Acyclovir Prophylaxis Predisposes to Antiviral-Resistant Recurrent Herpetic Keratitis

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(See the editorial commentary by James and Prichard on pages 1353–55.)

Purpose. Long-term acyclovir (ACV) prophylaxis, recommended to prevent recurrent herpes simplex virus type 1 (HSV-1) ocular disorders, may pose a risk for ACV-refractory disease due to ACV resistance. We determined the effect of ACV prophylaxis on the prevalence of corneal ACV-resistant (ACVR) HSV-1 and clinical consequences thereof in patients with recurrent HSV-1 keratitis (rHK).

Methods. Frequencies of ACVR viruses were determined in 169 corneal HSV-1 isolates from 78 rHK patients with a history of stromal disease. The isolates' ACV susceptibility profiles were correlated with clinical parameters to identify risk factors predisposing to ACVR rHK.

Results. Corneal HSV-1 isolates with >28% ACVR viruses were defined as ACVR isolates. Forty-four isolates (26%) were ACV-resistant. Multivariate analyses identified long-term ACV prophylaxis (≥12 months) (odds ratio [OR] 3.42; 95% confidence interval [CI], 1.32–8.87) and recurrence duration of ≥45 days (OR 2.23; 95% CI, 1.02–4.87), indicative of ACV-refractory disease, as independent risk factors for ACVR isolates. Moreover, a corneal ACVR isolate was a risk factor for ACV-refractory disease (OR 2.28; 95% CI, 1.06–4.89).

Conclusions. The data suggest that long-term ACV prophylaxis predisposes to ACV-refractory disease due to the emergence of corneal ACVR HSV-1. ACV-susceptibility testing is warranted during follow-up of rHK patients.

Keywords. recurrent keratitis; retrospective study; patients; acyclovir prophylaxis; acyclovir resistance; refractory disease; risk factors.

Herpes simplex virus type 1 (HSV-1) is a human alpha-herpesvirus that is endemic worldwide. The virus is typically acquired during early childhood via the orofacial route, leading to the establishment of a life-long latent infection of neurons located within the trigeminal ganglia. Intermittent reactivations lead to virus shedding and occasionally to recurrent disease [1]. HSV-1 causes a variety of diseases, ranging from mild herpes labialis to sight-threatening eye diseases [1]. Corneal HSV-1 infections, referred to as herpetic keratitis (HK), are a common infectious cause of visual impairment mainly due to their recurrent nature [2, 3]. HK manifests as infectious epithelial keratitis (IEK), which is characterized by superficial viral replication, or it can infect the underlying corneal stroma and cause herpetic stromal keratitis (HSK) [2]. Recurrent HSK may result in corneal blindness [4].

The drug of choice to treat HSV-1 infections is acyclovir (ACV). ACV is a prodrug selectively converted by HSV-1 thymidine kinase (TK) to ACV-monophosphate, allowing subsequent conversions by cellular kinases to its active form ACV-triphosphate, which blocks viral replication [3]. ACV is generally well tolerated, and its widespread use has resulted in a significant reduction in visual impairment caused by HSV-1-induced ocular diseases [1, 3]. However, ACV treatment may lead to the emergence of ACV-resistant (ACVR) HSV-1. All clinical HSV-1 isolates contain ACVR viruses, and the frequency of these viruses determines the isolate's ACV susceptibility phenotype: ACVR or ACV-sensitive (ACVS) [5–7].

The prevalence of ACVR HSV-1 is higher among
immunocompromised patients (up to 30%) compared with healthy individuals (<1%) and may cause severe HSV-1 disease if patients are not switched promptly to alternative antiviral therapies, including ganciclovir and TK-independent drugs such as foscarnet [8, 9].

In addition to symptomatic treatment, ACV prophylaxis is commonly prescribed for the prevention of recurrent herpetic eye diseases. The Herpetic Eye Disease Study group reported that ACV prophylaxis (twice daily, oral, 400 mg, for 12 months) significantly reduced the recurrence rate of both IEK and HSK in immunocompetent HK patients who had a disease episode within the preceding year [10, 11]. However, long-term ACV prophylaxis may pose a risk for ACV-refractory keratitis due to the selection for corneal ACV\textsuperscript{R} HSV-1 [5, 7, 12, 13]. In the current study, we determined the effect of ACV prophylaxis on the prevalence of ACV\textsuperscript{R} corneal HSV-1 and the clinical consequences in recurrent HK (rHK) patients with a history of stromal disease.

