Novel Method Based on Real-Time Cell Analysis for Drug Susceptibility Testing of Herpes Simplex Virus and Human Cytomegalovirus

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The plaque reduction assay (PRA) is the gold standard phenotypic method to determine herpes simplex virus (HSV) and human cytomegalovirus (HCMV) susceptibilities to antiviral drugs. However, this assay is subjective and labor intensive. Here, we describe a novel antiviral phenotypic method based on real-time cell analysis (RTCA) that measures electronic impedance over time. The effective drug concentrations that reduced by 50% (EC_{50} s) the cytopathic effects induced by HSV-1 and HCMV were evaluated by both methods. The EC_{50} s of acyclovir and foscarnet against a reference wild-type (WT) HSV-1 strain in Vero cells were, respectively, 0.5 μ M and 32.6 μ M by PRA and 0.8 μ M and 93.6 μ M by RTCA. The EC_{50} ratios for acyclovir against several HSV-1 thymidine kinase (TK) mutants were 101.8 $\times$, 73.4 $\times$, 28.8 $\times$, and 35.4 $\times$ (PRA) and 18.0 $\times$, 52.0 $\times$, 5.5 $\times$, and 87.8 $\times$ (RTCA) compared to those for the WT. The EC_{50} ratios for acyclovir and foscarnet against the HSV-1 TK/DNA polymerase mutant were 182.8 $\times$ and 9.7 $\times$ (PRA) and >125.0 $\times$ and 10.8 $\times$ (RTCA) compared to the WT. The EC_{50} s of ganciclovir and foscarnet against WT HCMV strain AD169 in fibroblasts were, respectively, 1.6 μ M and 27.8 μ M by PRA and 5.0 μ M and 111.4 μ M by RTCA. The EC_{50} ratios of ganciclovir against the HCMV UL97 mutant were 3.8 $\times$ (PRA) and 8.2 $\times$ (RTCA) compared to those for the WT. The EC_{50} ratios of ganciclovir and foscarnet against the HCMV UL97/DNA polymerase mutant were 17.1 $\times$ and 12.1 $\times$ (PRA) and 14.7 $\times$ and 4.6 $\times$ (RTCA) compared to those for the WT. RTCA allows objective drug susceptibility testing of HSV and HCMV and could permit high-throughput screening of new antivirals.

Herpes simplex viruses 1 (HSV-1) and 2 (HSV-2) cause orolabial and genital infections as well as keratitis, encephalitis, and neonatal infections. Human cytomegalovirus (HCMV) is responsible for mononucleosis-like syndromes and organ-specific diseases in immunocompromised patients. All antiviral agents currently approved for the treatment of HSV and HCMV infections ultimately target the viral DNA polymerase (1). First-line antiviral agents for the treatment of HSV and HCMV infections include the nucleoside analogues acyclovir (ACV) and ganciclovir (GCV), respectively. Both drugs require a first phosphorylation by the thymidine kinase (TK) encoded by the *UL23* gene (HSV) or the phosphotransferase encoded by the *UL97* gene (HCMV) and two subsequent phosphorylations by cellular kinases to be converted into their active forms (2–4). The triphosphate forms compete with dGTP for incorporation into replicating DNA. Acyclovir triphosphate acts as a DNA chain terminator to inhibit viral replication, whereas ganciclovir triphosphate slows down DNA polymerization and eventually stops chain elongation. The pyrophosphate analogue foscarnet (FOS) is a second-line antiviral drug for the treatment of HCMV diseases and may also be used in the treatment of infections caused by nucleoside analogue-resistant HSV mutants. Foscarnet does not require any phosphorylation to be active (5). It directly inhibits the activity of the viral DNA polymerases encoded by *UL30* (HSV) and *UL54* (HCMV) genes. Foscarnet binds to the pyrophosphate binding site and blocks the release of pyrophosphate from the last nucleoside triphosphate added onto the growing DNA chain. Prolonged treatment with nucleoside analogues may be required to prevent or to manage HSV/HCMV infections in the immunocompromised host. Such prolonged antiviral therapy may result in the selection of viral isolates with reduced drug susceptibilities (6, 7).

The plaque reduction assay (PRA) is the gold standard pheno-

typic method to determine the susceptibilities of HSV isolates to antiviral drugs and is approved as a standard protocol by the Clinical and Laboratory Standards Institute (8). The PRA has also been standardized in a consensus protocol for HCMV to decrease high interassay and interlaboratory variabilities (9). In this assay, cells are infected with a constant viral inoculum. The virus is then allowed to grow in the presence of serial drug dilutions for 2 to 3 (HSV) to 7 to 8 (HCMV) days before the cells are fixed and stained. The viral plaques are then counted under an inverted microscope. The drug concentration that reduces the cytopathic effects by 50% compared to controls (without antivirals) is defined as the 50% effective concentration (EC_{50}). However, the PRA is subjective and labor intensive. The objectivity of the readout was improved in several phenotypic methods based on the detection of specific antigens (by enzyme immunoassays or flow cytometry) or DNA (by hybridization or real-time PCR) (reviewed in references 6 and 7).

