Thymidine Kinase Not Required for Antiviral Activity of Acyclovir Against Mouse Cytomegalovirus

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Received 24 February 1981/Accepted 12 May 1981

Previous studies of herpesvirus infections have indicated that a virus-specified thymidine kinase is required for the initial phosphorylation of acyclovir [acycloguanosine or 9-(2-hydroxyethoxymethyl)guanine] in the formation of acycloguanosine triphosphate. The latter compound accumulates in infected cells and competitively inhibits the viral DNA polymerase. We found that mouse cytomegalovirus, which does not express a thymidine kinase, was sensitive to the antiviral effects of acyclovir at a 50% inhibitory dose of approximately 0.23 μ M. Acyclovir was equally effective against mouse cytomegalovirus in normal 3T3 cells and in 3T3 cells deficient in cellular thymidine kinase. Furthermore, the activity of acyclovir could not be reversed by excess thymidine, which easily reversed the antiviral activity of acyclovir against herpes simplex virus. Using a high-pressure liquid chromatography technique that easily detected acycloguanosine triphosphate in cells infected with herpes simplex virus, we could not detect acycloguanosine triphosphate in mouse cytomegalovirus-infected cells. These experiments demonstrated that the activity of acyclovir against mouse cytomegalovirus is not dependent on a thymidine phosphorylation pathway. Additional experiments are underway to determine whether acycloguanosine triphosphate is produced by another pathway in concentrations sufficient to inhibit mouse cytomegalovirus DNA polymerase.

Many herpesviruses code for a unique thymidine kinase (TK) that has less stringent substrate specificities than the corresponding cellular TK, and the chemotherapeutic potential of this pathway has been exploited with a variety of thymidine analogs. Acyclovir [ACV; acycloguanosine or 9-(2-hydroxyethoxymethyl)guanine], which can be considered an analog of guanosine or deoxyguanosine, belongs to a new class of antiviral agents that are active against herpesviruses and are now entering clinical trials. Previous studies have shown that a virus-specified TK is required for efficient phosphorylation of this drug so that acycloguanosine triphosphate (acyclo-GTP), the active derivative of ACV, can accumulate in infected cells and prevent viral DNA replication (1, 2, 7, 8, 11, 12). We found that mouse cytomegalovirus (MCMV), which does not express a TK, is sensitive to the antiviral effects of ACV. We also found that the antiviral activity of this drug does not depend on a cellular thymidine phosphorylation pathway.

MATERIALS AND METHODS

Virus. The Smith strain of MCMV (ATCC VR-194) was obtained from the American Type Culture Collection, Bethesda, Md. Virus pools were prepared

by serial passage in mouse embryo fibroblast (MEF) monolayers. MCMV titers were determined by adsorbing 0.1-ml portions of serially diluted samples in quadruplicate onto confluent MEF monolayers in the 16-mm wells of TC-24 trays (Costar). After 1 h, the inocula were aspirated, and the cultures were overlaid with 0.9% methylcellulose in minimal essential medium (MEM) containing 5% newborn calf serum (NCS), 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer, and 35 μ g of gentamicin per ml and incubated at 37°C in 5% CO₂. After 6 days the overlays were removed, the monolayers were fixed in absolute ethanol and stained with 0.5% methylene blue, and the plaques were counted. A clinical isolate of herpes simplex virus (HSV) type 1 was obtained from the Virology Laboratory, Johns Hopkins Hospital. Virus pools were prepared by passage in primary rabbit kidney cultures, and titers were determined on monolayers of Vero cells.