**MATERIALS AND METHODS**

**Patients and Clinical Specimens**

At the Rotterdam Eye Hospital (REH; Rotterdam, The Netherlands), between 1981 and 2011, 169 corneal swabs from 78 immunocompetent rHK patients with a long history of stromal disease were obtained for diagnostic purposes; the swabs were stored at −80°C. The median follow-up time at the REH for rHK patients included in our study was 17.6 years with an interquartile range (IQR) of 12.2–30.1 years. The virus was grown and typed for HSV-1 as described previously [5]. HK was classified based on clinical criteria [2, 3]. Each enrolled patient provided consent for storage of their HSV-1 isolates for future testing. The study was performed according to the tenets of the Declaration of Helsinki and approved by the local ethics committee. Written informed consent for use of the archived HSV-1 isolates for the current study was obtained from all patients or, in case of death of the patient at start of the study, by the patient’s closest living relative.

**Study Design**

Information on ACV treatment duration and dose was recorded for all patients in this retrospective study. Scored parameters were age, gender, affected eye, sampling date, length of disease recurrence sampled, visual acuity (best spectacle-corrected vision), and ocular disease entity at presentation and at end of follow-up. Long-term ACV prophylaxis was defined as ≥12 months systemic ACV therapy, ranging from 200 to 4000 mg daily, which was prescribed to rHK patients with healed corneal lesions in response to a treatment for a prior disease recurrence [10]. The format of prophylaxis provided to the patients was ACV or valacyclovir. Corneal isolate dates were stratified according to before and after 2000. ACV prophylaxis was introduced as standard care for rHK patients at the REH in 2000.

The start of an HSV-1 keratitis recurrence was defined as the presentation of an isolated IEK characterized by a dendritic or geographic corneal ulcer or clinical symptoms related to HSK pathology, including cell infiltrates in corneal stroma or anterior chamber and corneal stroma thinning, edema, vascularization, or cornea pseudoguttata [2, 14]. The end date of an isolated IEK was defined as the date of complete closure of the corneal epithelium, after topical ACV treatment was discontinued. In case of an HSK-associated recurrence, the end date of a recrudescence HSV-1 epithelial defect was defined as the date of complete closure of the corneal epithelium combined with the resolution of the aforementioned HSK-associated clinical parameters observed at the start of the disease recurrence. This combined with the return to both prerreurrence topical steroid treatment (ie, dexamethasone phosphate 0.1% or fluorometholone) and ACV prophylaxis. Whereas IEK lesions typically heal within 2 weeks with ACV treatment [15], HSV-1 epithelial defects in corneas of patients with a history of stromal disease require combined treatment using both ACV and steroids. In the latter patient group, resolution of corneal lesions generally takes longer than 3–4 weeks (Remeijer, L; unpublished data). Consequently, we considered disease recurrence duration to be ≥45 days, which is >3 times the normal duration of an isolated IEK episode (ie, 2 weeks), as ACV-refractory HK. The ACV susceptibility profile of the corneal isolates was unknown at the time of ACV prescription, and the authors did not have access to the results of the ACV susceptibility assays during assembly of the clinical data.

**ACV Susceptibility Assays**

The percentage of ACV\textsuperscript{R} viruses in corneal HSV-1 isolates was determined by the plating efficiency assay as described elsewhere [16, 17]. Briefly, serial 10-fold dilutions of corneal HSV-1 isolates were inoculated onto monkey kidney cells (ie, Vero cells) in culture medium consisting of Dulbecco’s modified eagle medium supplemented with 5% heat-inactivated fetal bovine serum and antibiotics (all from Gibco). After adsorption at 37°C for 1 hour, the inoculum was removed and cells were overlaid with culture medium containing 2% v/v methylcellulose (Sigma) supplemented with no ACV or 20 µmol/L ACV (Roche) and cultured for 3 days and 10 days at 37°C, respectively. The ACV concentration and culture period were chosen to ensure that only preexisting ACV\textsuperscript{R} viruses formed plaques [5]. The percentage of ACV\textsuperscript{R} viruses in each isolate was calculated by dividing the number of virus plaques obtained in the presence of 20 µmol/L ACV by the number of plaques obtained after culture in the absence of ACV [5, 7].

