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Inhibitory action of acyclovir (ACV) and penciclovir (PCV) on plaque formation and partial cross-resistance of ACV-resistant varicella-zoster virus to PCV

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Abstract

Penciclovir has potent antiviral activity against varicella-zoster virus (VZV). We have characterized the inhibitory effects of penciclovir and acyclovir on the plaque formation of cell-free VZV and cross-resistance of acyclovir-resistant VZV to penciclovir. The apparent effective concentration for 50% plaque reduction (EC_{50}) of penciclovir determined on the third day was significantly lower than that determined on the fourth or fifth day. The size of plaques was smaller in the presence of penciclovir than in the presence of acyclovir. The effective concentrations for 50% reduction of the number of infected cells per plaque were 1.40 and 5.00 $\mu g/ml$ for penciclovir and acyclovir, respectively. Thus penciclovir suppressed spread of infection within developing plaques more efficiently than acyclovir. Five acyclovir-resistant VZV strains with altered DNA polymerase selected by acyclovir were examined for cross-resistance to penciclovir. They were 11- to 18-fold more resistant to ACV than the parent strain, but only 4- to 5-fold more resistant to PCV. Penciclovir-triphosphate carrying the 3′-hydroxyl group of 2′-deoxyribose might have better affinity to the altered viral DNA polymerase than acyclovir-triphosphate without the 3′-hydroxyl group.

Keywords: Acyclovir; Penciclovir; Plaque formation; Cross-resistance; Varicella-zoster virus

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1. Introduction

Varicella-zoster virus (VZV) infection sometimes causes life-threatening disease, especially in immunocompromised hosts. VZV infection has been prevented by Oka varicella vaccine (Takahashi et al., 1975; Takahashi, 1992) and zoster immune globulin (ZIG) (Brunell et al., 1969), and has been treated with ZIG (Brunell et al., 1969), chemotherapeutic drugs acyclovir (ACV) (Elion et al., 1977; Crumpacker et al., 1979; Biron and Elion, 1980), vidarabine (Bryson and Connor, 1976; Whitley et al., 1976)) and the biological response modifiers, interferon (Merigan et al., 1978) and transfer factor (Steele et al., 1980). Herpes zoster is, at present, mainly being treated with antiviral chemotherapy in an increasing number of immunocompromised hosts, including patients with the acquired immunodeficiency syndrome. ACV is the current treatment of choice for severe VZV infection. Sorivudine (Machida, 1986) and penciclovir (PCV) (Boyd et al., 1987), agents with potent anti-VZV action, are other potentially useful compounds. Famciclovir, the oral form of PCV, has recently been introduced in the United States and United Kingdom for the treatment of herpes zoster.

It is preferable to assess antiviral activity against cell-free VZV as is done for herpes simplex virus (HSV) and cytomegalovirus (Shiraki et al., 1992). We have determined the effective concentration for 50% plaque reduction (EC_{50}) of PCV by using cell-free virus, but its values fluctuated compared with ACV (Shiraki et al., 1993). We noticed that the size of plaques was smaller in the presence of PCV than in the presence of ACV at the same concentration.

ACV-resistant VZV strains have emerged following prolonged treatment of herpes zoster in immunocompromised patients, especially those with the acquired immunodeficiency syndrome (Pahwa et al., 1988; Hoppenjans et al., 1990; Jacobson et al., 1990; Linnemann et al., 1990). Therefore it is necessary to treat them with another efficient chemotherapeutic agent (Balfour, 1994). We have examined cross-resistance of ACV-resistant VZV strains with altered DNA polymerase activity to PCV. We have characterized the inhibition of VZV plaque formation by PCV and have monitored the susceptibility of ACV-resistant VZV to PCV by using cell-free virus.

