# Infection of Primary Human Fetal Astrocytes by Human Herpesvirus 6

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Human herpesvirus 6 (HHV-6) is a lymphotropic betaherpesvirus which productively infects human CD4+ T cells and monocytes. HHV-6 is the etiologic agent for exanthem subitum (roseola), and it is well-known that central nervous system complications occur frequently during the course of HHV-6-associated disease. In addition, HHV-6 has been associated with encephalitis or encephalopathy. However, very little is known about its tropism for neural cells. There are reports that HHV-6 may infect some glial cell lines, but whether it can infect any primary neural cells is not known. Our studies show that both HHV-6A (GS) and HHV-6B (Z-29) can infect highly purified primary fetal astrocytes in vitro. Infected cells showed cytopathic effects, forming giant syncytia. In dual immunofluorescence assays, the infected cells were detected by antibodies against the HHV-6 p41 nuclear antigen and glial fibrillary acidic protein, indicating that the infected cells are indeed astrocytes. PCR and Northern (RNA) blot analyses also confirmed that the astrocytes are infected by HHV-6. The progeny virus did not alter its host range and could reinfect T cells as well as primary astrocytes. These findings suggest that infection of primary human astrocytes may play a role in the neuropathogenesis of HHV-6.

Human herpesvirus 6 (HHV-6) is a recently discovered lymphotropic betaherpesvirus (3) which has been identified as the etiologic agent for exanthem subitum (roseola) (35) and as a major cause of acute febrile illness in young children (17, 27). HHV-6 isolates can be differentiated into two groups, variants A and B, on the basis of antigenic specificity, genetic polymorphism, and in vitro growth properties (1). Variant A isolates include those that were isolated from adults, such as the prototype GS strain that was isolated from a patient with a lymphoproliferative disorder (29). HHV-6 variant group B consists of most of the isolates from children with exanthem subitum (3, 12), but it also includes some isolates from adults, such as the prototype Z-29 strain isolated from a Zairian AIDS patient (7). HHV-6 has been implicated as an important cofactor in AIDS; it can infect many of the same cell types as human immunodeficiency virus type 1 (HIV-1), such as CD4+ T cells (2), and it can enhance replication of HIV and the cytopathic effects in cells that are dually infected by HIV and HHV-6 (14). HHV-6 has been shown to transactivate HIV regulatory elements (14, 18, 34), up-regulate CD4 antigen expression, and expand the host range for HIV infection (24). In addition, other studies have shown that HHV-6 can suppress the replication of HIV-1 in coinfected cells (8, 22). Recent attention has also focused on the roles of HHV-6 in causing neurological disease (23, 33). It has been suggested that HHV-6 invades the central nervous system (CNS) during active infection. HHV-6 DNA has been detected in the cerebrospinal fluid from patients with exanthum subitum, especially from those with febrile convulsive seizures (9, 21, 36). HHV-6

sequences have also been detected in CNS specimens from patients with multiple sclerosis, but the significance of finding sequences in multiple sclerosis patients is controversial (10, 31). HHV-6 has been associated with encephalitis or encephalopathy (5, 13, 19, 20, 32), with possible latent infection of the CNS (9), and with reactivation to cause febrile convulsions (17).

In spite of the neurological complications associated with HHV-6 infection, very little is known about its tropism for neural cells. Two reports have described the detection of HHV-6 in human brain tissues associated with fatal encephalitis (5, 13). More recently HHV-6 was detected in four of five children with HIV-1 encephalitis by in situ hybridization of postmortem brain tissues (28). HHV-6 signals were found in numerous oligodendrocytes and less frequently in astrocytes, microglia, and neurons (28). These recent studies suggest that HHV-6 infection of the CNS may play an important role in the neuropathogenesis of HIV-1 infection. Thus, there is a need to establish cell culture models to study HHV-6 infection of neural cells in vitro, and it is important to determine the cell types that are permissive for HHV-6 infection in the CNS.

