Inhibition of Purified Human and Herpes Simplex Virus-induced DNA Polymerases by 9-(2-Hydroxyethoxymethyl)guanine Triphosphate

EFFECTS ON PRIMER-TEMPLATE FUNCTION*

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The inhibition of highly purified herpes simplex virus (HSV)-induced and host cell DNA polymerases by the triphosphate form of 9-(2-hydroxyethoxymethyl)guanine (acyclovir; acycloguanosine) was examined. Acyclovir triphosphate (acyclo-GTP) competitively inhibited the incorporation of dGMP into DNA, catalyzed by HSV DNA polymerase; apparent $K_{m}$ and $K_{i}$ values of ddGTP and acyclo-GTP were 0.15 $\mu$M and 0.003 $\mu$M, respectively. HeLa DNA polymerase a was also competitively inhibited; $K_{m}$ and $K_{i}$ values of ddGTP and acyclo-GTP were 1.2 $\mu$M and 0.18 $\mu$M, respectively. In contrast, HeLa DNA polymerase b was insensitive to the analogue. The "limited" DNA synthesis observed when dGTP was omitted from HSV or a DNA polymerase reactions was inhibited by acyclo-GTP in a concentration-dependent manner. Prior incubation of activated DNA, acyclo-GTP, and DNA polymerase (a or HSV) resulted in a marked decrease in the utilization of the primer-template in subsequent DNA polymerase reactions. This decreased ability of preincubated primer-templates to support DNA synthesis was dependent on acyclo-GTP, enzyme concentration, and the time of prior incubation. Acyclo-GMP-terminated DNA was found to inhibit HSV DNA polymerase-catalyzed DNA synthesis. Kinetic experiments with variable concentrations of activated DNA and fixed concentrations of acyclo-GMP-terminated DNA revealed a noncompetitive inhibition of HSV-1 DNA polymerase. The apparent $K_{m}$ of 3'-hydroxyl terminated was $1.1 \times 10^{-7}$ M, the $K_{ii}$ and $K_{i}$ values of acyclo-GMP-terminated DNA in activated DNA were $8.8 \times 10^{-8}$ M and $2.1 \times 10^{-8}$ M, respectively. Finally, $^{3}$H-labeled acyclo-GMP residues incorporated into activated DNA by HSV-1 DNA polymerase could not be excised by the polymerase-associated 3',5'-exonuclease activity.

The nucleoside analogue, 9-(2-hydroxyethoxymethyl)guanine, is a potent and selective inhibitor of herpes simplex virus replication in vivo and in vitro (1, 2, 4). The first step in the conversion of acyclovir to the active metabolite, acyclo-GTP, is catalyzed exclusively by the HSV-induced deoxynucleotidyl kinase (1, 3). Acyclo-GTP competitively inhibited the incorporation of dGMP into DNA catalyzed by partially purified HSV DNA polymerase and host a-polymerase (1, 4). Acyclo-$^{14}$C[GMP was shown to be incorporated into DNA by HSV DNA polymerase and, at a slower rate, by a polymerase, in vitro (4).

Acyclo-GMP incorporated into primer termini would prohibit subsequent primer elongation owing to its lack of an extendable 3' hydroxyl moiety. The suggestion has been made (4) that the inhibition of HSV DNA polymerization by acyclo-GTP might be analogous to the inhibition of Escherichia coli DNA polymerase I by 2',3'-dideoxythymidine 5'-triphosphate (5). That is, HSV DNA polymerase inhibition might be due to an effect concomitant with or subsequent to acyclo-GMP incorporation. To date, an examination of the primer-template effects of acyclo-GTP, using highly purified DNA polymerases, has not been reported.

Like E. coli DNA polymerase I and several other prokaryotic DNA polymerases, HSV DNA polymerase possesses a 3',5'-exonuclease activity (6-9) which may remove mismatched or fraudulent nucleotides incorporated into DNA. A previous report showed that acyclovir treatment of cells, which contained the HSV-specified deoxynucleotidyl kinase, caused an accumulation of very short nascent DNA fragments (9). These low molecular weight fragments, which contained acyclo-$^{3}$H[GMP residues at their 3'-termini, could not be chased into high molecular weight material even after the drug was removed (9). The potential ability of the HSV DNA polymerase 3',5'-exonuclease activity to remove 3'-terminal acyclo-GMP residues is important mechanistically and pharmacologically.