2. Materials and methods

2.1. Cells and viruses

Human embryonic lung cells were grown and maintained in Eagle's minimal essential medium supplemented with 10 and 2% fetal bovine serum (FBS), respectively. As VZV strains, we used the parent Kawaguchi strain which was plaque-purified 6 times as cell-free virus (Shiraki et al., 1983, 1985, 1992) and the ACV-resistant virus strains thereof (A1, A2, A3, A6, and A8), with altered DNA polymerase activity, selected by passages in the increasing concentrations of ACV (Shiraki et al., 1983, 1990).

2.2. Antiviral drugs

Antiviral drugs used were ACV (kindly supplied by Nippon Wellcome, Osaka, Japan), and PCV (by SmithKline Beecham Pharmaceutical, London, UK). Phosphonoacetic acid (PAA) was purchased from ICN Inc.

2.3. Preparation of cell-free virus

VZV-infected monolayers exhibiting 50–70% cytopathic effect (CPE) were treated with 0.04% EDTA in phosphate-buffered saline (PBS) and suspended in SPGA medium (PBS containing 5% sucrose, 0.1% Na glutamate and 10% FBS) (Shiraki and Takahashi, 1982; Shiraki et al., 1983, 1985, 1992). The cell suspension was treated for 40 s in an ultrasonic disrupter, or frozen and thawed 3 times with vigorous pipetting followed by centrifugation at 3000 rpm for 20 min. The supernatant was frozen at −85°C as a cell-free virus stock.

2.4. Plaque reduction assay

The susceptibilities of viruses to ACV and PCV were determined by examining the EC_{50} (Biron and Elion, 1980; Crumpacker et al., 1979; Shiraki et al., 1983, 1990, 1992). Briefly, confluent cell monolayers in 60 mm plastic dishes in duplicate or triplicate were infected with 100 plaque forming units (PFU) of cell-free virus for 1 h and incubated in the maintenance medium containing PCV or ACV (0, 0.2, 0.5, 1, 2, and 5 μg/ml). After the appearance of CPE, the cells were fixed with 5% neutral formalin and stained with methylene blue, and the number of plaques was counted with a dissecting microscope. The EC_{50} was determined graphically. To examine the effect of the incubation period on the EC_{50} values of PCV and ACV, the infected culture treated with drugs were incubated for 3, 4, and 5 days. The Student's *t*-test was used to evaluate the statistical significance of the difference in the EC_{50} values among the incubation periods and probability values ($P < 0.05$) were considered to be statistically significant. Concerning the ACV-resistant viruses, the cells were fixed and stained on the fourth day, and the EC_{50} was determined.

2.5. Determination of the mean number of infected cells per plaque

Two sets of duplicate or triplicate assay dishes were infected with 100 PFU of cell-free virus and incubated in the presence of PCV and ACV (0, 0.2, 0.5, 1, 2, and 5 μg/ml). One set was fixed and stained for the determination of the EC_{50} values. The other was used for the infectious center assay to determine the number of infected cells in each dish (Shiraki et al., 1992). The dish was treated with trypsin and 0.04% EDTA in PBS, and the cells were suspended in 2 ml of maintenance medium. Then the cell suspension was serially diluted and inoculated onto HEL cell monolayers. The number of plaques were determined as described above and the number of infected cells in each dish was estimated by the dilution. The mean number of infected cells per plaque was determined by dividing the number of infected cells by the number of plaques. The

effective concentration for 50% reduction of the number of infected cells per plaque ($ECIP_{50}$) was determined graphically. The difference in the number of infected cells per plaque was analyzed by two-way analysis of variance (ANOVA).

3. Results

3.1. Effect of incubation periods on EC_{50} values of PCV and ACV determined by cell-free virus

The apparent EC_{50} values of PCV and ACV were determined on the third, fourth, and fifth days after infection in 5 independent experiments. EC_{50} values of PCV for the Kawaguchi strain were 1.29 ± 0.29, 1.97 ± 0.45, and 2.93 ± 0.64 $\mu g/ml$ on the third, fourth, and fifth day, respectively, and those of ACV were 1.21 ± 0.28, 1.45 ± 0.22, and 1.63 ± 0.36 $\mu g/ml$ on the third, fourth, and fifth day, respectively. Thus, the apparent EC_{50} of PCV determined on the third day was significantly lower than that determined on the fourth or fifth day, while that of ACV did not significantly increase from the third

