In Vitro and In Vivo Resistance of Herpes Simplex Virus to 9-(2-Hydroxyethoxymethyl)guanine (Acycloguanosine)

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In vitro passage of the Patton strain of herpes simplex virus type 1 (HSV-1-P) in the presence of acycloguanosine (ACG) led to the emergence of a highly drug-resistant strain (HSV-1-P-ACG-R). Over 1% of virions in 6 of 15 HSV strains tested were able to form plaques in the presence of 10 μmol of ACG on first exposure to the drug. Therefore, there exists among HSV strains a broad range of susceptibilities to ACG, and some strains contain particles which are partially resistant to ACG before ever contacting the drug. HSV-1-P-ACG-R was partially resistant to iododeoxyuridine; conversely, iododeoxyuridine-resistant virus was highly resistant to ACG. ACG-resistant virus (HSV-1-P-ACG-R) was equal to its parent strain (HSV-1-P) in susceptibility to adenine arabinoside. The HSV-1-P-ACG-R strain produced corneal lesions in rabbits which were completely refractory to topical treatment with 1% unguent ACG, but responsive to 3% ACG. Partially resistant HSV could be isolated from the eyes of rabbits infected with ACG-susceptible virus and treated topically with either 1 or 3% ACG for 6 days.

Elion et al. (2) reported that 9-(2-hydroxyethoxymethyl)guanine (acycloguanosine, ACG) is nontoxic for normal cells, yet has an inhibitory activity against the KOS strain of herpes simplex virus type 1 (HSV-1) “greater than that of any hitherto known compound.” This high selectivity for HSV inhibition appears to be due to the fact that HSV thymidine kinase specifically phosphorylates ACG, whereas normal cell thymidine kinase does not significantly (3). ACG does not appear to be metabolized or substantially modified when given to normal mice, rats, or dogs (P. de Miranda, H. C. Krasny, S. Good, D. A. Page, T. H. Creagh, and G. B. Elion, Intersci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., abstr. no. 66, 1978) in contrast to other antiviral compounds. Several recent reports have shown that ACG is highly effective in the treatment of herpetic infections in experimental animals (9, 11) and humans (4, 5). Excep for the use of known thymidine kinase-deficient mutant strains of HSV by Elion et al. (2) and Fyfe et al. (3) in their demonstration of the mode of action of ACG, there has been only one brief report by Field et al. (H. J. Field, G. K. Darby, and P. Willy, Intersci. Conf. Antimicrob. Agents Chemother. 19th, Boston, Mass., abstr. no. 257, 1979) on in vitro induction of ACG-resistant HSV. There have been no reports of drug-resistant HSV strains emerging during in vivo exposure to ACG, nor have there been reports of treatment failures in animals or humans attributable to emergence of drug-resistant virus.

In this paper we present observations concerning: (i) the generation of HSV resistance to ACG by exposure of virus to increasing concentrations of the drug in vitro; (ii) the refractivity of corneal lesions, experimentally induced by an ACG-resistant strain of HSV, to drug treatment; (iii) wide variations in the susceptibilities of naturally occurring, recently isolated, and laboratory strains of HSV to ACG; and (iv) the susceptibility cross-resistance pattern of an ACG-resistant HSV strain to iododeoxyuridine (IUDR) and adenine arabinoside (Ara-A).

MATERIALS AND METHODS

Cell cultures. Viruses were grown and titrated at 36°C in human fetal fibroblasts derived from fetal tissues in our laboratory and used for virus work before the fifth passage. Cells were grown and maintained in basal medium Eagle (BME; Auto-Pow, Flow Laboratories) supplemented with 0.112% sodium bicarbonate, 2 mM l-glutamine (GIBCO Laboratories, Grand Island, N.Y.), 2 mg of neomycin (Mycifradin sulfate, Upjohn Co.) per 100 ml and 5 to 20% calf serum (KC Biologicals Inc., Lenexa, Kan.). Five percent BME, as described hereafter, would indicate medium containing 5 ml of calf serum in a total volume of 100 ml.

