Frequency of Acyclovir-Resistant Herpes Simplex Virus in Clinical Specimens and Laboratory Isolates

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The proportion of acyclovir (ACV)-resistant herpes simplex virus (HSV) isolates in clinical specimens and laboratory isolates was determined. HSV isolates in clinical specimens and laboratory isolates were cultured in the absence or presence of 5 µg of ACV per ml. The frequency of ACV-resistant HSV was calculated as (average virus titer in the wells with ACV)/(average virus titer in the wells without ACV). The mutation frequency of HSV type 1 isolates in clinical samples (directly from patient lesions) was $7.5 	imes 10^{-4}$ ± $2.5 	imes 10^{-4}$ (mean ± standard error), and that of HSV type 2 isolates was $15.0 	imes 10^{-4}$ ± $4.6 	imes 10^{-4}$. The mutation frequencies of isolates derived in the laboratory from these clinical samples were very similar. Similarly, the 50% inhibitory concentrations for isolates in clinical samples and laboratory isolates were identical. The frequencies of ACV-resistant HSV types 1 and 2 were in a narrow range of $7.5 	imes 10^{-4}$ to $15.0 	imes 10^{-4}$ among isolates in clinical specimens and did not change for laboratory-derived pools of viral isolates.

Acyclovir (ACV) is widely used for the treatment of primary and recurrent herpes simplex virus (HSV) and varicella-zoster virus infections because of its very favorable therapeutic ratio. Since 1982, $2.0 	imes 10^6$ kg of ACV and other nucleoside analogues has been distributed, with more than 50% of that amount distributed in the United States. ACV is a nucleoside analogue of guanine that is preferentially phosphorylated to ACV monophosphate by viral thymidine kinase and that is then further phosphorylated to ACV triphosphate by cellular enzymes. ACV triphosphate inhibits viral DNA polymerase and is incorporated into viral DNA, ultimately preventing elongation of viral DNA (7). HSV develops resistance predominantly (95%) as a result of mutations in genes that code for thymidine kinase, but resistance can also result from mutations in DNA polymerase (1, 3, 4, 9, 16).

ACV-resistant variants have been isolated from clinical specimens obtained before ACV was introduced (13). These variants are also readily detected in pools of laboratory strains of ACV-sensitive HSV. Mutation frequencies of $2.7 	imes 10^{-6}$ to $1.0 	imes 10^{-3}$ for HSV type 1 and $5.0 	imes 10^{-5}$ to $8.0 	imes 10^{-3}$ for HSV type 2 were detected, and these studies indicate that some proportion of HSV growing in cell culture is always resistant to ACV, even when the inoculum is considered to be ACV-sensitive HSV. Mutation frequencies of 2.7

MATERIALS AND METHODS

Viral specimens. Clinical samples from the Diagnostic Virology Laboratory of the University of Colorado Health Sciences Center were randomly chosen for study. Samples had been stored in a −70°C freezer in the prior year. ACV-resistant and ACV-sensitive strains were used as controls. HSV1S-115 is a diagnostic ACV-sensitive laboratory standard ($2.5 	imes 10^{7}$ PFU/ml) for which the 50% inhibitory concentration (IC50), as determined multiple times (in human fibroblasts), is $984 ± 0.4$ µg/ml. HSV1R-5 ($7.3 	imes 10^{7}$ PFU/ml) is an ACV-resistant standard isolate for which the IC50 is $16.17 ± 8.1$ µg/ml.

Virus isolation. HSV isolates were prepared from clinical specimens submitted to the laboratory by inoculation into tube cultures of human embryonic lung fibroblasts (passaged between 15 and 20 times). Tube cultures were observed for the presence of the HSV cytopathic effect by optical microscopy daily for 5 days. Almost all isolates were identified within 3 days. HSV isolates were confirmed and typed with a fluorescein isothiocyanate-conjugated monoclonal antibody (PathoDx Herpes typing kit; Diagnostic Products Co., Los Angeles, Calif.).

