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Murine cytomegalovirus DNA polymerase: purification, characterization and role in the antiviral activity of acyclovir

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Summary

Murine cytomegalovirus (MCMV) neither induces a viral thymidine kinase (TK) nor enhances the activity of a cellular TK. Nevertheless, MCMV is highly susceptible to 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV). The cellular TK is neither responsible for phosphorylation of ACV nor its anti-MCMV activity. This is clear from the findings that little ACV triphosphate is formed in MCMV-infected mouse embryo fibroblasts (MEF) and that the replication of MCMV is inhibited equally well by ACV in TK^+ and TK^- cells. Even if trace amounts of ACV triphosphate would be formed by enzymes other than TK, and ACV triphosphate would be responsible for the anti-MCMV activity of ACV, then the MCMV DNA polymerase ought to be highly sensitive to ACV triphosphate. To examine this possibility, the MCMV DNA polymerase was partially purified and characterized. The apparent K_i value of the MCMV DNA polymerase for ACV triphosphate indicates that the sensitivity of the MCMV DNA polymerase to ACV triphosphate is equivalent to that of the HSV DNA polymerase. Therefore, the trace amounts of ACV triphosphate that are formed in MCMV-infected MEF seem to be insufficient to inhibit MCMV DNA polymerase and may not play a key role in the anti-MCMV activity of ACV.

Acyclovir (ACV); ACV triphosphate; Murine cytomegalovirus (MCMV); DNA polymerase; Kinetic constant

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Introduction

9-(2-Hydroxyethoxymethyl)guanine (acyclovir, aciclovir, ACV) has been used to treat patients with herpes simplex virus (HSV) type 1 (HSV-1) or type 2 (HSV-2), or varicella-zoster virus (VZV) infection (Dorsky and Crumpacker, 1987). The antiviral activity of ACV depends on two virus-coded enzymes, thymidine kinase (TK) and DNA polymerase. The viral TK phosphorylates ACV more efficiently than the cellular TK (Fyfe et al., 1978). As a result, ACV triphosphate accumulates in the virus-infected cells, but not in the uninfected cells (Elion et al., 1977; St. Clair et al., 1980). ACV triphosphate inhibits DNA synthesis by competing with dGTP (Derse et al., 1981) and it leads to chain termination when incorporated into DNA (Furman et al., 1984). The viral DNA polymerase is more sensitive to the inhibitory action of ACV triphosphate than the cellular DNA polymerase (Elion et al., 1977).

HSV and VZV mutants lacking TK activity are resistant to ACV (Coen and Shaffer, 1980; Schnipper and Crumpacker, 1980; Biron et al., 1982). In addition, human cytomegalovirus (HCMV) which does not encode a specific TK (Zavada et al., 1976; Estes and Huang, 1977) requires a high concentration of ACV to be inhibited (Crumpacker et al., 1979). Thus, the antiviral effect of ACV usually depends on the virus-coded TK. In contrast, the Smith strain of murine cytomegalovirus (MCMV) does not induce TK activity both in a TK^+ and in a TK^- cell which is resistant to 5-bromodeoxyuridine (BUdR) (Muller and Hudson, 1977; Eizuru et al., 1978; Burns et al., 1981). The virus does not stimulate a TK^- cell to incorporate exogenous thymidine (Muller and Hudson, 1977). Furthermore, the replication of MCMV is not inhibited by BUdR in a TK^- cell (Muller and Hudson, 1977; Eizuru et al., 1978; Burns et al., 1981). Thus, MCMV neither induces viral TK activity nor enhances cellular TK activity. Yet, MCMV shows high susceptibility to ACV in vitro and in vivo (Burns et al., 1981; Wingard et al., 1981; Glasgow et al., 1982).

We considered two possibilities to account for the susceptibility of MCMV to ACV in the absence of TK activity. First, trace amounts of ACV triphosphate may be formed, to which the MCMV DNA polymerase would be extraordinarily sensitive. Second, if the MCMV DNA polymerase is as sensitive to ACV triphosphate as HSV DNA polymerase, ACV must function via a unique mechanism.

We first investigated the susceptibility of MCMV to ACV, partially purified the MCMV DNA polymerase and determined its kinetic constants for ACV triphosphate.

Materials and Methods

Chemicals and radiochemicals

ACV was generously supplied by Dr. K.L. Powell, Wellcome Research Laboratories, Beckenham, U.K. ACV triphosphate was a gift from Dr. H.

