Original article

Equine herpesvirus type 1 (EHV-1) replication in primary murine neurons culture

**J. Cymerys[1], T. Dzieciątkowski[3], A. Słońska[1], J. Bierła[2],
A. Tucholska[1], A. Chmielewska[1], A. Golke[1], M.W. Bańbura[1]**

[1] Division of Virology, Department of Preclinical Science
[2] Division of Physiology, Department of Physiological Studies, Faculty of Veterinary Medicine,
Warsaw University of Life Science (SGGW), Ciszewskiego 8, 02-786 Warsaw, Poland
[3] Chair and Department of Medical Microbiology, Faculty of Medicine, Medical University of Warsaw,
Chałubińskiego 5, 02-004 Warsaw, Poland

Abstract

Equine herpesvirus-1 (EHV-1) infections cause significant economic losses for equine industries worldwide as a result of abortion, respiratory illness, and neurologic disease in all breeds of horses. The occurrence of abortions caused by EHV-1 has repeatedly been confirmed in Poland, but neurological manifestations of the infection have not been described yet. Also it is unknown how the infection of neurons with non-neuropathogenic strains is regulated. To further understand the virus-neuron interaction we studied two strains of EHV-1 in murine primary neuron cell cultures. Both strains were isolated from aborted fetuses: Rac-H, a reference strain isolated by Woyciechowska in 1959 (Woyciechowska 1960) and Jan-E isolated by Bańbura et al. (Bańbura et al. 2000). Upon infection of primary murine neuronal cell cultures with Jan-E or Rac-H strains, a cytopathic effect was observed, manifested by a changed morphology and disintegration of the cell monolayer. Positive results of immunofluorescence, nPCR and real-time PCR tests indicated high virus concentration in neurons, meaning that both EHV-1 strains were likely to replicate in mouse neurons *in vitro* without the need for adaptation. Moreover, we demonstrated that some neurons may survive (limited) virus replication during primary infection, and these neurons (eight weeks p.i.) harbour EHV-1 and were still able to transmit infection to other cells.

Key words: EHV-1, latency, neuron, neurotropism, neuropathogenicity

Introduction

Equine herpesvirus type 1 (EHV-1), a member of the α-*Herpesvirinae* subfamily, is a major causative agent of upper respiratory tract infections, neurological disorders and abortion in horses. Similarly to other α-herpesviruses (e.g. HHV-1, HHV-2, VZV, PRV, BHV-1), it is neurotropic and causes latent infections in natural host (Dehlon et al. 2002, Ch'ng et al. 2005). It was currently proved that the latent form of EHV-1 is present in neurons of the trigeminal ganglion and can be reactivated. Neuronal infection is very important for the epidemiology of equine herpesvirus abortions (Sauerbrei et al. 2002, Regge et al. 2006). It remains unclear, however, how direct damage to neurons may contribute to the neuronal form of infection,

Correspondence to: J. Cymerys, e-mail: jcymerys@op.pl, tel.: (22) 593 60 26

as neurological symptoms originate mainly from virus replication in the endothelium of small blood vessels and related minor extravasations and thrombosis (Bryans et al. 1989, Sauerbrei et al. 2002). Although many studies have been devoted to the pathogenesis of various clinical forms of EHV-1 infection, it is still not fully explained how EHV-1 can damage cells.

Studies on productive and latent EHV-1 infections in the natural host are limited due to difficulty in finding "immunologically naive" test animals that have not been in contact with this virus. Therefore, pathogenesis of EHV-1 infection and other phenomena associated with productive and latent infections mostly studied in animals models. An excellent model is the BALB/c mouse, because it has been demonstrated that EHV-1 infection in this mouse strains is similar to that of the natural host (Awan et al. 1990, Walker et al. 1999, Gosztonyi et al. 2009). Accordingly, in the acute form, viremia developes, the virus replicates in the respiratory system and virus tropism to fetuses can be observed in pregnant females. In the latent phase of infection, the virus is found in the nervous tissue. As in the natural host, EHV-1 causes latent infection in mice with viral DNA in the olfactory bulbs (Baxi et al. 1996, Marshall et al. 1997), trigeminal ganglion and mononuclear cells (Baxi et al. 1996). Productive or latent infection relates not only to neurons in the olfactory bulbs, but to neurons in other parts of the rhinencephalon (Bańbura et al. 2003). In addition, infection was accompanied by some aspects of neuronal degeneration, but it remains unclear how these changes develop.

