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Association of human herpesvirus 6 with the demyelinative lesions of progressive multifocal leukoencephalopathy

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Progressive Multifocal Leukoencephalopathy (PML) is a primary demyelinating disease of the central nervous system occurring almost exclusively in individuals with impaired cell-mediated immunity. The JC polyoma virus has been accepted as the etiologic agent of PML. Using a two-step *in-situ* polymerase chain reaction procedure to amplify and detect genomic DNA of human herpesvirus-6 (HHV6) in formalin-fixed paraffin-embedded archival brain tissues, a high frequency of infected cells was consistently detected in PML white matter both within and surrounding demyelinative lesions and HHV6 genome was found mainly within oligodendrocytes. Lesser amounts of HHV6 genome were detected in most normal, AIDS, and other neurological disease control tissues. Immunocytochemistry for HHV6 antigens showed actively infected nuclei of swollen oligodendrocytic morphology only within the demyelinative lesions of PML but not in adjacent uninvolved tissue. In addition, no HHV6 antigens were detectable in control tissues including brains of individuals with HIV-1 encephalopathy but without PML. Double immunohistochemical staining for JC virus large T antigen and HHV6 antigens demonstrated co-labeling of many swollen intralesional oligodendrocytes in the PML cases. The evidence suggests that HHV6 activation in conjunction with JC virus infection is associated with the demyelinative lesions of PML.

Keywords: Progressive Multifocal Leukoencephalopathy; Human Herpesvirus 6; JCV; co-infection; demyelination; pathogenesis

Introduction

Progressive Multifocal Leukoencephalopathy (PML) is a primary demyelinating disease of the central nervous system (CNS) generally considered to be caused by direct lytic infection of oligodendrocytes by a human polyoma virus designated JC virus (JCV) (Padgett *et al*, 1971; Major *et al*, 1992; Aksamit, 1995). Originally described in 1958 as a rare condition associated with chronic lymphocytic leukemia and Hodgkin's lymphoma (Anstrom *et al*, 1958), a possible viral etiology was suggested the following year when inclusion bodies were observed within the nuclei of damaged oligodendrocytes (Cavanaugh *et al*, 1959). Subsequent electron microscopic studies revealed particles resembling polyoma virus that were prevalent in the nuclei of inclusion-bearing oligodendrocytes (ZuRhein, 1969). Cultivation of a unique human polyoma virus was accomplished by Padgett and colleagues (Padgett *et al*, 1971) by inoculation of primary human fetal glial cell cultures with brain extracts of a patient dying with PML. Subsequent studies using

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JCV-specific immunocytochemistry (ICC) and *in-situ* hybridization (ISH) have led to the conclusion that JCV is the exclusive pathogen in patients with PML (Walker and Padgett, 1983).

PML occurs almost invariably in individuals with impaired cell-mediated immunity (Berger *et al*, 1987; von Einseidel *et al*, 1993). The advent of the AIDS epidemic resulted in a marked increase in the incidence of PML; up to 4% of AIDS patients develop PML and in half of these it is the initial AIDS defining illness (Berger *et al*, 1998). Seroepidemiologic studies have established that JCV has a worldwide distribution. In the United States and Europe, 60–80% of adults possess antibodies to JCV, but infection is asymptomatic in the vast majority (Aksamit, 1995; Walker and Padgett, 1983). Most patients with PML have pre-existing antibody to JCV and do not show a rise in antibody titer during progression of the disease. Together with the lack of IgM antibody in the serum and CSF and the nearly ubiquitous presence of profound defects in T-cell immunity, these findings have suggested that reactivation of latent JCV infection is involved in the pathogenesis (Padgett and Walker, 1983; Walker and Padgett, 1983; Brooks and Walker, 1984).

The pathology of PML is characterized by multifocal microscopic and macroscopic demyelinative lesions seen most typically in the subcortical white matter near the gray-white matter junction (Major *et al*, 1992; Aksamit, 1995). Enlarged oligodendrocytes with swollen nuclei two to three times larger than normal are present and oriented circumferentially at the advancing edge of the lesion. These are often accompanied by hypertrophic astrocytes of bizarre morphology and large numbers of lipid-laden macrophages within the lesions. Examination by electron microscopy reveals the nuclei of infected oligodendrocytes packed with electron-dense papovavirus particles of approximately 40 nm in diameter. The clinical presentation of PML is that of an indolent but progressive neurological deterioration commonly characterized by hemiparesis, visual field deficits, and dementia. Median survival is 6 months and more than 90% of patients die within 1 year of diagnosis (Berger *et al*, 1998).

In previous studies on AIDS encephalopathy (Saito *et al*, 1994, 1995), we detected the presence of HIV-1 and HHV6 nucleic acids in brains of children by *in-situ* hybridization (ISH). To gain sensitivity, we developed a powerful two step *in-situ* polymerase chain reaction procedure (ISPCR) which detected both active and latent HIV-1 DNA (Sharer *et al*, 1996). Since PML is now mainly found in the context of AIDS, it was of interest to modify this procedure to provide increased sensitivity for detection of HHV6 DNA in archival autopsy and biopsy brain specimens from patients with PML, HIV-1 encephalopathy and controls. In particular, we used our two-step ISPCR procedure to investigate whether HHV6 may also be associated with PML in cases both with and without underlying HIV-1 infection.