A previous report has suggested that HHV-6 can abortively infect a glioblastoma cell line in vitro (4), but it has not been determined whether HHV-6 can infect any nontransformed, neuroepithelium-derived cells. In this study, we have tested the infectivity of HHV-6 in nontransformed human astrocytes. Astrocyte cultures used for HHV-6 infection were prepared from first-trimester human fetal specimens (46 to 83 days of gestation). Fetal CNS tissue was obtained from the Human Embryology Laboratory, University of Washington, Seattle. Procedures for procurement and use of this human fetal CNS tissue were approved and monitored by the University of Miami School of Medicine's Medical Sciences Subcommittee for the Protection of Human Subjects. The CNS tissue from each fetal

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specimen was processed separately and independently, as were subsequent cell cultures; there was no pooling of CNS tissue from distinct fetal specimens. These cells were grown in a serum-free, defined medium (B16) enriched with 5 ng of basic fibroblast growth factor per ml for optimal growth of astrocytes and for the suppression of fibroblast growth (Fig. 1A) (25). By immunofluorescence assay, these cultures homogeneously expressed glial fibrillary acidic protein (GFAP), the definitive marker for astrocytes (Fig. 1B). Most importantly, cultures expressed little or no fibronectin or prolyl hydroxylase, both markers for fibroblasts (Fig. 1C). Fewer than 50 fibronectin-positive cells could be counted per 10,000 GFAP-positive cells. Moreover, cultures continued to express GFAP with little or no fibronectin when monitored at each subculture and during HHV-6 infection. With supplemented B16 medium we were able to maintain highly purified human fetal astrocyte cultures, allowing subsequent unambiguous experimental determination of HHV-6 growth in astrocytes in vitro.

**Human primary astrocytes can be infected by HHV-6.** In order to study the susceptibility of astrocyte cultures to HHV-6 infection, the astrocytes were either cocultivated with HHV-6A strain GS-infected HSB-2 T cells (HSB-2/GS) or infected with cell-free GS virus at a $10^3$ 50% tissue culture infective dose $(TCID_{50})/10^6$ cells. For coculture, the astrocytes were incubated with HHV-6-infected HSB-2 T cells at a 1:1 ratio overnight and then washed extensively to remove the T cells. For cell-free HHV-6 infection, the astrocytes were incubated with the cell-free virus for 2 h at 37°C, washed three times with phosphate-buffered saline, and cultured with astrocyte medium. Astrocytes that were either cocultivated with HSB-2/GS or infected with cell-free GS virus formed giant syncytia with multiple nuclei (Fig. 2A), indicating that HHV-6 infection of astrocytes induces cell fusion and cytopathic effects. In the cocultivated cultures, syncytia tended to appear more quickly than in the GS-infected cultures and numerous syncytia could be observed within 2 days. The cultures that were infected by cell-free virus developed syncytia more slowly but usually by 3 days postinfection. Similar results were obtained with HHV-6B strain Z-29 (data not shown). However, with HHV-6B Z-29, the titer of the viral stock used was about 100-fold lower than that of GS, and only a $10^{1.7}$ $TCID_{50}/10^6$ cells was used for infection. For Z-29 infection compared with HHV-6A, fewer syncytia and less cytopathic effect were observed, perhaps because of the lower titer of the input Z-29 viral stock. Alternatively, Z-29 infection of the astrocytes may be less efficient.