Therefore, the aim of this study was to develop an *in vitro* murine model for natural infection of neurons with EHV-1 and determine replication differences, if any, between attenuated Rac-H strain and field isolate (Jan-E).

Materials and Methods

Virus

Two strains of equine herpesvirus (EHV-1) from the virus collection of the Virology Laboratory of the Department of Preclinical Sciences were used. Jan-E strain, a field isolate from an aborted fetus (mare Ezelda, Janów Podlaski stud, Poland) and identified by PCR using gB-specific primers (Borchers and Slater 1993) and reference strain Rac-H (isolated from mare Heraldia, Racot stud, Poland) had been passaged through a series of cell cultures, and defined as "pantropic, non-pathogenic" (Nugent et al. 2006). The viruses were propagated in equine dermal (ED) and Vero cell lines, grown in Eagle's medium (Serum and Vaccine Plant, Lublin) supplemented with 10% fetal bovine serum (Gibco) and antibiotics/antimycotic solution (Sigma).

Nerve Cell Cultures

Mice genetically susceptible to EHV-1 infection (Awan et al. 1990, Gosztonyi et al. 2009) Balb/c (H-2d) were used to establish primary culture of murine neurons. Balb/c mice aged 6-8 weeks were purchased from the Centre for Experimental Medicine (Warsaw) and were treated in accordance with the guidelines regarding the use and care of laboratory animals. All procedures involving live animals were approved by a local ethics committee and conformed to applicable international standards (decision no. 79/2006).

Pregnant female mice (16-19 days after mating) were sacrificed in general anaesthesia by cervical dislocation. Fetuses were removed and decapitated for brain collection. Cerebral hemispheres were isolated from the fetal brains, washed three times in cold HBSS solution (10x Hanks Buffer; Gibco-Invitrogen), and then incubated in 2.5% trypsin solution (Gibco-Invitrogen) at 37ºC for 15 minutes. Brain cell suspension was washed three times in warm HBSS solution and dissociated with a pipette. The cells were then counted and suspended in inoculating fluid (plated onto poly-L-lysine, or poly-D-lysine with laminin – coated coverslides at a density of 5×10^3 to 10^4 neurons per well). Murine neurons were cultured in B-27 Neuron Plating Medium consisting of neurobasal medium, B-27 supplement, 200mM of glutamine, 10mM of glutamate and penicillin/streptomycin antibiotics (Gibco-Invitrogen) with 10% supplement of fetal and equine serum (Gibco-Invitrogen). Neuronal cultures were maintained at 37ºC with 5% CO_2. After four days part of the medium was replaced with Neuron Feeding Medium (B-27 Neuron Plating Medium without glutamate).

Inoculation of Cells

Primary murine neuronal cells were infected with Rac-H or Jan-E strains of EHV-1 at 10^5 $CCID_{50}$/ml in culture medium. After incubation at 37ºC for one hour, the inoculum was aspirated and fresh culture medium was added.

Monoclonal antibody for IF staining

Monoclonal mouse-anti-neurofilament-160/200 antibody (Sigma) and murine anti-β-tubulin mAb (Sigma) were used to detect neuronal markers and were visualised with secondary Texas Red-conjugated goat anti-mouse antibody (Molecular Probes) or anti-mouse IgG-FITC (Sigma). In addition, staining with specific mouse mAb anti-Tau (Tau-5, neurons), anti-GFAP (astrocytes) and anti-CNP (oligoden-

drocytes) (Merck), was carried out to confirm the cell type. Immune complexes were then visualised with secondary anti-mouse IgG-R-phycoerythrin or anti-mouse IgG-FITC (Sigma). Polyclonal porcine fluorescein isothiocyanate (FITC)-labelled anti-EHV-1 antibody (RPK Gamakon conjugate; Mevak), was used to detect viral antigens. Cell nuclei were stained with Bisbenzimidine/Hoechst 33258, 7-aminoactinomicin D (Sigma), or using mounting medium for fluorescence with DAPI (Biokom), as instructed by the manufacturer.