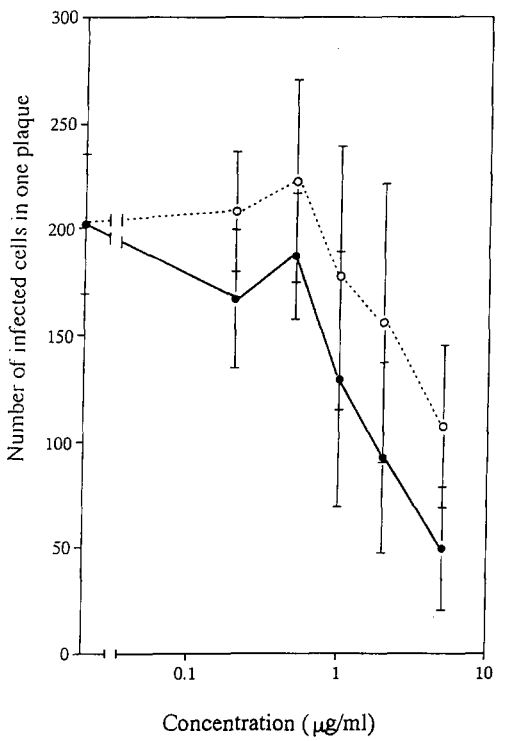

Fig. 1. Comparison of the mean number of infected cells in one plaque formed in the presence of PCV and ACV on the fourth day. Mean values for PCV- and ACV-treated cultures are indicated as closed and open circles, respectively, and the ranges of their S.D. values are expressed as bars.

till the fifth day. Thus the apparent EC_{50} value of PCV increased with the incubation period.

3.2. Number of infected cells in the plaques formed in the presence of PCV and ACV

As we observed that the size of plaques was smaller in PCV-treated cultures than in ACV-treated cultures, we determined the number of infected cells per plaque formed in the presence of PCV or ACV in order to compare their plaque sizes in 5 independent experiments. As shown in Fig. 1, the mean numbers of infected cells per plaque were significantly smaller in the presence of PCV than in the presence of ACV at any concentration by two-way analysis of variance (ANOVA) ($F_{1,48} = 12.997$, $P = 0.001$) as assessed on the fourth day after infection.

Fig. 2 shows the changes in the plaque number, the mean number of infected cells per plaque at various incubation times. The mean number of infected cells per plaque increased with the incubation period in the untreated culture. In contrast, the mean number of infected cells was apparently reduced with time in PCV- or ACV-treated

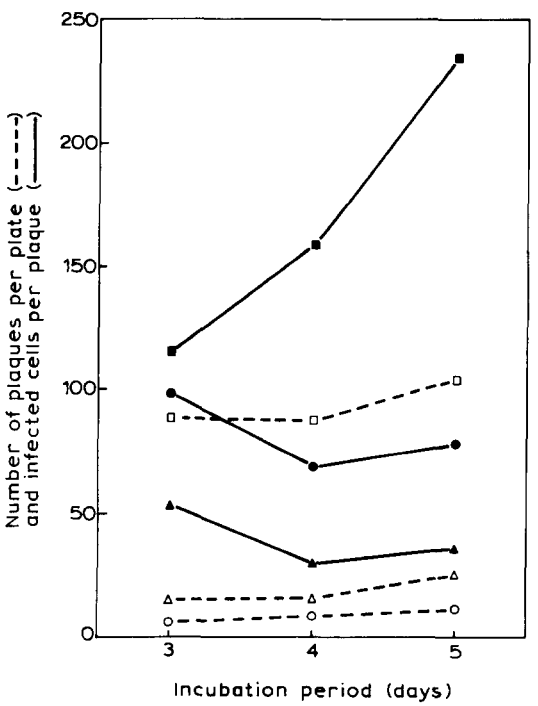

Fig. 2. Changes in the plaque number, the mean number of infected cells per plaque at various incubation times. The plaque number and the mean number of infected cells per plaque are determined on the third, fourth, and fifth day. Untreated control cultures (□, ■) the cultures treated with ACV at 5 μg/ml (○, ●), or with PCV at 5 μg/ml (△, ▲). Open symbols connected by dotted lines and closed symbols connected by solid lines indicate the plaque number and the mean number of infected cells per plaque, respectively. The difference in the number of infected cells per plaque was analyzed by two-way analysis of variance (ANOVA).