Results

ISPCR for HHV-6 genome in brain tissues of patients with PML

Seven cases of AIDS-associated PML and four cases of PML associated with other conditions were examined by two-step ISPCR for HHV6. Large numbers of HHV6 infected cells (50–100/20× field) were consistently seen in 10/12 sections (derived from 11 cases) of PML white matter (Figure 1A). One PML case averaged 10–20 HHV6 infected cells/20× field. A small surgical specimen from another case revealed less than 10 HHV6 infected cells/20× field, but autopsy tissue from the same case demonstrated 50–100 HHV6-containing cells/20× field. In all cases, the highest frequencies of staining for HHV6 DNA were observed within white matter with relatively less virus seen in adjacent gray matter (Figure 1B). The prevalence and localization of HHV6 in four cases of non-AIDS-associated PML were indistinguishable from that seen in AIDS-associated PML.

The majority of HHV6 signal appeared as nuclear staining within cells of typical 'fried-egg' oligodendrocyte morphology (Figure 1C, small arrow), while many nearby oligodendrocytes remained uninfected (block arrows). These cells were not immunoreactive with antibodies to CD3 or CD68. Many fields included several swollen cells (Figure 1C, large arrow) that are considered pathognomonic for JCV infected oligodendrocytes (Major *et al*, 1992; Aksamit, 1995). In gray matter, HHV6 genomic DNA signal was often observed in neuronal cytoplasm and in neuronal satellite cells (arrow) (Figure 1D). In some cases, HHV6 signal was also found in the nuclei of many perivascular mononuclear cells (Figure 1E, arrow). Further staining of this section with monoclonal antibodies to CD3 and CD68 indicated that both lymphocytes and monocytes were infected (data not shown).

There was consistent absence of HHV6 signal in human fetal brain sections that were included as negative controls in each ISPCR experiment (Figure 1F). In separate experiments (not shown), the biotinylated HHV6 probes hybridized strongly to primary cord blood lymphocytes that had been infected with HHV6. No hybridization signal was detected in any brain sections from which the biotinylated probes had been omitted.

ISPCR for HHV-6 genome in AIDS and other neurological disease brains

As a control for HHV6 in AIDS-associated PML brain, we probed brain sections of 18 children and

adults with HIV-1 encephalopathy but without clinical or pathologic signs of PML (Table 1). Ten of 18 patients with AIDS encephalopathy demonstrated <10 HHV6 infected cells/20$\times$ field in white matter, a similar percentage to that seen in non-AIDS controls. The remainder showed higher frequencies ranging as high as 50–100 cells/20$\times$ field in 4/18 cases. Staining for HHV6 genomic DNA was seen predominantly in oligodendrocytes and to a much lesser extent in macrophages, astrocytes, and neurons.

The relationship between HHV6 genome and active HIV-1 infection was next investigated by ISPCR for HHV6 followed by immunocytochemistry