The overall ACV susceptibility phenotype of a selected set of corneal isolates was determined by an HSV-1–specific real-time quantitative polymerase chain reaction (qPCR) assay as
described elsewhere [12, 18]. In brief, Vero cells were infected with 100-fold diluted corneal HSV-1 isolates. After adsorption at 37°C for 1 hour, the inoculum was removed and cells were incubated, in triplicate, with different concentrations of ACV diluted in culture medium. Twenty-four hours after inoculation, cells were lysed and the viral DNA load in the cell lysate was determined by an HSV-1–specific qPCR as described elsewhere [12, 18]. The median ACV inhibitory concentration (IC_{50}) of each isolate was defined as the concentration of ACV that reduced the number of viral copies by 50% compared with the control-infected cells without ACV. Isolates were considered ACVR at >1 µmol/L [12, 18].

**Statistical Analyses**

All statistical analyses were run using SPSS software (IBM SPSS Statistics 20). A 1-phase exponential association approach was used to assess the correlation between data obtained by qPCR IC_{50} and plating efficiency assays. The Mann–Whitney test was used to compare gender, visual acuity, follow-up time, and recurrence rates between patient groups. Spearman correlation tests were used to assess correlations among the percentage of ACVR viruses in corneal HSV-1 isolates, duration of disease recurrence, isolate date, and the duration of ACV prophylaxis before corneal sampling. Generalized estimating equations tests were used to identify risk factors. All statistically significant covariates from the univariate analyses were included for multivariate testing. Wilcoxon signed ranks test was used to compare the ACV susceptibility phenotype of sequential isolates. All statistical tests were significant when the P value was < .05.

**RESULTS**

To gain insight into the effect of long-term ACV prophylaxis on the prevalence of corneal ACVR HSV-1, we determined the frequencies of ACVR viruses in 169 corneal HSV-1 isolates from 78 immunocompetent rHK patients. Disease severity at time of sampling varied from mild IEK to necrotizing stromal keratitis. Thirty-five of 78 patients (44.5%) were female, the overall median age at presentation was 59.3 years (IQR 45.2–70.1 years), and 43.6% of affected eyes were left eyes. For 70 patients, 2 (n = 53) or at least 3 (n = 17) sequential isolates obtained during an HK recurrence from the same cornea were available for ACV susceptibility testing. The mean time interval between sequential isolates was 4.8 years (range 0.1–22.3 years). Nineteen patients (24.4%) did not receive ACV prophylaxis in the 12 months prior to sequential sampling of their corneas and are hereafter referred to as ACV treatment-naive rHK patients. For patients receiving ACV prophylaxis within 12 months prior to sampling, the mean time on therapy was 21.5 months (range 0.7–178.4 months). The median duration of disease recurrence was 41 days (IQR 24–70 days) and the median recurrence rate was 0.9 recurrences per year (IQR 0.6–1.2 recurrences/year). Sixty-nine patients received topical steroid therapy (93.2%) at the time of sampling; the steroid treatment regimen for 5 patients was unknown.

**Prevalence of ACVR HSV-1 in Corneal Isolates From rHK Patients**

We determined the percentage of ACVR viruses in all cornea isolates by the plating efficiency assay [16, 17]. The threshold of the percentage of ACVR viruses resulting in an overall ACVR phenotype of the corresponding isolate was determined by measuring the ACV IC_{50} values of a subset of corneal HSV-1 isolates (n = 36) by an HSV-1–specific qPCR-based assay. The IC_{50} cutoff value for ACVR isolates has previously been defined as >1 µmol/L ACV [12, 18]. The combined data demonstrated that isolates with >28% ACVR viruses had an overall ACVR phenotype (Figure 1A). In total, 44 of 169 (26%) corneal HSV-1 isolates were defined as ACV resistant. Uniform sequential ACVR isolates were identified in 8 patients and ACV^S isolates were identified in 43 patients. ACV susceptibility changed over time from ACV^S to ACVR in 12 patients or ACVR^S to ACV^S in 9 patients. Twenty-seven patients (34.6%) had an ACVR HSV-1 isolate at least once.

