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SHORT COMMUNICATION

Inhibition of Murine Leukemia Virus Replication in Cell Culture and Spleen Focus Formation in Mice by Polyadenylic Acids

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SUMMARY

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Poly(adenylic acid), poly(2'-*O*-methyladenylic acid), and poly(2'-*O*-ethyladenylic acid) moderately inhibit the synthesis of Moloney murine leukemia virus in cultured JLS-V9 cells. Moreover, they are potent inhibitors of spleen focus formation by Friend murine leukemia virus in mice. Both in cell cultures and in the animal system, the order of inhibitory potency observed is poly(2'-*O*-ethyladenylic acid) > poly(2'-*O*-methyladenylic acid) > poly(adenylic acid). This order is identical with the one we have noted for inhibition of the RNA-directed DNA polymerases of these two viruses. While the molecular basis of inhibition of viral replication is not yet known, our results are compatible with the notion that RNA-directed DNA polymerase may be a drug target *in vivo*.

Synthesis of a DNA copy of the template RNA ("provirus") is apparently required for infection and transformation of mammalian cells by RNA tumor viruses (1). Thus viral RNA-directed DNA polymerase, or reverse transcriptase, which catalyzes this DNA synthesis, plays an obligatory role in viral infection (1). This is substantiated by the lack of infectivity of a reverse transcriptase-deficient mutant, $RSV_\alpha(O)$, of Rous sarcoma virus (2). Furthermore, an apparent correlation exists between inhibition of reverse transcriptase and focus formation activity of murine leukemia-sarcoma virus by certain rifamycin derivatives (3). These studies were prompted by the possibility that selective inhibitors of reverse transcriptase would be therapeutically useful antiviral agents.

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Single-stranded polynucleotides have been shown to inhibit viral reverse transcriptase *in vitro* and virus replication in cell culture (4–9). Taking advantage of the relative resistance of 2′-*O*-alkylated polynucleotides to nucleolytic degradation, we recently investigated the inhibitory properties of these polynucleotides. We noted that poly(A), poly(2′-*O*-methyladenylic acid), poly(2′-*O*-ethyladenylic acid), and poly(dA) inhibit the reverse transcriptase activity of Moloney murine leukemia virus with little or no effect on the activity of partially purified cellular DNA polymerases (8, 10). These polynucleotides also inhibit the reverse transcriptase activity of Friend murine leukemia virus.[1] Analysis of the kinetics of inhibition gave the following order of inhibition: poly(2′-*O*-ethyladenylic acid) > poly(2′-*O*-methyladenylic acid) > poly(A) > poly(dA). We have now extended these studies to cell culture and animal systems and report here that these polynucleotides inhibit the synthesis of Moloney murine leukemia virus in cultured cells (JLS-V9) and are potent inhibitors of the spleen focus-forming activity of Friend murine leukemia virus in mice.

Moloney murine leukemia virus was obtained from the culture fluid of virus-infected mouse bone marrow JLS-V9 cells (11) grown in RPMI-1640 medium plus 10% fetal calf serum. The polycythemic strain of Friend murine leukemia virus was provided by Dr. J. S. Horoszewicz and was obtained as a filtered (0.45 nm) perfusate of spleens from virus-infected DBA/2 mice. The stock preparation contained 7×10^4 focus-forming units/ml. Poly(A) and oligo(dT)$_{12-18}$ were purchased from Miles Laboratories, and poly(2′-*O*-methyladenylic acid) and poly(2′-*O*-ethyladenylic acid) were synthesized as previously described (12).

DNA polymerase assays were performed in a reaction mixture (50 or 100 μl) containing 0.05 M Tris-HCl (pH 7.9), 0.06 M NaCl, 0.001 M $MnCl_2$, 0.02 M dithiothreitol, 0.05% NP-40, 50:5 μM poly(A):oligo(dT), 10 or 50 μM [^{3}H]dTTP (2200–6600 cpm/pmole), and a purified virus preparation (8) or the harvested culture medium containing virus (25 or 50 μl). The mixture was incubated at 37°, and aliquots were withdrawn at specified times and precipitated with trichloracetic acid. Radioactivity incorporated into acid-insoluble material was counted. For the purpose of this report, the titer of Moloney murine leukemia virus is expressed in terms of RNA-directed DNA polymerase activity units. One unit of polymerase activity is equivalent to the incorporation of 100 pmoles of TMP into acid-insoluble material in 30 min at 37° with 50:5 μM poly(A):oligo(dT) as a template. The RNA-directed DNA polymerase activity of murine leukemia virus preparations has been shown to be directly proportional to their titer of plaque-forming units as scored by XC assays (13, 14).

