ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1995, p. 1287–1291 Vol. 39, No. 6
0066-4804/95/$04.00+0

Antiviral Susceptibility Testing with a Cell Line Which Expresses β-Galactosidase after Infection with Herpes Simplex Virus

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Received 12 December 1994/Returned for modification 16 January 1995/Accepted 8 April 1995

Despite increasing concern about drug-resistant herpes simplex virus (HSV), antiviral susceptibility testing is not routinely performed by most clinical virology laboratories. This omission is in large part because the most widely accepted method, the plaque reduction assay (PRA), is cumbersome to perform and results are rarely available in time to influence treatment. We report here the development of a sensitivity test for HSV which utilizes a cell line (VeroICP6LacZ#7) that expresses β-galactosidase activity after infection with HSV such that infected cells can be detected by histochemical staining. We designed an assay in which 10-fold dilutions of virus stocks with undetermined titers were inoculated onto VeroICP6LacZ#7 cells in a 24-well tissue culture dish. Forty-eight hours after infection, the cell monolayers were histochemically stained. Plaques appear blue against a clear background and are thus easily visualized at 48 h. As with the standard PRA, the 50% inhibitory concentration (IC_{50}) was reported as the concentration of an antiviral drug that reduces the number of plaques by 50%. Evaluation of 10 well-characterized laboratory strains and 12 clinical HSV isolates showed that the IC_{50} determined by this method correlated in all instances with the IC_{50} determined by the PRA. This method is easy to use and eliminates the need to determine the titer of the virus, and results are available within 48 h of the detection of the virus. VeroICP6LacZ#7 cells are a useful tool for performing HSV antiviral susceptibility testing and could be used in a number of different formats to facilitate the identification of drug-resistant isolates of HSV.

Infections with herpes simplex viruses types 1 and 2 (HSV-1 and -2) (collectively termed HSV) account for a significant amount of morbidity each year, especially in immunocompromised patients such as newborns, leukemia patients, transplant recipients, and patients with AIDS. Acyclovir is the drug of choice for the treatment of mucocutaneous HSV infections, but a number of recent reports have described HSV isolates that are resistant to acyclovir (8, 10, 17, 19). Drug-resistant HSV infections occur primarily in immunocompromised patients who have been administered an antiviral drug for long periods either for prophylaxis (e.g., organ transplant recipients) or for chronic suppressive therapy (e.g., patients with AIDS) (9, 11). There are a number of antiviral susceptibility assays, but the plaque reduction assay (PRA) is generally considered to be a reference standard (7). Unfortunately, the PRA is labor-intensive and relatively slow, since the titer of the virus isolate has to be determined and formation of plaques takes 3 days. Several protocols have been developed to streamline the PRA or to use the reduction of viral cytopathic effects as an endpoint (15, 19). The dye uptake assay uses neutral red to stain the uninfected cells in a multiwell plate. Although this method is relatively simple to use, it is very sensitive to the virus inoculum and its results do not directly correlate with the PRA (16). Another strategy has been to quantitate the amount of viral proteins or nucleic acids as a measure of the inhibition of virus replication by a given antiviral agent. The enzyme-linked immunosorbent assay to quantify viral proteins has the advantage of being easily automated, but it is sensitive to the virus inoculum and the quality of the antisera used (1, 2, 12). A commercially available DNA hybridization method (24) (Hybrywix; Diagnostic Hybrids, Inc., Athens, Ohio) has been used with success, but in its present format it requires the use of radioactive probes.

We report here the development of a modified PRA for antiviral susceptibility testing for HSV which utilizes a cell line (VeroICP6LacZ#7) that is stably transformed with the *Escherichia coli lacZ* gene under the control of an HSV-1 early promoter. VeroICP6LacZ#7 cells express measurable β-galactosidase only after infection with HSV-1 or -2, and infected cells can be detected by histochemical staining, with a chromogenic β-galactosidase substrate. Antiviral agents such as acyclovir and foscarnet which inhibit HSV DNA synthesis do not block β-galactosidase expression in VeroICP6LacZ#7 cells, but in susceptible isolates, these drugs prevent viral replication and formation of blue-stained plaques. We designed a protocol for using VeroICP6LacZ#7 cells which, unlike the standard PRA, does not require that the titer of the virus stock be determined and allows the determination of the susceptibility of an isolate within 48 h of virus growth. We demonstrated the utility of this assay, using both laboratory strains and clinical isolates of HSV.

