Cellular localization of human herpesvirus-6 in the brains of children with AIDS encephalopathy

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Human herpesvirus-6, the etiologic agent of exanthem subitum, is a ubiquitous virus that infects almost all children by the age of 2 years and that has previously been shown to be neuroinvasive. These characteristics suggest that human herpesvirus-6 may be important in the neuropathogenesis of acquired immune deficiency syndrome (AIDS) in children. To address this hypothesis, we evaluated postmortem pediatric brain tissues for the presence of human herpesvirus-6 infection. Using in situ hybridization with a digoxigenin-labeled DNA probe for the large tegument protein gene of human herpesvirus-6, we detected nuclear signals in postmortem brain tissue from 4/5 children with human immunodeficiency virus-1 encephalitis. Human herpesvirus-6 DNA was found in numerous oligodendrocytes of the white matter and less frequently in astrocytes, macrophages, microglia and neurons. The human herpesvirus-6 positive cells detected by in situ hybridization were not immunoreactive either for human herpesvirus-6 early nuclear phosphoproteins or for surface glycoproteins associated with productive infection. Only rare human herpesvirus-6 infected cells were found in age-matched control brain tissues. No human herpesvirus-6 infected cells were found in human fetal brain tissue. These data suggest that human herpesvirus-6 is more extensively disseminated in neural cells in the presence of human immunodeficiency infection and immunodeficiency in pediatric AIDS patients, and it may contribute to the pathogenesis of AIDS encephalopathy.

Keywords: human herpesvirus-6 (HHV-6); human immunodeficiency virus type 1 (HIV-1); oligodendrocyte; in situ hybridization; AIDS

Introduction

Human herpesvirus-6 (HHV-6) is a recently discovered herpesvirus that has been isolated from peripheral blood lymphocytes of patients with lymphoproliferative disorders and human immunodeficiency virus type 1 (HIV-1) infection (Salahuddin et al, 1986; Tedder et al, 1987; Downing et al, 1987). It is the causative agent of exanthem subitum (Yamanishi et al, 1988) as well as other febrile illnesses in children (Pruksananonda et al, 1992), and it has been associated with seizures (Hall et al, 1994). Serological studies have shown that HHV-6 is a ubiquitous virus that infects more than 90% of all children before the age of 2 years (Hall et al, 1994; Okuno et al, 1989; Saxinger et al, 1988; Levy et al, 1990a). Recently, two distinct variants of HHV-6 have been identified: HHV-6 A and HHV-6 B (Schirmer et al, 1991; Ablashi et al, 1991; Aubin et al, 1991). In the US, HHV-6 B has been frequently isolated in a population of infants with symptomatic primary viral infection (Dewhurst et al, 1992, 1993). After the primary infection, there is lifelong viral persistence, possibly in a latent state. Thus, most adults continue to harbor detectable levels of viral DNA in both peripheral blood (Gopal et al, 1990; Kondo et al, 1991) and the oropharynx, including the salivary glands (Levy et al, 1990a; Gopal et al, 1990; Fox et al, 1990). In immune suppressed individuals, HHV-6 infection can be reacti-
vated from this latent or low-level productive state, giving rise to active infection with viremia (Okuno et al., 1990).

The brain is a common site for persistent viral infections (Johnson, 1982), including herpesviruses, and it is of interest that neurological complications occur frequently during the course of exanthem subitum (Moller, 1956). Evidence for entry of HHV-6 into the central nervous system (CNS) during primary infection includes intrathecal production of anti-HHV-6 antibodies (Ishiguro et al., 1990) as well as detection by polymerase chain reaction (PCR) of HHV-6 DNA in cerebrospinal fluid from children (Asano et al., 1992; Yoshikawa et al., 1992; Caserta et al., 1994; Kondo et al., 1993) as well as in post-mortem brain tissue (Asano et al., 1990). Taken together, these observations suggest that HHV-6 is able to invade the CNS during acute infection and persist. In the light of similarities between HHV-6 and other herpesviruses, including herpes simplex virus type 1 (HSV-1) (Fraser et al., 1981; Karlin et al., 1994), it is possible that HHV-6 may establish a persistent or latent infection in cells of the CNS.

