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Emergence of resistance to acyclovir and penciclovir in varicella-zoster virus and genetic analysis of acyclovir-resistant variants

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Abstract

We have characterized the differential actions of acyclovir and penciclovir against varicella-zoster virus (VZV) in cell culture by comparing the frequency of appearance of resistant viruses followed by their characterization. Cells were infected with cell-free virus and the cultures were successively treated with increasing concentrations of acyclovir or penciclovir. Drug-resistant viruses were selected in the presence of 6 μg/ml of acyclovir or penciclovir. The emergence frequency of resistant viruses was significantly higher following acyclovir exposure than following penciclovir exposure (Fisher's exact test, $P < 0.0001$), possibly reflecting virus growth differences under these experimental conditions. Based on antiviral drug susceptibility and thymidine kinase (TK) activity assays, 11 acyclovir-resistant variants from seven experiments using three virus strains (Kawaguchi strain, Oka varicella vaccine strain and a clinical isolate from a zoster patient) were found to be TK-deficient. Sequence analysis of TK-deficient variants of the Kawaguchi strain revealed deletions that caused frameshifts, resulting in premature termination in the TK gene.

Keywords: Acyclovir; Penciclovir; Resistance; Sequence analysis; Thymidine kinase; Varicella-zoster virus

1. Introduction

Varicella-zoster virus (VZV) infections have been mainly treated with acyclovir (ACV) (Elion et al., 1977 Crumpacker et al., 1979 Biron and

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Elion, 1980) and its oral prodrug valaciclovir. However, newer drugs, such as sorivudine (BVaraU; Machida and Sakata, 1984 Machida, 1986), penciclovir (PCV; Boyd et al., 1987 Boyd et al., 1993), and its oral producing form famciclovir, have also been introduced in the clinic. These drugs are mainly used for the short-term treatment of acute or recurrent VZV infections. However, an increasing number of chronic VZV infections in immunocompromised hosts, such as patients in the late phase of human immunodeficiency virus (HIV) infection, require repeated and/or long-term treatment. Under such conditions, emergence of drug-resistant viruses is unavoidable. ACV-resistant VZV strains have emerged following prolonged treatment of VZV infection in immunocompromised patients (Pahwa et al., 1988 Hoppenjans et al., 1990 Jacobson et al., 1990 Linnemann et al., 1990 Safrin et al., 1991 Talarico et al., 1993 Boivin et al., 1994 Lyall et al., 1994 Snoeck et al., 1994). Therefore, determination of the mechanism of resistance may help in designing strategies to diminish the appearance of drug-resistant VZV (Balfour, 1994).

In this paper, we report on the anti-VZV activity of ACV and PCV with particular reference to the emergence of drug-resistant strains. Both drugs are currently used to treat VZV infections. The development of ACV- and PCV-resistant VZV strains in vitro was engendered with increasing ACV and PCV concentrations that are similar to those achievable in plasma following administration to patients. We have previously reported that ACV and PCV have a similar anti-VZV activity in plaque reduction assays. The effective concentrations for 50% plaque reduction ($EC_{50} \pm$ S.D.) of ACV and PCV were 1.21 ± 0.28 and 1.29 ± 0.29 μg/ml, respectively (Hasegawa et al., 1995). Similar anti-VZV activity of ACV and PCV has also been reported by Boyd et al. (1993). Therefore, selection of resistant viruses was attempted using the same concentrations of both drugs possessing comparative anti-VZV activity. Resistant virus was selected only from ACV-treated cultures. These variants were characterized in detail and found to be thymidine kinase (TK)-deficient. This was consistent with clinical ACV-resistant viruses with preference of mutation in the TK gene relative to the DNA polymerase gene (Larder and Darby, 1984).

2. Materials and methods

2.1. Cells and viruses

Human embryonic lung cells were grown and maintained in Eagle's minimum essential medium supplemented with 10% and 2% fetal bovine serum, respectively. The Kawaguchi strain of VZV (Takahashi et al., 1975) was cloned six times serially by using cell-free virus. The cell-free virus stock (Shiraki et al., 1983, 1985) was prepared by freezing and thawing followed by sonication in SPGC medium (Shiraki et al., 1992; phosphate-buffered saline supplemented with 5% sucrose, 0.1% sodium glutamate and 10% fetal bovine serum) within six passages from the last cloning in this experiment. The Oka varicella vaccine strain of VZV (Takahashi et al., 1974) and a clinical isolate obtained from a skin lesion of a 74-year-old male patient with zoster were inoculated into the cells. The cell-free virus stocks were prepared in the same way. The TK-deficient strain was derived from the Kawaguchi strain used in this study.