Cells. MEF cultures were prepared from 18-day-old ICR mouse embryos by overnight trypsinization at 4°C. The cells were suspended in MEM supplemented with 10% NCS and 35 μ g of gentamicin per ml, seeded into 75-cm² flasks (Costar), and grown to confluency at 37°C in 5% CO₂. Secondary MEF cultures for MCMV assays were prepared by trypsinizing and seeding 200,000 cells into 16-mm wells (TC-24; Costar) in MEM supplemented with 10% NCS and gentamicin, and these cultures were used when they were confluent. 3T3 cells and a TK-deficient line of 3T3 cells (designated 3T3-TK⁻) were obtained from Howard

Green, Cambridge, Mass., and were maintained in MEM and Dulbecco MEM, respectively; both of these media were supplemented with 10% NCS and gentamicin. Periodically, the 3T3-TK⁻ cells were passaged in the presence of 25 μ g of 5-bromo-2'-deoxyuridine (BUdR) per ml to remove revertants. Vero cells were maintained in MEM containing 10% NCS and gentamicin.

Drug. ACV was generously supplied by the Burroughs Wellcome Co., Research Triangle, N.C., as a lyophilized powder. A 10^{-2} M solution in distilled water was passed through a 0.2- μ m filter and then diluted in medium to the appropriate concentration. Solutions were prepared fresh for each experiment. Acyclo-GTP was a gift from G. Elion, Burroughs Wellcome Co. BUdR was obtained from Sigma Chemical Co., St. Louis, Mo.; after reconstitution with water and passage through a 0.2- μ m filter, BUdR was kept at -70°C until it was used.

TK assay. Confluent monolayers of 3T3-TK⁻ cells in 25-cm² flasks were mock-infected or infected at a multiplicity of infection of 1 with either MCMV or HSV type 1. After 12 h the monolayers were scraped off the plastic surfaces, combined with the cells floating in the media, and washed once with Tris-saline; the cell pellets were frozen at -70°C until they were assayed. Before they were assayed, the cell pellets were suspended in 0.5 ml of sodium phosphate buffer (pH 6.0) and sonicated at 4°C for 60 s with a probe sonicator (Heat Systems Ultrasonics, Plainview, N.Y.) set at 30% of maximal intensity. The disrupted cells were centrifuged for 1 min at $12,000 \times g$ in a refrigerated RC-5 centrifuge (Ivan Sorvall Inc., Newtown, Conn.), and each supernatant was assayed for TK activity in quadruplicate as described by Preston (10) (pH 6, 30°C , 5 h). The protein content of each specimen was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

In vitro assay of antiviral activity. Confluent monolayers of MEF, 3T3, and 3T3-TK⁻ cultures were infected in quadruplicate at a multiplicity of infection of 0.5 to 1. After adsorption for 1 h, MEM supplemented with 10% NCS, gentamicin, and different concentrations of ACV or thymidine or both was added. Preliminary studies showed that one replication cycle took place in 32 h and that the plateau extended until 40 h. Virus was harvested at 36 h, and virus cultures were frozen and stored at -90°C until titers were determined. The 50% inhibitory dose was determined from a plot of virus yield (expressed as a percentage of virus yield in the absence of the drug) versus logarithmic increasing doses of drug. Preliminary studies showed no reduction in the number of viable (trypan blue-excluding) cells incubated in media containing ACV at levels of $\leq 100 \mu\text{M}$ for 36 h under the conditions of this assay. Furthermore, thymidine uptake by subconfluent MEF and 3T3 cultures was not affected 24 h after incubation with 10 times the 50% inhibitory dose of ACV.

High-pressure liquid chromatography technique to detect acyclo-GTP. Confluent MEF in 25-cm² flasks were infected with HSV or MCMV at a multiplicity of infection of 4. After 1 h of virus adsorption, the unadsorbed virus was removed, MEM containing 10% NCS and 0.5 mM ACV was added, and

