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Human herpesvirus type 6 indirectly enhances oligodendrocyte cell death

Hong Kong, Quinton Baerbig, Laine Duncan, Nick Shepel, and Michael Mayne

Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba, Canada and Division of Neurovirology and Neurodegenerative Disorders, St. Boniface Hospital Research Centre, Winnipeg, Manitoba, Canada

Accumulating evidence suggests that human herpesvirus type 6 (HHV-6) plays a pathogenic role in diseases of the central nervous system including multiple sclerosis (MS). Recent studies have indicated that HHV-6 DNA is detected with high frequency in MS lesions compared to normal-appearing white matter, implicating a role for HHV-6 in MS pathogenesis. It appears that T cells, which infiltrate into the brain in MS patients, and resident oligodendrocytes harbor HHV-6 virus in MS lesions. Because T cells infected with HHV-6 have elevated proinflammatory gene expression, we hypothesized that HHV-6 could be indirectly cytotoxic to glial cells, including oligodendrocytes. Supernatants from SupT1 cells infected with HHV-6 variant A (GS or U1102) or variant B (Z29) significantly reduced MO3.1 cell proliferation by 75% ± 10%, 78% ± 8% or 51% ± 9%, respectively. HHV-6 viral supernatants (GS or U1102 or Z29) significantly increased MO3.1 or primary human oligodendrocyte precursor cells (OPCs) cell death, whereas primary human fetal astrocytes were not affected. Removal of HHV-6 virions or proteins by trypsin treatment from culture supernatants did not reverse the loss in oligodendrocyte proliferation or viability. Supernatants from HHV-6 GS or U1102 cultures were significantly more cytotoxic to MO3.1 cells or OPCs compared to supernatants from T cells infected with Z29. Dying oligodendrocytes did not have an apoptotic-like phenotype and toxicity was not inhibited by general inhibitor of apoptosis, ZVAD. Further, oligodendrocytes had minimal caspase-3 activation even in the presence of staurosporine, suggesting that cell death followed caspase-independent pathways. These results indicate that HHV-6 is indirectly cytotoxic to oligodendrocytes and that cell death is driven primarily by caspase-independent pathways. *Journal of NeuroVirology* (2003) **9,** 539–550.

Keywords: cell death; human herpesvirus type 6; multiple sclerosis; oligodendrocytes; toxicity

Introduction

Human herpesvirus type 6 (HHV-6), a member of the beta human herpesvirus family, was first isolated from the peripheral blood of immunocompromised patients with lymphoproliferative disorders (Salahuddin *et al*, 1986). Although reactivation or new infection of HHV-6 is associated with the pathogenesis of several diseases, including human immunodeficiency virus (HIV) infection (Grivel *et al*, 2001), multiple sclerosis (Akhyani *et al*, 2000; Berti *et al*, 2000; Clark, 2000), and seizures in children and adults (Uesugi *et al*, 2000; Wainwright *et al*, 2001), the only disease in which HHV-6 has been clearly defined as the causative agent is *exanthem subitum* (roseola enfantum), a pediatric fever and skin rash in children (Yamanishi *et al*, 1988). HHV-6 is highly prevalent in the general population, with greater than 90% being seropositive (Campadelli-Fiume *et al*, 1999; Di Luca *et al*, 1996). HHV-6 reactivation is common in immunocompromised

Address correspondence to Dr. M. Mayne, Department of Pharmacology and Therapeutics, University of Manitoba, R4050 St. Boniface Hospital Research Centre, Winnipeg, Manitoba, Canada, R2H 2A6. E-mail: mmayne@cc.umanitoba.ca

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individuals and, like the closely related cytomegalovirus (CMV), has been associated with organ, bone marrow, and peripheral blood cell transplantation failure and engraftment inhibition (for reviews, see Campadelli-Fiume *et al* [1999], Yoshikawa and Asano [2000]). Recently, it was determined that HHV-6 infection was more frequent and the viral load was higher in cord blood stem cell transplant recipients with prior primary HHV-6 infection (Sashihara *et al*, 2002). HHV-6 infection has been associated, in rare cases, with central nervous system (CNS) complications, including neuroinflammation, febrile seizures, encephalitis, or encephalopathy (Yoshikawa and Asano, 2000).

Two major viral subgroups of HHV-6 have been defined and are designated variants A and B. Although there is significant DNA sequence homology between the two variants, each has distinctive genomic, antigenic, and biological properties (Ablashi *et al*, 1991; Aubin *et al*, 1991; Pellett *et al*, 1993). The prototypical HHV-6B variant is Z29, which was isolated from a Zairian acquired immunodeficiency syndrome (AIDS) patients (Black *et al*, 1989). Variant B can be further subcategorized into groups 1 and 2. HHV-6B is found primarily in the peripheral blood, saliva, and lymph nodes of healthy individuals, and has been detected in serum of children with *roseola* (Yamanishi *et al*, 1988). HHV-6A is detected less frequently than HHV-6B in healthy adults. A highly studied, lab-adapted strain of HHV-6A is U1102, a strain that was isolated from a Ugandan AIDS patient (Salahuddin *et al*, 1986). Variant A is found primarily in the skin, brain, and cerebrospinal fluid (CSF). Little is known about the epidemiological and geographical distribution of HHV-6A (Clark, 2000). A greater neurotropism of the HHV-6A variant has been suggested based on the detection of HHV-6A in the CSF of children and adults (Hall *et al*, 1998) and that it has been detected recently in the CNS of AIDS patients with areas of demyelination (Blumberg *et al*, 2000). Increased HHV-6A–specific immune responses as well as the detection of HHV-6A–specific DNA sequences in the serum, urine, and peripheral blood lymphocytes of multiple sclerosis (MS) patients support an involvement of the HHV-6A variant in this disorder (Akhyani *et al*, 2000; Kim *et al*, 2000; Soldan *et al*, 2000).