Machida, Yamasa Shoyu Co., Ltd., Choshi. Nonradioactive deoxyribonucleoside triphosphates (dATP, dCTP, dTTP and dGTP) were purchased from Sigma Chemical Co., St. Louis, MO. [^{3}H]dTTP (42 Ci/mmol) and [^{3}H]dGTP (10 Ci/mmol) were obtained from ICN Radiochemicals, Irvine, CA.

Cells and viruses

Mouse embryo fibroblasts (MEF) were prepared by trypsinization of 18- to 20-day-old embryos of ICR mice. A SV40-transformed mouse cell line, mks-A TU-7 (Kurimura et al., 1972), and its BUdR-resistant derivative, TU-7 BU, (Eizuru et al., 1978) were also used. MEF, mks-A TU-7, TU-7 BU and African green monkey kidney (Vero) cells were grown in Eagle's minimum essential medium (Nissui pharmaceut. Co., Tokyo) supplemented with 5% heat-inactivated calf serum and 60 μg/ml kanamycin. The Smith strain of MCMV was propagated in MEF, and plaque-titrated under 2% methylcellulose overlay and 5% CO_2 atmosphere. HSV-1 (KOS strain) was also grown and plaque-titrated in Vero cells.

Yield reduction assay of MCMV

The cell lines, mks-A TU-7 and TU-7 BU, served as TK^+ and TK^- cell lines, respectively. Cells were inoculated with MCMV at a multiplicity of infection (MOI) of 0.2 per cell and incubated in the presence of various concentrations of ACV for 40 h. Infectious viruses were quantified as PFU per ml. Virus yield is expressed as a percentage of control (without ACV).

Analysis of nucleoside triphosphate pools by high-pressure liquid chromatography (HPLC)

MEF were infected with HSV or MCMV at a MOI of 2 PFU and incubated in the presence of 0.5 mM of ACV for 8 and 24 h for HSV and MCMV, respectively. The infected cells were washed with phosphate-buffered saline (PBS) and treated with 9 vol. of 3.5% perchloric acid (Elion et al., 1977). The acid-soluble fraction was neutralized with 1 N KOH and centrifuged at 14000 rpm for 5 min. The supernatant was analyzed by HPLC (655 system, Hitachi Co., Tokyo) on a Partisil 10 SAX column (Whatman Inc., Clif., NJ). The elution was carried out at a flow rate of 1 ml/min with a linear gradient of KH_2PO_4 from 0.1 to 1.0 M (pH 3.5). Absorbance was monitored at 254 nm.

Partial purification of DNA polymerase

All steps of purification were carried out at 0–4°C.

MCMV-induced DNA polymerase Confluent monolayers of MEF were inoculated with MCMV at a MOI of 20. The infected cells were collected,

suspended in an extraction buffer [20 mM Tris.Cl (pH 7.5) and 0.5 mM dithiothreitol (DTT)], and disrupted by ultrasonication. The crude extract was treated with an equal volume of 3.4 M NaCl, 10 mM EDTA, and 1 mg of bovine serum albumin (BSA, Sigma) per ml. The mixture was left on ice for 40 min and centrifuged at 18 000 rpm for 20 min in an RPS50-2 rotor (Hitachi). The supernatant was dialyzed against buffer A [50 mM Tris·Cl (pH 7.5), 0.5 mM DTT, 0.2% Nonidet P-40, and 20% glycerol] and the dialysate was clarified by sedimentation at 15 000 rpm for 20 min in a 4N-II rotor (Tomy Seiko Co., Ltd., Tokyo).

The supernatant was batch-adsorbed to DEAE-cellulose (Whatman), preequilibrated in buffer A by the method of Nishiyama et al. (1983), and the mixture was packed into a column (1.2 × 9 cm). Elution was carried out with a 50 ml gradient of 0–0.3 M KCl in buffer A. An aliquot from each fraction was assayed for DNA polymerase activity in the presence or absence of 150 mM $(NH_4)_2SO_4$. The fractions eluting at 0.16–0.28 M were pooled and dialyzed against buffer B [50 mM Tris·Cl (pH 6.8, instead of pH 7.5 for the HSV-induced DNA polymerase reported by Powell and Purifoy, 1977), 0.5 mM DTT, 20% glycerol, and 0.2 M KCl]. The dialysate was batch-adsorbed to phosphocellulose (Whatman) which was preequilibrated with buffer B containing 500 μg of BSA per ml. The mixture was packed into a column (1.2 × 7 cm) and eluted with a 50 ml gradient of 0.2–0.45 M KCl in buffer B. The DNA polymerase activity of each fraction was assayed as described for the DEAE-cellulose column fractions. The fractions eluting at 0.35–0.39 M KCl were pooled, supplemented with BSA at a concentration of 500 μg/ml, desalted by gel filtration on a sephadex G-50 column (2.5 × 10 cm) and stored at −80°C as a sample of the MCMV-induced DNA polymerase.

