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Primary cultures of murine neurons for studying herpes simplex virus 1 infection and its inhibition by antivirals

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Summary. – Herpes simplex virus 1 (HSV-1) establishes life-long latency in peripheral neurons, where productive replication is suppressed. To study the specific relationship between the virus and peripheral neurons that would not be affected by other cells usually present in *in vivo* systems, we present an *in vitro* model system based on primary cultures of murine neurons. This model system can be used for characterization of various virus strains and testing of cytotoxicity and inhibitory activity of acyclovir (ACV), cidofovir (CDV) and other antivirals.

Keywords: herpes simplex virus 1; human herpesvirus 1; murine neurons; primary culture; acyclovir; cidofovir; cytotoxity

Introduction

The herpes simplex virus 1 (HSV-1) belongs to the *Alphaherpesvirinae* subfamily of the *Herpesviridae* family. The ability of most alphaherpesviruses to establish and maintain life-long latent infection in peripheral neurons is fundamental for their survival and function as human pathogens. During latency, the infectious virus and viral transcripts are undetectable, except for the latency-associated transcripts, important for efficient virus reactivation (Bloom *et al*., 2010). Reactivation of HSV-1, caused by diverse stimuli, i.e. trauma, fever, UV-light, certain hormones and cytokines, coexistent diseases or immunosuppressive therapy, leads to the development of clinical signs, and the virus becomes present at mucocutaneous sites, appearing in skin vesicles or mucosal ulcers (Roubalova *et al*., 2000; Sauerbrei *et al*., 2010). HSV-1 infections can be treated efficiently by the application of antiviral compounds, which selectively interfere with viral DNA synthesis. Several drugs on the basis of ACV, CDV penciclovir or foscarnet have already been approved (Dzieciątkowski *et al*., 2007; Sauerbrei *et al*., 2010). The treatment of choice in humans with HSV-1 is ACV but considering the ACV-resistant infections, other antiviral drugs, such as CDV, with different mechanisms of action could be used. So far, it is not possible to eliminate HSV-1 definitely, but with the appropriate treatment, it is possible to shorten the duration of symptoms, to extend the recurrence period, and to reduce the risk of infection.

Taking into account the neurotropism of HSV-1, we tested the potential of primary cultures of murine neurons for the analysis of their infection with various HSV-1 strains/isolates and inhibition of this infection with antivirals.

Materials and Methods

Virus strains. Four HSV-1 strains from the virus collection of the Chair and Department of Medical Microbiology, Medical University of Warsaw were used: (i) McIntyre strain – laboratory standard strain sensitive to acyclovir ($TCID_{50}$/ml = 2,76

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Abbreviations: ACV = acyclovir; CDV = cidofovir; HSV-1 = herpes simplex virus 1; RT-CES = real-time cell electronic sensing cells; p.i. = post infection

x 10^6), (ii) ACV – sensitive isolate ($TCID_{50}$/ml = 7,34 x 10^6), (iii) ACV – resistant isolate ($TCID_{50}$/ml = 6,21 x 10^5), (iv) PT366 strain – laboratory standard strain acyclovir-resistant ($TCID_{50}$/ml = 8,76 x 10^5). The viruses were propagated in Vero cell line (ATCC, No. CRL1587) grown in Eagle's Minimum Essential Medium (MEM, Gibco).