MATERIALS AND METHODS

Cells. African Green monkey kidney cells (Vero and CV-1 cells) were obtained from the American Type Culture Collection. The VeroICP6LacZ#7 cell line was generated in a manner similar to that of the previously described BHKICP6LacZ5 cell line (23). Briefly, Vero cells were transfected following a Lipofectin transfection protocol (Gibco BRL, Gaithersburg, Md.) with two plasmids: pMONhygro, which contains the *E. coli hygroB* gene under the control of the simian virus 40 early promoter, and pYBICP6LacZ, which contains the *E. coli lacZ* gene under the control of the HSV-1 ICP6 promoter. Hygromycin-resistant clones were selected with hygromycin (500 mg/ml, total weight/volume;

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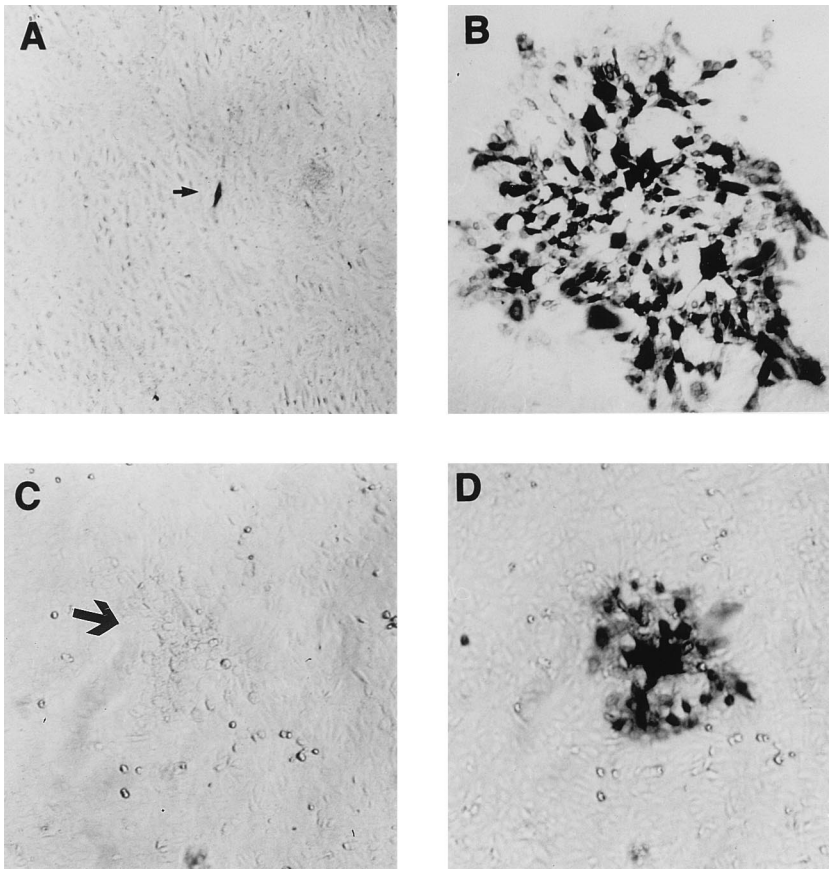

FIG. 1. Photomicrographs of HSV-infected VeroICP6LacZ#7 cells histochemically stained for β-galactosidase. (A) Infection with HSV-1 (KOS) at a low MOI (0.001 PFU per cell) and stained at 24 h. A single stained cell can be seen in the center of the field (arrow). (B) Infection with HSV-1 (KOS) at a low MOI (0.001 PFU per cell) and stained at 48 h. (C) Infection with a clinical isolate of HSV. An HSV plaque at 48 h after infection appears as a cluster of cells exhibiting cytopathic effects (arrow). (D) Plaque shown in panel C after histochemical staining for β-galactosidase activity.

Boehringer Mannheim, Indianapolis, Ind.). A clone which consistently displayed more than 95% blue cells after infection with HSV and staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was chosen and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and hygromycin (100 μg/ml).