The effect of polyadenylic acids on infection of cultured JLS-V9 cells by Moloney murine leukemia virus was studied as follows. Duplicate samples of cell monolayers in 75 cm^2 plastic dishes (approximately 70% confluent, about 20×10^6 cells) were treated with DEAE-dextran (25 μg/ml, 1 hr), washed with phosphate-buffered NaCl solution, and incubated for 2 hr with 5 ml of a 5 μg/ml solution of polynucleotide in RPMI-1640 medium. Control cells received 5 ml of RPMI-1640 medium alone. Cells were washed with phosphate-buffered NaCl, exposed for 1 hr to 5 ml of the Moloney murine leukemia virus preparation containing 10 units/ml of polymerase activity (approximately 8.7×10^5 plaque-forming units/ml, XC assay), washed with phosphate-buffered NaCl, and reincubated for 12 hr with 10 ml of the polynucleotide solution (5 μg/ml in RPMI-1640 medium plus 10% fetal calf serum). Control cells received medium without polynucleotide. Cells were then washed with phosphate-buffered NaCl and incubated with 10 ml of fresh RPMI-1640 medium plus 10% fetal calf serum but without polynucleotide. The medium was harvested at specified times, and virus content was monitored by assaying for polymerase activity of virions present in the medium. All operations were performed at 37°.

To determine the effect of polyadenylic acids on infection of mice by Friend murine

[1] S. K. Arya, unpublished observations.

leukemia virus, an appropriate dilution of virus stock was mixed with polynucleotide or phosphate-buffered NaCl and incubated at 37° for 30 min. A 0.5-ml aliquot (without further dilution) was then given intravenously to 8-week-old DBA/2 male mice. Mice were killed 9 days after infection, and the number of spleen foci was counted (15, 16).

Figure 1 shows the effect of polyadenylic acids on Moloney murine leukemia virus infection of cultured JLS-V9 cells. By 48 hr after infection, poly(A) (5 μg/ml) reduced the content of progeny virus in the harvested medium by about 30% relative to the control untreated cultures. Corresponding reduction by poly(2'-*O*-methyladenylic acid) and poly(2'-*O*-ethyladenylic acid) was 35% and 40% of the control value, respectively. The control cultures continued to produce virus at high levels for 72 hr while virus production in treated cultures declined further. The content of virus in the medium harvested from treated cultures at 72 hr had declined by about 40–50% relative to the control (inset, Fig. 1).

These effects may be due to a direct inhibition of replication of Moloney murine leukemia virus in cultured cells. Among other explanations which we have considered were (a) the presence (in the medium) of an inhibitory concentration of free, extracellular polyadenylic acids at the time of harvest (which would give low estimates of virus content when scored by polymerase assays), (b) a possible cytotoxic effect of polyadenylic acids on the cultured cells, (c) interference with the uptake of virus by cells, (d) formation of an extracellular, noninfective virus:polynucleotide complex, (e) inhibition of release of virus to the medium, and (f) interferon induction.

We could exclude the first possibility, since medium harvested from treated cells 24 and 48 hr after mock infection did not contain an inhibitory concentration of free polyadenylic acid. The medium from treated cells (as described above but mock-infected) was added to a polymerase assay mixture containing purified Moloney murine leukemia virus and no inhibition of exogenously added Moloney murine leukemia virus was detected. We could also eliminate the second possibility, since polyadenylic acids (5 μg/ml) showed no effect on the growth rate of cultured JLS-V9 cells. In addition, we noted no effects on growth rates when cells were plated at a relatively low density and grown in the continuous presence of polynucleotide; this diminishes the possibility that polymer degradation products significantly influenced cell growth. Others have also reported that, at concentrations up to 500 μg/ml, poly(A) does not affect the growth of mouse embryo cells in culture (5, 6); also, Tennant *et al.* (5) noted no significant effects of poly(A) on DNA synthesis in Swiss mouse embryo cells.

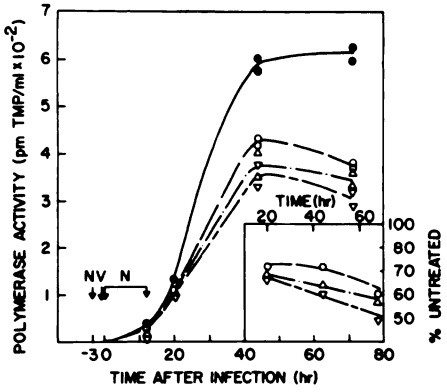

FIG. 1. *Effect of polyadenylic acids on infection of cultured JLS-V9 cells by Moloney murine leukemia virus*