HSV-induced DNA polymerase The HSV DNA polymerase was partially purified from an extract of the HSV-infected Vero cells by column chromatography with a combination of DEAE-cellulose and phosphocellulose according to the method of Powell and Purifoy (1977).

Host cell DNA polymerase α An extract of uninfected MEF cells was applied onto a DEAE-cellulose column. The DNA polymerase eluting at 0.06–0.22 M KCl was supplemented with BSA, desalted and stored as a sample of the DNA polymerase α.

Standard assay of DNA polymerase activity

The DNA polymerase activity was assayed by the method of Mar et al. (1985) with a slight modification. The standard reaction mixture consisted of polymerase buffer [20 mM Tris·Cl (pH 8.0), 10 mM $MgCl_2$, 0.4 mM DTT and 10 μg of BSA]; 10 μg of activated salmon testis DNA (Sigma); 100 μM each of dATP and dCTP; either 100 μM dGTP and 5 μM [^{3}H]dTTP (0.12 Ci/mmol) or 100 μM dTTP and 5 μM [^{3}H]dGTP (0.12 Ci/mmol); 10 μl of enzyme in a total

volume of 50 μl. An activated salmon testis DNA was prepared by the method of Yoshida et al. (1974). The mixture was allowed to react at 37°C and the reaction was terminated at the appropriate times by adding 50 μl solution of 0.1 M EDTA and 2 mg/ml BSA. The mixture received 100 μl of 10% trichloroacetic acid. The acid-precipitate was dissolved in 2 N NH_4OH and the radioactivity was counted by liquid scintillation counter (LSC-3500, Aloka Co., Ltd., Tokyo). One unit of DNA polymerase was defined as the amount of enzyme catalyzing the incorporation of 1 pmol of dTMP or dGMP into DNA per min at 37°C.

Assay of DNase activity

The reaction mixture (50 μl) consisted of polymerase buffer, 10 μg of [^{3}H]-labelled salmon testis DNA (13 000 dpm) and a DNA polymerase sample. A viral DNA polymerase sample was assayed in the presence of 150 mM $(NH_4)_2SO_4$, and a DNA polymerase α sample in the absence of $(NH_4)_2SO_4$. Salmon testis DNA was labelled with [^{3}H]dGTP and [^{3}H]dTTP according to a protocol of the Multiprime DNA labelling systems (Amersham International plc, Buckinghamshire). The reaction mixture was allowed to react at 37°C for 20 min, and the reaction was stopped as described for the DNA polymerase assay. The acid-soluble radioactivity was determined by liquid scintillation counter.

Determination of the kinetic constants, K_m and K_i

The reaction mixture consisted of polymerase buffer; 10 μg of activated salmon testis DNA; 100 μM each of dATP, dTTP and dCTP; four different concentrations of [^{3}H]dGTP (0.5, 1.0, 2.0 and 4.0 μM); various concentrations of ACV triphosphate; either 0.3 units of the HSV DNA polymerase, 1.0 unit of the MCMV DNA polymerase or 1.0 unit of the DNA polymerase α. The HSV DNA polymerase was assayed in the presence of 100 mM $(NH_4)_2SO_4$, the MCMV DNA polymerase in the presence of 150 mM $(NH_4)_2SO_4$ and the cellular DNA polymerase α in the absence of $(NH_4)_2SO_4$. The mixture was allowed to react for 30, 20 and 15 min for the HSV DNA polymerase, MCMV DNA polymerase and DNA polymerase α, respectively. The incorporation rate of [^{3}H]dGMP into DNA at the various concentrations of dGTP in the absence of ACV triphosphate was determined and the data obtained were used to determine the K_m value by drawing classical Lineweaver-Burk plots. The apparent K_i for ACV triphosphate was estimated by replotting the slopes of Lineweaver-Burk plots which were obtained in the presence of various concentrations of ACV triphosphate. Similar experiments were done to examine the effect of ACV itself on MCMV DNA polymerase.