The real-time cell analysis (RTCA) system allows dynamic real-time, label-free, and noninvasive analysis of cellular events

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TABLE 1 Drug susceptibility testing of wild-type and drug-resistant HSV-1 strains by the plaque reduction assay and real-time cell analysis^a

HSV-1 (phenotype)	Mutation in gene		EC ₅₀ (μM [fold change]) ^b			
			By PRA ^c		By RTCA ^d	
	UL23	UL30	ACV	FOS	ACV	FOS
C119629 (WT)	ND	ND	0.5 (1.0)	32.6 (1.0)	0.8 ± 0.1 (1.0)	93.6 ± 36.7 (1.0)
H25 (WT)	ND	ND	1.2 (2.4)	38.9 (1.2)	0.4 ± 0.3 (0.5)	129.7 ± 43.3 (1.4)
C170753 (ACV ^r)	R176W	ND	50.9 (101.8)	29.4 (0.9)	14.5 ± 7.5 (18.0)	92.0 ± 16.9 (1.0)
5123376433 (unknown)	S180N	ND	36.7 (73.4)	24.0 (0.7)	41.5 ± 8.4 (52.0)	98.0 ± 10.8 (1.0)
C74106 (ACV ^r)	R222H	ND	14.4 (28.8)	23.2 (0.7)	4.4 ± 0.7 (5.5)	151.9 ± 37.4 (1.6)
C114093 (ACV ^r)	R281stop	ND	17.7 (35.4)	27.2 (0.8)	70.2 ± 10.6 (87.8)	78.9 ± 11.9 (0.8)
C169997 (ACV ^r /FOS ^r)	add G at 430 nt (stop 304)	A605V	91.4 (182.8)	316.0 (9.7)	>100.0 (>125.0)	1014.0 ± 262.0 (10.8)

^a PRA, plaque reduction assay; RTCA, real-time cell analysis; WT, wild type; EC₅₀, 50% effective concentration; ACV, acyclovir; FOS, foscarnet; r, resistant; ND, not detected; nt, nucleotides.

^b Fold change compared to the WT HSV-1 C119629 reference strain value. Fold changes in bold correspond to drug resistance, which is typically defined by a 3-fold increase in the EC₅₀ compared to that for the WT determined by PRA.

^c Results are representative of two independent experiments.

^d Results are the means ± SD of three to four independent experiments.

(10–12). This system measures the electronic impedance using gold microelectrode sensor arrays integrated in a special cell culture plate (called an E-plate). The application of a low alternative current signal leads to the generation of an electric field between the electrodes due to media electrolytes, which is impeded by the presence of cells. The factors influencing the impedance (referred to as the cell index [CI]) are the number of cells seeded in the well, the way they interact, the quality of interaction of the cells with the microelectrodes, and the overall morphology of the cells (13). The use of the RTCA technology has already been reported for monitoring the cytopathic effects induced by a series of viruses belonging to different families as well as for the determination of antibody-neutralizing activity (14–18).

In the present study, the RTCA system was used to monitor the cytopathic effects induced by HSV-1 and HCMV strains in permissive cells. Indeed, these two herpesviruses differ in the cytopathic effects they produced with HSV-1 inducing mostly cell lysis and HCMV remaining mainly associated with cells. To validate the use of this technology as a phenotypic assay, the susceptibilities of wild-type (WT) and drug-resistant HSV-1 and HCMV strains to nucleoside (ACV or GCV) and pyrophosphate (FOS) analogues were compared with those obtained by the standard PRA. In addition, the replicative capacities of some HSV-1 and HCMV strains were evaluated using the same real-time assay.

MATERIALS AND METHODS

Cells. African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (ATCC CCL-81; Manassas, VA). Vero

cells and human foreskin fibroblasts (HFFs) were grown and maintained in minimal essential medium (MEM) (Gibco/Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (Wisent, Inc., St-Bruno, QC, Canada).

Antiviral compounds. Ganciclovir and foscarnet were purchased from Sigma-Aldrich (St. Louis, MO). Acyclovir was obtained from Hospira (Montreal, QC, Canada).

Viral strains. A panel of wild-type and drug-resistant HSV-1 isolates obtained from our clinical virology laboratory was used in this study. Table 1 shows that sequencing analysis of the UL23 gene of HSV-1 strain C170753 indicated an arginine to tryptophane change at position 176 that confers resistance to ACV (19). A serine to asparagine change was detected at position 180 of the TK protein of HSV-1 strain 5123376433, but the role of this mutation in drug resistance is unknown. Strain C74106 harbored an arginine to histidine change at position 222 of the TK enzyme that was reported to induce resistance to ACV (20). HSV-1 strain C114093 contained the change of an arginine by a stop codon at position 281 of the TK that was associated with resistance to ACV (21). No mutations were detected in the UL30 gene of these four strains. The addition of a G at nucleotide 430 of UL23 gene leads to a stop codon at position 304 in HSV-1 strain C169997 and was reported to confer resistance to ACV (21). This strain also had the change A605V in the UL30 gene that was associated with resistance to FOS (22). No mutations were detected in the UL23 and UL30 genes of HSV-1 strains C119629 (reference) and H25. The following HCMV laboratory strains were used in this study: the WT AD169 reference strain, the GCV-resistant strain XbaF, and the GCV- and FOS-resistant strain VQA3. Table 2 shows that the sequencing analysis of HCMV strain XbaF indicated the deletion of amino acids 590 to 593 in the UL97 gene that was associated with resistance to GCV (3), whereas no mutation was detected in the UL54 gene. The HCMV strain VQA3 had a

TABLE 2 Drug susceptibility testing of wild-type and drug-resistant HCMV strains by the plaque reduction assay and real-time cell analysis^a

HCMV (phenotype)	Mutation in gene		EC ₅₀ (μM [fold change]) ^b			
			By PRA ^c		By RTCA ^d	
	UL97	UL54	GCV	FOS	GCV	FOS
AD169 (WT)	ND	ND	1.6 (1.0)	27.8 (1.0)	5.0 ± 2.0 (1.0)	111.4 ± 16.2 (1.0)
XbaF (GCV ^r)	del 590–593	ND	6.1 (3.8)	21.3 (0.8)	41.2 ± 24.9 (8.2)	83.8 ± 29.5 (0.8)
VQA3 (GCV ^r /FOS ^r)	C603W	F412C, L802M	27.5 (17.1)	335.8 (12.1)	73.3 ± 33.2 (14.7)	517.0 ± 207.0 (4.6)

^a PRA, plaque reduction assay; RTCA, real-time cell analysis; WT, wild type; EC₅₀, 50% effective concentration; ACV, acyclovir; FOS, foscarnet; r, resistant; ND, not detected.

