A Rapid Assay to Screen for Drug-Resistant Herpes Simplex Virus


Herpes simplex virus (HSV) is a highly prevalent human pathogen that is especially problematic for immunocompromised hosts [1]. A number of antiviral agents have become available, but acyclovir (Glaxo-Wellcome, Research Triangle Park, NC) remains the drug of choice for the treatment of most HSV infections [2]. Resistance to acyclovir can appear during treatment, but because of the self-limited nature of HSV infections in normal hosts, acyclovir resistance is not a clinically significant problem in most cases [3]. However, immunosuppressed patients rely on specific antiviral therapy for clinical improvement, and resistance can emerge, especially when these patients have been taking antiviral agents for long periods [3–5]. In these cases, an alteration in antiviral therapy is necessary for successful outcome; therefore, the identification of acyclovir-resistant isolates has become increasingly important [3, 6].

A number of assays are available to identify HSV isolates that are resistant to antivirals such as acyclovir, but the plaque reduction assay (PRA) is generally considered to be the reference standard [7–10]. A major advantage of the PRA is that it utilizes the exquisite sensitivity of the viral plaque, which is the consequence of a single virus particle initiating infection of a single cell. Unfortunately, the PRA is relatively cumbersome to perform, since it involves growing the virus isolate, determining its titer by plaque assay, and performing the plaque assay in the presence of multiple concentrations of the antiviral. Each of these steps takes 2–3 days, and PRA results are usually not available for 7–10 days, by which time treatment decisions often have been already made on the basis of the clinical response.

Recently, we described a modified PRA based on the use of a genetically engineered Vero cell line that expresses β-galactosidase only after infection with HSV [11]. In this report, we describe our results with a CV1-derived cell line (CV19 cells) that expresses higher levels of β-galactosidase after HSV infection. We tested the performance of the CV19 cells in a rapid screening assay, the modified PRA (ELVIRA; Diagnostic Hybrids, Athens, OH), the format of which allows an isolate to be scored as sensitive or resistant to acyclovir within 48 h of the time the culture is positive [12].

Material and Methods

CV19 cells. CV1 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Clone CV19 was isolated in an identical manner as the previously described VerolCl-P6LacZ cells [11]. CV19 cells were maintained in Dulbecco's MEM supplemented with 10% fetal calf serum and hygromycin B (100 μg/mL; Boehringer Mannheim, Indianapolis).

Viral sensitivity testing. A standard PRA was done by using CV1 cells as previously described [11]. An IC₅₀ of ≥2 mg/mL was used as the cutoff for scoring an isolate as resistant to acyclovir.

The modified PRA was performed as follows: Confluent CV19 cells were prepared in 24-well plates. Twelve wells were used for each virus being tested, so that 2 virus isolates, or 1 isolate and a control, could be tested per plate (figure 1A). The supernatant of a positive culture was diluted 10-fold to 10⁻⁵. The four highest dilutions (10⁻₂ through 10⁻⁵) were used, and for each dilution, three 250-μL aliquots were inoculated into 3 wells. After a 90-min incubation at 35°C in 5% CO₂, medium alone or medium containing an antiviral was added to each well. Therefore, for each dilution of virus, there was 1 well with no drug, 1 well with acyclovir (Sigma, St. Louis) at 2 μg/mL, and 1 well with foscarnet (Sigma) at 300 μg/mL. Pooled human immune globulin (Amour Pharmaceutical, Kankakee, IL) was added to the medium (0.25 μL/mL) to limit spread of the virus to surrounding cells and to allow the formation of discrete plaques.

After 36–48 h, the plates were histochemically stained for β-galactosidase and read macroscopically or using an inverted light microscope under low magnification (×40). The lower dilutions
often contained an excessive viral inoculum, and the plaques were too numerous to count. The row (dilution) that produced 10–100 plaques in the well without antiviral was used to score sensitivity. If the number of plaques in the acyclovir well was >50% of the number seen in the absence of acyclovir, the virus was considered resistant. We included a well with a high concentration of foscarnet (300 μg/mL) to provide a control in which viral replication was completely inhibited. The modified PRA was performed and scored blind to the results of the standard PRA.

