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THYMIDINE KINASE WITH ALTERED SUBSTRATE SPECIFICITY OF ACYCLOVIR RESISTANT VARICELLA-ZOSTER VIRUS

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SUMMARY The acyclovir resistant mutant of varicella-zoster virus ACV-R (A 8) induced the same level of thymidine kinase activity in infected cells as the parent Kawaguchi strain. However, it induced less deoxycytidine kinase activity and did not induce phosphorylating activity for the nucleotide analogue, 9-(2 hydroxy-ethoxymethyl)-guanine-(acyclovir). Another acyclovir resistant mutant, ACV-R (A 4), which is cross-resistant to phosphonoacetate and is thought to be a viral DNA polymerase mutant, induced the same level of phosphorylating activities for thymidine, deoxycytidine and acyclovir as the parent strain. The altered substrate specificity of thymidine kinase induced by ACV-R (A 8) is concluded to confer resistance to acyclovir on ACV-R (A 8).

The nucleotide analogue 9–(2-hydroxy-ethoxymethyl)-guanine (acyclovir; ACV) has been shown to be a potent inhibitor of herpesvirus replication. Extensive studies on ACV have been carried out with herpes simplex virus (HSV) (Coen et al., 1980; Elion et al., 1977; Field et al., 1980; Schnipper et al., 1980). To be an effective inhibitor in infected cells, ACV has to be converted to its triphosphate, which disrupts virus DNA synthesis. Thymidine kinase (TK) coded by the virus, not the cell, is responsible for phosphorylation of ACV, and triphosphate ACV interferes selectively with viral DNA synthesis by inhibiting virus coded DNA polymerase more efficiently than the cell DNA polymerase. Thus two enzymes coded by the virus are closely related with the effectiveness of ACV, and mutations of these two virus genes, the TK and DNA polymerase genes, confer resistance to ACV. With regard to the TK gene, most of resistant strains acquire resistance by losing the ability for induction of TK, but another type of resistant strain that induces TK with altered substrate specificity has recently been recognized (Darby et al., 1981; Larder et al., 1983). Such mutants can phosphorylate thymidine (TdR) but not ACV. This type of mutant seems to be more important from the clinical viewpoint, because it may be as virulent as the wild type virus,

Key words: VZV, Thymidine kinase, ACV, altered substrate specificity

whereas the mutant that lacks TK is known to be relatively avirulent (Darby et al., 1980; Field and Darby, 1980; Tenser et al., 1979).

ACV inhibits varicella-zoster virus (VZV) replication (Biron and Elion, 1980). VZV is also known to induce virus specific TK and DNA polymerase (Hackstadt and Mallavia, 1978; Miller and Rapp, 1977; Ogino et al., 1977). ACV-resistant mutants of VZV are expected to appear in the same manner as those to HSV. We recently isolated eight ACV resistant mutants. Two of them were suspected to induce TK with altered substrate specificity (Shiraki et al., 1983). Biron et al. (1982) also isolated and preliminarily characterized ACV resistant mutants. In this work, we characterized an ACV-resistant strain of VZV (ACV-R (A8)) that was suspected from previouss studies to induce TK with altered substrate specificity, and compared it with the wild strain and another ACV-resistant strain ACV-R (A4). ACV-R (A4) induces TK with the same substrate specificity as that induced by the parent strain and has a mutation in DNA polymerase that confers resistance to ACV, and is cross-resistant to phosphonoacetic acid (Shiraki et al., 1983).

The parent VZV strain used was purified by six plaque transfers of the Kawaguchi strain (Shiraki et al., 1983). ACV resistant mutants were isolated by growing the parent virus in the presence of increasing concentrations of ACV and finally plaque purified in the presence of 100 μM of ACV. ED_{50} of ACV for parent, ACV-R (A4) and ACV (A8) are 4.6, >100, and >100 μM, respectively, and those of BUDR are 2.0, 1.45 and 1.6 μg/ml, respectively (Shiraki et al., 1982). After the appearance of cytopathic effects in more than 70% of the cells, human embryonic lung (HEL) cells infected with VZV were washed with phosphate buffered saline (PBS) and recovered after treatment with 0.1% EDTA in PBS. The cells were washed three times with PBS, suspended in 50 mM Tris-HCl (pH 8.0) containing 150 mM KCl and 3 mM 2-mercaptoethanol at a concentration of 5×10^6 cells/ml, and then sonicated in an ice bath. The sonicate was centrifuged at 100,000 g for 60 min at 4 C and the resulting supernatant was used as an enzyme preparation. The mixture for assay of TK in a final volume of 250 μl, contained 0.2 μCi of ^{14}C-TdR (57 mCi/mmol. Amersham), 5 mM ATP, 5 mM $MgCl_2$, enzyme extract, and 50 mM Tris-HCl (pH 8.0) to give a final volume of 250 μl. For assays of deoxcytidine kinase (CK) and ACV phosphorylating activities, 0.2 μCi of ^{14}C-deoxycytidine (CdR) (48.7 mCi/mmol. Amersham) and 0.2 μCi of ^{14}C-ACV (53/8 mCi/mmol, a gift from Japan Wellcome Co., Ltd.) respectively, were used, instead of ^{14}C-TdR. The mixtures were incubated at 38 C for 15 min and the reaction were stopped by immersing the mixtures in a boiling water bath for 2 min. The amount of phosphorylated radioactivity was determined by DEAE disc method (Ogino et al., 1977). The enzyme activities were linear under the conditions used.