Detection of ACV-resistant virus. Vero cells were obtained from the American Type Culture Collection (ATCC LCL), and six-well plates were seeded with Vero cells ($5 	imes 10^5$ cells per well in 2 ml). Cells were cultured in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO2 for 24 to 48 h until they were confluent. Thawed clinical specimens or virus isolates in 10-fold serial dilutions in DMEM containing 2% FBS were inoculated into quadruplicate wells (0.5 ml/well). After 1 h the inoculum was removed, 2.5 ml of a 0.4% agarose overlay containing DMEM plus FBS and no ACV or 5 µg of ACV per ml was added to duplicate wells, and the plates were incubated at 37°C. The plates were observed daily by optical microscopy until the characteristic HSV cytopathic effect was evident, the
The isolates in clinical specimens and those for laboratory isolates. Correlation of plaque-purified resistant (IC\textsubscript{50}, 5.85 g/ml) and sensitive (IC\textsubscript{50}, 0.61 g/ml) HSV isolates was calculated as (average virus titer in the wells with ACV)/ (average virus titer in the wells without ACV). Standard ACV-resistant and ACV-sensitive strains were included in each assay.

Antiviral susceptibility testing. Antiviral susceptibility testing was performed by PRAs with Vero cells in 12-well culture plates with a 0.4% agarose overlay (18). After the addition of HSV and adsorption (as described above), agarose containing ACV (9, 3, 1, 0.33, or 0 μg/ml) was added to triplicate wells. The cultures were incubated for 2 days, fixed with formalin, and stained with crystal violet as indicated above. The IC\textsubscript{50} was defined as the ACV concentration that reduced the plaque number by 50% from that in the untreated control wells. The IC\textsubscript{50} was calculated graphically from the plaque data. Standard ACV-resistant and ACV-sensitive strains were included in each assay.

Relationship between proportion of mutants in a sample and IC\textsubscript{50}. The titers of plaque-purified resistant (IC\textsubscript{50}, 5.85 μg/ml) and sensitive (IC\textsubscript{50}, 0.61 μg/ml) HSV isolates were determined. A reconstruction experiment was performed by studying these viruses alone and mixtures of the two viruses with proportions of sensitive to resistant virus that varied from 10,000:1 to 1:500. The total virus titer of each mixture was the same. Each mixture was then subjected to simultaneous studying these viruses alone and mixtures of the two viruses with proportions of sensitive to resistant virus that varied from 10,000:1 to 1:500. The total virus titer of each mixture was the same. Each mixture was then subjected to simultaneous

Mutation frequencies for clinical isolates. The average mutation frequency for HSV type 1 was 7.5 × 10\textsuperscript{-4} ± 2.5 × 10\textsuperscript{-4} (mean ± standard error) for virus in clinical specimens and 12 × 10\textsuperscript{-4} ± 4.2 × 10\textsuperscript{-4} for the corresponding laboratory isolates (Table 1). The difference in the mutation frequency between isolates in clinical specimens and laboratory isolates was not significant (P = 0.09). The average mutation frequencies for HSV type 2 in clinical specimens and laboratory isolates of 15.0 × 10\textsuperscript{-4} ± 4.6 × 10\textsuperscript{-4} and 9.3 × 10\textsuperscript{-4} ± 3.0 × 10\textsuperscript{-4}, respectively, were not significantly different (P = 0.06). The difference in mutation rates between HSV type 1 and 2 isolates in clinical specimens or laboratory isolates was not significant (P = 0.08 and 0.27, respectively).

The average IC\textsubscript{50} for HSV type 1 were 0.89 μg/ml for isolates in clinical specimens and 0.85 μg/ml for laboratory isolates. There was no significant difference between these IC\textsubscript{50}s (P = 0.27). The average IC\textsubscript{50} for HSV type 2 isolates in clinical specimens and laboratory isolates were 1.08 and 1.02 μg/ml, respectively, and there was no significant difference (P = 0.27). The difference in average IC\textsubscript{50} between HSV type 1 and type 2 isolates in clinical specimens or laboratory isolates was not significant (P = 0.16 and 0.13, respectively). The mutation frequency was not closely correlated with the average

RESULTS

Defining an assay to determine prevalence of ACV-resistant mutants. ACV-resistant mutants were detected by a modification of the procedure of Hall et al. (10). All HSV isolates or pools of HSV isolates from clinical specimens contained a variety of mutants with intermediate levels of resistance to ACV. These mutants grew slowly in the presence of the inhibitor but could be detected by a plaque assay if sufficient time had elapsed (Fig. 1). Thus, the duration of an assay used to study ACV-resistant mutants in a pool must be sufficiently long to permit detection of most mutants. Our decision to define the mutant phenotype by a 10-day assay was a result of a compro-

![Image](http://jcm.asm.org/)

FIG. 1. Plaque formation in the presence or absence of ACV as a function of time. ■ ACV at 5 μg/ml; □, no ACV.
IC₅₀ (R² values, 0.1059 for HSV type 1 and 0.2314 for HSV type 2). This may be because mutation was always a low-frequency event among the isolates studied, and the IC₅₀ determined by PRA does not change significantly until mutants make up 10% of the inoculum being tested (Fig. 2).

