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Inhibition of Cellular α and Virally Induced Deoxyribonucleic Acid Polymerases by the Triphosphate of Acyclovir

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The effect of the triphosphate of 9-(2-hydroxyethoxymethyl)guanine (acyclovir, acycloguanosine) on cellular α deoxyribonucleic acid (DNA) polymerases (DNA nucleotidyltransferases), DNA polymerases of several members of the herpes group, vaccinia virus DNA polymerase, and Friend leukemia virus ribonucleic acid-dependent DNA polymerase was examined. Several viruses, which were found to be susceptible to acyclovir, were found to induce DNA polymerases which were sensitive to acyclovir triphosphate (acyclo-GTP). Human cytomegalovirus and the H29R strain of herpes simplex virus type 1, however, were found to be relatively insusceptible to acyclovir, even though their induced DNA polymerases were inhibited by low concentrations of acyclo-GTP. The amount of acyclovir anabolized to acyclo-GTP was significantly lower for human cytomegalovirus and H29R than for the more susceptible viruses. Vaccinia virus and Friend leukemia virus induced DNA polymerases which were insensitive to inhibition by low concentrations of acyclo-GTP, anabolized little acyclovir to acyclo-GTP, and were found to be insensitive to inhibition by acyclovir. Uninfected WI-38 cells were not susceptible to inhibition by acyclovir, anabolized little acyclovir to acyclo-GTP, and had an α DNA polymerase which was insensitive to inhibition by low concentrations of acyclo-GTP.

Considerable effort has been devoted to the discovery of chemical compounds which inhibit viral replication without affecting the normal cell functions. Elion et al. (8) and Schaeffer et al. (19) reported that the purine analog 9-(2-hydroxyethoxymethyl)guanine (acyclovir, acycloguanosine) is a potent antiherpetic agent which possesses extremely low cytotoxicity to uninfected cells. Acyclovir is an inhibitor of herpes simplex virus (HSV) deoxyribonucleic acid (DNA) synthesis, whereas the synthesis of Vero cell DNA is only partially inhibited at acyclovir concentrations several-hundred-fold greater than the 50% effective dose (ED_{50}) for HSV type 1 (HSV-1) (11).

Acyclovir is anabolized to the monophosphate form by the viral thymidine kinase (8, 12), after which it is converted to the diphosphate form by cellular guanosine monophosphate kinase (W. H. Miller and R. L. Miller, J. Biol. Chem., in press) and then to the triphosphate form, apparently by cellular enzymes. Elion et al. (8) and Furman et al. (11) reported that acyclovir triphosphate (acyclo-GTP) is an inhibitor of HSV-1 DNA polymerases (DNA nucleotidyltransferases) and, to a lesser extent, of cellular α DNA polymerases.

This report examines the ability of acyclo-GTP to inhibit the activity of several virus-induced and two cellular α DNA polymerases. The viruses represented include HSV-1 and -2, human cytomegalovirus (HCMV), vaccinia virus, and an avian ribonucleic acid (RNA) tumor virus. Correlations are made between the degrees of sensitivity of the enzymes to acyclo-GTP, ED_{50}'s (expressed as micromolar acyclovir) for the viruses and cells, and amounts of acyclovir anabolized to acyclo-GTP by the virus-infected and uninfected cells.

MATERIALS AND METHODS

Viruses and cell cultures. HeLa S-3 cells (obtained from W. K. Joklik, Department of Microbiology, Duke University Medical Center, Durham, N.C.) were grown in suspension on Joklik-modified minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (Sterile Systems, Inc., Logan, Utah). Vero and WI-38 cells (American Type Culture Collection, Rockville, Md.) and L-929 cells (obtained from Naomi Cohn of these laboratories) were grown in Eagle minimum essential medium (GIBCO) with 10% heat-inactivated fetal calf serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Eveline and FG-10 cells (gifts of Alphonse Langlois, Department of Surgery, Duke University) were grown in Dulbecco-modified Eagle medium (GIBCO) and McCoy 5-A medium (GIBCO), respectively. The medium was supplemented with 10% heat-inactivated fetal calf serum,

50 U of penicillin per ml and 50 μg of streptomycin per ml.

Vaccinia virus was obtained from W. K. Joklik, Duke University Medical Center. HSV-1 strains H29 and H29R (a mutant resistant to acyclovir) were gifts of D. J. Bauer, Wellcome Research Laboratories, Beckenham, England. HCMV strain AD-169 was obtained from the American Type Culture Collection. The MS and 333 strains of HSV-2 were gifts of Earl Kern, Department of Pediatrics, University of Utah College of Medicine, Salt Lake City, Utah, and J. S. Pagano, Cancer Research Center, University of North Carolina, School of Medicine. Chapel Hill, N.C., respectively.

