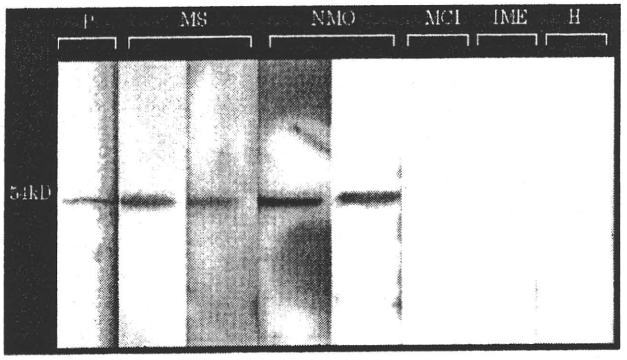

Fig. 2. Immunoblotting of human phosphoglycerate mutase 1 (PGAM1) full-length recombinant protein with GST. The recombinant protein (0.28 μg) was applied to each well. P, 1:300-diluted anti-PGAM1 monoclonal antibody (Abnova); MS, 1:1500-diluted sera from patients with multiple sclerosis; NMO, 1:1500-diluted sera from patients with neuromyelitis optica; MCI, 1:1500-diluted sera from patients with multiple cerebral infarctions; IME, 1:1500-diluted sera from patients with infectious meningoencephalitis; H, 1:1500-diluted sera from healthy controls.

analyzed on a MALDI TOF/TOF instrument, AXIMA Performance (Shimadzu). By utilizing information on the *x–y* positions of spotted samples on AccuSpot, autoexperiments using AXIMA Performance were performed to analyze the samples on the plates. Every autoexperiment and protein identification were performed using an integrated software, Kompact Ver.2.8. Protein identification was carried out using the MS/MS ion search database, Mascot (http://www.matrixscience.com/; Matrix Science Ltd.).

2.6. Immunoreactivity of sera from patients with various neurological diseases against human PGAM1 full-length recombinant protein

We examined the anti-PGAM1 antibodies in sera from 21 MS, 13 NMO, 21 PD, 20 MCI, to 19 IME patients, and 17 healthy controls by 1DE immunoblotting using the commercially available human PGAM1 full-length recombinant protein with GST (Abnova). Immunoblotting was carried out as described in Section 2.3. The screening dilution of sera from all patients and healthy controls was 1:1500.

2.7. Statistical analysis

We used the chi-square test with Yates' continuity correction to assess the difference in the prevalence of the anti-PGAM1 antibody between groups. Differences were considered significant at $P<0.05$.

3. Results

3.1. Screening and identification of target antigen of MS patients' autoantibodies (Fig. 1)

We examined the target antigen that reacted selectively with MS patients' sera. We detected by 1DE immunoblotting an approximately 30 kDa band corresponding to a protein that reacted with antibodies in sera from two out of five MS patients, but not with sera from five healthy controls. The same sample was subjected to 2DE, and one spot (observed MW/p*I*: 26,000/6.9) with a similar molecular weight reacted with the sera from these two MS patients but not with the sera from the healthy controls. We analyzed this spot by MALDI TOF-MS. This protein spot was identified as PGAM1 (accession number, P25113; score/coverage identification (%), 660/40; number of matched peptides, 11; theoretical MW/p*I*, 28,948/6.67).

3.2. Immunoreactivity of sera from patients with various neurological diseases against human PGAM1 full-length recombinant protein (Figs. 2 and 3)

To investigate whether the anti-PGAM 1 antibody is specific for MS, we examined this autoantibody in sera from patients with various neurological diseases (21 MS patients, 13 NMO patients, 21 PD patients, 20 MCI patients, and 19 IME patients) and 17 healthy controls by 1DE immunoblotting using the human PGAM1 full-length recombinant protein with GST (Figs. 2 and 3). As a result, the positivity rates were 81% (17 of 21) in MS patients, 77% (10 of 13) in NMO patients, 24% (5 of 21) in PD patients, 30% (6 of 20) in MCI patients, 32% (6 of 19) in IME patients, and 24% (4 of 17) in healthy controls. Statistically, the prevalence of the anti-PGAM1 antibody was significantly higher in patients with MS than in patients with PD ($P<0.001$), MCI ($P<0.003$), and IME ($P<0.005$), and in healthy controls ($P<0.002$). The prevalence of the anti-PGAM1 antibody was also significantly higher in patients with NMO than in patients with PD ($P<0.008$), MCI ($P<0.03$), and IME ($P<0.04$), and in healthy controls ($P<0.02$). These findings indicate that the anti-PGAM1 antibody has a stronger correlation with MS and NMO than with PD, MCI, IME, and being healthy.

4. Discussion

We identified PGAM1 as the target antigen of autoantibodies in sera from the MS patients by proteomics-based analysis. Western blotting analysis using the human PGAM1 recombinant protein

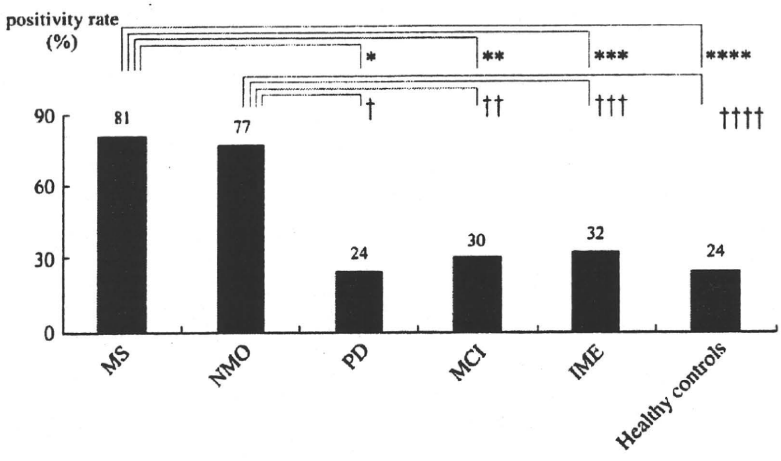

Fig. 3. Prevalence of antibodies against human phosphoglycerate mutase 1 (PGAM1) full-length recombinant protein. MS, patients with multiple sclerosis; NMO, patients with neuromyelitis optica; PD, patients with Parkinson's disease; MCI, patients with multiple cerebral infarctions; IME, patients with infectious meningoencephalitis. *$P<0.001$, **$P<0.003$, ***$P<0.005$, ****$P<0.002$, †$P<0.008$, ††$P<0.03$, †††$P<0.04$, and ††††$P<0.02$.

showed that the prevalence of the anti-PGAM1 antibody is much higher in not only patients with MS, but also those with patients with NMO, than in those with other neurological diseases and in healthy controls. To the best of our knowledge, this is the first study that elucidated the relationships between the anti-PGAM1 antibody and CNS autoimmune diseases. Lu et al. (2008) reported that the prevalence of the anti-PGAM1 antibody is much higher in patients with autoimmune hepatitis (AIH) than in those with other hepatic diseases and in healthy subjects. AIH is a rare liver disease and is characterized by hypergammaglobulinemia even in the absence of cirrhosis, characteristic autoantibodies, and a favorable response to immunosuppressive treatment (Zachou et al., 2004; Zolfino et al., 2002). Although the etiology of this disease is as yet unknown, the presence of several circulating autoantibodies such as the anti-nuclear antibody, anti-smooth muscle antibody, anti-liver kidney microsome type 1 antibody, and anti-liver cytosol type 1 antibody, which are serological markers for diagnostic criteria (Alvarez et al., 1999), suggests the important role of humoral mechanisms in AIH. There are several reports on MS patients with the complication of AIH (Pulicken et al., 2006; Takahashi et al., 2008; Ferrò et al., 2008). de Seze et al. (2005) reported that the prevalence of AIH seems to be about tenfold higher in patients with MS than in the general population. The anti-PGAM1 antibody can be generated in an immunological background common to both autoimmune CNS diseases and AIH.

