



Detection of Herpesviridae in postmortem multiple sclerosis brain tissue and controls by polymerase chain reaction

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Objective: To test for the presence of herpesviruses in postmortem brain samples from multiple sclerosis patients and controls using polymerase chain reaction.

Background: Herpes simplex virus, varicella-zoster virus, Epstein-Barr virus, cytomegalovirus, and human herpesvirus-6 are common viruses capable of persistence and latency. All have been detected in the CNS.

Methods: Active and inactive plaque tissue, unaffected white matter (WM) and gray matter (GM) from MS cases, and WM and GM controls (Alzheimer's disease, Parkinson's disease and non-neurological disease) were screened for the herpesvirus by PCR.

Results: (1) 37% of the MS cases were positive for herpes simplex virus (HSV). Twenty-eight percent of controls cases were positive for HSV. Forty-one percent of active plaques were positive for HSV in contrast to only 20% of inactive plaques (Sanders *et al.*, 1996). (2) 57% of the MS cases and 43% of the control cases were positive for HHV-6. Thirty-two percent of the active plaques contained HHV-6 compared to 17% of inactive plaques. (3) 43% of the MS cases and 32% of the control cases were positive for VZV. Fourteen percent of the active plaques and 10% of the inactive plaques were positive for VZV. (4) 27% of MS cases and 38% of control cases were positive for EBV. Five percent of the active plaques were positive for EBV and 10% of the inactive plaques were positive. (5) 16% of the MS cases and 22% of the controls were positive for CMV. Nine percent of the active plaques and 10% of the inactive plaques were positive. We also compared MS WM and GM with controls and found no significant difference.

Conclusions: HSV, HHV-6, and VZV were present in a greater frequency of MS cases compared to controls; however, no statistical differences were noted. The presence of herpesvirus in all tissue makes an etiologic association to MS uncertain. Cellular localization of virus and its relationship to pathology and latency may reveal an association.

Keywords: human herpesvirus 6; Epstein-Barr virus; varicella-zoster virus; cytomegalovirus; demyelination

Introduction

Multiple sclerosis (MS) is a disease of unknown etiology characterized by multifocal demyelination and polyphasic inflammation. It is hypothesized that MS is caused by a persistent virus in the central nervous system (CNS) inducing demyelination by direct infection of oligodendrocytes or indirectly by cytokine production. In order to determine an association between virus and MS, it is necessary to perform a systematic screening of different tissue types from MS and non-MS patients utilizing the most specific and sensitive techniques

available, namely polymerase chain reaction/Southern blot detection (PCR). There are seven human herpesviruses: herpes simplex 1 (HSV1), herpes simplex 2 (HSV2), cytomegalovirus (CMV), Epstein-Barr virus (EBV), Varicella-Zoster virus (VZV), human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7). They share biologic properties that are amenable to causing primary demyelination as seen in MS. The viruses are capable of establishing latency within the host and may reactivate under various conditions. Persistence lasts the lifetime of the host. Herpesviruses are endemic to the human population. Most infect the host at an early age. All viruses have been associated with neurological disease (with the exception of HHV-7).

HSV

Herpes simplex virus is one of the most common human pathogens; 70–80% of the population have antibodies to HSV1 (Whitley, 1990). Latent virus may be located in sensory ganglia and the CNS (Stevens, 1994). HSV can induce multifocal demyelination in mice characterized by preservation of axons and an inflammatory infiltrate (Kristensson *et al*, 1979; Hill, 1983). HSV has been isolated from the CSF during the 'first attack' of a MS patient (Bergstrom *et al*, 1989) and found in the CNS of an MS patient (Gudnadottir *et al*, 1964). Immunoreactivity against HSV-2 was seen in three of 31 MS postmortem brains. Reactivity was restricted to glial cell nuclei in or near the lesions (Martin *et al*, 1988). Our study has determined that 46% of the MS cases and 28% of the control cases had samples positive for HSV. Forty-one percent of active MS plaques were positive for HSV in contrast to 20% of inactive plaques, 24% of the MS WM, 14% of the MS GM. Twenty-eight percent of control WM and 7% of control GM were positive for HSV (Sanders *et al*, 1996). Although these frequencies were not statistically different, the presence of virus in a higher percentage of active plaques compared to other tissue types warrants further investigation.