Virus propagation. HSV-1 strains H29 and H29R and the MS and 333 strains of HSV-2 were propagated in Vero cells as described previously (6, 8, 11). Stocks of vaccinia virus were prepared in L-929 cells and stored at −70°C. HCMV was propagated in WI-38 cells. The infected cells were frozen and stored in liquid nitrogen. Friend leukemia virus (FLV) was produced in Eveline cells (20). After centrifugation to remove cells and cellular debris, the released virus was pelleted, resuspended in 0.01 M tris(hydroxymethyl)aminomethane (pH 7.2)–0.02 M ethylenediaminetetraacetic acid–0.01 M NaCl (TNE) and repelleted through a 20% sucrose (in TNE) pad. The pelleted FLV was then layered on a 10 to 50% sucrose gradient and centrifuged in an SW 27 Beckman rotor for 5 h at 27,000 rpm. The sharp virus band was collected and diluted in TNE, and the virus was repelleted.

Plaque reduction and cytotoxicity assays. Virus titrations and plaque reduction assays for HSV-1 and HSV-2 were performed in Vero cells as described by Collins and Bauer (7). HCMV was assayed in WI-38 cells. The cells were stained on day 9 after infection. Vaccinia virus was titrated and assayed in L-929 cells (17). FLV was assayed in FG-10 cells as reported by Bassin et al. (2). ED_{50}'s for WI-38 and FG-10 cells were performed as described previously (10).

Chemicals and radiochemicals. Deoxyribosyladenosine triphosphate (dATP), deoxyribosylcytosine triphosphate (dCTP), deoxyribosylguanosine triphosphate (dGTP), and deoxyribosylthymidine triphosphate (dTTP) were obtained from PL Biochemicals, Inc. Dithiothreitol (Cleland reagent) and calf thymus DNA were obtained from Calbiochem-Behring Corp. [^{3}H]dTTP was obtained from ICN Chemical and Radioisotope Division. Acyclo-GTP was prepared as described previously (11). [8-^{14}C]acyclovir was synthesized in these laboratories by Jeffrey Scharver by unpublished procedures.

Purification of polymerase. The H29 and H29R strains of HSV-1, the MS and 333 strains of HSV-2, vaccinia virus, HCMV, and WI-38 α and L-929 α DNA polymerases were isolated and identified as described previously (8, 11, 13, 21). FLV RNA-dependent DNA polymerase was isolated by homogenization and centrifugation of purified virions as described by Hurwitz and Leis (14).

Polymerase assays. DNA polymerase assays were carried out as described by Elion et al. (8) and Furman et al. (11). Briefly, the reaction mixture (50-μl total volume) contained, unless otherwise indicated, 50 μM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.5), 12 mM $MgCl_2$, 0.1 mM each dCTP, dATP, dGTP, and [^{3}H]dTTP (specific activities as stated in the figure legends), 1.2 mM dithiothreitol, 0.25 mg of activated calf thymus DNA per ml, and 12.5 μl of enzyme. After incubation at 37°C, 20 μl of the reaction mixture was transferred to Whatman DE-81 paper and processed by the procedure of Altman and Lerman (1).

RNA-dependent DNA polymerase was assayed by using the conditions described by Battula and Loeb (3). Activated calf thymus DNA served as the template. After incubation at 37°C, 20 μl of the 50-μl reaction mixture was transferred to DE-81 paper and processed as described above.

Chemical analyses. The acyclo-GTP content of infected and uninfected cells exposed to acyclovir was determined by high-pressure liquid chromatography. Cells infected with HSV-1, HSV-2, vaccinia virus, and FLV were treated with [^{14}C]acyclovir beginning at 1 h postinfection and harvested after 7 h of treatment. HCMV-infected WI-38 cells were treated for 7 h with [^{14}C]acyclovir at 24 h postinfection. WI-38 and L-929 cells were also exposed to [^{14}C]acyclovir for 7 h. All cells were extracted and analyzed as described previously (8).

The concentration of protein of the partially purified DNA polymerase samples was determined colorimetrically by the method of Bradford (5).

RESULTS

Inhibition of viral and cellular growth by acyclovir. The effect of acyclovir on viral and cell growth was determined. The results (Table 1) indicate that the H29 strain of HSV-1 was the most susceptible of the viruses studied. The resistant strain, H29R, was approximately 100-fold less susceptible to acyclovir than was H29. The MS and 333 strains of HSV-2 had similar ED_{50}'s of 1.4 μM acyclovir. With ED_{50}'s of greater than 100 μM, HCMV, FLV, and vaccinia virus were essentially resistant to inhibition by acyclovir. Of the two cell lines studied, WI-38 was the more resistant, having an ED_{50} of greater than 1,000 μM, whereas the ED_{50} for L-929 cells was 50 μM.