Table 1
Susceptibility (EC_{50}) of ACV-resistant VZV strains

Strain	ACV	PCV	PAA	ACV/PCV
Wild type	1.21 ± 0.28	1.29 ± 0.29	5.50 ± 0.22 [a]	0.9
A1	16.50 ± 1.44	5.45 ± 2.03	10.40 ± 0.85	3.0
A2	13.50 ± 4.35	6.00 ± 2.41	22.00 ± 2.83	2.3
A3	16.69 ± 4.72	5.61 ± 1.61	46.50 ± 2.12	3.0
A6	21.53 ± 5.47	5.48 ± 1.30	1.75 ± 0.07	3.9
A8	19.50 ± 5.56	6.03 ± 1.52	1.43 ± 0.04	3.2

EC_{50} values were determined by 5 independent experiments and are expressed as the mean EC_{50} ± S.D. (μg/ml).
[a] EC_{50} has been reported previously (Shiraki et al., 1990).

cultures, while the number of plaques slightly increased with time. The mean number of infected cells per plaque was smaller in the PCV-treated culture than in ACV-treated culture as shown in Fig. 2. This indicated that some of the infected foci were not counted on the third day as plaques in the culture treated with PCV because their sizes were too small to be counted as plaque. These infected foci became large enough to be counted as plaques on the fifth day.

The mean $ECIP_{50}$ was 1.40 μg/ml for PCV and 5.00 μg/ml for ACV from 5 experiments. These results indicated that PCV inhibited the spread of infection in the plaque more efficiently than ACV, resulting in the formation of smaller plaques by PCV than ACV.

3.3. Susceptibility of acyclovir-resistant viruses to penciclovir

Susceptibilities of ACV-resistant viruses to PCV are summarized in Table 1. Both PAA-sensitive and PAA-resistant ACV-resistant VZV strains were used for the susceptibility assay (Shiraki et al., 1983). Both types of ACV-resistant viruses were 11- to 18-fold more resistant to ACV than the parent strain, but only 4- to 5-fold more resistant to PCV, while wild-type VZV was as susceptible to PCV (EC_{50}, 1.29 μg/ml) as to ACV (EC_{50}, 1.21 μg/ml). A1, A2, and A3 are resistant to PAA, and A6 and A8 are sensitive to PAA as reported previously. Susceptibility of ACV-resistant virus to PCV was not affected by PAA sensitivity. EC_{50} of PCV for ACV-resistant VZV was lower than that of ACV.

4. Discussion

This experiment was designed to characterize the differential effect of PCV and ACV on plaque formation and cross-resistance between them by using cell-free virus (Shiraki et al., 1992). Therefore we used 6 times plaque-purified cell-free virus (Shiraki et al., 1983) to minimize the variability due to the virus strain, and, accordingly, any differences in susceptibility that may result from the assay method rather than the heterogeneity of virus population. The difference of plaque size might be assessed only

by using cell-free virus infection and this indicated the advantage of an assay using cell-free virus over cell-associated virus as an inoculum.

Similarity in EC_{50} values of PCV and ACV for wild VZV strains has been reported (Boyd et al., 1987). The EC_{50}s of both PCV and ACV for the Oka varicella vaccine strain and Kawaguchi strain range between 0.68 and 1.3 μg/ml (Shiraki et al., 1993). We characterized the difference of the plaque size formed in the presence of PCV and ACV by using cell-free virus. The size of plaque was smaller in the PCV-treated cultures than in the ACV-treated cultures ($P = 0.001$). This causes the discrepancy in the EC_{50} and $ECIP_{50}$ of PCV and ACV. Our results indicated that PCV inhibited spread of infection within the plaque more efficiently than ACV.