Immunofluorescent Staining Procedure

After washing in PBS, cells were fixed in 100% methanol at -20°C for five minutes. Then slides were washed again and blocked with 1% BSA. Antibodies were always diluted in PBS: primary antibody: 1:100, FITC-conjugated secondary antibody: from 1:50 to 1:100, Texas red-conjugated secondary antibody: 1:50, and RPK Gamakon conjugate: 1:16. Cells were incubated with antibody at 37°C for one hour and with RPK Gamakon at 37°C for 30 minutes. Washings were performed with PBS at room temperature for 2x5 minutes.

Cells on coverslips were examined under the BX-60, Olympus microscope and in FV-500, Olympus confocal microscope using FluoView software.

Nested PCR

Viral DNA in murine neurons was detected by nested polymerase chain reaction (*nested*PCR; nPCR) using EHV-1 specific primers (specific for the B (gB) EHV-1 glycoprotein gene; custom synthesised by GIBCO), both external and internal as described previously (Borchers et al. 1993). Amplification was performed with 1 μg of DNA isolated from infected murine neurons using High Pure Viral Nucleic Acid Kit® (Roche Diagnostics), as instructed by the manufacturer. DNA from non-infected murine neurons served as negative control, whereas DNA extracted from ED cells infected with a Rac-H EHV-1 strain served as positive control. Amplification products fractionated in agarose gel (0,8%) were analysed using Quantity One software (Versa Doc Imaging System).

Real-Time PCR

The quantity of EHV-1 DNA in all samples was estimated using real-time PCR technique (RT-PCR; qPCR) with fluorescent TaqMan probes, complementary for the sequence within the amplified prod-

uct. Tests were run on the LightCycler 2.0 instrument (Roche Diagnostics®) using an in-house quantitative method (Dzieciątkowski et al. 2009). Jan-E EHV-1 strain serial dilutions were used as reaction standards and DNA from non-infected ED cells served as negative control. Each sample was amplified with internal control (positive control of the amplification process) and tested in independent duplicates.

Results

Characteristics of Primary Murine Neuronal Cell Culture Derived From Mouse Brain

A primary neuronal cell culture was prepared as described in Materials and Methods. Monoclonal mouse-anti-neurofilament-160/200 antibody (Sigma) and murine anti-β-tubulin mAb (Sigma) were used as neuronal markers (Fig 1). In addition, staining with specific mouse mAb against anti-Tau (Tau-5, neurons), anti-GFAP (astrocytes) and anti-CNP (oligodendrocytes) (Merck) was carried out to confirm the type of cultured cells (data not shown). The viability and condition of neuronal cells were also determined. In a culture consisting of large adherent neuronal cell bodies, about 20% of neurons were shown to be non-viable, usually featuring no axonal growth and shrinked nucleus, while viable neurons showed axonal growth and a well-defined nucleus. A dense, intact fibre network, fanning radially out of the neuronal cell body (perikaryon) through the dendrites and axon into the cell membrane was also observed (Fig. 1).