Results

Effect of cellular TK on the anti-MCMV activity of ACV

The effect of the cellular TK on the anti-MCMV activity of ACV was examined with mks-A TU-7 (TK^+) and its BUdR-resistant derivative, TU-7 BU (TK^-) (Fig. 1). MCMV yields in TK^+ and TK^- cells after the one-step replication cycle were reduced in a similar, dose-dependent manner. The 50% effective doses (ED_{50}) of ACV were 0.80 μM for mks-A TU-7 and 0.85 μM for TU-7 BU. Thus, the susceptibility of MCMV to ACV did not depend on the cellular TK, supporting the results reported by Burns et al. with a different set of cell lines (Burns et al., 1981).

Formation of ACV triphosphate

HPLC analysis of an acid-soluble fraction of the HSV-infected MEF showed an accumulation of ACV triphosphate at 50 min of retention time (Fig. 2A). Accumulation of ACV diphosphate was detected at 30 min. In contrast, neither ACV triphosphate nor ACV diphosphate was detected spectroscopically in the eluates of extracts from MCMV-infected cells (Fig. 2B). The limit of detection was 6 pmole per 10^6 cells.

Partial purification of DNA polymerases

If trace amounts of ACV triphosphate formed by enzymes other than TK would be responsible for the inhibitory action of ACV, the MCMV DNA polymerase should be highly sensitive to ACV triphosphate. To determine if

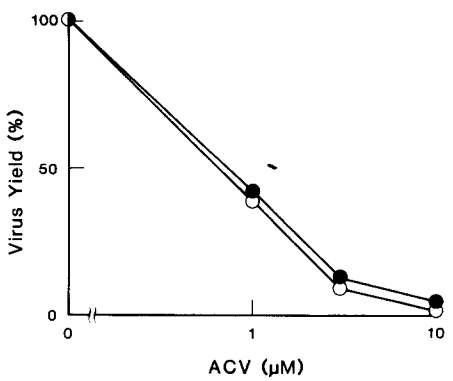

Fig. 1. Effect of ACV on MCMV replication in TK^+ and TK^- cells. MCMV-infected mks-A TU-7, TK^+ (○), and TU-7 BU, TK^- (●), were treated with various concentrations of ACV or untreated for 40 h. The virus yield was determined by plaque formation and is expressed as a percentage of the value in the absence of ACV.

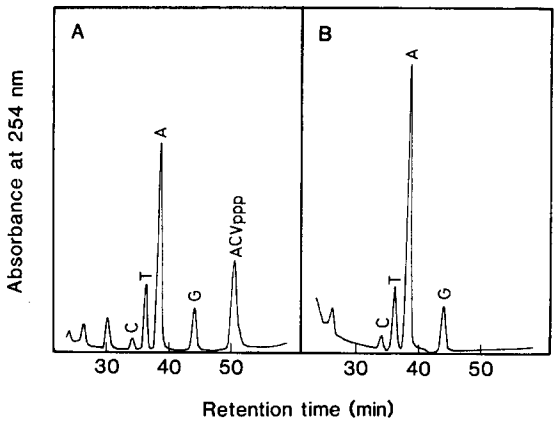

Fig. 2. Elution profiles of virus-infected cell extracts. Acid-soluble fraction of HSV (A)- or MCMV (B)-infected cells was analyzed by HPLC. The absorbance at 254 nm was monitored and elution profiles from 20 to 60 min of retention time are presented.

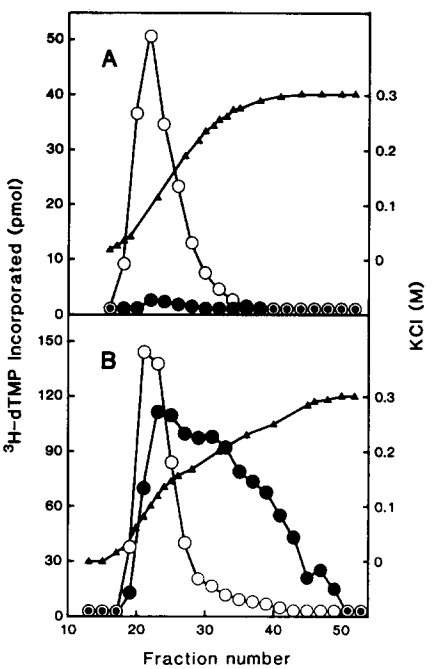

Fig. 3. DEAE-cellulose chromatography of the extracts of uninfected MEF (A) and MCMV-infected MEF (B). High-salt extracts of uninfected and MCMV-infected MEF cells were batch-adsorbed to DEAE-cellulose, and elution was achieved out with a gradient of 0–0.3 M KCl in buffer A. DNA polymerase activity of each fraction was assayed with the reaction mixture containing [^{3}H]dTTP in the presence (●) or absence (○) of 150 mM $(NH_4)_2SO_4$. KCl concentration (▲) of column eluates was determined by conductivity measurements.