Cells. Balb/c (H-2^d) mice genetically susceptible to HSV-1 infection were used to establish primary culture of murine neurons, as described before (Cymerys *et al*., 2010). Cells were plated onto poly-L-lysine or poly-D-lysine with laminin-coated coverslides at a density of 5x10^4 neurons per well (3.6 cm^2). Primary murine neurons were cultured in B-27 Neuron Plating Medium, consisting of neurobasal medium, B-27 supplement, 200 mmol/l of glutamine, 10 mmol/l of glutamate and penicillin/streptomycin antibiotics with supplement of fetal bovine (5%) and equine serum (5%) (Gibco Life Technologies). Cultures were maintained at 37°C in 5% CO_2. Primary murine neurons cultures (10^5 cells per well/3,6 cm^2) were infected with each HSV-1 strain for 60 min at 37°C. After adsorption, the inoculum was aspirated and fresh culture medium was added. Afterwards, cells were incubated for 24, 48, 72, 96, and 120 hrs at 37°C in 5% CO_2. To investigate the ACV and CDV antiviral activity, the culture of primary murine neurons was infected with HSV-1 for 60 min at 37°C and after adsorption the inoculum was aspirated and fresh culture medium containing ACV (32 µg/ml and 128 µg/ml) and CDV (16 µg/ml and 64 µg/ml) was added. Cells were incubated for 24, 48, 72, 96, and 120 hrs at 37°C in 5% CO_2.

Real-time cell electronic sensing (RT-CES). Evaluation of antiviral activity and cytotoxicity of ACV and CDV in primary murine neurons was performed using the xCELLigence system, according to the manufacturer protocol (Roche Applied Science and ACEA Biosciences). Primary murine neurons were seeded in 16-well plates with a glass bottom coated with capillary gold electrodes and incubated at 37°C in 5% CO_2. Neurons were plated at the density of approximately 5x10^4 cells per well. Cell viability was continuously monitored by detecting changes in the impedance calculated as a dimensionless parameter – Cell Index (CI) (Golke *et al*., 2012).

Immunofluorescence (IF) test for viral antigen. The presence of viral antigen was determined by direct IF, using FITC-conjugated Polyclonal Rabbit Anti-Herpes Simplex Virus 1 serum (Dako, dilution 1:200). Cell nuclei were counter-stained with Bisbenzimidine/Hoechst 33258 or DAPI (Sigma-Aldrich Chemicals Co., 2 µg/ml), according to the manufacturer's protocols. Cells cultured on coverslips were examined under Leica SD AF confocal microscope using MetaMorph software (Kawa.Ska, Poland).

Real-time PCR. For the detection and quantification of HSV-1 DNA, a real-time PCR assay with fluorescent TaqMan probe, complementary for the sequence lying within amplified product was used. Viral DNA was isolated from 200 µl of appropriate material. DNA isolation was performed using High Pure Viral Nucleic Acid Kit® (Roche Diagnostics), according to the manufacturer's protocols. Tests were run on the LightCycler 480 instrument (Roche Diagnostics) with modified