HHV-6

HHV-6 is a common virus with more than 50% of the population being seropositive (Levy *et al*, 1990; Levine *et al*, 1992). Virus persists in a latent form in the salivary glands and peripheral blood monocytes in normal adults (Jarrett, 1992). Exanthem subitum (roseola infantum) is the only disease known to be definitely caused by HHV-6. The virus has been detected in brain tissue by PCR in 11 out of 13 cases (85%) of normal adults (Luppi *et al*, 1994). HHV-6 has been associated with areas of demyelination in AIDS and bone marrow transplant patients at autopsy. Infected cells were only seen in areas of demyelination and appeared to be astrocytes and microglial cells. There was relative sparing of axons, absence of inflammation, and astrogliosis in adjacent white matter areas (Drobyski *et al*, 1994; Knox and Carrigan, 1995). HHV-6 has been detected by PCR in the acellular fraction of CSF in four of 36 MS patients, and two of 27 neuro-AIDS patients compared to none of the control patients (Liedtke *et al*, 1995). HHV-6 has been detected in 78% of MS and 74% of control brain samples by PCR. This study also found HHV-6 in the oligodendrocytes of MS patients but not controls and found a higher concentration of infected cells in areas of demyelination compared to unaffected white matter (Challoner *et al*, 1995).

VZV

Seroprevalence for VZV is 100% in temperate climates (Preblud *et al*, 1984). Latent virus has been detected in 78% of trigeminal ganglia examined and

53% of thoracic ganglia (Mahalingam *et al*, 1990; Liedtke *et al*, 1993). Latent virus can reactivate (in the absence of immunosuppression) and infect meningeal cells without producing zosteriform lesions (Echevarria *et al*, 1994). Multifocal plaques of active demyelination without inflammation have been noted in VZV leukoencephalitis (Lentz *et al*, 1993; Amlie-Lafond *et al*, 1995). There are similarities in the epidemiology of MS and VZV infection. Varicella is rare in tropical climates and there is a high susceptibility of individuals moving to more temperate climates (Venkitaraman *et al*, 1984; Kiersem and Jepsen, 1990). There is a north-south gradient for varicella in the United States, similar to that seen for MS. These results taken together present a loose correlation between the incidence of varicella and MS (Ross and Cheang, 1995).

EBV

Seroprevalence for EBV is between 50% and 100%, depending on urban development (Liebowitz and Kieff, 1993). EBV persists in B-lymphocytes in a latent form (Miyashita *et al*, 1995). EBV has been isolated from the CNS from patients with meningoencephalitis (Halsted and Chang, 1979; Trovato *et al*, 1994) and has been detected by PCR from the CSF or CNS of patients with other neurological complications (Pedneault *et al*, 1992; Landgren *et al*, 1994). Higher than normal frequencies and concentrations of EBV antibodies have been detected in MS patients (Haahr *et al*, 1992). In same-gender twins, serum antibody levels to measles, mumps and Epstein-Barr viruses were more elevated in the patients with MS, compared to their healthy co-twins (Kinnunen *et al*, 1990). EBV DNA has been detected by PCR in two patients with childhood MS (Pedneault *et al*, 1992). Age of EBV infection/mononucleosis symptoms has been linked to a greater risk for MS (Lindberg *et al*, 1991; Martyn *et al*, 1993). Four of five patients who had neurologically complicated primary EBV infection and developed progressive or relapsing neurologic deficits, were later diagnosed with MS (Bray *et al*, 1992).

CMV

Frequency of seropositivity for CMV is between 40 and 100% of the population depending on geographical location and urban development (Ho, 1991). The virus may cross the blood brain barrier as viremia or via infected lymphocytes. Pathological features of CMV infection in the CNS include microglial nodules, vasculitis, foci of demyelination, infarcts and cytomegalic ventricular ependymal cells. All cell types may be infected with CMV (Wiley *et al*, 1986; Morgello *et al*, 1988). CMV antigens may be present in cells without any cytopathological effect (Wiley and Nelson, 1988). These diseases have been reported for patients with apparently normal immune systems although they

are noted more frequently for immunosuppressed patients (Gilden, 1993).

This study was designed to determine the role of the human herpesviruses in the etiology of MS. The working hypotheses are as follows: (1) MS is a complex disease, due to more than one etiology (autoimmune, viral, molecular mimicry). (2) A subset of cases is due to persistent viral infection. (3) Virus is located in areas of active demyelination and may be located at lower frequencies or in a latent form in inactive plaques, normal-appearing white matter and normal-appearing gray matter. This is the first systemic examination of different tissue types from different diseases to elucidate an association of virus with primary demyelination using PCR/Southern blot.

Results

All viruses were found in all disease subgroups (Figure 1) and in all tissue types (Figure 2). Herpes simplex virus was included in our screening of the post-mortem MS tissue. The data for HSV has been published [Sanders, 1996 #1541]; therefore, a brief summary of background and results has been included in the introduction section.