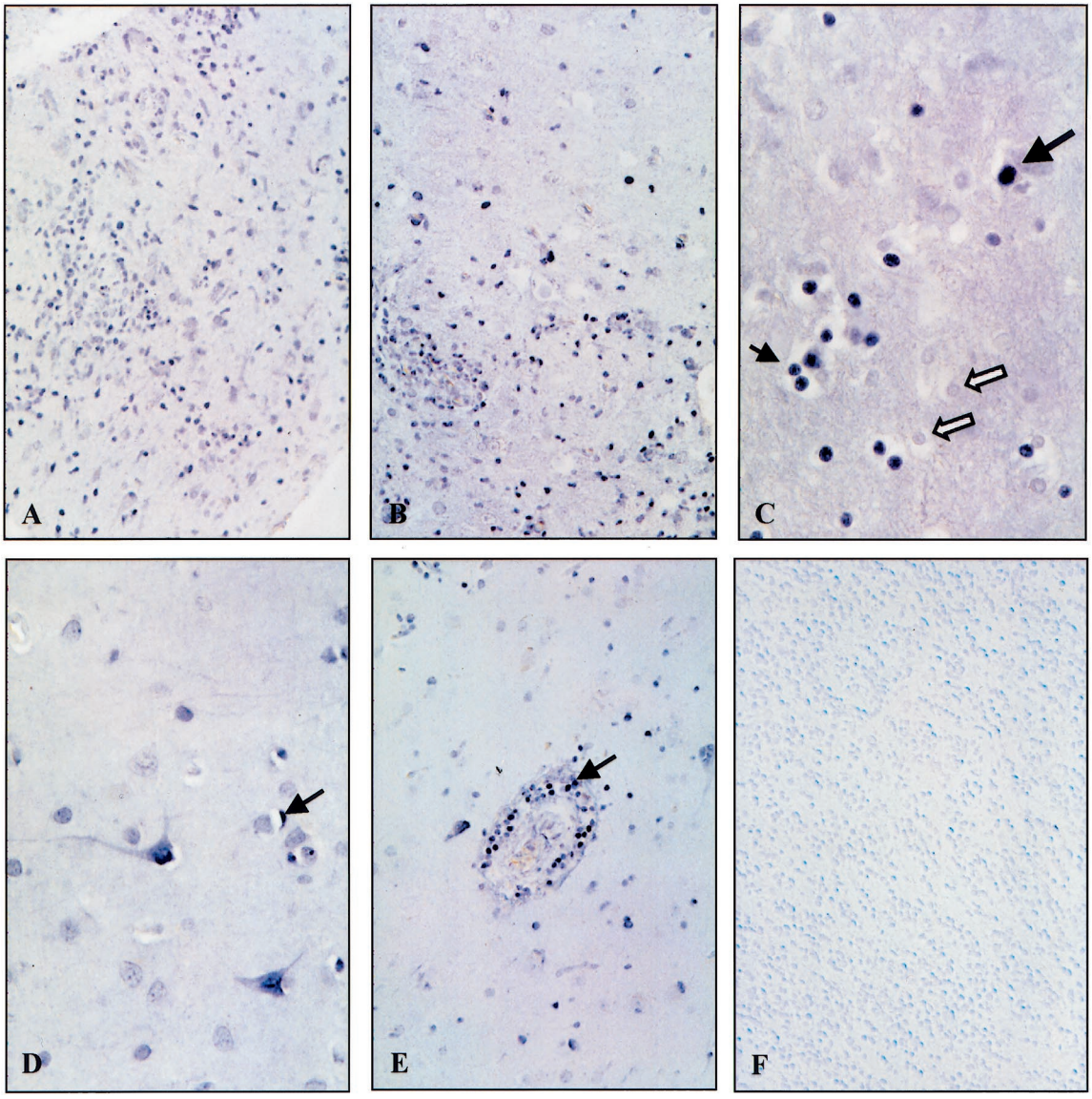

Figure 1 Amplification and localization of HHV6 genomic DNA in archival, formalin-fixed PML and control brain sections. Two-step ISPCR was performed as detailed in Materials and methods, with internal incorporation of digoxigenin-dUTP during PCR followed by *in-situ* hybridization with HHV6-specific biotinylated probes. Signal development was by standard ABC immunocytochemistry, with DAB/nickel stain (black). (**A**) A typical case of PML white matter demonstrating a very high frequency (>100 positive cells) of cells with nuclear HHV6 genomic DNA signal. (20$\times$ field; original magnification 60$\times$). (**B**) A second heavily infected case of PML, at the white/gray matter border: Cells containing HHV6 genome are less frequent in gray matter (top right). (20$\times$ field; original magnification 60$\times$). (**C**) Higher magnification of white matter from the same case as in (**B**); HHV6 genome signal is present in many oligodendrocytic nuclei (small arrow, left). A grossly swollen satellite oligodendrocytic nucleus characteristic of PML is at top right (large arrow), Uninfected oligodendrocytes of normal size are also present throughout the section (block arrows). (Original magnification 120$\times$). (**D**) HHV6 genome is present in gray matter within neurons and neuronal satellite cells of undetermined type (arrow) in a third case of PML. (Original magnification 120$\times$). (**E**) HHV6 genome is prevalent in perivascular mononuclear cells (arrow) in a different field of case (**B**). (Original magnification 60$\times$). (**F**) ISPCR amplification of HHV6 DNA is not detected within sections of human fetal brain employed as a negative control. (Original magnification 60$\times$).

for HIV-1 p24 antigen. There was no correlation between the numbers and types of HIV-1 p24 positive cells and that of HHV6 positive cells. HIV-1 p24 antigen was found only within macrophages/microglia, as previously observed (Sharer *et al*, 1985; Saito *et al*, 1994, 1995), and several cases with severe HIV-1 encephalitis and numerous p24 positive macrophages demonstrated less than ten HHV6 infected cells/20× field by ISPCR (data not shown).

Nine of 11 child and adult non-immunocompromised individuals without HIV-1 infection had less than 10–20 HHV6 infected white matter oligodendrocytes/20× field (Table 1). Diagnoses included one post-surgical glioblastoma, one with Parkinson's disease, three cases of hypoxia/ischemia, one intracerebral hemorrhage, one stroke and two with multiple congenital defects. Ten to 20 HHV6 infected cells/20× field were noted in one case each of adult stroke and child hypoxia/ischemia. Twenty to 50 HHV6-infected cells/20× field were observed in one case each of amyotrophic lateral sclerosis and Alzheimer's disease. No non-immunocompromised control case contained greater than 50 HHV6 positive cells/20× field.

Immunocytochemistry for HHV6 p41+p101 proteins

Although ISPCR detects genomic DNA with high sensitivity, it does not distinguish between latent and active infection. In order to study HHV6 gene expression and its relationship to the demyelinative lesions of PML, we employed ICC with monoclonal antibodies to either HHV6 p41 (the major DNA binding problem) or p101 (the major antigenic virion protein). In some experiments these two monoclonals were used together for increased signal strength. As was also observed with ISPCR, immunostaining with both HHV6 monoclonal antibodies consistently gave no signal in human fetal brain (Figure 2A).

In PML white matter there was staining of HHV6 antigens within perilesional white matter cells, and dense staining of many oligodendrocytes (Figure 2B), including grossly swollen oligodendrocytes (Figure 2B, large arrow; compare Figure 1C). Unstained cells of oligodendrocytic morphology but of more normal size are indicated for comparison (block arrows). In Figure 2C, a section from a different case stained with hematoxylin and eosin to better reveal cell morphology, several swollen oligodendrocytes (pathognomonic for JCV infection) are evident (boxed). When a subjacent serial section with the identical field of PML white matter was immunostained for HHV6 p41 and p101 antigens, the same swollen oligodendrocytes (boxed) were intensely stained (Figure 2D). Some hypertrophic astrocytes (large arrow) are less densely stained, while unstained macrophages (small arrow) and oligodendrocytes (block arrow) are also visible nearby.