^b Fold change compared to the WT HCMV AD169 reference strain. Fold changes in bold correspond to drug resistance, which is typically defined by a 3-fold increase in the EC₅₀ compared to that for the WT determined by PRA.

^c Results are representative of two independent experiments.

^d Results are the means ± SD of three to four independent experiments.

cysteine to tryptophan change at position 603 in the *UL97* gene that was associated with resistance to GCV (23, 24). This strain also harbored the mutations F412C and L802M in the *UL54* gene that confer resistance to GCV alone (23) and to both GCV and FOS (23, 25), respectively. No mutations were detected in the *UL97* and *UL54* genes of HCMV strain AD169.

Drug susceptibility testing by PRA. The susceptibilities of the different HSV-1 and HCMV strains to antiviral agents were, respectively, evaluated in Vero cells or HFFs by the use of the PRA (8, 9). Newly confluent cells seeded in 24-well plates were inoculated with 40 PFU and incubated for 90 min at 37°C in a 5% CO₂ atmosphere. Triplicate wells of infected cells were incubated with increasing concentrations of ACV, GCV, and FOS in MEM plus 2% FBS containing 0.4% SeaPlaque agarose (Lonza, Rockland, ME) for 3 (HSV-1) or 7 (HCMV) days. Cells were fixed and stained, and the number of PFU was counted under an inverted microscope. The number of PFU was plotted against the logarithm of antiviral concentrations, and a dose-response inhibition curve was then fitted with a least-squares method by the use of GraphPad Prism version 5.00 (GraphPad Software, Inc., San Diego, CA). The EC₅₀s of the different antiviral agents were extrapolated from the adjusted curves for each viral strain.

Cell proliferation assays by RTCA. To determine the optimal cell seeding density for an efficient infection with HSV and HCMV strains, the proliferations of Vero cells and HFFs seeded at different densities in 96-well E-plates were monitored by the use of the RTCA system (xCELLigence; ACEA Biosciences, Inc., San Diego, CA). Cells were seeded at densities of 5.0×10^3 to 4.0×10^4 per well (Vero) or 2.5×10^3 to 1.0×10^4 per well (HFFs) in a volume of 200 μ l of MEM plus 10% FBS and incubated at 37°C in a 5% CO₂ atmosphere. The CI values were calculated automatically by RTCA software version 2.0 and recorded every 30 min for 130 h (Vero) or 240 h (HFFs).

Virus-induced cytopathic effects by RTCA. To determine the optimal viral inoculum to be used in drug susceptibility testing, the cytopathic effects induced by increasing inocula of WT HSV-1 and HCMV strains were, respectively, monitored in Vero cells and HFFs. Cells were seeded in 96-well E-plates at a density of 1.5×10^4 per well (Vero) and 7.5×10^3 per well (HFFs), and the CI values were recorded every 30 min for 48 h (Vero) or 72 h (HFFs) (i.e., until they were in the late exponential or in the early stationary growth phase). As recommended by the manufacturer, the CI values were between 0.5 and ideally 1 (or greater) before cells were infected with the virus. Newly confluent Vero cells and HFFs were infected for 90 min with 150 μ l of serial 2-fold dilutions of the WT HSV-1 C119629 (from 25 to 200 PFU) and HCMV AD169 (from 200 to 1,600 PFU) reference strains, respectively. Viral suspensions were removed, and cells were washed once with phosphate-buffered saline (PBS) and incubated with fresh culture medium (MEM plus 2% FBS). According to the instructions of the manufacturer, the CI values were normalized by dividing the CI values at each time point by the CI value recorded at the last time point before infection of cells by using RTCA software version 2.0. Normalized CI values were recorded for an additional 92 h (HSV-1) and 168 h (HCMV).

Drug susceptibility testing by RTCA. Newly confluent Vero cells were infected for 90 min with an inoculum of each HSV-1 strain (i.e., C119629, H25, C170753, 5123376433, C74106, C114093, and C169997) that reduced the normalized CI value by approximately 80% compared to that of the noninfected controls after 72 h. Newly confluent HFFs were infected for 90 min with an inoculum of each cell-associated HCMV strain (i.e., AD169, XbaF, and VQA3) that decreased the normalized CI value by 60 to 70% compared to that of the noninfected controls after 7 days. Viral suspensions were removed, and cells were washed once with PBS. Four wells of infected cells were incubated with increasing concentrations of ACV, GCV, and FOS in MEM plus 2% FBS. Normalized CI values were then recorded for 92 h (HSV-1) and 228 h (HCMV) postinfection. Normalized CI values obtained on day 3 (HSV) or day 7 (HCMV) postinfection were selected for the determination of the EC₅₀s as the inhibition

curves showed a clear separation at these time points. Normalized CI values were plotted against the logarithm of antiviral concentrations. A normalized dose-response inhibition curve was then fitted with a least-squares method by the use of RTCA software version 2.0. The normalization consists of attributing the “top” value of the equation to the mean normalized CI value measured for control cells without drug at the same time point. The EC₅₀s of the different antiviral agents were extrapolated from the adjusted curves for each viral strain.

Viral replicative capacity testing by RTCA. Newly confluent Vero cells or HFFs (5 wells per time point) were inoculated with selected WT and drug-resistant viral strains at a multiplicity of infection (MOI) of 0.001 and incubated for 90 min. The viral suspension was removed and replaced by fresh culture medium (MEM plus 2% FBS). Normalized CI values were then recorded every 30 min for 60 h (HSV-1) or 216 h (HCMV) postinfection.