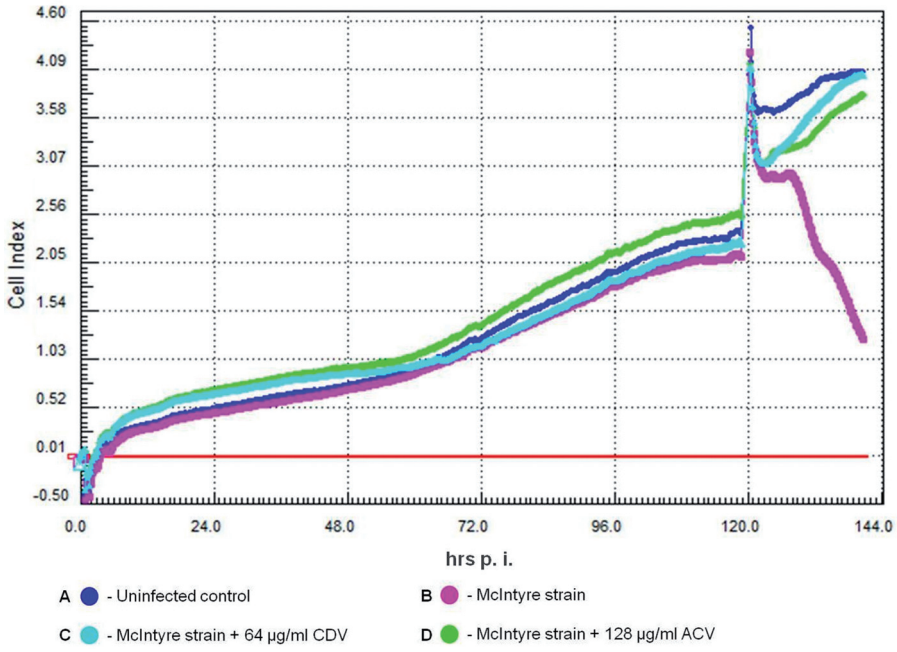

Fig. 1

RT-CES monitoring of the infection of murine neuronal cultures with HSV-1 and its inhibition by ACV and CDV

in-house method described below (Midak-Siewirska *et al.*, 2010). A conservative region of HSV-1 genome encoding the viral glycoprotein B (gB) gene has been chosen (GenBank: AB 297670), and a set of primers, as well as a probe labeled with the fluorophore reporter JOE on 5′-end and with BHQ-1 quencher on its 3′-end (Oligo*), were developed. Reaction was performed using TaqMan Master Kit* (Roche Diagnostics). Final reaction mixture contained 5 µl of isolated viral DNA, 3.25 µmol/l of HSV1_A primer [5′-ATC CAC ACC TTA TCG TTT TTG T-3′], 3.25 µmol/l HSV1_B primer [5′-CGT AAC GCA CGC TAG GGT-3′] and 1.50 µmol/l HSV1_JOE probe [5′-JOE – GGC GGT TGG TCC AGA CGC –BHQ1-3′], in a total volume of 20 µl. Fluorescence levels were detected at 560 nm wavelength, specific for JOE fluorophore dye.

Statistical analysis. Each analysis was performed at least in triplicate. The results were statistically evaluated using Student's t-test. Statistical differences were interpreted as significant at $P < 0.05$ and highly significant at $P < 0.01$.