the infected cells were incubated at 37°C in 5% CO_2 for different lengths of time. The medium was then decanted, and the cells were trypsinized into suspension, washed with phosphate-buffered saline, and extracted with 0.5 ml of 3.5% perchloric acid at 4°C . An internal standard of ITP (16 μ l of a 1 mM solution) was added, and the acid-soluble extract was neutralized by adding 0.1 ml of 1 M phosphate buffer and approximately 0.35 ml of 1 N KOH and then centrifuged. The supernatant was frozen, concentrated to dryness with a lyophilizer (The VirTis Co., Inc., Gardiner, N.Y.), and dissolved in 0.75 ml of water. Analyses were performed with a Waters high-pressure liquid chromatograph fitted with a Whatman Partisil PXS 10/25 SAX column (0.46 by 25 cm). Elution was with a linear gradient of 0.02 to 1.0 M KH_2PO_4 (pH 3.5) for 80 min at a flow rate of 60 ml/h, and UV absorption at 254 nm was monitored. Nucleotide peaks were identified by comparison with the elution patterns of control nucleotide samples, including acyclo-GTP, as well as by comparison with previously described patterns (1, 7). Reconstruction experiments demonstrated that we could detect ≥ 25 pmol of acyclo-GTP in cell extracts, and we estimated that we could have detected this compound if it had been present at ≥ 6 pmol per 10^6 cells or at an intracellular concentration of $\geq 2 \times 10^{-7}$ M (assuming that 10^6 cells have a volume of 0.005 ml).

RESULTS

Lack of viral TK activity. To eliminate cellular TK activity, cultures of 3T3-TK⁻ cells were used for direct assays of MCMV TK activity. Infection with MCMV resulted in no discernible TK activity above background levels, whereas TK activity increased 75-fold after infection with HSV (Table 1). Repeated assays of our Smith strain of MCMV failed to demonstrate virus-specific TK activity.

As an indirect test for the presence of virus TK, one-step replication cycles of MCMV were carried out in 3T3-TK⁻ cell monolayers in the presence of different concentrations of BUdR. Because BUdR is phosphorylated by either cellular or viral TK and subsequently is incorporated into viral DNA, where it decreases virus infectivity, productive infection of 3T3-TK⁻ cells with MCMV in the presence of BUdR would strongly suggest the lack of an MCMV-encoded TK. The controls which we used were (i) MCMV

TABLE 1. TK activity of 3T3-TK⁻ cells infected with MCMV or HSV

Infecting virus	TK activity (pmol/mg per h) ^a
None	18.8 $\pm$ 1.0
MCMV	20.2 $\pm$ 1.2
HSV	1,360 $\pm$ 29

^a Picomoles of [³H]thymidine phosphorylated per milligram of protein per hour.

replication cycles in 3T3 cell monolayers and (ii) HSV type 1 replication cycles in 3T3-TK⁻ cell monolayers (both in the presence of BUdR). The time of harvest after MCMV infection was 36 h, and the time of harvest after HSV infection was 24 h. As expected (Fig. 1), BUdR at a concentration of 2.5 μ g/ml greatly reduced the yield of HSV type 1 in 3T3-TK⁻ cells and reduced the yield of MCMV in TK-containing 3T3 cells. In contrast, we observed no significant effect on the yield of MCMV in 3T3-TK⁻ cells even at 25 μ g of BUdR per ml, again indicating the lack of an MCMV-specified TK.

Effect of ACV on the one-step replication cycle of MCMV. As Fig. 2 shows, ACV was effective against MCMV in MEF (50% inhibitory dose, 0.26 μ M), 3T3 cells (50% inhibitory dose, 0.23 μ M), and 3T3-TK⁻ cells (50% inhibitory dose, 0.21 μ M). In 3T3-TK⁻ cells neither cellular nor viral TK was present, yet the antiviral activity of ACV was undiminished.

Thymidine reversal. More evidence that ACV was not activated by a thymidine phosphorylation pathway in MCMV infections came from our inability to reverse the effect of ACV against MCMV with excess thymidine under conditions that caused a substantial reversal of anti-HSV activity in MEF cultures (Fig. 3). At a concentration of ACV (0.5 μ M) that reduced the HSV yield by 99% in the one-step replication cycle, the presence of 20- and 200-fold excesses of thymidine resulted in 14- and 34-fold increases in virus yields. In contrast, at a concentration of

ACV (1.0 μ M) that reduced the MCMV yield by 98%, 10- and 100-fold excesses of thymidine had little influence on the antiviral activity.

