Biochemical Characterization of a Virus Isolate, Recovered from a Patient with Herpes Keratitis, That Was Clinically Resistant to Acyclovir

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In vitro susceptibility assays of herpes simplex virus (HSV) do not necessarily correlate with treatment outcome. An HSV type 1 (HSV-1) isolate, N4, recovered from a patient who presented with herpes keratitis with localized immunosuppression, was characterized for susceptibility. Although the 50% inhibitory concentration (IC50) for this isolate was less than the accepted breakpoint for defining resistance to acyclovir (<2.0 µg/mL), the following lines of evidence suggest that the isolate was acyclovir resistant: (1) the clinical history confirmed that the infection was nonresponsive to acyclovir; (2) the in vitro susceptibility was similar to that of a thymidine kinase (TK)–negative, acyclovir-resistant virus SLU360; (3) the IC50 of acyclovir was more than 10 times the IC50 for an acyclovir-susceptible control strain; (4) plaque-purified clonal isolates were resistant to acyclovir (IC50s, >2.0 µg/mL); and (5) biochemical studies indicated that the HSV-1 N4 TK was partially impaired for acyclovir phosphorylation. Although residue changes were found in both the viral tk and pol coding regions of HSV-1 N4, characterization of a recombinant virus expressing the HSV-1 N4 polymerase suggested that the TK and Pol together conferred the acyclovir-resistance phenotype.

Antiviral-resistant herpes simplex virus (HSV) is prevalent in the immunocompromised population [1, 2], and there is a risk that HSV infections will become chronic in these individuals [3–7]. A number of in vitro assays are used to assess the susceptibility of HSV to antiviral agents [8]; however, there are limitations to how accurately antiviral-resistant HSV strains can be identified. These limitations largely result from the absence of an accepted panel of well-characterized standard HSV strains, including acyclovir-resistant and acyclovir-susceptible phenotypes, for global use; the failure of researchers to consistently include the same drug-susceptible control virus in each assay also contributes to these limitations. To date, the plaque reduction assay (PRA) remains the only assay in which a correlation has been established between the laboratory-determined IC50 and the clinical outcome of disease [9].

In fact, an in vitro breakpoint for defining acyclovir resistance has been set at 2.0 µg/mL on the basis of the correlation between the IC50 and the clinical response to antiviral therapy [9–12]. “Resistance to penciclovir” has been defined by the following criteria: (1) an IC50 that is >10-fold greater than the IC50 of penciclovir-susceptible control virus used in a particular assay, or (2) as an IC50 of >2.0 µg/mL [13, 14]. The aforemen-
tioned criteria were evaluated in the present study, to determine whether an isolate was resistant to acyclovir or penciclovir.

We report the isolation of an acyclovir-resistant HSV type 1 (HSV-1) virus strain that was cultured from a corneal sample obtained from a patient who was nonresponsive to acyclovir treatment. The IC₅₀ for the patient’s isolate was <2.0 μg/mL when tested against acyclovir, which suggests that the isolate was acyclovir-susceptible; however, the IC₅₀ was >10-fold greater than the IC₅₀ of the drug-susceptible control virus when tested using the same assay, which suggests that the virus was resistant to acyclovir. We present evidence to confirm that the phenotype was resistant to acyclovir.

MATERIALS AND METHODS

Patient treatment. Drugs used for therapy were dexamethasone phosphate (eyedrops; 0.1% solution given 1–3 times per day), acyclovir ointment (topical ointment, given either 5 times per day for treatment of acute infection or 1 time per day for maintenance therapy), oral acyclovir (400 mg 2 times per day), foscarnet iv solution used topically (6 g/250 mL), and trifluridine eye drops (ophthiole eyedrops; 10 mg/mL 2 times per day).

HSV isolation. Corneal ulcer smear specimens were obtained and inoculated into duplicate shell vials that contained fibroblasts in 1 mL of culture medium (Dulbecco modified Eagle medium with 10% fetal calf serum and antibiotics). Shell vials were centrifuged at 700g for 30 min and were incubated overnight at 37°C. For 1 week, the cultures were examined daily for cytotoxic effect, and the cell suspension was frozen and thawed to release cell-associated virus when infection was complete. The resulting virus preparation was stored at −80°C. The virus was typed using an immunofluorescence assay, according to the manufacturer’s recommendations (DAKO). Two plaques, representing the clonal isolates of N4-1 and N4-2, were picked randomly and purified 3 times, to homogeneity, for characterization.