Statistical analyses. Differences in the replicative capacities of WT and drug-resistant viruses were compared with a two-way analysis of variance (ANOVA) by using GraphPad Prism software version 5.00. A *P* value of ≤ 0.05 was considered statistically significant.

RESULTS

Optimization of cell density. The densities of the Vero cells and HFFs to be used in the drug susceptibility and viral replicative capacity testing of HSV-1 and HCMV strains by RTCA were optimized based on the proliferation characteristics obtained by seeding various amounts of cells in E-plates. Figure 1A shows that Vero cells seeded at densities of 1.0×10^4 and 2.0×10^4 per well reached the late exponential or the early stationary growth phase after 48 h. These cells showed no obvious decrease in CI values after an additional incubation of 72 h, which corresponds to the time period required for HSV-1 to induce cytopathic effects. We thus determined that the optimal seeding density of Vero cells was 1.5×10^4 per well to monitor the cytopathic effects induced by HSV-1 strains.

Figure 1B shows that HFFs seeded at 7.5×10^3 and 1.0×10^4 per well were in the late exponential or in the early stationary growth phase after 72 h. Thereafter, no major decrease in CI values was observed for approximately 168 h, which corresponds to the period of productive infection of HFFs by HCMV. We thus selected the optimal seeding density of HFFs as 7.5×10^3 per well to monitor the cytopathic effects induced by HCMV strains.

Real-time monitoring of virus-induced cytopathic effects. The cytopathic effects induced by WT HSV-1 C119629 and HCMV AD169 reference strains were, respectively, monitored in Vero cells and HFFs by RTCA. Figure 2A shows the time evolution of the normalized CI values for Vero cells infected or not with different inocula of HSV-1 C119629. A slow decline in the normalized CI values was observed over time in noninfected Vero cells and probably reflected morphological changes or a loss of cell adherence. In infected cells, the decrease in the normalized CI values was more pronounced from 25 to 72 h postinfection and resulted from virus-mediated cytopathic effects. Normalized CI values recorded for cells infected with HSV-1 decreased with time and according to the inoculum. As HSV causes cell lysis, infection with this virus induces a marked decrease in normalized CI values. Thus, a viral inoculum that reduced the normalized CI values by 80% compared to that of noninfected cells on day 3 postinfection was selected for each HSV strain (i.e., 70, 25, 300, 400, 800, 40, and 100 PFU per well for the C119629, H25, C170753, 5123376433, C74106, C114093, and C169997 strains, respectively) to perform drug susceptibility testing.

Figure 2B shows the time evolution of the normalized CI values

FIG 1 Real-time monitoring of Vero cell (A) and human foreskin fibroblast (B) proliferation. Cells were seeded at different densities in E-plates, and their proliferation was monitored over time by recording the cell index. The results are the means of four replicate wells and are representative of three independent experiments.

for HFFs infected or not with HCMV strain AD169. The normalized CI values of noninfected HFFs were unchanged throughout the incubation period, suggesting that there were no major morphological changes or loss of cell adherence. Virus-induced cytopathic effects developed from 10 to >168 h postinfection, depending on the viral inoculum used for the infection of cells. In our experiments, we used cell-associated HCMV for infection of HFFs. We observed that the use of a high viral inoculum for infection led to a rapid increase in normalized CI values due to the addition of cells to the system (data not shown). Normalized CI values then returned to the original values within 20 h following the infection. For example, a slight increase in normalized CI values is seen at 1,600 PFU in Fig. 2B. As HCMV remains mainly cell associated, infection with this virus does not markedly decrease normalized CI values. To perform drug susceptibility testing with the different HCMV strains, a viral inoculum that reduced normalized CI values by 60 to 70% compared to that of noninfected cells on day 7 postinfection was thus selected (i.e., 1,200, 800, and 800 PFU per well for strains AD169, XbaF, and VQA3, respectively).

Comparison of antiviral drug susceptibilities by PRA and RTCA. The susceptibilities of WT and drug-resistant HSV-1 and HCMV strains to antiviral agents were evaluated by PRA and RTCA. Table 1 shows that the WT reference strain HSV-1 C119629 had EC_{50} s for ACV and FOS of 0.5 μ M and 32.6 μ M, respectively, by the PRA. The WT HSV-1 strain H25 had EC_{50} s for ACV and FOS of 1.2 μ M and 38.9 μ M, respectively. The respective ACV EC_{50} s of HSV-1 strains C170753, 5123376433, C74106, and C114093 were 101.8-, 73.4-, 28.8-, and 35.4-fold higher than that of the reference strain C119629, whereas the EC_{50} s of FOS remained almost similar to that of the WT. The EC_{50} s of ACV and FOS against the strain C169997 were 182.8- and 9.7-fold higher than those of the WT,

FIG 2 Real-time monitoring of the cytopathic effects in Vero cells or human foreskin fibroblasts (HFFs) infected with different inocula of HSV-1 strain C119629 (A) or HCMV strain AD169 (B). Vero cells were seeded at a density of 1.5×10^4 per well and infected 48 h later with HSV-1 C119629. HFFs were seeded at a density of 7.5×10^3 per well and infected 72 h later with HCMV AD169. The cell index values were recorded every 30 min for a total period of 140 h and 240 h for HSV-1 and HCMV, respectively. The cell index values were normalized at the last time point before infection of cells. Results are the means of four replicate wells and are representative of three independent experiments.

respectively. By the RTCA, the WT reference HSV-1 strain C119629 had EC_{50} s of 0.8 μ M and 93.6 μ M against ACV and FOS, respectively. The WT strain H25 had EC_{50} s for ACV and FOS of 0.4 μ M and 129.7 μ M, respectively. The respective ACV EC_{50} s of HSV-1 strains C170753, 5123376433, C74106, and C114093 were 18.0-, 52.0-, 5.5-, and 87.8-fold higher than that of the reference strain C119629 whereas no increase in EC_{50} s was seen for FOS. The EC_{50} s of ACV and FOS against strain C169997 were >125.0- and 10.8-fold higher than those of the WT, respectively. Figure 3 shows typical susceptibility testing for selected WT (i.e., C119629) and drug-resistant (i.e., C114093 and C169997) HSV-1 strains to ACV.