2.2. Drugs

ACV was purchased from Sigma (St. Louis, MO) and PCV was kindly provided by SmithKline Beecham Pharmaceuticals (Surrey, UK). Phosphonoacetic acid (PAA) was purchased from ICN (Costa Mesa, CA), and sorivudine (BVaraU) was kindly supplied by Yamasa Shoyu (Choshi, Japan).

2.3. Determination of the stock viruses for drug susceptibility

Plaque forming units (PFU), 10^3 per 0.2 ml of stock virus of the three virus strains (Kawaguchi strain, Oka varicella vaccine strain and a clinical isolate), were inoculated into six sets of monolayer cultures of human embryonic lung cells on plastic dishes (6 cm in diameter) for each strain.

The infected cells were then incubated in the presence of 6 μg/ml of ACV for more than 6 days with medium replacement at, at least, 3-day intervals. The infected cells were then transferred after trypsinization onto cells with 18 plastic dishes for each strain, and incubated in the presence of 6 μg/ml of ACV for an additional 12 days with medium replacement at, at least, 3-day intervals. Similarly, the stock viruses were subjected to treatment of two sets of the infected monolayer culture with 6 μg/ml of PCV.

2.4. Emergence of drug-resistant variants

The cells in a 25-cm^2 flask were infected with 1000 PFU of cell-free VZV (Kawaguchi strain, Oka varicella vaccine strain, a clinical isolate) and cultured in the presence of 2 μg/ml of ACV or PCV for 6 days. The infected cells were then transferred onto new cells in a 150-cm^2 flask and cultured in the presence of 4 μg/ml of ACV or PCV for an additional 7 days. After these 7 days, the infected cells were transferred onto new cells in a 150-cm^2 flask and cultured for a further 11 days in the presence of 6 μg/ml of ACV or PCV. Finally, the resulting infected cells were transferred onto new cells in six plastic dishes (6 cm in diameter) and cultured in the presence of 6 μg/ml of either drug for 1 week. The infected cells were subcultivated after trypsinization with medium replacement at, at least, 3-day intervals. For one of the 12 experiments (experiment 8 in Table 1), where ACV was increased gradually at 2, 4 and 6 μg/ml as described above, PCV was used at lower concentrations of 1, 3 and 6 μg/ml. This sequential treatment was also performed in cultures infected with 1000 PFU of Kawaguchi strain supplemented with 5 PFU of the TK-deficient strain derived from the Kawaguchi strain (AR-2 in Table 2). Drug-resistant viruses were selected by covering the infected cell colony with a metal cylinder and the infected cells were trypsinized and subcultured without contamination of the other infected cells.

2.5. Plaque reduction assay

The susceptibilities of each strain to ACV, PCV, PAA and BVaraU were determined by examining the EC_{5O} values (Crumpacker et al., 1979 Biron and Elion, 1980 Shiraki et al., 1983, 1990, 1992 Hasegawa et al., 1995). Briefly, confluent cell monolayers in plastic dishes (6 cm in diameter), in duplicate, were infected with 100 PFU of the cell-free viruses for 1 h and cultured in medium containing the antiviral drug at the indicated concentrations (ACV, PCV, PAA: 0, 0.5, 1, 2, 5, 10

Table 1
Emergence frequency of ACV- or PCV-resistant viruses in ACV- or PCV-treated cultures

Experiment	Strains	Number of plaques[a]	
		Penciclovir	Acyclovir
1	Kawaguchi strain	0	506
2	Kawaguchi strain	0	1992
3	Kawaguchi strain	0	>2000
4	Kawaguchi strain	0	>2000
5	Kawaguchi strain	0	21
6	Kawaguchi strain	0	>2000
7	Kawaguchi strain	0	0
8	Kawaguchi strain[b]	0	>2000
9	Oka vaccine strain	0	>2000
10	Oka vaccine strain	0	>2000
11	Clinical isolate	0	>2000
12	Clinical isolate	0	7
13	Kawaguchi strain + TK[-c]	23	>2000

[a] Drug-resistant virus emergence following ACV-treatment by the Fisher's exact probability test ($P<0.0001$).