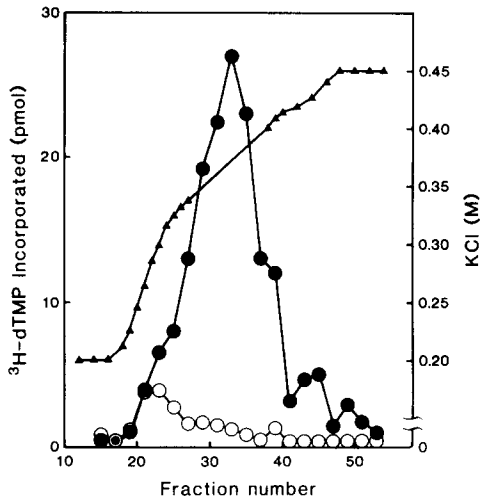

Fig. 4. Phosphocellulose chromatography of MCMV-induced DNA polymerase for further purification. Fractions from 27 to 44 eluted from DEAE-cellulose column of the extract of MCMV-infected cells (Fig. 3B) were pooled out and dialyzed against buffer B. The dialysate was batch-adsorbed to phosphocellulose and elution was carried out with a gradient of 0.2–0.45 M KCl. DNA polymerase activity in the fractions was assayed in the presence of 150 mM $(NH_4)_2SO_4$ (●) and in the absence of $(NH_4)_2SO_4$ (○). KCl concentration of column eluate (▲) is also presented.

this is the case, viral DNA polymerase was partially purified from MCMV-infected MEF and then subjected to kinetic studies.

Purification of MCMV DNA polymerase was carried out with a combination of DEAE-cellulose (Fig. 3B) and phosphocellulose (Fig. 4) column chromatography. The HSV DNA polymerase from HSV-infected Vero cells and the cellular DNA polymerase from uninfected MEF cells were also purified and used as the controls. The HSV DNA polymerase was purified in a similar fashion as the MCMV DNA polymerase, and the DNA polymerase α was obtained by DEAE-cellulose column chromatography (Fig. 3A). The activity was assayed in the presence of 150 mM and 100 mM $(NH_4)_2SO_4$ for the MCMV DNA polymerase and the HSV DNA polymerase, respectively. In contrast, the activity of DNA polymerase α was assayed in the absence of $(NH_4)_2SO_4$. The specific activity was 3540, 238, and 125 units per mg protein for the MCMV DNA polymerase, DNA polymerase α and HSV DNA polymerase, respectively. DNase activity was not detected in the enzyme samples under conditions used to measure DNA polymerase activity.

Characteristics of MCMV-induced DNA polymerase

Since this is the first report on the partial purification of the MCMV DNA polymerase, we examined the effect of salts and pH on the enzyme activity and its chromatographic properties. MCMV DNA polymerase activity was stimulated 8.7-fold by 200 mM $(NH_4)_2SO_4$ (Fig. 5A) and 2.1-fold by 250

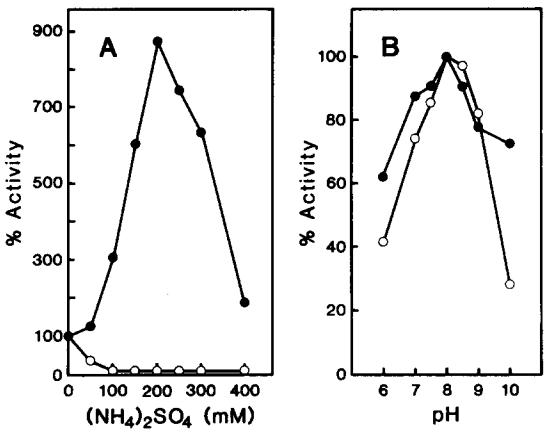

Fig. 5. Effect of $(NH_4)_2SO_4$ (A) and pH (B) on the activity of MCMV DNA polymerase (●) and cellular DNA polymerase α (○). (A) DNA polymerase activity was determined with the reaction mixture containing [^{3}H]dGTP in the presence of variable concentrations of $(NH_4)_2SO_4$ at pH 8.0. The activity in the presence of $(NH_4)_2SO_4$ is expressed as a percentage of the value in the absence of $(NH_4)_2SO_4$. (B) DNA polymerase activity was determined with the reaction mixture at various pH values. The activity is expressed as a percentage of the maximum value.

mM KCl (data not shown). The optimal pH was found to be 8.0 for the MCMV DNA polymerase (Fig. 5B). From the DEAE-cellulose column loaded with the extract of MCMV-infected cells, the DNA polymerase activity eluted at 0.08–0.28 M KCl (Fig. 3B). These fractions were considered to contain MCMV DNA polymerase because such an activity was not detected in the eluate of columns loaded with uninfected cell extract (Fig. 3A). MCMV DNA polymerase was eluted at 0.35–0.39 M KCl from phosphocellulose columns (Fig. 4). The properties are similar to those reported for other herpesvirus DNA polymerases (Table 2).

