Growth Inhibition by Acycloguanosine of Herpesviruses Isolated from Human Infections

clyde s. crumpacker,† lowell e. schnipper,† john a. zaia,† and myron j. levin

divisions of infectious disease† and oncology,† department of medicine and thorndike laboratory of
harvard medical school, beth israel hospital, boston, massachusetts 02215, and division of clinical
microbiology, sidney farber cancer institute, boston, massachusetts 02215

received for publication 8 february 1979

inhibition by acycloguanosine (acg) of plaque formation by herpes simplex
virus types 1 and 2 (hsv-1 and hsv-2), varicella-zoster virus, and cytomegalovirus
was studied. seventeen clinical isolates of hsv-1 were inhibited by acg at
a mean 50% inhibitory dose (id50) of 0.15 ± 0.09 µm. the mean id50 for 10 isolates
of hsv-2 was 1.62 ± 0.76 µm, and for four isolates of varicella-zoster virus it was
3.75 ± 1.30 µm. the id50's for two cytomegalovirus isolates were 100 and 160 µm,
and for four additional isolates of cytomegalovirus no end point (id50) was reached
at 200 µm. acg at a concentration of 200 µm had no effect on deoxyribonucleic
acid synthesis in human fibroblast cells and only inhibited thymidine incorpora-
tion by vero cells by one-third. these studies demonstrated the antiviral activity
of acg against clinical isolates of hsv-1, hsv-2, and varicella-zoster virus and
the lack of toxicity to monkey or human cells in culture at concentrations which
markedly inhibited these viruses. acg had little effect on cytomegalovirus at
concentrations in excess of 100 µm.

the human herpesviruses cause a wide spectrum of acute and recurrent diseases which vary
in severity from mild to life threatening (4, 6, 8, 17, 25). although a large number of agents have
been investigated, only two of these, adenine arabinoside and interferon, have been shown in
clinical trials to favorably alter the course of herpes simplex virus (hsv) and varicella-zoster
virus (vzw) infections (1, 13, 15, 23, 24). nevertheless, severe sequelae and treatment failures
do occur in spite of the use of these agents, and attempts to treat visceral involvement by in-
creasing the dose are likely to be complicated by myelo- and immunosuppressive side effects (2,
5, 10, 11, 22, 24). a new antiviral agent, acycloguanosine [acg; 9-(2-hydroxyethoxymethyl)
guanine], is active against human herpesviruses, both in vitro and in animals infected with hsv
(7, 20). acg requires a virus-specific thymidine kinase for conversion to acg-triphosphate, an
inhibitor of viral deoxyribonucleic acid (dna) polymerase (7). this property makes the com-
 pound very selective for virus-infected cells, and acg has a high in vitro therapeutic ratio.
the available information on the susceptibility of herpesviruses to acg is limited and is primarily
derived from the testing of laboratory strains. in this report the susceptibilities of isolates from
human infections with hsv, vzw, and cytomegalovirus (cmv) to acg are described.

materials and methods

cells. primary rabbit kidney cell cultures were prepared by a multiple extraction method (4). a cell
strain of human foreskin fibroblasts (350 q) derived
by m. myers was used at passages 17 to 22 (21). these
cell cultures were maintained in dulbecco modified
egle medium supplemented with 2% fetal calf serum
and antibiotics (penicillin, 250 u/ml; streptomycin,
250 µg/ml). the vero line of african green monkey
kidney cells was obtained from microbiological asso-
ciates (bethesda, md.) and maintained in medium 199
containing 2% fetal calf serum and antibiotics. cell
lines were free of mycoplasmas when tested on hay-
flick medium.

viruses. (i) hsv-1. hsv type 1 (hsv-1) isolates
were obtained from patients with recurrent facial-oral
herpes labialis. virus was isolated in primary rabbit
kidney cell cultures, and a pool was prepared by low-
multiplicity passage in vero cells. pools were har-
vested and frozen at -70°c when the cell sheet showed
complete cytopathic effect.

(ii) hsv-2. hsv-2 isolates were obtained from
patients with herpes genitalis or neonatal herpes
infection, and pools were prepared as described above.
hsv isolates were identified as hsv-1 or hsv-2 by a
simplified microneutralization technique (21).

(iii) vzw. clinical isolates of vzw were obtained
from patients with varicella or herpes zoster. vzw was
isolated, and a pool was prepared in human fibroblasts.
a laboratory strain (cp 5262; center for disease con-
trol, atlanta, ga.) was also studied. cell-free vzw
was prepared by scraping the cell sheet once when cyto-
pathic effect occurred in 50% of cells, sonic treatment
were cells plates (Falcon). Serum and cells in above, Packaging, Cambridge, 0.05 in concentration of 10^6 cells per ml in medium containing 8% dimethyl sulfoxide and 30% serum. VZV was identified by typical cytopathic effect growth characteristics, and the histological appearance of fixed slides.

