INHIBITION OF RNA DIRECTED DNA POLYMERASE OF MURINE LEUKEMIA VIRUS BY 2'-O-ALKYLATED POLYADENYLIC ACIDS†

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SUMMARY: Poly(2'-O-alkyl)adenylic acids strongly inhibit the RNA-directed DNA polymerase of Moloney murine leukemia virus (MLV) but have no significant effect on the activity of E. coli and murine spleen DNA polymerase. Poly(2'-O-methyl A) and poly(2'-O-ethyl A) show an apparent competitive inhibition of poly(A):oligo-(dT)-directed MLV polymerase. Poly(A), poly(2'-O-methyl A), poly(2'-O-ethyl A), and poly(dA) yield kinetics of inhibition of poly(C):oligo(dG)-directed MLV polymerase which are inconsistent with a simple competitive inhibition with respect to the template. The following order of strength of inhibition is observed: poly(I) > poly(2'-O-ethyl A) > poly(2'-O-methyl A) for the poly(A):oligo(dT)-directed reaction, and poly(2'-O-ethyl A) > poly(2'-O-methyl A) > poly(A)>> poly(dA) for the poly(C):oligo(dG)-directed reaction.

Virions of RNA tumor viruses contain a RNA-directed DNA polymerase which catalyzes the synthesis of a DNA copy of the resident or endogenous RNA (see reference 1 for review). The synthesis of DNA copy or "provirus" is believed to be an obligatory step in infection and cell transformation by these viruses (2,3). Thus, a specific inhibitor of viral RNA-directed DNA polymerase may yield a chemotherapeutically useful antiviral agent.

The viral DNA polymerase also catalyzes the polymerization of deoxyribonucleotides using exogenous polynucleotides as templates in the presence of poly- or oligodeoxyribonucleotides as primers (4,5). However, unprimed polynucleotides do not serve as templates (4,5). To the contrary, single stranded polynucleotides inhibit the viral DNA polymerase directed by endogenous as well as exogenous template:primer; the strength of inhibition depending on the base composition of the polynucleotide (6-8). Some of these polynucleotides reportedly inhibit virus replication in tissue culture (9,10). It has been suggested

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that single stranded polynucleotides may be specific inhibitors of viral DNA polymerase with little or no effect on bacterial and mammalian DNA polymerases (6,7).

In an effort to develop polymers with greater selectivity of inhibition and enhanced resistance to nucleolytic degradation, we have initiated a study of the inhibitory properties of polynucleotides which have been modified at 2'-hydroxy position (11,12). These studies also may contribute towards understanding the mechanism of action of oncornaviral DNA polymerase. We report here the results of our studies of inhibition of Moloney murine leukemia virus (MLV) DNA polymerase by 2'-O-alkylated polyadenylic acids.

MATERIALS AND METHODS: Murine leukemia virus (Moloney) was obtained from culture fluid of Moloney murine leukemia virus infected mouse bone marrow cell line JLSV-9 grown on RPMI-1640 medium plus 10% fetal calf serum. The culture fluid was harvested on a continuous basis. It was clarified by centrifugation (500 x g for 10 min) and centrifuged at 36,000 x g for 3 hours. The pellet of crude virus was purified by rate-zonal gradient centrifugation (5-20% sucrose in 0.005 M Tris-HCl [pH 8.4]-0.001 M EDTA, 25 min at 96,000 x g) followed by equilibrium gradient centrifugation (15-60% sucrose in Tris-EDTA buffer, 17 hr at 83,000 x g), and dialysis against 0.01 M Tris-HCl (pH 7.4). E. coli DNA polymerase was obtained by purification of a commercial preparation of E. coli DNA polymerase (P. L. Biochemicals) by successive chromatography on DEAE- and phosphocellulose (13). Murine DNA polymerase was a gift from Dr. M.J. Evans (RPMI).

Poly[d(A-T)](15·1s), poly(A)(8·1s), poly(C)(4·6s), poly(dA)(5·2s), and poly(I)(MW>100,000 daltons) were obtained from Miles Laboratories, Inc., Elkhart, Indiana. Oligo$(dT)_{12-18}$ and oligo$(dG)_{12-18}$ were purchased from P.L. Biochemicals, Inc., Milwaukee, Wisconsin. Poly(2'-O-methyl A)(7s) and poly(2'-O-ethyl A)(9s) were prepared by polymerization of 2'-O-alkyl adenosine diphosphates as described by Tazawa et al. (11). Tritium labeled thymidine triphosphate (50 C/mmole) and deoxyguanosine triphosphate (6.5 C/mmole) were obtained from Schwartz/Mann, Orangeburg, N.Y.