[b] In experiment 8, where ACV was increased gradually at 2, 4 and 6 μg/ml, PCV was used at lower concentrations of 1, 3 and 6 μg/ml.

[c] The sequential treatment was also performed in cultures infected with 1000 PFU of Kawaguchi strain supplemented with 5 PFU of TK-deficient strain AR-2 (Table 2) derived from Kawaguchi strain in experiment 2.

Table 2
Susceptibilities of the acyclovir-resistant strains to antiviral agents[a]

Strains	ACV[b]	PCV[b]	PAA[b]	BvaraU[b]
AR-1a	7.2 ± 1.9	8.6 ± 2.0	3.9 ± 0.5	>1
AR-1b	7.8 ± 0.9	8.7 ± 0.3	2.8 ± 0.7	>1
AR-2	8.6 ± 1.7	11.8 ± 2.6	2.9 ± 1.0	>1
AR-3	7.1 ± 0.7	10.2 ± 1.5	3.0 ± 0.9	>1
AR-4	7.4 ± 0.7	11.4 ± 0.5	3.2 ± 1.1	>1
AR-5a	8.3 ± 2.1	9.1 ± 1.7	3.0 ± 1.0	>1
AR-5b	7.5 ± 0.8	10.2 ± 1.0	3.3 ± 1.3	>1
AR-9a	11.0 ± 3.0	11.6 ± 4.5	2.5 ± 0.3	>1
AR-9b	10.1 ± 3.7	10.2 ± 5.5	2.2 ± 0.3	>1
AR-11a	11.3 ± 1.9	11.0 ± 0.8	5.0 ± 0.1	>1
AR-11b	11.7 ± 1.1	10.8 ± 0.6	5.2 ± 0.1	>1
Kawaguchi strain	1.2 ± 0.0	1.6 ± 0.1	4.9 ± 1.7	0.001 ± 0.0001
Oka vaccine strain	0.9 ± 0.2	2.2 ± 0.5	3.0 ± 0.4	0.001 ± 0.0002
Clinical isolate	1.1 ± 0.5	2.1 ± 0.4	5.9 ± 1.1	0.001 ± 0.0001

[a] ACV, acyclovir; PCV, penciclovir; PAA, phosphonoacetic acid; BVaraU, 1-β-D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil.
[b] Susceptibilities (EC_{50}: μg/ml) of ACV-resistant strains to ACV, PCV, PAA, and BVaraU are expressed as the mean values ± S.D. calculated from more than three experiments.

and 20 μg/ml; BVaraU: 0, 0.2, 0.5, 1, 2, 5 ng/ml and 1 μg/ml). After 5 days of incubation, the number of plaques was directly determined under the dissecting microscope. Each EC_{50} value from more than three independent experiments was determined graphically.

2.6. TK assay

TK activity of each strain was measured by the method reported previously (Shiraki et al., 1985). When more than 70% of the cells showed a cytopathic effect, the cells were washed with phosphate-buffered saline (PBS) and treated with 0.04% EDTA in PBS. The cell suspension was collected and washed with 50 mM Tris–HCl (pH 8.0) buffer containing 150 mM KCl and 3 mM 2-mercaptoethanol (2-ME) by low-speed centrifugation, and suspended in this buffer at a cell concentration of $2–4 \times 10^6$ cells/ml. These cells were sonicated in an ice bath, and centrifuged at 30 000 rpm for 30 min at 4°C. The supernatant was used as an enzyme extract for the TK activity assays. An extract of mock-infected cells was prepared similarly. The reaction mixture (250 μl) contained 2.0 μCi [*methyl*-^{3}H]-thymidine (20 Ci/mmol; NEN DuPont Research Products, Boston, MA), 5.0 mM ATP, 5.0 mM $MgCl_2$, the enzyme extract and 50 mM Tris–HCl (pH 8.0). The reaction was conducted at 37°C for 5 min and was stopped by immersing the reaction tubes in a boiling water-bath for 2 min. The amount of phosphorylated thymidine was determined by the DEAE-cellulose disc method (Ogino et al., 1977 Shiraki et al., 1985).