HHV-6

MS Incidence of positive cases within the entire MS group was 57% (21/37). Twenty-one percent (26/109) of the MS samples were positive for HHV-6. Thirty-two percent (7/22) of active plaques were positive for the virus, 17% (5/29) of inactive plaques were positive while 25% (9/37) of normal appearing WM and GM were positive. There was no significant difference among these frequencies ($p=0.67$).

Controls For the control tissue, 43% (16/37) of the cases gave positive signals. Thirty-eight percent (5/13) of the cases without neurological disease (NND) and 50% (6/12) of Alzheimer's disease (AD) cases had positive tissue samples. Forty-two percent (5/12) of the Parkinson's disease (PD) cases were positive for HHV-6. There was no significant difference among control subgroups ($p=0.84$).

Twenty percent (29/147) of control samples were positive. Seventeen percent (7/47) of the AD tissue samples were positive for HHV-6, 11% (6/52) and 12% (6/49) of the NND and PD samples were positive. Thirteen percent of the WM samples and 13% of the GM samples were positive. There was no significant difference between the frequency of positive samples within these subsets ($p=0.88$).

MS vs non-MS controls Fifty-seven percent of the MS patients samples had samples positive for HHV-6; 43% of the entire non-MS controls were positive for HHV-6. There was no significant difference

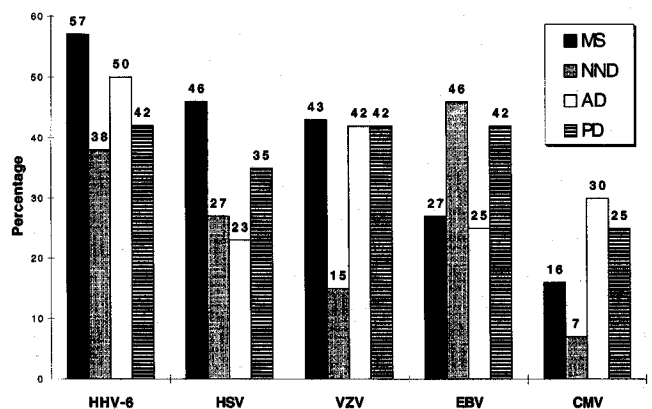


Figure 1 Percentage of patients from different disease subgroups positive for different herpesviruses. MS=Multiple sclerosis; NND=Non-neurologically diseased controls; AD=Alzheimer's disease controls; PD=Parkinson's disease control. HHV-6=Human Herpesvirus 6; HSV=Herpes simplex virus; VZV=Varicella-zoster virus; EBV=Epstein-Barr virus; CMV=Cytomegalovirus. Note: Herpes simplex virus data (Sanders *et al*, 1996) included for comparison.

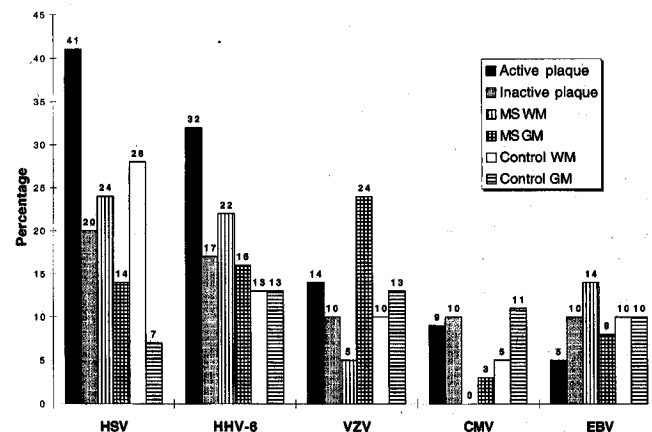


Figure 2 Frequency of different tissue types positive for different herpesviruses. HSV=Herpes simplex virus; HHV-6=Human Herpesvirus 6; VZV=Varicella-zoster virus; CMV=Cytomegalovirus; EBV=Epstein-Barr virus. Note: Herpes simplex virus data (Sanders *et al*, 1996) included for comparison.

between these groups ($p=0.24$). Virus was found in 25% and 16% of the MS WM and GM samples, respectively, and was found in 13% and 13% of the non-MS WM and GM respectively. There was no significant difference among these frequencies ($p=0.40$).

VZV

MS The MS group contained 43% (16/37) of cases positive for VZV. Fourteen percent (18/125) of the MS samples contained viral DNA. Fourteen percent (3/22) of the active plaques were positive for VZV, 10% (3/29) of the inactive plaques were positive

and 5% (2/37) and 24% (9/37) of normal appearing WM and GM were positive. There was no significant difference among these frequencies ($p=0.014$).