Phosphoglycerate mutase is a glycolytic enzyme that catalyzes the interconversion of 3- and 2-phosphoglycerate with 2, 3-bisphosphoglycerate as the primer of the reaction (Fothergill-Gilmore and Watson, 1989). In mammalian tissues, PGAM exists in three isozymes, composed of homodimeric and heterodimeric combinations of two different subunits, type M (muscle form, PGAM2) and type B (brain form, PGAM1). The homodimer MM form is mainly expressed in the muscle; the BB form in the brain, kidney and liver; and the heterodimer MB form in the heart (Omenn and Cheung, 1974; Zhang et al., 2001). A previous study showed that PGAM1 is induced after hypoxia, which would occur in patients with cerebral infarction (Takahashi et al., 1998). In this study, the positivity rate of the anti-PGAM1 antibody in patients with MCI is not significantly higher than those in patients with other neurological diseases and in healthy controls. This finding suggests that an immunological background is important for production of the anti-PGAM 1 antibody.

In conclusion, the results of this study suggest that the anti-PGAM1 antibody is not only a marker of AIH but also a nonspecific marker of CNS autoimmune diseases. However, further studies are required to assess the presence of the anti-PGAM1 antibody in a large cohort of patients, including those with other autoimmune-mediated diseases, and controls.

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Serial cerebrospinal fluid neurofilament concentrations in bacterial meningitis

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ABSTRACT

Neurofilament (NF) is one of the major cytoskeleton proteins of neurons. We investigated the concentrations of the heavy subunit of NF (NF-H) in cerebrospinal fluid (CSF) as biomarkers of neuronal injury in bacterial meningitis. Concentrations of NF-H in CSF of 26 children with bacterial meningitis and in 16 control subjects were measured by ELISA. The CSF NF-H levels were elevated in 22 of the 26 children (85%) with bacterial meningitis. The peak CSF NF-H level occurred at a median period of 10.5 days after onset of illness (range, 1 to 35 days). The peak CSF NF-H levels of the patients with neurological sequelae ($n=4$) were significantly higher than those without sequelae ($n=22$) (7.06 vs. 2.46 ng/mL as median, $p=0.048$). There was no significant difference in CSF NF-H levels between patients with and without severe neurological sequelae up to day 14 of illness, but the CSF NF-H levels in patients with sequelae were significantly higher than in those without sequelae after day 14 of illness (2.04 vs. 1.19 ng/mL as median, $p=0.024$). We suggest that neuronal injury occurs in bacterial meningitis regardless of the presence or absence of neurological sequelae.

1. Introduction

Neurofilament (NF) is a major structural element of neurons and is composed of three subunits: the light (NF-L), medium (NF-M) and heavy (NF-H) subunits [1]. NF is a specific biomarker for axonal injury, degeneration and neuronal loss, and detection of NF in cerebrospinal fluid (CSF) provides information on the degree of neuronal injury [1]. The phosphorylated forms of NF-H are resistant to proteases and are particularly concentrated in larger diameter axons [1]. It has been reported that NF-L or NF-H in CSF is increased in neurological diseases, including multiple sclerosis, hydrocephalus, subarachnoid hemorrhage, brain damage after cardiac arrest, AIDS dementia complex, Parkinsonian syndromes, amyotrophic lateral sclerosis, and Guillain–Barré syndrome [2–11].

Bacterial meningitis remains a serious and life-threatening disease. Antibiotics and adjunctive dexamethasone therapy improve the prognosis, but the condition can result in both severe neurodisability and milder motor and psychometric impairment. In this study, we determined serial CSF NF-H concentrations to evaluate neuronal injury in pediatric patients with bacterial meningitis.

2. Materials and methods

Informed consent was obtained from the parents of the patients and controls in the study. The protocol was approved by the Institutional Review Board of Yamaguchi University Hospital.

2.1. Bacterial meningitis

Ninety-six CSF samples were obtained from 26 children (13 females and 13 males, mean age: 1.1 years old, range: 2 days to 4 years old) with bacterial meningitis on admission to Yamaguchi University Hospital from August, 1990 to August, 2007 (Table 1). The day of onset of fever was considered to be day 1 of illness. Serial CSF samples were obtained from patients (mean: 3.7 times, range: 2 to 9 times), with the initial CSF sample obtained during days 1 to 15 (median: 1.0 days) of illness. Samples were stored at −80 °C until assay. CSF cultures from patients with bacterial meningitis yielded *Haemophilus influenzae* ($n=17$), *Streptococcus pneumoniae* ($n=3$), *Escherichia coli* ($n=3$), Group B *Streptococcus* ($n=2$), and methicillin-resistant *Staphylococcus aureus* ($n=1$). The patients were treated with multiple antibiotics that were effective against these bacteria, and adjunctive dexamethasone therapy was performed in 16 of the 26 patients according to the standard method (0.6 mg/kg/day in four intravenous doses) [12]. Administration of dexamethasone was started before the first administration of antibiotics. Four patients had severe neurological sequelae, including motor paresis ($n=2$), mental retardation ($n=1$), and sensorineural hearing impairment ($n=1$). The relationships between

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CSF NF-H levels and CSF cell counts and total protein levels were investigated at the time of specimen collection.

2.2. Control subjects

The control subjects were 16 afebrile and noninfectious children (7 females and 9 males, age: 2 days to 4 years old, median: 1.7 years old), including 10 patients with epilepsy, 3 with gait disturbance, 2 with psychomotor delay, and 1 with clubfoot (Table 1). CSF samples were obtained for routine analysis and all the controls had normal CSF cell counts. There was no significant difference in age or gender between patients with bacterial meningitis and controls by Mann–Whitney *U* test or χ^2 test.

2.3. Determination of CSF NF-H concentrations

The CSF concentrations of NF-H were measured with a phosphorylated NF-H ELISA kit (EnCor Biotechnology Inc., Gainesville, FL, USA). An anti-NF-H monoclonal coating antibody was adsorbed onto polystyrene microwells. NF-H in the samples or standard bound to the adsorbed antibodies and the NF-H/antibody complex was detected with an alkaline phosphatase-conjugated secondary antibody. The amount of captured NF-H was measured using an ELISA plate reader based on a color reaction. The detection limit was 0.10 ng/mL.