To confirm infection of the astrocytes by HHV-6, an immunofluorescence assay for specific viral antigens was performed. Cultures that were infected by cell-free GS or Z-29 were fixed with paraformaldehyde (25) at 5 days postinfection and then analyzed with a monoclonal antibody against an HHV-6 early nuclear antigen, p41 (11, 16). Most of the staining appeared to be localized to the syncytia, and the multinuclei were very brightly stained (Fig. 2B). Scattered individually stained cells also appeared throughout the culture, suggesting that there are many more infected cells than those that formed syncytia. The infected cells were simultaneously stained with an anti-GFAP polyclonal antibody. The astrocyte cultures, including the infected cells, expressed GFAP, indicating that the infected cells are indeed astrocytes (Fig. 2C). However, GFAP staining was weaker in syncytia, and there appeared to be condensation of GFAP-containing intermediate filaments. Infection was also monitored by another monoclonal antibody, 2D6, specific against the HHV-6 surface glycoprotein gp82-gp105 (6). Infected cells showed diffuse surface staining of the cytoplasm, both in cells forming syncytia and in single cells (data not
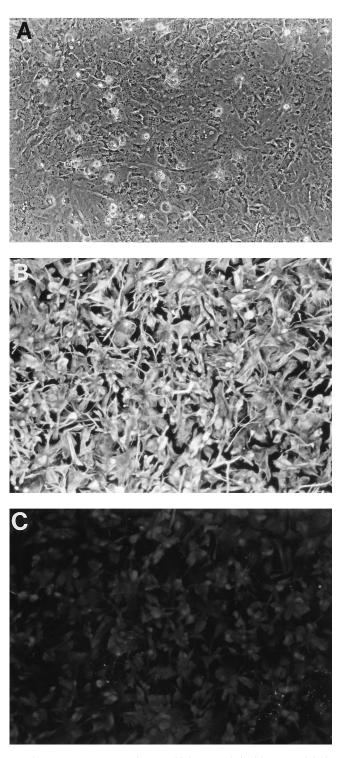


FIG. 1. Human astrocyte cultures at third passage derived from rostral CNS tissue maintained in B16 medium supplemented with basic fibroblast growth factor. (A) Phase-contrast micrograph; (B) immunofluorescence staining of astrocyte culture for GFAP; (C) immunofluorescence staining for fibronectin. Primary antibodies included rabbit polyclonal anti-GFAP (DAKO, Carpinteria, Calif.) and mouse monoclonal anti-human fibronectin (Boehringer Mannheim, Indianapolis, Ind.). Secondary antibodies included goat anti-rabbit immunoglobulin G conjugated to rhodamine and goat anti-mouse immunoglobulin G conjugated to fluorescein (both from Boehringer Mannheim). Magnification, ×110.
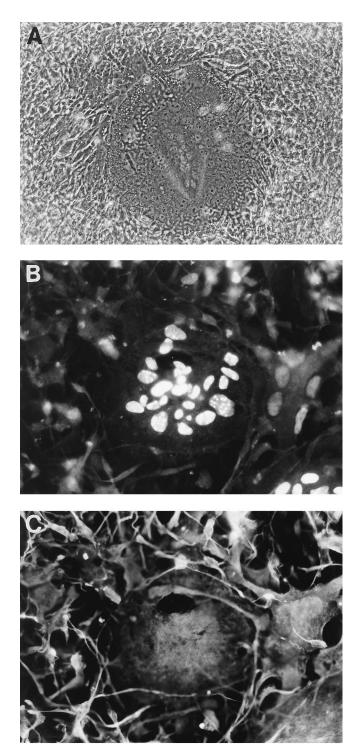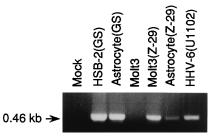
FIG. 3. PCR amplification of HHV-6 B701 gene fragment from total DNA extracted from various infected astrocyte cultures and T-cell lines. The cultures were infected with cell-free HHV-6, and the amplified product was analyzed on a 1.2% agarose gel. The 0.46-kb product (indicated on the left) represents the amplified B701 gene fragment. Astrocyte (GS) and astrocyte (Z-29) represent DNA extracted from astrocytes infected with cell free HHV-6 strains GS and Z-29, respectively. DNA from T-cell lines HSB-2 and Molt-3 infected by GS and Z-29, respectively, and HHV-6A strain U1102 viral DNA were used as positive controls. Mock and Molt-3 represent DNA from uninfected astrocytes and T cells, respectively.



FIG. 2. Human astrocyte cultures at 5 days postinfection with $10^3$ TCID$_{50}$ of HHV-6 GS. (A) Phase-contrast micrograph of the infected culture showing giant multinucleated syncytia; (B) immunofluorescence staining of infected cells with monoclonal antibody against p41 nuclear protein of HHV-6; (C) immunofluorescence staining of infected culture shown in panel B for GFAP. Secondary antibodies are described in the legend to Fig. 1. Magnification, ×100 (A) and ×220 (B and C).

shown). Our immunofluorescence studies thus suggest that viral proteins, including both early and late gene products, were expressed in astrocytes that are infected by HHV-6. To determine further the viral protein expression, uninfected and cell-free HHV-6A (GS)-infected astrocytes were labeled with [$^{35}$S]methionine for 20 h, and at 7 days postinfection, lysates were used for radioimmunoprecipitation reactions with various HHV-6A monoclonal antibodies according to methods described previously (6). Monoclonal antibodies immunoprecipitated viral early proteins and glycoproteins from infected cells (data not shown), thus confirming viral replication in astrocytes.