**Identification of Risk Factors for Corneal ACVR HSV-1**

Statistical analyses identified parameters that were not associated with corneal ACVR HSV-1. These factors were interrecurrence interval (P = .56), recurrence rate (P = .22), age (P = .30), and gender (P = .74). Patients were stratified based on clinical picture. Stromal involvement at time of corneal sampling was not significantly associated with corneal ACVR HSV-1 (P = .17).

Several clinical parameters were associated with corneal ACVR HSV-1 (Figure 1 and Table 1). A weak correlation was observed between the duration of ACV prophylaxis before corneal sampling and the percentage of ACVR viruses in the corresponding corneal HSV-1 isolates (Figure 1B) and the duration of the sampled recurrence (Figure 1C). Additionally, the percentage of ACVR viruses in corneal HSV-1 isolates was weakly correlated with the duration of the disease recurrence sampled (Figure 1D) and the year in which the corresponding corneal HSV-1 isolate was obtained (Figure 1E). Univariate analyses revealed the following predisposing risk factors: isolate date after 2000, duration of disease recurrence for ≥45 days, and long-term ACV prophylaxis (Table 1). Multivariate analyses identified both duration of disease recurrence of ≥45 days (odds ratio [OR] 2.23; 95% confidence interval [CI], 1.02–4.87) and long-term ACV prophylaxis prior to corneal sampling (OR 3.42; 95% CI, 1.32–8.87) as independent risk factors for an ACVR phenotype of a corneal isolate (Table 1).

Next, we analyzed parameters associated with ACV-refractory rHK, herein defined as duration of disease recurrence of ≥45 days. Univariate analyses demonstrated that the isolate date after 2000, long-term ACV prophylaxis prior to corneal sampling,
and a corneal ACVR HSV-1 isolate were risk factors for ACV-refractory rHK (Table 2). Multivariate analyses identified the ACV phenotype of a corneal HSV-1 isolate as an independent risk factor for ACV-refractory rHK (OR 2.28; 95% CI, 1.06–4.89; Table 2). The data suggest long-term ACV prophylaxis as a risk factor for corneal ACVR HSV-1. Subsequent detection of ACVR HSV-1 in rHK is a risk factor for ACV-refractory disease.

Longitudinal Effect of ACV Prophylaxis on ACVR Corneal HSV-1
We determined the longitudinal effect of ACV prophylaxis on the ACV susceptibility phenotype of sequential corneal HSV-1 isolates for the individual rHK patients. The ACV susceptibility phenotype of sequential isolates did not change significantly in rHK patients who were either ACV treatment naive (n = 25, P = .40) or received ACV prophylaxis on all sampling dates (n = 24, P = .68). For 24 patients (30.8%), corneal HSV-1 isolates were obtained during alternating ACV treatment regimens. The prevalence of corneal ACVR HSV-1 did not change in patients from whom only the first isolate was obtained during ACV prophylaxis (n = 6, P = .44). Notably, patients who initially were ACV treatment naive and had ACV prophylaxis during consecutive sampling dates had an increased prevalence of corneal ACVR HSV-1 (n = 18, P = .02). The data suggest that ACV prophylaxis of rHK patients predisposes them for corneal ACVR HSV-1 over time.

DISCUSSION
ACV prophylaxis significantly reduces the recurrence rate of HK [10]. This is of particular benefit to HK patients with a
Abbreviations: ACV, acyclovir; ACVR, ACV resistant; ACVS, ACV-sensitive; CI, confidence interval; HSV-1, herpes simplex virus type 1; OR, odds ratio.

history of stromal involvement as they are at increased risk of developing recurrent HSK, which can lead to corneal blindness [11]. The current study suggests that long-term ACV prophylaxis is an important risk factor for ACV-refractory rHK due to the emergence of corneal ACV R HSV-1. Thirty-four of 76 (44.7%) corneal HSV-1 isolates obtained during a breakthrough recurrence from rHK patients who received >12 months ACV prophylaxis were ACV R (Figure 1B).