2.4. Statistical analysis

Values lower than the detection limit were taken to be 0.05 ng/mL (half of the detection limit). Differences were analyzed using a Mann–Whitney *U* test and correlations were determined by Spearman's correlation coefficient test. *P*-values less than 0.05 were taken to be significant. Calculations were performed using SPSS v. 12.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

The median CSF NF-H concentration in controls was 0.05 ng/mL (range:<0.1–0.84 ng/mL) and all values in controls were lower than 0.94 ng/mL, the normal upper limit [13,14]. There was no significant correlation of the CSF NF-H concentration with age in our controls. Serial CSF NF-H concentrations in the 26 patients with bacterial meningitis are shown in Fig. 1. The peak CSF NF-H levels in the patients were significantly higher than those in controls ($p<0.001$) and the peak level occurred at a median of 10.5 days after onset of illness (range: 1 to 35 days). The peak CSF NF-H levels of patients with severe neurological sequelae were significantly higher than for those without sequelae (median, range; 7.06, 5.16–13.89 vs. 2.46,<0.10–26.67 ng/mL, $p=0.048$). There was no significant difference in CSF NF-H levels between patients with and without severe neurological sequelae up to day 14 of illness (median, range; 1.38, 0.37–1.84 vs. 0.88,<0.10–26.67 ng/mL, $p=0.822$), but the CSF NF-H levels in patients with sequelae were significantly higher than in those without sequelae after day 14 of illness (median, range; 2.04, 0.75–13.89 vs. 1.19, 0.23–4.82 ng/mL, $p=0.024$). There were no significant differences in peak CSF NF-H concentrations between patients who did ($n=16$) and did not ($n=10$) receive adjunctive dexamethasone therapy (median, range; 2.45,<0.10–7.74 vs. 6.98, 0.86–26.67 ng/mL, $p=0.077$), or between patients who tested positive ($n=17$) and negative ($n=9$) for *H. influenzae* in the CSF culture (median, range; 2.56,<0.10–8.80 vs. 6.46, 0.86–26.67 ng/mL, $p=0.220$). The CSF NF-H concentrations showed no correlation with CSF cell counts or total protein levels in patients with bacterial meningitis.

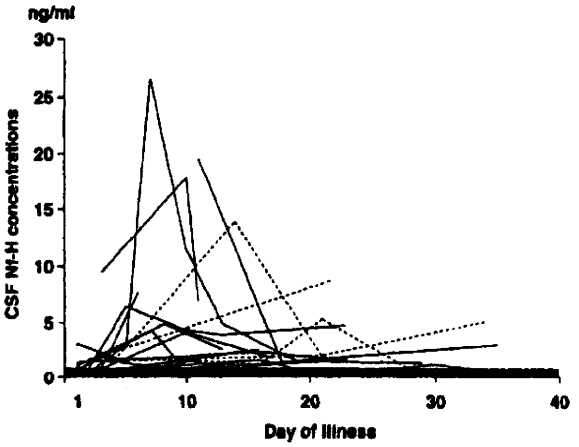

Fig. 1. Serial CSF NF-H concentrations in 26 children with bacterial meningitis. Dot lines indicate patients with severe neurological sequelae. The shaded area shows the normal value (<0.94 ng/mL).

Table 1
Clinical characteristics of the children with bacterial meningitis and controls.

	Bacterial meningitis $N=26$	Controls $N=16$
Age (median, range)	8 months, 2 days–4 yr	1.7 yr, 2 days–4 yr
Sex (female: male)	13: 13	7:9
Primary causative bacteria	*Haemophilus influenzae*	17
	Streptococcus pneumoniae	3
	Escherichia coli	3
	GBS	2
	MRSA	1
Outcome	Normal	22
	Motor paresis	2
	Mental retardation	1
	Hearing impairment	1
CSF NF-H concentrations (ng/mL; median, ranges)	3.08, <0.1–26.67	0.05, <0.1–0.84

GBS, Group B *Streptococcus*; MRSA, methicillin-resistant *Staphylococcus aureus*.

4. Discussion

Measurement of NF is useful for confirmation of neuronal injury, evaluation of therapeutic effect, prediction of prognosis, and differential diagnosis [2–11]. However, there are only a few reports of NF in pediatric disorders, including perinatal asphyxia, cerebral white matter abnormalities and subacute sclerosing panencephalitis [15–17]. Here, we provide the first report of CSF NF levels in bacterial meningitis. Our data show that these levels were elevated in most patients regardless of the presence or absence of neurological sequelae. However, the peak CSF NF-H levels of patients with severe neurological sequelae were significantly higher than those without sequelae. These findings suggest that CSF NF-H levels may reflect the severity of neuronal damage in bacterial meningitis, in which such damage is common.

NF-H levels tend to increase several days after onset of acute disease. A significant increase in these levels is typically seen 7 days after subarachnoid hemorrhage [18], and peak levels have been observed 3 days after experimental spinal cord injury and 2 days after experimental traumatic brain injury [13]. Our results showed peak CSF NF-H levels at a median of 10.5 days after onset of bacterial meningitis. The CSF NF-H levels in patients with severe neurological sequelae were significantly higher than in those without sequelae after day 14 of illness, but up to day 14 there was no significant difference in CSF NF-H levels between patients with and without sequelae. These findings suggest that the late rise in CSF NF-H levels might be related to secondary brain damage as a complication of meningitis. In addition, the peak CSF NF-H levels were not related to adjunctive dexamethasone therapy. This therapy suppresses acute inflammation, whereas CSF NF-H levels reflect neuronal damage followed by acute inflammation. These results suggest that dexamethasone therapy

during the early phase did not affect the late peak of CSF NF-H levels. There was also no significant difference in CSF NF-H levels based on the type of causative bacteria in our patients, but a further large-scale study is necessary to clarify the relationship between CSF NF-H levels and causative bacteria in bacterial meningitis.

Bacterial meningitis can have severe neurological sequelae in 12 to 29% of survivors, and milder impairment of neurological function occurs in another 15 to 38% [19]. Based on previous reports and our present data, most patients with bacterial meningitis have neuronal damage and may develop severe neurological sequelae and milder impairment of neurological function. Therefore, careful long-term clinical follow-up and comprehensive developmental assessments are necessary for patients with bacterial meningitis to evaluate sequelae. We also conclude that CSF NF-H concentrations are elevated in most patients with bacterial meningitis.

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Acute Encephalopathy Associated With Influenza C Virus Infection

To the Editors:

Influenza C virus infection is considered to be milder than the infections caused by influenza viruses A and B; there are no reports of severe complications associated with influenza C virus infections.[1] Influenza C virus is distributed worldwide, and the seropositive rate in people aged above 10 years is approximately 100%. However, the clinical diagnosis of type C influenza is complicated by the rarity of specific symptoms and the dearth of facilities equipped with the resources for performing efficient viral isolation. Here we report the first case of acute encephalopathy associated with influenza C virus infection.