Recently, Knox and Carrigan have demonstrated productive HHV-6 infection in lung, lymph node, spleen, liver and kidney of young adult patients who died with acquired immune deficiency syndrome (AIDS) (Knox and Carrigan, 1994). Both HIV-1 and HHV-6 are neuroinvasive, and hence HHV-6 may interact with HIV-1 in the CNS, particularly in pediatric subjects who might be expected to have a primary HHV-6 infection before the age of 2 years. In order to investigate the possible role of HHV-6 in the pathogenesis of AIDS encephalopathy, we examined previously characterized brain tissue from pediatric AIDS patients for the presence of HHV-6 infection.

Results

Microscopic findings

Neuropathological findings from the five cases are included in Table 1. No cells bearing nuclear or cytoplasmic inclusions were seen in any of the cases.

In situ hybridization for HHV-6 of control cells and tissues

The digoxigenin-labeled HHV-6 DNA probe hybridized strongly to cord blood mononuclear cells (CBMC) that had been infected with HHV-6 (Figure 1a). No hybridization signal was detected in uninfected cord blood cells (Figure 1b). HHV-6 DNA was rarely found in brain sections from the HIV-1 negative, immunocompetent children (Figure 1c), while human fetal brain tissues had no cells containing HHV-6 DNA (Figure 1d). Lack of cross hybridization of this probe with human cytomegalovirus (CMV) was confirmed by performing in situ hybridization on a human CMV-infected retinal xenograft (DiLoreto et al., 1994), with negative results (Data not shown).

In situ hybridization for HHV-6 of HIV-1 infected tissues

Positive hybridization for HHV-6 nucleic acid sequences was obtained in four of the five HIV-1 infected cases (Table 2). The results of this study indicate that HHV-6 is a potential etiologic agent of AIDS encephalopathy.

Table 1 Summary of cases examined

<table>
<thead>
<tr>
<th>Patient Age (yrs)</th>
<th>Sex</th>
<th>Clinical and neuropathological features</th>
<th>PCR HIV</th>
<th>ICC p24</th>
<th>ISH HHV-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>Progressive encephalopathy, severe myelopathy; Severe HIV-1 encephalomyelitis; vacuolar myelopathy</td>
<td>(+)</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>Progressive encephalopathy, terminal viral pneumonitis; White matter pallor, mineralizations in basal ganglia</td>
<td>(+)</td>
<td>0</td>
<td>2+</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>Probable progressive encephalopathy, with cerebral atrophy on CT; Moderately severe HIV-1 encephalitis</td>
<td>(+)</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>Progressive encephalopathy; Moderately severe HIV-1 encephalitis; pontine leukoencephalopathy</td>
<td>(+)</td>
<td>1+</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>Severe progressive encephalopathy; Severe HIV-1 encephalomyelitis</td>
<td>(+)</td>
<td>3+</td>
<td>1+</td>
</tr>
</tbody>
</table>

0 = No cells positive, 1+ = 1 to 10 positive cells/20x field, 2+ = to 40 positive cells/20x field, 3+ = Greater than 40 positive cells/20x field
Figure 2. HHV-6 in situ hybridization of HIV-1 infected tissue sections. (a) Subcortical white matter from case 2 demonstrating numerous HHV-6 positive nuclei identified by NBT/X-phos chromogen. Note also HHV-6 negative nuclei (eosin counterstain, X 120). (b) Subcortical white matter from case 1 demonstrating HHV-6 nuclear staining in cells with morphologic features of oligodendrocytes (eosin counterstained, X 380). Similar positive hybridization for HHV-6 nucleic acid sequences was obtained in cases 3 and 5, with the majority of positive nuclei occurring in the white matter.

