, thus permitting the use of a much higher concentration of poly(vinyluracil) than that used for inhibition of MLV.

In vitro effects on RNA-dependent DNA polymerase

The inhibitory effect of single-stranded polynucleotides on the DNA polymerase present in virions of oncornaviruses has been well documented (2). To determine if vinyl polymers could affect the *in vitro* activity of the DNA polymerase of MLV as well, we performed two sets of experiments. In the first set, we followed the conditions used by Brockman *et al.* (16) for the study of inhibitors of low molecular weight. Under these conditions, virus particles are only partially disrupted by detergent and endogenous template is used. The results are summarized in Fig. 2. Poly(U) partially inhibits the reaction, whereas poly(A) increases the incorporation of deoxyribonucleoside triphosphates into the macromolecular fraction. Both vinyl analogs are without any distinct influence. In the other set of experiments, the conditions of Baltimore and Smoler (17) were used, where the use of a higher concentration of detergent more fully disrupts the virus particles. The results are summarized in Fig. 3. With endogenous template only, the incorporation is negligible even when all four deoxyribonucleoside triphosphates are present. When exogenous template [(poly(A)] and primer oligodeoxythymidylic acid ($dT_{\overline{14}}$) were used, there was effective incorporation; this reaction is inhibited by both poly(vinyluracil) and poly(U). Poly(vinyladenine) by itself, or with the $dT_{\overline{14}}$ primer, does not serve as a template, but when poly(vinyladenine) was added to mixtures containing poly(A) and $dT_{\overline{14}}$, the incorporation of [^{3}H]TTP was stimulated. The increase was dependent on the amount of analog added, but not in any simple proportionate way (Fig. 3). This effect of vinyl polymers probably is not due to any direct regulation of the intrinsic concentration of $dT_{\overline{14}}$, as our experiments were done under conditions where changes in oligomer concentration cause only very minor changes in rate of synthesis (17).

DISCUSSION

The evidence for adenine-rich sequences in the 70S RNA of oncogenic RNA viruses and the obvious requirement of these regions for RNA-dependent DNA polymerase activity (18–21) suggest means by which the activity of this enzyme may be blocked. Thus, it was shown that in *in vitro* systems, the DNA polymerase from MLV can be inhibited completely by poly(U), and to a lesser degree by other single-stranded polynucleotides (2). This result may indicate that the affinity of the enzyme for poly(U) is higher than its affinity for poly(A); however, one cannot determine from the results whether the observed inhibition by poly(U) is due to the binding of the polynucleotide to the active site of the enzyme, or whether it may be due to a binding to the template leading to a change in the secondary structure of the recognition sites.

The observed *in vitro* inhibition of DNA polymerase activity of MLV by poly(vinyluracil) is probably due to template–inhibitor interaction, rather than to enzyme–inhibitor interaction. We have found that poly(vinyluracil) binds through base-pair formation to poly(A), and that this binding is a specific process (7). The stimulatory effect of poly(vinyladenine) on the virion DNA polymerase activity *in vitro* is not explainable.

The effects of poly(vinyluracil), poly(vinyladenine), poly(U), and poly(A) on acute MLV infection appears to be specific. No apparent changes in the growth of the host cells or the rate of their DNA synthesis were observed; furthermore, preliminary experiments indicate that treatment with a representative polymer did not affect the adsorption of the virus substantially. Also, interference through the interferon mechanism can be eliminated. Single-stranded polynucleotides are very poor interferon inducers (22); vinyl polymers, being neutral polymers, do not induce interferon at all (Pitha, unpublished). Inhibitory effects are maximal in the early stages of viral infection and the inhibition is very selective; no effects on the replication of vesicular stomatitis virus or Sindbis virus were observed.

The mechanism of inhibition of MLV replication by polynucleotides and their vinyl analogs *in vivo* might be due to interference with those steps in viral replication that are catalyzed by the virion RNA-dependent DNA polymerase. However, there is an apparent lack of correlation between the observations *in vivo* and the experiments *in vitro*, which were done without the addition of an exogenous template. This discrepancy may be due to differences in accessibility; the conditions of the reaction *in vitro* may not allow the polymers to penetrate inside the virus particle. The possibility that poly(vinyluracil) and poly(vinyladenine) could directly inhibit the functional expression of poly(A)-rich sequences present in high amount in 70S RNA from MLV must also be considered. Both cellular RNA (heterogeneous and messenger) (23, 24) and the mRNA of the examined lytic viruses (25, 26) contain some poly(A)-rich stretches, although probably the percentage is lower than in the MLV genome (18). Therefore, the assumption that the observed inhibition is related to interference with the functions of poly(A) sequences could be applied only in a differential way.

In conclusion, we would like to point out that although the inhibitory effects of vinyl polymers in this system were lower than the ones observed for the corresponding polynucleotides, their action may be generally more selective. Since vinyl polymers do not serve as a template for any enzymatic reaction and do not bind to ribosomes (8) less interference with the cellular metabolism than in the case of polynucleotides may be expected. They are not enzymatically hydrolyzable; in some systems, this may be an important requirement, as the uptake of macromolecules seems to be directly proportional to their size. Small fragments, even when effective *in vitro*, would not show any activity *in vivo* simply because they would never get inside the cell.

NOTE ADDED IN PROOF

The effects of 1 mM poly(A) and 1 mM poly(vinyladenine) on the number of infectious centers at 24 hr after infection and virus yield at 36 hr after infection were also determined for single-step infection of mouse-embryo cells with MLV. With poly(A), the number of infectious centers and the virus

yield were inhibited 100 and 90%, respectively, with the 2-hr prior treatment; these values fell to 60 and 80% with the 6- to 12-hr treatment. With the poly(vinyladenine) prior treatment there was a 40% inhibition of infectious centers and 30% inhibition of virus yield; whereas the 6- to 12-hr treatment showed a 50 and 40% decrease, respectively. These results correlate well with the inhibition of virus plaques *in vitro;* i.e., poly(A) exerts its maximal inhibitory effect when added before or during the early stages of virus replication, while the inhibitory effect of poly(vinyladenine) is not specific for an early step.

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