Viruses. The following strains of HSV were used: (i) HSV-1 strains Patton (courtesy of Fred Rapp), MacIntyre (American Type Culture Collection, Rockville, Md.), KOS (our own isolate), EL 12, EL 15, EL 19, 225, and 227 (clinical isolates, the latter two cour-
tesy of Hubert Malherbe); (ii) HSV-2 strains Benefield (courtesy of Amos Palmer), G (American Type Culture Collection, Rockville, Md.), EL 1, EL 3, EL 4, EL 6, and EL 7 (clinical isolates).

Viral plaque titrations. The titer of all HSV strains was determined by a plaque titration method (12). Tissue culture dishes (35 by 10 mm, Falcon) were seeded with cells and used for assays when they were approximately 75% monolayer. Volumes (0.2 ml) of logarithmic dilutions of each virus strain were inoculated onto each of two tissue culture dishes and adsorbed for 1 h with intermittent shaking, the inoculum was removed, and 2 ml of 5% BME containing 0.5% human immune serum globulin (USP Immuglobin, Travenol Laboratories Inc., Costa Mesa, Calif.) was added. After a 48-h incubation period at 36°C in a 5% CO₂ atmosphere, the overlay medium was removed, and the cell sheets were stained with a 0.05% aqueous crystal violet solution. The number of plaques was counted with a Nikon profile projector which magnified the dishes 10×. The duplicates were averaged, and the number of plaque-forming units was calculated.

Antiviral drugs: determination of the ED₉₀. BW-248-U [9-(2-hydroxyethoxymethyl)]guanidine, MW, 211; courtesy of Burroughs-Wellcome Co., Research Triangle Park, N.C.,] and Ara-A (kindly provided by Lois Allen) and IUDR (Sigma Chemical Co., St. Louis, Mo.) were used throughout this study. A stock solution of ACG was prepared freshly by dissolving 2.11 mg in 0.5 ml of 0.1 N NaOH and adding 9.5 ml of 5% BME. The IUDR stock solution was prepared by dissolving 1 g of IUDR in 10 ml of dimethyl sulfoxide and storing small portions in the dark at −20°C. The Ara-A stock solution was prepared by dissolving 10 mg of Ara-A in 1 ml of dimethyl sulfoxide. Appropriate dilutions of each drug were made in 5% BME containing 0.5% human immune serum globulin just before usage. For 50% effective dose (ED₉₀) titrations, all titrations were done at least in duplicate. Tissue culture dishes (35 by 10 mm) with approximately 75% cell monolayer were inoculated with approximately 50 plaque-forming units of HSV per 0.2 ml, and the virus was adsorbed for 1 h, with intermittent shaking. After removal of the inoculum, 2 ml of 5% BME with 0.5% immune globulin and threefold dilutions of the appropriate drug were added. One set of dishes received no drug and was later used for the "no-drug control." After a 48-h incubation period at 36°C in a 5% CO₂ atmosphere, the overlay medium was removed, the cells were stained as described above, and plaques were counted. After averaging the counts of replicate plates, the number of plaques emerging in the presence of each drug dilution was calculated as a percent of the control as follows:

(average number of plaques with drug/average number of plaques without drug) × 100

Each drug titration was plotted on semi-logarithmic graph paper. The ED₉₀ concentration was that amount of drug per milliliter of overlay medium that inhibited the plaque numbers by 50%, compared with the no-drug controls, and this was read off the graphic plot of the data.

