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*Case Report*

# Characterisation of Penciclovir Resistant Acyclovir Sensitive Herpes Simplex Virus Type 2 Isolated From an AIDS Patient†,**

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A heterogeneous herpes simplex virus type 2 (HSV-2) population was characterised from an AIDS patient with relapsing genital ulcer. The isolate had an unusual antiviral spectrum, showing resistance to penciclovir and susceptibility to acyclovir. Two viral populations were plaque purified, one resistant and the other susceptible to both antiviral drugs. The resistant clone was deficient in thymidine kinase (TK) activity and a nucleotide substitution, thymine for cytosine, at position 153 was identified in its *TK* gene. This mutation resulted in an amino acid change, arginine to tryptophan, in the ATP binding site. In the deficient mutant, a loss of virulence was observed in mice. ***J. Med. Virol. 73:60–64, 2004.*** © 2004 Wiley-Liss, Inc.

**KEY WORDS:** thymidine kinase (TK); antiviral; mixed population

Herpes simplex virus type 2 (HSV-2), formally *Human herpesvirus* 2, member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Simplexvirus* is cause of common viral infections, and may produce serious diseases. Both acyclovir and penciclovir are analogues of the natural nucleoside deoxyguanosine, and they are potent and selective inhibitors of many members of the family *Herpesviridae* [Elion et al., 1977; Boyd et al., 1987]. Oral prodrugs of penciclovir (famciclovir) and acyclovir (valaciclovir) were subsequently developed to improve their oral bioavailability, although oral formulations of acyclovir are widely used. The mechanism of action of acyclovir and penciclovir against HSV implies an initial drug phosphorilation by viral thymidine kinase (TK). Acyclovir/penciclovir monophosphate is further phosphorilated by cellular enzymes to bi- and tri-phosphate form, which is a competitive inhibitor of viral DNA polymerase [Elion, 1982].

Although HSV infections have been treated with nucleoside analogues for over two decades, only 0.1–0.6% of immunocompetent persons show acyclovir resistance. Isolates obtained from immunocompromised patients show a lower susceptibility having an IC 50% greater than 2 μg/ml to acyclovir or penciclovir in 5–6% of the isolates [Christophers et al., 1998; Oram et al., 2000]. Viral resistance to acyclovir is related to one of the following mechanisms: complete loss of TK activity ($TK^-$), decreased activity of TK, altered substrate specificity TK, and altered substrate specificity DNA polymerase.

Characterisation of acyclovir-resistant HSV isolates has been carried out using laboratory and clinical isolates [Gaudreau et al., 1998]. Two active centres of the HSV-1 TK enzyme, including a nucleotide (ATP) binding site and a nucleoside binding site have been described [Darby et al., 1986]. Several mutations have been reported concerning resistant isolates; they included single point mutations as well as frameshift mutations within homopolymers of Gs and Cs [Gaudreau et al., 1998].

In this article, we studied a heterogeneous virus clinical isolate of HSV-2 with an unusual susceptibility pattern, showing resistance to penciclovir and susceptibility to both acyclovir and foscarnet.

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## MATERIALS AND METHODS

### Viruses

The HSV-2 clinical strain that exhibited resistance to penciclovir, but not to acyclovir, was isolated from a perianal ulcer of a male patient suffering from AIDS. He received three courses of acyclovir treatment to control his recurrent genital ulcer. He had never been treated with penciclovir or famciclovir; even more, he had never received acyclovir or penciclovir cream for genital or labial herpes simplex. The ulcer healed well after the first oral cycle 200 mg, five times a day during 10 days. A first recurrence also responded well to the same oral treatment. A second recurrence did not heal well with oral acyclovir alone, requiring 500 mg of acyclovir every 8 hr intravenously administered during 14 days, followed by oral acyclovir at 200 mg, five times a day for 10 days. The HSV-2 isolate corresponded to the second recurrence.

HSV-2 333, a TK-positive ($TK^+$) strain which TK's sequence is available from the National Library of Medicine (GenBAnk accession number M29942), was used as a control for genetic studies and TK assays. The HSV-1 TK-negative ($TK^-$) strain Δ305, carrying a 700 bp deletion in the *TK* gene [Post and Roizman, 1981], was used as a control for the TK assays and viral susceptibility assays.

### Virus Susceptibility Assays

Susceptibility studies to acyclovir (Glaxo-SmithKline, UK), penciclovir (Glaxo-SmithKline), and foscarnet (Astra-Zeneka, Portugal) were carried out in triplicate by plaque reduction assay onto Vero cell monolayers [Collins et al., 1982; Sibrack et al., 1982]. HSV-1 Δ305, a $TK^-$ strain, was used as a resistant control strain. Acyclovir resistance was defined by $IC_{50}$ values >2 μg/ml. Penciclovir resistance was defined by $IC_{50}$ values higher than 3.9 μg/ml, three times the mean $IC_{50}$ for penciclovir obtained with our HSV-2 isolates [Bacon et al., 2002; Bacon et al., 2003]. Foscarnet resistance was defined by $IC_{50}$ values >100 μg/ml.