Cell lines and virus strains. MRC-5 (American Type Culture Collection), a diploid human embryonic lung cell line, and 143 thymidine kinase (TK)− (American Type Culture Collection), a human osteosarcoma cell line, were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics. Cell lines were centrifuged at 700g for 30 min and were incubated overnight at 37°C. For 1 week, the cultures were examined daily for cytotoxic effect, and the cell suspension was frozen and thawed to release cell-associated virus when infection was complete. The resulting virus preparation was stored at −80°C. The virus was typed using an immunofluorescence assay, according to the manufacturer’s recommendations (DAKO). Two plaques, representing the clonal isolates of N4-1 and N4-2, were picked randomly and purified 3 times, to homogeneity, for characterization.

RESULTS

Patient history. A 71-year-old immunocompetent man who had diabetes mellitus and disciform herpetic keratitis had presented to the hospital with recurrent episodes of disciform herpetic keratitis (episodes 1–5) and epithelial keratopathy (episodes 6–8) since 1993. Acute therapy (duration, ≤7 days) with topical dexamethasone and acyclovir (both given 5 times per day) was routinely used to manage the corneal inflammation and viral infection (figure 1). To minimize recurrences while the patient was receiving steroid treatment, maintenance therapy (long-term topical application of acyclovir at a lower dose and frequency than that used during acute therapy) was used as a preventative measure after episodes 3 (treatment with topical acyclovir and dexamethasone for 9 months), 7 (treatment with topical triflurorothymidine and dexamethasone for 2 months), and 8 (treatment with oral acyclovir and dexameth-
Figure 1. Treatment history for a patient with herpes simplex virus isolate N4. This schematic displays the treatment courses for recurrences of herpetic disciform keratopathy (episodes 1–5) and epithelial keratopathy (episodes 6–8) since 1993. Treatment therapies included topical or oral acyclovir (ACV), topical dexamethasone (Dex), topical trifluorothymidine (TFT), and iv foscarnet solution applied topically. The isolate characterized in this article was recovered during episode 8. Dashed line, acute therapy (duration, ≤7 days); solid line, longer-term maintenance therapy (duration of treatment is given in detail in the “Results” section); ■, points in time when the patient was nonresponsive to topical acyclovir treatment and when therapy with acyclovir was discontinued.

Table 1. Antiviral susceptibility of herpes simplex virus type 1 (HSV-1) N4, as determined by use of the plaque reduction assay in MRC-5 cells.

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<th>Virus</th>
<th>Penciclovir</th>
<th>Acyclovir</th>
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<tbody>
<tr>
<td>HSV-1 F</td>
<td>0.14</td>
<td>0.06</td>
</tr>
<tr>
<td>HSV-1 N4b</td>
<td>0.53</td>
<td>0.89</td>
</tr>
<tr>
<td>HSV-1 SLU360c</td>
<td>9.7</td>
<td>0.81</td>
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NOTE. IC<sub>50</sub>, 50% inhibitory concentration.

a Acyclovir-susceptible wild-type strain.
b Test sample.
c Acyclovir-resistant, thymidine kinase–deficient strain.

asone for 1 year). Resistance to acyclovir was suspected during 2 of the epithelial recurrences (episodes 6 and 8) because of the failure of treatment to impact lesion pathology and clearance effectively (figure 1). Treatment with trifluorothymidine was initiated in these instances. The virus isolate characterized here was cultured during episode 8, during which time the patient was nonresponsive to treatment with topical acyclovir ointment (Zovirax) and trifluorothymidine therapy.