Viruses. HSV-1 (KOS strain) was obtained from M. Challberg (National Institutes of Health, Bethesda, Md.). A thymidine kinase (TK) deletion mutant (*dl*sptk) derived from HSV-1 (KOS) has a 360-bp deletion in the *tk* gene and was a gift of D. Leib (Washington University, St. Louis, Mo.) (4). HSV-2 (strain 333) was kindly provided by D. Galloway (University of Washington). D. McCleron (Burroughs Wellcome, Research Triangle Park, N.C.) generously provided us with seven well-characterized viral isolates (SC16, SC16-S1, DM2.1, BW-S, BW-R, KOST, and PAAr5) (5, 14, 22). Clinical specimens were provided by G. Storch (Laboratory of Clinical Virology, St. Louis Childrens Hospital, Washington University School of Medicine, St. Louis, Mo.). Several of the clinical isolates came from patients with AIDS who had been on acyclovir for a long time. The identity of all isolates as HSV was confirmed by a nontyping immunofluorescence assay. Typing was not performed.

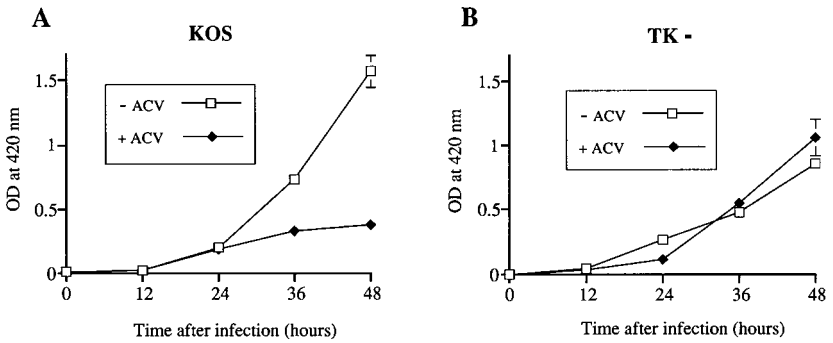

FIG. 2. Effect of acyclovir on β-galactosidase activity in VeroICP6LacZ#7 cells after infection with HSV-1 at a low MOI (0.001). (A) Time course of β-galactosidase activity after infection with an acyclovir-susceptible strain (KOS) in the presence or absence of acyclovir (ACV; 2 μg/ml). The increase in β-galactosidase activity between 24 and 48 h is due to viral replication and a second round of infection. (B) Time course for cells infected with an acyclovir-resistant strain (TK deletion mutant [TK^-] *dl*sptk). β-Galactosidase activity was measured by a colorimetric assay as described in Materials and Methods. Each point represents the mean of three separate samples, and error bars represent standard deviations from the mean; where not visible, the error bars were smaller than the symbols. OD, optical density.