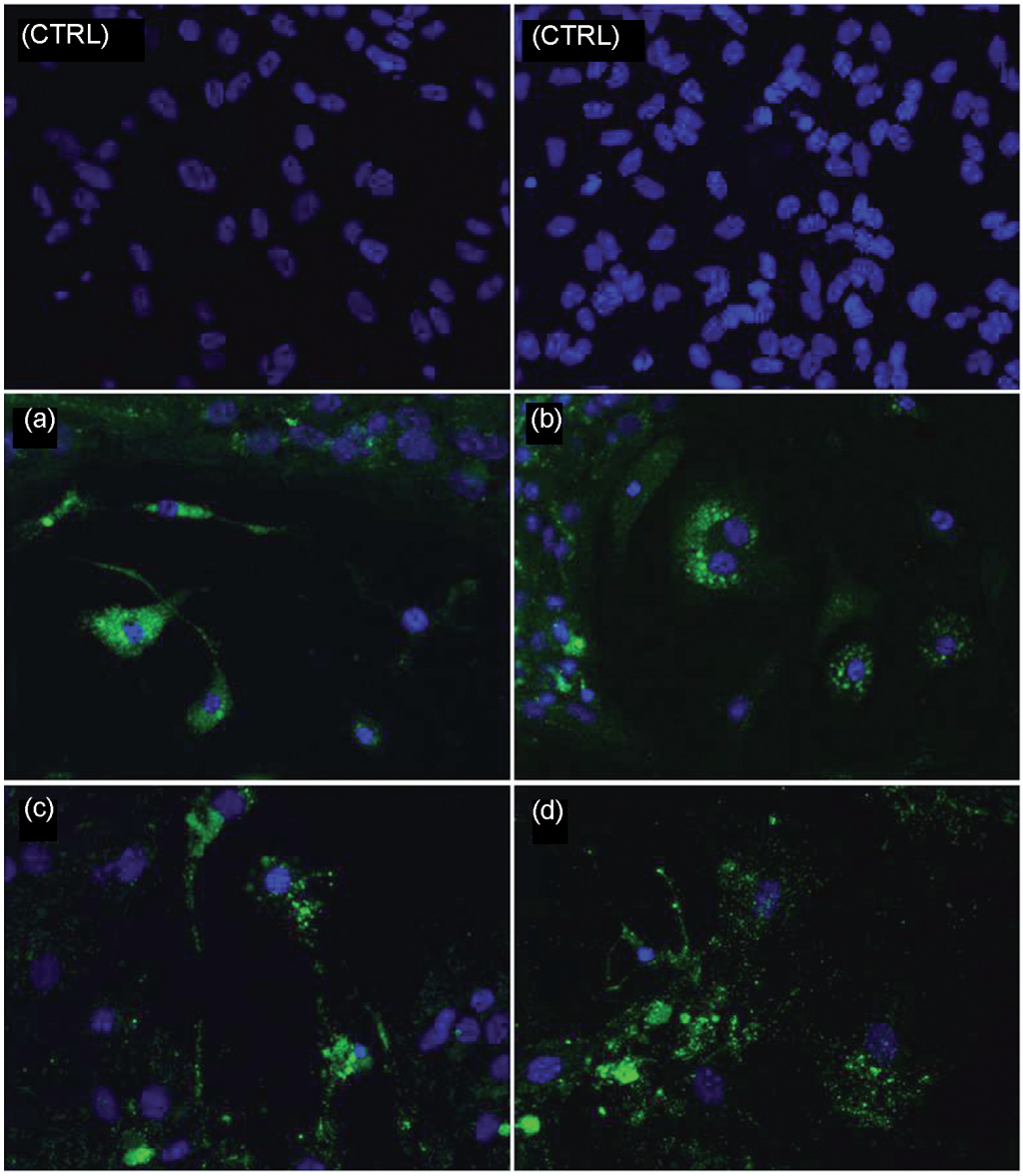

Fig. 2

Formation of viral antigen in cultures of murine neurons infected with various strains/isolates of HSV-1

Direct immunofluorescent assay, confocal microscopy. Uninfected controls (CTRL), McIntyre strain (a), ACV-sensitive isolate (b), ACV-resistant isolate (c), PT366 strain (d). Magnification 200x.

Results and Discussion

We have demonstrated that HSV-1 was able to replicate in cultured murine neurons without the need for adaptation and for that reason they constitute a good model for investigating the mechanisms of HSV-1 neurovirulence and interactions between the virus and neuronal cells. HSV-1 antigens were observed in infected cells regardless of the strain used for infection (Fig. 2). The signal was emitted mostly from the cytoplasmic compartment and more seldom from the nucleus. Moreover, we have observed a decrease of viral antigens in infected murine neurons (24 hrs post infection (p.i.)) in the presence of the ACV and CDV (Fig. 3).

In order to evaluate the cytotoxicity of ACV and CDV, the RT-CES assay using xCELLigence system was performed. Primary murine neurons were treated with increasing concentrations of ACV and CDV. Concentrations of antiviral drugs, which have not shown a toxic effect on neurons, have been selected for further investigation. Application of concentrations higher than 128 μg/ml in the case of ACV and 64 μg/ml in the case of CDV, resulted in a significant decrease of CI values, indicating cell death and detachment. Additionally, without the addition of ACV or CDV, we have observed a significant decrease of CI values at 24 hrs p.i., however this effect has not been observed in the presence of either ACV or CDV. In order to estimate the antiviral activity of ACV and CDV, neurons were infected with HSV-1 (McIntyre strain) and subsequently treated with the highest concentration of the drug that had no cytotoxic effect on neurons (Fig. 1). The results obtained from real-time PCR have confirmed that both ACV and CDV can suppress HSV-1 replication, this was also manifested by the fact that the amount of viral DNA did not exceed the level of viral DNA introduced into the cell culture during infection (Fig. 4). Furthermore, we can assume that the ACV and CDV caused inhibition of productive viral replication, but had no impact on the latent infection in neurons. We have shown a significant decrease in the amount of viral DNA in the culture medium at 24 hrs p.i., at a time when, during a productive infection, progeny virions should be released from the cell (Fig. 5).

