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Differential tropism of human herpesvirus 6 (HHV-6) variants and induction of latency by HHV-6A in oligodendrocytes

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Human herpesvirus 6 (HHV-6) is a ubiquitous β-herpesvirus associated with a number of clinical disorders. Two closely but biologically distinct variants have been described. HHV-6 variant B causes the common childhood disease exhanthem subitum, and although the pathologic characteristics for HHV-6 variant A are less well defined, HHV-6A has been suggested to be more neurotropic. We studied the effect of both HHV-6 variants in an oligodendrocyte cell line (MO3.13). Infection of M03.13 was monitored by cytopathic effect (CPE), quantitative TaqMan PCR for viral DNA in cells and supernatant, reverse transcriptase-polymerase chain reaction (RT-PCR) to detect viral RNA, and indirect immunofluorescence (IFA) to detect viral protein expression. HHV-6A infection induced significantly more CPE than infection with HHV-6B. HHV-6B induced an abortive infection associated with a decrease of the initial viral DNA load over time, early RNA expression, and no expression of viral antigen. In contrast, infection with HHV-6A DNA persisted in cells for at least 62 days. During the acute phase of infection with HHV-6A, intracellular and extracellular viral load increased and cells expressed the viral protein IE-2 and gp116/54/64. No HHV-6A RNA or protein was expressed after 30 days post infection, suggesting that HHV-6A formed a latent infection. These studies provide *in vitro* support to the hypothesis that HHV-6 can actively infect oligodendrocytes. Our results suggest that HHV-6A and HHV-6B have different tropism in MO3.13 cells and that an initially active HHV-6A infection can develop latency. Differences between HHV-6A and -6B infection in different neural cell types may be associated with different neurological diseases. *Journal of NeuroVirology* (2005) **11,** 384–394.

Keywords: human herpesvirus 6; latency; MO3.13; multiple sclerosis; oligodendrocytes

Introduction

Human herpesvirus 6 (HHV-6), a member of β-herpesviridae, was first isolated from patients with lymphoproliferative disorders in 1986. Seroepidemiological studies demonstrate that at least 90% of the healthy population is seropositive for HHV-6 (Levy *et al*, 1990), suggesting that HHV-6 can establish latency in immunocompetent hosts. Two variants of HHV-6 (HHV-6A and HHV-6B) have been described based on genomic, antigenic, and biological differences (Ablashi *et al*, 1991). The genomes of HHV-6A and HHV-6B range in length from 160 to 170 kb and encode approximately 100 proteins (Isegawa *et al*, 1999). The overall nucleotide sequence identity between HHV-6A and HHV-6B is approximately 90% (Dominguez *et al*, 1999). Eighteen open reading frames are unique to either HHV-6A or HHV-6B. Due to the close sequence identities between the two

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viruses and the absence of serological assays that can easily discriminate between the two variants, HHV-6A and HHV-6B are often treated as a single virus. However, it has been argued that differences in the biology, cellular tropisms, and restriction endonuclease profiles of the two variants are sufficient to classify them as distinct herpesviruses rather than as variants of the same virus (Dominguez *et al*, 1999).

The HHV-6B variant has been identified as the causative agent of *exanthem subitum*, a common childhood disease, characterized by fever, followed by a rash (Yamanishi *et al*, 1988), and accounts for the majority of symptomatic HHV-6 infections in infants (Dewhurst *et al*, 1993; Hall *et al*, 1998). However, the HHV-6A variant has yet to be clearly associated with a particular disease (Braun *et al*, 1997). Although HHV-6B has been detected more frequently in the cerebrospinal fluid (CSF) of children and adults than HHV-6A, patients who are infected with HHV-6A are more likely to have HHV-6A DNA present in their CSF, suggesting an increased neurovirulence of HHV-6A compared to HHV-6B (Hall *et al*, 1998). Additionally, the HHV-6A variant has been isolated from the CNS of acquired immunodeficiency syndrome (AIDS) patients with areas of demyelination (Knox and Carrigan, 1995).

The role of HHV-6 in neurological disease is poorly understood. Neurological complications such as febrile and afebrile seizures, encephalitis, and meningitis sometimes occur after primary HHV-6 infection (Caserta *et al*, 1994; Hall *et al*, 1994; Jones *et al*, 1994; Suga *et al*, 1993; Yanagihara *et al*, 1995; Zerr *et al*, 2002). Reactivation of HHV-6 in adults is believed to be responsible for encephalitis in immunocompromized hosts such as bone marrow transplant, organ transplant, and stem cell recipients (Drobyski *et al*, 1994; Wainwright *et al*, 2001; Zerr *et al*, 2001). Furthermore, a neuropathogenic role for HHV-6 has been suggested based on the development of a variety of disorders associated with active HHV-6 infection, including fulminant demyelinating encephalomyelitis, subacute leukoencephalitis, necrotizing encephalitis, progressive multifocal leukoencephalopathy, and chronic myelopathy (Carrigan *et al*, 1996; Mackenzie *et al*, 1995; Mock *et al*, 1999; Novoa *et al*, 1997; Wagner *et al*, 1997). More recently, HHV-6B viral proteins have been detected in astrocytes from the hippocampus of patients with mesial temporal lobe epilepsy (MTLE) (Donati *et al*, 2003). *In vitro* studies of HHV-6–infected astrocytes demonstrated that HHV-6A and HHV-6B have differential tropism (Donati *et al*, unpublished observations) where HHV-6B has been shown to be associated with latency in an astrocytic cell line (Yoshikawa *et al*, 2002).