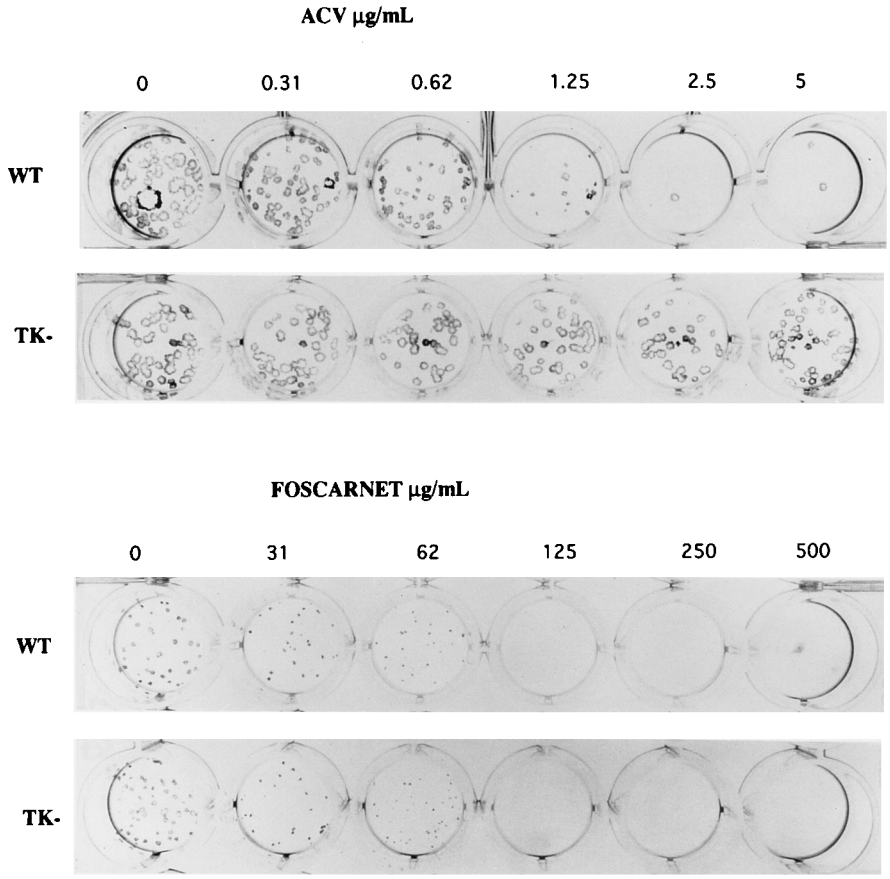

FIG. 3. Macroscopic view of the results of acyclovir (ACV) and foscarnet susceptibility testing of two laboratory strains of HSV-1: the wild type (WT; KOS) and the TK deletion mutant virus (TK$^-$; *dl*sptk) with VeroICP6LacZ#7 cells. The assay is described in Materials and Methods. A 24-well plate was used, but only the rows in which the dilution of virus resulted in 20 to 100 plaques in the absence of an antiviral agent are shown. The strain of virus inoculated is indicated on the left of each row, and the concentration of the antiviral agents is indicated above each column. Low concentrations of acyclovir inhibit plaque formation by wild-type virus (IC_{50}, 0.5 μg/ml) but have no effect on the formation of plaques by *dl*sptk virus. Foscarnet inhibits plaque formation by both viruses equivalently (IC_{50}, 20 μg/ml). Plaques in the top two rows appear larger because they were stained at 72 h after infection, whereas the bottom two rows were stained at 48 h.

Viral susceptibility testing. For analysis of clinical isolates, frozen first-passage stocks were thawed and grown on Vero or CV-1 cells until complete destruction of the cell monolayer occurred. The supernatant from a low-speed centrifugation was then used as the virus inoculum. In some instances, supernatants from the primary viral culture vials were used. No virus titers were determined. Eighty to ninety percent confluent VeroICP6LacZ#7 cells were prepared in 24-well plates. Four 10-fold dilutions of the test virus of unknown titer, starting with a 100-fold dilution, were inoculated in each well of the four rows of the plate (250 μl per well). After incubation for 2 h at 37°C in 5% CO_2, twofold dilutions of the antiviral compound were added to each well of the six columns. For acyclovir, the concentrations tested were 5, 2.5, 1.25, 0.62, 0.31, and 0 μg/ml. For foscarnet, concentrations of 500, 250, 125, 62, 31, and 0 μg/ml were tested. Pooled human immunoglobulin (0.4%) (Gammar; Armour, Kankakee, Ill.) was added to the media to limit the spread of the virus to surrounding cells and allow the virus to form discrete plaques. After 48 h at 37°C in 5% CO_2, the plates were histochemically stained for β-galactosidase activity and read macroscopically or by using a dissecting microscope. After 2 h of staining, plaques appear blue against a clear background of unstained, uninfected cells. Cell monolayers can be left in staining solution for up to 24 h without adversely affecting the results. The 50% inhibitory concentration (IC_{50}) was the concentration of antiviral drug that reduced the number of plaques by 50% as determined from a plot of the percent of control plaques versus the drug concentration. To avoid the problem of an excessive viral inoculum, the row scored was the one which produced between 10 and 100 plaques in the well to which no antiviral agent was added. Virus isolates for which the IC_{50}s were greater than 3 μg of acyclovir per ml were considered resistant; those whose IC_{50}s were less than 3 μg/ml were considered susceptible (18). All isolates were tested at least twice. The standard PRA was done on Vero cells for the laboratory strains and on CV-1 cells for the clinical isolates. The clinical isolates were submitted blindly to our test.

β-Galactosidase assay. The colorimetric assay for β-galactosidase activity was done on whole-cell lysates using 1 mg of *o*-nitrophenyl-β-D-galactopyranoside (Sigma, St. Louis, Mo.) per ml as a substrate in a standard assay (21). A 300-μl lysate was made from 100% confluent cells in wells of a 24-well plate. One hundred microliters of the lysate was used in the assay.

Histochemical staining. Cell monolayers were washed one time with 1 ml of phosphate-buffered saline (PBS; pH 7.2) before being fixed in 2% formaldehyde–0.4% glutaraldehyde in PBS for 5 min. After two washings with PBS, cells were incubated at room temperature in staining solution (X-Gal [Sigma; 1 mg/ml], 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS). The staining solution was made up fresh before use with concentrated stocks of each reagent.