We also examined cross-resistance of ACV-resistant VZV strains to PCV. They were partially sensitive to PCV. Both PAA-sensitive and PAA-resistant ACV-resistant VZV strains might be similar to the ACV-resistant HSV strains reported earlier (Field et al., 1981; Larder and Darby, 1985). Both sorts of ACV-resistant VZV strains were shown to be about 2- to 4-fold less resistant to PCV than ACV, while wild type VZV was as susceptible to PCV (EC_{50}, 1.29 μg/ml) as to ACV (EC_{50}, 1.21 μg/ml). Thus the sensitivity to PCV was not influenced by PAA sensitivity.

The anti-VZV action of PCV and ACV has been characterized as follows. Both are phosphorylated by viral thymidine kinase (TK) and inhibit viral DNA synthesis (Elion et al., 1977; Biron and Elion, 1980; Boyd et al., 1987). ACV and PCV triphosphates are competitive inhibitors of viral DNA polymerase, become incorporated into viral DNA and prevent DNA chain elongation. PCV-triphosphate levels are higher, and persist for a longer time, than the ACV-triphosphate levels inside infected cells (Vere Hodge and Perkins, 1989; Earnshaw et al., 1992; Bacon and Schinazi, 1993; Vere Hodge and Cheng, 1993). K_i values of HSV-1 DNA polymerase for ACV-triphosphate and PCV-triphosphate are 0.07 and 8.5 μM, respectively, when dGTP is used as substrate (Earnshaw et al., 1992). Yet, EC_{50}s of ACV and PCV for HSV-1 are similar because of higher concentration and longer maintenance of PCV-triphosphate in the infected cells (Boyd et al., 1987; Earnshaw et al., 1992; Vere Hodge and Cheng, 1993). These biochemical differences might reflect in the inhibition of plaque formation by VZV. PCV suppressed spread of infection for a longer period than ACV, resulting in the formation of smaller plaques.

Although ACV-resistant HSV are generally resistant to PCV, some strains remain sensitive to PCV (Boyd et al., 1987; Boyd et al., 1993). ACV-resistant clinical VZV isolates containing a TK mutation are reported to be resistant to PCV in the assay using cell-associated virus (Talarico et al., 1993). EC_{50} values of PCV for the parent and TK-deficient viruses were 1.2 and 4.5 μg/ml (Shiraki et al., 1993), thus similar to the EC_{50} of PCV for ACV-resistant VZV strains with altered DNA polymerase. Thus TK-deficient virus with complete resistance to ACV exhibited partial resistance to PCV and we assumed that contribution of TK insensitivity to PCV may be less pronounced than insensitivity of VZV to ACV (Shiraki et al., 1990). Our assay using cell-free virus may reveal a different aspect of cross-resistance of ACV-resistant VZV strains (including a TK-deficient strain) to PCV that was not detected by using cell-associated virus (Talarico et al., 1993). Our results indicated that cell-free ACV-resistant VZV strains with altered DNA polymerase were more sensitive to PCV than to ACV. We assume

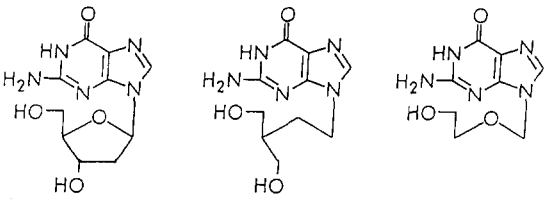

Fig. 3. Structural formulae of deoxyguanosine, penciclovir, and acyclovir.

that PCV carrying the 3′-hydroxyl group of 2′-deoxyribose might have relatively better affinity to such mutant forms of viral DNA polymerase than ACV missing this 3′-hydroxyl group (Fig. 3).

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