JLS-V9 cells were treated with DEAE-dextran (25 μg/ml, 1 hr), washed with phosphate-buffered NaCl, and incubated for 2 hr with the test polynucleotide (5 μg/ml). Cells were then washed with phosphate-buffered NaCl and exposed to a preparation of Moloney murine leukemia virus for 1 hr. They were washed with phosphate-buffered NaCl and reincubated with polynucleotide (5 μg/ml) for 12 hr. The polynucleotide-containing medium was then removed, and cells were incubated in fresh medium without polynucleotide. Control cultures were treated identically but received medium devoid of polynucleotide. The medium was subsequently harvested at specified times. Virus content was estimated by assaying for reverse transcriptase activity of virions in the harvested medium as described in the text. ●——●, untreated cultures; O- - -O, poly(adenylic acid)-treated cultures; Δ----Δ, poly(2'-*O*-methyladenylic acid)-treated cultures; ▽ -·-·▽, poly(2'-*O*-ethyladenylic acid)-treated cultures. N, polynucleotide; V, virus.

TABLE 1

Effect of polyadenylic acids on spleen focus formation by Friend murine leukemia virus

Eight-week-old male mice (groups of seven) were injected intravenously with a 0.5-ml suspension of virus in phosphated-buffered NaCl or virus plus polynucleotide. The mice were killed 9 days later, and the number of spleen foci formed was counted.

Polynucleotide	Foci per spleen at various polynucleotide concentrations			
	0	0.17 μg/0.5 ml	0.85 μg/0.5 ml	1.7 μg/0.5 ml
	No. (mean ± SE)			
None	76.8 ± 2.4			
Poly(adenylic acid)		44.5 ± 3.4	32.8 ± 1.3	23.5 ± 3.8
Poly(2′-*O*-methyladenylic acid)		39.3 ± 10.5	17.8 ± 2.6	13.3 ± 1.5
Poly(2′-*O*-ethyladenylic acid)		27.2 ± 3.0	19.5 ± 4.5	5.2 ± 3.0

The other possibilities, although unlikely, cannot yet be excluded. Tennant *et al.* (7) reported that poly(A) and poly(2′-*O*-methyladenylic acid), at 10 μg/ml, do not affect the uptake of labeled Moloney murine leukemia virus by cultured mouse embryo cells; exposure of Moloney murine leukemia virus to poly(A) also did not decrease viral infectivity if the drug was later removed (5). The induction of interferon as an explanation for our results seems unlikely, since single-stranded polynucleotides are known to be poor inducers of the interferon response (17).

The effect of polyadenylic acids on induction of spleen focus formation in mice by Friend murine leukemia virus is shown in Table 1. These compounds diminish the spleen focus-forming activity of Friend murine leukemia virus by 2–15-fold in the dosage range studied. For example, even at a dose of polynucleotide of less than 1 μg/mouse, the spleen foci were reduced more than 2-fold relative to animals which did not receive polynucleotide. Poly(2′-*O*-ethyladenylic acid) showed a greater apparent inhibition of spleen focus formation than did poly(2′-*O*-methyladenylic acid) and poly(A). The polynucleotides alone did not display any apparent effect on the spleens of normal animals. The basis for this potent inhibitory effect against Friend murine leukemia virus is not yet known. Since we have observed the same order of inhibition among the polyadenylic acids for reverse transcriptase *in vitro* (8), viral synthesis in cell culture, and spleen focus formation in mice, it is possible that inhibition of reverse transcriptase may be a factor responsible for these effects. However, other possible explanations, such as inactivation of a spleen focus-forming virus component of Friend murine leukemia virus, general augmentation of the immune response, alteration in the sensitivity (or pool size) of spleen target cells, and interferon induction, also require investigation. Recently Tennant *et al.* (18) reported that poly(2′-*O*-methyladenylic acid) inhibits tumor development (and death) induced by Moloney murine sarcoma-leukemia virus complex in newborn mice. They also noted that poly(2′-*O*-methyladenylic acid) enhances the antibody response of newborn mice to endogenous leukemia virus envelope antigens; this result suggests that the antiviral effect of poly(2′-*O*-methyladenylic acid) may be mediated through enhancement of the immune response.

Independent of the mechanism, our results suggest that, at least under certain conditions, polyadenylic acids inhibit the functions of RNA tumor viruses both in cell culture and in the intact animal; poly(2′-*O*-ethyladenylic acid) appears to be more effective in inhibition than other polyadenylic acids thus far studied. Further effort is now warranted to determine whether the breadth of antiviral action can be extended, and whether the basis for such an inhibition in fact resides in inactivation of the DNA polymerase of murine RNA tumor virions.

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