In vitro evaluation of HSV-1 susceptibility to antiviral drugs is based on the determination of the viral replication inhibition in the presence of antiviral drug. *In vivo* experiments are time-consuming and the ability to manipulate the host processes is limited. In conclusion, presented *in vitro* model utilizing cultured primary murine neurons provides a simple and effective method to investigate the molecular mechanisms underlying HSV-1 sensitivity to antiviral drugs. The proposed model could also facilitate distinction of specific virus-neuron relationship from general consequences of immune response mediated by non-neuronal support cells in live animals (Kobayashi *et al.*, 2012). It is worth mentioning, that systems based on impedance measurements, like xCELLigence, can be successfully used in virological studies to investigate the antiviral activity of chemotherapeutics allowing not only to determine the cytotoxic concentration of the drug, but also to observe its action in a particular

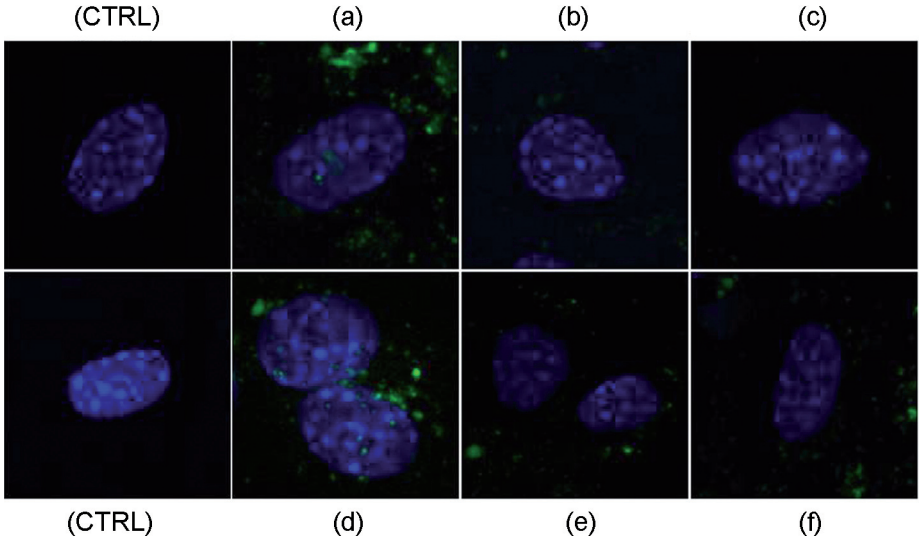

Fig. 3

The inhibition of viral antigen formation in HSV-1-infected cultures of murine neurons by ACV and CDV

Direct immunofluorescent assay, confocal microscopy. Uninfected controls (CTRL). Infection with ACV-resistant isolate (a-c), CDV none (a), 16 μg/ml (b) and 64 μg/ml (c). Infection with ACV-sensitive isolate (d-f), ACV none (d), 32 μg/ml (e), and 128 μg/ml (f).

