Spontaneous Reactivation of Thymidine Kinase–Deficient, Acyclovir-Resistant Type 2 Herpes Simplex Virus: Masked Heterogeneity or Reversion?

Joseph J. Sasadeusz and Stephen L. Sacks

Division of Infectious Diseases, Department of Medicine, University of British Columbia, Vancouver, Canada

Herpes simplex virus (HSV) strain 1737, acyclovir-resistant and uniformly thymidine kinase–deficient (tkD) by all conventional assays, clinically reactivated in an AIDS patient in the absence of antiviral drug pressure. Investigation of its neurovirulence and latency characteristics in a mouse model using a tkD plaque isolate (1737-14), however, yielded a neurovirulent, homogeneous, acyclovir-sensitive, tk wild type (tkWT) strain (1737-14ME), while trigeminal ganglia from a surviving animal yielded a heterogeneous tkD/tkWT population (1737-14/10iB). Heterogeneity may have arisen due to selection of a preexisting tkWT subpopulation or to genetic reversion. “Ultralow” levels of tk, undetectable by conventional means, may be sufficient for reactivation while retaining the acyclovir-resistant phenotype. A possible mechanism for spontaneous reactivation of 1737 is in vivo complementation between heterogeneous tk populations. Eradication of acyclovir-resistant, tkD virus does not ensure subsequent reactivations to be acyclovir-sensitive, and alternating antivirals may be required for effective therapy.

Genital herpes simplex virus (HSV) infections continue to be a major public health problem [1–3]. For the immunocompetent, recurrences are self-limiting, but in the immunocompromised, untreated mucocutaneous HSV infections can be chronic and progressive [4, 5]. Acyclovir is a potent and selective antiviral that has been the reference standard of treatment against HSV. Resistance has been associated with significant clinical disease progression in ~5% of immunocompromised hosts [6–10]. Antiviral resistance has been associated with significant clinical disease progression in ~5% of immunocompromised hosts [11–15]. With one notable exception [16], immunocompetent and acyclovir-naive patients have been spared clinically significant resistance [17, 18].

A deficiency in viral thymidine kinase (tk), the enzyme required for selective initial phosphorylation of acyclovir before cellular kinases convert it to the active triphosphate, accounts for the overwhelming majority of instances of resistance [19]. Detection of tk activity at low levels by existing techniques may lead to difficulty in discrimination between tk-deficient (tkD) and tk-negative (tk-) phenotypes. For this discussion, tk- refers to that subset of isolates in which tk activity is not present (lack of tk activity is absolute), while tkD refers to isolates in which tk activity is undetectable by conventional means. There may be uncertainty concerning subthreshold enzyme activity expressed by tkD isolates. Isolates that are tkD have been shown to be able to establish and maintain latency but demonstrate a loss of efficiency of latency reactivation as well as diminution of neurovirulence in mice [20–25]. Virulence reductions of such strains has been used to explain the relative absence of acyclovir resistance in the immunocompetent [19]. There has been only one other report of spontaneous clinical reactivations of acyclovir-resistant HSV-2 in AIDS patients. This was an observation of drug-free first reactivations after foscarnet therapy in a clinical trial that did not assess tk activities or viral heterogeneity [26]. Accordingly, it was of interest to extensively characterize an acyclovir-resistant HSV-2 isolate obtained from an AIDS patient with genital herpes who was receiving no antiviral therapy at the time.