In all, 11 sections of white matter from eight individuals with PML were examined for immunoreactivity to HHV6. In 9/11 sections, there was intense staining of HHV6 antigens within perilesional white matter cells, particularly oligodendrocytes. The two remaining sections demonstrated only weak staining within astrocytes, but extensive pathology and tissue damage had left few remaining oligodendrocytes for examination. As one control on the specificity of ICC, serial sections from eight different PML patients that stained intensely for HHV6-p41 and p101 proteins by DAB/Ni chromogen were stained for the irrelevant antigen CAM 5.2, a human cytokeratin which is not found in the normal CNS; no staining was observed in PML brain, whereas a simultaneously processed multiorgan 'sausage' slide containing small sections of liver, kidney, lung, etc., was appropriately stained (not shown). A distinct chromogen system, streptavidin-biotin with HRP and AEC was used by different personnel in a second laboratory as a control for HHV6 staining with DAB/Ni; the results were the same.

The detection of HHV6 within enlarged oligodendrocytes by both immunostaining and ISPCR in PML brains, suggested the possibility that these cells were co-infected with JCV. To test this possibility, we used double immunocytochemistry for the HHV6 p41 protein and the JCV large-T antigen. Double staining of several enlarged oligodendrocytes was revealed by an orange-red (AEC) stain for JCV large-T antigen against a black (nickel-DAB) stain for HHV6 p41, resulting in a brown reaction product (Figure 2E, large arrows and inset). This observation directly demonstrates co-infection of PML white matter oligodendrocytes by HHV6 and JCV. Normal appearing oligodendrocytes without immunostain are seen in the same field (block arrows). In contrast, coinfection with HIV-1 (as assayed by staining for p24 antigen) and JCV was observed only in rare astroglial and microglial cells (not shown). No staining for HHV6 antigens was detected in either the four cases of HIV-1 encephalopathy without PML or in control brains from non-immunocompromised individuals.

Table 1 Frequency of HHV6 positive cells in White Matter by ISPCR

	Positive sections/Number of sections examined				
Signal frequency	*PML*	*Adult AIDS*	*Adult controls*	*Pediatric AIDS*	*Pediatric controls*
◆◆◆◆	10/12	4/12	0/7	0/6	0/4
◆◆◆	0/12	3/12	2/7	1/6	0/4
◆◆	1/12	0/12	1/7	0/6	1/4
◆	1/12	5/12	4/7	5/6	3/4

Key: ◆◆◆◆: 50–100 positive cells/20× field.
◆◆◆: 20–50 positive cells/20× field.
◆◆: 10–20 positive cells/20× field.
◆: <10 positive cells/20× field.