PRA. An HSV-1 isolate, HSV-1 N4, was recovered from the cornea of the aforementioned patient, who had not responded to acyclovir treatment; the isolate was subsequently cultured. The isolate was then tested for susceptibility to acyclovir and penciclovir by use of the PRA. HSV-1 F and HSV-1 SLU360, 2 control viruses that represent known acyclovirus-susceptible and acyclovir-resistant virus standards, respectively, were tested in parallel with HSV-1 N4. To define an isolate as “drug resistant in MRC-5 cells,” it must have an IC<sub>50</sub> of >2.0 μg/mL, according to the established breakpoint for defining acyclovir resistance. Alternatively, according to the aforementioned “10-fold criterion,” if one uses HSV-1 F as the drug-susceptible control strain, the IC<sub>50</sub> of acyclovir must be >0.6 μg/mL and the IC<sub>50</sub> of penciclovir must be 1.4 μg/mL, given that the IC<sub>50</sub>s for HSV-1 F were 0.06 μg/mL for acyclovir and 0.14 μg/mL for penciclovir.

HSV-1 N4 IC<sub>50</sub>s were <2.0 μg/mL for both acyclovir and penciclovir in MRC-5 cells (table 1). By use of this same assay, HSV-1 SLU360, which expresses TK activity of <1% (data not shown) and which is characterized as a TK-negative virus, had an IC<sub>50</sub> of acyclovir that was similar to that of HSV-1 N4; however, it was still less than the 2.0 μg/mL breakpoint for acyclovir resistance. The IC<sub>50</sub>s of penciclovir also remained less than the breakpoints of 2.0 μg/mL and 10-fold resistance. As has been reported in studies published elsewhere [19], the IC<sub>50</sub>s...
of penciclovir were generally greater than those of acyclovir when tested in MRC-5 cells. Furthermore, HSV-1 N4 remained highly susceptible to bromovinyldeoxyuridine, another TK-activated antiviral, with IC_{50} values of <0.01 \mu g/mL. PRA analyses of the 2 clonal isolates from HSV-1 N4 (N4-1 and N4-2) revealed that IC_{50} values of acyclovir were >6.25 \mu g/mL, whereas the IC_{50} of penciclovir for the same samples remained <2.0 \mu g/mL (data not shown).

**Biochemical assays.** To determine whether HSV-1 N4 or the clonal isolates N4-1 and N4-2 expressed a mutant TK polypeptide, viral gene expression was examined by means of Western blot analysis. Total cell lysates that appeared after virus infection were probed with antiserum specific to the viral TK polypeptide (figure 2). HSV-1 N4 and the clonal isolates produced a full-length 43-kDa protein that reacted with TK antiserum and that was similar in size and expression level to the protein generated from an acyclovir-susceptible HSV-1 F virus infection. A parallel blot that used antiserum against the viral DNA Pol showed that levels of polymerase expressed by the drug-susceptible and test viruses were similar (data not shown). Viruses that contained frameshift mutations in the tk gene would be expected to express truncated TK and/or extended products, although acyclovir resistance can also occur by virtue of point mutations within the tk coding region without altering the size or expression level of the polypeptide.

Most acyclovir-resistant mutations that have been characterized to date are TK negative. Accordingly, a TK biochemical assay was performed to determine whether HSV-1 N4 produced a functional TK protein. Mock infection with TK-negative–infected human osteosarcoma 143 cells and extracts of cells that were infected with the known TK-negative deletion mutation, HSV-1 SLU360 [20], were below the limit of detection of the TK assay (TK activity, <0.3%). Extracts of cells infected with wild-type HSV-1 F control virus were set at 100%. HSV-1 N4 and the 2 clonal isolates (N4-1 and N4-2) phosphorylated thymidine at levels similar to those of wild-type HSV-1 (figure 1). Specifically, the HSV-1 N4 mixture exhibited 99% TK activity; the clonal isolates and wild-type HSV-1 exhibited 82% and 74% TK activity, respectively.

The ability to phosphorylate thymidine is an indicator of TK function and primary phosphorylation. HSV-1 N4 was susceptible to bromovinyldeoxyuridine, which suggests that HSV-1 N4 is competent either at transferring the second phosphate group onto this antiviral agent or at secondary phosphorylation. However, it remained possible that the potential for impairment within the nucleoside phosphorylation–activation pathway could be acyclovir specific. To address this possibility, the ability of the HSV-1 N4 TK to phosphorylate 3H-labeled acyclovir and penciclovir was examined. Drug-susceptible HSV-1 phosphorylated acyclovir 5-fold more effectively than did the HSV-1 N4 isolate or the 2 plaque-purified clonal isolates, which indicates that HSV-1 N4 expresses an altered TK protein product with impaired acyclovir recognition. HSV-1 N4 did not identifiably differ from drug-susceptible HSV-1 with regard to phosphorylation of penciclovir.