Figure 1. HHV-6 in situ hybridization controls. (a) HHV-6 infected cord blood cells show intense blue nuclear signal (X 120). (b) Uninfected cord blood cells have no nuclear staining (X 120). (c) Control brain tissue from 8-year-old girl with acute intracerebral hemorrhage: arrow indicates rare cell containing HHV-6 nucleic acid in nucleus (X 240). (d) 19 week gestation human fetal brain contains no HHV-6 positive nuclei (X 120). (All in situ hybridization for HHV-6 with NBT/X-phos, eosin counterstain).
Figure 3  Cellular localization of HHV-6 in neural cells by combined DNA in situ hybridization and immunocytochemistry. (a) and (b) Oligodendrocytes containing nuclear signal for HHV-6 DNA in cerebral white matter, case 1 (arrows). (Immunocytochemistry for galactocerebroside with New fuchsin red chromogen and in situ hybridization for HHV-6 with NBT/X-phos blue chromogen, no counterstain. a; × 420, b; × 450). (c) HHV-6 signal in nucleus of neuron in putamen, case 1 (arrow). The identity of the cell is based on its shape and location. Positive small nucleus of oligodendrocyte is also present (in situ hybridization for HHV-6 DNA, NBT/X-phos chromogen, eosin counterstain, × 380).

Figure 4  Cellular localization of HHV-6 in neural cells by combined DNA in situ hybridization and immunocytochemistry. (a) and (b) White matter, case 2. In most of the regions, astrocytes (in red) are negative for HHV-6 nuclear signal (in blue), although a rare astrocyte is positive (arrow). (Immunocytochemistry for GFAP with New fuchsin and in situ hybridization for HHV-6 DNA with NBT/X-phos, no counterstain. (a) × 420, (b) × 260 (c) Cerebral cortex, case 1. A few microglial cells have nuclei that are positive for HHV-6 signal (arrow). (Immunocytochemistry for CD68 with New fuchsin and in situ hybridization for HHV-6 DNA with NBT/X-phos, no counterstain. × 330).
infected cases (Table 1), with the majority of positive nuclei occurring in the white matter (Figure 2a,b). These cells appeared to be mainly oligodendrocytes, on the basis of morphology. The oligodendrocyte lineage of these cells was confirmed by combined immunocytochemistry for Galactocerebroside and in situ hybridization for HHV-6 viral nucleic acids (Figure 3a,b). Positive signal for HHV-6 also occurred, although less frequently, in nuclei of neurons (Figure 3c), astrocytes and macrophages/microglia. Each of these cell types was also confirmed by combined immunocytochemistry and in situ hybridization (Figure 4a-c). Coinfection with HHV-6 and HIV-1 could also be demonstrated in a small number of cells in case 1 and 5 by combined immunocytochemistry for HIV p24 and in situ hybridization for HHV-6 DNA (Figure 5).

Immunocytochemistry for HHV-6
No cells in any of the five pediatric AIDS cases were immunoreactive for either HHV-6 early nuclear phosphoproteins, viral surface glycoproteins or the 101kDa major immunoreactive virion protein, although staining for HIV-1 p24 was positive in all. HHV-6 infected CBMC expressed all HHV-6 antigens, consistent with productive infection, while uninfected CBMC were negative. Control, HIV-1 negative tissues were completely negative for HHV-6 antigens.

Genetic characterization of HHV-6 in HIV-1 infected brain tissues
In the two patients (case 1 and case 2) in whom frozen CNS tissue was available, we observed two bands of 515bp and 235bp, indicating the presence of the HHV-6 B strain, after nested PCR amplification and Hind III digestion (Figure 6). In the other three cases, DNA was extracted from paraffin embedded tissue but was of insufficient quality for restriction enzyme analysis. We did not detect HHV-6 DNA by PCR in GNS tissue from the control cases.

Discussion
In this study, we have shown that HHV-6 gene sequences are found considerably more frequently, in cells of the developing CNS, in cases where HIV-
1 infection was present than in controls, a previously
unreported finding. Only 10–30% of children
born to HIV-1-infected mothers become infected,
mostly perinatally, but clinical disease appears
in many of these children before the age of 2 years
(Scott et al., 1989; Rossi et al., 1992), and the CNS
may be heavily infected (Shaw et al., 1985). AIDS
eнцеphalopathy is common, and is characterized by
neuropathological changes (Sharer et al., 1986) and
by developmental delays and/or loss of motor mile-
stones and intellectual abilities acquired previously
(Blanche et al., 1990; Epstein et al., 1986). Because
HHV-6 infection is acquired postnatally, after the
waning of maternal immunity, extensive dissemina-
tion of HHV-6 in the CNS may occur in response to
inflammatory processes of HIV-1 encephalitis
(Genis et al., 1992; Gelbard et al., 1994) or due to
coincident immunodeficiency.