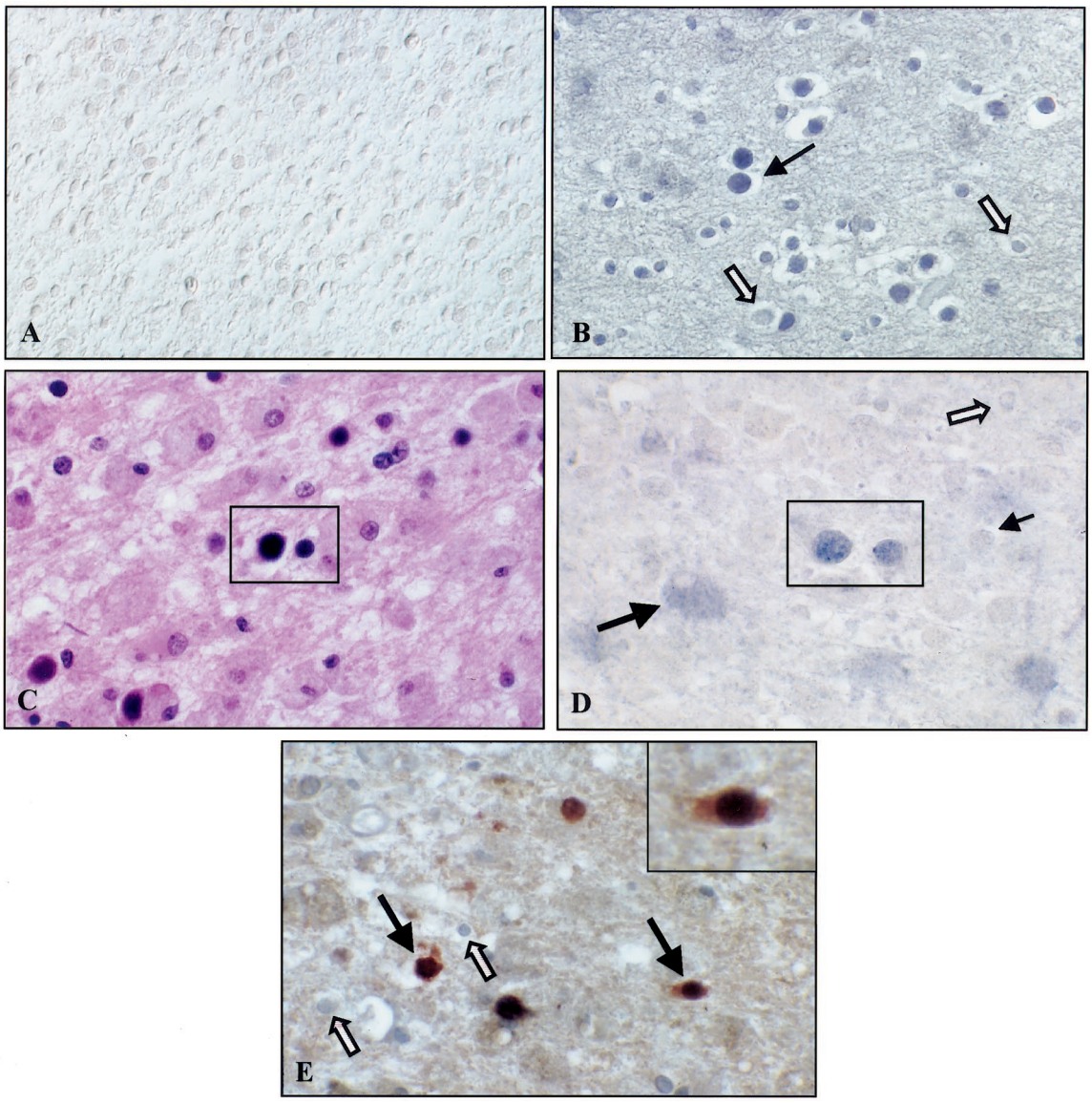

Figure 2 Localization of HHV6 and JCV gene expression in PML and control brains. Signal development for HHV6 p41 and p101 antigens employed standard ABC immunocytochemistry with DAB/Nickel stain (black). Immunostaining for JCV large-T antigen was with AEC counterstain (orange-red). (**A**) Immunocytochemistry for HHV6 p41 and p101 proteins in human fetal brain. Immunostaining yields no signal in this negative control tissue. (Original magnification 60×). (**B**) Immunocytochemistry for HHV6-p41 and p101 proteins in PML brain white matter. Signal is present mainly in swollen oligodendrocytes (large arrow), suggesting HHV6 co-infection of JCV-infected cells, and possibly in normal sized oligodendrocytes. Unstained oligodendrocytes of normal size may also be seen in the same field (block arrows). (Original magnification 120×). (**C**) Hematoxylin and Eosin stained serial section from PML white matter demonstrating several swollen oligodendrocytes characteristic of JV virus infection (boxed). (Original magnification 120×). (**D**) Adjacent serial section from the same PML case shown in (**C**). The section has been immunostained for HHV6 p41 and p101 antigens. Intense staining of swollen oligodendrocytes (same cells boxed in (**C**)) is demonstrated, with lesser staining of a reactive astrocyte cytoplasm (large arrow). An unstained macrophage (small arrow) and oligodendrocyte (block arrow) are also indicated. (Original magnification 120×). (**E**) Double immunocytochemistry for the HHV6 p41 protein (DAB/Nickel stain: black) and the JCV large-T antigen (AEC counterstain: orange-red) in PML thalamus. Note dual signal (brown) in swollen oligodendrocytes (large arrows); unstained oligodendrocytes are also present (block arrows). A red-orange rim (JCV) appears around black stained (HHV6) oligodendrocyte nuclei (inset). (Original magnification 120×; inset magnification 225×).

Discussion

These studies demonstrate the presence of high frequencies of HHV6 DNA in white and gray matter of individuals with PML, often in association with the demyelinative lesions. ISPCR was chosen as our primary tool for its capacity to both identify essentially all infected cells as well as to localize

them in relation to the neuropathologic lesions. As in our previous studies (Saito *et al*, 1994, 1995; Sharer *et al*, 1996), ICC was used in concert with nucleic acid hybridization techniques, to identify gene products resulting from active infection. In studies of HHV6, a virus with 102 open reading frames whose expression remains incompletely understood (Gompels *et al*, 1995; Braun *et al*, 1997) immunocytochemical methods are constrained by the large number of potential target antigens, of which only a subset may be present.

Our two-step ISPCR method is somewhat similar to that popularized by Bagasra *et al* (1997), but the differences are important. First, we use digoxigenin-labeled dNTPs during the PCR step and biotinylated dUTP to make the probes. We find that the highly hydrophobic digoxigenin moiety helps anchor the otherwise diffusible PCR product in the tissue, but cannot be used as a target for signal development due to an unavoidable background artifact (Gressens and Martin, 1994; Sallstrom *et al*, 1993); the second step of *in-situ* hybridization using biotinylated probes confers specificity and permits signal development by conventional avidin-biotin immunocytochemistry. Second, we use very slow thermocycling, with long ramps and annealing/extension times, to permit penetration of primers and Taq polymerase through the crosslinked matrix of archival tissue. Third, we use multiple sets of primers/probes to increase the signal-to-noise ratio. DNA amplification by this technique is demonstrated by our consistent observation that signal in oligodendrocytes and other cells subjected to two-step ISPCR is usually very intense, and specificity of amplification was confirmed by the presence of nearby unstained cells of the same types. In contrast, cells stained by ICC exhibited a wide range of signal intensities (see Materials and methods for a further discussion of these issues and controls).

By ISPCR, a high frequency (50–100 cells/20× field) of HHV6 infected cells, morphologically identified primarily as oligodendrocytes, was found within and immediately surrounding the lesions in 10/12 sections from 11 patients with PML. Although all adult and pediatric control tissues also demonstrated HHV6 infected cells, again primarily oligodendrocytes, they generally (9/11) had less than 20 HHV6 containing cells/20× field. Among patients with HIV-1 encephalopathy but without PML, the majority (10/18), had less than ten HHV6 positive cells/20× field; only 4/18 had a frequency similar to that seen in the PML patients (50–100 positive cells/20× field). Precise quantitative comparisons are problematic because of differences in age and details of processing between samples. However, a larger number of swollen oligodendrocytes, considered pathognomonic for JCV infected cells, could be double labeled for both the JC virus large-T antigen and the HHV6 p41 protein, thus demonstrating dual infection of these cells.