Replication of EHV-1 in Cultured Neurones

Neuronal cell cultures were infected with Jan-E or Rac-H EHV-1 strains (Materials and Methods). Regardless of the strain used to infect neuronal cell culture, a cytopathic effect (CPE) could be observed (Fig. 2). For Jan-E EHV-1 strain, the CPE was manifested by changed morphology of cells and nuclei degeneration (Fig. 2D), whereas interference with neuronal viability by the Rac-H EHV-1 strain, resulted, in addition to a changed morphology of cells and disintegration of their nuclei, in cytoplasm vacuolation and cells lysis, which led to plaque formation (Fig. 2E). An off-laboratory Jan-E strain induced changes that were well visible as early as within 48-72 hours after infection. On the other hand, the reference strain yielded a similar result within 96-120 hours. A direct immunofluorescence test confirmed the presence of EHV-1 antigen in the infected cells (Fig. 3, 4). The signal was emitted mostly from the cytoplasmatic
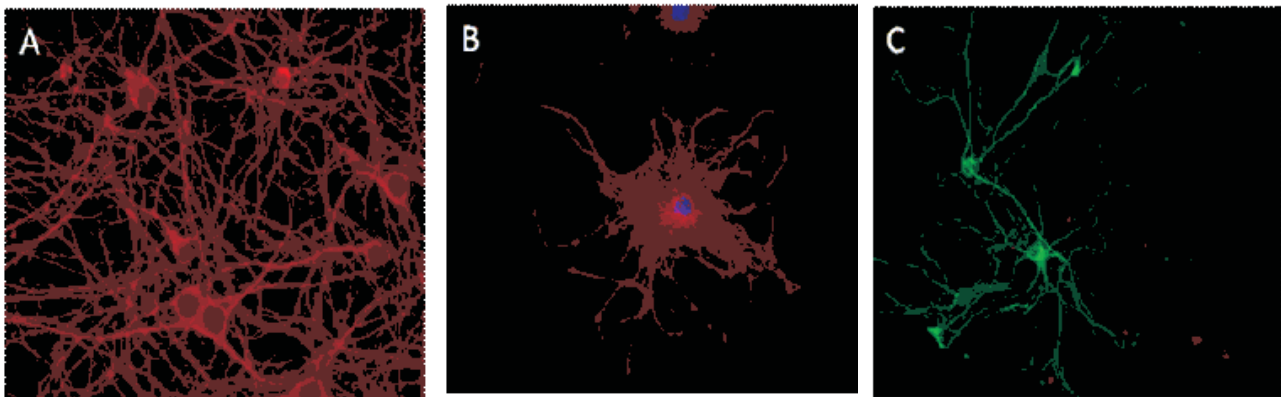
Fig. 1. Primary culture of murine neuronal cells. Non-infected cells form a network of β-tubulin fibres (A, B) – red colour, nuclei – blue colour; neurofilaments stained green (C). Microscope magnification: 60x.
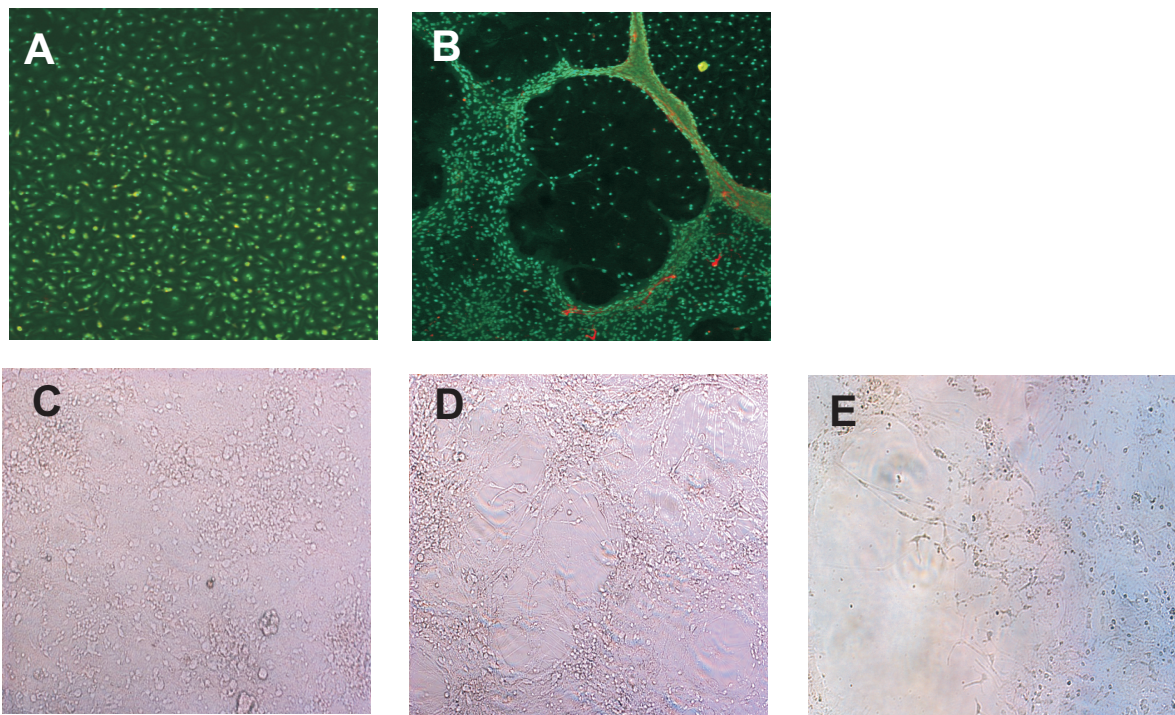