DNA polymerase assays were performed in a reaction mix (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 dithiothrietol, 0.05% NP-40, 10 µg/ml purified murine leukemia virus protein, 50 µM [^{3}H]thymidine triphosphate (2,600 cpm/pmole) and 10-100:1-10 µM poly(A):oligo(dT) (molar ratio, 10:1) or 50 µM [^{3}H]deoxyguanosine triphosphate (2,600 cpm/pmole) and 2-20:0.2-2 µM poly(C):oligo(dG) (molar ratio, 10:1). Under these conditions the enzyme concentration is limiting and the concentration of deoxyribonucleotides is in excess. For poly[d(A-T)]-directed reaction, 0.006 M $MgCl_2$ replaced $MnCl_2$ and included 100 µM deoxyadenosine triphosphate. The detergent, NP-40, was omitted from reaction mix with partially purified DNA polymerases. The template:primer complexes were prepared by mixing the appropriate polynucleotide and oligonucleotide in 0.01 M Tris-HCl (pH 7.2) containing 0.1 M NaCl or 0.01 M NaCl, incubating at 37° for 30 minutes followed by slow cooling. The reaction mix was incubated at 37°, and the radioactivity incorporated into acid-insoluble material was determined by withdrawing 10 or 20 µl aliquots at different times and spotting on Whatman 3 MM filter paper discs. The discs were processed according to Bollum (14) and counted.

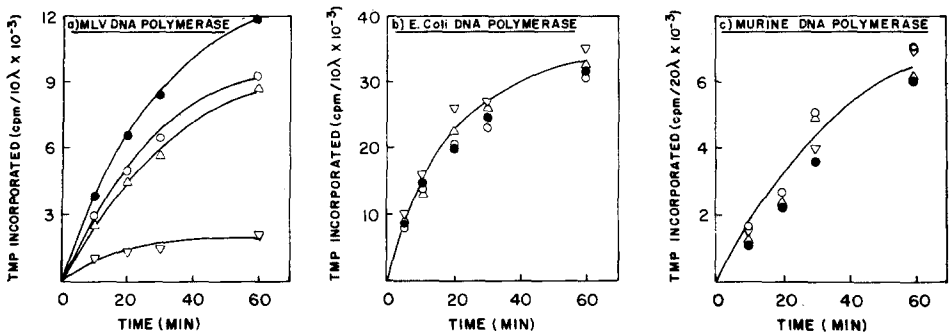

Figure 1. Effect of polyadenylic acids on DNA polymerase activity from (a) murine leukemia virus, (b) E. coli, (c) mouse spleen. The reaction mix contained 0.05 M Tris-HCl (pH 7.9), 0.06 M NaCl, 0.006 M $MgCl_2$, 0.02 M dithiothrietol, 100 µM dATP, 50 µM [^{3}H]dTTP (2600 cpm/pmole), 50 µM poly[d(A-T)] and appropriate enzyme preparation. -●-●-, poly[d(A-T)]; -o-o-, poly[d(A-T)] plus poly(A) (10 µM); -Δ-Δ-, poly[d(A-T)] plus poly(2'-me A) (10 µM); -∇-∇-, poly[d(A-T)] plus poly(2'-et A) (10 µM).

To obtain kinetic constants, the data were plotted according to Linweaver-Burk (15). The complexity of the enzyme system, where at least three binding sites (template, primer, and precursor/substrate) are conceived, is obvious. Since no adequate kinetic model for enzyme:template:primer:substrate system is available, a simplified model is used where the template:primer is employed as substrate (in the presence of excess deoxyribonucleotide precursor), and the incorporation of the precursor is taken as a measure of template:primer utilization. Accordingly, the K_M and V_{max} were determined from the intercepts, and K_I's from the slopes of the lines for inhibited and uninhibited reactions.