Controls Thirty-two percent (12/37) of the control cases gave positive signals. Fifteen percent (2/13) of NND cases had positive tissue samples. Forty-two percent (5/12) of the AD cases had samples positive for VZV. Forty-two percent (5/12) of the PD cases were positive for VZV. There were no significant differences among control subgroups ($p=0.27$).

Eleven percent of the non-MS tissue samples were positive for VZV (16/147). Fifteen percent of the AD samples contained viral DNA (7/47). Eight percent of the NND samples were positive (4/52), and 10% of the PD samples were positive (5/48). Statistically significant difference was not reached for the frequency of positive samples within these groups ($p=0.51$). Ten percent (11/109) of the WM samples were positive for the virus; 13% (5/38) of the GM samples had virus present.

Table 1 MS patient information, listed by increasing duration of disease

HSB#	Age/ Gender	Duration (yrs)	Cause of death	Autolysis (h)
735	39/M	4	Pneumonia	6
894	38/M	5	Pneumonia	60
944	33/F	6	Septicemia	22
644	37/F	8	Cardiac arrest	11
793	58/F	8	Pneumonia	5
802	48/F	8	Pneumonia	10
573	50/F	9	Overdose/suicide	15
678	40/M	10	Cardiac arrest	5
536	31/M	11	Cardiac arrest	25
1864	37/F	11	Anoxia	12
1035	66/M	12	Pneumonia	10
511	40/M	13	Respiratory failure	12
1238	36/F	13	G.I. bleeding	19
1134	51/M	14	Septicemia	35
1862	69/M	14	Septicemia	9
2190	70/M	14	Respiratory failure	9
1011	44/M	16	Pneumonia	4
1337	59/F	16	MS	14
1291	44/F	19	Cardiac arrest	7
996	41/F	20	Fibrosarcoma	14
888	50/M	21	Pneumonia	8
1145	42/M	22	Anoxia	31
344	42/M	23	Pneumonia	6
845	45/M	23	Drowning	28
1588	46/F	23	Respiratory failure	24
1061	50/F	24	MS	12
934	50/F	25	Pneumonia	11
1174	65/M	27	Septicemia	12
640	51/M	28	Pneumonia	23
1446	51/F	29	Pneumonia	4.5
1270	67/F	30	Septicemia	23
1668	59/F	32	Septicemia	9
909	70/F	40	Undetermined	31
1088	64/F	40	Renal failure	11
579	61/M	41	Pneumonia	12
338	65/M	44	Pneumonia	6
1779	69/F	47	Lung cancer	6

MS vs non-MS controls Forty-three percent of the cases with MS had samples positive for VZV; 32% of the entire non-MS controls were positive for VZV. There was no significant difference between these groups ($p=0.34$). Virus was found in 24% and 5% of the MS WM and GM samples, respectively, and was found in 10% and 13% of the non-MS WM and GM samples. There was no statistically significant difference between these groups ($p=0.06$).

EBV

Multiple sclerosis Twenty-seven percent (10/37) of the MS patients had samples positive for EBV. Ten percent (12/125) of the MS tissue samples were positive for EBV. Five percent (1/22) of the active plaques were positive for EBV. Ten percent (3/29) of the inactive plaques were positive for EBV. Fourteen percent (5/37) and 8% (3/37) of normal appearing WM and GM were positive, respectively. There was no significant difference among these frequencies ($p=0.67$).

Controls For the control cases, 38% (14/37) gave positive signals. Forty-six percent (6/13) of NND cases, 25% (3/12) of the AD cases and 42% (5/12) of the PD cases were positive for EBV. There was no difference among the frequency of positivity for the control cases ($p=0.52$).

Ten percent of all of the control samples gave a positive signal for EBV (15/147). Six percent (3/48) of the AD samples were positive for CMV. Twelve percent (6/51) of the NND samples were positive, and 13% (6/47) of PD samples were positive. There were no significant differences among control subgroups ($p=0.54$). On the average, 10% (11/109) of WM samples were positive; 10% (4/38) of GM samples were positive.

MS vs non-MS controls The MS group showed 27% of the cases had samples positive for EBV while 38% of the non-MS controls were positive for EBV. There was no significant difference between these groups ($p=0.32$). Virus was found in 14% and 8% of the MS WM and GM samples and was found in 10% and 10% of the non-MS WM and GM samples, respectively. There was no significant difference among these groups either ($p=0.90$).