HHV-6 has been considered an attractive candidate as a possible etiologic agent in multiple sclerosis (MS), a chronic demyelinating disease, for several reasons. (1) Primary infection with HHV-6 usually occurs during the first few years of life and the involvement of HHV-6 with MS is consistent with epidemiological evidence in MS, suggesting exposure to an etiologic agent before puberty (Kurtzke, 1995; Yamanishi *et al*, 1988). (2) HHV-6, particularly the HHV-6A variant, is highly neurotropic (Hall *et al*, 1998). Primary infection with HHV-6 occasionally results in neurologic complications, including meningitis and menigoencephalitis, encephalitis, and febrile seizures (Asano *et al*, 1992; Hall *et al*, 1994; Ishiguro *et al*, 1990). (3) HHV-6 is pleiotropic and infects cells of both lymphoid and nonlymphoid origin. The pleiotropism of HHV-6 could explain abnormalities observed in both the immune and nervous systems of patients with MS. (4) As one of the fundamental properties of herpesviruses is their tendency to reactivate from a latent state, the same factors that often lead to herpesvirus reactivation, such as stress and infection with another agent, have also been associated with MS exacerbations. One hypothesis for how HHV-6 could lead to antimyelin autoreactivity is MS includes the necessity of the virus to reactivate from latency in oligodendrocytes. Unfortunately, the mechanisms by which HHV-6 achieves latency and reactivation are poorly understood (Kondo *et al*, 2002; Yasukawa *et al*, 1999a, 1999b).

The association of HHV-6 with MS is supported by immunological and molecular studies. HHV-6 DNA is detected more frequently in oligodendrocytes in acute-phase lesions and in MS plaques compared to normal appearing white matter (Cermelli *et al*, 2003; Challoner *et al*, 1995; Goodman *et al*, 2003; Opsahl and Kennedy, 2005). HHV-6 reactivation also correlates with MS disease activity and with detection of gadolinium-enhancing lesions on magnetic resonance imaging (MRI) (Chapenko *et al*, 2003). The highly neurotropic A variant rather than the B variant may play a role in this disease (Soldan *et al*, 2000) based on an increased lymphoproliferative response to the HHV-6A variant and the unique amplification of HHV-6A DNA in the peripheral blood mononuclear cells (PBMCs), serum, and urine of MS patients (Akhyani *et al*, 2000). This indicates that the two variants may have different tropisms for different brain cells. Therefore, future studies concerning the putative association of HHV-6 with MS must consider variant specific tropisms and immunology.

As there is a paucity of information studying tropisms and growth characteristics of HHV-6 variants in glial cells, we directly compared the effects of infection with both variants of HHV-6 in an oligodendrocyte cell. The oligodendrocyte cell line M03.13 was used as a target for HHV-6 infection due to difficulties in obtaining and propagating sufficient quantities of purified human adult oligodendrocytes. M03.13 cells are a hybrid of oligodendrocytes and rhabdomyosarcoma that express 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), galactosyl cerebroside (GalC), myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein

(MAG) (Buntinx *et al*, 2003; McLaurin *et al*, 1995). Although the use of this cell model is limiting, it has been considered a reasonable surrogate to study human primary oligodendrocytes (Arbour *et al*, 1999a, 1999b; Kong *et al*, 2003; Plumb *et al*, 2002). The results presented in this article will demonstrate different growth characteristics of HHV-6 variants in MO3.13 cells suggesting that variants of HHV-6 may have different functional consequences for infected human glial cells.

Results

Morphology and viral growth characteristics

Differences between HHV-6A and HHV-6B infection in MO3.13 were initially based on induction of cytopathic effects and changes in cell morphology. During acute infection with HHV-6B, no change in cell proliferation with little change in cell morphology was observed compared to mock-infected MO3.13. Brightfield images of HHV-6B infected cultures at day 7 show cells morphologically similar to mock infected cells (Figure 1B). In contrast, acute infection with HHV-6A was associated with many cells becoming rounded, a loss of cellular extensions (a characteristic of oligodendrocytes), and high levels of cell death (Figure 1A). These morphological changes and induction of cytopathology suggested that HHV-6A, relative to HHV-6B, induced a more active, lytic infection in HHV-6A infected MO3.13 cells.

MO3.13 cells were infected with HHV-6A and HHV-6B and growth characteristics of each virus were monitored throughout infection. Viral loads in cell lysates and cell-free supernatants were quantified by HHV-6A– and HHV-6B–specific real-time TaqMan polymerase chain reaction (PCR). During the first 2 days of infection with HHV-6A, viral load in both cell lysate and supernatant decreased (Figure 2A). By day 3, HHV-6A viral load peaked in HHV-6A–infected cell lysates (1.3 log increase) followed 2 days later by a peak in HHV-6A viral load in supernatants (a 1.2 log increase) (Figure 2A). These observations were suggestive of active HHV-6A viral replication in virus-infected MO3.13 cells wheras detection of increased HHV-6A viral loads in supernatants indicated release of viral particles. Following the early increase in viral replication, cell lysate viral load stabilized and remained at a level of 1 copy/10 cells for the duration of infection (62 days). HHV-6A viral loads in supernatant decreased after the initial spike at day 5 and were undetectable after 14 days (Figure 2A). The presence of viral DNA in cells without viral DNA in supernatant suggested the development of a latent infection. During latency, there was no active viral DNA replication because the intracellular viral DNA load remained steady and we were unable to detect viral DNA in cell culture supernatant. This last observation suggests the virus the virus no longer replicated and produced viral particles.