While re-activated CNS infections, particularly
JCV and CMV, have been implicated as co-factors in
AIDS in adults (Atwood et al., 1993), the number of
reported opportunistic infections in the CNS of
children with HIV-1 infection is substantially less
than for adults (Epstein et al., 1986; Sharer et al.,
1986). Indeed, Achim et al. (1994) demonstrated by
PCR that HHV-6 was not present in the CNS of adult
AIDS patients. Although more extensive studies are
required to draw definitive conclusions, the pres-
ence of HHV-6 gene sequences in four/five pediatric
AIDS brains examined (it is possible that case 4
never acquired systemic HHV-6 infection) suggests
that HHV-6 is an important opportunistic agent in
pediatric AIDS and warrants further study. We spec-
ulate that the more extensive dissemination of
HHV-6 in the CNS in children than in adults may
reflect the combined effects of (1) primary HHV-6
infection as opposed to reactivation, (2) persistent
HHV-1 infection of the nervous system and (3) coin-
cident immunodeficiency at the time of HHV-6
infection.

Recently, we and others have demonstrated, in
postmortem CNS tissues from children who died
with severe HIV encephalopathy, that HIV-1 pro-
duces a 'restricted' infection in astocytes (Saito
et al., 1994; Tornatore et al., 1994) in addition to the
well-known productive infection of
macrophages/microglia (Sharer, 1992). In vitro
experiments have suggested that glial cells are sus-
ceptible to HHV-6 infection (Tedder et al., 1987;
Ablashi et al., 1987; Levy et al., 1990b), but little is
known about its cellular tropism in human brain.
Recently, Drobskyi et al. (1994) reported a case of
fatal encephalitis due to HHV-6 B infection or re-
activation in a bone marrow transplant recipient.
HHV-6 B was the only virus type identified in two
of our five patients and this finding is consistent
with previous observations in children with prima-
ry HHV-6 infection (Dewhurst et al., 1992, 1993). In
Drobskyi's case, most of the HHV-6-infected cells
appeared to be astrocytes, with lesser infection of
oligodendrocytes, even though most of the infected
cells and tissue damage occurred in the white
matter. In contrast, we found HHV-6 DNA in numer-
ous oligodendrocytes of the white matter, but less fre-
quently in astrocytes, macrophages and microglia,
and also in neurons. Thus, these data demonstrate
that HHV-6 infects a broader spectrum of neural
cells than HIV-1 in vivo. However, it remains to be
determined whether astrocytic or oligodendroglial
infection by HHV-6 contributes to white matter
pathology in pediatric AIDS patients.

Interestingly, we did not find evidence for
expression of HHV-6 antigens with any of the HHV-
6 specific antibodies currently available, even in tis-
sues where strong signals were obtained by in situ
hybridization. This negative finding suggests that
HHV-6 in neural cells is latent during late stages of
AIDS encephalopathy, although expression of unas-
sayed HHV-6 gene products has not been ruled out.
Minimal virus production demonstrated by culture,
immunocytochemistry, or electron microscopy only
weeks after acute herpes virus encephalitis is not
uncommon (Nicolli et al., 1991). We speculate that
active HHV-6 infection occured at an earlier time,
perhaps during primary viral infection, and that
this led to viral dissemination in the CNS, with sub-
sequent viral persistence.