There is accumulating immunological, molecular, and histological evidence suggesting that HHV-6 is involved in the pathogenesis of MS (Ablashi *et al*, 1998; Akhyani *et al*, 2000; Blumberg *et al*, 2000; Cermelli *et al*, 2003; Challoner *et al*, 1995; Friedman *et al*, 1999; Ongradi *et al*, 1999; Sanders *et al*, 1996; Soldan *et al*, 1997; Soldan and Jacobson, 2001; Tejada-Simon *et al*, 2002). Despite this association, there is little understanding of the mechanisms by which HHV-6 may be involved with lesion development or demyelination. HHV-6 induces proinflammatory gene expression during infection of T cells (Mayne *et al*, 2001) and specific proinflammatory cytokines are directly cytotoxic to human oligodendrocytes and oligodendrocyte precursor cells (for review, see Diemel *et al* [1998], Levine *et al* [2001]). Thus, we hypothesized that HHV-6 may be indirectly toxic to glial cells (bystander effect) and focused our studies on the indirect actions of HHV-6 on astrocytes and oligodendrocytes. Our aim was to determine the cytotoxic actions of supernatants from HHV-6–infected SupT1 cells on glial cell types because: (1) oligodendroyctes are the major producers of myelin within the CNS and are the major cell type lost in MS lesions (Levine *et al*, 2001; Ludwin, 1997; Pouly and Antel, 1999), and (2) astrocytes are known to protect oligodendrocytes (Corley *et al*, 2001). We report here that supernatants from SupT1 cells infected with HHV-6 selectively induced oligodendrocyte cell death whereas astrocytes were not affected.

Results

Viral cultures

At 7 days post subculturing, approximately 50% ± 8% of Z29-infected cells or 10% ± 4% of GS or U1102 displayed cytopathic effect (CPE), in which there was significant swelling of infected cells as reported previously (Mayne *et al*, 2001). In Z29-infected cultures, CPE-positive cells were always gp116 positive and no CPE was detected in gp116-negative cells (not shown). All HHV-6A–infected SupT1 cells (GS or U1102) that were CPE positive were also gp116 positive. However, the majority of SupT1 cells infected with HHV-6A did not display CPE but were gp116 positive (not shown). Taqman analysis of SupT1 cells infected with GS or U1102 showed that 7 days following subculturing, supernatants had $5 \times 10^4 \pm 2 \times 10^3$ DNA copies/million cells and cells contained $6 \times 10^4 \pm 5 \times 10^3$ DNA copies/million cells (average of GS and U1102). Analysis of SupT1 cells infected with Z29 showed that after 7 days of culture, supernatants had $6 \times 10^6 \pm 3 \times 10^4$ copies/million cells and cells contained $8 \times 10^7 \pm 4 \times 10^5$ DNA copies/million cells.

Human oligodendrocyte precursor cells are primarily O4 positive

Because MO3.1 cells are a hybrid of astrocytes and oligodendrocytes and they express several markers of human astrocytes (McLaurin *et al*, 1995), these cells represent a limited model for the study of mature oligodendrocytes. Accordingly, we purchased human oligodendrocyte precursor cells (OPCs) from Sciencell (San Francisco, CA) and cultured them *in vitro* for 3 days using differentiation medium supplied by Sciencell. Following 3 days of differentiation, approximately 80% of cells were of oligodendrocyte lineage (approximately 70% O4 positive and 10% (CNPase) positive with minimal (GFAP) reactivity; Figure 1). The majority of the cells were bipolar morphologically. OPCs were not

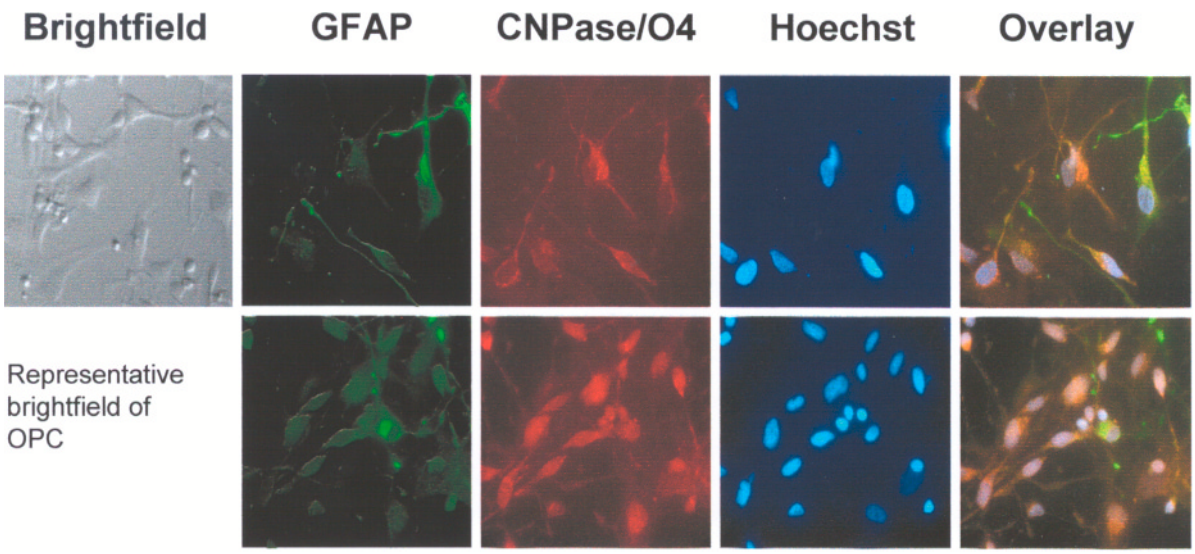


Figure 1 Neural precursor cells differentiate into cells of oligodendrocyte lineage. Neural cells from 20- to 24-week human fetal tissue were selected for nestin and A2B5 expression (Sciencell, San Francisco, CA). Cells were differentiated for 3 days and fixed using 4% paraformaldehyde. Brightfield analysis shows that the majority of the cells are bipolar in nature, with few polydendritic branches characteristic of mature oligodendrocytes. A small percentage of cells were positive for the mature oligodendrocyte marker CNPase (*upper panel*; depending on the fetal source, approximately 10% of the culture). The majority of the cells were O4 positive (approximately 80%; shown on *lower panel*) and limited numbers of cells (less than 10%) were GFAP positive. In our hands, the cells could be used in only one experiment as cultures extended past 10 days became highly contaminated with rapidly dividing astrocytes (GFAP positive cells; not shown). Magnification 20×.

cultured in the presence of fetal bovine serum (FBS) in order to limit astrocyte differentiation.

HHV-6 culture supernatant reduced MO3.1 cell proliferation

Experiments were conducted to determine if supernatants from HHV-6–infected SupT1 cells altered MO3.1 proliferation. Supernatants that contained equal amounts of Z29, GS, or U1102 virus (500,000 HHV-6 DNA copies/ml; as determined by Taqman) were placed onto MO3.1 cells for up to 5 days. Although supernatant from uninfected SupT1 cells did not reduce MO3.1 proliferation, supernatant from SupT1 cells infected with Z29, GS, or U1102 significantly reduced proliferation by $50\% \pm 7\%$, $74\% \pm 8\%$, and $76\% \pm 9\%$, respectively (Figure 2**A**). Because the Z29 HHV-6 cultures contained higher viral DNA copies/ml than U1102 or GS, cultures, normalization of the number of HHV-6 DNA copies within supernatants led to a dilution of the Z29 culture supernatants. In order to ensure that our observations were not due to a dilution artifact, we repeated our experiments using supernatants that contained Z29 viral titres reduced to levels similar in U1102 and GS cultures (by collecting supernatants from Z29 cultures 3 days post subculturing and determining HHV-6 DNA levels by Taqman). Despite using this approach, supernatants from U1102 or GS cultures continued to show increased anti-proliferative actions on MO3.1 cells compared to Z29 cultures (Figure 2**B**).