(iv) CMV. CMV isolates were obtained from bone marrow transplant recipients with febrile illnesses or from normal individuals with a CMV mononucleosis syndrome. Cell-free and cell-associated virus pools were prepared as described for VZV from virus grown on 350 Q cells. Two laboratory strains (Davis and AD 169) were also studied. CMV was identified by growth characteristics and fluororescent-antibody staining of infected cultures (12).

All virus pools were titered by plaque titration on Vero (HSV-1 and HSV-2) or 350 Q (VZV and CMV) cells, using methyl cellulose overlay (4).

Drugs. ACG (BW 248U) was supplied by the Burroughs Wellcome Co. (Research Triangle Park, N.C.) with the cooperation of the Antiviral Substances Program of National Institute of Allergy and Infectious Diseases. A 10 mM stock solution of ACG was prepared in distilled water and stored at -20°C.

Determination of antiviral activity. (i) HSV. To determine anti-HSV activity, monolayers of Vero cells (seeded with 3 x 10^5 cells in 3 ml) were prepared in 6-well (35 mm) plastic tissue culture plates (Linbro Div., Flow Laboratories, Inc., Rockville, Md.). Three days later, confluent monolayers were inoculated with approximately 100 plaque-forming units in 0.2 ml of medium containing ACG. After 1 h at 37°C, the inoculum was removed and 3 ml of 1% methyl cellulose was added. Methyl cellulose was prepared in medium containing 2% serum and appropriate concentrations of ACG. After 5 days at 37°C in 10% CO_2-90% air, the monolayers were fixed with acetic acid-methanol (1:3) and stained with 1% crystal violet, and the plaques were counted. The amount of ACG required to reduce plaques by 50% (ID_{50}) from the number present in the control wells without drug was calculated directly from the nonlinear plot relating surviving plaques in the presence of increasing concentrations of ACG in the medium. No attempt was made to obtain linearity.

(ii) VZV. The ID_{50} of VZV was determined by a similar technique, using 4-day-old confluent monolayers of 350 Q cells (seeded with 6 x 10^5 cells in 3 ml). After 4 days of incubation, the infected cells were fixed with 10% Formalin, the methyl cellulose was removed, and the plates were washed with water. The monolayers were stained with 0.03% methylene blue, and the plaques were counted.

(iii) CMV. The ID_{50} of CMV was determined as above, using 4-day-old confluent monolayers of 350 Q cells in 24-well cluster tissue culture plates (Costar, Data Packaging, Cambridge, Mass.). Virus was added in 0.05 ml, and 1 ml of 1% methyl cellulose containing serum and ACG was added at 1 h. The plaque number was determined at 7 days after infection.

Toxicity assay. Vero or 350 Q cells were added (10^5 cells in 0.5 ml) to 96-well flat-bottom microtiter plates (Falcon). After 24 h of incubation, while the wells were subconfluent, 0.05 ml of ACG solution or diluent alone was added. Three hours later, [3H]methyl-midine (0.25 μCi per well; 2 Ci/mmol) was added in 0.05 ml. After 18 h, the monolayers were drained, and 0.05 ml of trypsin-ethylenediaminetetraacetate solution was added (37°C, 15 min), followed by 0.05 ml of 0.5% sodium dodecyl sulfate. The contents of the wells were harvested with a multiple automated sample harvester (Microbiological Associates), using water. The counts per minute retained on the glass fiber filters were determined in a scintillation spectrometer.

RESULTS

The susceptibilities to ACG of 17 clinical isolates of HSV-1 were determined in Vero cells. The ID_{50}’s ranged from 0.06 to 0.35 μM, with a mean ID_{50} of 0.15 ± 0.09 μM (Fig. 1). The ID_{50}’s for 10 isolates of HSV-2 ranged from 0.46 to 3.0 μM, with a mean of 1.62 ± 0.76 μM. Four isolates of VZV were inhibited by ACG at concentrations ranging from 1.5 to 5.0 μM, with a mean of 3.75 ± 1.30 μM. Cell-free and cell-associated VZV were inhibited by similar concentrations of ACG (Fig. 1). Six isolates of CMV were resistant to ACG; four failed to show 50% plaque reduction at 200 μM, and two isolates had ID_{50}’s of 100 and 160 μM.

Typical kinetics of plaque reduction by ACG for each of the human herpesviruses tested are shown in Fig. 2. These curves (Fig. 2) show the result of single determinations on single isolates. Similar kinetics were obtained in multiple determinations on all of the isolates. Only for CMV was there persistence of plaques at a concentration of ACG greater than 10 μM. Cell-free and cell-associated VZV were inhibited with similar kinetics (data not shown).

DNA synthesis by the mammalian cells used for the assay of antiviral effect was determined in the presence of ACG (Table 1). At a concentration of 200 μM, no inhibitory effect on human

**Fig. 1. ACG ID_{50}’s for clinical isolates of herpesvirus. Symbols: ○, ID_{50} for all isolates except for cell-free VZV; □, ID_{50} for cell-free VZV; |—|, mean ID_{50} for each group of virus tested.**
fibroblasts was noted. The incorporation of [3H] thymidine into the DNA of monkey cells, however, was decreased by ACG at concentrations as low as 50 μM. At an ACG concentration of 200 μM, DNA synthesis by Vero cells was decreased by 33%.