**Molecular analyses of HHV-6-infected cells.** To further confirm infection of the astrocyte cultures by HHV-6, DNA was extracted 7 days postinfection from astrocytes that were infected with cell-free HHV-6 and analyzed for HHV-6 sequences by PCR. The infected cells were digested with 0.05% trypsin-EDTA and then with 5 U of DNase for 30 min at 37°C before DNA extraction in order to eliminate virus particles adsorbed onto the surface of the cells. The purified DNA was then tested for the presence of an immediate-early gene B701 (16) of HHV-6 by PCR (15). The sequence of this gene is conserved between both A and B strains (34), and the primer set should amplify a 460-bp fragment (Fig. 3). The primer set used was 5′-TACATTATGAAGTCTTGC-3′ and 5′-CTCA AGTATGACGTATC-3′, and the reaction conditions were, for 30 cycles, 94°C for 1 min, 50°C for 1 min, and then 72°C for 2 min. DNA extracted from HHV-6A strain GS-infected astrocytes showed an intense PCR-amplified band of 460 bp; similar bands were detected in DNA obtained from GS-infected HSB-2 T cells, HHV-6B strain Z-29-infected astrocytes, Molt-3 T cells, and purified HHV-6A strain U1102 viral DNA. No viral DNA was detected in mock-infected astrocytes or Molt-3 T cells. Even though the PCR amplification performed was not quantitative, much more viral DNA was amplified from GS-infected astrocytes than from Z-29-infected cells when the same total number of infected cells were used (Fig. 3). This result is consistent with our infectivity studies, which show less extensive cytopathic effect with Z-29.

**Northern blot analyses of HHV-6-infected astrocytes.** To further confirm that HHV-6 genes are expressed in infected astrocytes, Northern (RNA) blot analysis was carried out with the p41 early gene probe (11, 16, 37). The p41 gene codes for an accessory protein which binds to viral DNA polymerase (11, 16, 37). Multiple RNA species are transcribed from this gene
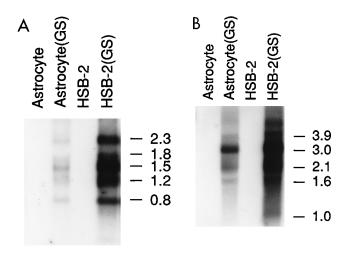
FIG. 4. Northern blot analysis of cell-free GS-infected astrocytes and HSB-2 T cells. Total RNA was analyzed on a 1.2% formaldehyde gel, transferred, and then hybridized to a labelled HHV-6 p41 (A) or gp105 (B) probe. RNA (10 μg) from uninfected HSB-2, astrocytes, and GS-infected astrocytes was used. For positive control GS-infected HSB-2 cells, only 1 μg of RNA was used. The numbers on the right represent the sizes (in kilobases) of different RNA species.



FIG. 5. Estimation of HHV-6 titer produced from infected astrocytes. Astrocytes were infected with $10^3$ $TCID_{50}$ of cell-free HHV-6A (GS) virus, and the viral titer was determined at 1, 4, and 7 days postinfection by using HSB-2 T cells as the indicator cells. Each time point represents results from at least three independent experiments; error bars indicate standard deviations. The titer is expressed as log $TCID_{50}$/ml of culture supernatant from the infected astrocyte culture.

locus. Five different RNA species, with sizes of 2.3, 1.8, 1.5, 1.2, and 0.8 kb, were detected in GS-infected HSB-2 T cells. Similar RNA species were detected in cell-free GS-infected astrocyte cultures (Fig. 4A). On Northern blots, the intensity of the hybridized RNA species was much weaker in the extracted RNA from infected astrocytes than in an equivalent amount of RNA from T cells. This difference again suggests that infection or viral RNA expression was less efficient in astrocytes than in T cells. In spite of weaker signals, the viral RNA species expressed are identical for both cell types, suggesting that there are no gross differences in the p41 viral gene expression between the two cell types.