In contrast to the observed growth characteristics of HHV-6A in MO3.13, infection with HHV-6B did not induce active viral replication or release of viral particles. HHV-6B viral loads in cell lysates and supernatant from HHV-6B infected cultures steadily decreased over time with undetectable levels in cells at day 32 and in supernatant at day 14 (Figure 2B). HHV-6B–infected MO3.13 cells were followed for over 60 days with no detectable virus in either cell lysates or supernatants (data not shown). These results suggest that unlike the lytic effects observed with HHV-6A infected MO3.13 cells, HHV-6B infection of these cells was abortive.

Viral antigen expression

Changes in cell morphology, induction of cell death, and increased viral load during acute HHV-6A infection suggested an early active viral infection whereas infection with HHV-6B appeared abortive. These results were supported by immunofluorescence microscopy showing expression of viral-specific proteins in HHV-6A–infected cells. Infection of MO3.13 with HHV-6A for 7 days induced expression of the HHV-6A–specific IE-2 antigen (green staining; Figure 1C). Such patch-like staining of IE-2 within cell nuclei was consistent with a previous report by Arsenault *et al* (2003). At 7 days post infection, HHV-6A–infected cells also expressed the structural nonvariant specific antigen gp116/54/64 (red; Figure 1C). In contrast, HHV-6B–infected cells were negative for the nonvariant specific gp116/54/64 at 7 days post infection (Figure 1D). Mock-treated MO3.13 were negative for IE-2 and gp116/54/64 (data not shown). JJahn T cells infected with HHV-6A were used as a positive control for IE-2 and gp116/54/64. JJhan infected with HHV-6A expressed IE-2 and gp116/54/64 (Figure 1E). Uninfected JJahn T cells were used as a negative control and did not stain for either IE-2 or gp116/54/64 (Figure 1F).

Detection of HHV-6 mRNA during acute HHV-6 infection

To confirm that acute infection with HHV-6A was active as indicated by increased viral load and viral antigen expression, we detected HHV-6–specific mRNA by reverse transcriptase (RT)-PCR in MO3.13 at 5 days post infection. MO3.13 infected with HHV-6A expressed mRNA for the HHV-6–specific gene U83 (Figure 3). Although infection of MO3.13 with HHV-6B was abortive and was associated with decreasing viral load (Figure 2B) without viral protein expression (Figure 1D), HHV-6B–infected cells expressed mRNA for U83 confirming that they were acutely infected (Figure 3).

Development of a latent infection of MO3.13 with HHV-6A

At day 30 post infection, the viral load in HHV-6A–infected MO3.13 cell lysates was approximately

