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# SUSCEPTIBILITIES OF PHOSPHONOACETIC ACID AND ACYCLOVIR RESISTANT VARICELLA-ZOSTER VIRUS MUTANTS TO 9-β-ARABINOFURANOSYLADENINE AND 1-β-ARABINOFURANOSYLCYTOSINE

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SUMMARY The DNA polymerase activity, and susceptibilities to 9-β-D-arabinofuranosyladenine(ara-A) and 1-β-arabinofuranosylcytosine(ara-C) of a phosphonoacetic acid resistant mutant (PAA-R) of varicella-zoster virus (VZV) selected in the presence of PAA were examined. The DNA polymerase activity of PAA-R was inhibited less than that of the parent strain by PAA in vitro. PAA-R was resistant to acyclovir and also to both ara-A and ara-C. The susceptibilities to ara-A and ara-C of four acyclovir resistant mutants selected in the presence of acyclovir, and also resistant to PAA, were examined. Two variants were resistant, one was slightly resistant, and one was sensitive to both drugs. These cross-resistances and susceptibilities of VZV variants to PAA, ACV, ara-A and ara-C should be considered in chemotherapy of VZV infections.

## INTRODUCTION

Several potent and selective anti-herpesvirus drugs with actions that depend on virus-specified enzyme activities have been recently developed. These drugs can be classified into two categories: one related to thymidine kinase (TK), and the other to DNA polymerase. Variants with different susceptibilities to drugs have been isolated and characterized in many laboratories. Studies of resistant mutants have provided valuable informations about the mechanisms of drug resistance (Larder and Darby., 1984). Acyclovir (ACV)-resistance in herpes simplex virus (HSV) has been explained by mutations of the TK and DNA polymerase genes (Coen and Schaffer, 1980;

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Coen et al., 1982; Larder and Darby, 1984; Schnipper and Crumpacker, 1980). We have isolated phosphonoacetic acid (PAA)-resistant (PAA-R) and ACV-resistant mutants and shown that some of the ACV resistant variants were also resistant to PAA (Shiraki et al., 1980). The DNA polymerase activity induced by VZV has been reported to be selectively inhibited by PAA (Mar et al., 1978; Miller and Rapp, 1977). In the present study, we first compared the inhibitions by PAA of DNA polymerases induced by parent and PAA-R viruses to determine the mechanism of resistance of VZV to PAA. The mechanism of the antiviral activity of ara-A is not clear, but it has been reported that mutation of the viral DNA polymerase gene can confer resistance to ara-A in HSV (Coen et al., 1982; Eriksson and Oberg, 1979). The sensitivities to ara-A and ara-C of VZV variants resistant to PAA and ACV were examined to obtain clues to the sites of action of these drugs and also of the actions of these drugs in chemotherapy of varicella-zoster virus infections.

## MATERIALS AND METHODS

### 1. *Cells*

Human embryonic lung (HEL) cells were propagated in a mixture of equal volumes of Eagle's minimal essential medium (MEM) and medium 199, supplemented with 10% calf serum and were maintained in the same medium with 3% calf serum.

### 2. *Viruses*

The Kawaguchi strain of varicella-zoster virus (VZV) was propagated in HEL cells and cell-free virus was plaque-purified 6 times and stocked as cell-free virus in SPGC medium (Asano and Takahashi, 1978) at −70 C. Twenty-three other wild strains of VZV isolated from varicella patients were used for comparison.

### 3. *Isolation of drug-resistant mutants*

The isolation of drug-resistant mutants was described previously (Shiraki et al., 1983). Briefly; cell-free virus was inoculated onto HEL cells and after appearance of the 50% cytopathic effect (CPE), infected cells were inoculated onto uninfected HEL cells at a ratio of 1:5 and maintained in the presence of a drug. A PAA-resistant mutant (PAA-R) was isolated by passage of the virus in increasing concentrations of the drug (10 $\mu g$, 20 $\mu g$, 50 $\mu g$, 75 $\mu g$ per ml) with 3 passages in each concentration until the appearance of CPE. When 50% of the monolayer showed CPE, the infected cells were washed once with ice-cold phosphate buffered saline (PBS), treated with 0.1% EDTA in PBS and sonicated in SPGC medium. The sonicate was centrifuged at 2,000 g for 10 min at 4 C and the supernatant was used for plaque purification. After plaque-purification twice, the obtained virus was used as PAA-R. ACV-resistant mutants were isolated in presence of increasing concentration of the drug (20 $\mu$M, 50 $\mu$M, and 100 $\mu$M) by 3 passages at each concentration until the appearance of CPE. After plaque purification twice in the presence of 100 $\mu$M of ACV, the mutants were used as ACV-resistant mutants (ACV-R (A1) to (A4)).