RESULTS

Vero cells were stably transformed with an ICP6::*lacZ* cassette, and hygromycin-resistant colonies were isolated as described in Materials and Methods. Hygromycin-resistant cell lines were then analyzed for β-galactosidase activity before and after infection with HSV-1 (KOS). More than 95% of the cells of one clone (VeroICP6LacZ#7) expressed β-galactosidase at 24 h after infection at a multiplicity of infection (MOI) of 5, as assayed by histochemical staining, but they displayed no staining above the background level in the absence of infection (data not shown). All further studies were performed with VeroICP6LacZ#7 cells. At early time points (12 to 24 h) after infection at a low MOI (0.001 PFU per cell), individual infected cells express β-galactosidase in a background of unstained, uninfected cells (Fig. 1A). After 48 h, the virus spreads

to and infects surrounding cells and forms a blue-staining plaque (Fig. 1B). For certain HSV isolates, plaques were not visible at 48 h prior to histochemical staining (Fig. 1C) but were easily visualized after staining (Fig. 1D).

We next evaluated the effect of inhibition of DNA synthesis on β-galactosidase expression of HSV-infected VeroICP6 LacZ#7 cells by measuring the β-galactosidase activity of infected-cell lysates. Not unexpectedly, we found that after infection at an MOI of 5, β-galactosidase activity was unaffected by treatment with an inhibitor of DNA synthesis (e.g., acyclovir) since the ICP6 promoter is an early promoter and thus is expressed prior to viral DNA replication (data not shown) (25). After infection at a low MOI (0.001), VeroICP6LacZ#7 cells express very low levels of β-galactosidase activity which, as shown in Fig. 2, increases between 24 and 48 h after infection. Using a low MOI, we infected VeroICP6LacZ#7 cells with either an acyclovir-susceptible (KOS) or -resistant (TK^- deletion mutant, *dl*sptk) HSV-1 in the presence or absence of acyclovir. The increase of β-galactosidase activity is prevented by acyclovir after infection with an acyclovir-susceptible virus but not after infection with a resistant virus (Fig. 2).

We next used the VeroICP6LacZ#7 cell line in an anti-HSV drug susceptibility assay that could be performed without prior knowledge of the titer. The assay was first demonstrated by performing acyclovir susceptibility testing on previously characterized susceptible and resistant laboratory strains of HSV-1. Figure 3 shows the rows of the 24-well plate which were inoculated with a dilution of virus that resulted in 10 to 100 plaques in the absence of drug. The results were consistent with the expected phenotype of these strains, i.e., the number of plaques formed on VeroICP6LacZ#7 cells by the KOS strain was reduced by greater than 50% at low concentrations of both drugs, whereas plaque formation of a TK deletion mutant (*dl*sptk) was inhibited by foscarnet and not at all by acyclovir. We next used this assay to perform acyclovir susceptibility testing on 10 well-characterized laboratory strains and 12 clinical isolates (see Table 1). IC_{50} data from the PRA showed that there were 13 acyclovir-susceptible and 9 acyclovir-resistant strains according to the definitions of susceptible (IC_{50} of <3 μg/ml) and resistant (IC_{50} of ≥ 3 μg/ml) of Safrin et al. (18). IC_{50} data determined with the VeroICP6LacZ#7 cells showed a 100% correlation with the PRA results.

TABLE 1. Comparison of acyclovir susceptibility testing results of the standard PRA and the VeroICP6LacZ#7 cell assay

Virus type and strain[a]	TK phenotype[b]	Acyclovir IC_{50} (μg/ml) for test[c]	
		PRA[d]	VeroICP6LacZ#7[e]
Laboratory strains			
HSV-1 (KOS)	Positive	0.30	0.50
TK^- virus (*dl*sptk)	Deficient	>20	>5
HSV-2 (333)	Positive	0.50	0.62
SC16	Positive	0.6[f]	0.55
SC16-S1	Altered	17.4[f]	>5
DM2.1	Deficient	>100[f]	>5
BW-S	Positive	0.6[f]	0.4
BW-R	Deficient	12[f]	>5
KOST	Altered	20[f]	>5
PAAr5	Positive	4.8[f]	>5
Clinical strains			
1	NT[g]	0.24	0.5
2	NT	2.5	1.2
3	NT	1	1.2
4	NT	1.20	1.2
5	NT	1	2
6	NT	≥20	>5
7	NT	10	>5
8	NT	≥20	>5
9	NT	<0.15	<0.3
10	NT	<0.15	<0.3
11	NT	<0.15	<0.3
12	NT	<0.15	<0.3