Fig. 2. Primary culture of murine neuronal cells, non infected (A, C) and infected with Jan-E (D; 48 hpi) and Rac-H (E; 96 hpi) EHV-1 – cythopathic effect (CPE; B; 96 hpi). Microscope magnification: 20x.

region and more seldom from the nucleus (Fig 3, 4). We found florescence to be most intense in those cell regions which were already damaged.

Viral DNA Replication in Cultured Neurones

To quantitate viral DNA, cultured cells were infected with Jan-E or Rac-H EHV-1 strains, and at various time points (24, 72, 144 hpi and 15 dpi) representative sample was taken for viral DNA isolation.

Virus-specific DNA was amplified with nestedPCR. After the second round of amplification distinct bands typical of EHV-1, measuring 1.29 kbp, were present. Bands were not found in negative control (Fig. 5). Products of amplification of DNA isolated from plasma using EHV-1-specific qPCR method, expressed as a presence of exponential fluorescence gains, were detected in all samples. Obtained quantitative results were at medium level and ranged from $2,24 \times 10^3$ to $1,49 \times 10^5$ $TCID_{50}$ (Table 1).
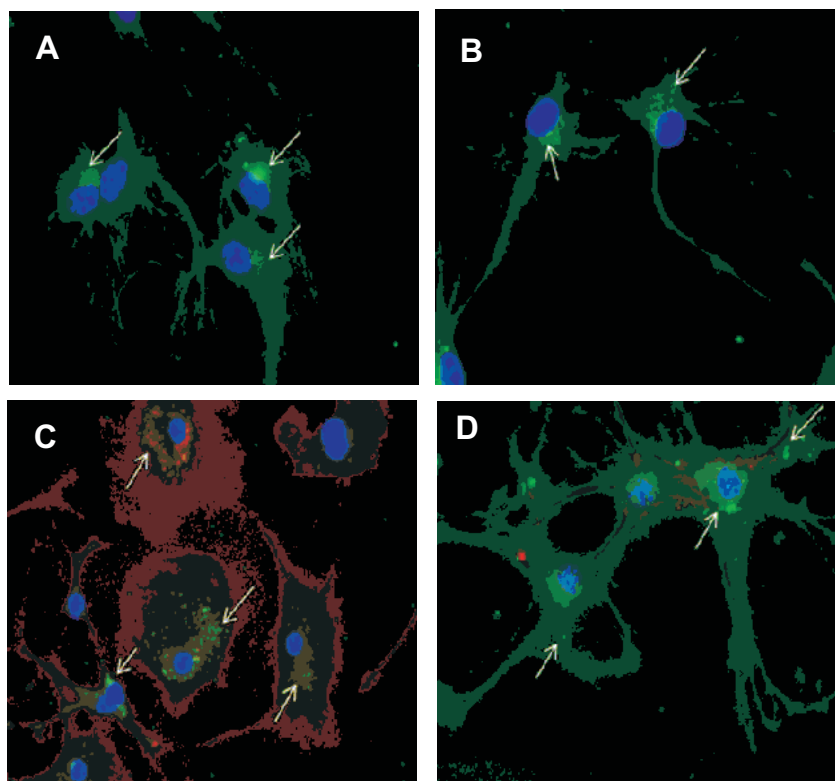
Fig. 3. Primary culture of murine neuronal cells infected with Jan-E strain in 24 (A), 48 (B) and 96 (C, D) hours after infection, respectively. Viral antigen (→) and nuclei are stained green and blue, respectively. Microscope magnification: 200 x.
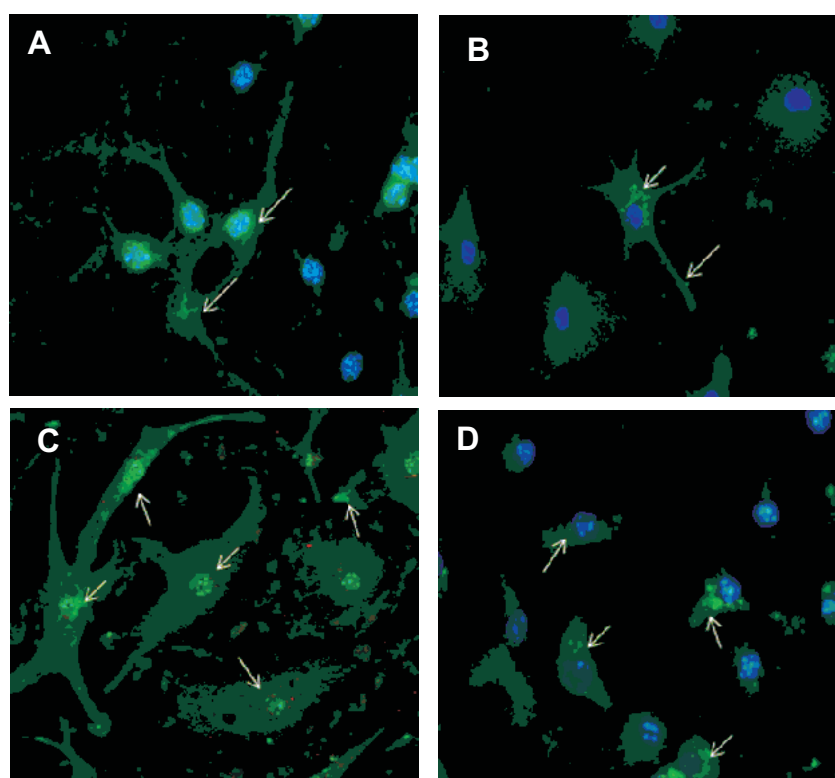