PAA was purchased from ICN Pharmaceuticals Inc. (Plainview, N.Y.) and ara-C from Sigma Chemical Co. (St. Louis, Mo), and ACV and ara-A were gifts from Japan Wellcome Co. (Mino, Osaka, Japan).

### 4. *Measurement of sensitivity of virus to drugs*

Monolayers of HEL cells in 60 mm plastic petri dishes were inoculated with 100 plaque forming units (PFU)/dish of virus in 0.2 ml SPGC medium. After incubation for 1 h to permit absorption, 5 ml of maintenance medium and the required concentration of drug were added. The drug concentrations used were 5, 10, 25, 50 and 75 $\mu g$ of PAA/ml, and 0.005, 0.01, 0.1, 1 and 2 $\mu$M of ara-C. After the appearance of CPE, the cells were fixed and stained and plaques were counted. The effective doses for 50% plaque reduction ($ED_{50}$) was determined from plots of reduction in plaque number against the log of the drug concentration in $\mu g$/ml or $\mu$M. Experimental errors were within 15%.

### 5. *Preparation and assay of viral DNA polymerase*

When more than 70% of the monolayer cells showed CPE (about 48 h after infection), the cells were harvested in EDTA-PBS and washed three times with Tris buffered saline (TBS); 25 mM Tris-HCl pH 8.0 with 100 mM KCl). Cells were treated with TBS containing 0.5% Nonidet P40 (Sigma Chemical Co.) and centrifuged at 2,000 g

for 10 min at 4 C. The pellet (nuclear fraction) was suspended in column buffer (25 mM Tris-HCl pH 8.0, 100 mM KCl, 5 mM 2-mercaptoethanol, 20% glycerol (V/V) (Coen and Schaffer, 1980), at a concentration of $10^7$ cell/ml, and sonicated in an ice bath for 3 min. The extract was centrifuged at 100,000 g for 60 min at 4 C, and part of the supernatant was absorbed with an equal volume of the $\gamma$-globulin fraction of anti-HEL-cell serum overnight at 4 C and dialyzed against the column buffer. The absorbed and unabsorbed extracts were used as crude enzyme extracts. Actively growing uninfected HEL cells were treated in exactly the same way to prepare cellular DNA polymerase.

The reaction mixture in a final volume of 250 $\mu$l contained 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 $\mu$g of bovine serum albumin, 50 $\mu g$ of activated calf thymus DNA, 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 0.01 mM dTTP, 1.5 $\mu$Ci of $^3$H-dTTP (109 Ci/mmol) and 75 $\mu$l of enzyme extract. The reaction was conducted at 37 C for 30 min and terminated by adding trichloroacetic acid. The precipitated material was collected on glass filter paper and its radioactivity was counted. The incorporation of radioactivity was linear with time for at least 1 h under the conditions used.

For inactivation of DNA polymerase by antiserum, the enzyme extract was mixed with $\gamma$-globulin fraction of antiserum and incubated overnight at 4 C. The mixture was then assayed for enzyme activity as described above.

6. *Phosphocellulose column chromatography*

A 10 ml column of base- and acid-washed phosphocellulose (Whatman P-11) was equilibrated with column buffer (25 mM Tris-HCl (pH 8.0), 100 mM KCl, 5 mM 2-mercaptoethanol, 20% glycerol (V/V)) and washed with 10 mg of bovine serum albumin dissolved in column buffer. The bound DNA polymerase was eluted with 90 ml of a linear gradient of 0.1 to 1.0 M KCl in the column buffer. The KCl concentration was determined by measurement of conductivity.