To evaluate the results of the TK assays, the amounts of VZV antigens in the enzyme extracts were determined by enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Rennard et al., 1981). Briefly, the extracts used for the TK assay were fixed on the plastic ELISA plate. Anti-VZV guinea pig serum and anti-guinea pig-IgG goat IgG fraction conjugated with horseradish peroxidase (whole molecule) (Organon Teknika, West Orange, NJ) were used as the first and second antibody, respectively. The amount of VZV antigen was determined using the ELISA color reagent kit (DENKA SEIKEN, Tokyo, Japan).

2.7. Sequencing of the TK gene

VZV DNA was extracted from nucleocapsids as reported previously (Shiraki et al., 1991a,b).

Briefly, VZV-infected cells (90–100% infected) were harvested in 20 mM Tris–HCl (pH 8.0) buffer containing 10 mM EDTA and 1% Triton X-100, and centrifuged at 3000 rpm for 20 min and 27 000 rpm for 1 h at 4°C. The pellet was suspended in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) and treated with 10 μg/ml of RNase for 1 h at 60°C, and then treated with 0.3% sodium dodecyl sulphate and proteinase K at 50 μg/ml at 37°C for 24 h followed by phenol extraction. VZV DNA suspended in TE buffer was amplified by polymerase chain reaction (PCR) with the two primers corresponding to the sequence comprising the open reading frame of the VZV TK gene (Davison and Scott, 1986 Mori et al., 1988): 5′ end, 64650 CCTCGACGTACGTTATCAAT-64669; 3′ end, 65891-CGCGAGTATGACAATGTGTA-65872. In this amplification, 1 μg of the VZV genomic DNA was applied to the mixture (50 μl) which was also supplemented with 120 mM Tris–HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.001% bovine serum albumin, 200 μM (each) deoxynucleotide, 1.0 μM (each) primer, and 1.25 units of KOD DNA polymerase (TOYOBO, Osaka, Japan). The reactions, repeated for 50 cycles, were programmed at 98°C for 15 s, at 68°C for 15 s and at 72°C for 55 s. Each PCR product was then processed with phenol and chloroform, and resuspended with TE for determination of the DNA sequence.

DNA sequencing was performed by using Auto Sequencer Core Kit (TOYOBO) with the primers corresponding to the sequence of the VZV TK fragment. All primers were conjugated with fluorescein isothiocyanate at the 5′-end. The PCR product (0.05–0.1 μg) was subjected to cycle sequencing. The sequencing reaction, repeated for 24 cycles, was performed as follows: denaturation at 95°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 84 s. After this cycle sequencing reaction, 6 μl of each sample was applied to a 6.0% Long Ranger gel (FMC BioProducts, Rockland, ME). The result of this cycle sequencing reaction was analyzed with the ALF DNA Sequencer (Pharmacia Biotech, Uppsala, Sweden). The DNA sequence was determined from both strands independently.

2.8. Statistical analysis

The differences between the emergence frequencies of resistant viruses to ACV and PCV were analyzed by the Fisher's exact test. Nucleotide change observed in the TK gene was analyzed by exact binominal probability. A *P*-value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Determination of stock viruses for drug susceptibility

Stock virus, containing 1000 PFU per 0.2 ml of SPGC medium of the three virus strains (Kawaguchi strain, Oka varicella vaccine strain and a clinical isolate), was subjected to treatment of the infected cultures with 6 μg/ml of ACV or PCV for more than 18 days, after which the infected cells were transferred onto new cells and the medium was replaced at, at least, 3-day intervals. No ACV-or PCV-resistant virus was observed. Stock virus (at least 6000 PFU) of the three virus strains was found not to contain any detectable ACV-resistant virus under these experimental conditions.