The patient (age, 2 years and 4 months) presented with hyperpyrexia. Several hours after the onset of hyperpyrexia, the patient had a generalized convulsion that lasted for approximately 10 minutes, after which the patient exhibited severely disturbed consciousness and symptoms of compensatory shock. His body temperature was 42.0°C.

The pharyngeal and nasal swabs collected on admission tested positive for the influenza C virus but negative for the other viruses. The serum hemagglutination-inhibition titer of antibodies against the isolated virus increased from less than 8-fold at the onset of hyperpyrexia to 128-fold on day 24.

CSF analysis revealed a normal cell count. The cytokine profile on admission revealed markedly elevated serum and CSF concentrations of interleukin (IL)-6 (1527.2 pg/mL and 951.3 pg/mL, respectively) and IL-10 (582.3 pg/mL and 49.3 pg/mL, respectively).

Diffusion-weighted imaging of the brain, performed on day 7, revealed diffuse high-intensity signals over the subcortical white matter. Diffusion-weighted imaging performed on day 24 revealed that the high-intensity signals indicating dendritic forms had disappeared; however, mild diffuse brain atrophy persisted.

Acute encephalopathy with prolonged febrile seizure and late reduced diffusion (AESD) has been suggested to be associated with infection by some viruses (eg, influenza A, influenza B, and human herpes virus type 6).[2] AESD is considered the primary form of excitotoxicity-induced acute encephalopathies. The MRI findings obtained in the present case are compatible with those noted in patients with AESD. AESD usually exhibits a biphasic clinical course, with status epilepticus at the onset. However, our patient underwent a monophasic clinical course, and status epilepticus was not noted. This could be attributable to the immediate intensive care that the patient received during the early phase. We considered that the diagnostic criteria for AESD were satisfied in the present case.

The invasion, uncoating, and proliferation mechanisms of the influenza C virus are fundamentally identical to those of the influenza A virus,[3] and we can assume that the influenza C encephalopathy is not associated with any unique pathophysiology. A unique feature of our case is the concomitant elevation in the CSF levels of IL-6 and IL-10. The marked elevation in the patient's IL-10, which is not observed in common AESD,[4] indicated CNS inflammation.

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Original article

Serum and cerebrospinal fluid levels of cytokines in acute encephalopathy associated with human herpesvirus-6 infection

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Abstract

Human herpesvirus-6 (HHV-6) is a causative agent of exanthema subitum. The immunological pathogenesis of acute encephalopathy associated with HHV-6 infection is still unclear. We measured the concentrations of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-6, IL-10, and soluble TNF receptor 1 (sTNFR1) in serum and cerebrospinal fluid (CSF) during the acute stage in 15 infants with acute encephalopathy and 12 with febrile seizures associated with HHV-6 infection. The serum IL-6, IL-10, sTNFR1, CSF IL-6, and sTNFR1 levels of infants with encephalopathy who had neurological sequelae ($n = 9$) were significantly higher than those with febrile seizures ($p = 0.011$, 0.043, 0.002, 0.029, and 0.005, respectively). In acute encephalopathy, serum IL-6, sTNFR1, and CSF IL-6 levels in infants with neurological sequelae were significantly higher than those without ($n = 6$) neurological sequelae ($p = 0.043$, 0.026, and 0.029, respectively), and serum IFN-γ, IL-6, IL-10, and sTNFR1 levels were significantly higher than those in the CSF ($p = 0.037$, 0.037, 0.001, and 0.021, respectively). There were no significant differences in serum or CSF cytokine levels between infants who were positive for HHV-6 DNA in the CSF ($n = 6$) compared to those who were negative ($n = 9$). We suggest that cytokines mediate the pathogenesis of acute encephalopathy associated with HHV-6 infection, and that the elevated levels of serum IL-6, sTNFR1, and CSF IL-6 are important for predicting neurological sequelae.

Keywords: Cytokine; Encephalopathy; Human herpesvirus-6; Interleukin-6; Soluble tumor necrosis factor receptor

1. Introduction

Human herpesvirus-6 (HHV-6) is well-known as the causative agent of exanthema subitum, a common infectious disease in infants [1,2]. Exanthema subitum is characterized by an abrupt rise in temperature to as high as 40 °C, followed in 2–4 days by a rapid drop in temperature that coincides with the appearance of an erythematous maculopapular rash that persists for 1–3 days [3]. HHV-6 infection occasionally accompanies neurologic complications, including febrile seizures and acute encephalitis/encephalopathy [3]. These neurologic complications may be caused by direct invasion into the central nervous system (CNS) [3–5] and secondary immune-mediated CNS injury [6–10]. However, the immunological pathogenesis of the acute encephalopathy remains unclear. There has been very little research on the outcome of HHV-6 encephalitis/encephalopathy [4,11]. To determine the role of cytokines in the pathogenesis of acute encephalopathy associated with HHV-

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6 infection, we determined the concentrations of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-6, IL-10, and soluble TNF receptor 1 (sTNFR1) in the serum and cerebrospinal fluid (CSF) of affected infants, compared to infants with febrile seizures associated with HHV-6 infection. In addition, the relationships between the presence of HHV-6 DNA identified by the polymerase chain reaction (PCR) and cytokine levels in the CSF of infants with acute encephalopathy were investigated.

2. Patients and methods

2.1. Acute encephalopathy associated with HHV-6 infection

Informed consent was obtained from the parents of infants enrolled in this study. CSF and serum samples were obtained from 15 infants (seven males and eight females, aged from 3 to 19 months: median, 12 months) showing exanthema subitum clinically with HHV-6 encephalopathy on admission to our hospital and four collaborating research hospitals from January 2001 to June 2007 (Table 1). We divided the infants with the disease into two groups, i.e., those who had neurological sequelae (Group 1, $n = 9$), and those who survived (Group 2, $n = 6$). The criteria for the diagnosis of acute encephalopathy associated with HHV-6 infection were: (1) clinical symptoms and signs compatible with acute encephalopathy, which was defined as a febrile disorder with an alteration of consciousness and slow activity on electroencephalography lasting for more than 24 h after an acute onset, and no bacteria or fungi on CSF cultures, all other neurologic, vascular, metabolic, endocrine, toxic, and drug-induced disorders having been excluded; and (2) isolation of HHV-6 from the throat, and/or a 4-fold increase in the antibody titer determined by the fluorescence antibody test. The day of fever onset was considered as the first day of illness. Neurologic symptoms, including alternation of consciousness and/or seizures, occurred on days 1–2 of the illness. The duration of initial seizures in infants was 29 ± 18 min (mean ± SD; range, 5–60 min). The duration of consciousness disturbance of the infants was 3 to more than 60 days. CSF and serum samples were taken from the infants on days 1–6 of the illness. The neurologic prognoses of the affected infants were made by pediatric neurologists 6–24 months after the onset of encephalopathy.