CMV

Multiple sclerosis Sixteen percent (6/37) of the MS cases had at least one sample positive for CMV. Six out of 125 (5%) MS samples were positive for CMV. Nine percent (2/22) of the active plaques were positive for CMV; 10% (3/29) of the inactive plaques were positive for CMV. None of normal appearing WM and 3% (1/37) of the GM samples were positive. There was no significant difference among these frequencies ($p=0.16$).

Controls For the control tissue, 22% (8/37) gave positive signals. Eight percent (1/13) of the cases without neurological disease, 33% (4/12) of AD cases and 25% (3/12) of the PD cases were positive for CMV. No significant difference was found among these subsets ($p=0.28$).

Seven percent (11/162) of the control samples were positive for CMV. Two percent (1/56) of the NND samples were positive, 9% (5/55) of the AD samples were positive for CMV, and 10% (5/51) of PD samples were positive. On the average, 5% (6/118) of the WM samples were positive; 11% (5/44) of the GM samples were positive.

MS vs non-MS controls Sixteen percent of the MS cases had samples positive for CMV; 22% of the

entire non-MS controls were positive for CMV. There was no significant difference between these groups ($p=0.35$). Virus was found in none of the MS WM samples and found in 3% of the MS GM samples. CMV was found in 5% and 11% of the non-MS WM and GM samples. There was no significant difference among these groups ($p=0.07$).

Discussion

The epidemiology of MS, immune abnormalities seen in MS patients, and the existence of viral diseases that result in demyelination support a viral etiology of MS. Despite all the research, no virus has been clearly and consistently associated with

Table 2 Control patient information, sorted by diagnosis

<i>Non-neurologically diseased patients:</i>				
<i>HSB#</i>	<i>Age/Gender</i>	<i>Duration (yrs)</i>	<i>Cause of death</i>	<i>Autolysis (h)</i>
648	52/F	n/a ¹	accidental O.D.	22
1645	70/F	n/a	cardiorespiratory failure	8
1732	72/M	n/a	cardiorespiratory failure	19
1736	76/M	n/a	cardiorespiratory failure	17
1768	71/M	n/a	pulmonary embolism	25
1775	69/M	n/a	cardiorespiratory failure	17
1818	59/M	n/a	cardiorespiratory failure	15
1846	73/M	n/a	pneumonia	26
1903	70/M	n/a	cardiorespiratory failure	13
1933	48/M	n/a	cardiorespiratory failure	15
2098	undetermined	n/a	undetermined	undetermined
2169	undetermined	n/a	undetermined	undetermined
2181	68/M	n/a	lung cancer	7
<i>13 NND Cases</i>				
<i>Alzheimer's Disease Patients</i>				
1702	76/F	15	cardiorespiratory failure	5
1723	77/F	undetermined	undetermined	12
1733	80/M	6	cardiorespiratory failure	19
1739	84/M	6	pneumonia	8
1801	75/F	4	pneumonia	19
1979	83/M	6	pneumonia	13
2162	75/M	12	cerebral infarct	5
2173	79/F	3	pulmonary embolism	5
2175	83/M	undetermined	undetermined	7
2182	92/F	8	cardiorespiratory failure	10
2186	83/F	8	undetermined	9
2188	80/M	3	cardiorespiratory failure	20
<i>12 AD Cases</i>				
<i>Parkinson's Disease Patients</i>				
1315	74/M	13	cardiorespiratory failure	10
1567	76/M	undetermined	undetermined	19
1593	79/M	17	cardiorespiratory failure	5
1613	85/M	23	cerebral infarct	7
1746	78/M	12	undetermined	5
1747	75/M	undetermined	renal failure	5
1767	72/M	27	cardiorespiratory failure	5
1810	65/M	19	multiple myeloma	7
1889	76/M	18	adenocarcinoma	15
2163	83/F	11	cardiorespiratory failure	9
2174	61/M	16	pulmonary embolus	12
2177	80/M	23	lung cancer	13

¹ n/a= not applicable

the disease. This could be due to the lack of sensitivity of the techniques used or the lack of systemic screening, on a case by case basis, of MS tissue and controls for all the possible candidate viruses.

HHV-6

Fifty-seven percent of the MS cases had at least one sample positive for HHV-6. Active plaques had the highest percentage of positive samples (32%) compared to 17% of the inactive plaques; 25% of the normal appearing WM, and 16% of the GM, respectively. The higher prevalence of virus present in active plaques may be due to the lymphotropic nature of HHV-6. All active plaques contain B cells, T cells, and macrophages. However, since not all active plaques were positive, this would suggest that these cells are not the source of positive signal. Since statistical significance was not reached, a clear association between HHV-6 and primary demyelination as seen in MS active plaques was not established.