3.2. Comparison of the frequency of emergence of drug-resistant viruses

The cells were infected with 1000 PFU of cell-free virus from the virus stock and the cultures were successively exposed to increasing concentration of ACV or PCV (2, 4 and 6 μg/ml). The infected cultures were subcultured while keeping the infected cells spreading before the destruction of the cell cultures (by the cytopathic effect). Finally, viruses were selected from the infected cultures that were treated with 6 μg/ml of ACV or PCV. Table 1 shows the results of the 12 experiments using Kawaguchi strain and Oka varicella vaccine strain as well as a clinical isolate. In 11 out of 12 experiments, resistant virus was selected only from the cultures treated with ACV. In experiment 8, where ACV was increased gradually

to 2, 4 and 6 μg/ml, PCV was used at lower concentrations of 1, 3 and 6 μg/ml. Even under these conditions, resistant virus was selected only from the ACV-treated culture (Table 1). In the 12 experiments, PCV-resistant virus was not selected. The difference in the emergence frequency of viruses resistant to ACV and PCV was statistically significant by the Fisher's exact test ($P < 0.0001$). When 5 PFU of TK-deficient strain (AR-2 in Table 2) was mixed with the inoculum, drug-resistant viruses were observed in both ACV- and PCV-treated cultures (Table 1). The seven ACV-resistant strains obtained from ACV-treated cultures from the five repeated experiments using Kawaguchi strain, the two ACV-resistant strains obtained from the Oka vaccine strain and the two ACV-resistant strains obtained from the clinical isolate were characterized further.

3.3. Susceptibilities of all virus variants to antiviral agents

Table 2 summarizes the susceptibilities, expressed as the mean EC_{50} calculated from more than three experiments, of the 11 ACV-resistant variants to the four antiviral drugs. AR-1a and AR-1b, AR-2, AR-3, AR-4, AR-5a and AR-5b, AR-9a and AR-9b, AR-11a and AR-11b were selected from experiments 1, 2, 3, 4, 5, 9 and 11, respectively (Table 1). The 11 variants lost sensitivity to ACV, PCV and BVaraU, while the parent strains were susceptible (Table 2). Although the EC_{50} of ACV and PVC for the 11 variants was rather low, this may have been influenced by the assay. Resistance of the 11 variants to BVaraU was rather high. This is characteristic of TK-deficient VZV (Machida and Sakata, 1984 Machida, 1986 Shiraki et al., 1990). Also, the 11 variants were as susceptible to the polymerase-dependent antiviral agent PAA as the parent strains. This indicated that the 11 variants were resistant to ACV, PCV and BVaraU as a result of a mutation in the TK gene. This was verified by the TK assay and sequencing.

3.4. TK activity of ACV-resistant viruses

Table 3 shows the TK activity and the amount of VZV antigen in the enzyme extracts from cells infected with the parent strains and 11 ACV-resistant variants. The amounts of VZV antigen in cells infected with ACV-resistant variants were similar to those in cells infected with the parent strains as determined by ELISA (Table 3). The TK activities of the 11 ACV-resistant variants were far less than the activities expected from the amounts of VZV antigen in

Table 3
TK activity induced in cells infected with ACV-resistant strains

Strains	TK activity (%)[a]	Amount of VZV antigen (%)[b]
Experiment 1		
Kawaguchi strain	100	100
Uninfected cells	3	0
AR-1a	1	119
AR-1b	3	110
AR-2	2	122
AR-3	4	119
AR-4	3	74
AR-5a	1	156
AR-5b	2	58
Experiment 2		
Oka vaccine strain	100	100
Uninfected cells	16	0
AR-9a	7	108
AR-9b	5	106
Experiment 3		
Clinical isolate	100	100
Uninfected cells	5	0
AR-11a	7	118
AR-11b	5	86

[a] The mean TK activity of eight samples is expressed as the per cent activity of cells infected with the parent strains (Kawaguchi strain, $38\,528 \pm 7559$ dpm; Oka vaccine strain, 9799 ± 1540 dpm; clinical isolate, $40\,962 \pm 3493$ dpm).

[b] The amount of VZV antigen in the TK enzyme extracts was assessed by ELISA and determined by optical density (OD_{450}). The optical density of the uninfected cells was subtracted from that of the infected cells. The mean of more than three samples is expressed as per cent of parent strain-infected cells (Kawaguchi strain, 0.139 ± 0.051; Oka vaccine strain, 0.147 ± 0.015; clinical isolate, 0.159 ± 0.009).