Previous reports on immunocompetent patients with nonocular HSV-1 infections demonstrated that antiviral treatment was not associated with an increased risk for the emergence of ACV R HSV-1 [13, 19–21]. Nonetheless, ACV R HSV-1 isolates have been obtained after prolonged ACV treatment and appeared to correlate with treatment failure [22, 23]. Moreover, ACV resistance can revert when therapy is discontinued [22]. Kriese et al noted that ACV resistance in an immunocompetent individual developed in the setting of immunosuppressive drugs and suboptimal ACV treatment [22]. The host’s immune status and, in case of HK, the infected organ most likely contribute to the discrepancies found. In otherwise healthy individuals with nonocular HSV-1 infections, such as herpes labialis and genital herpes, virus replication in infected tissues is largely controlled by local immune responses in which ACV therapy has a supportive role [19–21, 24, 25]. In contrast to the oral and genital mucosa, the cornea is an immunoprivileged site where local immune responses are reduced, thereby preserving visual acuity [26]. Consequently, the role of a local immune response in controlling corneal infections is more restricted, resulting in a favorable environment for an ACV therapy–driven enrichment of corneal ACV R HSV-1 [26].

Asymptomatic shedding of infectious HSV-1 and HSV-1 DNA in tears of healthy individuals without a history of herpetic ocular diseases has been described [27–29]. A recent study performed on tear samples collected twice daily for 30 consecutive days demonstrated HSV-1 DNA in tears of 47 (94%) of 50 healthy study participants at least once [29]. Studies document that HSV-1 reactivation more commonly leads to asymptomatic corneal HSV-1 shedding compared with concomal disease [27–29]. Notably, valacyclovir treatment (500 mg daily) of healthy individuals did not reduce the asymptomatic HSV-1 shedding rate and viral DNA load in tears during treatment for 30 days [30]. Corneal HSV-1 isolates contain a

Table 1. Risk Factors for Acyclovir-Resistant Corneal Herpes Simplex Virus Type 1 Isolates of Patients With Recurrent Herpetic Keratitis

<table>
<thead>
<tr>
<th>Variablea</th>
<th>ACV S Isolatesb</th>
<th>ACV R Isolatesb</th>
<th>OR 95% CI</th>
<th>PValue</th>
<th>OR 95% CI</th>
<th>PValue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate obtained after 2000</td>
<td>59 (48%)</td>
<td>34 (79%)</td>
<td>4.16 1.43–12.11</td>
<td>.009</td>
<td>2.69 .91–7.93</td>
<td>.073</td>
</tr>
<tr>
<td>Duration of disease recurrence ≥45 d</td>
<td>41 (33%)</td>
<td>27 (63%)</td>
<td>3.00 1.53–5.91</td>
<td>.001</td>
<td>2.23 1.02–4.87</td>
<td>.045</td>
</tr>
<tr>
<td>Long-term ACV prophylaxis</td>
<td>31 (25%)</td>
<td>26 (61%)</td>
<td>4.38 1.81–10.60</td>
<td>.001</td>
<td>3.42 1.32–8.87</td>
<td>.012</td>
</tr>
</tbody>
</table>

ACV resistance of corneal HSV-1 isolates was determined by the plating efficiency assay. Isolates containing >28% ACV S viruses were considered ACV resistant.

a Long-term ACV prophylaxis refers to systemic ACV use for ≥12 months before date of corneal sampling.

b Number and percentage of corneal HSV-1 isolates with the indicated ACV susceptibility phenotype. Paired clinical parameters were available for 123 ACVS and 43 ACVR HSV-1 isolates.

c Generalized estimating equations were used to determine the ORs. The 95% CIs and P values are shown.