Table 2 shows that the WT reference strain HCMV AD169 had EC_{50} s for GCV and FOS of 1.6 μ M and 27.8 μ M, respectively. The EC_{50} s of the HCMV strain XbaF were 3.8- and 0.8-fold those of the WT for GCV and FOS, respectively. The EC_{50} s of GCV and FOS against strain VQA3 were 17.1- and 12.1-fold higher than those of the HCMV reference strain, respectively. By the RTCA, the WT HCMV strain AD169 had EC_{50} s of 5.0 μ M against GCV and of 111.4 μ M against FOS. The EC_{50} s of HCMV strain XbaF were 8.2- and 0.8-fold those of the reference strain AD169 for GCV and FOS, respectively. The EC_{50} s of GCV and FOS against strain VQA3 were 14.7- and 4.6-fold higher than those of the WT, respectively. Figure 4 shows typical susceptibility testing for the WT and drug-resistant HCMV strains to GCV.

Real-time monitoring of viral replicative capacity. The replicative capacities of selected WT and drug-resistant HSV-1 and HCMV strains in permissive cells were evaluated by RTCA. Vero

FIG 3 Real-time monitoring of the susceptibilities of selected wild-type and drug-resistant HSV-1 strains to acyclovir. Vero cells were seeded at a density of 1.5×10^4 per well and infected 48 h later with HSV-1 strains C119629 (A), C114093 (B), and C169997 (C) strains. Cells were incubated for 90 min, and the viral suspension was removed and washed with PBS. Infected cells were incubated with increasing concentrations of acyclovir. The cell index values were recorded every 30 min for a total period of 140 h and normalized at the last time point before infection of cells. The EC_{50} s were determined on day 3 postinfection. The results are the means of four replicate wells and are representative of three independent experiments.

cells were infected with the different HSV-1 strains (i.e., C119629, C114093, and C169997) at an MOI of 0.001, and normalized CI values were recorded for 60 h. **Figure 5A** shows that the viral replicative capacity of the TK/DNA polymerase mutant (i.e., C169997) was reduced by 30% and 50% ($P < 0.001$ for both) compared to that of the WT strain at 48 h and 60 h postinfection, respectively.

HFFs were infected with the different HCMV strains (i.e., AD169, XbaF, and VQA3) at an MOI of 0.001, and normalized CI values were recorded for 9 days. **Figure 5B** shows that the replicative capacity of HCMV strain VQA3 was altered compared to those of the WT and XbaF strains. The viral replicative capacity of the former UL97/DNA polymerase mutant was reduced by 44%, 51%, and 50% ($P < 0.001$ for all) at days 7, 8, and 9 postinfection, respectively, compared to that of the WT.

DISCUSSION

The common basis of phenotypic assays is the determination of virus growth inhibition in the presence of an antiviral drug. After appropriate periods of incubation with the antiviral, the reduction

FIG 4 Real-time monitoring of the susceptibilities of wild-type and drug-resistant HCMV strains to ganciclovir. Human foreskin fibroblasts were seeded at a density of 7.5×10^3 per well and infected 72 h later with HCMV strains AD169 (A), XbaF (B), and VQA3 (C). Cells were incubated for 90 min, and the viral suspension was removed and washed with PBS. Infected cells were incubated with increasing concentrations of ganciclovir. The cell index values were recorded every 30 min for a total period of 300 h and normalized at the last time point before infection of cells. The EC_{50} s were determined on day 7 postinfection. The results are the means of four replicate wells and are representative of three independent experiments.

in cytopathic effects or plaque formation is evaluated either microscopically or colorimetrically (26). However, this assay is time-consuming and is based on a somewhat subjective readout. Here, we describe a novel method for the real-time monitoring of the cytopathic effects induced by HSV and HCMV strains in permissive cell lines and drug susceptibility testing based on the measurement of impedance shifts by microelectronic sensor arrays integrated at the bottom of cell culture plates.

The RTCA technology uses the inherent morphological and adhesive characteristics of cells as a physiologically relevant and quantitative readout for various cellular assays (10). This impedance-based label-free technology is noninvasive and allows the performance of kinetics measurements (11, 12, 27). Due to intrinsic growth characteristics, the cell density and the culture time period required to reach an appropriate growth phase at which time cells can be infected with the different viruses should be optimized (28). In our study, the optimal seeding densities for Vero cells and HFFs were determined to be 1.5×10^4 and 7.5×10^3 per

FIG 5 Replicative capacities of selected wild-type and drug-resistant HSV-1 (A) and HCMV (B) strains determined by real-time cell analysis. Vero cells or human foreskin fibroblasts were infected with the different HSV-1 or HCMV strains at a multiplicity of infection of 0.001. The cell index (CI) values were recorded during 60 h (HSV-1) or 9 days (HCMV) and normalized at the last time point before infection of cells. Data are presented as 1/normalized CI values measured at each indicated time point. Results are the means $\pm$ standard deviations of five determinations and are representative of one independent experiment. Statistical analyses were performed using a two-way ANOVA. Statistically significant results are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

well, respectively. Cells seeded at these densities reached the late exponential or the early stationary growth phase after 48 h (Vero cells) or 72 h (HFFs).