Formation of acyclo-GTP. Using the column and buffer systems for high-pressure liquid chromatography described by Elion et al. to demonstrate acyclo-GTP in HSV-infected cells

FIG. 2. Effect of ACV on the one-step replication cycle of MCMV in cultures of MEF (x), 3T3 cells (●), and 3T3-TK⁻ cells (○).

FIG. 1. Effect of BUdR on virus yield. Symbols: ○, MCMV grown in 3T3-TK⁻ cells; ●, MCMV grown in 3T3 cells; x, HSV type 1 grown in 3T3-TK⁻ cells.

FIG. 3. Reversal of antiviral activity of ACV with thymidine. One-step replication cycles of HSV (open bars) and MCMV (solid bars) were performed in MEF cultures (control yield, 100%). ACV was added at concentrations of 0.5 μ M (HSV cultures) and 1.0 μ M (MCMV cultures) to achieve 99 and 98% yield reductions, respectively. The effect of thymidine on the virus yields of the ACV-treated cultures is shown.

and in varicella-zoster virus-infected cells (1, 7), we were able to reproduce the results of these workers for HSV in our MEF cultures (Fig. 4a and b). However, in the presence of 500 μ M ACV, MCMV-infected MEF did not show a spectroscopically identifiable peak of acyclo-GTP at 8.5 h after infection (Fig. 4c) or at 10, 12, 21, and 24 h after infection (data not shown).

DISCUSSION

The studies of Elion and co-workers have established clearly several facts concerning ACV in relation to infection of cells with HSV (7, 8, 11). In uninfected cells ACV is minimally phosphorylated. A deoxypyrimidine kinase (TK) converts ACV to acycloguanosine monophosphate, and other kinases then rapidly convert this compound to acyclo-GTP. HSV-infected cells contain a virus-encoded TK which is able to utilize ACV more efficiently than the cellular TK, resulting in an accumulation of acyclo-GTP in infected cells. Studies on the partially purified viral DNA polymerase show that the activity of this enzyme is considerably more sensitive to the inhibitory action of acyclo-GTP than cellular (HeLa) DNA polymerase. Furthermore, acyclo-

GTP can serve as a substrate for the viral DNA polymerase and can be incorporated into viral DNA, where it may lead to chain termination. The non-phosphorylated form of ACV has no effect on the viral and cellular DNA polymerases (7). Taken together, the actions of the phosphorylated derivative of ACV result in a large therapeutic index for ACV.

The antiviral activity of ACV appears to be dependent on a viral TK capable of phosphorylating the drug and has been demonstrated for HSV types 1 and 2, monkey B virus, varicella-zoster virus (11), and equine herpesvirus type 1 (Burns, unpublished data), all of which code for TK. Significantly, the efficacy of this drug in vitro against human cytomegalovirus, which does not code for TK, is much less than its efficacy against the TK-positive herpesviruses; activity against human cytomegalovirus requires at least 100 times the drug concentration required for activity against HSV (4). Additional evidence for the dependence of the antiviral effect of ACV on a virus-encoded TK comes from genetic studies. Many ACV-resistant mutants of HSV have been selected; with few exceptions, emergence of resistance to ACV has been correlated with decreasing TK activity, and the resistance mutations were located in the viral TK gene (2, 12).