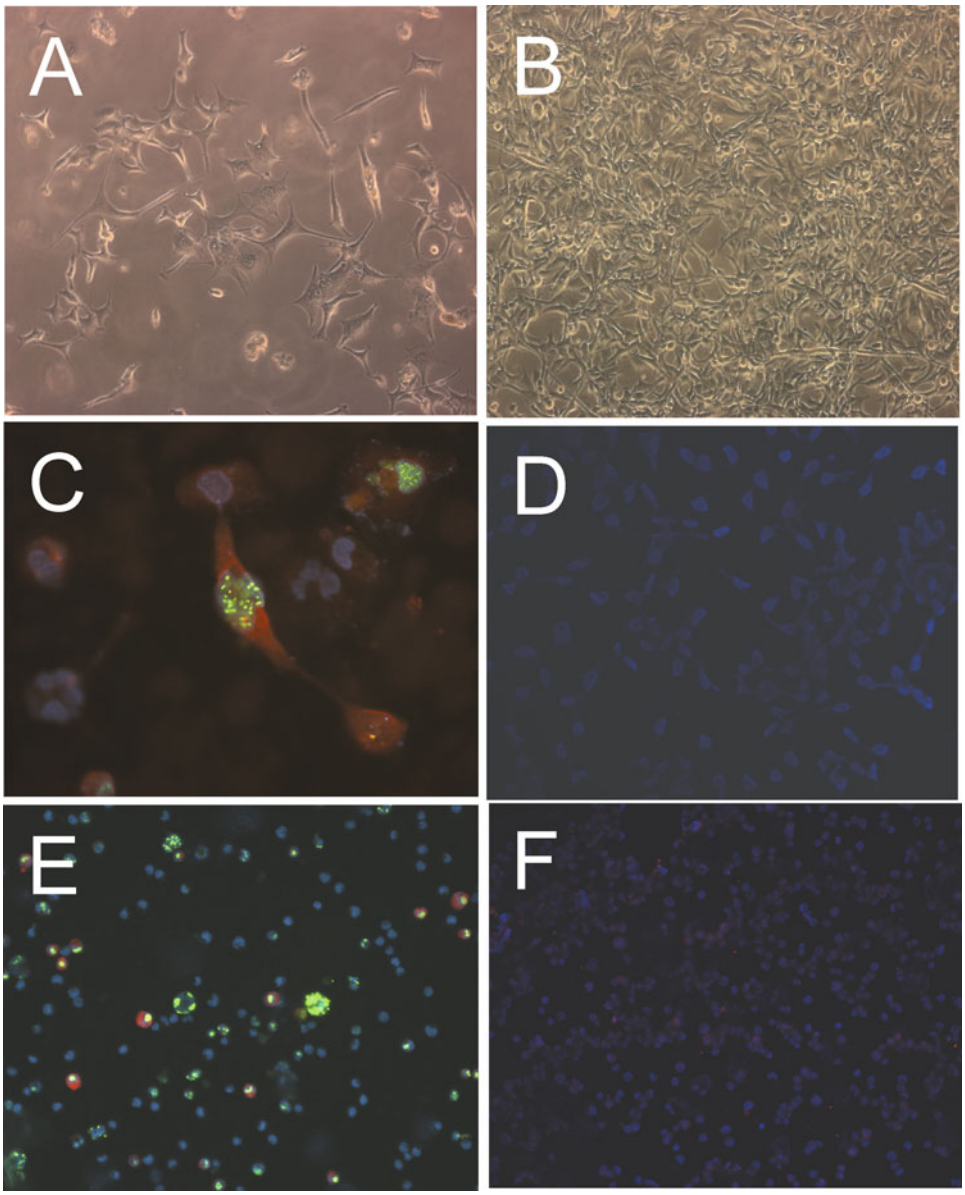

Figure 1 Infection of MO3.13 with HHV-6A induces cytopathic effects (CPEs) and viral antigen expression. Infection of HHV-6A (**A**) induces significantly more cell death than infection with HHV-6B (**B**) at 7 days post infection (brightfield images; 20× objective). MO3.13-infected cells were fixed and stained for HHV-6 viral antigens (HHV-6A variant specific IE-2 and gp116/54/64 and specific for both variants of HHV-6) and were counterstained with DAPI to reveal cell nuclei (blue). Seven days post infection, MO3.13 cells infected with HHV-6A (**C**) are positive for IE-2 as indicated by staining of nuclear granules (green; 63× objective) and for the structural antigen gp116/54/64 (red). Seven days post infection, MO3.13 cells infected with HHV-6B (**D**; 63× objective) are negative for gp116/54/64. JJhan infected with HHV-6A were positive for IE-2 (green; 20× objective) and gp116/54/64 (red) (**E**) and uninfected JJahn were negative for both IE-2 and gp116/54/64 (**F**; 20× objective).

10^5 viral copies/10^6 cells and no viral DNA was detected in supernatants (Figure 2A), suggesting that the early active viral infection had developed latency. Presence of the complete viral DNA genome during this late phase of infection with HHV-6A was indicated by primary PCR of immediate early, early and late HHV-6 genes (U16/U17, U27, U31, U39, U57, and U94) (Figure 4A). At day 34 post infection, the viral load in cell lysate was 3×10^5 viral copies/10^6 cells (Figure 2B) and all six HHV-6 genes were detected by primary PCR (Figure 4A). At day 60 post infection, the viral load in cell lysate persisted at 4×10^4 viral copies/10^6 cells (Figure 4B) and by primary PCR we were able to detect U27 (Figure 4C). Latent HHV-6 infection has been defined as detectable viral DNA without expression of viral RNA (Yasukawa *et al*, 1999a). Confirming the development of latency, no viral protein or viral mRNA was detected in HHV-6A–infected MO3.13 at 30, 38, and 47 days post infection (data not shown).

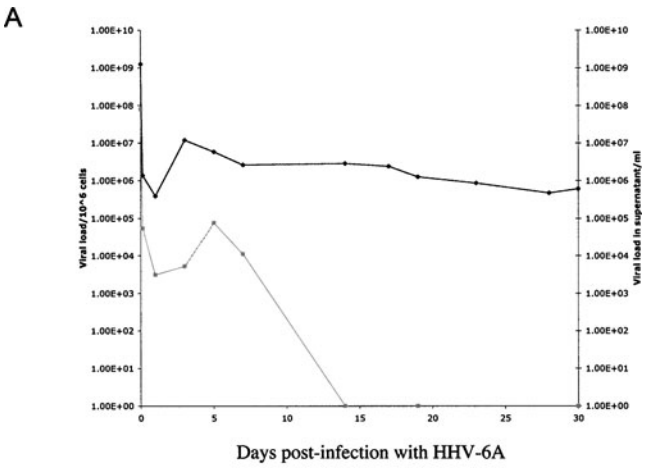

Figure 2 HHV-6A and 6B demonstrate different growth characteristics in MO3.13 cells. Viral DNA was quantitated in cell lysate (bold) and cell-free supernatant (thin line) by real-time TaqMan PCR. **A**, Infection of HHV-6A in MO3.13 resulted in an increase of the viral load in cell lysate from day 1 to day 3 post infection and an increase in viral load in supernatant from day 3 to day 5 post infection. Viral load remained stable at approximately 10^6 viral copies/10^6 cells after day 7. **B**, Viral load in cell lysate and supernatant of cultures infected with HHV-6B decreased over time and abrogated by day 32.