After infection of cells with HSV-1 and HCMV strains, the normalized CI values, which reflect the cytopathic effects, decreased as a function of the amount of input virus and time of incubation. The decrease in normalized CI values (e.g., at a similar viral inoculum of 200 PFU) was slower for HCMV than for HSV-1 WT strains, as expected from their respective growth rates in classical plaque assays. HSV-1 and HCMV also differ in the cytopathic effects they induce in permissive cells; HSV-1 causes mainly cell lysis, whereas HCMV remains mostly associated with cells. These different behaviors have thus influenced the optimal viral infection conditions selected to perform susceptibility testing with these two viruses. For HSV-1 strains that cause cell lysis and induce a marked decrease in normalized CI values, a viral inoculum that reduced normalized CI values by 80% compared to that of noninfected cells on day 3 postinfection was chosen. To avoid the addition of a large number of cells during infection with HCMV strains and to take into account the fact that most viruses remain cell associated (and thus do not markedly decrease normalized CI values with increased infection), a viral inoculum that reduced normalized CI values by 60 to 70% compared to that of noninfected cells on day 7 postinfection was selected.

After the conditions for cell proliferation and viral infection were defined, the susceptibilities of the WT reference and drug-resistant HSV-1 and HCMV strains to antiviral agents determined by RTCA were compared to those determined by the standard PRA. The effects of antiviral agents on the cytopathic effects induced by HSV-1 and HCMV strains in permissive cell lines were monitored by the increase in normalized CI values compared to that of infected cells incubated in the absence of drug. An optimal time point after cell infection allowing a clear separation of the dose-response inhibition curves at the different antiviral concentrations was selected to accurately determine the EC_{50} s. For HSV-1 and HCMV strains, normalized CI values recorded for each antiviral concentration on day 3 or 7 postinfection, respectively, were used to calculate the EC_{50} s. These time points correspond roughly to those at which EC_{50} s were determined by the standard PRA. Overall, optimal standardization of cell proliferation and viral infection conditions as well as selection of an optimal time point for EC_{50} determination allowed reduction in intra- and interassay variations in drug susceptibility testing for HSV-1 and HCMV strains by RTCA. There was a good agreement between the two methods for the discrimination of drug-susceptible and drug-resistant HSV-1 and HCMV strains. However, a higher number of HSV-1 and HCMV strains should be tested by RTCA before the thresholds for the EC_{50} s that define resistance to each antiviral drug are definitively set. The ACV EC_{50} s of the different HSV-1 strains did not rank in the same order by PRA and RTCA. This discordance is probably related in part to the different readouts of these two methods (plaque versus cell density and adherence). The ACV EC_{50} s of HSV-1 strain 5123376433 determined by PRA and RTCA also suggest that the unknown S180N mutation probably might be associated with resistance to this drug. The S180N mutation is not located in a conserved region of the TK gene. Therefore, additional studies such as recombinant phenotyping and enzymatic assays are needed to confirm the role of this mutation in ACV resistance (6).

Thymidine kinase is not essential for HSV replication in most tissues and cultured cells (21, 29). Mutations in this protein are thus not expected to result in altered viral replicative capacity (30). Similarly, substitutions or small deletions in the *UL97* gene had no major impact on the viral replicative capacities of HCMV strains (31–34). In contrast, isolates with *UL30* or *UL54* DNA polymerase mutations conferring drug resistance usually exhibit an attenuated or slow-growth phenotype in cell culture compared to those of their WT HSV (35) or HCMV (36, 37) counterparts. The replicative capacities of HSV-1 and HCMV strains that harbored mutations in the DNA polymerase were reduced compared to those of their respective WT strains when determined by RTCA. Furthermore, we have already demonstrated that there was good concordance between viral replicative capacity determined by RTCA and genome copy numbers in cell lysates measured by real-time PCR for recombinant WT and mutant HSV-1 strains in Vero cells (38). The RTCA technology has the advantages of requiring less work and a shorter manipulation time to evaluate the viral replicative capacities of HSV-1 and HCMV strains than the measurement of virus yields by classical plaque assays or intracellular genome copy numbers by real-time PCR.

The major limitation of the RTCA-based method for drug susceptibility and viral replicative capacity testing of HSV and HCMV relates to the equipment needed to perform the analyses. Indeed, the xCELLigence platform is composed of a workstation

supporting a 96-well microtiter detection device (placed inside a cell culture incubator) that is connected to an electronic sensor analyzer and to a computer equipped with the RTCA software. Moreover, this system requires special 96-well microtiter plates that are coated with gold microelectrode sensor arrays (E-plates).

In conclusion, determination of the susceptibilities of HSV and HCMV strains to antiviral agents by the RTCA system is more objective and requires less work and hands-on time than the PRA. In contrast to the PRA, which is an endpoint assay and allows assessment only after a defined time point, RTCA provides measurements throughout the incubation period. This system may be useful for analyzing the effects of drugs acting on different viral or cellular targets. Similarly, transient effects of drugs on cell viability could be identified by real-time monitoring of the cell status, an advantage compared to endpoint assays (27, 39). Despite some limitations, the RTCA technology is a powerful and reliable tool that could be useful in high-throughput screening of new antiviral compounds against HSV and HCMV as it allows concomitant determination of drug susceptibility and cytotoxicity.