In PML, HHV6 genome was found by ISPCR both in and around the lesions, while HHV6 proteins were found predominantly at the active margins of demyelinative lesions and were not seen outside of the lesional areas. Using ICC, HHV6 gene expression in PML white matter was detected primarily in abnormal oligodendrocytes; intense staining for HHV6 proteins was found within perilesional oligodendrocytes in at least one section from all patients with PML. The number of cells stained by ICC was less than that stained by ISPCR, suggesting that not all HHV6 genes were expressed. HHV6 DNA was also identified within neuronal cytoplasm and neuronal satellite cells as well as astrocytes and macrophages both in and around demyelinated lesions. Perivascular infiltrates of HHV6-infected mononuclear cells were also sometimes observed. These observations imply that either retrograde axonal transport or a hematogenous route of CNS entry of HHV6-infected mononuclear cells may occur in PML, but this will need to be addressed in future studies.

Retrograde neuronal transport clearly occurs with other herpes family viruses such as Herpes Simplex and Varicella Zoster, while transport to the CNS via mononuclear leukocytes is postulated to occur in both Cytomegalovirus and Epstein–Barr virus encephalitides. Further, the results of both *in-vitro* and *in-vivo* studies have demonstrated HHV6 infection of monocytes, macrophages, and lymphocytes, most abundantly CD4 positive lymphocytes (Takahashi *et al*, 1998). Similarly, in PML, JCV-infected B lymphocytes have been identified in some brain samples and *in-situ* DNA hybridization studies of brain tissue have shown many JCV-infected cells surrounding blood vessels (Houff *et al*, 1988; Major *et al*, 1990; Gallia *et al*, 1997).

Four of the 11 cases of PML we examined occurred in individuals with underlying diseases other than AIDS; however, no notable differences in HHV6 were seen between cases with or without co-existing HIV-1 infection by either ISPCR or ICC. Further, no white matter staining for HHV6 antigens was seen in either control brains or in brains with HIV-1 encephalopathy but without PML. This is consistent with the observations of Saito *et al* (1995) who examined post-mortem brains from children dying with AIDS encephalopathy and found HHV6 nucleic acids in 4/5 patients by *in-situ* hybridization, primarily in white matter oligodendrocytes, but no evidence for HHV6 gene expression by immunocytochemistry. This is also analogous to the results of a previous study (Kuchelmeister *et al*, 1993) in which the presence or absence of HIV-1 had little effect on the neuropathology of PML lesions. Since the prevalence and localization of HHV6 in cases of non-AIDS associated PML were indistinguishable from those seen in AIDS-associated PML, we conclude that HIV-1 has little effect on CNS lesions associated with HHV6.

Table 1 summarizes the frequency of HHV6 positive cells/20 × field found in PML, in brains of adult and pediatric AIDS patients without PML, and in adult and pediatric controls. Among the conditions examined in this study, PML clearly has the highest prevalence of HHV6. Table 1 also makes it clear that the highest frequencies run from bottom right to top left, suggesting that the degree of HHV6 infection increases directly with patient age and with the degree of immunocompromise. This may add perspective to studies of HHV6 in patients that have given widely variable results. In one study (Knox and Carrigan, 1995) where demyelinating lesions were found at autopsy in 4/6 unselected adults with AIDS, immunocytochemical staining with a rabbit anti-HHV6 serum and an HHV-6B specific monoclonal antibody demonstrated infected cells in areas of demyelination but not in histologically normal areas. In contrast, Achim *et al* (1994) found evidence for HHV-6 DNA by solution PCR in only 1/45 adult AIDS cases with and without encephalitis. HHV6 is commensal and neurotropic and its reactivation may produce a broad range of manifestations ranging from asymptomatic latency to extensive white matter demyelination and severe encephalitis. Acquisition of HHV-6 occurs in nearly 100% of children by the age of 3 years (Pruksananonda *et al*, 1992). HHV6 causes transient febrile convulsions among 12–15-month-old children (Hall *et al*, 1994); 29% had HHV6 only in CSF, suggesting that the CNS may provide a site of viral latency (Caserta *et al*, 1994). PCR studies of immunocompetent adults dying of unrelated causes also suggest that healthy persons may silently harbor HHV6 in the CNS (Luppi *et al*, 1995; Clark *et al*, 1996).

The significance of the strong association between the demyelinative lesions of PML and high frequencies of HHV6 genome are not yet entirely clear; while the association might be construed as a mere artifact or an epiphenomenon of CNS injury, this seems unlikely on three counts. First, early HHV6 gene expression as assayed by immunocytochemistry for the viral p41 protein was seen only in the PML cases. Second, it was confirmed primarily to the swollen JCV-inclusion bearing intralesional oligodendrocytes generally considered pathognomonic of PML; cases of HIV-1 encephalopathy without PML as well as other neurological disease controls demonstrated HHV6 genome but not protein expression. Third, high frequencies of HHV6 protein expression as well as genome were found in surgical biopsy specimens as well as autopsy tissues in both AIDS and non-AIDS related PML cases.