Drug studies in HSV-infected rabbit eyes. Adult New Zealand white rabbits were used through-out this work. The multiple microtrophination technique of Jones and Al-Hussaini (5) and Jones et al. (7) was employed for animal inoculations. Multiple sites on the cornea of each eye were independently inoculated with an HSV suspension containing 5 × 10⁶ plaque-forming units per ml. These microinoculations were made by gentle pressure from, and rotation of the end of, a 1-mm-diameter glass capillary tube filled with inoculum. This produced small rings of slight epithelial damage which were simultaneously flooded with virus from the end of the capillary tube; these rings became the sites of herpetic lesions. Infections were allowed to progress for either 4 or 3 days (experiments 1 and 2, respectively) before ACG treatment was begun. Three percent unguent ACG and the drug-free vehicle (in which the drug was suspended) were obtained from the Burroughs-Wellcome Co. One percent ACG ointment was prepared in our own laboratory by thorough mixing of the drug in warm petrolatum (USP). The drug-free vehicles were used as a placebo treatment.

Rabbits were inoculated with the appropriate drug-susceptible or drug-resistant virus; on either day 3 or 4 of infection (depending on the experiment), their eyes were stained with a 1% aqueous solution of bengal rose and examined with slit-lamp illumination by one of us (R.H.P.) in single-masked fashion. The lesions were scored from 0 to 5, according to severity. On day 3 or 4, animals inoculated with a given virus were divided into two equal groups, the groups being selected to have almost identical average lesion scores; one group of animals was then used for ACG treatment, and the other received placebo treatment. Ointment was applied into the eyes four times per day through either day 9 or 10 of viral infections. Ophthalmic lesions were scored periodically as described above. Five to seven animals were used in each group, giving a total of 10 to 14 infected eyes for evaluation in each experiment. The mean lesion score for each treatment group was calculated at each observation time and plotted graphically (see Fig. 3 and 4).

RESULTS

Emergence of ACG-resistant virus in vitro. During the course of determining inhibitory concentrations of ACG with various laboratory strains of HSV, we observed that a few plaques of Patton strain HSV-1 emerged in the presence of 9 to 27 μmol of ACG (see Fig. 1A). However, no plaques formed in the presence of drug concentrations greater than 27 μmol. This HSV-1 strain was serially passaged through increasing concentrations of ACG (6, 18, and 54 μmol of ACG, respectively). The emerging virus was then passaged twice more through 54 μmol of ACG, divided in small volumes, and stored at −70°C. This virus was found to produce cytopathic effects rapidly, to have a titer of 3.2 × 10⁷ plaque-forming units per ml and to show complete in vitro resistance to 162 μmol of ACG (see Fig. 1B), i.e., there was no detectable decrease.
in plaque numbers or size at this drug concentration. This strain of resistant virus was designated HSV-1-P-ACG-R to distinguish it from the original Patton strain from which it was derived, which was designated HSV-1-P.

Initial impressions were that different HSV strains varied greatly in the amount of virus able to replicate in the presence of 10 μmol of ACG. This was somewhat surprising in view of the earlier report by Elion et al. (2) that ACG inhibited one HSV strain at 0.1 μmol. To study this further, eight strains of HSV-1 and seven strains of HSV-2 were plaque titrated in the absence and presence of 10 μmol of ACG to determine what fraction of the total infectious virus was capable of forming plaques in the presence of this drug concentration. Table 1 shows that the percent drug-resistant virus present in these strains (which had never before been exposed to ACG) ranged from <0.001 to 28%. Of the 15 different HSV strains, 6 (40%) showed the presence of resistant virus at the 1% or greater level. The strain showing the highest percentage of virus capable of plaque formation in the presence of 10 μmol of ACG strain 227 was a fresh clinical isolate from the face of a patient who denied ever having been treated with antiviral drugs. Our initial impressions that HSV strains vary greatly in their resistance to ACG on initial exposure were, therefore, confirmed. These data show no distinctive differences in the patterns of drug susceptibility or resistance between HSV-1 or -2. Clearly, the KOS strain was among the most, if not the most, susceptible of the 15 strains studied.