Case by case analysis revealed that 43% of the non-MS cases had at least one sample positive for HHV-6. The AD cases had the highest prevalence rate (50%) compared to PD cases (42%) and NND cases (38%). Considering the seroprevalence rates and the ability of the virus to cross the blood brain barrier, prevalence rates approaching 50% are reasonable. The slightly higher prevalence could be due to the increased average age of the AD group and the age-compromised blood brain barrier (Garton *et al*, 1991). There was an equal amount (13%) of white and gray matter samples positive for the virus. Therefore, HHV-6 does not seem to have a predilection for a single tissue or cell type.

Despite the number of neurological diseases associated with HHV-6 infection, few studies have been published determining the prevalence of the virus in the CNS of normal and neurologically diseased brains. Luppi *et al* found HHV-6 in 85% (11/13) of postmortem brains from cases without neurological disease (Luppi *et al*, 1994). Challoner *et al* found viral DNA in 78% of their MS group and

74% of their control group. In addition, immunocytochemical staining localized virus to oligodendrocytes in areas of demyelination (Challoner *et al*, 1995). The differences in these rates compared to ours may be due to their use of nested primers which are used to increase sensitivity. However, since our sensitivity rate was never less than 10 copies/ μ l and often 1 copy/ μ l, it is unlikely that our lower rates were due to differences in sensitivity. Sample differences or the technical difficulties in performing nested PCR without contamination could explain the differences. Since HHV-6 is known to trans-activate HIV and augment damage, co-infection of HHV-6 and another, as yet, uncharacterized virus could lead to CNS pathology by a similar mechanism. It is believed that further investigation of cellular location and latency state of HHV-6 in our cases is warranted.

VZV

VZV was found in 43% of the cases with MS. Fourteen percent of active plaques and 10% of the inactive plaques were positive for VZV. Twenty-four percent of the MS GM samples were positive while only 5% of the MS WM samples were positive. The virus detected in active plaques may be in a replicating state whereas that in other tissue types could be in a latent state. Another possibility is that low levels of active VZV in GM may not lead to cytopathological damage but rather lead to down regulation of 'luxury' proteins involved in myelin compaction at the axolemma/sheath junction. This would augment the demyelination by allowing macrophages to respond to abnormal myelin. The greater frequency of VZV in the GM could reflect latent virus in the GM neurons. Activation may lead to transmission of virus to oligodendrocytes and/or astrocytes leading to cytological damage in the form of vasculopathy or demyelination. Virus could also be present in the endothelial cells of the small blood vessels found in a greater amount in the gray matter.

There was a greater percentage of AD and PD patients with virus present compared to NND cases. This may be due to increased viremia due to age or

Table 3 Primer sequences used in PCR amplification to screen for presence of herpesviruses

Virus	Sequence	Product length	Sequence obtained from:
HSV	5'-TTg AAg Cgg TCg gCg gCg TA -3'	148	Dr Fred Lakeman (Univ. Alabama, Birmingham)
HSV	5'-gCA TCg TCg Agg Agg Tgg A -3'		
CMV	5'-Cag CAC CAT CCT CCT CTT CCT CTgg -3'	435	Dr Harry Vinters (UCLA)
CMV	5'-CCA AgC ggC CTC TgA TAA CCA AgC C -3'		
EBV	5'-TgT CTg ACg Agg ggC CAg gTA CAg gAC -3'	245	Dr Harry Vinters
EBV	5'-gCA gCC AAT gCA ACT Tgg ACg TTT TTg -3'		
VZV	5'-ATg TCC gTA CAA CAT CAA CT -3'	267	Oligo 4.0 software
VZV	5'-CgA TTT TCC AAa AgA gAC gC -3'		
HHV-6	5'-gAT CCA Tgg TCg TCT TTC CAC g -3'	384	Dr Harry Vinters
HHV-6	5'-gTg ATg TAC gTg gCC gTC TCC Tg -3'		

disease-related blood brain barrier breakdown. The frequency of VZV positive trigeminal ganglia has been shown to increase with age (Liedtke *et al*, 1993). Fifty percent of individuals over the age of 80 develop herpes zoster (Gilden and Vafai, 1989). Age or disease-related immunosuppression may allow for virus to reactivate and enter the CNS. However there was no statistically significant difference among the groups. For all the non-MS cases, there was only a slight difference in the frequency of positive WM samples and GM samples (10% and 13%, respectively).