Discussion

Our aim in this study was to investigate the growth characteristics of HHV-6 in oligodendrocytes. Because of the difficulty in culturing and propagating human primary oligodendrocytes, we compared infections of both HHV-6A and -6B variants in the well-characterized oligodendrocyte cell line MO3.13 (Buntinx *et al*, 2003; McLaurin *et al*, 1995). Our results demonstrated differential growth characteristics for the two variants of HHV-6 in MO3.13. Infection with HHV-6A was associated early with an active and lytic infection in MO3.13 and later with latency in over 60 days of culture. In contrast, infection with HHV-6B was abortive and self-limiting.

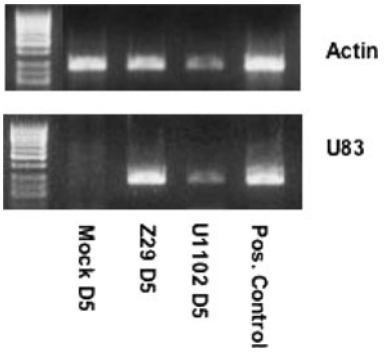

Figure 3 MO3.13 cells acutely infected with HHV-6 express viral RNA. At 5 days post infection, MO3.13 infected with HHV-6A (U1102) and HHV-6B (Z29) were positive for the HHV-6–specific gene U83. HHV-6A–infected human astrocytes were used as a positive control for U83 RT-PCR.

Although HHV-6 is a commensal pathogen in the CNS of the healthy population (Challoner *et al*, 1995; Luppi *et al*, 1995), reactivation of latent HHV-6 infection in the brain has been implicated in the development of neurologic disease (Dockrell and Paya, 2001). However, *in vitro*, little is known about infection characteristics and tropisms involved in induction of latency of HHV-6 variants A and B in neural cells and differences in cellular tropisms may be important for development of disease. That both variants can populate the brain has been demonstrated though they may differ in preference of cell type for latency and replication. Studies have suggested that HHV-6A is more commonly associated with MS whereas HHV-6B has been detected in brain specimens from epilepsy patients (Donati *et al*, 2003; Soldan *et al*, 2000).

Our results in MO3.13 cells demonstrate that HHV-6A induced significant cell death not seen in cells infected with HHV-6B. Viral load in both cells and supernatant declined steadily in HHV-6B–infected cells, suggesting that the virus did not actively replicate or produce and release viral particles. Although HHV-6 viral mRNA was detected at 5 days post infection, the infection with HHV-6B abrogated after 32 days. Presence of viral mRNA during the acute infection with HHV-6B confirms that these cells can be infected but the infection was not sustained. In addition, we were unable to detect viral antigen at 7 days post infection with HHV-6B. The steady decline of viral load and lack of viral antigen expression demonstrated that HHV-6B was associated with a nonproductive infection in MO3.13 cells.

Differences may exist between premature oligodendrocytes like MO3.13 and mature adult oligodendrocytes in their permissiveness to HHV-6B infection. Albright and colleagues detected HHV-6B viral particles in adult oligodendrocytes by electron microscopy (Albright *et al*, 1998); however, only expression of the non-structural immediate early protein IE1 and not of the structural glycoprotein gp60/10 was