Case Report

The patient was a 45-year-old woman with a history of genital HSV since 1984 that she episodically self-medicated with oral acyclovir. In 1985, she was found to be positive for human immunodeficiency virus antibody. Her human immunodeficiency virus disease was subsequently manifested by cervical carcinoma, recurrent pelvic infections, and two episodes of Pneumocystis carinii pneumonia. By September of 1989, her CD4 cell count was 300/\( \mu \)L and she was admitted after an episode of genital herpes unresponsive to 4 weeks of oral acyclovir (up to 800 mg orally every 8 h) and 3 weeks of intravenous acyclovir (10 mg/kg every 8 h). Clinical isolates from this outbreak were acyclovir-resistant and tkD and included 1106 (acyclovir ID50 8.4 \( \mu \)g/mL; tk activity, 0.6%). Treatment was changed to foscarnet, 60 mg/kg every 8 h for 14 days, with full clinical and virologic resolution of lesions. A brief recurrence in November with acyclovir-sensitive virus responded to oral acyclovir. Further acyclovir-resistant, tkD
The patient was then prescribed foscarnet prophylaxis in reducing recurrences soon followed in January, April, and May 1990, each in the setting of acyclovir therapy and also responding to foscarnet. The patient was readmitted with neurologic complications and was noted again to have an acyclovir-resistant, tk\(^{-}\) genital recurrence (clinical isolate 1737: acyclovir ID\(_{50}\) 6.6 µg/mL; tk activity, 9.7%). She was treated for only 3 days because of foscarnet-induced symptomatic hypocalcemia but had a satisfactory clinical and virologic response and was discharged receiving no further antiviral treatment. In September 1990, she sustained another recurrence that was acyclovir-sensitive (clinical isolate 1773: acyclovir ID\(_{50}\) 0.11 µg/mL; tk activity, 198.6%). The patient died in December 1990 of causes unrelated to her genital HSV infection.

**Materials and Methods**

**Virologic studies.** Clinical isolates were obtained on human foreskin fibroblasts and identified and typed as described [27]. Virus stocks were grown in Vero cell monolayers and then titered by plaque reduction on Vero cell monolayers to determine ID\(_{50}\)s compared with drug-free wells using 24-well plates inoculated with 50 pfu/well overlaid with medium containing drug dilutions and anti-HSV-1 polyclonal antibody (Dako, Glostrup, Denmark), as modified from a previously described technique [28]. Resistance to acyclovir and foscarnet was defined as an ID\(_{50}\) > 2.0 and > 100 µg/mL, respectively. A single round of 20 plaque purifications was done by a modification of a previously described technique [29], using three dilutions in 6-well plates overlaid with medium containing 0.5% agarose.

**tk activities were determined by measuring the uptake of 1-β-d-arabinofuranosyl-ethyl-5-(2-12\(^{3}\)Iiodovinyl)uracil ([\(1^{23}\)I]VaraU; 2200 Ci/mmol) by human foreskin fibroblasts infected with the strain of interest compared with wild type HSV-2 reference strain G (American Type Culture Collection, Rockville, MD) and tk\(^{-}\) reference strain ACG4 [gift of Don Coen, Harvard Medical School, Boston]. Polystyrene tissue culture tubes (16 × 125 mm) containing ~3 × 10\(^3\) fibroblasts in 2.0 mL of 5% MEM were inoculated at an MOI of 0.1 and 2 × 10\(^6\) pfu of \([1^{23}\)I]VaraU 12 h before the assay. After media removal, monolayers were washed 4 times with 4 mL of PBS, pH 7.4, at 37°C and removed with trypsin, and counts were expressed as a percentage of uptake compared with that of control strain G.

Plaque autoradiography was done by a modification of the method of Martin et al. [30], using 100 pfu inoculated onto 60 × 15 mm Petri dishes seeded with 2 × 10\(^6\) Vero cells or 4 × 10\(^3\) 143B cells (cellular tk\(^{-}\) ). After a 1-h adsorption, monolayers were overlaid with 0.5% methylcellulose-containing media and incubated for 3–4 days. The overlays were removed, and media containing 0.5 µCi of [\(1^{23}\)I]iododeoxyctydine or [\(^{3}^{14}\)C]thymidine (to exclude thymidine kinase–altered phenotype) was added to Vero or 143B cells, respectively, for a further 4 h. Cells were fixed with 10% formalin, stained with 0.5% crystal violet, washed twice with PBS, and air-dried. Circumferential rims were then removed, and monolayers were exposed to radiographic film (Kodak X-Omat XAR) for 5 days and developed. The number of plaques on monolayers was then compared with the number with incorporated radionuclide on autoradiographs as designated by the formation of dark rims at the periphery of plaques.