[a] For descriptions of the viruses, see Materials and Methods.
[b] The TK phenotypes for strains SC16 to PAAr5 were provided by D. McCleron (Burroughs Wellcome) (5, 14, 22).
[c] All isolates were tested at least twice (by both methods). See Materials and Methods for the definition of the IC_{50}. The values shown are means. Standard deviations were all less than 15% of the mean.
[d] The PRA was done with Vero cells (laboratory strains) or CV-1 cells (clinical isolates).
[e] The highest concentration of acyclovir tested was 5 μg/ml.
[f] The IC_{50}s for strains SC16 to PAAr5 were provided by D. McCleron (Burroughs Wellcome) (5, 14, 22).
[g] NT, not tested.

DISCUSSION

Resistance to antiviral agents is likely to become an increasing therapeutic problem for clinicians. In particular, the widespread and prolonged use of acyclovir for patients with AIDS has promoted the emergence of acyclovir-resistant HSV (10, 19). There also have been a number of reports of infections with foscarnet-resistant HSV, sometimes associated with resistance to both of these drugs (20). Acyclovir is inactive until it is phosphorylated by HSV TK and subsequently by cellular kinases. Acyclovir triphosphate then inhibits DNA elongation by the viral DNA polymerase (6). Foscarnet does not require phosphorylation but acts by directly inhibiting the viral DNA polymerase (13). HSV can became resistant to these antiviral agents through several mechanisms. In most cases of acyclovir resistance, the virus is deficient in TK. Certain isolates of HSV can produce a TK with lowered affinity for acyclovir. Cells infected with such isolates phosphorylate acyclovir less efficiently than cells infected with wild-type strains. Finally, mutations in the HSV DNA polymerase can confer resistance to acyclovir and foscarnet (3).

As more anti-HSV drugs become available and if, as expected, the problem of resistance to these drugs becomes more common, clinicians will have to rely more on results of drug susceptibility testing. Studies have shown a good clinical correlation between in vitro resistance and failure of therapy in immunodeficient patients, such as those with AIDS and organ transplant recipients (19). Unfortunately, antiviral susceptibility testing has not achieved the rapidity and simplicity of antibacterial susceptibility testing. Direct analysis of the viral genotype by PCR can identify known mutations that confer resistance to antiviral agents, but isolation of the virus remains the only way to identify resistance due to new mutations. There is a need, therefore, for more-rapid assays which use infectious virus.

The antiviral susceptibility test described in this report is essentially a rapid PRA that uses a cell line that expresses β-galactosidase after infection with HSV. Results correlate completely with a standard PRA, but there are several advantages to this method. One of the main advantages is that results are available within 48 h of isolation of the virus. This speed is because of the elimination of the time-consuming step of determining the titer and because stained plaques on VeroICP6 LacZ#7 cells can be easily seen after only 48 h. Plaques appear as clusters of blue-stained cells which can be enumerated macroscopically. Also, the blue-stained plaques at 48 h are small and discrete, which allows the use of the small wells of a

24-well plate. The use of a multiwell plate makes it convenient to inoculate dilutions of the virus in the same plate in order to obtain an adequate number of plaques per well. In contrast, the standard PRA involves negative staining which is not as easy to visualize as our staining and many viral isolates do not form plaques within 48 h (Fig. 1C). Also, in the standard PRA plaques need to be large to be able to be easily visualized by negative staining and thus it is difficult to obtain a sufficient number of well-isolated plaques in the wells of a 24-well plate. Other advantages of our method are that it does not require the use of radioactive probes, monoclonal antibodies, or any equipment not currently used in clinical virology laboratories.

There are a number of other protocols in which this cell line could be used to perform susceptibility testing. Automated assays using 96-well plates and measurement of β-galactosidase activity on cell lysates as an index of antiviral activity would be very useful to high-volume laboratories. In settings with a high incidence of resistance, a screening assay on directly inoculated clinical specimens might enable therapy, from its start, to be more precisely targeted. We are currently investigating these possibilities.

ACKNOWLEDGMENTS

We thank David Leib (Washington University) for the gift of the TK deletion virus, *dl*tspk, Paul Hippenmeyer (Monsanto Corp., St. Louis, Mo.) for plasmid pMONhygro, Gregory Storch for the clinical isolates, and Daniel McCleron (Burroughs Wellcome) for the drug-resistant HSV isolates.

Isolation of VeroICP6LacZ#7 cells was supported by funds provided by Diagnostic Hybrids, Inc.

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