Viruses. HSV-1 (KOS strain) was obtained from M. Challberg (NIH, Bethesda, MD). A thymidine kinase (tk) deletion mutant (dlspkt), derived from HSV-1 KOS and with a 360-bp deletion in the tk gene, was a gift of D. Leib (Washington University, St. Louis).

Forty-six HSV isolates were evaluated blindly by both the modified and the standard PRA. Thirty-nine of the isolates had been submitted to the Clinical Virology Laboratory, St. Louis Children’s Hospital (Washington University), for acyclovir susceptibility testing, and frozen medium from the primary culture was available. For these isolates, untittered, first-passage stocks, stored at −80°C, were thawed. After low-speed centrifugation, the supernatant, diluted as described above, was used as the virus inoculum. Several of the clinical isolates came from patients with AIDS who had been treated with acyclovir for long periods. Typing was not performed. Another 7 well-characterized isolates were obtained from D. McCleron (Burroughs Wellcome, Research Triangle Park, NC).

Results

To evaluate the performance of the CV19 cells, we performed a standard PRA on well-characterized laboratory strains using the CV19 cells. As expected, complete inhibition of plaque formation was seen in the presence of acyclovir (2 μg/mL) or foscarnet (300 μg/mL) with a tk− HSV-1 KOS; a tk− (dlspkt) HSV-1, however, showed the same number of plaques in the presence and absence of acyclovir but no plaques in the presence of foscarnet (data not shown). The IC50 for

Figure 1. Example of modified plaque reduction assay (PRA) results from 2 HSV isolates; acyclovir-sensitive (#1) and acyclovir-resistant (#2). Modified PRA was performed as described in Materials and Methods. A, Macroscopic view of plate histochemically stained 48 h after inoculation of isolate. Drug added to well is shown above each column (−, no drug; ACV, acyclovir at 2 μg/mL; FOS, foscarnet at 300 μg/mL). Dilution of isolate is shown on left of each row. Arrow on right indicates dilution (10−4) used to score each isolate. B, Photomicrograph of low-magnification (×40) view of cell monolayers in wells from 10−4 dilution of isolate 1 (a–c) and 2 (d–f). Plaques appear as cluster of blue (black in this photograph) cells. Arrowhead shows stained, single infected cell. Bar = 100 μm.
acyclovir for strain KOS (<0.1 μg/mL) and dlsttk (>20 μg/mL) was the same when the PRA was performed using CV1 and CV19 cells (data not shown).

Forty-six clinical isolates that had previously undergone standard PRA to determine susceptibility to acyclovir were evaluated by modified PRA. Twenty-three isolates (50%) were resistant to acyclovir (IC_{50} ≥ 2) in the standard PRA and the other 23 were sensitive. Figure 1B shows a typical example of how the test appears under low-power (×40) light microscopy. In the absence of drug or with drug-resistant isolates, plaques are seen as clusters of blue cells. In the presence of inhibitory concentrations of the drug, single blue (dark) cells are seen (Figure 1Bb, arrowhead).

Among the 46 isolates tested, 44 showed agreement between the results of standard and modified PRA. One isolate did not form discrete plaques in the modified PRA, and therefore could not be scored. One clinical isolate with an IC_{50} for acyclovir of 3.5 μg/mL by standard PRA was scored as sensitive by modified PRA. The test identified 96% of the clinical specimens correctly as sensitive or resistant. The prevalence of resistance to acyclovir (50%) of the isolates we tested is comparable to the overall prevalence of resistance of isolates submitted for drug sensitivity testing to our Clinical Virology Laboratory. Excluding the isolate that could not be scored by modified PRA, and using standard PRA as reference standard, the modified PRA had a sensitivity of 95.5%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 95.8%.