Demyelination in PML is widely believed to result from cytolysis of oligodendrocytes as a consequence of JCV infection. However JCV infection of human glial cells *in vitro* may produce only subtle cytopathic effects (Padgett *et al*, 1971; Major and Vacante, 1989; He *et al*, 1996). Moreover JCV, like HHV6, may occur as a commensal virus of normal human brain; one study using nested PCR demonstrated JCV in brain tissues from 28/28 patients with PML but also in 6/13 patients without either AIDS or PML (Ferrante *et al*, 1997). Several studies have noted a requirement for co-infection: infection of human fibroblasts with hCMV and JCV results in cytolysis (Heilbronn *et al*, 1993), while co-infection with HHV6 increased expression of the JCV-related papillomavirus HPV in human genital epithelial cells (DiPaolo *et al*, 1996). HHV6 itself requires co-infection with HHV7, a closely related human herpesvirus, for cytolytic activity in human mononuclear cells (Katsafanas *et al*, 1996; Levy, 1996). Such virus-virus interactions leading to enhanced neuropathological effect have also been described *in vivo*; C58 or AKR strain mice must express C-type retrovirus within neurons in order to permit subsequent infection and destruction of these cells by lactate dehydrogenase virus (Pease and Murphy, 1980). In the present study, the finding in all PML brains of HHV6 and JCV co-infection with active viral protein expression in swollen lesional oligodendrocytes, raises the possibility that this co-infection may be associated with oligodendrocyte death. The possibility that other herpes family viruses may also be present remains to be defined by our two-step ISPCR technique. In light of the presence of HHV6 and JCV co-infected oligodendroglial cells in PML, the neuropathological changes may represent a more complex interaction than has been previously appreciated.

The recently published failure (Hall *et al*, 1998) of either intravenous or intrathecal cytarabine to improve survival in PML, underscores the need for consideration of alternative therapeutic strategies. Recent case reports suggest a possible clinical benefit of cidofovir, a drug with both potent anti-herpes and anti-polyoma virus activity, in treatment of patients with PML (Blick *et al*, 1998). Several other drugs, including foscarnet and ganciclovir as well as, more recently, cidofovir, are presently in use against infections caused by the related Beta-Herpesvirus cytomegalovirus, while newer, more potent agents have been described against polyomaviruses (Andrei *et al*, 1997) and herpesviruses (Takahashi, 1998). It is hoped that our data may provide a rationale for consideration of drugs active against herpesviruses in the treatment of PML which remains, at present, a nearly uniformly fatal disease.

Materials and methods

Patient materials

Archival formalin-fixed paraffin-embedded adult PML and AIDS brain tissues were obtained mainly from surgical and post-mortem collections at the University of Rochester School of Medicine and

Dentistry; and pediatric AIDS brains were provided by Dr Leroy Sharer from his collection at the UMDNJ-New Jersey Medical School (Newark). Additionally, sections from four PML brains were obtained from University of Iowa archives, and blocks from two adult AIDS brains were obtained from the University of Edinburgh (a kind gift of Dr Jeanne Bell). Where tissue size permitted, at least ten fields were counted in the involved area and the average number of HHV6 infected cells/20× field was scored visually. Processed tissue sections were always examined by several authors, but the final evaluation and interpretation of signals was performed by a senior neuropathologist (JM Powers).

Serial sections of 4 microns in thickness, alternately processed for ISPCR or immunocytochemistry or stained with hematoxylin and eosin, were employed to facilitate morphological identification of infected cells. Both surgical and autopsy brain tissues from adults with PML and either AIDS or a variety of other underlying diseases including chronic lymphocytic leukemia, lymphoma, breast cancer, and bronchiolitis obliterans (n=12 sections from 11 patients) were studied. As controls, brains from both adults and children with AIDS encephalopathy but without histopathologic evidence of PML, were examined (n=18). Other neurological disease controls (n=11) included brains from patients with Parkinson's disease (1), Alzheimer's disease (1), amyotrophic lateral sclerosis (1), cerebrovascular disease (1), hypoxia/ischemia (3), intracerebral hemorrhage (1), multiple congenital defects (2), and glioblastoma (1). Negative controls were brain tissues from normal 12–16-week human fetuses, while the positive control utilized HHV6-infected cord blood lymphocytes. Tissue sections were fixed on slides with Vectabond (Vector Corp.) and heated at 55°C for 1 h.

Immunocytochemistry

Avidin-biotin immunocytochemistry was performed by standard techniques as previously described (Saito *et al*, 1994, 1995), using alternatively Vector ABC Elite kits with horseradish peroxidase (HRP) complexed secondary antibody and diaminobenzidine (DAB) chromogen with nickel which gives a black reaction product (Vectastain elite, Vector Corp.), or Vector ABC-alkaline phosphatase complexed secondary antibody with new fuchsin chromogen (DAKO, Carpenteria, CA, USA) which give a red reaction product compatible with DAB/Ni staining.

Monoclonal antisera against HHV6 antigens were directed against p41 (Virotech International, Rockville, MD, USA), the major DNA binding protein (Zhou *et al*, 1997) and against p101 (Chemicon International, Temecula, CA, USA), the major antigenic protein of HHV6B (Pellett *et al*, 1993). Monoclonal anti-p24 (ABI) was used to identify HIV-1 infected cells. For identification of JCV large T antigen, a rabbit polyclonal antiserum directed against the related polyomavirus SV40 (Access Biomedical, San Diego, CA, USA) was used. Antibodies against cellular antigens included GFAP (Dakopatts) (astrocytes), CD68 (Dakopatts) (activated microglia and macrophages). CNPase (Boehringer-Mannheim) (oligodendrocytes) and CD3 (Dakopatts) (pan T cell). Monoclonal antibodies against an irrelevant antigen (anti-human cytokeratin CAM 5.2; Becton-Dickinson) were used as a control.