Both the Vero cells and the human fibroblast cells were viable and continued to increase in number daily when counted for 7 days after the addition of ACG to the media. Thus, the ACG did not appear to have a delayed effect on host cell DNA synthesis which would not be detected during an 18-h exposure to [3H] thymidine. This method of assaying for cell cytotoxicity only looks at the effects of the drug on DNA synthesis and does not assess possible inhibition of protein or ribonucleic acid synthesis. DNA synthesis appeared to be the most sensitive parameter of macromolecular synthesis to examine, as ACG is a nucleoside analog whose primary effects have been shown to be on viral DNA synthesis (7).

**DISCUSSION**

Schaeffer et al. showed that ACG inhibited the ICI laboratory strain of HSV-1 at an ID<sub>50</sub> of 0.10 μM (20). Three other laboratory strains (KOS, McIntyre, and H29) have been used in studies on the drug, but their ID<sub>50</sub>'s were not reported (7). We find that all HSV-1 isolates from human disease are similarly susceptible (mean ID<sub>50</sub> = 0.15 μM). Their inhibitory range of 0.06 to 0.35 μM indicates that naturally occurring resistance to ACG by HSV-1 is uncommon. The mean ID<sub>50</sub> for 10 strains of HSV-2 was 1.62 μM, a value 10-fold higher than the value of 0.14 μM reported by Schaeffer et al. (20). Both studies were carried out by plaque reduction in Vero cells. Part of the discrepancy is explained by the range of susceptibility of the isolates we studied. Although HSV-2 is less susceptible to ACG, it is still inhibited by levels readily attained in human serum during parenteral administration (R. E. Keeney, personal communication). The greater susceptibility to ACG of HSV-1, compared with HSV-2, resembles the relative susceptibility observed with other antiviral compounds, such as 5, 6-dihydro-5-aza-thymidine (19) and adenosine arabinoside (14).

Although it has been stated that VZV and CMV are inhibited by ACG (20), the data have not been published. We observed that clinical isolates of VZV are rarely inhibited by clinically attainable levels of ACG and that cell-free and cell-associated viruses are equally susceptible. Studies on CMV isolates indicate that the ID<sub>50</sub>'s for four isolates are greater than 200 μM and that the ID<sub>50</sub>'s for two others are 100 and 160 μM.

The exact reason why CMV is not susceptible to ACG at the noncytotoxic concentrations used is not apparent. The fact that CMV is not known to induce a virus-coded thymidine kinase suggests that CMV-infected cells do not convert ACG to the active form, ACG-triphosphate, and thus viral DNA replication is not inhibited. In HSV-infected cells, ACG-triphosphate has been shown to be a noncompetitive inhibitor of virus-induced DNA nucleotidyltransferase (7). These results might be further evidence that CMV does not require its own thymidine kinase for replication, that it utilizes a cell thymidine kinase which does not convert ACG to an active inhibitor, or that it replicates without any requirement for thymidine kinase at all. An alternative explanation is that the CMV-induced DNA nucleotidyltransferase is very resistant to the action of ACG-triphosphate.

**ACG exerts antiviral effect on HSV-1, HSV-2,**

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HERPESVIRUS INHIBITION BY ACYCLOGUANOSINE

Vol. 15, 1979

and VZV at levels which appear to be nontoxic for mammalian cells. The mean ID50 of HSV-1 in Vero cells is 0.15 μM, whereas 50 μM ACG depresses DNA synthesis by Vero cells only moderately; thus, the therapeutic index in Vero cells is at least 150. These findings strongly support previous results showing that ACG is a relatively nontoxic antiviral agent which is very effective in inhibiting HSV-1, HSV-2, and VZV. In its apparent lack of toxicity, ACG resembles several other antiviral compounds, including 5-iodo-5-azino-2,5-dideoxyuridine (18), 1-β-D-arabinofuranosylcytosine (3, 9), and 5,6-dihydro-5-azathymidine (19). ACG appears to have much less inhibiting effect on DNA synthesis in uninfected cells than does adenine arabinoside (16). On the basis of our in vitro studies, it is unlikely that CMV disease will be affected by ACG therapy.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Sherie Gibson and Joan Bedinghaus in performing the assays. This work was supported by Public Health Service (PHS) contract no. AI-52530 from the Antiviral Substance Program of the National Institute of Allergy and Infectious Diseases (NIAID) and the National Dental Institute and PHS clinical center grant no. CA 10589 from the National Cancer Institute (NCI). C.S.C. is the recipient of career development award no. 5-K04-CA001393 from the NCI. J.A.Z. is the recipient of young investigator award no. AI 14373 from the NIAID.

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