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Short Communication

Negative association of Epstein-Barr virus or herpes simplex virus-1 with tumefactive central nervous system inflammatory demyelinating disease

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Central nervous system (CNS) demyelination has been suggested to be associated with infections caused by the Epstein-Barr virus (EBV) or herpes simplex virus (HSV)-1. CNS inflammatory demyelinating disease (IDD) rarely presents as a large lesion. We evaluated samples of serum and cerebrospinal fluid (CSF) by enzyme-linked immunosorbent assay to detect recent infection with these viruses and analyzed CSF and brain specimens by polymerase chain reaction (PCR) or immunohistochemical studies for evidence of these viruses in three patients with biopsy-proven CNS IDD. The results of PCR tests for EBV and HSV in CSF or brain specimens were negative. Elevated anti-EBV or -HSV antibody levels were not found in serum or CSF in any patient. Immunohistochemical studies showed that IDD lesions were negative for latent membrane protein (LMP)-1, Epstein-Barr nuclear antigen (EBNA)-2, and EBNA noncoding RNA (EBER)-1. These results suggest a negative association between CNS IDD and EBV or HSV. *Journal of NeuroVirology* (2010) **16,** 466–471.

Keywords: Epstein-Barr virus; herpes simplex virus; inflammatory demyelinating disease; multiple sclerosis; viral infection

Introduction

Central nervous system (CNS) inflammatory demyelinating disease (IDD) rarely presents as a large lesion, referred to as a tumefactive demyelinating lesion (Dagher and Smirniotopoulos, 1996). Such lesions are often misdiagnosed as neoplastic lesions. Although the etiology of CNS IDD remains unclear, most patients respond to corticosteroid therapy (Kepes, 1993). CNS demyelination has been suggested to be associated with viral infection (Sonneville *et al*, 2009; Menge *et al*, 2005). Epstein-Barr virus (EBV)-infected memory B cells are altered in patients with autoimmune diseases (Ascherio *et al*, 2001), and a pathogenetic association between multiple sclerosis and EBV-infected lymphocytes has been reported (Franciotta *et al*, 2008; Serafini *et al*, 2007). In one study, herpes simplex virus (HSV) or related antigens were associated with CNS demyelination on pathological examination in 31 patients with multiple sclerosis (Martin *et al*, 1988), but the association between HSV-1 and CNS demyelination has not been studied subsequently. Whether viral infection is associated with CNS IDD remains an open question. We therefore evaluated samples of serum, cerebrospinal fluid (CSF), and brain specimens for evidence of recent infections with these viruses in patients with biopsy-proven CNS IDD.

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Results

Pathological findings of CNS IDD

In all three patients, biopsy demonstrated characteristic features of active inflammatory demyelinating disease accompanied by hypercellular lesions with myelin loss (Figure 1). Cell infiltration of the brain parenchyma, perivascular lesions, and reactive gliosis were evident. Luxol-fast blue (LFB) staining showed myelin loss. The inflammatory demyelination was not restricted perivenular lesions, which