2.2. Febrile seizures associated with HHV-6 infection

Febrile seizures associated with HHV-6 infection were defined as seizures with fever and impaired consciousness lasting less than 24 h without neurological sequelae, with HHV-6 infection being verified by the above mentioned method. Twelve infants were enrolled (six males and six females, aged from 6 to 25 months: median, 13 months), as shown in Table 1. The duration of febrile seizures of the infants was 12 ± 13 min (mean ± SD; range, 5–40 min). Serum and CSF were obtained from 10 and 9 of the 12 infants, respectively. CSF and serum samples were obtained from all infants on the onset day of febrile seizures. CSF cell counts were 1.9 ± 1.5/μl (mean ± SD; range, 0–4/μl), and CSF pro-

Table 1
Data from 15 infants with acute encephalopathy and febrile seizures associated with HHV-6 infection.

Patient no./age/sex	Day of onset[a]	Sampling day	Duration of initial seizures (min)	Duration of consciousness disturbance (days)	CSF findings		Neurological sequelae
					Cell (/μl)	Protein (mg/dl)	
Group 1, Infants with acute encephalopathy who had neurologic sequelae							
1/18 mo/F	2	2	40	14	1	11	Right hemiplegia
2/13 mo/F	2	2	15	40	1	14	MeR
3/11 mo/F	1	4	45	21	1	9	Left hemiplegia
4/13 mo/F	2	4	30	17	2	20	MeR
5/7 mo/M	1	4	30	30	1	13	MeR, Epi
6/9 mo/F	2	6	20	>60	1	17	Severe tetraplegia
7/12 mo/M	2	2	60	>60	1	10	Severe tetraplegia
8/6 mo/F	2	2	60	>60	1	21	Severe tetraplegia, Epi
9/9 mo/F	2	2	5	14	3	26	MeR, Epi
Group 2, Infants with acute encephalopathy who did not have neurologic sequelae							
10/15 mo/M	2	2	30	7	1	19	–
11/11 mo/M	1	2	40	5	1	28	–
12/12 mo/F	2	5	30	21	3	16	–
13/13 mo/M	1	1	20	7	2	22	–
14/3 mo/M	1	3	5	4	1	18	–
15/19 mo/M	1	4	10	3	2	14	–
Group 3, Infants with febrile seizures							
$N = 12$	1.4 ± 0.5	1.0	13 ± 12	–	1.9 ± 1.5	22 ± 5	–

The day of fever onset was considered the first day of illness. MeR, mental retardation; Epi, epilepsy.
[a] Day of onset of acute encephalopathy or febrile seizures.

tein levels were 22 ± 5 mg/dl (mean ± SD; range, 15–30 mg/dl).

2.3. Control subjects

The control subjects for the serum cytokine levels were 37 healthy children (17 males and 20 females, aged from 3 months to 3 years; median, 17 months). The control subjects for the CSF levels of cytokines were 17 afebrile and noninfectious children with neurological disorders, such as psychomotor delay, epilepsy, etc. (eight males and nine females, aged from 3 months to 3 years; median, 18 months). CSF samples were obtained from these subjects on routine analysis, and all exhibited normal CSF cell counts.

2.4. Determination of cytokine and sTNFR1 concentrations

The concentrations of serum and CSF IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 were measured with a cytometric bead array (CBA) kit (BD PharMingen, San Diego, CA, USA) according to the manufacturer's instructions, as described previously [12–14]. Data analysis was performed using GraphPad Prism software (GraphPad Prism Software, San Diego, CA, USA). Briefly, a CBA comprises a series of beads exhibiting discrete fluorescence intensities at 670 nm. Each series of beads is coated with a monoclonal antibody against a single cytokine, and a mixture of six series of beads can detect six cytokines in one sample. A secondary phycoerythrin-conjugated monoclonal antibody stains the beads proportionally to the amount of bound cytokine. After fluorescence intensity calibration and electronic color compensation procedures, standard and test samples were analyzed with a FACScan flow cytometer equipped with CellQuest software (BD PharMingen). Data were transferred to GraphPad Prism for transformation and analysis. Starting with standard dilutions, the software performed log transformation of the data, and then fitted a curve to 10 discrete points using a four-parameter logistic model. A calibration curve generated for each cytokine was used to determine the cytokine concentrations of the samples. The lower detection limits for IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 were 7.1, 2.8, 2.6, 2.6, 2.5, and 2.8 pg/mL, respectively.

The concentrations of sTNFR1 in serum and CSF were determined using an sTNFR1 ELISA kit (Bender Medsystems, Vienna, Austria), with a detection limit of 0.05 ng/mL.

2.5. Determination of HHV-6 DNA in the CSF

HHV-6 DNA in CSF samples from 15 infants with acute encephalopathy associated with HHV-6 infection was examined by the nested PCR method, as described previously [15].

2.6. Statistical analysis

All data were log-transformed to obtain an approximately normal distribution. The differences between groups were analyzed using the *t*-test. Correlations were analyzed using Pearson's correlation coefficient. The *p*-values less than 0.05 were considered significant. Analyses and calculations were performed using SPSS-12.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Serum concentrations of cytokines

The serum IL-6 levels in infants with acute encephalopathy who had neurological sequelae were significantly higher than those without such sequelae, those with febrile seizures, and those in controls ($p = 0.043$, $p = 0.011$, and $p < 0.001$, respectively) (Fig. 1 and Table 2). The serum IL-6 levels in infants with acute encephalopathy who did not have neurological sequelae and those with febrile seizures were significantly higher than those in controls (both $p = 0.002$) (Fig. 1). The serum IL-10 levels in infants with acute encephalopathy who had neurological sequelae were significantly higher than those with febrile seizures and those in controls ($p = 0.043$ and $p < 0.001$, respectively) (Fig. 1). The serum IL-10 levels in infants with acute encephalopathy who did not have neurological sequelae were significantly higher than those in controls ($p < 0.001$) (Fig. 1). The serum sTNFR1 levels in infants with acute encephalopathy who had neurological sequelae were significantly higher than those without neurological sequelae, those with febrile seizures, and those in controls ($p = 0.026$, $p = 0.002$, and $p = 0.003$, respectively) (Fig. 1 and Table 2). There were significant correlations among serum IL-6, IL-10, and sTNFR1 levels in infants with HHV-6 infection, including acute encephalopathy and febrile seizures (IL-6 and IL-10, $p < 0.001$; IL-6, and sTNFR1, $p = 0.005$; IL-10, and sTNFR1, $p = 0.013$) (Fig. 2). There were no significant differences in serum IFN-γ, TNF-α, IL-2, or IL-4 levels among infants with acute encephalopathy with/without neurological sequelae, those with febrile seizures, and controls.