There has been no systematic study examining different tissue types from different neurological diseases. Only one study has examined postmortem tissue for presence of VZV by PCR. This study found no VZV viral DNA in temporal cortex of 24 schizophrenic, suicide and normal patients (Alexander *et al*, 1992). The differences between their results and ours could be explained by their small sample number or by methodological differences. Sensitivity level was described for the HSV primer pair used in the study but not for the VZV primer pair. Our study did not show a clear association between VZV and MS; however, its role in primary demyelination should be further examined due to the demyelination seen in VZV encephalitis cases and virus present in a subset of our MS patients.

EBV

Twenty-seven percent of cases with MS were positive for EBV. There was higher frequency of virus found in inactive plaques (10%) and WM (14%) than active plaques (5%) or GM (8%). Since EBV has not been localized to any cell in the CNS, it is difficult to comment on the presence of virus in inactive plaque tissue, WM or GM. The low level of infection within the total MS sample and the lack of significant difference between tissue types suggest that EBV does not play a role in active demyelination.

There was a slightly higher percentage of positive cases within the NND (46%) and PD (42%) groups compared to the AD group (25%). Since EBV is likely to enter the brain as viremia, it would be more likely to be found at a higher frequency in the older patients (AD and PD). Most of the patients had only one sample positive (usually WM). This could be due to the as yet unknown location of the virus. However, the total percentage of non-MS patients (38%) with EBV present in CNS samples is not surprising considering the seroprevalence of adults (>95%).

This study is the first to determine the presence of EBV in the CNS of MS, PD, AD, and NND patients by PCR. No EBV RNA was detected in MS plaques by *in situ* hybridization (Hilton *et al*, 1994). One study has used PCR to detect EBV in CNS samples. Pedneault *et al* (1992) screened biopsy samples from 24 patients of various diagnoses suggestive of

EBV infection. Viral DNA was found in two of two childhood MS cases. Perhaps this unusual clinical presentation represents a subset of MS patients with primary demyelination being due to EBV infection. Also of interest was EBV DNA detected in two of two progressive multifocal leukoencephalopathy (PML) cases. Co-infection of a chronic lymphocytic leukemia patient with JC virus and EBV has been reported. In this case, virus was located in the leukemia B cells disseminated in areas of demyelination (Farge *et al*, 1994). It is intriguing that EBV has been found in a demyelinating disease known to be due to another virus. Perhaps co-infection increases the degree of myelin damage.

Although no association was found for MS, the baseline frequency of EBV for all disease groups is important for future studies in the role of EBV infection in CNS disease and certainly leads to the next question: the cellular location of EBV within the brain.

CMV

CMV was detected in 16% of the cases with MS. A slightly higher frequency of virus was found in active plaque tissue (9%) and in inactive plaque tissue (10%) compared to the unaffected WM (0%) and GM (3%); however, there was no significant difference among these subsets. The higher frequency of virus present in plaque tissue could be due to the compromised blood-brain barrier found in areas of MS lesions. It could also be brought in via T or B cells found in the plaque regions. Molecular mimicry could be a mechanism of demyelination; antibodies to glycosphingolipids of peripheral nerve myelin have been detected in humans with CMV infection (Ogawa-Goto *et al*, 1994). There could be a similar response to CNS myelin proteins and this may initiate demyelination or exacerbate the direct destruction of CMV infection. However, until specific cellular localization is performed, one must be conservative designating direct causation to the virus.

Twenty-two percent of the non-MS cases were positive for CMV. The low level of positivity for all samples is expected considering the seroprevalence of CMV. A slightly lower prevalence was seen for the non-neurologically diseased group (8%). This difference may suggest some role of the virus in the neurodegenerative diseases but, more likely, is a result of altered blood-brain barrier function due to age or disease in the AD and PD populations. The older patients would have greater generalized blood-brain barrier breakdown and thus a greater chance of CMV entering the CNS. There was no significant difference in the presence of the virus in WM or GM. Since virus has been detected in all cell types within the brain, this is not unexpected.

Few studies have screened CNS tissue for the presence of CMV. One study did not detect CMV by PCR/Southern blot detection in any of eight

schizophrenic, suicide and normal control samples (Alexander *et al*, 1992). The amplification was only done for 35 cycles and detection sensitivity was not mentioned in the text. Thus, their negative results could be due to virus present at low levels below their sensitivity cut-off. Another study did find CMV by *in situ* hybridization in seven of ten Rasmussen's encephalitis cases and two of 46 controls (Power *et al*, 1990). Their results indicate that CMV may be etiologically involved in RE and that a subset of other disorders may also harbor the virus. Examination by PCR, a much more sensitive technique, may have revealed virus in their other control samples.