### TK Assay

TK activity was assayed indirectly [Prieto et al., 1991], by measuring the uptake of [$^3$H] thymidine ([$^3$H]TdR 82.2 Ci/mmol; New England Nuclear, Boston, MA) in DNA from 143 cells ($TK^-$ cell line from human osteosarcoma) [Campione-Piccardo et al., 1979]. Monolayers with $5 \times 10^4$ cells were inoculated with 10 multiplicity of infection (moi) of the clinical isolates, purified clones, the $TK^+$ and $TK^-$ strains, and labelled with 1 μCi [$^3$H]TdR for 24 hr after infection. Cultures were lysed with 0.5 % SDS and 10 mM NaOH, and precipitated with ice cold 10% TCA. Precipitates were collected by filtration through glass-fibre filters and counted in toluene-based scintillation fluid with a scintillation counter (Beckman LS 5000 TD). Results of TK activity were expressed as a percentage to $TK^+$ HSV-2 333.

### Viral Plaque Purification

Viral stocks grown from the initial clinical isolate were purified by single plaque isolation. Single plaques were isolated by inoculating suitable dilutions of virus onto confluent Vero monolayers in 24-well tissue culture plates (Costar, Cambridge, MA). Virus was allowed to adsorb at 37°C for 1 hr before being overlaid with 1 ml of agarose (0.5%) in minimum essential medium. After 48 hr, the infected wells were stained by adding 50 μl of neutral red (0.001 g/ml). They were then examined, 24 hr later, for single isolated plaques. Three rounds of plaque purification were done in absence of acyclovir pressure and sensitivity and TK assays were carried out.

### Genetic TK Analysis

DNA from infected Vero cells was extracted by phenol–chloroform [Prieto et al., 1991]. Amplification of the viral *TK* gene coding strand was done in a DNA thermal cycler (PTC-200 M. J. Research) using primers based on published GenBAnk sequences for HSV-2 strain 333 (accession number M29942). PCR was performed with 2 μg of template and 50 pmol of each 5′- CGA ACG CCT TGT AGA AGC -3′, 5′-CCT TCC GTT CGG GCT TCC-3′ as external primers of *TK* gene. Polymerase chain reaction products were purified (DNA PCR preps, Promega, Madison, WI) and sequenced using an automatic DNA sequencing system. Five primers spanning the entire coding strand of the *TK* gene were utilised for DNA sequencing. Results were compared with known TK sequences from the 333 (HSV-2) reference strain. The viral TK sequences were confirmed by sequencing of cloned *TK* genes in pGEM T Easy plasmid (Promega).

### TK Assay From Transformed Bacteria

Viral TK genes obtained from recombinant pAGEM T easy plasmids were expressed in *E. coli* (strain KY893) using pUC18 as expressing vector of the fusion βgal-viral TK protein, obtaining the plasmids pU1S harbouring TK gene of S-1a virus and pU4R harbouring *TK* gene of R-101 virus (see below). Transformed bacteria were grown onto culture agar plates containing uridine 25 μg/ml, thymidine 50 μg/ml, and 5-fluor-uracil (5-FU) 25 μg/ml. Only bacteria transformed with the functional *TK* gene grow on this medium since *E. coli* KY893 $TK^-$ is unable to grow on selection medium containing 5-fluouracil [Igarashi et al., 1967; Martín-Hernández and Tabarés, 1991].

### Animal Studies

Groups of four BALB/c female mice were used to calculate lethal dose 50% (LD50) and infective 50% (ID50). To synchronise the estrus cycle in the mice and produce a uniform susceptibility to HSV-2 challenge, the animals were treated with subcutaneous medroxyprogesterone (Depo-Progevera; Upjohn, Belgium) (2 mg each mouse) 4 days before challenge [Kriesel et al., 1996]. Mice were anaesthetised before

inoculation with 3 mg of intraperitoneal ketamine (Parke-Davis, Ireland) and 0.3 mg of intraperitoneal xylacine (Ronpun; Bayer, Germany). About 50 μl of viral suspension were absorbed onto Dacron pledges and placed in vagina for 30 min. Viral suspensions were carried out beginning with $10^7$ pfu/ml. Mice were observed daily for external genital inflammation and survival during 2 weeks.

## RESULTS

### Antiviral Susceptibility

Acyclovir, penciclovir, and foscarnet susceptibilities for the clinical isolate, expressed as the mean $IC_{50} \pm$ standard deviation were as follows: Acyclovir, $0.66 \pm 0.25$ μg/ml; penciclovir, $4.2 \pm 0.34$ μg/ml; and phoscarnet, $17.63 \pm 2.47$ μg/ml. This strain might be considered susceptible to acyclovir, but resistant to penciclovir.