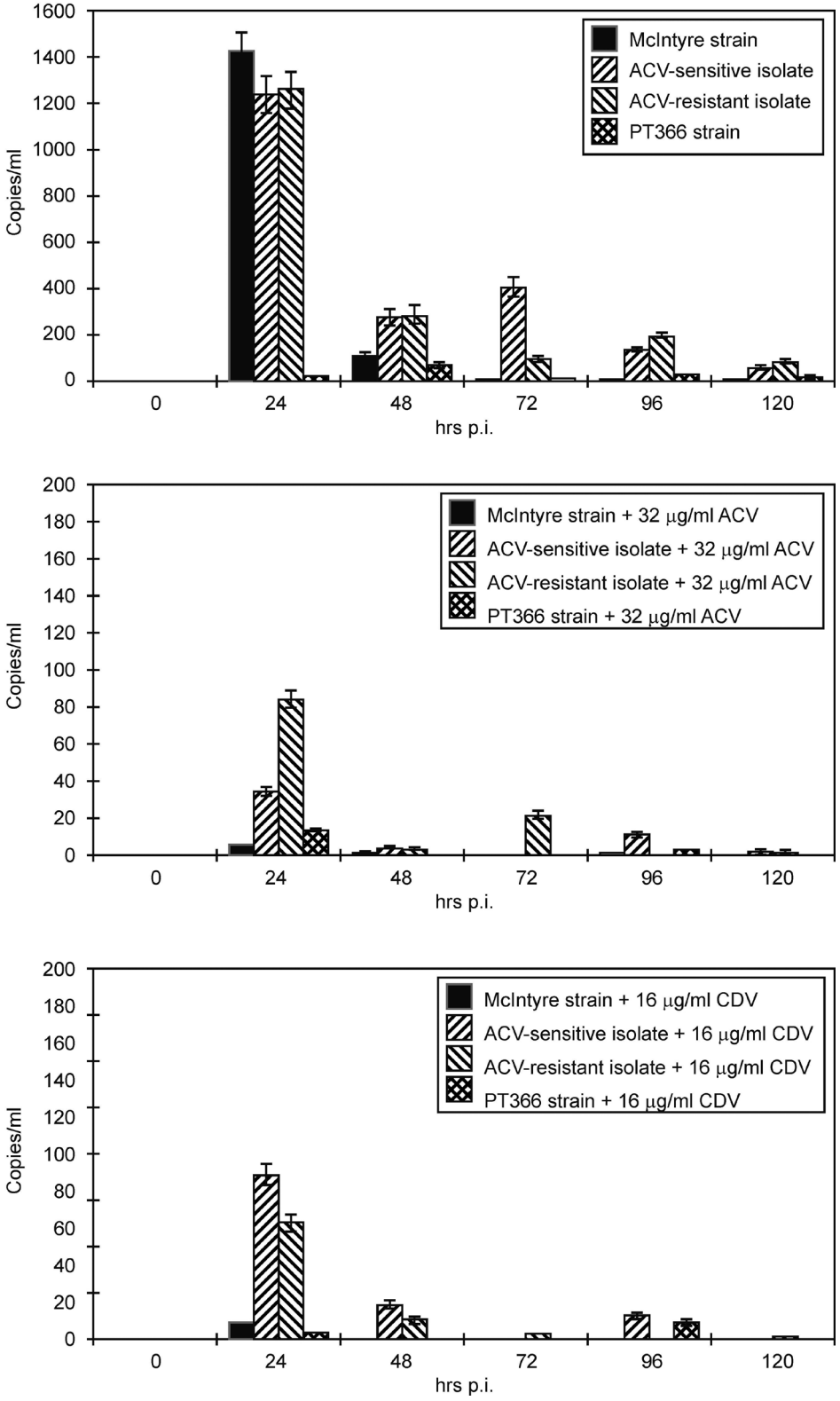

Fig. 4

The levels of viral DNA in cultured murine neurons infected with various strains/isolates of HSV-1 and effects of ACV and CDV

Real-time PCR.