HHV-6 culture supernatant reduced MO3.1 viability

Following 1 day of culture, MO3.1 cells treated with supernatant from uninfected SupT1 cells had $3\% \pm 5\%$ trypan blue–positive cells, which was not significantly different from untreated MO3.1 controls ($3\% \pm 4\%$). However, treatment using supernatants from Z29 cultures induced $12\% \pm 4\%$, GS had $41\% \pm 6\%$ and U1102 had $39\% \pm 5\%$ trypan blue–positive cells, indicating a significant amount of cell death. Reduction in MO3.1 viability induced by HHV-6 supernatants was not due to HHV-6 virions, as removal of HHV-6 virions by filtering did not reverse the loss in viability (Figure 3**B**). Filtering of supernatants removed greater than 99% of HHV-6 DNA from supernatants as determined by Taqman (filtered supernatants had $2 \times 10^2 \pm 2 \times 10^1$ copies/ml. HHV-6 DNA that was detected following filtering was likely naked DNA from nonviable HHV-6 virions because culturing of filtered supernatant from Z29- or U1102-infected SupT1 cells (prefiltered HHV-6 DNA levels $10^6 \pm 10^4$ copies/ml) on naïve SupT1 or MO3.1 cells for 5 or 10 days did not yield any gp116-positive cells as determined by immunofluorescence; not shown). Thus, there was no infectious virus in the filtered supernatants. The reduction in proliferation was also not protein mediated as trypsin treatment for 90 min at 37°C did not reverse cytotoxicity (Figure 3**B**). Trypsin inhibitor did not alter cell viability (not shown). However, the indirect cytotoxicity was heat-labile as boiling of filtered supernatants for 5 min significantly reversed the cytotoxic actions (Figure 3**B**).

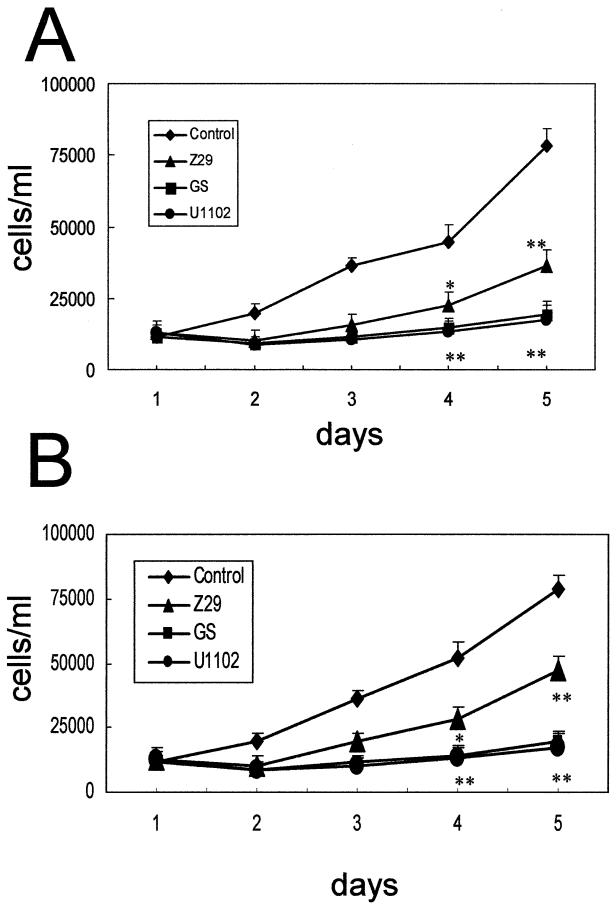


Figure 2 Supernatants from SupT1 cells infected with HHV-6 reduce MO3.1 proliferation. (**A**) Supernatants that contained equal amounts of HHV-6A or -6B DNA copies were placed onto MO3.1 cells and cell proliferation was determined over 5 days. Because HHV-6B (Z29) replicated more efficiently in SupT1 cells compared to HHV-6A variants, supernatants from Z29 cultures were diluted in order to normalize HHV-6 DNA copy number. Supernatants from HHV-6A variants reduced MO3.1 proliferation to a greater extent than Z29 cultures. (**B**) Viral cultures were adjusted such that approximately equal titres (as determined by Taqman) were present in the supernatant at time of collection. Thus, experiments were conducted in which nondiluted supernatants had equal amounts of HHV-6 DNA copies (approximately 10^4 copies/ml). Supernatants from U1102 and GS remained more efficient at reducing MO3.1 proliferation. Figure is representative of three experiments. $^{*}P < 0.01$; $^{**}P < 0.001$.

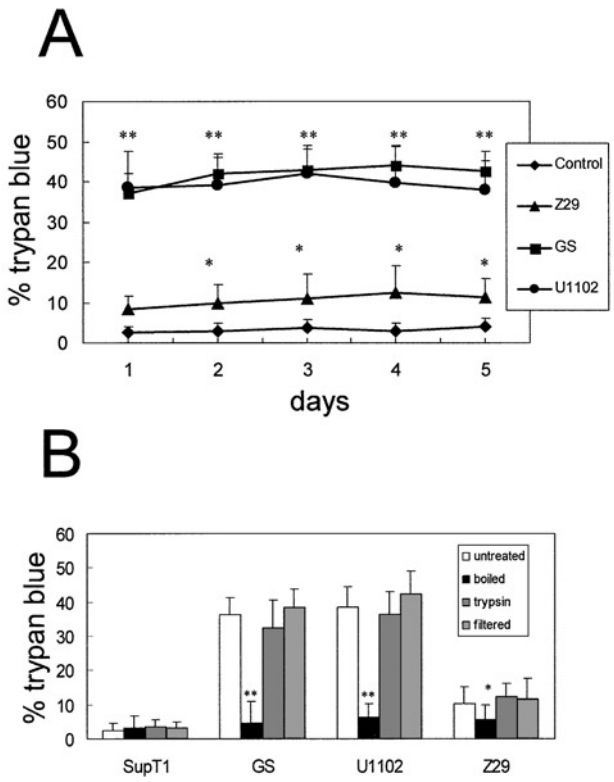


Figure 3 HHV-6 supernatants reduce MO3.1 viability and viral-induced toxicity is heat labile. (**A**) Experiments identical to those outlined in Figure 2 showed that supernatants from HHV-6A (GS or U1102) significantly reduced MO3.1 cell viability compared to supernatants from Z29-infected SupT1 cells or uninfected SupT1 controls. (**B**) Boiling of supernatants for 5 min reversed oligodendrocyte toxicity whereas trypsin treatment (30 min) or removing HHV-6 virions by filtering did not. Because boiled supernatants could not support MO3.1 proliferation (i.e., MO3.1 cells would not proliferate when cultured solely in boiled supernatant; not shown), experiments were conducted using 1 volume (50%) of boiled viral supernatants supplemented with 1 volume (50%) of RPMI and FBS (500,000 HHV-6 copies/ml). Data are representative of three independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$.