In the present communication, a further investigation of the inhibition by acyclo-GTP of highly purified HSV and HeLa cell DNA polymerases is presented. The primer-template effects of the drug were examined and the question of the ability of the HSV DNA polymerase-associated 3',5'-exonuclease activity to remove 3'-terminal acyclo-GMP residues was addressed.

MATERIALS AND METHODS

All chemicals used were of reagent grade or better. Deoxynucleoside triphosphates and calf thymus DNA were purchased from Sigma.

1 The abbreviations used are: acyclovir or acycloguanosine, 9- (2-hydroxyethoxymethyl)guanine; HSV, herpes simplex virus; HSV-1, herpes simplex virus type I; HSV-2, herpes simplex virus type II; acyclo-GMP, 9-(2-hydroxyethoxymethyl)guanine monophosphate; acyclo-GTP, 9-(2-hydroxyethoxymethyl)guanine triphosphate; ddGTP, 2',3'-dideoxy-GTP.
Removal of acyclo-GMP residues in DNA by the HSV-1 DNA polymerase-associated 3',5'-exonuclease activity was analyzed indirectly by measuring the template-dependent conversion of acyclo-[14C]GTP to acyclo-[14C]GMP or directly by measuring the release of acyclo-[14C]GMP from acyclo-[14C]GMP-terminated DNA. An HSV-1 DNA polymerase reaction (1 ml) was performed as described above for the synthesis of acyclo-[14C]GMP-terminated DNA except that 22 units of HSV-1 DNA polymerase (1 ml) was used. An aliquot taken from the nucleotide fraction of the Sephadex G-25 column chromatography was spotted using a PEI-cellulose plate (Brinkman Instruments) The aliquot, containing 1 x 10^6 cpm 14C-labeled acyclovir nucleotides, was applied with markers dGMP, dGDP, and dGTP and developed with 2 M acetic acid, 0.5 M LiCl. After drying, the plate was cut into 1 cm sections and placed in 10 ml of scintillation fluid and counted. An aliquot of the stock acyclovir was run in parallel as a standard. To determine directly the excision of acyclo-[14C]GMP residues from 3'-termini in DNA, a 0.2 ml reaction mixture containing: 50 mM Tris-Cl, pH 8.0, 4 mM MgCl2; 0.5 mM dithiothreitol; 0.5 mg/ml of bovine serum albumin; 12 ng of acyclo-[14C]GMP-terminated DNA (100 cpm/µg, prepared as described above); and 1.0 units of HSV-1 DNA polymerase was incubated for 45 and 90 min at 37°C.

RESULTS AND DISCUSSION

Inhibition by Acyclo-GTP—HSV-1 and HSV-2 DNA polymerase reactions in the presence of variable concentrations of [14C]GTP and varied, fixed concentrations of acyclo-GTP are presented in the form of Lineweaver-Burk plots in Fig. 1, A and B. Acyclo-GTP competitively inhibited the incorporation of dGMP into DNA catalyzed by DNA polymerase of either virus type. Apparent Ks, and Ks, values of dGTP and acyclo-GTP for both DNA polymerases were 0.15 µM and 0.003 µM, respectively. These kinetic constants are considerably lower than those previously reported (4), and may be due to the greater purity of the enzymes used here or the different reaction conditions employed. HeLa DNA polymerase was also competitively inhibited by acyclo-GTP and ddGTP, and acyclo-GTP for both DNA polymerases were 0.15 µM and 0.18 µM, respectively. In contrast, HeLa β-polymerase was not inhibited by acyclo-GTP concentrations up to 50 µM at 1.0 µM dGTP. Interestingly, the pattern of inhibition of these DNA polymerases by acyclo-GTP was the reverse of that observed with 2',3'-dideoxy-GTP. The latter compound specifically inhibited HeLa β-polymerase but it was only weakly inhibitory toward HSV DNA polymerase and HeLa α-polymerase, in agreement with the results of Zimmermann et al. (15). While acyclo-GTP and ddGTP are similar in their lack of 2'- and 3'-hydroxyl groups, the absence of the 2' and 3' carbon atoms in acyclo-GTP may allow a degree of flexibility which is prohibited by the rigid furanside ring structure of ddGTP.