Table 2. Risk Factors for a Disease Recurrence Duration of >45 Days

<table>
<thead>
<tr>
<th>Variablea</th>
<th>Recurrence &lt;45 d</th>
<th>Recurrence ≥45 d</th>
<th>Univariatec</th>
<th>Multivariatec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate obtained after 2000</td>
<td>45 (50%)</td>
<td>46 (68%)</td>
<td>2.09 .48–9.21</td>
<td>.049</td>
</tr>
<tr>
<td>Long-term ACV prophylaxis</td>
<td>27 (30%)</td>
<td>30 (44%)</td>
<td>1.81 .93–3.55</td>
<td>.083</td>
</tr>
<tr>
<td>ACV R HSV-1 isolate</td>
<td>16 (18%)</td>
<td>27 (40%)</td>
<td>3.00 1.53–5.91</td>
<td>.001</td>
</tr>
</tbody>
</table>

Abbreviations: ACV, acyclovir; ACV R, ACV resistant; CI, confidence interval; HSV-1, herpes simplex virus type 1; OR, odds ratio.

a Long-term ACV prophylaxis refers to systemic ACV use for ≥12 months before date of corneal sampling. ACV resistance of corneal HSV-1 isolates was determined by the plating efficiency assay. Isolates containing >28% ACV S viruses were considered ACV resistant.

b Paired clinical and laboratory parameters were available for 90 and 68 corneal HSV-1 isolates obtained from patients with the indicated disease recurrence duration.

c Generalized estimating equations were used to determine the ORs. The 95% CIs and P values are shown.
mixed virus population, consisting of both ACV$_S$ and ACV$_R$ genetically related variants [5]. Consequently, ACV prophylaxis may lead to the enrichment of ACV$_R$ HSV-1 during asymptomatic corneal shedding and subsequently to the development of ACV-refractory rHK. The presence of latent ACV$_R$ HSV-1 in the cornea-innervating trigeminal ganglion poses a life-long risk of subclinical corneal shedding and symptomatic HK recurrences involving reactivated ACV$_R$ HSV-1 [5,27–30].

Our study has 2 limitations. First, the study design did not allow for verification of patient compliance. Several patients reported adverse effects of long-term ACV prophylaxis, mainly gastrointestinal complaints and drowsiness, which may have affected their adherence to therapy. Patients’ noncompliance can influence the emergence of drug-resistant viruses during therapy [31,32]. Second, all rHK patients included had extensive clinical records indicating frequent rHK episodes with stromal disease and are probably at increased risk for ACV resistance due to their extensive ACV treatment history. Indeed, we noted that about one-third of the patients had an ACVR HSV-1 corneal isolate at least once, which is higher than reported previously for HK patients [5,12]. Furthermore, the inability to detect a correlation between the proportion of corneal ACV$_R$ HSV-1 and the severity of disease at the end of follow-up may also be due to the selection of severe rHK patients (data not shown). Future studies on a larger cohort of rHK patients, including patients with solely HSV-1 epithelial keratitis, are mandatory to address these issues.

In conclusion, the data presented here argue for the rationalized use of long-term ACV prophylaxis for rHK patients. The associated risk of the development of ACV-refractory disease due to the emergence of corneal ACV$_R$ HSV-1 over time should be considered when choosing the appropriate antiviral treatment in rHK patients with a history of stromal disease, a patient group prone to long and extensive antiviral and steroid treatment regimens. Moreover, the unapparent cost effectiveness of ACV prophylaxis to reduce ocular HSV recurrences implies that therapeutic decisions for rHK must be made on a case-to-case basis [33]. When the integrity of the eye is not at risk, the combined data favor the use of discontinuous ACV prophylaxis, that is, stopping treatment after a maximum of 1 year [10,11], and more aggressive therapeutic intervention of a subsequent recurrence. Multidrug therapy that combines different modes of drug actions is common in the treatment of other viral infections [34,35]. Combination therapy with anti-HSV drugs with discordant modes of action, such as systemic and topical ACV combined with topical foscarnet or trifluorothymidine, can be of significance [9]. This will potentially result in a shorter disease duration and might lower the risk of emergence of drug-resistant viruses during therapy. Finally, antiviral drug sensitivity testing of corneal swabs is indicated for rHK patients with recurrent disease during ACV prophylaxis to guide the rationalized selection of the appropriate alternative antiviral agents.

Notes

**Financial support.** This work was financially supported, in part, by Stichting Wetenschappelijk Onderzoek Oogziekenhuis Rotterdam (to F. B. v. L.).

**Potential conflicts of interest.** A. D. M. E. O. is a part-time employee of Viroclincs Biosciences BV (see also www.virosciencelab.org). The stated competing interest does not alter the author’s adherence to the policies on sharing data and materials. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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