Figure 2 shows the results of simultaneous

**Table 1. Fractions of various HSV strains which are resistant to 10-μmol concentrations of ACG**

<table>
<thead>
<tr>
<th>HSV type</th>
<th>HSV strain</th>
<th>Total virus titer (PFU × 10^6/ml)</th>
<th>Drug-resistant virus titer (PFU × 10^6/ml)</th>
<th>% Drug-resistant virus</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>227</td>
<td>2.4</td>
<td>0.67</td>
<td>20.0</td>
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<td>1</td>
<td>Patton-Original</td>
<td>26.0</td>
<td>1.6</td>
<td>6.2</td>
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<td>1</td>
<td>MacIntyre</td>
<td>27.0</td>
<td>1.3</td>
<td>4.8</td>
</tr>
<tr>
<td>2</td>
<td>EL-3</td>
<td>4.0</td>
<td>0.15</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>Palmer</td>
<td>0.40</td>
<td>0.015</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>EL-4</td>
<td>0.36</td>
<td>0.005</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>EL-7</td>
<td>0.11</td>
<td>&lt;0.001</td>
<td>&lt;0.91</td>
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<td>2</td>
<td>2-G</td>
<td>0.70</td>
<td>&lt;0.001</td>
<td>&lt;0.14</td>
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<tr>
<td>2</td>
<td>EL-6</td>
<td>0.79</td>
<td>&lt;0.001</td>
<td>&lt;0.13</td>
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<tr>
<td>1</td>
<td>EL-12</td>
<td>3.3</td>
<td>0.003</td>
<td>0.09</td>
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<td>1</td>
<td>EL-19</td>
<td>28.0</td>
<td>0.02</td>
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<td>&lt;0.001</td>
<td>&lt;0.07</td>
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<td>1</td>
<td>225</td>
<td>15.0</td>
<td>0.008</td>
<td>0.06</td>
</tr>
<tr>
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<td>EL-15</td>
<td>7.5</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1</td>
<td>KOS</td>
<td>90.0</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* All strains except Patton, MacIntyre, Palmer, and KOS were fresh isolates; none had ever been exposed to ACG.
* PFU, Plaque-forming units.
* Drug-resistant virus is defined as that which formed visible plaques when incubated for 48 h in the presence of 10 μmol of ACG.
ED$_{50}$ titrations of different virus strains and illustrates further the major differences between strains in drug susceptibility patterns, particularly in the concentration range of 1 to 10 $\mu$mol of ACG.

Ocular herpes induced in rabbits by ACG-resistant HSV. Rabbits were inoculated with the original Patton strain (HSV-1-P) and with the highly resistant strain (HSV-1-P-ACG-R) derived from that strain and then treated with ACG and a placebo. In experiment 1, the results of which are shown in Fig. 3, the resistant strain produced corneal lesions which were almost completely refractory to treatment with 1% unguent ACG (on day 7, the average lesion scores were 3.6 and 3.7 for ACG-treated and placebo-treated eyes). Lesions induced by the original strain responded well to the same ACG treatment (lesion scores in placebo- and drug-treated animals on day 7 were 4.3 and 1.8, respectively).

A second experiment was designed so as (i) to begin treatment 1 day earlier and (ii) to use 3% unguent ACG instead of 1%. The results are shown in Fig. 4. In this experiment, the lesions produced by the resistant strain were somewhat better controlled by ACG treatment than in experiment 1. Again, lesions induced by the original strain responded well to treatment. It appears, therefore, that starting therapy 1 day earlier and using 3% rather than 1% ACG ointment allows improved treatment of epithelial lesions produced by highly resistant virus.

Isolation of virus from rabbit corneas after ACG treatment. One rabbit from each of the two experiments described above was selected for viral culture. These were animals which (i) had been inoculated with the original Patton strain of virus, (ii) which had obvious corneal lesions, and (iii) which had been treated with ACG. Approximately 2 days were allowed after treatment had ceased before viral cultures were taken. Cultures were taken by gently stroking a dry cotton swab across the cornea. Virus was readily isolated from both animals, allowed to produce 3 to 4+ cytopathic effects in human fibroblast cells, aliquoted, and frozen at $-70^\circ$C. Both strains were then titrated, and ED$_{50}$ determinations were done on each isolate and the original Patton strain. In both cases, the virus recovered from the ACG-treated eyes was about three times more resistant to ACG (ED$_{50}$'s were three times greater in simultaneous assays) than the virus originally inoculated. More importantly, it is clear that 6 days of treatment with ACG at concentrations of either 1 or 3% did not lead to complete elimination of infectious virus from the eyes.