3.2. CSF concentrations of cytokines

The CSF IL-6 levels in infants with acute encephalopathy who had neurological sequelae were significantly higher than those without such sequelae, those with febrile seizures, and those in controls ($p = 0.029$, $p = 0.029$, and $p < 0.001$, respectively) (Fig. 3 and Table 2). The

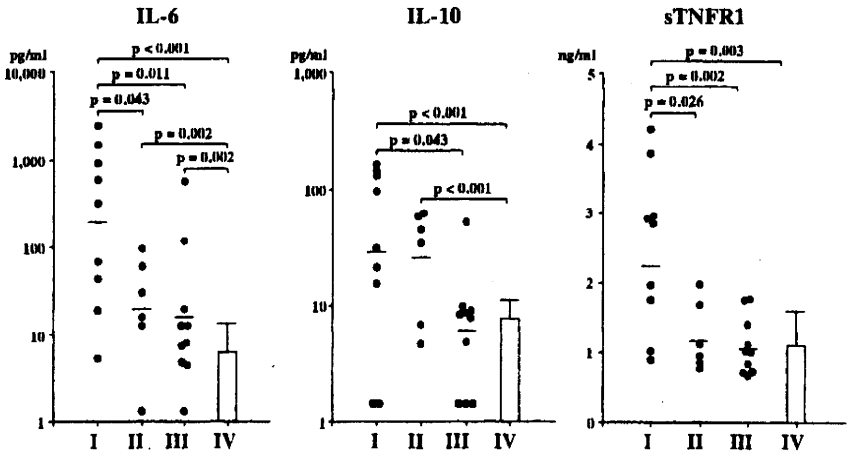

Fig. 1. Serum concentrations of IL-6, IL-10, and sTNFR1 in infants with acute encephalopathy and febrile seizures associated with HHV-6 infection, and controls. (I) Acute encephalopathy with neurological sequelae; (II) acute encephalopathy without neurological sequelae; (III) febrile seizures; (IV) controls. Horizontal lines show geometric means. Data of controls are presented as means + 1 SD. The shaded area indicates under the detection limits.

Table 2
Serum and CSF cytokine concentrations and CSF HHV-6 DNA in 15 infants with acute encephalopathy associated with HHV-6 infection.

Patient no.	Serum			CSF			HHV-6 DNA in the CSF (copies/ml)
	IL-6 (pg/ml)	IL-10 (pg/ml)	sTNFR1 (ng/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	sTNFR1 (ng/ml)	
Group 1, Infants with neurologic sequelae							
1	5.3	<2.8	1.75	15.1	<2.8	1.27	–
2	923.6	30.7	2.91	25.6	<2.8	1.88	168
3	18.9	<2.8	1.95	116.0	<2.8	1.22	–
4	42.5	15.2	1.01	49.0	<2.8	0.98	1240
5	67.6	20.8	0.88	73.1	<2.8	1.33	56
6	320.0	146.0	2.95	59.8	16.4	1.49	–
7	2461.3	131.0	2.84	27.4	18.3	1.94	102
8	597.4	97.1	4.21	15.9	6.8	0.49	–
9	1498.1	166.5	3.87	22.7	4.4	1.66	–
Group 2, Infants without neurologic sequelae							
10	59.1	58.0	0.94	39.4	9.2	0.81	–
11	94.7	44.0	0.85	10.6	<2.8	0.91	–
12	12.4	61.3	1.96	8.6	<2.8	1.15	–
13	<2.5	4.6	1.67	<2.5	4.6	1.15	52
14	15.9	34.0	1.11	18.9	<2.8	0.98	–
15	29.4	6.8	0.76	19.4	<2.8	0.85	1600

CSF IL-6 levels in infants with acute encephalopathy who did not have neurological sequelae and those with febrile seizures were significantly higher than those in controls ($p = 0.002$ and $p = 0.005$, respectively) (Fig. 3). The CSF sTNFR1 levels in infants with acute encephalopathy who had neurological sequelae were significantly higher than those with febrile seizures, and those in controls ($p = 0.005$ and $p = 0.048$, respectively) (Fig. 3). There were no correlations among CSF cytokine levels in infants with HHV-6 infection, including acute encephalopathy and febrile seizures. There were no significant differences in serum IFN-γ, TNF-α, IL-2, IL-4, or IL-10 levels among infants with acute encephalopathy with/without neurological sequelae, those with febrile seizures, and controls.

3.3. Relationships between serum and CSF concentrations of cytokines and clinical data

All five infants in whom all of the serum IL-6, sTNFR1, and CSF IL-6 levels were elevated, had neurologic sequelae (Table 2). Especially, three infants with severe tetraplegia (Patients 6, 7, and 8) exhibited high levels of serum IL-6, sTNFR1, and CSF IL-6. Four of the seven infants in whom two of serum IL-6, sTNFR1, and CSF IL-6 levels were elevated, had neurologic

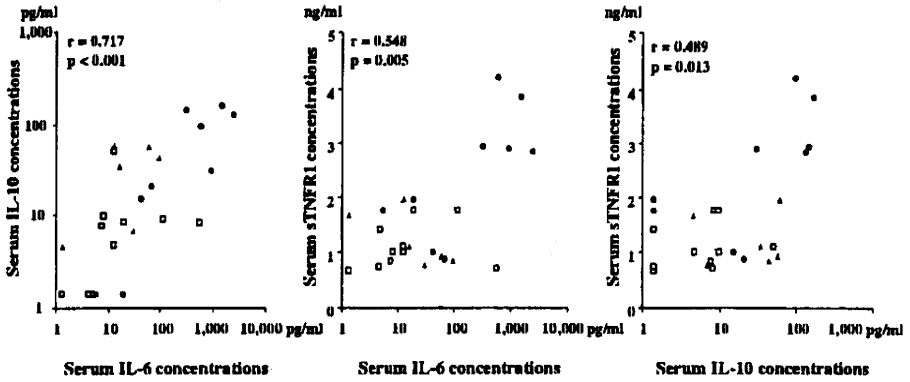

Fig. 2. The relationship among serum IL-6, IL-10, and sTNFR1 concentrations in infants with acute encephalopathy and febrile seizures associated with HHV-6 infection. Circles, triangles, and squares indicate acute encephalopathy with neurological sequelae, acute encephalopathy without neurological sequelae, and febrile seizures, respectively. *r*, Pearson's coefficient.

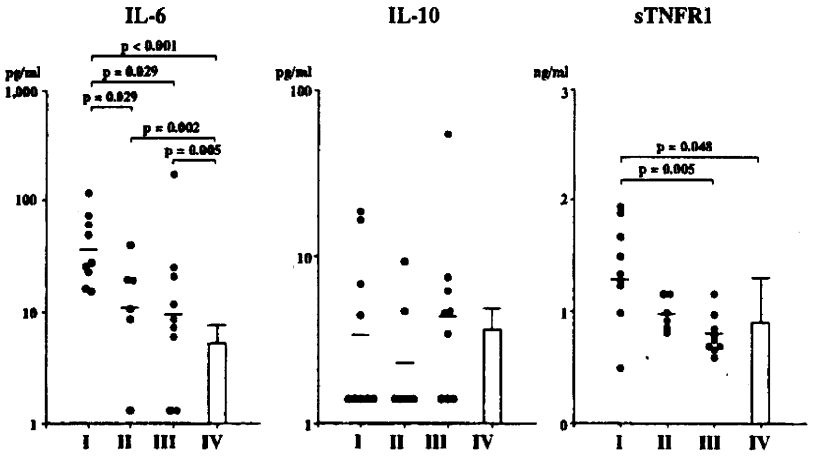

Fig. 3. CSF concentrations of IL-6, IL-10, and sTNFR1 in infants with acute encephalopathy and febrile seizures associated with HHV-6 infection, and controls. (I) Acute encephalopathy with neurological sequelae; (II) acute encephalopathy without neurological sequelae; (III) febrile seizures; (IV) controls. Horizontal lines show geometric means. Data of controls are presented as means + 1 SD. The shaded area indicates under the detection limits.

sequelae, but none of the three infants in whom one or none of those were elevated, exhibited such sequelae (Table 2).