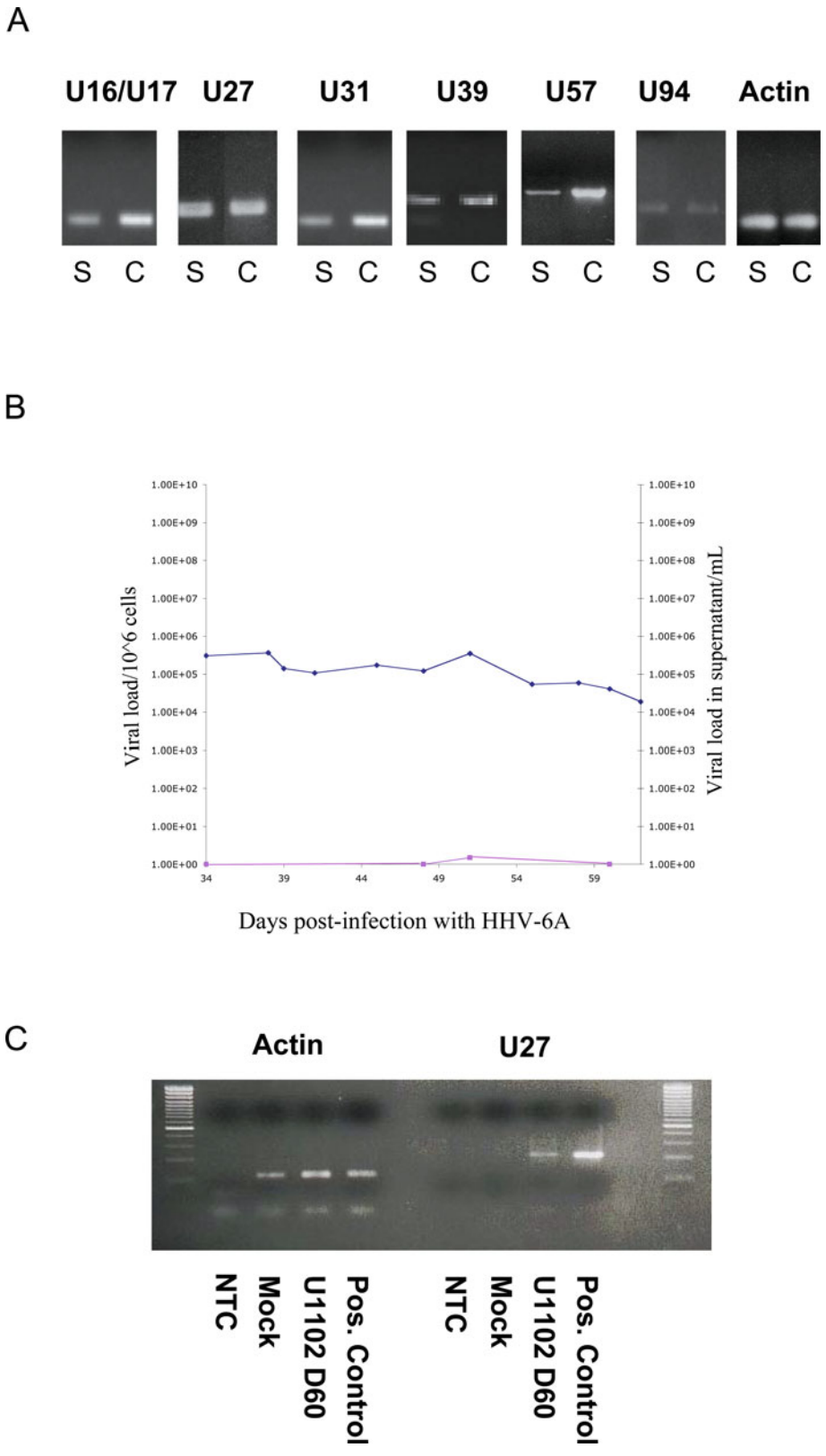

Figure 4 HHV-6A forms a latent infection in MO3.13 cells. **A**, At day 34 post infection with HHV-6A, DNA for six viral genes were detected by primary PCR (S = sample; C = positive control). **B**, Viral DNA was quantitated in cell lysate (bold) and cell-free supernatant (thin line) by real-time TaqMan PCR. Viral loads in cell lysates from HHV-6A infected MO3.13 cells persisted from 34 to 62 days post infection. Viral loads in supernatants were undetectable during this late phase of infection. **C**, At 60 days post infection, DNA for U27 was detected by primary PCR in mock-infected MO3.13 and U1102-infected MO3.13. (NTC, no template control.)

observed in HHV-6B infected adult oligodendrocytes or MO3.13 (De Bolle *et al*, 2005), which would suggest that no viral particles were formed. This suggests the presence of an intracellular blocking mechanism in MO3.13 that inhibits the viral life cycle to proceed to the formation of viral particles, a phenomenon that has previously been reported for cytomegalovirus (CMV) (Weinshenker *et al*, 1988). In our study, we were unable to detect HHV-6B viral protein expression or increased viral DNA in MO3.13 culture supernatant or cell lysates. M03.13 is a premature oligodendrocyte cell line and therefore has different characteristics than adult oligodendrocytes (Buntinx *et al*, 2003). Infected mature oligodendrocytes may produce HHV-6B viral particles and be more permissive to infection (Albright *et al*, 1998) whereas the infection aborts in premature cells. It is also possible that the inability of HHV-6B to produce viral particles in MO3.13 is due to the inherent properties of the cell line, because glial precursor cells have also been shown to be susceptible to HHV-6 infection *in vitro* (Dietrich *et al*, 2004).

In contrast to our observations with HHV-6B, infection of MO3.13 with HHV-6A induced high levels of cytotoxicity, increases in viral load in cell lysates (day 3) and cell-free supernatant (day 5), and viral antigen expression during the acute phase of infection. Viral load persisted for 62 days in cell lysates whereas viral load in supernatants was undetectable after day 14. Early increases in viral load in cell-free supernatant (day 5) indicated active viral replication and release of viral particles. Persistence of viral DNA in cells without detectable viral DNA in supernatant during the later stages of infection suggested the development of latency. This observation is further supported by a lack of viral antigen and viral RNA expression during the persistent phase of infection. RNA for the immediate early genes (U16/U17, U94) and the late gene U12 was undetectable by RT-PCR at days 30, 38, and 47 post infection (data not shown). Yasukawa and coauthors defined latency as presence of HHV-6 viral DNA without expression of viral RNA (Yasukawa *et al*, 1999a). Our results suggest that HHV-6A can form a latent infection in MO3.13 and thus provides *in vitro* support to the hypothesis that HHV-6 may persist in oligodendrocytes *in vivo*. The immediate-early gene U94 has been suggested to play a role in latency, but in our study we were unable to detect RNA for U94 or for the immediate-early gene U16/U17 and the late gene U12. It is unclear whether immediate-early genes are expressed in cells persistently infected with HHV-6. Expression of immediate early genes is not an inherent feature of latency in the related herpesvirus CMV (Kurz *et al*, 1999) but may represent the first step of reactivation (Hummel and Abecassis, 2002). *In vivo*, establishment of latency and reactivation episodes of recurrent infections are hallmarks of herpesvirus biology. Amongst herpesviruses, only human herpesvirus 1 (HSV-1) and HHV-6 have been demonstrated to establish latent infections in the brain (Caserta *et al*, 1994; Fraser *et al*, 1981). Compared to HHV-6B, HHV-6A is detected three times more frequently in brain tissue (Cuomo *et al*, 2001) and has been suggested to be more neurotropic *in vivo* (Rotola *et al*, 2004) and *in vitro* (De Bolle *et al*, 2005). The ability to actively infect oligodendrocytes and develop latency may account for more frequent detection of HHV-6A in brain tissue.