Formation of acyclo-GTP. Elion et al. (8) reported that the amount of acyclo-GTP formed by cells incubated in the presence of [^{14}C]acyclovir increased dramatically upon infection of the cells. The amount of acyclo-GTP formed was also found to be dependent upon the virus group and even upon the virus strain used when infecting the cells. To make a more meaningful correlation between ED_{50}'s and apparent K_i's it was, therefore, necessary to determine the amount of acyclovir anabolized to acyclo-GTP after infection by each of the virus strains studied.

When all of the virus-infected and uninfected cells were exposed to 100 μM [^{14}C]acyclovir, the

TABLE 1. *Apparent kinetic constants, acyclo-GTP formation, and inhibition of plaque formation*

Polymerase Source	Apparent K_m[a] (μM dGTP ± SE)	Apparent K_i[a] (μM acyclo-GTP ± SE)	Acyclo-GTP[b] (μM)	ED_{50}[c] (μM acyclovir)
HSV-1 (strain H29)	(0.38 ± 0.13)[d]	(0.08 ± 0.03)[d]	9.8	0.9[e]
HSV-1 (strain H29R)	0.62 ± 0.40	0.51 ± 0.34	0.80	84
HSV-2 (strain MS)	1.34 ± 0.28	0.45 ± 0.12	68	1.4
HSV-2 (strain 333)	1.17 ± 0.26	0.56 ± 0.16	74	1.4
HCMV (strain AD-169)	3.79 ± 0.70	0.25 ± 0.05	0.06	115 (WI-38)
Vaccinia virus	2.13 ± 0.38	3.69 ± 0.70	0.04	200 (L-929)
FLV	9.63 ± 1.07	27.60 ± 6.58	0.04	>100 (FG-10)
WI-38 cells	2.66 ± 0.05	1.86 ± 0.82	0.04	>3,000
L-929 cells	2.13 ± 0.33	2.80 ± 1.07	0.78	50

[a] The activity of the polymerase was measured by the incorporation of [^{3}H]dTTP (specific activities, 23.6 to 165 cpm/pmol). The reaction mixture contained 25 to 350 U of enzyme per ml, with a specific activity of 0.26 to 8.0 pmol/min per μg of protein. SE, Standard error.

[b] Cells were treated for 7 h beginning at 1 h postinfection with 100 μM [^{14}C]acyclovir. Micromolar concentrations were calculated by assuming a packed volume of 0.005 ml for 10^6 cells (8). All HSV-1 and HSV-2 were grown in Vero cells, HCMV was grown in WI38 cells, vaccinia virus was grown in L-929 cells, and FLV was grown in FG-10 cells.

[c] ED_{50}'s were determined in Vero cells except as indicated.

[d] Elion et al. (8); Furman et al. (11).

[e] This strain originally showed an ED_{50} of 0.1 μM, but presently shows an ED_{50} of 0.9 μM.

amount of acyclo-GTP formed was very much higher in those cells containing virus susceptible to acyclovir, e.g., H29, MS, and 333 (Table 1). The Vero cells containing H29R and uninfected L-929 cells made approximately 0.8 μM acyclo-GTP, whereas the amounts of acyclo-GTP in highly resistant cells and in HCMV-infected cells were below 0.06 μM. At concentrations of [^{14}C]acyclovir approximating the ED_{50}'s for the more susceptible viruses, the amounts of acyclo-GTP formed were between 0.4 and 0.8 μM (Table 2).

TABLE 2. *Formation of acyclo-GTP in Vero cells infected with HSV-1 and HSV-2*

Virus	Acyclovir in medium[a] (μM)	Acyclo-GTP[b] (μM)
HSV-1 (H29)	0.5	0.80
HSV-2 (MS)	1.0	0.70
HSV-2 (333)	1.0	0.40

[a] Cells were treated for 7 h beginning at 1 h postinfection.

[b] Micromolar concentrations were calculated by assuming a packed volume of 0.005 ml for 10^6 cells (8).

Inhibition of polymerase activity with acyclo-GTP. The effects of both acyclovir and its triphosphate on enzyme activity were studied. When the concentrations of the natural deoxynucleoside triphosphates were 100 μM, 20.9 μM acyclo-GTP inhibited the activity of 333 DNA polymerase by 66% after 15 min of incubation (Fig. 1), and 209 μM acyclo-GTP inhibited the enzyme activity by greater than 99%. On the other hand, 131 μM of the unphosphorylated compound had no effect on the same enzyme.