Effect of ACV triphosphate on MCMV DNA polymerase

The substrate specificity of MCMV DNA polymerase for dGTP was compared with that of the HSV DNA polymerase and cellular DNA polymerase α. DNA polymerase activity in function of variable concentrations of [^{3}H]dGTP is presented by a Lineweaver-Burk plot (Fig. 6). The K_m values obtained from the plots were 0.32, 0.50, and 0.77 μM for the MCMV DNA polymerase, HSV DNA polymerase and DNA polymerase α, respectively (Table I). In the presence of variable concentrations of ACV triphosphate, the Lineweaver-Burk plots showed typical competitive inhibition. The apparent K_i for ACV triphosphate determined from the replotted slopes were 73, 17 and 383 nM for MCMV DNA polymerase, HSV DNA polymerase and DNA polymerase α, respectively (Table 1). The K_i value for the MCMV DNA polymerase was comparable to that for the HSV DNA polymerase, indicating

TABLE 1

Kinetic constants of DNA polymerase α, MCMV DNA polymerase and HSV DNA polymerase

DNA polymerase	K_m[a] (μM)	K_i[b] (nM)
	dGTP	ACV triphosphate
Mouse α	0.77	383
MCMV	0.32	73
HSV	0.50	17

[a]The K_m value was obtained from the slope of the Lineweaver-Burk plot shown in Fig. 6; [b]The K_i value was determined by replotting the slopes of the Lineweaver-Burk plots shown in the inset of Fig. 6.

that sensitivity of the MCMV DNA polymerase to ACV triphosphate was not extraordinarily high.

Effect of ACV on MCMV DNA polymerase

We also examined whether unphosphorylated ACV may directly inhibit MCMV DNA polymerase. From the Lineweaver-Burk plots (Fig. 7), it is evident that no inhibition was observed even at a concentration of 50 μM ACV. This indicates that ACV itself is not inhibitory to MCMV DNA polymerase.

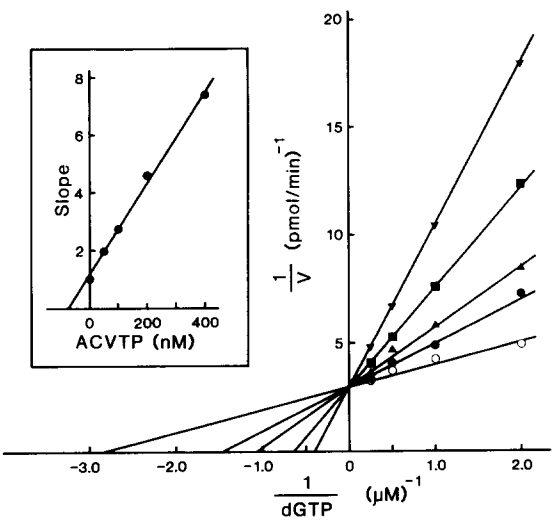

Fig. 6. Competitive inhibition by ACV triphosphate of MCMV DNA polymerase. The activity of MCMV DNA polymerase was assayed in the presence of various concentrations of [^{3}H]dGTP and ACV triphosphate at 0 (○), 0.05 (●) 0.1 (▲), 0.2 (■) and 0.4 (▼) μM. The incorporation rate of [^{3}H]dGTP into DNA is presented in the form of Lineweaver-Burk plots. The slopes of the plots were replotted as shown in the inset.

TABLE 2

A comparison of herpesvirus DNA polymerases

Virus	Optimal condition			Elution at M		Reference
	$(NH_4)_2SO_4$ (mM)	KCl (mM)	pH	DEAE-cellulose cellulose	Phospho-cellulose	
HSV-1	150	250		0.09–0.12(KPO_4)[a]	0.20(KPO_4)	Weissbach et al., 1973
	100	75–100	8.0–8.5	0.12(KCl)	0.21–0.27 (KCl)	Powell et al., 1977
VZV	60			0.18(NaCl)	0.32(NaCl)	Mar et al., 1978
	50		8.0		0.33(KCl)	Miller and Rapp., 1977
EBV	50		7.5		0.29(KCl)	Miller et al., 1977
HCMV	30–60	60–120		0.13–0.25(NaCl)	0.26(NaCl)	Huang et al., 1975
	100–140	200	8.0–8.5		0.28–0.35 (KPO_4)	Nishiyama et al., 1983
MCMV	200	250	8.0	0.08–0.25(KCl)	0.34(KCl)	This study

[a]HSV-induced DNA polymerase activity was eluted as two peaks.