Differences in HHV-6 cellular tropism may be important for development of neurologic disease. A recent report demonstrated presence of HHV-6B DNA and viral protein expression in astrocytes in mesial temporal lobe epilepsy (Donati *et al*, 2003). Viral protein expression indicates an active viral infection that may result from reactivation of latent HHV-6B. HHV-6A has been associated with the chronic inflammatory disease multiple sclerosis (Akhyani *et al*, 2000; Alvarez-Lafuente *et al*, 2004; Rotola *et al*, 2004; Soldan *et al*, 2000). HHV-6 infection has been associated with other demyelinating diseases of the central nervous system (CNS), including acute disseminated encephalomyelitis and demyelinating encephalomyelitis (Kamei *et al*, 1997; Novoa *et al*, 1997). Although the HHV-6 genome is prevalent in nervous tissue in normal controls, detection of viral DNA in oligodendrocytes, lymphocytes and microglia in acute-phase lesions in MS suggests that HHV-6 may be reactivated. MS is a multifactorial disease influenced by several genes, environmental factors, and dysregulated immune responses (Markovic-Plese and McFarland, 2001). Even if HHV-6 viral DNA is ubiquitous in oligodendrocytes in vivo, MS patients may not sufficiently control the virus. Although there is no conclusive evidence demonstrating any virus as the etiologic agent in multiple sclerosis, studies designed to understand further the tropisms and biology of HHV-6A and HHV-6B infection will help clarify the role these viruses play in neurologic disease.

Materials and methods

Viruses and cell lines

Human T-cell lymphoblast lines SupT-1 and JJhan were used to propogate HHV-6B variant (Z29) and HHV-6A variant (U1102), respectively. Cell-free inocula were obtained when the cytopathic effect (CPE) was >80%, normally at day 7 post infection. Cell suspensions were centrifuged for 10 min at 1200 rpm and the supernatants were harvested and centrifuged again for 10 min at 1200 rpm. Cell-free inocula were stored in 10-ml aliquots in −70°C. One aliquot was tested for viral DNA by real-time TaqMan PCR (described below). The inocula were not thawed more than once.

M03.13 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 25 mM glutamine,

Table 1 Primers and program cycles used for primary PCR and RT-PCR

ORF	*Nucleotide sequence* 5′ → 3′	*DNA (bp)*	*cDNA (bp)*	*PCR program (35 cycles)*
U12	R-primer CACTGTCATTGAGCTGTCCAA F-primer ACCACATGAGCACAAAATCG	327	238	94°C: 5 min; 94C: 30 s, 57C: 30s, 72C: 30 s; 72°C: 7 min
U16/U17	R-primer TTTAATTGGCCGAAAAGTCG F-primer CCTCTCCCCAGACAGAAACA	240	147	94°C: 4 min; 94°C: 1 min, 50°C: 2 min, 72°C: 2 min; 72°C: 7 min
U27	F-primer CGGGAACATAGAGAAACGAGAG R-primer GACAAAAACAAACATTCCGCC	209	209	94°C: 3 min; 94°C: 30 s, 52°C: 30 s, 72°C: 30 s; 72°C: 7 min
U31	R-primer GTGGATCCGACGTCCTACAAACAC F-primer GGAGAATCTTGTAAGTATATGGTC	834	834	94°C: 4 min; 94°C: 1 min, 50°C: 2 min, 72°C: 2 min; 72°C: 7 min
U39	R-primer CCATACCCTCCTCCTTTTCC F-primer CTTTCCTTATGCCACCAATCC	131	131	94°C: 3 min; 94°C: 30 s, 54°C: 30 s, 72°C: 30 s; 72°C: 7 min
U57	R-primer GCGTTTTCAGTGTGTAGTTCGGCAG F-primer TGGCCGCATTCGTACAGATACGGAGG	520	520	92°C: 3 min; 92°C: 30 s, 52°C: 30 s, 72°C: 30 s; 72°C: 7 min
U94	R-primer GCATACGTGCACCAATCATC F-primer ACGCTAAGCGGAGAATAAA	167	167	94°C: 3 min; 94°C: 30 s, 53°C: 30 s, 72°C: 30 s; 72°C: 7 min

and 100 IU/ml penicillin/streptomycin (McLaurin *et al*, 1995). SupT-1 and JJhan were maintained in RPMI 1640 medium containing 10% FBS, 25 mM L-glutamine, and 100 IU/ml penicillin/streptomycin.