HHV-6 supernatants dose-dependently reduced oligodendrocyte but not astrocyte viability

Using an automated fluorescent analysis procedure in which viable cells metabolized calcein-AM and could fluoresce following excitation with wavelength of 495 nm, we found that supernatants from SupT1 cells infected with HHV-6A variants dose-dependently reduced MO3.1 (Figure 4**A**) or OPC (Figure 4**B**) viability to a greater extent than supernatants from variant B (Z29) or uninfected controls. Independent of the HHV-6 viral copies amount used, removal of HHV-6 virions by filtering or removal of protein-based components within the supernatant by trypsin treatment did not reverse cytotoxicity whereas boiling did (not shown). In order to determine whether the cytotoxic actions of HHV-6 culture supernatants were toxic to other glial cells, we conducted additional experiments using primary fetal astrocytes. Although primary fetal astrocyte viability was reduced by approximately 10% ± 5% when treated with supernatants from uninfected SupT1 cells, filtered supernatants from SupT1 cells infected with HHV-6A or 6B did not alter astrocyte viability (Figure 4**C**), indicating that primary astrocytes were not susceptible to indirect cytotoxicty induced by HHV-6.

Supernatants from HHV-6–infected SupT1 cells elevate oligodendrocyte cell death

The loss in cell viability observed in the automated fluorescent-based could be due to a combination

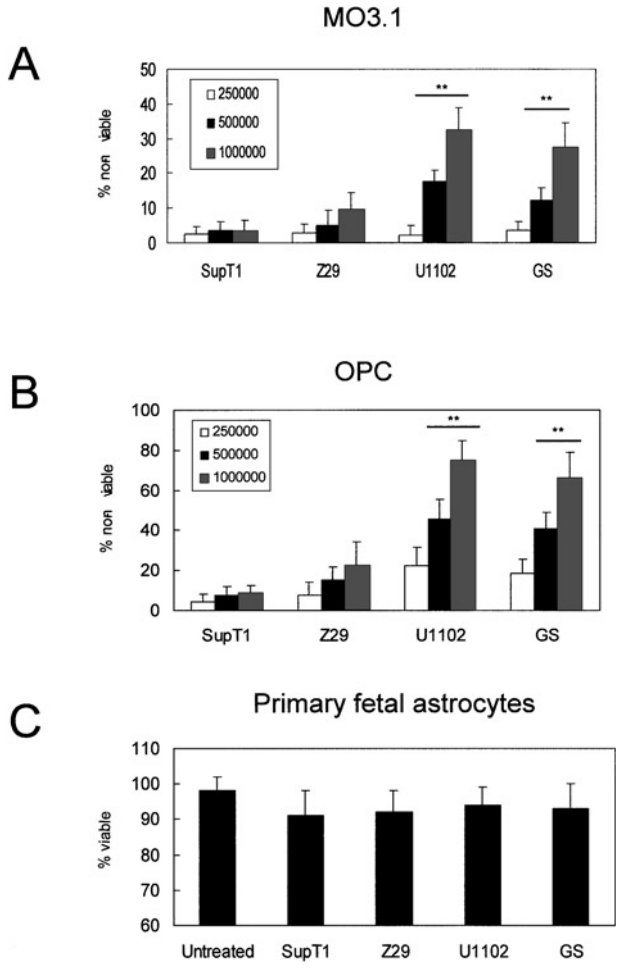


Figure 4 Supernatants from HHV-6–infected SupT1 cells reduce oligodendrocyte but not astrocyte viability. Automated fluorescence analysis showed that supernatants from SupT1 cells infected with GS or U1102 dose-dependently reduced MO3.1 (**A**) and OPC (**B**) viability. However, viral supernatants (1,000,000 copies/ml of HHV-6 DNA) did not alter primary fetal astrocyte viability (**C**). Percent viable or nonviable cells are reported per HHV-6 DNA copies following 4 days of culture. Data are representative of three independent experiments. $^{**}P < 0.001$.

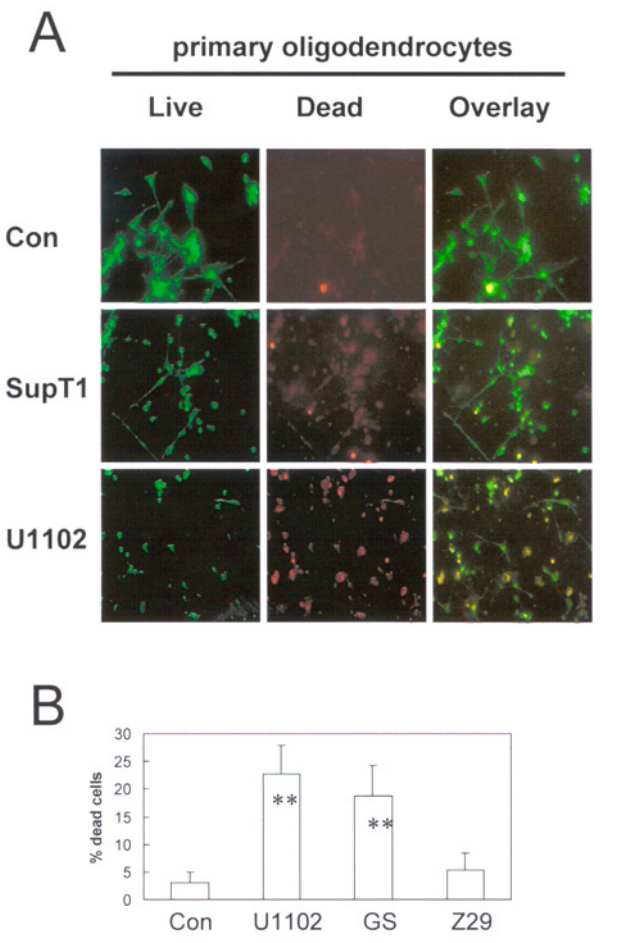


Figure 5 Supernatants from HHV-6–infected T cells induce oligodendrocyte cell death. (**A**) OPC or MO3.1 were treated with viral supernatant for 2 days and percent of live versus dead cells was determined (OPC shown). Representative fluorescent images show an increase in ethidium-positive staining in primary oligodendrocytes that were treated with U1102 supernatants (*bottom panels*) compared to untreated (Con) or cells treated with supernatants from uninfected SupT1 cells (SupT1). (**B**) Supernatants from SupT1 cells infected with U1102, GS, or Z29 significantly increased OPC cell death as determined by Live/Dead Assay. Dead cells are reported per 500,000 HHV-6 DNA copies/ml supernatant. Data are representative of three independent experiments. $^{**}P < 0.001$. Magnification 63×.