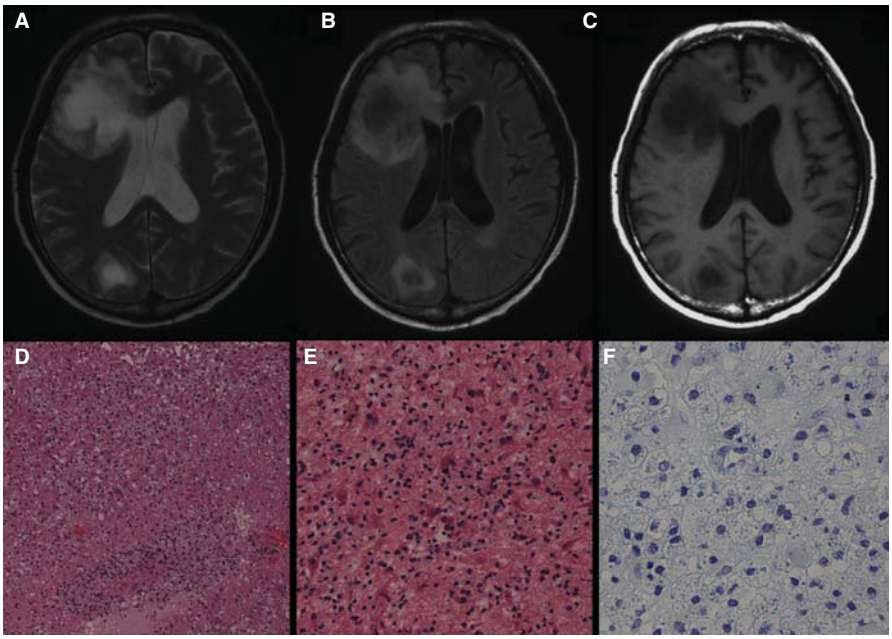

Figure 1 Neuroimaging and pathological findings of a central nervous system inflammatory demyelinating lesion in patient 1. The central nervous system inflammatory demyelinating lesion showed high signal intensity on T2-weighted (**A**) and fluid-attenuated inversion recovery (**B**) images and low signal intensity on T1-weighted images (**C**). Cell infiltrations in the brain parenchyma and perivascular lesions with reactive gliosis were evident (**D** and **E**; hematoxylin-eosin staining). Macrophages and lymphocytes were evident in the demyelinating lesions (**B**; hematoxylin-eosin staining). Luxol-fast blue (LFB) staining (**F**) showed myelin loss with lipid-laden macrophages. They contain LFB stain–positive myelin debris within their cytoplasm. Materials employed in this study (i.e., 20% formalin-fixed and paraffin-embedded specimens) were retrieved from the surgical pathology files of the Pathology Section of Nara Medical University Hospital, Kashihara City, Nara, Japan.

can be pathologically distinguished from acute disseminated encephalomyelitis (Hart and Earle, 1975). The results of immunohistochemical tests were negative for the SV40 in patients 1 and 2 and for the major capsid protein VP1 of JC virus in patient 1.

Relation of CNS IDD to recent viral infection

The results of polymerase chain reaction (PCR) tests for HSV-1 and EBV in CSF were negative in all three patients (Table 1). There were no significant differences in serum titers of EBV viral capsid antigen (VCA) (5.1 ± 3.559 versus 4.2 ± 2.165; P =.291) or EBV nuclear antigen (EBNA) immunoglobulin G (IgG) (2.93 ± 1.172 versus 2.5 ± 1.507; P =.847) between the patients with CNS IDD and the control subjects. EBV VCA and EBNA IgM antibody were not detected in serum samples from any patient or control subject. IgG and IgM antibodies for VCA and EBNA in CSF were not detected in the control subjects and were minimally detected in the patients with CNS IDD (titer: 0.1), with no significant difference between the two groups. As for HSV-1 infection, there were no significant differences in serum titers of HSV-1 IgM antibodies (0.63 ± 0.217 versus 0.61 ± 0.175; P =.89) between the patients with CNS IDD and control subjects. The median titer of serum HSV-1 IgG antibody in the three patients was less than that of the control subjects (2.0 versus 56.3), with no significant difference between the two groups. IgG and IgM antibody titers for HSV-1 in CSF did not differ significantly between the patients with CNS IDD and control subjects (0.20 ± 0 versus 0.33 ± 0.272, P =.27, and 0.42 ± 0.095 versus 0.39 ± 0.069, P =.55, respectively). The titers of each virus antibody in the control subjects are shown in Table 2.

Immunohistochemical analysis showed that all IDD lesions in the three patients were negative for latent membrane protein (LMP)-1, EBNA-2, and EBNA noncoding RNA (EBER)-1 (data not shown). The results of PCR tests for HSV-1 and EBV in the brain specimens of IDD lesions were negative in all patients.