Table 4
Sequence changes in the TK gene of ACV-resistant variants of Kawaguchi strain[a]

Strains	Nucleotide change[b]	Terminator codon[b]	X site
AR-1	376 (*del*:A)-377 (*del*:T)	163	
AR-2	72 (*del*:A)	38	5′-GG-X-3′
AR-3	72(*del*:A)	38	5′-GG-X-3′
AR-4	72 (*del*:A)	38	5′-GG-X-3′
AR-5	950 (*del*:C)	329	5′-X-CC-3′

[a] *del*, deletion.
[b] Nucleotide change in the TK gene was numbered from the initiation codon of A and the termination codon following frameshift was numbered from the initiation codon.

the enzyme extracts compared with those of the parent strains. Thus, the results indicated that the 11 ACV-resistant variants failed to induce viral TK activity and, therefore, could be considered as TK-deficient.

3.5. Analysis of DNA sequence of the TK gene

The open reading frame of the VZV TK gene is 1023 nudeotides long and encodes for a protein of 341 amino acids (Davison and Scott, 1986). The proposed ATP- and nucleoside-binding sites are defined by amino acids 12–29 and 129–145, respectively. The TK gene of the seven ACV-resistant variants of Kawaguchi strain and the parent Kawaguchi strain was sequenced. A direct comparison of the TK gene sequences of these virus strains to that of the Dumas strain in GenBank showed that each of the strains had a common S288L substitution (Mori et al., 1988 Sawyer et al., 1988 Lacey et al., 1991 Talarico et al., 1993). Our strains also had a nucleotide (C→T) change that would not result in an amino acid substitution, at position 183 in the parent Kawaguchi strain (Mori et al., 1988). The TK gene of the seven ACV-resistant variants contained either a single-nucleotide or double-nucleotide deletion (Table 4). Subsequently, these frameshift mutations created premature terminator codons in the TK gene and resulted in the expression of truncated TK molecules. The nucleotide deletions found in AR-1a and AR-1b as well as in AR-5a and AR-5b were identical, indicating that these pairs of variants could be considered as identical. Therefore, they were designated as AR-1 and AR-5, respectively (Table 4). The deletions observed were downstream from two sequential guanosine nucleotides (5′-GG-X-3′) in three out of the five ACV-resistant variants (AR-2, AR-3, AR-4). In one out of the five ACV-resistant variants (AR-5), the deletion occurred upstream from two sequential cytidine nucleotides (5′-X-CC-3′). The site for 5′-GG-X-3′ and 5′-X-CC-3′ of the TK DNA sequence (Kawaguchi strain) corresponded to nucleotides 72 and 950, respectively. Thus, these ACV-resistant strains contain a unique mutation in the TK gene.

4. Discussion

VZV is a highly cell-associated virus (Shiraki and Takahashi, 1982), and plaques are formed even without agarose or methylcellulose overlay. The cultures infected with herpes simplex virus (HSV) or cytomegalovirus, and treated with the antiviral drug at a concentration which permits partial virus growth, are completely destroyed by the cytopathic effect of the released viruses. In contrast, few variants of VZV formed in presence of the antiviral drugs at a concentration which permits partial virus growth are conserved in the culture without complete destruction by the replicated virus. Therefore, a minor population of variants of VZV can be obtained by the highly cell-associated nature of VZV infectivity.

Both ACV and PCV exhibit anti-VZV activity in a similar way: phosphorylation by viral TK and inhibition of viral DNA synthesis. ACV and PCV give similar levels of plasma concentration following intravenous administration of therapeutic doses to patients, and oral administration of therapeutic doses of ACV valaciclovir and famciclovir achieve similar plasma levels of ACV and PCV, respectively (Brigden and Whiteman, 1985 Pue and Benet, 1993) In our previous study, we found that the anti-VZV activity of ACV and PCV in vitro is similar as assessed by their EC_{50} values (Hasegawa et al., 1995). Based on similar pharmacokinetic plasma concentrations and anti-VZV activity, we used the same increasing con-

centrations of ACV and PCV to examine susceptibility of virus growth and the emergence of resistant viruses to either drug using three virus strains (Kawaguchi strain, Oka varicella vaccine strain, a clinical isolate). Interestingly, TK-deficient virus emerged in ACV-treated cultures, but not in PCV-treated cultures.