DNA polymerases catalyze a limited rate of DNA synthesis when a single deoxyxynucleoside triphosphate is omitted from the reaction (13, 14). The relative rates of DNA synthesis with a limited versus complete complement of dNTPs has previously been used to assess the processivity of DNA polymerases (10, 14). In order to determine possible effects of acyclo-GTP beyond the competitive inhibition of dGMP incorporation, we examined the influence of the drug on DNA synthesis limited by the omission of dGTP. As shown in Fig. 2, in the absence of dGTP, HSV DNA polymerase and HeLa α polymerase reactions were inhibited by acyclo-GTP, in a concentration dependent manner. Iso values for acyclo-GTP in this experiment were 0.003 µM for HSV-1 DNA polymerase and 0.4 µM for HeLa α-polymerase. These data indicate that acyclo-GTP inhibition of DNA synthesis involved more than the simple competitive inhibition of dGMP incorporation, and reflects an increase in the cycling time of the polymerase (14) which was possibly a consequence of acyclo-GMP incorporation into DNA.

Primer-Template Studies—In order to determine the ef-

2 D. Dene and Y-C. Cheng, data not shown.
The effects of acyclo-GMP incorporation on primer-template function, activated DNA was incubated with DNA polymerase (HSV or α) and acyclo-GTP for varied periods of time. The DNA polymerase activity was then destroyed and an aliquot of fresh DNA polymerase plus an excess of deoxyribonucleoside triphosphates were added and DNA synthesis was monitored.

Fig. 3 shows that with increasing times of prior incubation in the presence of acyclo-GTP, the subsequent ability of the DNA to support a normal rate of synthesis progressively declined. The rate and extent of this decrease was dependent on the amounts of enzyme and acyclo-GTP present in the prior incubation. HSV DNA synthesis was apparently more sensitive to these prior incubations than α-polymerase. The decreased rate of DNA synthesis on preincubated primer-template could be explained by a significant decrease in the concentration of 3'-termini available or, more likely, to a high affinity of DNA polymerase for a limited number of acyclo-GMP termini. To test the latter possibility, acyclo-GMP-terminated DNA was synthesized, as described under “Materials and Methods.” Ten per cent of the initial usable 3'-termini in this activated DNA were occupied by acyclo-GMP residues, as determined by the amount of acyclo-[3H]GMP incorporated in a parallel reaction. Fixed concentrations of this partially acyclo-GMP-terminated, “inhibitor,” DNA preparation were added to HSV-1 DNA polymerase reactions with varying amounts of activated DNA. The data are presented in the Lineweaver-Burk plot shown in Fig. 4A. The abscissa is the inverse concentration of activated DNA added; therefore the lines where inhibitor DNA is present are curved, due to the presence of additional 3'-hydroxyl termini in this preparation. When the DNA concentrations in Fig. 4A were corrected to include the total concentration of usable primer-template present and replotted, Fig. 4B was obtained. Unexpectedly, a noncompetitive inhibition pattern was reproducibly observed. This is possibly a reflection of the tight binding of DNA polymerase to acyclo-GMP termini. Alternatively, acyclo-GMP-terminated DNA may interact with HSV-1 DNA polymerase at a second DNA binding site. It should be noted that acyclo-GTP was not present in the inhibitor DNA preparation. The apparent K, of activated DNA was 5.2 μg/ml; K, and K, values of acyclo-GMP-terminated DNA, calculated from slope and intercept replots, were 0.1 μg/ml and 4.2 μg/ml, respectively. In terms of primer-termini, the K, of usable 3'-hydroxyl termini and the K, of acyclo-GMP termini were 1.1 × 10^{-7} M and 2.1 × 10^{-6} M, respectively. These data clearly indicate that acyclo-GMP-terminated DNA is itself a potent inhibitor of HSV-1 DNA synthesis; the kinetically derived affinity of HSV-1 DNA polymerase for acyclo-GMP termini is approximately 50