Inhibition of viral DNA polymerase activity by PAA was measured using the peak fraction of activity, which was identified as viral with anti-VZV serum. Measurements were made at the indicated concentration of PAA in salt-enhanced condition.

7. *Anti-HEL-cell serum and anti-VZV serum*

Anti-HEL-cell serum was obtained by repeated injections of sonically disrupted uninfected HEL cell extract together with Freund's complete adjuvant into rabbits. Anti-VZV serum was prepared in African green monkeys by repeated injections of VZV-infected monkey kidney cell extract with Freund's complete adjuvant. The neutralizing antibody titer of the serum to VZV was 1:1280 (Yamanishi et al., 1980). Both sera was dialyzed against column buffer before use.

## RESULTS

### 1. *DNA polymerase activity induced by VZV and its inhibition by PAA*

The enzyme extract from HEL cells infected with the parent Kawaguchi strain was applied to a phosphocellulose column and bound DNA polymerase activity was eluted with a linear gradient of 0.1 to 1.0 M KCl as described in the Materials and Methods. DNA polymerase activity in each fraction was assayed in the presence or absence of 100 mM $(NH_4)_2SO_4$ (Fig. 1a). The main peak was eluted with 0.32 M KCl and with 0.44 M KCl in the presence of $(NH_4)_2SO_4$, in the absence of $(NH_4)_2SO_4$ respectively. Since salt enhances the viral DNA polymerase activity and inhibits the cellular activity (Miller and Rapp, 1977), the peaks at 0.32 M and 0.44 M KCl were thought to represent viral and cellular $\beta$-DNA polymerase, respectively. The activity corresponding to $\alpha$-DNA polymerase, which is thought to be eluted at about 0.28 M KCl, was not observed in the present experiment since the nuclear fraction of infected cells was used as an enzyme extract (Miller and Rapp, 1977).

For further characterization of the induced DNA polymerase, the effect of antiserum on DNA polymerase activity was examined. Anti-VZV serum reduced the activity eluted at 0.32 M KCl to 13% of that measured in the presence of preimmune serum. Anti-HEL cell-serum reduced the activity eluted at 0.44 M KCl to 9% of that measured in the presence of preimmune serum. In addition, when the enzyme extract was absorbed with anti-HEL-cell serum before chromatography, the peak

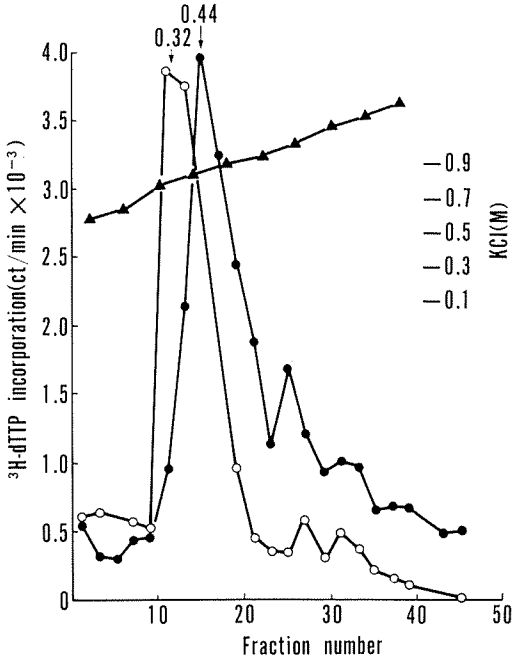


FIGURE 1.a *Phosphocellulose chromatography of the DNA polymerase of VZV-infected HEL cells. Sample (75 μl) of each fraction of elute were assayed for DNA polymerase activity in reaction mixture without added salt (●–●) and with 100 mM-$(NH_4)_2SO_4$ (○–○). The KCl concentration was determined by conductivity measurement (▲–▲).*