**Mouse neurovirulence studies.** Mice were inoculated by the intranasal route as described [31]. Briefly, 4-week-old female BALB/c mice were lightly anesthetized with methoxyflurane (Pitman-Moore, Mississauga, Canada). The appropriate virus inoculum was injected in 20 µL of 5% MEM. Inocula of 10\(^{-1}\)–10\(^{-4}\) pfu were used for each strain, and 6 mice were inoculated at each inoculum. Strains tested included plaque-purified isolates from the spontaneously reactivated tk\(^{-}\), acyclovir-resistant isolate (1737-14) and the subsequent posttreatment tk wild type (tk\(^{WT}\)), acyclovir-sensitive isolate (1773-5). Controls included the tk\(^{WT}\) reference strain G, tk\(^{-}\) reference strain ACG4, and sham-inoculated animals. Mice were then observed for evidence of encephalitis for 21 days. The brains of dying tk\(^{-}\)-inoculated mice were removed, homogenized, sonicated, and cocultivated on Vero cell monolayers. At 30 days, surviving mice were sacrificed. Trigeminal ganglia of 10 such mice inoculated with the two highest inocula of 1737-14 were removed, minced, and cocultivated on Vero cell monolayers. Output isolates from the animal experiments were recharacterized by susceptibility testing, tk uptake, and plaque autoradiography, as described above.

**Immunoprecipitation.** Identification of tk protein was done by immunoprecipitation using an anti-HSV-2 tk monoclonal antibody (provided by Ken Powell, Burroughs Wellcome, Beckenham, UK). Briefly, 60 × 15 mm culture dishes were inoculated with an MOI of 0.1 and adsorbed for 1 h. The infected monolayer was washed three times with 1 mL of methionine- and cysteine-free Dulbecco's MEM (DMEM), overlaid with 5 mL of medium containing a 9:1 ratio of methionine- and cysteine-free DMEM to regular DMEM, 4% dialyzed fetal calf serum, and 50 µCi/mL Trans\(^{3}^{25}\)S Label (1101 Ci/mmol; ICN Biomedicals, St. Laurent, Canada) and incubated overnight. The monolayer was then washed with 2 mL of ice-cold PBS. Cells were lysed with 1 mL of cold lysis buffer for 15 min on ice and removed with a cell scraper, and nuclei were removed by centrifugation at 15,000 rpm for 5 min at 4°C.

The supernatant was incubated with 100 µL of 10% NP-40, 10% Na deoxycholate, and 1% SDS together with 1/100 dilution of tk antibody and incubated overnight on ice. Immune complexes were collected by addition of 100 µL of a 10% suspension of Staphylococcus aureus cells, rocking at 4°C for 2 h, and centrifugation at 12,000 rpm for 1 min. The pellet was washed sequentially with 0.5 mL of the following solutions: wash buffer 1 (20 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 1% NP-40), wash buffer 2 (20 mM TRIS-HCl, pH 8.8, 150 mM NaCl, 1% NP-40, 0.2% SDS), and wash buffer 3 (20 mM TRIS-HCl, pH 6.8, 150 mM NaCl, 1% NP-40, 0.2% SDS). The pellet was then resuspended in 50 µL of SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 62.5 mM TRIS-HCl, pH 6.8, 10% glycerol, and 0.001% bromphenol blue), denatured at 100°C for 5 min, and electrophoresed at 25 mA for 4 h through a 6% stacking and 10% resolving acrylamide gel.
were then fixed in 40% methanol and 5% acetic acid, dried, and exposed to radiographic film.