**DISCUSSION**

The susceptibility of HSV to nucleoside analogues is commonly determined by PRAs (12, 17, 18). The determination by a PRA that a clinical isolate is resistant to the test drug requires that a large proportion of the HSV isolates in the pool be resistant to a defined concentration of that drug (Fig. 2). Thus, the PRA will not detect small changes in the proportion of isolates with a mutant phenotype in treated HSV lesions either during the course of therapy or after multiple courses of therapy. Such changes might be important when contacts are exposed to resistant HSV present in the peripheral lesions of an index patient. Similarly, if resistant virus enters the sensory ganglia of an index patient and if resistant virus can reactivate, then selection of resistant mutants in primary lesions might lead to an increase in the proportions of resistant HSV isolates in populations of patients who are likely to receive antiviral therapy.

In order to measure small changes in the proportion of mutant HSV isolates in a virus pool, we modified a method for the enumeration of small numbers of resistant virus in the
presence of large numbers of sensitive virus (10). The application of this assay resulted in two conclusions. First, the proportion of HSV isolates in lesions that might be selected for by the application of anti-HSV therapy is approximately 7.5 × 10⁻⁴ to 15.0 × 10⁻⁴ for both HSV type 1 and HSV type 2. Published results of studies that used similar methods applied to small numbers of laboratory-passaged HSV isolates indicate that the proportions of ACV-resistant HSV mutants are 1.5 × 10⁻⁴ to 10.0 × 10⁻⁴ for HSV type 1 (8, 10) and 0.5 × 10⁻⁴ to 5.0 × 10⁻⁴ for HSV type 2 (13). These isolates had not been exposed to antiviral drugs. Sarisky et al. (15) found that the mean mutation frequency for four HSV type 1 clinical isolates was similar, 3.0 × 10⁻⁴. However, they also found that four HSV type 2 clinical isolates had a mutation frequency of 8.0 × 10⁻³ (15). Their finding of a significantly higher mutation frequency for HSV type 2 may reflect several differences in experimental design. Perhaps most important is their use of a plaque-purified inoculum, whereas we used an inoculum that consisted of clinical isolates that closely represent the typical mix of phenotypes found in humans with HSV disease. We chose this starting point for our experiments in order to determine the resistance profiles that most closely mimic the resistance profiles for isolates in clinical situations. Sarisky et al. (15) also used MRC-5 cells and a different concentration of ACV to define mutants, and the number of clinical isolates that they examined was limited.

Second, the results presented in Table 1 demonstrate that the proportion of mutants present in a clinical specimen is not altered during the one or several cycles of replication that occur during the recovery of HSV in the laboratory in the absence of selection pressure. This being the case, the IC₅₀s for isolates in clinical specimens and their derived isolates were found to be very similar, and either of the values would accurately reflect the presence of mutants in a clinical setting.

ACV is now widely accepted as a safe and effective treatment for the management of HSV infections in normal and immunocompromised patients. A common concern with regard to the widespread use of any antiviral agent is the emergence of resistance. Although the appearance of ACV-resistant HSV was first described in 1982, the prevalence of ACV-resistant isolates has remained stable at less than 1% among immunocompetent hosts in the subsequent 18 years (1, 5). However, these epidemiological surveys were generally performed with isolates from recurrent HSV outbreaks or from primary episodes in patients not treated with antiviral agents. Thus, they may represent isolates that had been “archived” in patients from a preantiviral era. A more sensitive estimate of the trend in antiviral resistance might be obtained by determining the frequency of mutants in patients having their first recurrence after therapy for a primary infection (Y. K. Shin et al., unpublished data). This might also better define the acquisition of resistance in treated immunocompromised patients, particularly those with AIDS or bone marrow transplants, who have a 5 to 10% incidence of resistant HSV after treatment (6, 8, 9, 14). The information on resistance phenotypes presented above provides baseline information for such an endeavor.

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REFERENCES