Discussion

Although, in most cases, physicians do not treat serious bacterial infections without ordering antibiotic susceptibility testing on the causative organism, this is done routinely for HSV infections. A number of factors contribute to this: First, not all physicians have access to a clinical virology laboratory. Second, results of antiviral susceptibility testing are not usually available on a timely basis. Third, standards have not yet been established for antiviral susceptibility testing. Finally, the perception has been that there is not much need for rapid antiviral susceptibility testing.

This work is a part of our overall goal to reduce the effort and time required for the laboratory to perform antiviral susceptibility testing and, concomitantly, to lower the threshold for the clinician to order it. We envision a future in which, as is the case with antibacterial susceptibility testing, antiviral susceptibility testing is done routinely in clinical situations in which it is warranted. To accomplish this goal, many clinical and technical problems need to be solved.

In the present study, we addressed the technical issues. We approached the design of antiviral susceptibility testing with the following notions: (1) Although genotypic tests are valuable, phenotypic tests will always be necessary; (2) as obligate intracellular pathogens, viruses replicate only in cells, and therefore methods to evaluate the effect of antivirals on virus growth require cell culture methodology; (3) genetic modification of cells can improve and simplify cell culture–based methods; (4) plaque-based methods have certain advantages in antiviral susceptibility testing; and (5) a rapid screening test that gives sensitivity and resistance determinations can provide valuable information for the clinician and could dramatically reduce the number of isolates submitted to a more time-consuming IC_{50} assay.

The PRA has been the reference standard in antiviral susceptibility testing of HSV, and it is the only method for which a study has shown a clinical correlation between treatment outcome and in vitro assay results [13]. The plaque assay has been a critical tool in the field of HSV biology and has formed the cornerstone of studies of HSV replication and genetics, because it has allowed the quantitation of viral replication and the isolation of clonal strains and mutants [14]. The PRA is, therefore, highly grounded in HSV biology and has many advantages in evaluating the behavior of HSV in the presence of an antiviral. Alternative, less cumbersome, cell culture–based methods have been developed, but they have not received wide acceptance [7].

The modified PRA described here solves many of the problems inherent to the PRA while preserving the merits of a plaque-based assay. It provides results that are directly comparable to those of standard PRA and it has excellent sensitivity and specificity. It does not provide an IC_{50}, but it provides a qualitative determination that an isolate is either resistant or sensitive. This determination may be sufficient for the clinician to make the appropriate therapeutic decision. In addition, the modified PRA is easy to perform, and the materials and equipment it requires are readily available to any clinical virology laboratory. Although, for the purposes of the present study, the assay was designed for acyclovir susceptibility testing, susceptibility to virtually any anti-HSV drug could be tested.

The modified PRA could also be used to analyze isolates taken directly from patients. There are clinical circumstances in which a patient has had recurrent or persistent HSV infections—the diagnosis has already been confirmed but it is important to determine whether the ongoing infection is due to a drug-resistant virus. This would be an ideal situation in which to screen the specimen directly for resistance without amplification of the isolate. We have taken swab specimens from patient lesions and tested these specimens in the modified PRA. We found that ~70% of such specimens contained sufficient amounts of virus for the modified PRA (data not shown).

Finally, the modified PRA has the ability to identify a subpopulation of resistant viruses in a population that appears overall to be sensitive. For a number of isolates, we observed a reduction of >50% in the number of plaques in the acyclovir-containing well compared with the no-drug well, and thus the isolate was scored as sensitive. However, in these cases, a significant number of plaques were present in the acyclovir well, indicating the presence of a resistant subpopulation. The
modified PRA could provide the requisite tool with which to perform a clinical study to determine the significance of relative levels of resistant subpopulations, and perhaps in the future this would allow the laboratory to alert the physician of the emergence of a resistant population.

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References

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