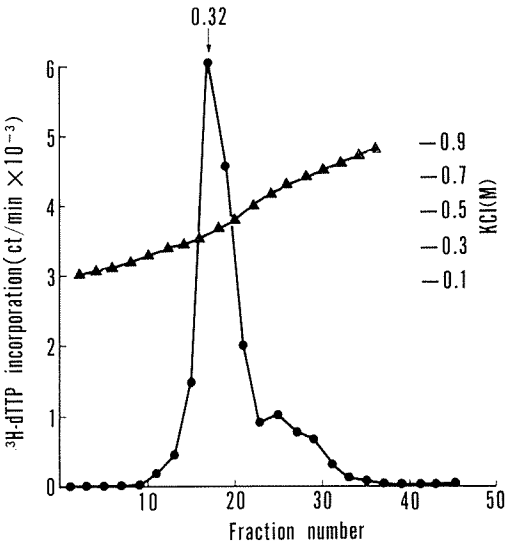


FIGURE 1.b *Phosphocellulose chromatography of the DNA polymerase of VZV-infected HEL cells. The enzyme extract was preincubated with anti-HEL-cell serum overnight at 4 C, dialyzed against the column buffer, and applied to the phosphocellulose column. DNA polymerase activity with added salt (●–●); KCl concentration (▲–▲).*

of activity at 0.32 M KCl was the same as that of the untreated preparation (Fig. 1b). These immunological findings support the conclusion that the activities at 0.32 M and 0.44 M KCl are viral and cellular DNA polymerase, respectively. The elution pattern of DNA polymerase activity from PAA-R-infected HEL cells and the effects of antisera on the activity were similar to those with enzyme from the parent strain.

The DNA polymerase activities induced by the parent and PAA-R strains were assayed in the presence of various concentration of PAA (Table 1). The enzyme preparation used in this experiment was the 0.32 M KCl fraction from the phosphocellulose column of anti-HEL-cell serum treated cell extract and the activity was assayed under salt enhanced conditions. The DNA polymerase activity of PAA-R-infected cells was inhibited far less than that of cells infected with the parent strain: the enzyme activity of the parent strain in the presence of 2 μg/ml of PAA was about 40% of that in the absence of PAA. The corresponding figure for the PAA-R strain was 97%, and 60% of the activity of the PAA-R strain remained even in the presence of 1,000 μg/ml of PAA.

## 2. *Susceptibilities of PAA-resistant variants to ara-A and ara-C*

The $ED_{50}$ values of ara-A and ara-C of various VZV strains are shown in Table 2. The $ED_{50}$ of ara-A for the parent Kawaguchi strain was 1.2 μM, while that for the PAA-R strain was 34 μM. The mean $ED_{50}$ value of ara-A for 24 wild strains of VZV was 3.14 μM (range 1.2 to 6.5; data not shown). The $ED_{50}$

TABLE 1. *Inhibition of VZV-induced DNA polymerase activity by PAA*

| Strain | DNA polymerase activity at various concentration of PAA ($\mu$g/ml) | | | | |
|---|---|---|---|---|---|
| | 0 | 2 | 15 | 150 | 1,000 |
| Parent strain | 5,778 (100)[a] | 2,212 (38.3) | 1,891 (32.7) | 1,533 (26.5) | 1,122 (19.4) |
| PAA-R strain | 3,816 (100) | 3,696 (96.9) | 4,007 (105) | 2,906 (76.2) | 2,396 (62.8) |

Extract from infected cells were treated with anti-HEL-cell serum and subjected to phosphocellulose column chromatography.
The fraction eluted with 0.32 M KCl was used as the enzyme preparation.
Enzyme activity was assayed in the presence of 100 mM $(NH_4)_2SO_4$.
[a] Figures in parenthese indicate percentages of the activity without PAA.

TABLE 2. *Drug susceptibilities of PAA- and ACV- resistant variants*

| Strain | $ED_{50}$ of Drug[a] | | | |
|---|---|---|---|---|
| | PAA[b] ($\mu$g/ml) | ara-A ($\mu$M) | ara-C ($\mu$M) | ACV[b] ($\mu$M) |
| Parent | 5.2 | 1.2 | 0.046 | 4.6 |
| PAA-R | 75 | 34 | 1.0 | >100 |
| ACV-R (A1) | 24 | 7.9 | 0.077 | >100 |
| ACV-R (A2) | >50 | 37 | 0.46 | >100 |
| ACV-R (A3) | >50 | 46 | 1.05 | >100 |
| ACV-R (A4) | 44 | 1.3 | 0.013 | >100 |

[a] See materials and methods for details. Figures are averages of values in three separate experiments.
[b] These data were reported previously (Shiraki et al., 1983).

of ara-C for the parent Kawaguchi strain was 0.046 $\mu$M, while that for PAA-R was 1.0 $\mu$M. The mean $ED_{50}$ of ara-C for 9 wild strain was 0.044 $\mu$M (range 0.022 to 0.075 $\mu$M, data not shown). Thus the PAA-R strain was cross-resistant to both ara-A and ara-C.