In infants with acute encephalopathy, serum IL-6 and sTNFR1 levels were correlated with the durations of consciousness disturbance (IL-6, $p = 0.025$ and $r = 0.575$; sTNFR1, $p = 0.003$ and $r = 0.716$), but not seizure durations, as shown in Tables 1 and 2. There were no correlations between CSF cytokine levels and the duration of seizures or consciousness disturbance in infants with acute encephalopathy.

Fig. 4 shows serum and CSF concentrations of IFN-γ, IL-6, IL-10, and sTNFR1 in infants with acute encephalopathy. The serum IFN-γ, IL-6, IL-10, and sTNFR1 levels were significantly higher than those in the CSF ($p = 0.037$, $p = 0.037$, $p < 0.001$, and $p = 0.021$, respectively).

3.4. HHV-6 DNA in the CSF of infants with acute encephalopathy

Six of the 15 infants were positive for HHV-6 DNA by PCR in the CSF (4 in Group 1 and 2 in Group 2), as shown in Table 2. There were no significant differences in serum or CSF cytokine levels between infants who were positive ($n = 6$) compared to those who were negative ($n = 9$) for HHV-6 DNA in the CSF.

4. Discussion

The pathogenesis of HHV-6 infection-associated neurologic complications remains unclear. In adult immunocompromised patients, HHV-6 encephalitis is followed by transplantation [16–19]. Our present study revealed that the serum IL-6, IL-10, sTNFR1, CSF

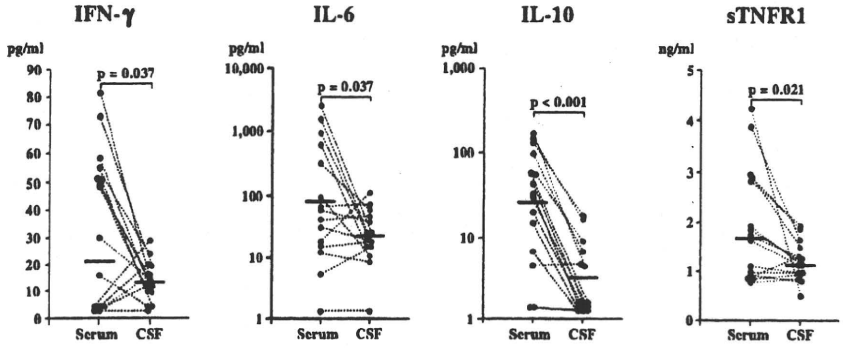

Fig. 4. Serum and CSF concentrations of IFN-γ, IL-6, IL-10, and sTNFR1 in infants with acute encephalopathy associated with HHV-6 infection. Dotted lines represent samples from the same infants on the same day. Horizontal lines show geometric means. The shaded area indicates under the detection limits.

IL-6, and sTNFR1 levels in infants with acute encephalopathy who had neurological sequelae were elevated. In particular, their serum IL-6, sTNFR1, and CSF IL-6 levels were significantly higher than those without neurological sequelae and those with febrile seizures, and the serum IFN-γ, IL-6, IL-10 and sTNFR1 levels were significantly higher than those of the CSF in acute encephalopathy. These findings suggest the existence of hypercytokinemia in acute encephalopathy. Previous studies have shown that sTNFR is an inhibitor and natural homeostatic regulator of the action of TNF-α, and that its level, rather than that of TNF-α, reflects the true biological activity of TNF-α [20–22]. TNF-α has a short half-life of 30 min but induces IL-6 [23], and this IL-6 inhibits TNF-α secretion [24]. This explains why TNF-α was not elevated while sTNFR1 was significantly elevated in the serum of affected infants. High levels of IL-6 and TNF-α in the serum may result in neuronal and vascular endothelial cell damage in acute encephalopathy. IL-10 as an anti-inflammatory cytokine decreases the production of IL-6 and TNF-α [25,26]. Therefore, we suggest that IL-10 is induced in response to the production of IL-6 and TNF-α in acute encephalopathy. IL-6 protects against excitotoxic and ischemic damage to neurons [27,28]. Therefore, IL-6 may be induced in the CSF to protect the neurons damaged by acute encephalopathy.

CSF IFN-γ levels were not elevated in all 15 infants with acute encephalopathy. We previously demonstrated that CSF IFN-γ levels were elevated in CNS disorders due to direct viral invasion, such as viral meningitis and herpes simplex encephalitis [29–31], but not in immune-mediated CNS disorders, such as acute disseminated encephalomyelitis, influenza-associated encephalopathy, acute encephalopathy following prolonged febrile seizures, and hemolytic uremic syndrome with encephalopathy [32–35]. IFN-γ, which is produced by NK cells, CD8+ and Th1 type CD4+ T lymphocytes, plays an important role in host defense against viral infection, and inhibits viral replication [36]. Therefore, IFN-γ elevating in the CSF may exert an inhibitory effect against viruses invading the CNS. Although HHV-6 DNA was positive in the CSF in 6 of the 15 infants with acute encephalopathy, their CSF IFN-γ levels were not elevated. With respect to CSF IFN-γ levels, we suggest that the main pathogenesis of acute encephalopathy associated with HHV-6 infection is not caused by the direct invasion of HHV-6 into the CNS. Our speculation supports several reports of acute encephalopathy following HHV-6 infection [6–11,37].

In infants with acute encephalopathy, the serum IL-6, sTNFR1, and CSF IL-6 levels in infants with were significantly higher than those without neurological sequelae, and serum IL-6 and sTNFR1 levels were correlated with durations of consciousness disturbance. These cytokine levels seemed to be related to the severity and prognosis in infants with acute encephalopathy. In cases whereby infants with acute encephalopathy associated with HHV-6 infection show elevated levels of serum IL-6, sTNFR1, and CSF IL-6, it is likely that they are at a high risk of developing neurological sequelae. However, a part of infants with acute encephalopathy associated with HHV-6 infection who developed neurological sequelae did not have elevated levels of cytokines in the serum or CSF. Therefore, serum or CSF cytokines' levels could not predict the outcome of all infants with acute encephalopathy associated with HHV-6 infection.