We have used a six-time plaque-purified Kawaguchi strain using cell-free virus with virus stock completion within six passages. It is possible that this virus stock contained ACV-resistant virus. However, it is difficult to envisage the presence of at least three kinds of mutations in the TK gene (Table 4) in this homogeneous virus stock. Moreover, ACV-resistant virus selected in this study was also resistant to PCV (Table 2), and it is difficult to imagine why TK-deficient virus in the virus stock would only propagate in ACV-treated cultures.

When the TK-deficient virus was mixed with the original virus inoculum, resistant virus was observed in both ACV- and PCV- treated cultures, as shown in Table 1. The fact that resistant virus could be isolated following from both drug treatment regimes, corroborates the absence of ACV-resistant virus in the virus stock.

We have performed an additional experiment to exclude the possibility of pre-existing ACV- or PCV- resistant virus in the virus stock. The Kawaguchi strain, the Oka vaccine strain and the clinical isolate were inoculated into dishes at 1000 PFU per dish and incubated in the presence of 6 μg/ml of ACV or PCV for more than 6 days. Then the infected cells were further subcultivated in the presence of 6 μg/ml of ACV or PCV. However, despite 12 days of culture, neither ACV- nor PCV-resistant virus was observed.

In this study, resistant viruses emerged in the ACV-treated cultures and not in the PCV-treated cultures when the infected cultures were successively treated with increasing concentrations of the drugs. This may be due to differences in the modes of anti-VZV action between ACV and PCV. Both ACV and PCV are phosphorylated by the viral TK, and ACV is more efficiently incorporated into viral DNA than PCV is (Vere Hodge and Cheng, 1993 Bacon et al., 1996). However, PCV is a better substrate for viral TK than ACV (Earnshaw et al., 1992). PCV triphosphate (TP) is formed at higher levels in infected cells than ACV triphosphate (TP) and the former maintains its intracellular concentrations for longer than the latter (Bacon et al., 1996). The half-life of PCV TP is longer than that of ACV TP in infected cells (Darby, 1994). PCV TP has a prolonged in vitro intracellular half-life (7–14 h) in VZV-infected cells. In contrast, the in vitro intracellular half-life of ACV-TP is substantially shorter (0.8 h) in VZV-infected cells (Crumpacker, 1996). Thus the difference in the half-life of PCV TP and ACV TP may cause a difference in the selective pressure on VZV replication between the PCV and ACV treatments. We have reported that in spite of similar EC_{50} values in the plaque reduction assay, the effective concentrations for 50% reduction of the number of infected cells per plaque are 1.40 and 5.00 μg/ml for PCV and ACV, respectively, and that PCV is more effective in suppressing the spread of infection than ACV, when the assay period is longer (Hasegawa et al., 1995). Thackray and Field (1996) have shown that famciclovir reduces latent virus in infected mouse ganglia more efficiently than valaciclovir. Thus, PCV might suppress virus growth more strongly than ACV, and this might permit the selection of TK-deficient virus only in ACV-treated cultures.

We have determined the mutations of five ACV-resistant variants of Kawaguchi strain in the TK gene. In four out of five ACV-resistant variants, the mutations were at the X site of 5′-GG-X-3′ or 5′-X-CC-3′. Such X sites of 5′-GG-X-3′ and 5′-X-CC-3′ are present in 124 sites out of 1023 nucleotides in the TK DNA sequence (Kawaguchi strain). Although the number of ACV-resistant viruses examined was limited, statistical analysis by exact binominal probability showed that the deletions occurred principally at the X sites of 5′-GG-X-3′ and 5′-X-CC-3′ (exact $P = 0.00095$). We have extended this analysis to the clinical isolates of ACV-resistant VZV in previously reported studies as shown in Table 5 (Sawyer et al., 1988 Talarico et al., 1993 Boivin et al., 1994). Mutational changes at the nucleotide at the X site of 5′-GG-X-3′ and 5′-X-CC-3′ were found in six out of 21 ACV-resistant clinical VZV strains isolated from patients following long-term treatment