We demonstrated that HHV-6 gene sequences and
HHV-1 antigens are infrequently co-localized in
macrophages, where both these viruses produce
active infection, in the CNS of pediatric patients.
Co-infection could also occur in microglia, but is
less likely in astrocytes or oligodendrocytes, or in
neurons where HIV-1 was identified by PCR in situ
in a single report (Nuovo et al., 1994). In vitro stud-
ies indicate that co-infection leads to activation of
HIV-1 long terminal repeat (LTR)-directed gene
expression (Hovart et al., 1989; Lasso et al., 1989;
Ensoli et al., 1989). Thus, although the frequency of
HIV-1/HHV-6 co-infection was low, the possibility
eexists that HHV-6 proteins may transiently activate
cellular or HIV-1 genes, particularly if productive
HHV-6 infection of neural cells occurs at the time of
primary infection. However, activation of HHV-6 by
HIV-1 gene products, such as tat (Frankel and Pabo,
1988), is less likely due to the unusual nature of the
HHV-6 promoter (Agulnick et al., 1994).

In summary, this study provides evidence that
HHV-6 is widely disseminated in neural cells in
postmortem pediatric AIDS brains, and could thus
trigger HIV-1 replication during the course of AIDS
encephalopathy in children. Further studies with
HHV-6/HIV-1 co-infections in animal model sys-
tems (Cvetkovich et al., 1992) are needed to estab-
lish the temporal relationship of HIV-1 and HHV-6
infections in brain tissue, which is the key to a
fuller understanding of the role of HHV-6 in the
pathogenesis of AIDS encephalopathy.
Materials and methods

**HIV-1 cases**

Brain tissue was obtained from five children, ranging in age from 6 months to 9 years, who died with perinatally acquired HIV-1 infection and AIDS. Four of these children had HIV-1 encephalitis, on neuropathological examination. The clinical and pathological findings in these children are shown in Table 1. Tissues were fixed in neutral buffered formalin for up to 4 weeks, and blocks were embedded in paraffin for pathological studies. Regions selected for study included cerebral cortex, cerebral white matter, basal ganglia and pons.

**Controls**

*Tissue controls*  Sections of formalin fixed brain tissue were obtained from an 8-year-old boy who died with Dandy-Walker malformation, an 8-year-old girl with acute intracerebral hemorrhage, and a 19 week old fetus. These cases were all negative for infection by HIV-1 as determined by paraffin PCR.

*Cell controls*  *HHV-6 infected and uninfected CBMC* were grown using media and cell culture conditions as previously described (Dewhurst et al, 1992). The cells were centrifuged at 250 X g for 10 min, resuspended in a small volume of PBS, placed on Vectabond-treated slides (Vector Laboratories, Burlingame, CA) and fixed for 20 min in 4% (wt/vol) paraformaldehyde in phosphate buffered saline (PBS).

**Preparation and PCR amplification of viral DNAs**

Genomic DNA was extracted from either frozen brain tissue or formalin-fixed, paraffin-embedded brain tissue sections using a proteinase K incubation extraction protocol similar to that described by Jackson et al (1990). A nested PCR protocol was used to amplify a previously described portion of the large tegument protein gene (LTP) from HHV-6 (Dewhurst et al, 1993). Genotyping of HHV-6 was carried out by using a Hind III restriction site polymorphism (Aubin et al, 1991; Dewhurst et al, 1993), which allows the identification of two distinct viral variants, HHV-6 A and HHV-6 B.

**Immunocytochemistry**

Avidin-biotin immunocytochemistry was performed as previously described (Saito et al, 1994) using ABC kits with an avidin-biotin-alkaline phosphatase complex (Vectastain elite, Vector) with New fuchsin chromogen (DAKO, Carpinteria, CA), which gives a red reaction product. Productive HHV-6 infection was identified by staining with mouse monoclonal antibodies and rabbit hyperimmune serum specific for putative early and late structural proteins of the virus. The following primary antibodies were used: anti-p41 monoclonal antibody 9A5D12 (directed against early nuclear phospho-protein; 1:1 dilution), monoclonal antibody 6A5D5, directed against viral surface glycoproteins gp116, gp64, gp54 (1:1 dilution) (Balachandran et al, 1989), UK 82 rabbit antiserum (directed against surface glycoproteins gp82, gp105; 1:400 dilution) (kindly provided by Dr Bala Chandran, University of Kansas Medical Center); and a monoclonal antibody C3108-103 (kindly provided by Dr Phillip Pellett, Centers for Disease Control and Prevention) directed against the 101KDa major immunoreactive virion protein of HHV-6 B (1:200 dilution) (Pellett et al, 1993).