Summary and future

The possible association of Herpesviridae with disease is difficult because of the high prevalence of these viruses within the human population and their ability to establish latency. Detection of the virus by a sensitive methodology is the first step in this study. However, presence of virus does not necessarily indicate causative role in a disease process. If the virus is in a latent state, even at high concentrations, it is incapable of producing infectious virion and causing damage. While no clear association was found between presence of any of the herpesviruses and MS tissue, the presence of HSV and HHV-6 in active plaques at a higher frequency rate than other tissue types warrants further investigation. In addition, a baseline frequency of virus within various neurologically diseased and non-neurologically diseased patient subgroups has been established.

The results raise many interesting questions as to the nature of the viruses within the brain: where is the virus located, its route of entry, its latency state and its association with brain pathology? Localization experiments must be done to determine which cells are infected and route of entry. Appropriate primers (LAT, ICP0) may be used to determine latency state. Finally, direct demonstration that virus causes primary demyelination in a susceptible animal due to infection of oligodendrocytes at the edge of an active plaque would permit a detailed study of pathogenesis. On the other hand, fulfilling this important Koch's postulate may never be possible given the species specificity of viruses. Hence, a successful clinical trial of an appropriate antiviral drug in MS would provide further evidence for a pathogenic role of virus in this disease (Lycke *et al*, 1996).

Materials and methods

Subjects/tissue samples

One hundred and twenty-five samples from 37 patients with MS were examined. Disease duration ranged from 4 to 47 years. The average age at death was 51 years old (Table 1). The control group varied

slightly between viruses. One hundred and forty-seven samples (for CMV, 161 samples) from 37 cases comprising of 12 Alzheimer's Disease (AD), 12 Parkinson's Disease (PD) and 13 cases with no neurological disease (NND) were used as controls. The average age of the AD cases was 80 years of age. The average age of NND cases was 66 years old, and the average age at death of the PD cases was 75 years old (Table 2).

Each tissue sample was characterized histologically and immunocytochemically for presence of myelin debris and immunological activation (Sanders *et al*, 1993). There were 22 active plaques; these were defined as plaque areas containing immunologically activated microglia and macrophages as determined by expression of the HLA-DR II molecule and containing macrophages filled with myelin debris (neutral lipids). There were 29 inactive plaques; these were plaque areas characterized by lack of active immunological expression or demyelination. Thirty-seven normal appearing white matter (WM) and gray matter (GM) samples were each used as internal control samples. Eight cases comprised of an active plaque, WM, and GM; 15 were an inactive plaque, WM, and GM. Fourteen cases were made up of both plaque types as well as WM and GM. Control tissue consisted of periventricular WM and cortical GM.

Positive controls to verify extraction and amplification efficiency were: DNA from EBV-infected lymphoblastoid cell line, CMV-infected fibroblast cell lines, VZV-infected fibroblast cell line (all cell lines from ATCC, Rockville, Md.), and an MS case (HSB# 1206) determined to have a strong signal for HHV-6 (G Burner, Pathogenesis Corp., Seattle, WA, personal communication).

Tissue preparation and PCR amplification was performed as described previously (Sanders *et al*, 1996). Optimal annealing temperature for each primer was determined empirically using the Stratagene Gradient Robocycler™ (Table 3). The following procedures were carried out to prevent any possible contamination leading to false positive results common to PCR: cryosectioning, nucleic acid extraction, reagent preparation and amplification and detection procedures were located in three separate buildings. All laboratories were irradiated daily by mercury UV lamps. Lab technicians were designated separate duties. All pipette tips, tubes, and gels used for analyzing PCR products were discarded in a waste receptacle containing a 0.1 M HCl solution; similarly, all non-porous surfaces within the labs were wiped down with dilute HCl to minimize carry-over into the other rooms. To identify false positive results, 25% reagent blank controls were included in each PCR thermocycling batch. Each specimen was run in triplicate; specimens exhibiting only one positive was suspected of being a false-positive and was re-run.

Statistics

Chi-square (with continuity corrected as needed) was used to compare frequencies of positive signals among groups with a cut off p value of 0.05 (Siegal, 1956).

There was no cross-reactivity between virus-specific primers and other herpesviruses. Purified DNA from each of the herpes viruses (Advanced Biotechnologies, Columbia, MD) was used as template for PCR amplification and Southern blot detection with each virus-specific primer set and probe. Only the appropriate virus gave a positive signal.

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