**Fig. 1. Competitive inhibition of HSV DNA polymerase and HeLa α-polymerase by acyclo-GTP.** The data are presented in the form of Lineweaver-Burk plots. HSV-1 (A) and HSV-2 (B) DNA polymerase reactions (0.1 ml) contained 50 mM Tris-Cl, pH 8.0; 4 mM MgCl₂; 0.5 mM dithiothreitol; 50 μg of bovine serum albumin; 0.2 mM KCl; 12 μg of activated calf thymus DNA; 0.1 mM each dATP, dCTP, dTTP, [3H]dGTP at 1.5 × 10⁶ cpm/mmol; 0.1 unit of HSV-1 or 0.17 unit of HSV-2 DNA polymerase; and acyclo-GTP at 0 ( ), 0.01 ( ), 0.02 μM ( ), 0.1 μM ( ), and 2 μM ( ) acyclo-GTP. Lines were drawn by the method of least squares analysis. K, values were determined from the slope replots shown in the inset.

**Fig. 2. Acyclo-GTP inhibition of DNA polymerization in the absence of dGTP.** A. HeLa DNA polymerase α reactions contained 50 mM Tris-Cl, pH 8.0; 8 mM MgCl₂; 0.5 mM dithiothreitol; 50 μg of bovine serum albumin; 25 μg of activated calf thymus DNA; 0.1 mM each dATP and dCTP; 20 μM [3H]dGTP at 70 cpm/mmol; 0.28 unit of α polymerase; and 0 ( ), 0.1 μM ( ), and 0.2 μM ( ) acyclo-GTP. B. HSV-1 DNA polymerase reactions (0.1 ml) contained 50 mM Tris-Cl, pH 8.0; 4 mM MgCl₂; 0.5 mM dithiothreitol; 50 μg of bovine serum albumin; 0.2 mM KCl; 12 μg of activated calf thymus DNA; 0.1 mM each dATP and dCTP; 20 μM [3H]dGTP at 400 cpm/mmol; 0.1 unit of HSV-1 DNA polymerase; and the indicated concentrations of acyclo-GTP, as μM. The per cent of the incorporation of [3H]dTMP observed in the presence, relative to the absence of acyclo-GTP is plotted versus the log of acyclo-GTP concentration.
times greater than for 3'-hydroxyl termini, in activated DNA.

Excision of Acyclo-GMP Residues in DNA—HSV DNA polymerase has been shown to possess a 3',5'-exonuclease activity (6-8), which may serve a proof-reading role in removing mismatched or fraudulent nucleotides incorporated into DNA. We have recently shown that this 3',5'-exonuclease activity can remove 3'-terminal araAMP residues in DNA in vitro (8). It was of interest, in elucidating the mechanism of action of acyclovir, to determine whether this activity could also remove acyclo-GMP residues from DNA. After incubations of activated DNA with acyclo-[14C]GTP and HSV-1 DNA polymerase, as described under “Materials and Methods,” [14C]acyclovir nucleotides were separated from the DNA product by gel filtration chromatography. Analysis of the nucleotides by thin layer chromatography revealed that no detectable amounts of acyclo-[14C]GMP were generated during the reaction. The acyclo-[14C]GMP-containing DNA was then used as the substrate for the HSV-1 DNA polymerase 3',5'-exonuclease activity. Again, liberation of acyclo [14C]GMP could not be detected, even after extensive incubations. These results indicated that 3'-terminal acyclo-GMP residues in DNA were not removed at a detectable rate by the HSV-1 DNA polymerase-associated exonuclease.