Fig. 4. Primary culture of murine neuronal cells infected with Rac-H strain in 48 (A,B), 96 (C) and 120 (D) hours after infection, respectively. Viral antigen (→) and nuclei are stained green and blue, respectively. Microscope magnification: 200 x.

1 –	control (negative)
2 –	cotrol (positive)
3 –	Jan-E 24hpi
4 –	Jan-E 72hpi
5 –	Jan-E 144hpi
6 –	Jan-E 15dpi
7 –	Rac-H 24hpi
8 –	Rac-H 72hpi
9 –	Rac-H 144hpi
10 –	Rac-H 15dpi

Fig. 5. Amplification of DNA isolated from neurons using PCR technique with EHV-1 specific starters.

Table 1. Results of real-time PCR quantification of EHV-1 DNA.

Sample number	Sample	qPCR quantitative result ($TCID_{50}$)
1.	Jan-E EHV-1 strain [24hpi]	$1.49 \times 10^{*}5$
2.	Jan-E EHV-1 strain [48hpi]	$9.01 \times 10^{*}4$
3.	Jan-E EHV-1 strain [72hpi]	$6.58 \times 10^{*}4$
4.	Jan-E EHV-1 strain [15dpi]	$1.75 \times 10^{*}4$
5.	RacH EHV-1 strain [24hpi]	$5.11 \times 10^{*}3$
6.	RacH EHV-1 strain [48hpi]	$3.85 \times 10^{*}4$
7.	RacH EHV-1 strain [72hpi]	$1.92 \times 10^{*}4$
8.	RacH EHV-1 strain [15dpi]	$2.24 \times 10^{*}3$