of reduction in cell proliferation or an increase in cell death. Experiments using trypan blue exclusion indicated that a significant proportion of MO3.1 cells were dying (Figure 3**A**). In order to confirm that increases oligodendrocyte death was occurring, MO3.1 or OPCs were treated with HHV-6 supernatant and Live/Dead Assay (Molecular Probes) was conducted. Viral supernatants from SupT1 cells infected with U1102, GS or Z29 significantly increased MO3.1 cell death (ethidium-positive cells) at 2 days by 22% ± 7%, 18% ± 5%, and 12% ± 6%, respectively. Identical experiments using primary oligodendrocytes determined that U1102, GS, or Z29 had 26% ± 6%, 23% ± 6%, and 11% ± 5% ethidium-positive cells, respectively (Figure 5).

Increased cell death is not mediated by glutamate and is primarily independent of apoptosis

We sought to clarify the mode of cell death induced by HHV-6. We chose not to focus on cytokine-mediated cell death as trypsin treatment of viral supernatnats did not reverse cytotoxicity (Figure 3), indicating that the primary cause of cytotoxicity was not protein mediated. However, boiling of supernatants did reverse cytotoxicity (Figure 3) indicating that the cytotoxic factor(s) are heat labile. Accordingly, we hypothesized that increased glutamate may mediate increased oligodendrocyte death. High-performance liquid chromatography (HPLC) measurements showed that HHV-6 infection (variant A or B) did not alter extracellular glutamate levels

Table 1 Extracellular glutamate levels in SupT1 cells infected with HHV-6

Condition	*Glutamate (mM)*	*SE*
Uninfected	0.819	0.02
GS	0.709	0.01
U1102	0.760	0.05
Z29	0.873	0.01

Table is representative of supernatants ($n = 3$) collected at day 7 after subculturing.

compared to uninfected controls (Table 1). Levels of extracellular glutamate in culture supernatants were below that required to induce cytotoxicity to MO3.1 or OPCs at 48 h ($LD_{50} = 40 \pm 8$ mM in MO3.1 and 25 ± 12 in OPCs). In addition, NBQX (10 μM) did not reverse HHV-6 GS–, U1102–, or Z29–induced cytotoxicity in MO3.1 (not shown).

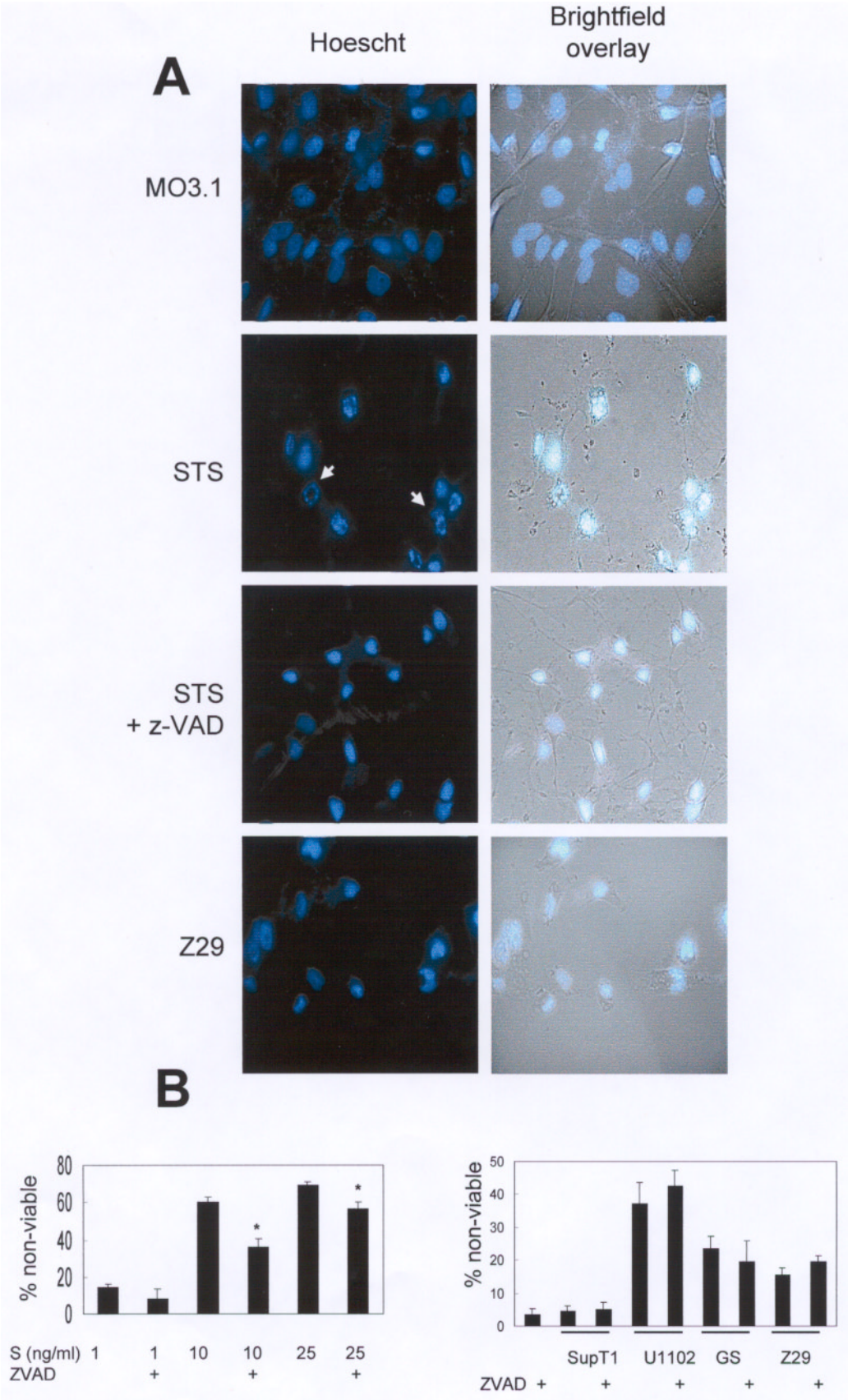
Next, we sought to determine if the mode of cell death was apoptotic. Hoescht staining of MO3.1 cells treated with HHV-6 supernatants (48-h treatment) indicated that minimal apoptotic-like nuclei were present (less than 2%), whereas staurosporine treatment of MO3.1 cells induced $80\% \pm 9\%$ cells to display apoptotic nuclei (Figure 6). We sought to further clarify whether cells were dying via apoptosis by treating oligodendrocytes with the general antiapoptotic peptide z-VAD FMK. Pretreatment however, did not protect MO3.1 cells from HHV-6–induced cell death (Figure 6**B**). In agreement, OPCs cell death was not protected by the apoptotic inhibitor z-VAD (not shown). In order to further determine the involvement of apoptosis in mediating cell death, we determined caspase-3 activation following treatment. Although treatment of MO3.1 cells with staurosporine (10 μg/ml for 24 h) induced a minimal activation of capsase-3 ($15\% \pm 10\%$ compared to untreated cells as determined by semiquantitative western analysis), supernatants from HHV-6–infected cultures did not induce any detectable caspase-3 activity (not shown).