Susceptibility of ACG-resistant virus to Ara-A and IUDR. HSV-1-P (the original strain) and HSV-1-P-ACG-R (ACG-resistant strain) were compared in their susceptibilities to three drugs: ACG, Ara-A, and IUDR. Table 2 shows their ED$_{50}$'s and the calculated fold increase in resistance of the HSV-1-P-ACG-R
strain to each drug (parent and resistant strains were titrated simultaneously in each experiment). The HSV-1-P-ACG-R strain was extremely resistant to ACG (as already demonstrated), unchanged in susceptibility to Ara-A, and partially resistant to IUDR (4.2-fold), when compared with its parent strain.

Susceptibility of IUDR-resistant virus to ACG. Since ACG-resistant virus was partially resistant to IUDR (Table 2), we sought to determine whether, conversely, IUDR-resistant virus is resistant to ACG. An HSV-1 strain (HSV-1-P) was passaged several times through ever-increasing concentrations of IUDR until a strain emerged whose plaques could form in the presence of 10 μg of IUDR per ml (it was 17 times more resistant than the parent strain). This strain (designated HSV-1-P-IUDR-R) was then tested to determine its susceptibility to ACG. The results (Fig. 5) indicate that IUDR-resistant virus is markedly resistant to ACG, i.e., the HSV-1-P-IUDR-R strain was over 100 times more resistant to ACG than its parent strain, HSV-1-P, and showed no evidence of plaque inhibition in the presence of 243 μmol of ACG.

**DISCUSSION**

We showed that 6 of 15 different herpesvirus strains (which consisted of types 1 and 2, 11 new isolates, and 4 old laboratory strains), initially (before drug exposure) had 1% or more infectious particles capable of forming plaques in the presence of 10 μmol of ACG. Serial in vitro passage of one of these strains through gradually increasing concentrations of ACG resulted in the emergence of a highly resistant strain (resistant to 162 μmol; see Fig. 1B). This ACG-resistant strain produced lesions in the corneas of rabbits which responded poorly to 1% unguent ACG; therefore, in vitro drug resistance paralleled in vivo resistance. However, beginning ACG treatment earlier and using 3% ACG produced a better response in rabbits (a slowing of lesion formation; see Fig. 4), which indicates that the in vitro-resistant virus strain was not totally resistant to

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**TABLE 2. Drug susceptibilities (ED50's) of the original HSV-1-P strain and the in vitro-derived ACG-resistant strain HSV-1-P-ACG-R**

<table>
<thead>
<tr>
<th>Drug</th>
<th>HSV-1-P</th>
<th>HSV-1-P-ACG-R</th>
<th>Fold resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACG</td>
<td>0.44</td>
<td>&gt;17</td>
<td>&gt;39</td>
</tr>
<tr>
<td>Ara-A</td>
<td>4.4</td>
<td>42</td>
<td>1.0</td>
</tr>
<tr>
<td>IUDR</td>
<td>1.05</td>
<td>4.4</td>
<td>4.2</td>
</tr>
</tbody>
</table>

* Average of two or three titrations each.