RESULTS: The kinetics of polymerization of deoxyribonucleotides in the absence and presence of poly(A), poly(2'-O-methyl A) [poly(2'-me A)], and poly(2'-O-ethyl A) [poly(2'-et A)] by poly[d(A-T)]-directed DNA polymerases from three sources is shown in Figure 1. The MLV DNA polymerase is inhibited by all three polyadenylic acids, whereas no inhibition is observed for E. coli and murine DNA polymerase. At least at the concentrations of polyadenylic acids tested under the assay conditions, these polymers are potent inhibitors of viral enzyme with no significant effect on bacterial and mammalian enzyme. Poly(2'-et A) shows considerably greater inhibition than poly(2'-me A) and poly(A) for MLV DNA polymerase.

Figure 2 shows a representative 1/v versus 1/s plot of poly(A):oligo(dT)-directed reaction of MLV polymerase (detergent disrupted virus) in the absence and presence of poly(2'-me A) and poly(2'-et A). These polyadenylic acids show

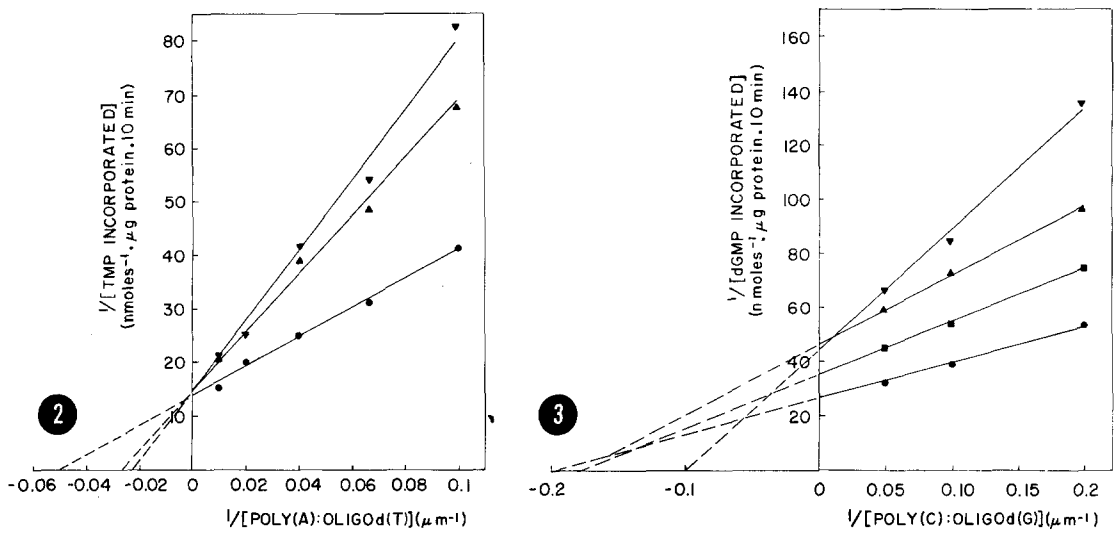

Figure 2. Kinetics of inhibition of poly(A):oligo(dT)-directed MLV DNA polymerase by polyadenylic acids. The reaction mix contained 0.05 M Tris-HCl (pH 7.9), 0.06 M NaCl, 0.001 M $MnCl_2$, 0.02 M dithiothrietol, 0.05% NP-40, 50 μM [^{3}H]dTTP (2600 cpm/pmole), 10 μg/ml purified MLV, and poly(A):oligo(dT) in the absence (-●-●-), and presence of 20 μM poly(2'-me A) (-▲-▲-), and 20 μM poly(2'-et A) (-▼-▼-).

Figure 3. Kinetics of inhibition of poly(C):oligo(dG)-directed MLV DNA polymerase by polyadenylic acids. The reaction conditions are the same as in Figure 2 except [^{3}H]dTTP was replaced by [^{3}H]dGTP and poly(A):oligo(dT) by poly(C):oligo(dG). Poly(C):oligo(dG) in the absence (-●-●-), and in the presence of 20 μM poly(A) (-■-■-), 20 μM poly(2'-me A) (-▲-▲-), and 20 μM poly(2'-et A) (-▼-▼-).

an apparent competitive inhibition of poly(A):oligo(dT)-directed reaction. The K_M of poly(A):oligo(dT) is found to be 20 μM, and K_I's of poly(2'-me A) and poly(2'-et A) to be 25 μM and 14 μM respectively. When unprimed poly(2'-me A) and unprimed or oligo(dT)-primed poly(2'-et A) were used as templates for detergent disrupted virions, no polymerization of TMP was observed. However, poly (2'-me A):oligo(dT) catalyzed low levels of polymerization. The K_M and V_{max} estimated from 1/v versus 1/s plots for poly(2'-me A):oligo(dT) were about 1.0 μM and 0.1 pmole/μg protein/min respectively (data not shown).