Discussion

EBV reactivation in demyelinating disease has been confirmed on the basis of viral DNA and serologic indices on PCR. In multiple sclerosis, an association of EBV with demyelinating lesions has been demonstrated by increased anti-EBV antibody levels (Ascherio *et al*, 2001) or by accumulations of EBV-infected B cells in postmortem brain tissue (Serafini *et al*, 2007). However, whether EBV is associated with disease activity in multiple sclerosis remains controversial. A recent study showed a

Table 1 Characteristics of three patients with central nervous system inflammatory demyelination lesions and the results of recent tests for Epstein-Barr virus and herpes simplex virus-1 infection

	Patient		
	1	2	3
Age/Sex	63/M	33/M	30/F
Age at symptom onset*	63	30	24
Symptoms*	Decreased consciousness, tetraplegia	Headache, hemianopsia, hemiplegia	Hemiplegia, visual loss
EDSS*	9.5	3.0	3.5
Intracranial multiple lesions on MRI	+	+	+
Spinal cord lesions on MRI	+	–	–
Treatments	Pulse therapy with intravenous methylprednisolone (1 g/day, 3 days, 4 times) oral prednisolone (40 to 30 mg/day, 60 days)	Pulse therapy with intravenous methylprednisolone (1 g/day, 3 days, 4 times)	Pulse therapy with intravenous methyl prednisolone (1 g/day, 3 days, 1 times) intravenous dexamethazone with taper (4 to 2 mg/day, 15 days)
Last follow-up EDSS	9.0	3.0	2.0
Duration of follow-up [months]	3	29	18
Disease phase	Monophasic	Relapsing-remitting	Relapsing-remitting
Aquaporin-4 antibodies	–	–	–
CSF			
White blood cells/μl	4	4	7
Protein mg/dL	29	59	35
Oligoclonal bands	–	–	–
HSV PCR	–	–	–
HSV IgM [ELISA]	0.36	0.37	0.53
HSV IgG [ELISA]	0.2	0.2	0.2
EBV PCR	–	–	–
EBV VCA IgM [ELISA]	0.0	0.0	0.1
EBV VCA IgG [ELISA]	0.1	0.0	0.0
EBNA IgG [ELISA]	0.1	0.0	0.1
Serum			
HSV IgM [ELISA] (0.48–0.97**)	0.83	0.4	0.67
HSV IgG [ELISA] (2–93.6**)	32.6	2	2
EBV VCA IgM [ELISA] (0–0**)	0.0	0.0	0.0
EBV VCA IgG [ELISA] (1.1–7.3**)	8.8	1.7	4.8
EBNA IgG [ELISA] (0.5–4.5**)	3.4	1.6	3.8

*At presentation of inflammatory demyelinating lesion.**The range of titers in controls subjects. EDSS = Expanded Disability Status Scale; CSF = cerebrospinal fluid; HSV = herpes simplex virus; EBV = Epstein-Barr virus; VCA = viral capsid antigen; EBNA = EBV nuclear antigen-1.

Table 2 Clinical characteristics and the titers for Epstein-Barr virus and herpes simplex virus-1 in control subjects.

			HSV-IgM		HSV-IgG		EBV VCA IgM		EBV VCA IgG		EBV-EBNA IgM		EBV-EBNA IgG	
Diagnosis	Age/Sex	CSF cells/ul	Serum	CSF	Serum	CSF	Serum	CSF	Serum	CSF	Serum	CSF	Serum	CSF
MND	50/M	1	0.97	0.33	62.80	0.35	0.0	0.0	1.1	0.0	0.0	0.0	2.4	0.0
Spastic paralysis	48/F	0	0.50	0.4	2.00	0.20	0.0	0.0	5.3	0.0	0.0	0.0	3.1	0.0
SCD	56/F	0	0.58	0.47	68.60	0.20	0.0	0.0	3.6	0.0	0.0	0.0	3.0	0.0
Periodic paralysis	57/M	2	0.48	0.49	93.60	0.93	0.0	0.0	3.9	0.0	0.0	0.0	4.5	0.1
PKC	27/M	1	0.70	0.32	56.30	0.20	0.0	0.0	7.3	0.0	0.0	0.0	0.5	0.0
TOS	22/F	1	0.59	0.33	2.00	0.20	0.0	0.0	6.0	0.0	0.0	0.0	3.5	0.1
Wilson disease	17/F	0	0.48	0.37	2.00	0.20	0.0	0.0	2.2	0.0	0.0	0.0	0.5	0.0