The Lineweaver-Burk plots obtained with the cellular α and viral DNA polymerases studied indicated competitive inhibition between dGTP and acyclo-GTP. Examples for polymerases induced by HSV-2 and vaccinia virus are given in Fig. 2. The concentration of acyclo-GTP used with vaccinia virus DNA polymerase was approximately 10-fold higher than that used with HSV-2. The apparent K_i's for acyclo-GTP and the apparent K_m's for dGTP (Table 1) were calculated by assuming normal enzyme kinetics (see Discussion). The apparent K_i's for acyclo-GTP for HSV-1, HSV-2, and HCMV DNA polymerases were 3- to 35-fold lower than the apparent K_i's for the cellular α DNA polymerases. The apparent K_i for L-929 α DNA polymerase was found to be 1.5-fold higher than the apparent K_i for WI-38 α DNA polymerase. FLV RNA-dependent DNA polymerase possessed the highest apparent K_i (27.60 ± 6.58 μM acyclo-GTP) of any of the DNA polymerases which were studied.

DISCUSSION

The Lineweaver-Burk plots obtained with these DNA polymerases, as with the DNA polymerases reported by Elion et al. (8) and Furman et al. (11), indicated that acyclo-GTP is a competitive inhibitor with respect to dGTP. However, the decreasing rate of [^{3}H]dTTP incorporation observed when acyclo-GTP was included

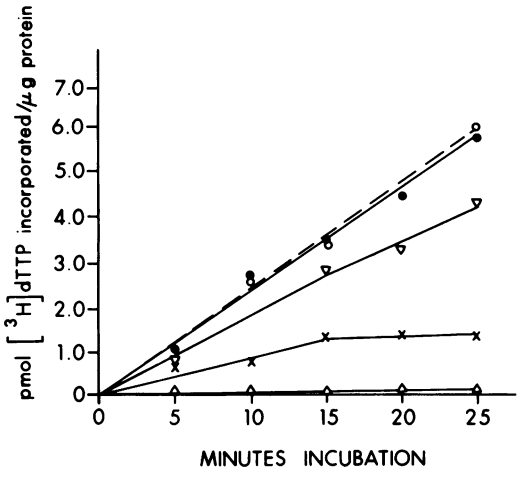

FIG. 1. *Effects of acyclovir and acyclo-GTP on HSV-2 (strain 333) DNA polymerase. Symbols: ●, no acyclo-GTP; ▽, 2.09 μM acyclo-GTP; ×, 20.9 μM acyclo-GTP; △, 209 μM acyclo-GTP; ○----○, 131 μM acyclovir. The activity of the polymerase was measured by the incorporation of [^{3}H]dTTP (specific activity, 138 cpm/pmol). HSV-2 DNA polymerase (20.4 U/ml) with a specific activity of 0.23 pmol/min per μg of protein was used.*

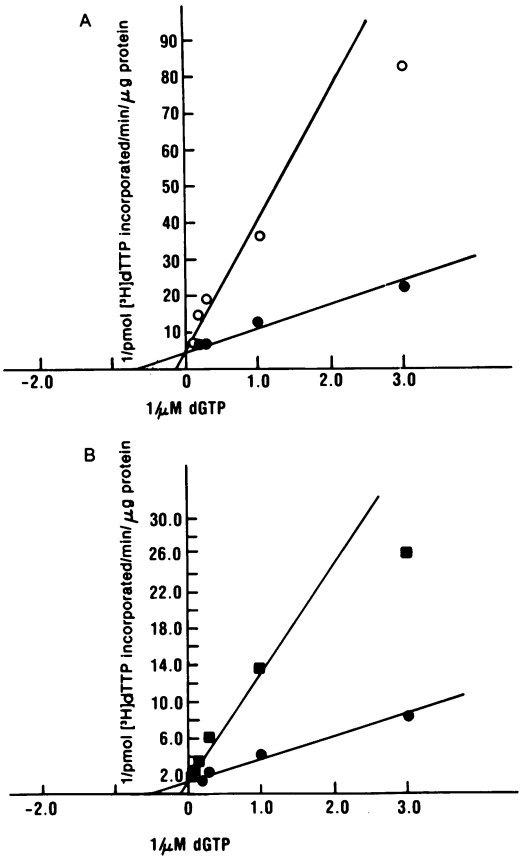

FIG. 2. *Lineweaver-Burk plots showing inhibition of (A) HSV-2 (strain MS) DNA polymerase and (B) vaccinia virus DNA polymerase. Symbols: ○, 2.09 μM acyclo-GTP; ■, 12.55 μM acyclo-GTP; ●, no acyclo-GTP. The activity of the polymerase was measured by the rate of incorporation of [^{3}H]dTTP. Specific activities: (A) 113 cpm/pmol, (B) 30.9 cpm/pmol. HSV-2 DNA polymerase (20.6 U/ml) with a specific activity of 0.39 pmol/min per μg of protein and vaccinia DNA polymerase (68 U/ml) with a specific activity of 1.4 pmol/min per μg of protein were used.*