Another 4 PAA resistant variants, which were originally isolated as ACV-resistant variants and named ACV-R (A1), ACV-R (A2), ACV-R (A3) and ACV-R (A4), were tested for resistance to ara-A and ara-C. All of them could induce viral thymidine kinase and their DNA polymerase genes were thought to be mutated (Shiraki et al., 1983). The ACV-R (A2) and ACV-R (A3) strains were resistance to both drugs. ACV-R (A1) was slightly resistant and ACV-R (A4) was sensitive to both drugs. The susceptibility to ara-A was parallel with that to ara-C in this study, suggesting that ara-A and ara-C have a common sites of action.

## DISCUSSION

Phosphonoacetic acid (PAA) is an effective inhibitor of growth of herpes simplex virus (HSV) and its effect is thought to be the consequence of its direct action on DNA polymerase (Hay and Subak-Sharpe, 1976; Honess and Watson, 1977). Replication of VZV was also inhibited by PAA and VZV-induced DNA polymerase activity was shown to be inhibited more than the cellular enzyme activity by PAA (Mar et al., 1978; Miller and Rapp, 1977). For comparison of the properties of PAA-R polymerase with those of the parent virus polymerase, the viral DNA polymerases were separated from cellular ones by phosphocellulose column chromatography. The DNA polymerase activities of HEL cells infected with the parent and PAA-R strains were both eluted at 0.32 M KCl and 0.44 M KCl when assayed in the presence and absence of $(NH_4)_2SO_4$, respectively. This elution profile is consistent with that reported previously (Miller and Rapp, 1977). Although

the purification of DNA polymerase by phosphocellular column chromatography is not complete, the activity eluted at 0.32 M KCl was regarded as virus-specific DNA polymerase activity while, that eluted at 0.44 M KCl was thought to be cellular $\alpha$-DNA polymerase, because the nuclear fractions of infected HEL cells were used as starting materials (Miller and Rapp, 1977). PAA at 2 $\mu g$/ml was found to inhibit more than 60% of the enzyme activity induced by the parent strain activity, but only about 3% of the enzyme activity induced by PAA-R. The $ED_{50}$ values of PAA for the parent and PAA-R strains viruses were 5.2 $\mu g$/ml and more than 75 $\mu g$/ml, respectively. These in vitro susceptibilities to PAA of the DNA polymerases induced by the parent and PAA-R strains correlated well with the susceptibilities of in vivo replications of the parent and PAA-R viruses, strongly suggesting that DNA polymerase of VZV confers the PAA-R strain with PAA-resistance.

Viral DNA polymerase is the target of various antiherpetic agents (Larder and Darby, 1984). PAA-R was shown to be cross-resistant to ara-A, ara-C and ACV which are known to have DNA polymerase as their target. Nishiyama et al., (1984) found that a PAA-resistant mutant of HSV-2 was also resistant to ara-A, ara-C and ACV. Coen et al., (1982) reported that ara-A resistance and PAA resistance markers of HSV-1 behaved as if they were clearly linked during marker transfer. However, not all PAA-resistant mutants of HSV are ara-A resistant (Coen et al., 1982). Moreover, all PAA-resistant mutants of HSV were cross-resistant to ACV, but not all ACV-resistant mutants of HSV were cross-resistant to PAA (Larder and Darby, 1984). We need to examine more PAA-resistant mutants of VZV besides PAA-R for further clarification of the relation between different drug resistant mutants of VZV. We previously isolated ACV-resistant mutants of VZV and found that some of them were cross-resistant to PAA (Shiraki et al., 1983). In the present study, four of them were examined for cross-resistance to ara-A and ara-C. A2 and A3 were resistant, A1 was slightly resistant, and A4 was sensitive to both drugs. This evidence paralleled between the susceptibilities to ara-A and ara-C suggests that these two drugs act on a common site. Crumpacker et al. (1982) showed that the DNA regions containing resistant mutations for PAA, ara-A and ACV were closely linked and clustered in a 2.6 kilobase pair region of the HSV-1 genome between map units 40.2 and 41.8. For clarification of the mechanism of drug resistance of VZV, marker transfer and rescue experiments on drug resistant mutants of VZV will be necessary, though because of the cell-associated nature of VZV experiments using free-virus or viral DNA are very difficult.