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REFERENCES

- Andrei G, de Clercq E, Snoeck R. 2009. Viral DNA polymerase inhibitors, p 481–526. In Cameron CE, Gotte M, Raney K (ed), *Viral genome replication*. Springer, New York, NY.
- Elion GB, Furman PA, Fyfe JA, de Miranda PA, Beauchamp L, Schaefer HJ. 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl) guanine. *Proc Natl Acad Sci U S A* 74:5716–5720. <http://dx.doi.org/10.1073/pnas.74.12.5716>.
- Sullivan V, Talarico CL, Stanat SC, Davis M, Coen DM, Biron KK. 1992. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. *Nature* 358:162–164. <http://dx.doi.org/10.1038/358162a0>.
- Littler E, Stuart AD, Chee MS. 1992. Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral ganciclovir. *Nature* 358:160–162. <http://dx.doi.org/10.1038/358160a0>.
- Oberg B. 1989. Antiviral effects of phosphonoformate (PFA, foscarnet sodium). *Pharmacol Ther* 40:213–285. [http://dx.doi.org/10.1016/0163-7258\(89\)90097-1](http://dx.doi.org/10.1016/0163-7258(89)90097-1).
- Piret J, Boivin G. 2011. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. *Antimicrob Agents Chemother* 55:459–472. <http://dx.doi.org/10.1128/AAC.00615-10>.
- Lurain NS, Chou S. 2010. Antiviral drug resistance of human cytomegalovirus. *Clin Microbiol Rev* 23:689–712. <http://dx.doi.org/10.1128/CMR.00009-10>.
- Swierkosz EM, Hodinka RL, Moore BM, Sacks S, Scholl DR, Wright DK. 2004. Antiviral susceptibility testing: herpes simplex virus by plaque reduction assay. Approved standard, vol 24. Clinical and Laboratory Standards Institute, Wayne, PA.
- Landry ML, Stanat S, Biron K, Brambilla D, Britt W, Jokela J, Chou S, Drew WL, Erice A, Gilliam B, Lurain N, Manischewitz J, Miner R, Nokta M, Reichelderfer P, Spector S, Weinberg A, Yen-Lieberman B, Crumpacker C. 2000. A standardized plaque reduction assay for determination of drug susceptibilities of cytomegalovirus clinical isolates. *Antimicrob Agents Chemother* 44:688–692. <http://dx.doi.org/10.1128/AAC.44.3.688-692.2000>.
- Solly K, Wang X, Xu X, Strulovici B, Zheng W. 2004. Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. *Assay Drug Dev Technol* 2:363–372. <http://dx.doi.org/10.1089/adt.2004.2.363>.
- Atienza JM, Yu N, Kirstein SL, Xi B, Wang X, Xu X, Abassi YA. 2006. Dynamic and label-free cell-based assays using the real-time cell electronic sensing system. *Assay Drug Dev Technol* 4:597–607. <http://dx.doi.org/10.1089/adt.2006.4.597>.
- Ke N, Wang X, Xu X, Abassi YA. 2011. The xCELLigence system for real-time and label-free monitoring of cell viability. *Methods Mol Biol* 740:33–43. http://dx.doi.org/10.1007/978-1-61779-108-6_6.
- Giaever I, Keese CR. 1993. A morphological biosensor for mammalian cells. *Nature* 366:591–592. <http://dx.doi.org/10.1038/366591a0>.
- Witkowski PT, Schuenadel L, Wiethaus J, Bourquain DR, Kurth A, Nitsche A. 2010. Cellular impedance measurement as a new tool for poxvirus titration, antibody neutralization testing and evaluation of antiviral substances. *Biochem Biophys Res Commun* 401:37–41. <http://dx.doi.org/10.1016/j.bbrc.2010.09.003>.
- Fang Y, Ye P, Wang X, Xu X, Reisen W. 2011. Real-time monitoring of flavivirus induced cytopathogenesis using cell electric impedance technology. *J Virol Methods* 173:251–258. <http://dx.doi.org/10.1016/j.jviromet.2011.02.013>.
- Tian D, Zhang W, He J, Liu Y, Song Z, Zhou Z, Zheng M, Hu Y. 2012. Novel, real-time cell analysis for measuring viral cytopathogenesis and the efficacy of neutralizing antibodies to the 2009 influenza A (H1N1) virus. *PLoS One* 7:e31965. <http://dx.doi.org/10.1371/journal.pone.0031965>.
- Teng Z, Kuang X, Wang J, Zhang X. 2013. Real-time cell analysis—a new method for dynamic, quantitative measurement of infectious viruses and antiserum neutralizing activity. *J Virol Methods* 193:364–370. <http://dx.doi.org/10.1016/j.jviromet.2013.06.034>.
- Ebersohn K, Coetzee P, Venter EH. 2014. An improved method for determining virucidal efficacy of a chemical disinfectant using an electrical impedance assay. *J Virol Methods* 199:25–28. <http://dx.doi.org/10.1016/j.jviromet.2013.12.023>.
- Burrell S, Bonnafont P, Hubacek P, Agut H, Boutolleau D. 2012. Impact of novel mutations of herpes simplex virus 1 and 2 thymidine kinases on acyclovir phosphorylation activity. *Antiviral Res* 96:386–390. <http://dx.doi.org/10.1016/j.antiviral.2012.09.016>.
- Stránská R, Schuurman R, Nienhuis E, Goedegebuure IW, Polman M, Weel JF, Wertheim-Van Dillen PM, Berkhout RJ, van Loon AM. 2005. Survey of acyclovir-resistant herpes simplex virus in the Netherlands: prevalence and characterization. *J Clin Virol* 32:7–18. <http://dx.doi.org/10.1016/j.jcv.2004.04.002>.
- Gaudreau A, Hill E, Balfour HH, Jr, Erice A, Boivin G. 1998. Phenotypic and genotypic characterization of acyclovir-resistant herpes simplex viruses from immunocompromised patients. *J Infect Dis* 178:297–303. <http://dx.doi.org/10.1086/515626>.
- Saijo M, Suzutani T, Morikawa S, Kurane I. 2005. Genotypic characterization of the DNA polymerase and sensitivity to antiviral compounds of foscarnet-resistant herpes simplex virus type 1 (HSV-1) derived from a foscarnet-sensitive HSV-1 strain. *Antimicrob Agents Chemother* 49:606–611. <http://dx.doi.org/10.1128/AAC.49.2.606-611.2005>.
- Chou S, Marousek G, Guentzel S, Follansbee SE, Poscher ME, Lalezari JP, Miner RC, Drew WL. 1997. Evolution of mutations conferring multidrug resistance during prophylaxis and therapy for cytomegalovirus disease. *J Infect Dis* 176:786–789. <http://dx.doi.org/10.1086/517302>.
- Chou S. 2010. Recombinant phenotyping of cytomegalovirus UL97 kinase sequence variants for ganciclovir resistance. *Antimicrob Agents Chemother* 54:2371–2378. <http://dx.doi.org/10.1128/AAC.00186-10>.
- Cihlar T, Fuller MD, Cherrington JM. 1998. Characterization of drug resistance-associated mutations in the human cytomegalovirus DNA polymerase gene by using recombinant mutant viruses generated from overlapping DNA fragments. *J Virol* 72:5927–5936.
- Cotarello M, Catalan P, Sanchez-Carrillo C, Menasalvas A, Cercenado E, Tenorio A, Bouza E. 1999. Cytopathic effect inhibition assay for determining the in-vitro susceptibility of herpes simplex virus to antiviral agents. *J Antimicrob Chemother* 44:705–708. <http://dx.doi.org/10.1093/jac/44.5.705>.
- Atienzar FA, Gerets H, Tilmant K, Toussaint G, Dhalluin S. 2013. Evaluation of impedance-based label-free technology as a tool for pharmacology and toxicology investigations. *Biosensors* 3:132–156. <http://dx.doi.org/10.3390/bios3010132>.
- Atienzar FA, Tilmant K, Gerets HH, Toussaint G, Speckaert S, Hanon E, Depelchin O, Dhalluin S. 2011. The use of real-time cell analyzer technology in drug discovery: defining optimal cell culture conditions and