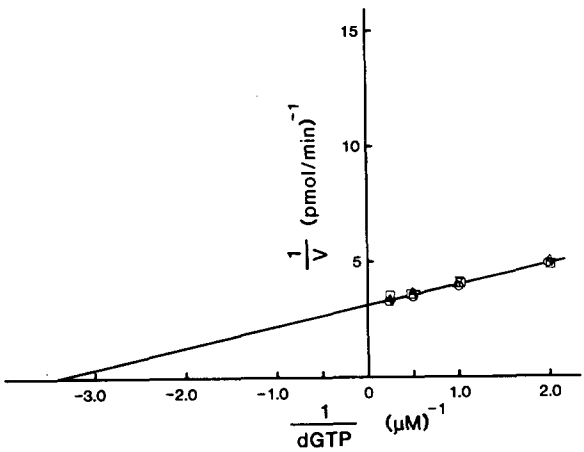

Fig. 7. Effect of ACV itself on MCMV DNA polymerase. The incorporation rate of [^{3}H]dGTP into DNA by MCMV DNA polymerase is determined in the presence of ACV at concentrations 0 (○), 0.2 (△) 2.0 (□) and 50 (▽) μM. The data are presented in the form of Lineweaver-Burk plots.

Discussion

DNA polymerases induced by HSV, VZV, HCMV and Epstein-Barr virus (EBV) are known to possess several common characteristics (Table 2). The activity of HSV-, VZV-, EBV- and HCMV-induced DNA polymerases is stimulated several fold at high salt concentration: 50–150 mM $(NH_4)_2SO_4$ and 60–250 mM KCl (Weissbach et al., 1973; Huang, 1975; Miller and Rapp, 1977;

Miller et al., 1977; Powell and Purifoy, 1977; Mar et al., 1978; Nishiyama et al., 1983). The MCMV DNA polymerase is also markedly stimulated by 200 mM $(NH_4)_2SO_4$ or 250 mM KCl. The optimal pH for the MCMV DNA polymerase is 8.0, which is in the range (7.5–8.5) of the optimal pH values noted for other herpesvirus DNA polymerases (Miller and Rapp, 1977; Miller et al., 1977; Powell and Purifoy, 1977; Nishiyama et al., 1983). The HSV DNA polymerase eluted at 0.12 M KCl from DEAE-cellulose and at 0.20–0.27 M KCl from phosphocellulose (Weissbach et al., 1973; Powell and Purifoy, 1977). The EBV- and VZV-induced DNA polymerases eluted from phosphocellulose at 0.29 M and 0.32–0.33 M KCl, respectively (Miller and Rapp, 1977; Miller et al., 1977; Mar et al., 1978). Similarly, the MCMV DNA polymerase eluted at 0.08–0.28 M KCl from DEAE-cellulose and at 0.37 M KCl from phosphocellulose, indicating that it possesses an equivalent molecular charge. Thus, the MCMV DNA polymerase shares certain properties with other herpesvirus DNA polymerases (Table 2).

The anti-MCMV activity of acyclovir can not depend on a viral TK, because MCMV does not induce a viral TK (Muller and Hudson, 1977; Eizuru et al., 1978; Burns et al., 1981). The anti-MCMV effect of ACV might depend on a cellular TK. However, this possibility was excluded by the findings that the replication of MCMV was inhibited by ACV equally well in TK^+ and TK^- cells, as described in this paper and previously by Burns et al. (1981). Thus, neither viral TK nor cellular TK seems to be required for the antiviral activity of ACV against MCMV.