**Results**

The acyclovir-resistant, tk<sup>0</sup> clinical isolate that reactivated spontaneously (1737) was characterized further to determine the reason for its reactivation ability. An acyclovir-sensitive isolate from the next reactivation (1773) served as an internal control. Initially, plaque isolations were done in an attempt to demonstrate heterogeneity within clinical isolates. Nineteen plaque isolates were successfully regrown from 1737 and recharacterized. Each was acyclovir-resistant with acyclovir ID<sub>50</sub> ranging from 2.1 to 17.1 μg/mL. Individual plaque isolates from both 1737 (1737-14: acyclovir ID<sub>50</sub>, 11.8 μg/mL; tk activity, 5.4%; foscarnet ID<sub>50</sub>, 30.9 μg/mL) and 1773 (1773-5: acyclovir ID<sub>50</sub>, 0.14 μg/mL; tk activity, 134.7%; foscarnet ID<sub>50</sub>, 34.0 μg/mL) were then selected for animal model studies. More rigorous assessments of heterogeneity by plaque autoradiography demonstrated 1737 and 1773 and their respective plaque isolates (1737-14 and 1773-5) all to be homogeneous populations (figure 1).

Because heterogeneity did not seem to underlie the reactivation ability of 1737, we attempted to determine whether this unusual characteristic of the isolate was paralleled by increased neurovirulence. Accordingly, 1737-14 was assessed for neurovirulence and latency characteristics in the mouse model. No animals administered an inoculum of >10<sup>2</sup> pfu of tk<sup>WT</sup> strains G or 1773-5 survived, while all animals inoculated with tk<sup>0</sup> strain ACGr4 and all sham-inoculated animals survived. As

![Figure 1](https://academic.oup.com/jid/article-abstract/174/3/476/902653)

*Figure 1.* Plaque autoradiographs from clinical and laboratory isolates of HSV-2. Strain G is tk wild type control and strain ACGr4 is tk-deficient (tk<sup>0</sup>) control. Upper 3 rows demonstrate plaques grown; lower 3 rows demonstrate corresponding autoradiographs. Plaques expressing tk activity appear as dark rims, while tk-deficient plaques fail to produce dark rims. A, 0.5 μCi of [<sup>35</sup>]<sup>3</sup>Iiododeoxycytidine used as tk probe in Vero cells. B, 0.5 μCi of [<sup>14</sup>C]thymidine used in tk<sup>0</sup> cell line (143B).
predicted by its tk<sup>-</sup> phenotype, 1737-14 overall showed reduced neurovirulence, with all animals administered inocula up to 10<sup>5</sup> pfu surviving. Surprisingly, however, 1 of 6 mice inoculated with 10<sup>6</sup> pfu died from encephalitis.

Recharacterization of this encephalitic output strain, 1737-14ME, showed it to be fully acyclovir-sensitive (acyclovir ID<sub>50</sub>, 0.12 μg/mL; tk activity, 154.9%), and plaque autoradiography demonstrated a homogeneous tk<sup>WT</sup> population (figures 1 and 2). In contrast, virus recovered by cocultivation from trigeminal ganglia of 2 of the surviving mice inoculated with 1737-14 yielded strains that were acyclovir-resistant and had reduced tk activities (1737-14/10<sup>6</sup>A; 1737-14/10<sup>7</sup>B). Strain 1737-14/10<sup>6</sup>A (acyclovir ID<sub>50</sub>, 9.0 μg/mL; tk activity, 8.9%; foscamet ID<sub>50</sub>, 11.8 μg/mL) was similar to the acyclovir-resistant, tk<sup>-</sup> phenotype of the input strain 1737-14, but strain 1737-14/10<sup>7</sup>B (acyclovir ID<sub>50</sub>, 15.2 μg/mL; tk activity, 53.5%; foscamet ID<sub>50</sub>, 9.7 μg/mL), despite acyclovir resistance, demonstrated a surprisingly high level of tk uptake. Plaque autoradiography demonstrated strain 1737-14/10<sup>6</sup>A to be a homogenous tk<sup>2</sup> population similar to the input strain, but strain 1737-14/10<sup>7</sup>B was an almost equal heterogeneous population of tk<sup>-</sup> and tk<sup>WT</sup> plaques (figures 1 and 2); this correlated with the unexpectedly high tk activity. Foscamet susceptibility excluded a mutation in the viral DNA polymerase locus.