Discussion

Although several studies have implicated that HHV-6 is pathogenic factor in MS (for review, see Cermelli and Jacobson [2000]), because it is highly difficult to isolate live virus from small amounts of biopsy or autopsy brain tissue, it remains speculative as to the absolute role of HHV-6 in the development and progression of MS. Because anti-HHV-6 immunoglobulin (Ig)G, IgA, and IgM antibodies are found with higher frequency in serum from MS patients (Gutierrez *et al*, 2002; Soldan *et al*, 1997), it remains possible that MS patients are susceptible to HHV-6 reactivation. In agreement with this hypothesis, it was reported recently that T cells recognizing the recombinant 101-kDa protein (101K) of HHV-6 occurred at significantly lower precursor frequency in MS patients than in control subjects (Tejada-Simon *et al*, 2002), suggesting that some MS patients may be unable to clear the virus effectively.

One way in which HHV-6 could be involved in lesion development is through indirect actions on bystander cells, including neurons, astrocytes, and oligodendrocytes. This hypothesis is based on our previous observation that HHV-6 infection of T cells induced a TH1-type immune response (Mayne *et al*, 2001). Here, we studied oligodendrocytes because of their importance in myelin production and that these cells are primarily lost during demyelination events associated with MS (Ludwin, 1996, 1997). Others have shown HHV-6 "footprints" in MS lesions in that HHV-6 antibodies (Challoner *et al*, 1995) or HHV-6 DNA (Blumberg *et al*, 2000; Cermelli *et al*, 2003; Goodman *et al*, 2003) was detected at high frequencies. Our experiments showed that HHV-6A was significantly more proficient at inhibiting oligodendrocyte proliferation compared to HHV-6B (Z29). In addition, our data indicated that HHV-6A and HHV-6B variants elevated oligodendrocyte but not astrocyte cell death, indicating that HHV-6 may selectively target oligodendrocytes within the glial cell population. Because filtering the HHV-6 virions from culture supernatants did not reduce oligodendrocyte cytotoxicity, these studies support our hypothesis that HHV-6 infections have a negative bystander effect on oligodendrocytes. As there were large contrasts in the actions of HHV-6 variants (A versus B) in reducing oligodendrocyte viability and inducing cell death, it is most likely that these findings are not merely a function of dying or activated T cells within the viral culture but more so a function of the specific HHV-6 variants. In support of this claim is the observation that variant A viruses GS and U1102 had lower levels of cytopathic effect (cell swelling) in SupT1 cells, despite that majority of the culture was gp116 positive, indicating that, under our growth conditions, variant A has a different pathogenic life cycle compared to variant B.

Figure 6 HHV-6–induced oligodendrocyte cell death is primarily caspase independent. (**A**) Hoescht staining combined with brightfield analysis indicated that supernatants from HHV-6 cultures did not induce apoptotic-like nuclei. Treatment of cells with staurosporine (10 ng/ml) for 24 h induced apoptotic-like nuclei in greater than 80% of cells. *White arrowhead* indicates apoptotic-like nuclei. Note the lack of apoptotic-like nuclei in MO3.1 cells treated with Z29 supernatant. Similar results were observed in MO3.1 cells treated with supernatants from U1102 or GS cultures (not shown). z-VAD treatment (50 μM) significantly reduced apoptotic nuclei. (**B**) Automated fluorescent analysis confirmed that staurosporine treatment (of 1, 10, or 25 ng/ml) of MO3.1 elevated cell death, which could be reduced by z-VAD. However, z-VAD treatment of MO3.1 for 30 min prior to addition of HHV-6 supernatants did not protect MO3.1 cells from cell death. Analysis was conducted at 48 h following the addition of viral supernatants. Percent of nonviable cells are reported per 500,000 HHV-6 DNA copies following 4 days of culture. $^{*}P < 0.001$.


Hoescht
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A
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STS + z-VAD
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SupT1 U1102 GS Z29
ZVAD + + + + +

Our finding that variant A was more potent in reducing cell proliferation compared to variant B is interesting because HHV-6A DNA is found with higher frequency in MS serum (Akhyani *et al*, 2000; Alvarez-Lafuente *et al*, 2002) and MS patients have increased lymphoproliferative responses to HHV-6A (Soldan *et al*, 2000). It has also been reported that HHV-6A has increased neurotropism compared to HHV-6B (Hall *et al*, 1998). Based on these observations, we are currently determining the extent to which HHV-6 (variant A in particular) inhibits the differentiation of the oligodendrocyte precursor cells. These experiments are important because reduced oligodendrocyte differentiation may affect remyelination events within an MS lesion (Lassmann, 1998). Controversy remains, however, as others have shown that lesion development is not associated with HHV-6A but rather HHV-6 in general or that there is no association between MS and HHV-6 detection (Enbom *et al*, 1999; Ongradi *et al*, 1999; Taus *et al*, 2000).