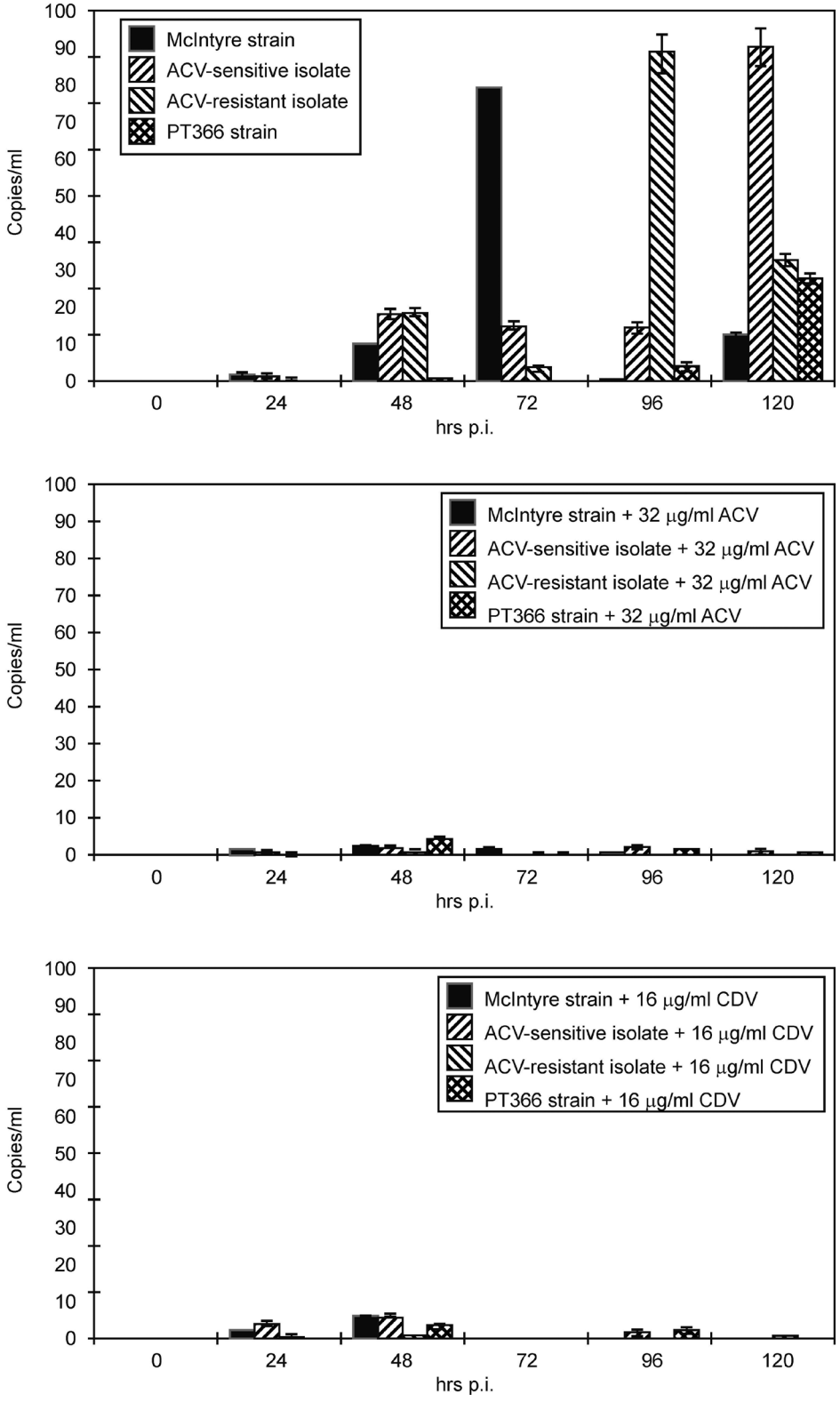

Fig. 5

The levels of viral DNA in the medium of cultured murine neurons infected with various strains/isolates of HSV-1 and effects of ACV and CDV

Real-time PCR.

type of cells in the real time (RT-CES). This type of analysis could provide much more data than conventional end-point analysis in a shorter time. The proposed model could be particularly useful for determining the differences in the susceptibility of clinical strains of HSV-1 to the antiviral drugs. Furthermore, RT-CES methods provide an excellent complement to real-time PCR, which is valuable for detection of HSV-1 due to its sensitivity and accuracy.

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