Acyclo-GTP inhibition of HSV DNA polymerization in vitro appears to be a consequence of acyclo-GMP incorporation into primer-template which is augmented by a strong competitive inhibition of dGMP incorporation. The observations that acyclo-GTP inhibited HSV DNA synthesis in reactions limited by the omission of dGTP and that prior

![Figure 3: Effect of prior incubation with acyclo-GTP on subsequent template-primer function. HSV-1 ( □ ) and HSV-2 ( ▲ ) DNA polymerase reactions contained 50 mM Tris-Cl, pH 8.0; 4 mM MgCl2; 0.5 mM dithiothreitol; 50 μg of bovine serum albumin; 0.2 mM KCl; 5 μg of activated calf thymus DNA; 0.1 mM each dATP and dCTP; 0.05 μM acyclo-GTP; and 0.15 unit of HSV-1 or HSV-2 DNA polymerase, in a volume of 0.1 ml. HeLa DNA polymerase a ( ■ ) reactions (0.1 ml) contained 50 mM Tris-Cl, pH 8.0; 8 mM MgCl2; 0.5 mM dithiothreitol; 50 μg of bovine serum albumin; 6 μg of activated calf thymus DNA; 0.1 mM each dATP and dCTP; 1.0 μM acyclo-GTP; and 0.3 unit of α polymerase. Parallel reactions were also performed in which acyclo-GTP was not included. At the times indicated on the figure, reactions were heated to 65 °C for 15 min then cooled slowly to 25 °C. DNA polymerase activity was assessed on these preincubated primer-templates after additions of dGTP to 0.14 μM plus [1H]dTMP (2.5 μM, 116 cpm/pmol) and 0.15 unit of HSV-1 or HSV-2 DNA polymerase or 0.1 μM dGTP plus [1H]dTMP (10 μM, 53 cpm/pmol) and 0.3 unit of HeLa α polymerase. The figure shows the per cent of [1H]dTMP incorporated into DNA after prior incubations with acyclo-GTP relative to prior incubations minus acyclo-GTP versus the time of prior incubation. The amounts of [1H]dTMP incorporated into DNA subsequent to prior incubations in the absence of acyclo-GTP were 18 pmol, 15 pmol, and 38 pmol for HSV-1, HSV-2, and HeLa α-polymerases, respectively. These values did not vary significantly with increasing times of prior incubation.

![Figure 4: Inhibition of HSV-1 DNA polymerase by acyclo-GMP-terminated DNA. The data are displayed in the form of Lineweaver-Burk plots. HSV-1 DNA polymerase reactions contained 50 mM Tris-Cl, pH 8.0; 4 mM MgCl2; 0.5 mM dithiothreitol; 50 μg of bovine serum albumin; 0.2 mM KCl; 0.1 mM each dATP, dCTP, dGTP; 2 μM [1H]dTMP (9380 cpm/pmol); activated calf thymus DNA as indicated; 0.1 unit of HSV-1 DNA polymerase; and 0 (ΟΟ), 2.5 ( ■ ■■), and 10 μg/ml ( ▲ ▲ ▲ ) of acyclo-GMP-terminated DNA preparation (synthesized as described under “Materials and Methods”). In A, the abscissa is the inverse concentration of activated DNA added to each reaction and does not include the usable primer-template present in the acyclo-GMP-terminated DNA sample. In B, the total concentration of usable activated DNA present in each reaction was calculated; that is, the activated DNA added plus 90% of the acyclo-GMP-terminated DNA present. Lines were drawn by the method of least squares analysis. In B, the actual concentrations of acyclo-GMP-terminated DNA are 0 (ΟΟ), 0.25 ( □ □ ), 0.5 ( ■ ■ ), and 1.0 μg/ml ( ▲ ▲ ▲ ) of acyclo-GMP-incubation with the analogue decreased primer-template functionality strongly support this view. In addition, the kinetically derived affinity of HSV-1 DNA polymerase for acyclo-GMP termini in DNA was approximately 50 times greater than for 3'-hydroxyl termini. Thus, the DNA product of acyclo-GMP incorporation was a potent inhibitor of further polymerization. The apparent inability of the HSV-1 DNA polymerase 3',5'-exonuclease activity to remove acyclo-GMP residues incorporated into DNA may contribute to the tight binding of the polymerase to these termini as well as to prolonging the inhibition of DNA polymerization.

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Herpes Simplex Virus DNA Polymerase

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