in the reaction mixture (Fig. 1) and preliminary evidence that acyclo-GTP incorporation may lead to enzyme or template inactivation or both (11) suggest that anomalies may exist in the kinetics of the inhibition. Therefore, only those datum points which represent the initial velocities were used in the Lineweaver-Burk plots, and inhibition constants were calculated by assuming normal enzyme kinetics. The DNA polymerases induced by HSV-1, HSV-2, and HCMV, having apparent K_i's which varied from 0.08 ± 0.03 to 0.56 ± 0.16 μM acyclo-GTP, were extremely sensitive to inhibition by acyclo-GTP. The DNA polymerases induced by vaccinia virus and FLV and the α DNA polymerases of WI-38 and L-929 cells, however, required much higher concentrations of acyclo-GTP to achieve the same degree of inhibition.

Vero cells infected with HSV-1 (strain H29) and incubated in the presence of a concentration of acyclovir twofold lower than the ED_{50} for H29 resulted in a cellular acyclo-GTP concentration 10-fold higher than the apparent K_i of the H29 DNA polymerase. In addition, when Vero cells infected with HSV-1 (strain H29R) or two strains of HSV-2 were incubated in a concentration of acyclovir approximating the respective ED_{50}'s, the resulting acyclo-GTP content approached the apparent K_i's of the induced DNA polymerases. Inasmuch as the viral thymidine kinase is the enzyme responsible for the initial phosphorylation of acyclovir (8, 12), and H29R thymidine kinase exhibits a 15-fold reduction in acyclovir phosphorylating activity compared with H29 (12), the resistance of H29R to acyclovir was not unexpected. The amount of acyclo-GTP formed in H29R cells at 100 μM (near the ED_{50}) was equal to the K_i for viral DNA polymerase.

Cells infected with HCMV or vaccinia virus anabolized little acyclovir to acyclo-GTP, and both viruses were found to be insusceptible to inhibition by acyclovir. The DNA polymerase induced by vaccinia virus was not as insensitive

to inhibition by acyclo-GTP as were the DNA polymerases induced by HSV-1 and -2 (eg., Fig. 2 and Table 1). The DNA polymerase induced by HCMV was as sensitive to inhibition by acyclo-GTP as were the DNA polymerases induced by HSV. The resistance of HCMV to acyclovir appears to be due primarily to the absence of a virus-specified thymidine kinase (9). Vaccinia virus, on the other hand, induces a thymidine kinase (15, 16). However, Fyfe et al. (12) showed that the thymidine kinase induced by vaccinia virus was unable to phosphorylate acyclovir. FLV RNA-dependent DNA polymerase was virtually unaffected by acyclo-GTP, and FLV-infected FG-10 cells anabolized little acyclovir to acyclo-GTP. FLV, not unexpectedly, was also found to be resistant to inhibition by acyclovir.

WI-38 cells were found to be extremely resistant to inhibition by acyclovir, whereas the ED_{50} for L-929 cells was only 50 μM. L-929 cells formed 20-fold more acyclo-GTP than did WI-38 cells when treated with the same concentration of acyclovir. The low ED_{50} for L-929 cells was consistent with the ED_{50}'s observed for other mouse cell lines (10, 18).

The fact that low concentrations of acyclo-GTP inhibited HSV-induced DNA polymerases although even large amounts of the unphosphorylated form of the compound had no effect on 333 DNA polymerase indicated that acyclovir must be phosphorylated to the triphosphate form before the activity of the viral DNA polymerase will be inhibited. Inhibitory concentrations of the triphosphate are dependent upon the formation of sufficient amounts of acyclovir monophosphate. Therefore, the viral susceptibility to acyclovir depends not only on the sensitivity of the virus-induced DNA polymerase to inhibition by acyclo-GTP, but also on the presence of a nucleoside kinase capable of phosphorylating acyclovir and on the presence of cellular enzymes which convert the monophosphate to the triphosphate.

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