Since expression of the p41 gene may not necessarily indicate that HHV-6 can actively replicate and express viral late genes in the infected astrocytes, Northern blot analysis with a late HHV-6 gene that encodes for the envelope-gp105 protein was also performed. This gene is expressed late in infection, and multiply spliced RNA species are expressed in this locus (26). The hybridization pattern of the RNA extracted from cell-free HHV-6A GS-infected astrocytes with the gp105 probe was similar to that from GS-infected HSB-2 T cells (Fig. 4B). Four different RNA species, with sizes of 3.9, 3.0, 2.1, and 1.6 kb, were detected. These four RNA species represent the multiply spliced RNA expressed from this locus late in infection (26). However, the 1.0-kb RNA species found in T cells were not detected in the infected astrocytes, suggesting either that the level of expression was too low to be detected or that there may be differences in splicing and posttranscriptional modifications of the RNA for this gene in different cell types. Nevertheless, the detection of spliced RNA strongly suggests that active viral replication can occur in GS-infected astrocytes.

**Replication kinetics of HHV-6 in astrocytes.** To demonstrate that infectious HHV-6 virus was produced by the infected astrocyte cultures, cells were infected with cell-free HHV-6A (GS) at a concentration of $10^3$ $TCID_{50}$/$10^6$ cells. The infected cells were washed and then incubated in culture medium at 37°C. Supernatants from these infected cell cultures, containing cell-free virus, were collected at 1, 4, and 7 days postinfection, and the infectious titer of the virus was determined in HSB-2 indicator cells (Fig. 5) (30). No virus was detected at
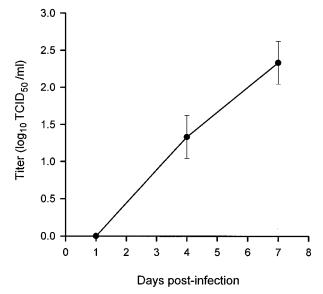
day 1 postinfection. The titer of the cell-free virus from the infected culture then increased with time, with about $10^{1.5}$ $TCID_{50}$/ml at day 4 postinfection and $10^{2.5}$ $TCID_{50}$/ml at day 7 postinfection. The infectious titer of cell-free virus was not determined after day 7 postinfection because the cumulative cytopathic effect destroyed the infected astrocytes. The kinetics of viral production corresponded well with the development of syncytia; syncytia were not detected at day 1 postinfection but started to appear at day 3 postinfection. Massive syncytia and cell death usually occurred by about 7 days postinfection. The increases in virus titer and cytopathic effects with time strongly suggest that infectious virus was produced in astrocytes as a result of active HHV-6 replication. The ability of the viruses from the astrocyte culture to reinfect T cells indicates that the host range specificity of HHV-6 has not been altered by passaging through astrocytes. Nevertheless, the titer of virus obtained from the infected astrocyte cultures is about 100-fold lower than the quantity of HHV-6A (GS) obtained from T cells (HSB-2 or J-Jhan) with similar $TCID_{50}$ inoculum doses and infection procedures. This lower virus titer is consistent with the level of viral gene expression as detected by PCR and Northern blot analyses. Previous studies have shown that HHV-6 infection of a glioblastoma cell line leads to a noncytopathic infection with a transient low level of viral synthesis (4). In contrast, infection of our nontransformed, low-passage astrocyte cultures with HHV-6 led to a productive cytopathic infection. It is not clear at this point whether latent infection may occur, but our results indicate that the effects of HHV-6 infection in transformed cell lines may not necessarily reflect those that may occur in primary or nontransformed cell cultures or in vivo. It will also be important to determine if primary human neurons and/or human oligodendrocytes can be infected by HHV-6. Our results nevertheless suggest that lytic infection of astrocytes may play a role in the neurological complications seen in children with acute HHV-6 infection.

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