The Smith strain of MCMV is the same strain reported by two other laboratories to be TK negative (6, 9), and we confirmed this by a direct assay for TK activity in infected cells (Table 1). Furthermore, this virus replicates in 3T3-TK⁻ cells in the presence of 25 μ g of BUdR per ml (Fig. 1), conditions which inhibit HSV replication since the viral TK phosphorylates BUdR, leading to its accumulation in viral DNA. We also found that trifluorothymidine, which is phosphorylated to its active form by cellular or viral TK, inhibits MCMV growth in 3T3 cells but not in 3T3-TK⁻ cells (J. R. Wingard, R. K. Stuart, R. Saral, and W. H. Burns, submitted for publication). Therefore, we were surprised to find that infection by the TK-negative MCMV was sensitive to the antiviral activity of ACV in vitro. The 50% inhibitory dose of ACV for MCMV in MEF cells (0.26 μ M) is comparable to the 50% inhibitory doses reported for HSV type 1 (0.1 to 0.4 μ M) (4, 7) and HSV type 2 (0.4 to 2.0 μ M) (3) and lower than the 50% inhibitory dose for varicella-zoster virus (1.0 to 5.0 μ M) (1, 4).

We next considered whether a cellular TK might provide acyclo-GTP in sufficient quantities to inhibit the viral DNA polymerase. Since MCMV grows well in 3T3-TK⁻ cells, we used these cells to determine whether ACV is effective in a situation in which neither the cells nor

FIG. 4. Use of high-pressure liquid chromatography to detect acyclo-GTP. MEF cultures were infected at multiplicity of infection of 4 with HSV without ACV (a), HSV with 0.5 mM ACV (b), or MCMV with 0.5 mM ACV (c). ITP was used as an internal standard.

the virus produces TK and in which BUdR and trifluorothymidine are ineffective since they cannot be phosphorylated; we found that the 50% inhibitory doses in 3T3 cells (0.23 μ M) and 3T3-TK⁻ cells (0.21 μ M) were similar. Our inability to reverse the anti-MCMV effect of ACV with excess thymidine, which substantially reversed the anti-HSV effect of ACV, also suggests that TK activity is not necessary for the anti-MCMV activity of ACV.

Since the ultimate site of action of ACV in herpesvirus infections appears to be at the level of the viral DNA polymerase (7, 8), we examined whether in MCMV infections ACV was phosphorylated by cellular or viral enzymes other than TK (e.g., nucleoside kinases) and thus inhibited the DNA polymerase. Using a high-pressure liquid chromatography technique that easily detected acyclo-GTP in HSV and varicella-zoster virus infections, we detected no acyclo-GTP in MCMV-infected cells. Thus, ACV must be phosphorylated very inefficiently by such putative enzyme(s), and the DNA polymerase of MCMV apparently is inhibited by very low concentrations of acyclo-GTP. This appears to be the case for Epstein-Barr virus, for which a viral TK has not been identified, but the very sensitive viral DNA polymerase of this virus (K_i , 1.5×10^{-8} M) may be inhibited by the low levels of acyclo-GTP detected in Epstein-Barr virus-infected cells (3, 5). In these studies cellular TK may have been responsible for phosphorylating ACV, whereas we found no dependence on cellular TK.

To summarize, previous studies have suggested that with the possible exception of Epstein-Barr virus, ACV must be phosphorylated by a virus-encoded TK to acyclo-GTP to be effective against herpesvirus infections. In contrast, our studies demonstrate that ACV may express its antiviral activity against MCMV in the absence of viral or host TK activity and that another phosphorylating enzyme system must be involved. Additional biochemical and genetic studies to identify the enzyme(s) involved and to determine whether they are cellular or virus specified are underway and should provide important information for similar antiviral compounds under development, especially compounds to be used against those herpesviruses that lack a gene for TK (e.g., human cytomegalovirus and Epstein-Barr virus).

ACKNOWLEDGMENTS

We thank G. Elion, Burroughs Wellcome Co., for the gift of acyclo-GTP and John Hilton and Albert Owens, Jr., Johns Hopkins Oncology Center, for advice and encouragement.

This work was supported by Public Health Service grants CA06973 and CA17970 from the National Institutes of Health and by the Harley Howell Foundation.

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