### Characterisation of Viral TK Activities

We compared the TK activities of the clinical isolate with the TK activity of the reference strains. The clinical isolate showed about 40–50% of TK activity in relation to HSV-2 strain 333 (Fig. 1). Three possible explanations for this curious contrast between acyclovir and penciclovir susceptibilities and TK activity were considered: a reduced expression of TK, an expression of a TK with altered substrate affinity, or the presence in the patient's lesion of a mixed population of $TK^+$ and $TK^-$ HSV-2. Plaque selection and purification of the clinical isolate virus showed to be a mixture of two viral populations, one susceptible to acyclovir/penciclovir (S-1a) and another resistant to acyclovir/penciclovir (R-101). The susceptible S-1a had a TK activity similar to that of the reference strain. Finally, the resistant R-101 was deficient in TK activity (Fig. 2).

### *TK* Gene Analysis

The DNA sequences of PCR-amplified *TK* genes from S-1a, R-101 isolates were analysed and related to HSV-2 333 reference strain (Table I). There was only one mutation site among the two plaque-purified strains of

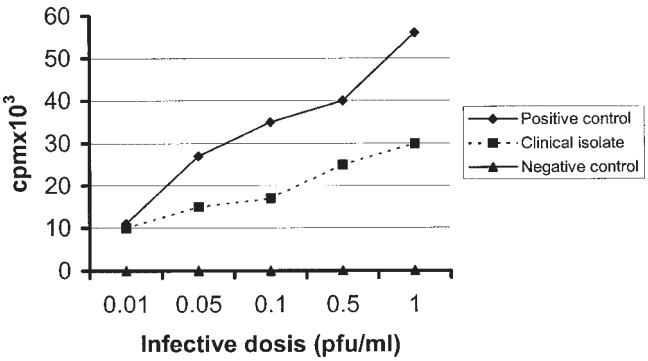


Fig. 1. Thymidine kinase (TK) activity of the HSV-2 clinical isolate. TK activity was measured indirectly by [$^3$H] dTdR incorporation to DNA and related to viral infectivity.

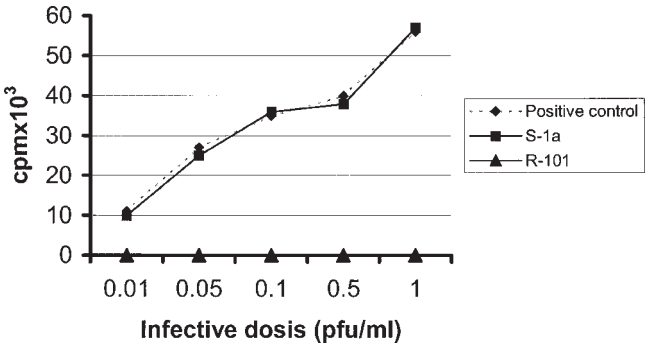


Fig. 2. TK activities of the HSV-2 plaque-purified viruses (S-1a and R-101) from the clinical isolate. TK activity was measured indirectly by [$^3$H] dTdR incorporation to DNA and related to viral infectivity.

the clinical isolate that affected the nucleotide binding site, which includes from position 151 to position 189 [Darby et al., 1986]. It led to a change from arginine (in the $TK^+$ purified S-1a and reference $TK^+$) to tryptophan (in the $TK^-$ purified R-101). There were other four variations that affected the two plaque purified clones of the clinical isolate versus the reference strain. These additional changes did not affect the nucleotide binding site nor the nucleoside binding site. The functional TK activity of plaque purified clones was checked by expression in TK-deficient *E. coli* [Hiraga et al., 1967]. These cells were transformed with pU1S and pU4R harbouring the TK sequences obtained from the two plaque-purified virus from the clinical isolate (S-1a and R-101, respectively). Bacteria transformed with the plasmid which contained the TK sequence obtained from the S-1a clone grew on selective TK medium, showing that the cloned *TK* gene was functional and expressed TK activity, while bacteria transformed with the one containing the TK sequence obtained from the R-101 clone did not grow. The two cloned genes were further sequenced, confirming the sequences obtained with the PCR amplifications.