Note. HSV = herpes simplex virus; EBV = Epstein-Barr virus; VCA = viral capsid antigen; EBNA = EBV nuclear antigen-1; CSF = cerebrospinal fluid; MND = motor neuron disease; SCD = spinocerebellar degeneration; PKC = paroxysmal kinesigenic choreoathetosis; TOS = thoracic outlet syndrome.

negative association between the reactivation of late EBV infection and relapsing-remitting multiple sclerosis (Torkildsen *et al*, 2008). In our patients with CNS IDD, elevated anti-EBV antibody levels and evidence of EBV on PCR were not found in serum, CSF, or brain specimens, and EBV proteins were negative in the CNS IDD lesions. As for HSV-1, anti-HSV-1 antibodies were not increased in serum or CSF, and HSV-1 was negative in CSF or brain specimens from CNS IDD lesions on PCR. These findings do not support an association of EBV or HSV-1 infection with CNS IDD. A more recent study provided no evidence that varicella-zoster virus, which is a herpesvirus, is a relevant antigen in multiple sclerosis (Burgoon *et al*, 2009). More recently the relation of EBV with multiple sclerosis trends to be very much in question (Willis *et al*, 2009). Our results also suggest a negative association of CNS IDD with EBV or HSV-1 but the confirmation will require larger cohort studies.

Materials and methods

We studied three patients with biopsy-confirmed CNS IDD, all of whom were given a definitive diagnosis of multiple sclerosis according to McDonald's revised criteria (Polman *et al*, 2005). One patient with a monophasic clinical course (patient 1) showed new lesions on follow-up T2-weighted images obtained 3 months after the onset of neurologic symptoms. Two patients (patients 1 and 2) had clinical relapses (two and five times, respectively). Clinical information was obtained from detailed medical records. All patients had a circumscribed hyperintense lesion exceeding 30 mm in diameter on fluid-attenuated inversion recovery (FLAIR) and T2-weighted images, associated with neurological signs and symptoms (Figure 1). No patient had complete resolution of these abnormalities on magnetic resonance imaging after steroid treatment. The results of serologic and radiologic investigations ruled out the following diseases and conditions: cerebral thromboembolism and hemorrhage, cerebral venous sinus thrombosis, cerebral aneurysm, cerebral arteriovenous malformation, mitochondrial encephalopathy, diabetic encephalopathy, hepatic encephalopathy, systemic lupus erythematosus, antiphospholipid antibody syndrome, sarcoidosis, and Hashimoto's encephalopathy. Moreover, bacterial and fungal infections were excluded by CSF culture, and syphilis was excluded by serologic testing. Tuberculous CNS infections were excluded by CSF culture and single PCR. No patient had preceding symptoms of infection or abnormalities restricted to the limbic system on magnetic resonance imaging, which would strongly suggest viral encephalitis. The clinical information is summarized in Table 1.

Biological analysis

Recent viral infections were evaluated in the three patients with CNS IDD. Samples of blood and CSF were obtained on days 27 and 35 after the first attack in patients 1 and 2, respectively. These patients clinically showed a progressive course, and these samples were obtained after greater neurological deterioration than that after the initiation of the first attack. Samples of blood and CSF were obtained on day 10 after the second attack in patient 3. All blood and CSF samples were stored at $-80^{\circ}C$ before analysis. Levels of EBV viral capsid antigen (VCA) IgG and IgM and nuclear antigen-1 (EBNA-1) IgG were measured by an enzyme-linked immunosorbent assay (ELISA), performed according to the manufacturer's instructions (Denka Seiken, Tokyo, Japan). IgG-specific and IgM-specific antibodies against HSV-1 in serum and CSF were measured by ELISA. EBV or HSV-1 infections were also evaluated in serum and CSF samples from seven control subjects without CNS inflammatory or autoimmune disorders (motor neuron disease, spastic paralysis, spinocerebellar degeneration, periodic paralysis, paroxysmal kinesigenic choreoathetosis, thoracic outlet syndrome, Wilson disease). These control subjects were all admitted to our neurological institute and reviewed by our neurological conference. No control subject had abnormal intracranial signals on magnetic resonance imaging (MRI) or had received immunosuppressive, immunomodulating, or antiviral medications. There was no significant difference in age between the patients with CNS IDD and the control subjects (42 ± 18.3 and 39.6 ± 17.0 years; $P = .85$). Detailed information on the control subjects is shown in Table 2.