- assay reproducibility with different adherent cellular models. *J Biomol Screen* 16:575–587. <http://dx.doi.org/10.1177/1087057111402825>.
29. Morfin F, Souillet G, Bilger K, Ooka T, Aymard M, Thouvenot D. 2000. Genetic characterization of thymidine kinase from acyclovir-resistant and -susceptible herpes simplex virus type 1 isolated from bone marrow transplant recipients. *J Infect Dis* 182:290–293. <http://dx.doi.org/10.1086/315696>.
 30. Sergerie Y, Boivin G. 2006. Thymidine kinase mutations conferring acyclovir resistance in herpes simplex type 1 recombinant viruses. *Antimicrob Agents Chemother* 50:3889–3892. <http://dx.doi.org/10.1128/AAC.00889-06>.
 31. Emery VC, Cope AV, Bowen EF, Gor D, Griffiths PD. 1999. The dynamics of human cytomegalovirus replication in vivo. *J Exp Med* 190:177–182. <http://dx.doi.org/10.1084/jem.190.2.177>.
 32. Chou S, Meichsner CL. 2000. A nine-codon deletion mutation in the cytomegalovirus UL97 phosphotransferase gene confers resistance to ganciclovir. *Antimicrob Agents Chemother* 44:183–185. <http://dx.doi.org/10.1128/AAC.44.1.183-185.2000>.
 33. Chou S, Waldemer RH, Senters AE, Michels KS, Kemble GW, Miner RC, Drew WL. 2002. Cytomegalovirus UL97 phosphotransferase mutations that affect susceptibility to ganciclovir. *J Infect Dis* 185:162–169. <http://dx.doi.org/10.1086/338362>.
 34. Gill RB, Frederick SL, Hartline CB, Chou S, Prichard MN. 2009. Conserved retinoblastoma protein-binding motif in human cytomegalovirus UL97 kinase minimally impacts viral replication but affects susceptibility to maribavir. *Virology* 496:100–108. <http://dx.doi.org/10.1016/j.virol.2008.05.010>.
 35. Bestman-Smith J, Boivin G. 2003. Drug resistance patterns of recombinant herpes simplex virus DNA polymerase mutants generated with a set of overlapping cosmid and plasmids. *J Virol* 77:7820–7829. <http://dx.doi.org/10.1128/JVI.77.14.7820-7829.2003>.
 36. Baldanti F, Underwood MR, Stanat SC, Biron KK, Chou S, Sarasini A, Silini E, Gerna G. 1996. Single amino acid changes in the DNA polymerase confer foscarnet resistance and slow-growth phenotype, while mutations in the UL97-encoded phosphotransferase confer ganciclovir resistance in three double-resistant human cytomegalovirus strains recovered from patients with AIDS. *J Virol* 70:1390–1395.
 37. Cihlar T, Fuller MD, Mulato AS, Cherrington JM. 1998. A point mutation in the human cytomegalovirus DNA polymerase gene selected in vitro by cidofovir confers a slow replication phenotype in cell culture. *Virology* 248:382–393. <http://dx.doi.org/10.1006/viro.1998.9299>.
 38. Piret J, Goyette N, Eckenroth BE, Drouot E, Gotte M, Boivin G. 2015. Contrasting effects of W781V and W780V mutations in helix N of herpes simplex virus 1 and human cytomegalovirus DNA polymerases on antiviral drug susceptibility. *J Virol* 89:4636–4644. <http://dx.doi.org/10.1128/JVI.03360-14>.
 39. Limame R, Wouters A, Pauwels B, Fransen E, Peeters M, Lardon F, De Wever O, Pauwels P. 2012. Comparative analysis of dynamic cell viability, migration and invasion assessments by novel real-time technology and classic endpoint assays. *PLoS One* 7:e46536. <http://dx.doi.org/10.1371/journal.pone.0046536>.