**Preparation of digoxigenin-labeled DNA probe for in situ hybridization**

A DNA probe was prepared by labeling HHV-6 LTP DNA fragments with digoxigenin 11-dUTP (Boehringer Mannheim, Indianapolis, IN) by PCR (Seibl et al, 1990), using previously described primers (Dewhurst et al, 1993) and the plasmid pZVH14 containing an 8.7-kb Hind III fragment of HHV-6 GS strain DNA (Josephs et al, 1986). The amplification conditions were those used previously (Dewhurst et al, 1993), except that dTTP was partially (30%) substituted with dig-11-dUTP.

**In situ hybridization**

Five μm thick brain tissue sections were placed onto Vectabond-coated glass slides and baked at 50°C for 1 h. The sections were dehydrated in three changes of Propar (Anatech, Battle Creek, MI) and rehydrated in graded ethanols. After rehydration in PBS, the sections were treated with proteinase K (2μg ml⁻¹) in 20mM Tris-HCl pH7.5/2mM CaCl₂ for 30 min at 37°C. Sections were washed in PBS and then acetylated in 0.25% acetic anhydride/0.1M triethanolamine (pH8.0) for 10 min. The hybridization mixture consisted of digoxigenin-labeled probe (1:200 dilution), 50% formamide, 2XSSC, 50mM Tris-HCl pH 7.5, 2XDenhard's solution, 1mM EDTA, 2% dextran sulfate, fragmented salmon-sperm DNA and tRNA. Hybridization mixture(30μl) was applied to the sections and sealed with high pressure vacuum grease under a coverslip. Probe and target DNAs were simultaneously denatured by placing the sections on an 80°C heating block for 10 min. Hybridization was carried out overnight at 40°C in a sealed humidified container. The post-hybridization washes consisted of one wash in 50% formamide/2XSSC, one wash in 2XSSC, and one wash in 0.2XSSC at 40°C for 15min. Hybridized DNA was detected by using an anti-digoxigenin Fab fragment conjugated to alkaline phosphatase (AP) followed by detection of specifically bound AP using nitroblue tetrazolium (NBT) and X-phosphate (X-phos) chromogen (Boehringer Mannheim) that gives a blue tetrazolium pigment, with an eosin counterstain. The same procedure was used on HHV-6 infected and uninfected CBMC except deparaffinization.
**Combined immunocytochemistry and in situ hybridization**

*In situ* hybridization was combined with immunocytochemistry for simultaneous detection of HHV-6 nucleic acid sequences and either cell specific antigens or HIV-1 antigens. Tissue sections were first Immunostained using the alkaline phosphatase reaction with new fuchsin substrate, followed by *in situ* hybridization as described above. The following primary antibodies were used: polyclonal anti-glia fibrillary acidic protein (GFAP) antibody (Dako) as a marker for astrocytes; monoclonal anti-galactocerebroside antibody (Boehringer Mannheim) as a marker for oligodendrocytes; monoclonal anti-CD68 antibody (Dako) as a marker for activated macrophages and microglia; polyclonal anti-PGP 9.5 antibody (UltraClone, Isle of Wight, UK) as a marker for neurons: polyclonal anti p24 antibody (ABT, Cambridge MA) as a marker for productive HIV-1 infection.

**Acknowledgments**

We thank Dr Robert Gallo for the gift of plasmid pZVH14, we also thank Drs Philip Pellett and Bala Chandran for the gift of primary antibody to HHV-6 (C3108 103 and 9AD512, 6AD5, UK82) respectively. We acknowledged Kim McIntyre, Nurcan Ergin, Kirk Dzenko, Bojun Chen, He Wang and especially Harold James, for expert technical assistance. This work was supported by grants 770226-14-PF (YS) from the Pediatric AIDS Foundations, RO1 AI30202-02 (CBH) from the NIAID, KO4 Al01240 (SD) from the NIAID and PO1 NS31492-02 (LGE, BMB, and LRS) from the NINDS. Dr Saito is a Pediatric AIDS Foundation scholar.

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