Since the viral TK is absent in the MCMV-infected MEF and the cellular TK does not contribute to phosphorylation of ACV (Davidson et al., 1981; St. Clair et al., 1987), ACV triphosphate may be assumed to be formed to a limited extent by enzymes other than TK. However, no ACV triphosphate was detected by HPLC in an extract from MCMV-infected cells. The detection limit was 6 pmol per 10^6 cells, which corresponds to 1.2 μM when calculated by assuming a packed volume of 5 μl for 10^6 cells (Elion et al., 1977). For two reasons, the amount of ACV triphosphate which may be produced from ACV at an effective concentration against MCMV (ED_{100}: 5–10 μM) is considered to be much less than 1.2 μM. First, since no peak of ACV triphosphate was detected by HPLC, the actual concentration of ACV triphosphate in the extract must be less than the detection limit. Second, the extent of formation of ACV triphosphate is dependent upon the concentration of ACV in the medium. Actually, ACV triphosphate levels (8.7 pmol/10^6 cells; 1.7 μM) in uninfected Vero cells exposed to 500 μM ACV are 50-fold higher than those (0.17 pmol/10^6 cells; 0.034 μM) obtained in cells exposed to 5 μM ACV (Elion et al., 1977). Similarly, the concentration of ACV triphosphate in MCMV-infected MEF cells exposed to 5–10 μM ACV must be significantly less than the levels in cells exposed to 500 μM ACV. Thus, only trace amounts of ACV triphosphate could be present in the MCMV-infected cells.

The amount of ACV triphosphate present in Vero cells infected with TK^- mutants of HSV-1 was comparable to that in the uninfected Vero cells (Elion et

al., 1977; St. Clair et al., 1980). TK^- HSV mutants are insensitive to ACV, which is evident from a 100-fold higher ED_{50} against TK^- HSV than against the parental strain (St. Clair et al., 1980). Thus, trace amounts of ACV triphosphate in the TK^- HSV-infected cells should be insufficient to inhibit the HSV DNA polymerase. The amount of ACV triphosphate formed in the MCMV-infected MEF may be similar to that formed in the uninfected and TK^- HSV-infected cells. Should the MCMV DNA polymerase be extraordinarily sensitive to the inhibitory action of ACV triphosphate, such trace amounts of ACV triphosphate could inhibit MCMV DNA synthesis. This possibility was examined by determining the apparent K_i of the MCMV DNA polymerase for ACV triphosphate. The K_i values were assessed for the MCMV- and HSV-induced DNA polymerases which were purified to a similar extent. The K_i values obtained, 73 nM for the MCMV DNA polymerase and 17 nM for the HSV DNA polymerase, demonstrate that the sensitivity to ACV triphosphate of the MCMV DNA polymerase is equivalent to that of the HSV DNA polymerase. Therefore, trace amounts of ACV triphosphate in the MCMV-infected cells are insufficient to inhibit the MCMV DNA polymerase. In other words, it is unlikely that ACV triphosphate is responsible for the anti-MCMV activity of ACV.

A similar reasoning may apply to the inhibitory effect of ACV on EBV. Although EBV encodes a TK gene (Littler et al., 1986) and induces a specific TK in the infected cells (Gabhan et al., 1984; Stinchcombe and Clough, 1985; De Turenne-Tessier et al., 1986), apparent phosphorylation of ACV has not been observed (Lin et al., 1986). Despite lack of formation of sufficient amounts of ACV triphosphate, synthesis of EBV DNA is inhibited (Colby et al., 1980). This has been attributed to the high sensitivity of the EBV DNA polymerase to ACV triphosphate (Datta et al., 1980). However, the K_i value of the EBV DNA polymerase is equivalent (Frank et al., 1985) to or higher (Allaudeen et al., 1982) than that of the HSV DNA polymerase. These results indicate that the EBV DNA polymerase is not highly sensitive to ACV triphosphate and, again, trace amounts of ACV may not be responsible for the anti-EBV effect of ACV, as is the case with its anti-MCMV effect.

Although it is unlikely that ACV triphosphate plays a key role in the anti-MCMV activity of ACV, the mechanism of the anti-MCMV effect of ACV is actually unknown. The analysis of resistant mutants may offer a possibility to elucidate this mechanism. ACV-resistant mutants of MCMV show cross-resistance to phosphonoacetic acid (PAA) (Sandford et al., 1985; Kumura, unpublished data). Since most PAA-resistant mutants of HSV have been shown to possess mutations in the DNA polymerase gene (Purifoy et al., 1977), the target enzyme for the anti-MCMV action of ACV may still be the DNA polymerase. It is not excluded that an ACV metabolite, other than ACV triphosphate, is inhibitory to the MCMV DNA polymerase. Unmodified ACV has no inhibitory effect on the activity of MCMV DNA polymerase. The putative ACV metabolite responsible for the inhibition of MCMV replication remains to be determined. In addition, the gene(s) responsible for ACV

resistance should be identified so as to determine the target enzyme(s) for the anti-MCMV activity of ACV.

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