Our experiments indicated that viral supernatants reduced MO3.1 cell proliferation by approximately 50% (Z29) and by 75% (GS or U1102). The reduction in the MO3.1 proliferation is likely due to reduced differentiation and increased cell death. Although this is the first report to document that HHV-6 supernatants increase oligodendrocyte cell death, the antiproliferative actions of HHV-6 has been demonstrated before in T cells (Horvat *et al*, 1993). HHV-6–infected T cells have reduced levels of interleukin (IL)-2 production, which plays a critical role in T-cell proliferation (Flamand *et al*, 1995), and we have observed in our SupT1 tissue cultures that IL-2 mRNA is reduced as determined by microarray analysis (Mayne *et al*, 2001). Oligodendrocytes respond to IL-2 by stimulating proliferation and maturation (Otero and Merrill, 1997). Although we have not attempted to "rescue" the oligodendrocyte cultures by treatment with IL-2, it is unlikely that the reduction in oligodendrocyte viability is entirely due to a reduction in IL-2 because with trypsin treatment, the HHV-6 supernatants did not reverse cytotoxicity, indicating that the cytotoxic factor is most likely not protein based. This observation led us to eliminate other possible protein cytotoxic candidates, including, proinflammatory cytokines such as tumor necrosis factor (TNF)-α and Fas ligand, both of which have been implicated in mediating oligodendrocyte cell death associated with MS (D'Souza *et al*, 1996; Selmaj *et al*, 1991; Selmaj and Raine, 1988).

We do not know the specific mechanism that causes loss in oligodendrocyte viability or increased cell death. However, our experiments on both MO3.1 and OPCs have ruled out a potential role for glutamate-mediated cytotoxicity. We hypothesized that glutamate may be involved because activated immune cells produce elevated levels of glutamate by deamidating glutamine via glutaminase (Werner *et al*, 2000, 2001). We also postulated that glutamate could be a source of cytotoxicity as recent studies have shown that NBQX attenuates excitotoxic injury in developing white matter (Follett *et al*, 2000), and NBQX protected oligodendrocytes in an experimental model of MS, experimental allergic encephalomyelitis (Pitt *et al*, 2000). However, NBQX, which blocks AMPA/kainate glutamate receptors that are primarily expressed on oligodendrocytes (Matute *et al*, 2001), did not protect MO3.1 or OPC cells treated with HHV-6 viral supernatants. Our studies suggested, however, that oligodendrocyte cell death induced by HHV-6 supernatants most likely follows caspase-independent pathways. This is based on the observations that z-VAD did not protect oligodendrocytes from HHV-6 toxicity and HHV-6 supernatants did not elevate caspase-3 activity in oligodendrocytes (not shown). Our studies cannot completely rule out a role for apoptosis, as others have observed that oligodendrocyte cell death can occur in a caspase-independent fashion (D'Souza *et al*, 1996). Our conclusion could be due in part to impaired caspse machinery in our model cell line MO3.1 (i.e., even the strong apoptotic agent, staurosporine, induced only minimal caspase-3 activity). Nonetheless, we rarely observed apoptotic-like nuclei in oligodendrocytes treated with supernatants from HHV-6, but rather swollen cells, suggesting that cell death is necrotic.

In summary, findings reported here indicated that *in vitro*, HHV-6 through indirect mechanisms enhanced oligodendrocyte cell death. Determination of the factors the mediate the oligodendrocyte toxicity is of critical importance and will strengthen our understanding of the indirect actions of HHV-6. Ultimately, it will be important to confirm these observations *in vivo* and determine if HHV-6 virus can block remyelination via antiproliferative actions on oligodendrocyte precursor cells or directly kill oligodendrocytes and promote demyelination.

Materials and methods

HHV-6 virus and cytopathic effects

The human T-cell lymphoblast line SupT1 was used to propagate the HHV-6A variants (GS and U1102) and the HHV-6B variant (Z29) as described previously (Mayne *et al*, 2001). SupT1 cell culture conditions and input multiplicity of infection were determined previously and infected and uninfected cell culture (5×10^5 cells/ml) was performed as described (Soldan *et al*, 2000). HHV-6 infection was confirmed by immunofluorescence assay (IFA; see below) and Taqman analysis (see below). Except where noted, supernatants were collected at 7 days after passage. On the day of collection, greater than 50% of the Z29-infected SupT1 cells exhibited CPE. GS and U1102 cultures exhibited minimal CPE (<15%) on the day of collection. Following supernatant collection, the number of viable SupT1 cells were determined using trypan blue exclusion. Supernatants

were centrifuged at 600 × *g* for 10 min in order to remove cell debris or were filtered using a polyvinylidene difluoride 0.1-micron filter (Fisher Scientific, Mississauga, ON) and frozen at −80°C until taken for Taqman assay and used in experiments. Supernatants were thawed once. Where indicated, HHV-6 cultures were normalized so that approximately equal amounts of HHV-6 DNA copies were present per ml of culture supernatant. In order to reduce the amount of HHV-6 DNA copies in Z29 cultures to levels observed in U1102 and GS cultures (approximately 50,000 copies/ml) the amount of time for infection was reduced by approximately 2 days. This enabled us to maintain a low-level Z29 viral titre in Z29 cultures without significantly reducing the amount of SupT1 cells present in the culture.

Real-time quantitative PCR and analysis of HHV-6 DNA

Taqman primers, probe sets, and standard controls used for amplification of HHV-6 variants A and B were described previously (Nitsche *et al*, 2001). β-Actin primers and probe were used for internal calibration (Mayne *et al*, 2001). Standard curve for β-actin was generated following two-fold serial dilution of genomic DNA purified from normal donor peripheral blood mononuclear cells (PBMCs). Each nanogram of DNA contains approximately 333 copies of the β-actin gene. Five hundred nanograms of sample DNA was used as template and analyzed by Bio-Rad iCycler (Bio-Rad, Mississauga, ON). Standard and sample DNA were always amplified in triplicate in the same 96-well reaction plate as outlined previously (Mayne *et al*, 2001).

Cell lines and primary cells

Human SupT1 cells were maintained as outlined previously (Mayne *et al*, 2001), with the exception that cells were maintained in 2% FBS. The reduction in FBS did not affect the growth of HHV-6 viral cultures compared to viral cultures maintained in 5% FBS. We maintained our HHV-6 cultures in 2% FBS because our experimental procedures required a minimal level of FBS to maintain the SupT1 cell line and not cause extensive nonspecific fluorescence in our cell viability or cytotoxicity assays (see below). The oligodendrocyte-like cell line MO3.1 was cultured as described (McLaurin *et al*, 1995). Human OPCs were obtained commercially (Sciencell, San Francisco, CA). Nestin- and A2B5-positive OPCs (10×10^6) from 20- to 24-week-old human fetal brain tissue were shipped by overnight courier on wet ice and cultured immediately on 96-well plates (Life Technologies, Burlington, ON) coated with poly D-lysine (85 μg/ml) (Sigma, St. Louis, MO) at a concentration of 400,000 cells/ml. For immunofluorescence imaging, OPCs were cultured on Permanox slides (Falcon LabTek, Mississauga, ON) coated with poly D-lysine (85 μg/ml). In our hands, we have determined that following 3 days of *in vitro* culture in differentiation medium (provided by Sciencell) at 37°C supplemented with 5% CO_2, approximately 10% ± 10% of the cellular population was CNPase positive (with small variation between fetal specimens). The majority of the remaining cells (approximately 80%) are of oligodendrocytic lineage, O4 positive (Ludwin, 1997), and small percentages were GFAP positive or of astrocytic lineage. Primary human fetal astrocytes (weeks 12 to 18) were cultured and maintained as described previously (Chen *et al*, 1997). Because the source of primary cells were fetal in origin, all experiments were conducted a minimum of 3 times.