TABLE I. Genetic Analysis of HSV-2 Thymidine Kinase (*TK*) Genes From the Two Plaque-Purified Viruses (S-1a, $TK^+$; and R-101, $TK^-$) of the Clinical Isolate and a HSV-2 (Strain 333)

| Nucleotide position | Strain | Codon | Amino acids |
|---|---|---|---|
| 79, 80, 81 | Reference 333 | gcg | Ala |
| | S-1a | acg | Thr |
| | R-101 | acg | Thr |
| 85, 86, 87 | Reference 333 | tcc | Ser |
| | S-1a | gcc | Ala |
| | R-101 | gcc | Ala |
| 151, 152, 153 | Reference 333 | cgg | Arg |
| | S-1a | cgg | Arg |
| | R-101 | tgg | Trp |
| 232, 233, 234 | Reference 333 | aat | Asn |
| | S-1a | gat | Asp |
| | R-101 | gat | Asp |
| 418, 419, 420 | Reference 333 | ttg | Leu |
| | S-1a | ttt | Phe |
| | R-101 | ttt | Phe |

### Analysis of Viral Virulence

In the mouse genital herpes model, the R-101 strain, TK-plaque purified strain, was considerably less virulent than the S-1a $TK^+$ strain. The viral dose that killed 50% of mice (LD50) was $10^5$ pfu/ml for the S-1a strain and more than $10^7$ pfu/ml for the $TK^-$ R-101 strain. The dose that infected 50% of mice (ID50) was $10^4$ pfu/ml for the S-1a strain and $5 \times 10^5$ pfu/ml for the R-101 strain.

## DISCUSSION

To our knowledge there are no existing reports of HSV isolates with both penciclovir resistance and acyclovir susceptibility, but dissociated susceptibility in the inverse manner, acyclovir resistance and penciclovir susceptibility has been described in relation to TK altered phenotypes or DNA polymerase mutations [Pelosi et al., 1998]. On the other hand, our dissociated susceptibility was only apparent and was due to the presence of a mixed population of virus. The complete TK activity of the susceptible population and the abolished TK activity of the resistant population made very unprobable the presence of an altered fenotype TK.

It has long been recognised that laboratory strains and clinical isolates of HSV contain mixtures of wild type and acyclovir/penciclovir-resistant virus and, thus contain pre-existing drug-resistant TK variants (6–8 mutants per $10^4$ plaque forming viruses) [Parris and Harrington, 1982; Sarisky et al., 2000; Sarisky et al., 2001; Shin et al., 2001]. The fidelity of the HSV DNA replication process is directly responsible for this naturally occurring variation [Hall and Almy, 1982] Errors in the viral DNA are introduced spontaneously during DNA replication, not requiring the presence of the antiviral drug. However, the exposure to an antiviral compound may induce selective selection of a resistant variant, leading to the enrichment of a pre-existing drug-resistant virus. It is also known that in HSV-2 susceptible isolates, $IC_{50}$ to penciclovir are slightly greater than to acyclovir [Safrin and Phan, 1993; Cassady and Whitley, 1997]; so, it is not surprising that a mixed population can reach a resistant cut off for penciclovir without reaching one for acyclovir.

Multiple mechanisms associated with changes in the viral *TK* gene can account for HSV resistance to guanosine analogues. Most resistant isolates contain a frameshift mutation within homopolymer repeats of Gs, Cs and much less frequently As, indicating the presence of mutational "hot spots" within the viral *TK* gene [Sasadeusz et al., 1997; Gaudreau et al., 1998]. Addition and deletion of nucleotides are presumed to cause a frameshift reading and consequently the production of an inactive truncate protein [Oram et al., 2000]. Point mutations affecting the proposed active sites, nucleoside and nucleotide binding sites, are known to lead to abolished, altered, or low producer TK mutants. The importance of lysine and threonine at codons 62 and 63, respectively, has been demonstrated [Liu and Summers, 1988]. In other α-herpesvirus there is an abolition of TK activity with only a single base mutation that leads to an aminoacid substitution in the ATP proposed binding site [Prieto et al., 1991]. On the other hand, variations in codon 60, in the ATP proposed binding site, where valine in the 333 reference strain (published GenBAnk accession number M29942) is replaced by methionine in the G strain (published GenBAnk accession number AF466703) lead to no activity changes; but in this case both aminoacids are apolar. The change from arginine (polar amino acid) to tryptophan (non-polar aromatic amino acid) must cause serious conformational alterations in the ATP binding site and may explain the abolition of TK activity with only one nucleotide substitution.

Results of our virulence studies in a mouse model suggest that the $TK^-$, acyclovir resistant strain, is less virulent than the $TK^+$ one, and confirm that generally, TK deficient viruses are less virulent than wild-type viruses [Tenser et al., 1979; Darby et al., 1981; Oliver et al., 1989].

In conclusion, this case of antiviral resistance showed several unusual facts. First, the apparently dissociated susceptibility between acyclovir and penciclovir; second, the TK activity, around 50%, which suggested a TK low producer or a TK with altered substrate affinity and was caused by a mixed population; and third, a loss of viral TK activity caused by a single nucleotide substitution in the two clinical isolate populations which resulted in the change from a polar aminoacid to an aromatic one in the ATP-binding site.

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