Discussion

Equine herpesvirus-1 (EHV-1) is an important equine viral pathogen which, in addition to causing respiratory disease, can also result in abortion and/or neurological illness. Symptoms of neurological disorders vary in severity and range from mild ataxia to paraplegia. The significance of both productive and latent EHV-1 infection of nervous system cells in the mouse model, either *in vivo* or *in vitro*, has not been fully explained and published data are not entirely consistent. Based on histopathology, neurological EHV-1 related disorders are not considered to be a direct result of viral infection of neuronal cells, but rather the consequence of ischemia, haemorrhage or inflammation of the neural parenchyma, which may lead to cerebrospinal vasculitis accompanied by leakage and thrombosis (Yamada et al. 2008). A key aspect to the life cycle of α-herpesviruses is their neurotropic behavior. Sensory neurons of the trigeminal ganglion are important target cells for many α-herpesviruses (including HHV-1 PRV and BHV-1), and constitute major sites of latent infections (Slater et al. 1994, Ch'ng et al. 2005, Regge et al. 2006).

The occurrence of abortions caused by EHV-1 has repeatedly been confirmed in Poland (Rola et al.

1997, Bańbura et al. 1998), but neurological manifestations of the infection have not been found yet. The neuropathogenic mechanism of the virus infection has been partially explained by others (Nugent et al. 2006, Allen 2007, Perkins et al. 2009, Smith et al. 2009). All neuropathogenic EHV-1 strains have been found to have a point mutation in the DNA polymerase-encoding gene. Epidemiological studies have shown that a single-nucleotide polymorphism in the EHV-1 DNA polymerase gene is associated with outbreaks of severe neurological disease in horses. Reverse genetic experiments further demonstrated that a $G_{2254} A_{2254}$ nucleotide mutation introduced in neurovirulent strain Ab4, which resulted in an asparagine for aspartic acid substitution ($D_{752} N_{752}$), rendered the virus nonneurovirulent in the equine [A_{2254}/752N – "non-neuropathogenic"; G_{2254}/752D – "neuropathogenic"] (Allen 2007, Perkins et al. 2009, Smith et al. 2009, Vissani et al. 2009).

It is also unknown how the infection of neurons with non-neuropathogenic strains is regulated. It should be noted here that the terms "neuropathogenicity" (in its clinical sense) and "neurotropism" do not carry the same meaning. "Neuropathogenicity" should be understood as the ability to cause pathological changes in the nervous tissue and related clinical symptoms, whereas "neurotropism" is the ability to infect a particular type of cells: nerve cells. Accordingly, "non-neuropathogenicity" does not mean that the virus is unable to replicate and remains latent in nerve cells.

Two EHV-1 strains were used to infect mouse neurons *in vitro*. The results obtained may support the hypothesis outlined above that both pathogenic (Jan-E) and non-pathogenic (Rac-H) EHV-1 strains are capable of replicating in neuronal cells. Positive qPCR and nPCR results indicated the presence of virus in neurons 24 h after infections with both the reference and the field strain although the virus load of Rac-H EHV-1 strain was lower than that of Jan-E strain. Interestingly, some differences were also observed in how and when nerve cell metabolism is damaged following infection with Rac-H or Jan-E EHV-1 strains, i.e. cytopathic effect. Jan-E, the field

strain, induced CPE within 48 to 72 hours after murine neuronal cells infection. The reference EHV-1 strain yielded a similar result within 96 to 120 hours. It may depend on different virulence observed among various EHV-1 strains. It is known that due to the repeated passage of the Rac-H strain *in vitro* some parts of the viral genome have been deleted, making the virus significantly less virulent. Therefore the studies presented were carried out using both the reference and the field strains.