Immunoprecipitation demonstrated 1773-5 to produce a full-length 40-kDa tk protein identical to that of tk<sup>WT</sup> reference strain G, while 1737-14 produced a truncated 28-kDa protein but no full-length tk product; this is consistent with a protein lacking tk activity (figure 3).

**Discussion**

This is the first report that documents, characterizes, and provides the mechanistic basis for a spontaneous clinical reactivation of an acyclovir-resistant HSV isolate that tests as tk<sup>-</sup> by all conventional assays. Resistance was acquired during the treatment of previous episodes and then reactivated without selection pressure by continuing antiviral administration. Complete eradication of HSV from the affected area between episodes was well-documented, demonstrating that latent acyclovir resistance had been induced.

The behavior of this isolate and its reactivation in the patient appeared paradoxical in the face of current dogma suggesting that tk<sup>-</sup> HSV should not be able to reactivate. Although tk uptake studies may have suggested a low level of activity, this assay has proved insensitive in discriminating between low and absent activity. Plaque autoradiography demonstrated a homogeneous tk<sup>-</sup> population, while immunoprecipitation showed it to produce a truncated protein in the absence of any detectable full-length tk protein, this more accurately reflecting its phenotype. Despite its apparent absence of tk activity, however, 1737-14 resulted in mouse encephalitis with a pure tk<sup>-</sup> population, while trigeminal ganglia latency was due to mixed tk phenotypes. It is very likely that the tk activity demonstrated in the neurovirulence studies also enabled clinical isolate 1737 to reactivate. This work further enhances the principle that tk activity is required for reactivation of HSV.

It has been previously documented that the tk activity of an isolate correlates well with the proportion of tk<sup>WT</sup> virus within a mixed population [30] and that mutants with low levels of tk activity can reactivate from latency [32]. These data suggest that very sensitive tk assays challenge old phenotypes and demonstrate that the levels of tk expression necessary for reactivation are quantitatively much lower than previously appreciated. Indeed, these data demonstrate that tk activity may fall below the threshold of conventional assays. In this case, tk activity was amplified using passage in the animal model.

![Figure 2](https://academic.oup.com/jid/article-abstract/174/3/476/902653) Derivation of HSV-2 plaque isolate 1737-14 tracing input and output strains from mouse neurovirulence studies. Schematic demonstrates how reactivated tk-deficient (tk<sup>-</sup>) clinical isolate 1737 underwent plaque purification to produce plaque isolate 1737-14, both of which appear as homogeneous tk<sup>-</sup> populations by plaque autoradiography. After inoculation of 1737-14 into mouse model, neurovirulent brain-derived output strain, 1737-14ME, was homogeneous tk wild type (tk<sup>WT</sup>), while latent virus strains successfully recovered from trigeminal ganglia of 2 surviving mice (1737-14/10<sup>6</sup>A and 1737-14/10<sup>7</sup>B) consisted of homogeneous tk<sup>-</sup> and heterogeneous tk<sup>WT</sup>/tk<sup>-</sup> populations, respectively.
Mock-infected lane represents cellular protein. Arrowhead at left points to a prominent band at 40 kDa produced by 1773-5, which is identical to that produced by tk-wild-type (tk\(^{WT}\)) reference strain G. Kpn\(\Delta\)333 is a reference tk-deficient deletion mutant and does not produce full-length product. Arrowhead at right points to truncated 28-kDa tk protein produced by both 1737-14 and 1106-2. Light bands are of the same size as cellular protein in mock-infected lane and are identical to that produced by tk wild type (tk\(^{WT}\)).