A similar plot for poly(C):oligo(dG)-directed MLV polymerase in the absence and presence of poly(A), poly(2'-me A), and poly(2'-et A) is shown in Figure 3. All the three polyadenylic acids strongly inhibit the poly(C):oligo

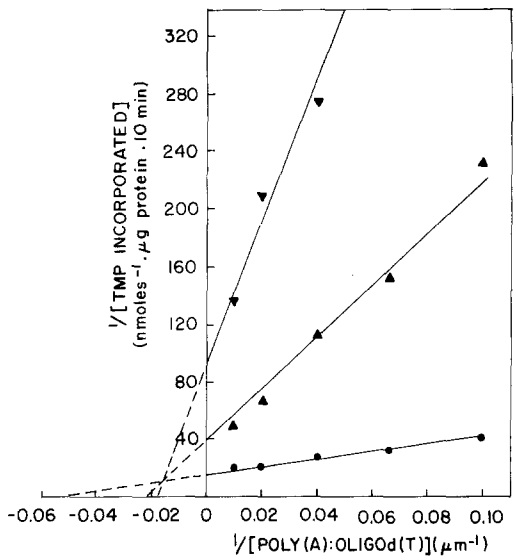

Figure 4. Kinetics of inhibition of poly(A):oligo(dT)-directed MLV DNA polymerase by poly(I). The reaction conditions are the same as in Figure 2. Poly(A):oligo(dT) in the absence (-●-●-), and in the presence of poly(I) (-▲-▲-, 5 μM; -▼-▼-, 20 μM).

(dG)-directed reaction. The kinetics of inhibition is rather complex. The K_M of poly(C):oligo(dG) calculated from the intercept is 5 μM and K_I's calculated from the slopes of the lines (independent of type of inhibition) of poly(A), poly(2'-me A), and poly(2'-et A) are 36 μM, 24 μM and 10 μM respectively. The K_I of poly(dA) for poly(C):oligo(dG)-directed reaction calculated from similar plots (not shown) was found to be 130 μM. The poly(I) inhibition of poly(A):oligo(dT)-directed reaction yields kinetics which also is inconsistent with a simple competitive inhibition (Fig. 4). The kinetic parameters from several such assays are catalogued in Table I. It is clear that poly(2'-et A) is a stronger inhibitor than poly(2'-me A) for poly(A):oligo(dT)-directed reaction. The relative strength of inhibition for poly(C):oligo(dG) reaction is poly(2'-et A)>poly(2'-me A)>poly(A)>>poly(dA). We have not investigated the effect of the size of the polyadenylic acids on the degree of inhibition. The sizes of the polyadenylic acids used in this study are roughly comparable (Materials and Methods). Poly(I) was also found to be a potent inhibitor of poly(A):oligo(dT)-directed MLV DNA polymerase. A systematic study of the effect

TABLE I

KINETIC PARAMETERS OF MLV DNA POLYMERASE

Assay	Template:Primer and Inhibitor	K_M (μM)	V_{max} (pm/μg/min)	K_I (μM)
1.	Poly(A):oligo(dT)	20	7.1	-
	Poly(A):oligo(dT)+Poly(2'-me A) (10 μM)	30	7.7	26
	Poly(A):oligo(dT)+Poly(2'-me A) (25 μM)	40	7.7	24
2.	Poly(A):oligo(dT)	20	7.2	-
	Poly(A):oligo(dT)+Poly(2'-et A) (5 μM)	26	7.1	17.2
	Poly(A):oligo(dT)+Poly(2'-et A) (10 μM)	37	6.8	10.4
	Poly(A):oligo(dT)+Poly(2'-et A) (20 μM)	46	6.8	14.3
3.	Poly(C):oligo(dG)	5.0	3.8	-
	Poly(C):oligo(dG)+Poly(A) (20 μM)	5.5	2.8	36
	Poly(C):oligo(dG)+Poly(2'-me A) (20 μM)	5.5	2.2	22
	Poly(C):oligo(dG)+Poly(2'-et A) (20 μM)	10.0	2.2	8.5
4.	Poly(C):oligo(dG)	4.5	6.6	-
	Poly(C):oligo(dG)+Poly(2'-me A) (10 μM)	5.2	5.7	26
	Poly(C):oligo(dG)+Poly(2'-et A) (10 μM)	5.5	4.5	11.4
5.	Poly(C):oligo(dG)	4.5	3.2	-
	Poly(C):oligo(dG)+Poly(dA) (10 μM)	4.5	2.8	130
	Poly(C):oligo(dG)+Poly(dA) (20 μM)	4.5	2.5	130
6.	Poly(A):oligo(dT)	20	6.6	-
	Poly(A):oligo(dT)+Poly(I) (5 μM)	45	2.2	0.9
	Poly(A):oligo(dT)+Poly(I) (20 μM)	55	1.1	1.1