Sequence determination of the tk coding region from the 2 clonal isolates showed 2 amino acid substitutions, compared with the tk genes from drug-susceptible HSV-1 F strains detailed in GenBank (http://www3.ncbi.nlm.nih.gov/). In HSV-1 N4-1 and N4-2, the methionine at residue 128 had changed to a leucine and the glycine at residue 240 had modified to glutamic acid. These changes are not directly within the proposed adenosine triphosphate or nucleoside-binding site domains of TK; nonetheless, they may alter acyclovir susceptibility, as indicated by poor 3H-acyclovir phosphorylation.

To assess whether these 2 residue substitutions were solely responsible for contributing to the acyclovir-resistant phenotype, a PRA was performed using HSV TK–expressing cells. Cells that constitutively expressed the HSV TK polypeptide can compensate for mutant viral TK, and PRAs performed using such cells can provide an immediate indication as to whether the Pol protein contains a mutation(s) that directly alters acyclovir resistance. The IC_{50} for HSV-1 N4 and the clonal isolates, which expressed functional TK activity in the biochemical assay, did not completely rescue susceptibility back to wild-type levels in HSV TK+ cells. This is unlike HSV-1 SLU360, which is known to be resistant to acyclovir because of production of a defective TK protein (table 2). Therefore, it remains possible that mutation(s) within the HSV-1 N4 pol coding region may confer additional partial resistance to acyclovir.

![Figure 2](https://example.com/figure2.png)
**Recombinant polymerase virus.** Sequence analysis of the pol coding region of the 2 clonal isolates identified 7 amino acid differences, compared with the following wild-type HSV-1 viruses detailed in GenBank (presented as wild-type amino acid, residue number, mutant amino acid): E-258-V, S-522-N, G-524-D, S-593-L, T-1086-M, P-1124-H, and A-1184-T. None of these changes reside in the 6 highly conserved regions of protein sequence similarity shared by HSV Pol and other DNA polymerases [21]. However, the serine-to-leucine change at residue 593 is located within region A, which is particularly conserved among DNA polymerases that are susceptible to certain antiviral drugs [21]. Because the TK-expressing cells only partially rescued the acyclovir-resistant phenotype, which suggests that HSV-1 N4 contains mutations in tk and pol genes, there was generation of a recombinant virus in which the LacZ–disrupted polymerase of HSV-1 HP66 was replaced with the HSV-1 N4-2 pol coding region to create a functional virus. Sequencing of the pol coding region from this recombinant confirmed that the residue changes present in N4-2 were maintained. Surprisingly, this recombinant virus was susceptible to both acyclovir and penciclovir (IC_{50}, <2.0 μg/mL), as determined using the PRA in MRC-5 cells. In addition, the virus was susceptible to foscarnet and bromovinyldeoxyuridine (data not shown).

**DISCUSSION**

The PRA continues to be the “gold standard” for susceptibility testing of HSV. For acyclovir, a correlation between in vitro IC_{50} and clinical outcome has been established [9]. An HSV-1 isolate was recovered from a patient who had herpes keratitis, cultured, and then tested using PRA; it was found to have an IC_{50} of acyclovir that was less than the accepted breakpoint for resistance (2.0 μg/mL). Acyclovir treatment did not resolve the infection, especially because it can be difficult to deliver antiviral concentrations that effectively treat ocular infections. Moreover, concurrent treatment with dexamethasone, in addition to subtherapeutic local antiviral concentrations, may have contributed to the selection of clinically resistant HSV. To address the apparent discrepancy between in vivo outcome and in vitro testing data, the clinical isolate and 2 clonal isolates were characterized for biological activity.