HHV-6 infection

M03.13 were seeded onto 6-well plates and onto permanox 2-well chamber slides at a density 40,000 and 20,000 cells, respectively. After an overnight incubation allowing cells to adhere, cultures were washed twice with PBS and infected with freshly thawed cell-free viral supernatant containing $10^{9.25}$ DNA viral copies/10^6 cells. After a 3-h incubation at 37°C in 5% CO_2, cultures were washed three times with PBS and fresh medium was added. At every time point cell cultures were checked for CPE, chamber slides were fixed, and supernatant and cells were harvested and stored at −70°C for DNA and RNA extraction. Seven days post infection, medium was changed every third day and cells were passaged with trypsin when they became confluent. Mock infections were carried out using supernatant from uninfected SupT-1 or JJhan. Infections of each HHV-6 variant were performed twice.

DNA extraction and quantitative real-time TaqMan PCR

DNA was extracted using the DNeasy tissue kit for cells and the Viral RNA kit for supernatant (Qiagen) as per manufacturer's instructions. DNA concentrations for cells were adjusted to 10 ng/μl. Ten microliter of DNA from cells and supernatants were applied to a 96-well TaqMan plate in duplicates. Quantitative real-time PCR specific for each of the two viral variants was performed as described (Donati *et al*, 2003). Primers and probes (Applied Biosystems) were specific for the immediate early (IE) gene U67 of variants A and B (Nitsche *et al*, 2001). In parallel, samples were normalized with human genomic β-actin DNA.

PCR analysis

Primary PCR was conducted using primers for U16/U17, U27, U31, U39, U57 and U94 (Table 1). β-Actin primers sequences were 5′-CACACTGTGC CCATCTACGA-3′ and 5′-CTCAGTGAGGATCTTCAT GAGGTAGT-3′, amplifying a 105-bp sequence. PCR conditions were as follows: initial denaturation, 92°C for 3 min, follows 35 cycles each of denaturation at 92°C for 30 s, annealing at 52°C for 30 s min, and extension at 72°C for 30 s; final extension was done for 7 min at 72°C.

Primer sequences and program cycles for HHV-6 genes are shown in Table 1. PCR product was subjected to electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

mRNA Amplification and RT-PCR for HHV-6 U83

Total RNA was extracted according to manufacture's protocol using RNeasy (Qiagen, CA). MessageAmp aRNA Kit (Ambion, TX) was used to amplify mRNA according to the manufacturer's instructions. HHV-6 U83 RT-PCR was performed with gene specific primers previously described U83-F 5′-GTCGACCATGTTCATTTGGCTTTTTATTGTT-3′ and U83-R 5′-ATGAATTCTCATGATTCTTTGTCTA ATTTC-3′ (Zou *et al*, 1999) with AccessQuick RT-PCR system (Promega, WI). Reverse transcription of mRNA to cDNA was performed at 48°C for 45 min followed by 2 min inactivation of the AMV reverse transcriptase at 94°C. PCR amplification was performed under the following conditions: at total of 40 cycles at 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min, and one final extension at 68°C for 7 min.

Viral RNA detection

RNA extractions for RT-PCR were performed using the RNeasy kit as per manufacturer's instructions (Qiagen). To remove viral DNA, RNA samples were digested with 1 U DNase I (Invitrogen) for 20 min at room temperature. First strand cDNA synthesis was carried out with 400ng of total RNA using the following conditions: 70°C for 5 min for random

primer annealing, 5 min on ice, annealing at 25°C for 5 min, first strand synthesis at 42°C for 50 min, and inactivation at 70°C for 15 min (Improm-II reverse transcription system, Promega, Madison, WI, USA). Absence of DNA was verified by PCR amplification without retrotranscription. β-Actin primers sequences were: 5′-CAAGAGATGGCCACGGCTGCT-3′ and 5′-TCCTTCTGCATCCTGTCGGCA-3′, amplifying a DNA fragment of 340 bp and a cDNA fragment of 275 bp. PCR conditions were: initial denaturation, 94°C for 3 min, 35 cycles each of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s; final extension for 2 min at 72°C. Primer sequences and program cycles for U12, U16/U17 and U94 are shown in Table 1.

Indirect immunofluorescence assay

Cells grown on 2-well slides were washed once with phosphate-buffered saline (PBS), fixed with 1:1 acetone/methanol at −20°C for 10 min, air-dried, and stored in −20°C. Thawed slides were washed once in PBS and blocked for 10 min with 3% bovine serum albumin diluted in PBS. HHV-6 infected cells were incubated for 60 min at room temperature with the nonvariant-specific anti-HHV-6 gp116/54/64 mouse monoclonal antibody diluted 1:100 (Advanced Biotechnologies, Columbia, MD) and immediate-early 2 (IE-2) HHV-6A specific monoclonal antibody diluted 1:40 (A kind gift from Louis Flamand, Laval University, Quebec, Canada). Slides were washed three times with PBS, once with distilled water, and incubated for 1 h at room temperature with alexa fluor-conjugated anti-mouse IgG1 and IgG2b diluted 1:1000 (Molecular Probes, Eugene, OR). Slides were washed three times with PBS, incubated with DAPI diluted 1:30 000, washed with distilled water, and covered with glass coverslips. HHV-6A–infected and –uninfected JJhan were used as positive and negative controls, respectively.

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