The phosphorylating activities of parent, ACV-R (A4) and ACV-R (A8) infected HEL cell extracts were assayed with TdR, CdR, and ACV (Table 1). Both ACV-R (A4) and ACV-R (A8) induced viral TK like to parent strain. Previously we reported (Shiraki et al., 1983) that TK activities induced by parent, ACV-R (A4) and ACV-R (A8) were immunologically distinct from cellular one, and that the phosphorylation of TdR by ACV-R (A8)-induced TK was not inhibited by the addition of ACV, whereas the TKs induced by the parent and ACV-R (A4) strains were inhibited by ACV. Consistent with these previous results, ACV-R (A8) induced TK did not phosphorylate ACV, while the TKs induced by the parent and ACV-R (A4) strains did phosphorylate ACV. These findings indicate that ACV-R (A8) induced TK has different substrate specificity from TK induced by the parent strain. Biron et al. (1982) reported the isolation of ACV resistant mutants of VZV. Their mutants phosphorylated ACV less than the parent virus but they did not discriminate between altered substrate

TABLE 1. *Phosphorylating activities induced in human embryonic lung cells after infection with VZV (strain Kawaguchi)*

Enzyme	Thymidine[a]	Deoxycytidine[a]	Acyclovir[a]	ratio $\left(\frac{\text{Deoxycytidine}^a}{\text{Thymidine}^a}\right)$
Parent	17,788	23,273	2,105	1.31
ACV-R (A-4)	15,785	19,610	2,127	1.24
ACV-R (A-8)	16,911	6,719	ND[b]	0.40

Enzyme activity is presented as CPM/15 min at 38 C.
Enzyme extracts contained 200–300 μg of protein.
[a] The activity of an extract of control uninfected cells was substrated from that in each assay.
[b] Not detectable.

specificity or reduced TK activity. The resistance of ACV-R (A4) to ACV was concluded to be due to alteration in DNA polymerase from previous experiments (Shiraki et al., 1983) and in the present study ACV-R (A4)-induced TK was shown to phosphorylate ACV as effectively as TK induced by the parent strain.

TK induced by VZV has been shown to have a broad substrate specificity and to be able to use CdR as a substrate (Hackstadt Mallavia, 1978; Ogino et al., 1977). The TKs induced by the parent and ACV-R (A4) strains phosphorylated CdR, their ratios of CdR phosphorylation to TdR phosphorylation being 1.31 and 1.24, respectively (Table 1). ACV-R (A8), however, phosphorylated CdR less, its ratio of phosphorylation of CdR to that of TdR being 0.40. Because of the cell-associated nature of VZV, it is difficult to infect cells with different virus strains under the same conditions and to compare their specific enzyme activities. But the ratio of the phosphorylating activities with different substrates is meaningful. The results on CdR phosphorylation also support the conclusion that TK of ACV-R (A8) has altered substrate specificity, which confers resistance to ACV on ACV-R (A8). ACV-resistant HSV mutants with altered substrate specificity have also been shown to have reduced activities to phosphorylate CdR (Larder et al., 1983). These results coincide with De Clercq's hypothesis that ACV is recognized as substrate by CdR kinase rather than the TK activity associated with the virus-induced TK (De Clercq, 1982), but, as the mutants in our experiment were selected by gradually increasing drug concentrations, whether the reduced CdR phosphorylating activity and the loss of ACV phosphorylating activity are due to a single or multiple mutants cannot be concluded at present.

In this communication, we describe a TK mutant of VZV that induces TK with altered substrate specificity, which confers resistance to ACV. As ACV is reportedly useful in treatment of patients with varicella-zoster virus (Biron and Elion, 1980), it is important to investigate the nature of ACV resistant mutants. The majority of recently developed selective anti-herpes nucleotide analogues, including ACV, have fundamentally similar mode of action in that they require conversion to an active phosphorylated form (De Clercq, 1982). In other words, drug susceptibility is deeply related with the spectrum of substrate specificity of enzymes concerned. Therefore, substrate specificity of VZV-induced TK is important research subject for anti-VZV chemotherapy. A purified enzyme fraction must be used for further characterization of TK, and studies on TK using other nucleoside analogues or other mutated TKs should provide more useful information on antiviral chemotherapy and the mechanism of resistance.

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