## REFERENCES


Asano, Y. and Takahashi, M. 1978. Studies on neutralization of varicella-zoster virus and serological follow-up of cases of varicella and zoster. Biken J. 21: 15–23.

Coen, D. M. and Schaffer, P. A. 1980. Two distinct loci confer resistance to acycloguanosine in herpes simplex virus type 1. Proc. Nat. Acad. Sci. USA 77: 2265–2269.

Coen, D. M., Furman, P. A., Gelep, P. T. and Schaffer, P. A. 1982. Mutations in the herpes simplex virus DNA polymerase gene can confer resistance to 9-$\beta$-D-arabinofuranosyladenine. J. Virol. 41: 909–918.

Crumpacker, C. S., Schnipper, L. E., Kowalsky, P. N. and Sherman, D. M. 1982. Resistance of herpes simplex virus to adenine arabinoside and E-5-(2-bromovinyl)-2-deoxyuridine: a physical analysis. J. Infect. Dis. 146: 167–172.

Elion, G. B., Furman, P. A., Fyfe, J. A., Miranda, P. D., Beauchamp, L. and Scaffer, H. J. 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl) guanine. Proc. Nat.

Acad. Sci. USA 74: 5716–5720.
Eriksson, B. and Oberg, B. 1979. Characteristics of herpesvirus mutant to phosphonoformate and phosphonoacetate. Antimicrob. Agents Chemother. 15: 758–762.
Hay, J. and Subak-Sharpe, J. H. 1976. Mutants of herpes simplex virus type 1 and 2 that are resistant to phosphonoacetic acid induce altered DNA polymerase activities in infected cells. J. Gen. Virol. 31: 145–148.
Honess, R. W. and Watson, D. H. 1977. Herpes simplex virus resistance and sensitivity to phosphonoacetic acid. J. Virol. 21: 584–600.
Larder, B. A. and Darby, G. 1984. Virus drug-resistance: mechanisms and consequences. Antiviral Res. 4: 1–42.
Mar, E. C., Huang, Y. S. and Huang, E. S. 1978. Purification and characterization of varicella-zoster virus-induced DNA polymerase. J. Virol. 26: 249–256.
Miller, R. L. and Rapp, F. 1977. Varicella-zoster virus-induced DNA polymerase. J. Gen. Virol. 36: 515–524.
Nishiyama, Y., Suzuki, S., Yamauchi, M., Maeno, K. and Yoshida, S. 1984. Characterization of an Aphidicolin-resistant mutant of herpes simplex virus type 2 which induces an altered viral DNA polymerase. Virology 135: 87–96.
Schnipper, L. E. and Crumpacker, C. S. 1980. Resistance of herpes simplex virus to acycloguanosine: role of viral thymidine kinase and DNA polymerase loci. Proc. Nat. Acad. Sci. USA 77: 2270–2273.
Shiraki, K., Ogino, T., Yamanishi, K. and Takahashi, M. 1983. Isolation of drug resistant mutants of varicella-zoster virus: cross resistance of acyclovir resistant mutants with phosphonoacetic acid and bromdeoxyuridine. Biken J. 26: 7–23.
Takahashi, M., Okuno, Y., Otsuka, T., Osame, J., Takamizawa, A., Sasada, T. and Kubo, T. 1975. Development of a live varicella vaccine. Biken J. 18: 25–33.
Yamanishi, K., Matsunaga, Y., Ogino, T., Takahashi, M. and Takamizawa, A. 1980. Virus replication and location of varicella-zoster virus antigens in human embryonic fibroblast cells infected with cell-free virus. Infect. Immun. 28: 536–541.