Antibodies and immunofluorescence

For IFA, OPCs that were cultured for 3 days on Permanox slides were washed twice with sterile phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. Anti-CNPase 1:2000 primary antibody (Sternberger Monclonal Antibodies, Lutherville, MD) or anti-O4 1:1000 primary antibody (Chemicon, Temecula, CA) was added to the specimens for 1 h at room temperature. Antibody detection was determined using the secondary antibody Alexa Fluor 546 (goat anti-mouse IgG H + L 1:400; Molecular Probes, Eugene, OR) for CNPase or O4. For HHV-6 detection, SupT1 cells infected with HHV-6 or MO3.1 cells treated with HHV-6 supernatants were incubated with a 1:40 dilution of a mouse monoclonal antibody (mAb) that recognized HHV-6 glycoprotein (gp) 116/64/54 (ABI, Columbia, MD, USA) for 30 min at 37°C. Antibody detection was determined using a fluorescein-labeled goat anti-rabbit IgG serum (Sigma, St. Louis, MO, USA) for 30 min at 37°C. Uninfected SupT1 cells were used as negative controls.

Cell viability and Live/Dead assays

Cell proliferation was determined by culturing trypan blue negative MO3.1 cells (10,000 cells/ml) in the presence of viral supernatant. Trypan blue–negative cells were counted on days 1 through 5. MO3.1 or OPC cell death was determined using the Live/Dead Assay (Molecular Probes). We modified the Live/Dead Assay in order to automate our determination of oligodendrocyte or astrocyte viability. Fluorescent analysis was conducted using an automated plate reader (Molecular Dynamics, Mississauga, ON). Briefly, MO3.1 (starting density 10,000 cells/ml) or OPC or astrocytes (starting density 200,000 cells/ml for oligodendrocytes or 50,000 cells/ml for astrocytes) were cultured in sterile poly D-lysine–coated black 96-well plates. MO3.1 were cultured overnight or OPCs for 3 days in order to ensure no bacterial contamination occurred. Primary astrocytes were cultured for 7 days after the removal of neurons and microglia as described

previously (Chen *et al*, 1997). Supernatants from HHV-6–infected SupT1 cells (2% FBS final concentration) were either filtered to remove any contaminating cells or debris (0.45-μm filter; Fisher Scientific, Mississauga, ON) and/or filtered to remove HHV-6 virions (approximately 160 μm in diameter [Roffman *et al*, 1990] using a 0.1-μm filter) and added to cell cultures. The removal of HHV-6 virus in supernatants was confirmed by conducting Taqman analysis of DNA preparations and by conducting infectious assays of filtered supernatants using uninfected SupT1 or MO3.1 cells. Supernatants, glutamate, or staurosporine (Sigma) were placed directly onto cell cultures. Cell viability was determined at day 2 and/or day 4 following addition of supernatants by bathing the cells with Hoescht 33342 (Molecular Probes) for 15 min, or for the automated assay, calcein-AM (2 μM) for 45 min. In order to ensure that calcein fluorescence was specific, cells were killed with 0.5% saponin (20 min) prior to calcein treatment. The involvement of proteins in mediating cytotoxicity was determined by boiling supernatants for 5 min and quick chilling at 4°C prior to addition to MO3.1 cells or supernatants were treated with trypsin (1 mg/ml; Sigma) for 30 min at 37°C. Following this treatment, trypsin inhibitor (1 mg/ml; Sigma) was added to the reaction in order to inhibit any direct cytotoxic actions of trypsin on MO3.1 cells. The involvement of glutamate, or in general caspases, was determined by treating MO3.1 with the AMPA/kainate antagonist NBQX (10 μM) 15 min prior to addition of HHV-6 supernatants or with the general caspase peptide inhibitor z-VAD FMK (50 μM) (Calbiochem, La Jolla, CA) for 30 min prior to the addition of viral supernatants. Caspase-3 activation was determined by Western analysis using anti–caspase-3 antibody (Santa Cruz; primary 1:1000) at 24 h following addition of staurosporine and at 48 h following addition of viral supernatants. Caspase-3 determination in MO3.1 cells could not be made at 48 h post addition of staurosporine because the majority of the cell population was dead. Percent nonviable or dead cells were reported per 500,000 HHV-6 DNA copies or as indicated.

Determination of extracellular glutamate

The HPLC conditions and reagents are described elsewhere (Cohen and Michaud, 1993; Strydom and Cohen, 1994) with some modifications. Cell culture medium (50 μl) was combined with an equal volume of chilled 4% trichloroacetic acid, mixed by vortex, and then centrifuged at 20,000 $\times$ *g* for 15 min at 4°C. Supernatants were diluted 1:15 with mobile phase A (consisting of sodium acetate 140 mM and triethylamine 17 mM at pH 5.1) and a 10-μl aliquot was derivitized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) using the AccQ-Fluor Reagent kit from Waters (Milford, MA). Gradient elution of amino acids at 1 ml/min was carried out with mobile phases A and B (60% acetonitrile). Gradient conditions were: initial 100% A, 6 min 90% A, 12 min 85% A, 19 min 78% A, 33 min 68% A, 45 min 100% B, 50 min 100% B, 60 min 100% A. The column (Nova-Pak C_{18}, 3.9 $\times$ 150 mm; Waters) was equilibrated with mobile phase A for 10 min between injections and maintained at 37°C. Fluorescence detection was carried out using excitation and emission wavelengths of 245 and 394 nm, respectively. Amino acid peaks were identified and quantified by comparison to standards that were prepared in the same fashion as the samples. Data collection and peak integration was carried out using Millennium software (Waters).

Statistics

All tests were examined by analysis of variance (ANOVA) or Student's *t* test and statistical significance was considered to be at the $P < .01$ level (Instat2, Graphpad Software, San Diego, CA).

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