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**FIG. 4. Results of ACG treatment of corneal lesions in rabbits. Three percent ACG ointment was used four times per day, beginning on day 3 after infection with HSV-1-P (A) and HSV-1-P-ACG-R (B). Placebo treatment involved use of the ointment base.**

**FIG. 5. ED50 titration of HSV-1-P (original virus) and HSV-1-P-IUDR-R (IUDR-resistant virus) in the presence of ACG.**
a high drug concentration administered in vivo. Three percent ACG, which is now being used in prelicensing clinical trials as a topical treatment for human herpes keratitis, is probably a wise choice of concentration for therapeutic purposes. It is fortunate that ACG demonstrates such low toxicity for delicate tissues (2), enabling clinicians to use high therapeutic concentrations of the drug without tissue damage.

ACG-resistant virus was partially resistant to IUDR, and IUDR-resistant virus was highly resistant to ACG. This cross-resistance pattern can probably be explained by the fact that the two drugs share a common mechanism of antiviral action, i.e., inhibition of HSV-coded thymidine kinase activity (3, 8). IUDR has several mechanisms of action not shared by ACG, including incorporation into viral deoxyribonucleic acid (8) and interference with viral assembly (8, 13), which explains why highly ACG-resistant HSV is only partially resistant to IUDR. Our present finding that Ara-A is highly effective against ACG-resistant virus in vitro (see Table 2), together with the report that Ara-A is effective in many patients resistant to IUDR treatment (10), suggests that Ara-A would be a logical second choice if clinical failures were encountered with either ACG or IUDR chemotherapy.

It is ironic that the first studies of Elion et al. (2) and Fyfe et al. (3) demonstrating the antiviral effects of ACG were done with the KOS strain of HSV-1, a strain which we have shown in the present work to be among the most susceptible of 15 randomly selected HSV strains. Also, the earlier in vitro susceptibility studies of HSV to ACG were done in Vero (simian) cells rather than in human cells. We have evidence that ED₅₀ values in Vero cells tend to be lower than in some human fibroblast cell lines (unpublished data). This combination of highly susceptible viral strain and cell type may partially account for the conclusion of Elion et al. that the inhibitory effect of ACG against HSV is “greater than any hitherto known compound.” Our studies with other HSV strains in human fibroblasts indicate that the potency of ACG, on a molar basis, is in the same range as that reported for IUDR and Ara-C (1).

The present findings, which show large variations between different HSV strains in their susceptibilities to ACG, are consistent with the observations of Collins and Bauer (1) in their studies of different HSV strains and five other drugs. They showed that different HSV strains can vary over a wide range (up to about 100-fold) in their susceptibilities to drugs other than ACG. In a preliminary communication (abstract), Collins and Bauer also reported a three-fold variation between different herpesvirus strains in their sensitivities to ACG (P. Collins and D. J. Bauer, 18th ICAAC, abstr. no. 71, 1978). It is clear, therefore, that the potency of ACG should be assessed against many different HSV strains to obtain an accurate evaluation of the drug activity. By assuming that our random sampling of 15 HSV strains is reasonably representative of those producing human disease and that some HSV strains circulating in the human population are, therefore, partially drug resistant, it will be interesting to see how successful topical ACG treatment is in patients infected with viruses resembling strain 227 (see Table 1), which was initially quite drug resistant. It will also be interesting to observe whether ACG is effective when used a second or third time in individuals with recurrent ophthalmic herpes, in view of our finding that virus can be isolated from rabbit eyes after treatment and that partial drug resistance develops in vivo after several days of exposure to ACG. This slight increase in drug resistance can hardly account for the persistence of infectious virus after 6 days of treatment with 3% ACG, a very high drug concentration.

It is predictable that ACG will be more effective against herpesvirus infections in tissues which are easily accessible to high concentrations of drug and correspondingly less effective against those which are less accessible. If clinical failures occur in ACG treatment of human herpes infections, it seems most likely that they would be due to one or a combination of the following: (i) inability to deliver sufficiently high drug concentrations to deeper sites of infection; (ii) partial natural resistance of the patient’s herpesvirus strain; (iii) repeated treatment of recurrent lesions, leading to selection and emergence of increasingly resistant strains of latent virus; or (iv) IUDR-resistant strains resulting from previous clinical failures with this drug.

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