Herpesviruses have developed multiple mechanisms that help them escape the host immune mechanisms, but the major strategy of survival continues to be the establishment of latency. Alphaherpesviruses, such as pseudorabies virus (PRV) and herpes simplex virus type 1 (HSV-1), have the ability to establish lifelong latent infection in sensory neurons innervating the primary site of replication (Geenen et al. 2005). *In vivo* studies in natural host and mice indicated that EHV-1 replicates in the neurons of the trigeminal ganglion and the olfactory bulbs, where latent infection is also established (Awan et al. 1990, Chesters 1997, Walker et al. 1999). Our earlier results have demonstrated, however, that in mice experimentally infected with the EHV-1 field strain productive and latent infection affected not only neurons in the olfactory bulbs, but also those in other parts of the rhinencephalon (Bańbura et al. 2000, Bańbura 2003). Moreover, infection was accompanied by signs of neuronal degeneration. Interestingly, the presence of viral DNA (Jan-E EHV-1 strain) was confirmed in mouse brain eight weeks after infection despite the absence of any virus reactivation factors. Moreover, detectable levels of viral RNA expression were found in latently infected neurons (Bańbura et al. 2000). Similar results were obtained *in vitro* in the studies presented. When infected, some murine neurons (approx. 60%) in the primary cell culture changed their shape and degenerated, although some remained unchanged and as such survived for more than eight weeks. In addition, positive results of immunofluorescence tests indicated the presence of EHV-1 antigens in the nucleus and cytoplasm of neurons, which implies that there may be constant, yet "limited" replication, followed by the release of viral progeny. These results were confirmed by infecting murine neurons culture with supernatant from EHV-1-infected culture incubated for eight weeks. Positive immunofluorescence indicated the presence of viral antigen in neurons (Cymerys et al. unpublished data). It is difficult to explain the constant detectable levels of viral transcripts, long after viral progeny has been generated and neurons have not been lysed. An open question remains whether neurons were infected persistently or latently in which case infected neurons may slowly degenerate due to the constant, yet limited, transcriptional activity of the virus. Considering the general properties of α-herpesviruses, it seems probable to assume that EHV-1 enters the neurons of the central nervous system, and it's genome remains transcriptionally active. Based on the present results it is tempting to speculate whether that two different forms of the virus may concurrently occur in a population of cultured murine neurons: virus capable of active replication, which leads to changed morphology and slow degeneration of neurons and the latent virus, in which virus replication is blocked and the transcriptional activity of the viral genome is limited only to the generation of *Latency Associated Transcripts* (LATs) or *Latency Related Regions* (LRs). Our results obtained are at least partially consistent with other studies, most of which relate to *in vitro* tests. It has been observed that TG and other neurons may survive (limited) virus replication during primary infection or reactivation, and enter or resume latency afterwards (Simmons and Tscharke 1992, Geiger et al. 1995, Perng et al. 2000, Aleman et al. 2001). Furthermore, it was observed that surviving TG neurons (96 hours p.i.) were still able to transmit infectious virus to other cells (Geenen et al. 2005). Other reports show that LAT can supress apoptosis (either *in vivo* or *in vitro*) and that this function may explain the importance of LAT in α-herpesvirus (for example HHV-1, BHV-1) latency and reactivation (Geiger et al. 1995, Perng et al. 2000, Jones 2003). It is worth adding that some phenomena associated with latent infection depend on the tissue where the virus resides in it's latent form. Other authors noticed that latency-related transcripts are transcribed from various areas of the genome, depending on the type of tissue. EHV-1 is latently present in the cell nuclei of trigeminal ganglion neurons, from where it can be reactivated, and the area of the genome region near the 3'-end of gene 63, which is homologue of IE 110 HSV-1 (IE; immediate-early) is transcriptionally active during latency (Slater et al. 1994, Baxi et al. 1996). Transcriptional activity of the EHV-1 genome during latent infection was also noted in peripheral blood leukocytes when no latency-associated transcripts (LATs) were present in the nervous tissue (Chesters 1997). However, the portion that partially overlaps with the 3'-end of gene 64, but not gene 63, is expressed in leukocytes.

Our study has provided some answers to a number of key EHV-1-related questions: field isolates at low *in vitro* passage Jan-E and reference strain at high passage Rac-H were able to replicate without the need for adaptation in mouse neurons that were cultured *in vitro*; some neurons survived infection and showed constant, yet limited, virus replication.

Taken together our results indicate that cultured murine neurons provide a good model for testing mechanisms of EHV-1 neurovirulence and virus-neuron interactions.

Acknowledgement

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