of base composition alone and in conjunction with 2'-O-alkylation on the inhibitory properties of polynucleotides has been undertaken.

DISCUSSION: Oncornaviral DNA polymerases catalyze the polymerization of deoxyribonucleotides using exogeneous nucleic acids as templates (4,5). Polyribonucleotide:oligodeoxyribonucleotide hybrids seem to be the best templates for these enzymes (4,5). For murine leukemia virus DNA polymerase, poly(C):oligo (dG) is a more efficient template than poly(A):oligo(dT) in the presence of Mn^{++} (5). Our results provide an explanation for this differential template efficiency. We find that the apparent affinity ($^1/K_M$) of poly(C):oligo(dG) for MLV polymerase is four times that of poly(A):oligo(dT).

Poly(A), poly(2'-me A), and poly(2'-et A) strongly inhibit MLV DNA polymerase directed by three templates tested; namely, poly[d(A-T)], poly(A):oligo

(dT) and poly(C):oligo(dG). These polyadenylic acids do not inhibit the poly [d(A-T)]-directed reaction of E. coli and murine spleen DNA polymerase. These results suggest that these polynucleotides may be specific inhibitors of the oncornaviral enzyme and could serve as diagnostic tools for characterizing the RNA-directed DNA polymerase activity in cells and tissues, for example, those of human neoplastic origin. However, the effect of these polynucleotides on DNA polymerases from several other mammalian tissues will need to be investigated before a definitive conclusion regarding specificity of inhibition can be reached (7).

Poly(2'-me A) and poly(2'-et A) show an apparent competitive inhibition of MLV DNA polymerase directed by poly(A):oligo(dT). This is in agreement with the competitive inhibition by poly(A) and poly(2'-me A) of poly [d(A-T)]-directed Rauscher murine leukemia virus DNA polymerase reported by Tuominen and Kenney (6). On the other hand, poly(2'-et A), poly(2'-me A), poly(A), and poly(dA) yield kinetics of inhibition of the poly(C):oligo(dG)-directed reaction which are not consistent with a simple competitive inhibition where the inhibitor competes with the template for the same binding site on the enzyme. Given the complexity of the enzyme system, the observed kinetics are perhaps not unexpected. This situation may be even more complex if the template binding site consists of subsites as has been proposed by Marcus et al. (16). Our results are not inconsistent with this proposal. Under such a system, polyadenylic acids will competitively inhibit the binding of poly(A):oligo(dT) to its binding site based on structural similarities. The binding site for poly (C):oligo(dG) will involve some elements of subsites which are not common to the poly(A):oligo(dT) site and which are not competed for by other polyadenylic acids. Such a model will be consistent with the complex kinetics observed for poly(I) inhibition of poly(A):oligo(dT)-directed reaction. Clearly, other explanations of our results, including template-specific conformational changes of the enzyme, are possible.

Regardless of the complex kinetics, our results demonstrate that

O-alkylation of 2'-hydroxy group of poly(A) increases its inhibitory potential for viral DNA polymerase; 2'-O-ethylation being more effective than 2'-O-methylation. Poly(dA) is the least effective inhibitor of this series suggesting that the 2'-oxygen function may be involved in the interaction of polyribonucleotides with the enzyme. Since 2'-O-alkylated polynucleotides are also more resistant to nucleolytic degradation (12), they may be potent inhibitors of *in vivo* viral replication and cell transformation. Some of these properties of 2'-O-alkylated polynucleotides are now under investigation.

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