In-situ *polymerase chain reaction*

Two-step *in-situ* polymerase chain reaction (ISPCR) was performed by modification of a method previously described for amplification and detection of HIV-1 genome in archival AIDS brain (Sharer *et al*, 1996). Multiple sets of HHV6 primers were used on each tissue section to increase signal-to-noise ratio. One set of primers (A: 5′-gtgatgtacgtggccgtctcctg+B: 5′-gatccatggtcgtctttccacg) corresponded to a 384 bp sequence of the large tegument protein (LTP) and was sometimes used together with a 151 bp nested inner set (H: 5′-cggtcaacgtgccgctatctata+I: 5′-cacgacatttataagggacc-cg) (Lindquester and Pellett, 1991). A second set of primers (J1: 5′-gtgtttccattgtactgaaaccggt+J2: 5′-taaacatcaatgcgttgcatacagt), corresponding to a non-overlapping 776 bp region of the LTP, as well as a nested inner set (MC1: 5′-ggatcgttgacgtctgtgtt+MC3: 5′-cgtcctgaccatgaatgaga) giving a 500 bp PCR product, were used in addition (Kondo *et al*, 1990, 1993). Other primer sets have been successfully used: it is not clear that nested primers provide an advantage. These primer sets have been previously tested against other herpes viruses including HHV7, without detectable amplification (Hall *et al*, 1994), and they do not give nonspecific amplification products in DNA extracted from human fetal brain, which is the only reliably HHV6-negative control tissue we have found.

Control experiments with ISPCR for both HHV6 and HIV-1 included omission of Taq polymerase, primers, and probes. In our two-step ISPCR protocol, only omission of probe resulted in no signal. In essence, ISPCR is a form of boosted ISH, so that omission of Taq or primers simply produces weaker signals in fewer cells, and the use of irrelevant primers has the same effect in our method. The background artifact (Gressens and Martin, 1994; Sallstrom *et al*, 1993) may be due to the presence of cellular RNA or DNA fragments acting as nonspecific primers; thus all cells subjected to ISPCR contain amplicons, and specificity must be conferred by the probe. For successful ISH, only 100–1000 target copies of DNA are required, thus the degree of amplification in ISPCR need not be nearly as great as in solution PCR, where nanograms of PCR product must be visualized on a gel. On the other hand, annealing and extension are highly inefficient in ISPCR, due to hindered penetration of

primers and Taq into the tissue matrix; therefore, slow thermocycling is required.

Three sets of biotinylated probes were made in standard 50 μl PCR reactions using each of the above primer sets in a separate reaction with HHV6-infected cellular DNA as a target and a deoxynucleotide labeling mix containing a 3 : 1 mixture of TTP : biotin-16-dUTP (Boehringer-Mannheim). Probes suitable for ISPCR were characterized by single bands with strongly reduced mobility on 1.2% agarose gel electrophoresis, when compared to control PCR products made with ordinary dNTPs. The concentration of each biotinylated PCR product was measured by comparison with λ/*Hind*III markers on the same gel.

For the first step of ISPCR, tissue sections baked on vectabond-treated slides were deparaffinized, rehydrated through graded EtOH, treated with proteinase K (2 μg/ml, 30 min at room temp) followed by washes in 0.1 M glycine and DEPC-treated water, and pre-incubated for 1 h at room temperature in 1 × PCR mix+1.5 mM Mg^{+2} (Boehringer-Mannheim). This was removed and replaced by 30 μl of 1 × PCR mix containing the three primer sets (at 50 mM), digoxigenin-11-dUTP labeling mix containing @200 μM deoxynucleotides [1 : 4 ratio of Dig-dUTP : TTP] and 0.5 U Taq polymerase (Boehringer-Mannheim). Slides were coverslipped and placed on the block of a Coy Slide Cycler with mineral oil overlay. The sections were denatured at 95°C for 2 min, then subjected to 25 cycles of denaturation at 94°C for 2 min, annealing at 50°C for 5 min and extension at 72°C for 10 min, with 2 min ramps between steps. Following thermocycling, the sections were post-fixed in freshly-made 4% paraformaldehyde/4 × SSC, and washed extensively in DEPC-treated water.

For the second step of ISH, tissue sections were pre-hybridized in 4 × SSC containing 50% formamide, 0.5% sarkosyl and 2 × Denhardt's solution for 60 min at room temperature. This was replaced by the same buffer containing an equimolar mixture (at 10 ng/ml) of the biotinylated probes described above, plus 10 μg/ml each of salmon sperm DNA, poly A, and yeast RNA; the slides were coverslipped and placed under mineral oil seal as before, then heated for 5 min at 95°C and incubated overnight at 42°C on the thermocycler block. The following morning the sections were extensively washed in PBS, endogenous peroxidases were quenched with 3% hydrogen peroxide in PBS for 5 min at room temperature, and again washed in PBS. Sections were then treated with Vector Elite ABC-HRP followed by DAB-nickel substrate until a dark color began to become apparent, then extensively washed in water. Coverslips were mounted in glycerol for viewing; aqueous mounting allowed for co-localization of HHV6 with a secondary target by subsequent ICC using alkaline phosphatase conjugated secondary antibodies with new fuchsin (or AEC chromogen with HRP).

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