Herein, we have designated such strains as "ultralow" tk producers. The distinction between isolates with very low levels of tk activity as opposed to true absolute tk deficiency remains confused. Our data suggest that the tk activity of such isolates may represent a continuum, based on the proportion of tk\(^{WT}\) virus, with the threshold for reactivation yet to be determined.

The ability of such a small tk\(^{WT}\) subpopulation to impart their characteristics on an overwhelmingly larger tk\(^{D}\) population to enable it a wider phenotypic expression by reactivating suggests that in vivo complementation between the populations may be occurring. If complementation explains these events, it would further suggest that this phenomenon would be capable of selectively dissociating the tk phenotypic expressions of reactivation and neurovirulence from others by still testing as tk\(^{D}\), acyclovir-resistant by all conventional means, because unmasked by the exquisite sensitivity of the animal model in amplifying virus or in vivo reversion to tk\(^{WT}\) took place. The natural frequency of reversion of the tk gene has been estimated at \(10^{-4}\) to \(10^{-6}\) [39]. Recent work has demonstrated that 1737-14 contains a frameshift mutation at a site that is a mutational "hot spot" [40], suggesting much higher reversion frequencies at such sites, combined with a possible growth advantage of tk\(^{WT}\) over tk\(^{D}\), may allow for the emergence of such subpopulations. If that is the case, then a plaque isolate completely devoid of tk activity may be unachievable from a clinical isolate regardless of the number of rounds of plaque purification.

The issue of whether tk activity can be dissociated from neurovirulence has also been recently debated. Erlich et al. [14] and Chatis and Crumpacker [41] reported a plaque-purified tk\(^{D}\) HSV isolate from an AIDS patient that, due to a single base substitution, produced a full-length protein and retained full neurovirulence. Plaque autoradiography in those studies, however, was done in a tk-producing cell line using a [\(^{14}\)C]thymidine overlay, potentially reducing assay sensitivity in demonstrating heterogeneity or a tk low-producer phenotype.

In addition, the combination of a full-length tk polypeptide and a base substitution that is not in any of the three described conserved binding domains [42] calls into question the significance of this mutation. Contrary to this, the isolate from the current report demonstrated neurovirulence, as predicted, to be directly linked to tk activity. The possibility that masked heterogeneity, as seen here, was present but not tested in the previous report must be considered and may have provided sufficient tk activity to explain the observed neurovirulence.

The clinician must appreciate that effective clinical and virologic eradication of mucocutaneous viral shedding through foscarnet (or alternative) therapy, followed by no antiviral treatment whatsoever, may still eventuate in reactivation of acyclovir-resistant isolates in a subsequent episode. Accordingly, the use of acyclovir may, in some cases, fail even for treatment of a new and unchallenged recurrence. Nevertheless, normal tk activity prevailed in the majority of untreated reactivations. Presumably, clones expressing tk were more efficient...
reactivators. That withdrawal of acyclovir pressure in this patient eventually led to an acyclovir-sensitive, tk<sup>WT</sup> reactivation further supports this hypothesis. Alternatively, tk<sup>WT</sup> reactivations may have originated from different latently infected ganglion sites altogether that had not been subjected to the development of resistance. Regardless of the underlying mechanism, for patients with refractory disease, this reversion of HSV over time to an acyclovir-sensitive population is reassuring in that, at least initially, acyclovir may be useful in such instances. Alternating acyclovir and foscarnet or other such combination may thus be a useful strategy in the control of resistant disease.

Acknowledgments

We thank Christopher H. Sherlock and the University of British Columbia Diagnostic Virology Laboratory for providing us with laboratory isolates of HSV and Ken Powell (Burroughs Wellcome, Beckenham, UK) for providing the HSV-2 anti-tk monoclonal antibody.

References