The statistical significance of differences between the patients and control subjects were evaluated with the use of Student's paired t test. P values of less than.05 were considered to indicate statistical significance.

The DNA polymerase genes of EBV and HSV-1 in the CSF samples were evaluated by real-time polymerase chain reaction (PCR). DNA in CSF was extracted using QIAamp DNA Blood Min Kits (Qiagen). A real-time quantitative PCR assay with a fluorogenic probe was performed with use of a TaqMan PCR kit (TaqMan PCR Core Reagents Kit with AmpliTaq Gold). Briefly, 250 ng of DNA from CSF was added to a PCR mixture containing 10 mM Tris (pH 8.3), 50 mM KCl, 10 μM EDTA, 5 mM $MgCl_2$, 100 μM dATP, dCTP, dGTP, and dTTP, 0.2 mM each primer, 0.1 μM fluorogenic probe, and 1.25 U of AmpliTaq Gold (PE Applied Biosystems). After activation of the AmpliTaq Gold for 10 min at $95^{\circ}C$, 45 to 50 cycles of 15 s at $95^{\circ}C$ and 1 min at $62^{\circ}C$ were carried out with an ABI PRISM 7700 Sequence Detection System. As for EBV, the PCR primers for this assay were selected in the BALF5 gene encoding the viral DNA polymerase (Baer *et al*, 1984). The upstream and downstream primer sequences were 5'-CGGAAGCCCTCTGGACTTC-3' and

5'-CCCTGTTTATCCGATGGAATG-3', respectively. A fluorogenic probe (5'-TGTACACGCACGA-GAAATGCGCC-3') with a sequence located between the PCR primers was used. As for HSV-1, the PCR primers for this assay were in the UL30 gene encoding the viral DNA polymerase of HSV-1 (Kimura *et al*, 1998). The upstream and downstream primer sequences were 5'-ACATCATCAACTTCGACTGG-3' and 5'-CTCAGGTCCTTCTTCTTGTCC-3', respectively. The fluorogenic probe consisted of an oligonucleotide sequence located between the PCR primers (5'-ATGGTGAACATCGACATGTACGG-3'), a 5'-reporter dye, and a 3'-quencher dye. EBV-infected cell DNA (P3HR-1) or HSV-1–infected cell DNA (VR-3) was used as a positive control. The real-time PCR for EBV and HSV-1 has a high sensitivity of 100% and 100%, respectively, and a high specificity of 100% and 99%, respectively (Drago *et al*, 2004). The smallest number of copies of each virus detectable on the real-time PCR was 100 copies/ml.

HSV-1 DNA or EBV DNA in the brain specimen from IDD lesions was extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen). The DNA polymerase genes of HSV-1 were evaluated by single PCR, as described previously (Cao *et al*, 1989). The DNA polymerase genes of EBV from the brain specimens were evaluated by real-time PCR, as described above.

Immunohistochemistry

Immunohistochemical studies were performed using anti-latent membrane protein-1 (LMP-1) monoclonal antibody (at a dilution of 1:100; DAKO, Tokyo, Japan) and anti-EBNA-2 antibody (at a dilution of 1:50 LMP-1) according to the Bond polymer method using the autoimmunostainer Bond MAX (Mitsubishi Chemical Medience, Tokyo, Japan). The pretreatment antigen retrieval steps were performed using Bond Epitope Retrieval Solution 1 (citrate-based pH 6.0 solution). In situ hybridization was done using EBER-1 detection (Leica, Tokyo, Japan) of the previous autoimmunostainer. Immunohistochemical studies were performed using anti-simian vacuolating virus 40 (SV40) T antigen (Ab3) (at a dilution of 1:50; PAb280, Calbiochem, USA) according to the Bond polymer method using the autoimmunostainer Bond MAX (Mitsubishi Chemical Medicine). Immunohistochemical studies using antibodies against the major capsid protein VP1 of JC virus were performed as described previously (Okada *et al*, 2002).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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