The extent of the increase in antiviral susceptibility between a drug-susceptible control virus and test viruses is generally predictive of clinical response to drugs [9]. A ∼4-fold increase in resistance (compared with drug-susceptible HSV) was found to be clinical correlation for treatment outcome [9]. The HSV-1 N4 clinical isolate was almost 15-fold more resistant to acyclovir than was the drug-susceptible control strain, HSV-1 F. Therefore, the failure of the HSV-1 N4 infection to respond to acyclovir and the difference in the extent of the increase in susceptibility (compared with wild-type HSV) together suggest that this isolate may be resistant to acyclovir.

Biochemical and in vitro susceptibility assays demonstrated that the clonal isolates (HSV-1 N4-1 and N4-2) were, in fact, resistant to acyclovir, with IC_{50s} that were significantly greater than 2.0 μg/mL. Although HSV-1 N4 showed a small deficiency in the ability to phosphorylate thymidine, phosphorylation of acyclovir was 5-fold less efficient than that of the wild-type virus. This TK-altered property of HSV-1 N4 can only partially explain the acyclovir-resistant phenotype, because TK-expressing cells did not completely rescue the defect(s). The susceptibility of HSV-1 N4 to acyclovir was similar in both MRC-5 cells and TK-expressing cells (table 1), whereas the acyclovir-resistant HSV-1 N4 variant SLU360, which expressed <1% TK activity, had an 8-fold lower IC_{50} in TK-expressing cells. Overall, these results suggest that either (1) the TK-altered protein produced by HSV-1 N4 functions in a dominant-negative manner, perhaps by dimerizing with and disrupting the wild-type TK activity present in Δ tk cells [22, 23]; and/or (2) the pol coding region also contributes to the acyclovir-resistant phenotype.

Sequence analysis identified mutations within the Pol of the 2 clonal isolates, and one of these mutations was within a region associated with sensitivity to antiviral agents (region A). It has been suggested that region A interacts with drugs and substrates to render viral DNA polymerases more susceptible than human Pol α to various antiviral drugs [21]. However, the HSV-1 N4 Pol recombinant virus remained susceptible to acyclovir and penciclovir, and this result is not compatible with HSV-1 N4 acyclovir resistance caused by an altered Pol interaction with acyclovir-triphosphate. Therefore, the biological mechanism to explain the observed antiviral resistance of the HSV-1 N4 isolates is unclear at present time. However, the data do suggest that either (1) the interplay of both mutated proteins (from HSV-1 N4) with acyclovir results in resistance that is sufficient enough to impair the clinical effect of therapy, or (2) resistance

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<tr>
<td>HSV-1 Fa</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>HSV-1 N4</td>
<td>0.09</td>
<td>0.67</td>
</tr>
<tr>
<td>HSV-1 N4-1</td>
<td>0.09</td>
<td>0.48</td>
</tr>
<tr>
<td>HSV-1 N4-2</td>
<td>0.08</td>
<td>0.39</td>
</tr>
<tr>
<td>HSV-1 SLU360c</td>
<td>0.04</td>
<td>0.10</td>
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</table>

**NOTE.** IC_{50}, 50% inhibitory concentration.

a Acyclovir-susceptible wild-type strain.
b Test sample.
c Acyclovir-resistant thymidine kinase–deficient strain.
of HSV-1 N4 to acyclovir is mediated by a region of the viral genome distinct from those previously proven to mediate resistance, namely \( tk \) and \( pol \).

The inability of the patient’s HSV-1 N4 lesion to respond to acyclovir treatment during episode 8 could be attributed either to (1) antiviral resistance or (2) an inability to achieve sufficient concentrations within the ocular infection to impact viral replication. The data we present suggest that susceptibility testing without implementation of appropriate control standards within each individual assay could result in the underidentification of drug-resistant viruses. Application of stringent resistance criteria, such as use of an IC\(_{50}\) that is 10-fold greater than that of a drug-susceptible control virus within a particular assay, in addition to the standard 2.0 \( \mu \)g/mL breakpoint, may help ensure that clinical laboratories sufficiently identify antiviral-resistant isolates, especially in the immunocompromised patient population. Furthermore, it would help facilitate improved disease management. Additional studies that would use clinical isolates (HSV-1 and HSV-2) are necessary to evaluate the impact of the proposed testing criteria on the detection of antiviral-resistant strains of HSV.

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