Table 5
Nucleotide changes in the TK gene of the reported clinical ACV-resistant isolates of VZV[a]

Reference	Strains	Nucleotide change	X site
Sawyer et al., 1988	VZV 7-1-3	389 (G→A)	
Talarico et al., 1993	V8807-3	681 (*del*:A)-682 (*del*:C)	
	V8811-4	76 (*del*:A)[b]	5′-GG-X-3′
	V9007-1	47-50 (*del*:ATTT)	
	V9012-4	681 (4el:A)-682 (*del*:C)	
	V8808-4	412 (T→C); 725 (C→T)	
	V8812-1	427 (A→G)	
	V8812-5	385 (G→A)	
	V8901-1	74 (A→G)	
	V8919-1	428 (G→A)	
	V9006-1	176 (A→G)	5′-GG-X-3′
Boivin et al., 1994	2A	71 (G→A)	
	3B	677 (*del*:A)-678(*del*:C)	
	5C	922 (G→C); 412 (T→C)	
	6D	682(*del*:C)-683(*del*:T)	
	8E	72 (*del*:A)	5′-GG-X-3′
	10G	493 (*add*: C)	5′-X-CC-3′
	11H	889(*add*:T)-890(*add*:C)	
	12H	72 (*add*: A)	5′-GG-X-3′
	16I	493 (*del*:C)	5′-X-CC-3′
	17J	987-990 (*add*:GAAA)	

[a] *del*, deletion; *add*, addition.
[b] A-76 deletion was regarded as identical to A-72 deletion, because both of them had adenosine homopolymer stretches between nucleotides 72 and 76.

with ACV. According to Davison and Scott (1986), such X sites are present in 125 sites out of 1023 nudeotides in the TK DNA sequence, statistical analysis by exact binominal probability showed that the mutations significantly occurred at the X site of 5′-GG-X-3′ and 5′-X-CC-3′ (exact $P = 0.026$).

Three of five ACV-resistant variants of the Kawaguchi strain possessed the same mutation (deletion of A-72) in the TK gene. This site could be a hot spot for mutation as there was no detectable ACV-resistant virus in the inoculum, the three strains were selected independently, and each experiment was performed at a different time point to avoid cross-contamination of virus. Mutation at this site of the reported clinical ACV-resistant isolates is observed in one or two of the ten isolates with TK mutation (Table 5) (Talarico et al., 1993 Boivin et al., 1994).

We have extended the analysis of such X sites of 5′-GG-X-3′ and 5′-X-CC-3′ to the clinical isolates of ACV-resistant HSV in the previously reported studies. Mutations at such X sites of clinical isolates of ACV-resistant HSV type 1 and type 2 were found in one out of two isolates, and eight out of 12 isolates, respectively (Kit et al., 1987 Chatis and Crumpacker, 1991 Nugier et al., 1991 Palu et al., 1992 Hwang et al., 1994 Sasadeusz et al., 1997).

Sasadeusz et al. (1997) have demonstrated that the majority of ACV-resistant HSV-2 clinical isolates contain frameshift mutations within two long homopolymer nucleotide stretches (seven G residues between nucleotides 433 and 439, six C residues between nudeotides 550 and 555) which function as hot spots for mutation within the HSV TK gene. Similarly, we have observed mutations at the sites next to the successive GG sequence in the TK gene of VZV, and they are widely distributed throughout the TK gene (Sasadeusz et al., 1997). Thus, it is interesting that mutations selected for VZV following ACV treat-

ment in this study were similar to those observed previously for HSV, although the length of the G stretches was shorter.

TK-deficient variants selected in ACV treatment may not be unique to ACV, and similar variants may be selected when PCV treatment permits continuous virus growth for a prolonged period as ACV treatment did in this study. Conventional treatment of VZV infection with ACV has been successful and has not induced ACV-resistant VZV at all (Ozaki et al., 1998). However, if inappropriate ACV treatment permits partial virus growth in the lesions as observed in this study, ACV-resistant viruses could be selected from immunocompromised patients undergoing prolonged treatment. This study indicates that appropriate ACV or PCV treatment, which inhibits virus growth completely, would avoid the appearance of drug-resistant virus, especially when immunocompromised hosts are submitted to prolonged anti-VZV therapy.

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