In conclusion, our results suggest that cytokines, such as IL-6, IL-10, and TNF-α, mediate the pathogenesis of acute encephalopathy associated with HHV-6 infection, and that the levels of serum IL-6, sTNFR1, and CSF IL-6 are related to the neurologic outcome.

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ORIGINAL COMMUNICATION

Serum matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 levels in non-herpetic acute limbic encephalitis

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Abstract The pathogenesis of non-herpetic acute limbic encephalitis (NHALE) has been not clear. Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase 1 (TIMP-1) play important roles in the function of the blood–brain barrier. We measured the serum concentrations of MMP-9 and TIMP-1 by using enzyme-linked immunosorbent assay (ELISA) in 23 patients with NHALE in the acute and convalescent stages. Serum MMP-9 concentrations and ratios of serum MMP-9/TIMP-1 were significantly higher (1) in patients with NHALE in acute and convalescent stages than in control patients (all $P < 0.001$); (2) in patients with NHALE at the acute stage compared with those at the convalescent stage ($P = 0.004$, and $P = 0.014$, respectively). In contrast, serum TIMP-1 concentrations were significantly lower in patients with NHALE in the acute and convalescent stages than in control patients (both $P < 0.001$) but did not differ in patients with NHALE in the acute and convalescent stages. Our preliminary study suggests that the prolonged imbalance of MMP-9 and TIMP-1 is associated with the pathogenesis of NHALE.

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Keywords Blood–brain barrier · Glutamate receptor ε2 · Matrix metalloproteinase-9 · Non-herpetic acute limbic encephalitis · Tissue inhibitor of metalloproteinase-1

Introduction

In Japan, non-herpetic acute limbic encephalitis (NHALE) has been identified as a new subgroup of limbic encephalitis [1, 17, 26]. The clinical picture of NHALE is similar to that of herpes simplex encephalitis (HSE). However, NHALE is not caused by herpes simplex virus (HSV) infection or a paraneoplastic disease process. Many patients with NHALE have a better neurological prognosis than those with HSE [1, 23]. Autopsies on patients with NHALE have demonstrated neuronal loss and severe gliosis with inflammatory cell infiltrations in the hippocampus and amygdala [20, 23]. However, the pathogenesis of NHALE is still unclear.

Matrix metalloproteinases (MMPs) are a family of enzymes that mediate the degradation of extracellular matrix proteins [3]. MMPs play important roles in normal and pathological processes, including embryogenesis; wound healing; inflammation; and the development of arthritis, cardiovascular diseases, pulmonary diseases, and cancer [2]. MMP-9, a member of this family which is capable of degrading collagen IV, is a major component of the basement membrane of the cerebral endothelium and promotes the migration of cells through tissues and across the blood–brain barrier (BBB) [19]. The activity of MMPs is controlled by specific tissue inhibitors of metalloproteinases (TIMPs) [24]. TIMP-1 has high affinity for MMP-9 [18]. We have reported imbalances in the ratio of MMP-9 to TIMP-1 in neurological diseases such as acute disseminated encephalomyelitis, subacute sclerosing

panencephalitis, influenza-associated encephalopathy, acute encephalopathy following prolonged febrile seizures, haemolytic uremic syndrome with encephalopathy, and perinatal asphyxia [5, 7, 9, 12, 25, 28, 29].

To investigate the roles of MMP-9 and TIMP-1 in the pathogenesis of NHALE, we determined the serum concentrations of MMP-9 and TIMP-1 in patients with NHALE in the acute and convalescent stages. Moreover, the presence of autoantibodies against the *N*-methyl-D-aspartate glutamate receptor (GluR) ε2 subunit was determined in the serum and cerebrospinal fluid (CSF) of affected patients, and the relationships between MMP-9 and TIMP-1 levels and the presence of these autoantibodies were analyzed.

Patients and methods

Informed consent was obtained from the families of the patients and controls enrolled in this study. The protocol was approved by the institutional review board of the National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders.

Non-herpetic acute limbic encephalitis

Serum samples were obtained from 23 patients with NHALE (6 men and 17 women, ages 15–79 years; median age 32 years) who were admitted to 20 collaborating research hospitals from April 2002 to August 2007 (Table 1). The criteria for diagnosis of NHALE were:

1 acute or subacute onset of neurological disorder with limbic-associated symptoms such as amnesia, delirium, panic, anxiety, and excitation;
2 absence of HSV DNA in CSF on nested polymerase chain reaction (PCR) assay and absence of HSV antibodies in CSF on enzyme-linked immunosorbent assay (ELISA);
3 visible lesions in the temporal lobe, especially in the hippocampus and amygdala, on magnetic resonance imaging (MRI) (Fig. 1);
4 absence of malignancy and paraneoplastic disorders;
5 absence of bacteria or fungi on CSF culture; and
6 exclusion of all other neurological, vascular, metabolic, endocrine, toxic, and drug-induced disorders.

Table 1 Clinical data of patients with NHALE and control subjects

	NHALE $N = 23$	Control subjects $N = 41$
Age (median, range)	32 years, 15–79 years	39 years, 15–78 years
Gender (male:female)	6:17	5:36

Serum samples obtained during the acute and convalescent stages were stored at −70°C. Convalescent-stage samples were obtained 21–247 days (median 94 days) after sample collection at the acute stage. These samples were collected during the convalescent stage, several weeks after the neurological status started to improve, or during the chronic stage, when the patient was still morbid. Immunological therapy was administered as follows: 17 of 23 patients were treated with corticosteroids, one patient was treated with intravenous immunoglobulin (IVIG), and three patients were treated with corticosteroids and IVIG.

Controls

The control subjects were 41 healthy adult volunteers (5 men and 36 women, aged 15–78 years; median, 39 years), as shown in Table 1. There were no significant differences in age or sex between the patients with NHALE and the controls as determined using the Mann–Whitney *U* test or the chi-squared test.

Determination of serum MMP-9 and TIMP-1 concentrations

The serum concentrations of MMP-9 and TIMP-1 were determined using sandwich-type ELISA kits (Amersham, Buckinghamshire, UK). A monoclonal coating antibody was adsorbed on to polystyrene microwells to bind MMP-9 or TIMP-1 in the samples or in the standard. A horseradish peroxidase-conjugated monoclonal antibody with neutralizing activity toward MMP-9 or TIMP-1 was added to bind to MMP-9 or TIMP-1 captured by the first antibody. A substrate solution, reactive with horseradish peroxidase, was then added to the wells to produce a colour reaction proportional to the amount of MMP-9 or TIMP-1, and the absorbance was measured. The detection limits were 2.5 ng/mL for MMP-9 and 2.4 ng/mL for TIMP-1. The assay for MMP-9 recognized both the pro and active forms of MMP-9.

Detection of autoantibodies against GluRε2 in serum and CSF

Serum and CSF levels of IgG and IgM autoantibodies against GluRε2 were measured, as described previously, in 22 of 23 patients with NHALE in the acute and convalescent stages [30].

Statistical analysis

The differences between the patients and controls were analysed using the Mann–Whitney *U* test. The differences in the results obtained during the acute and the convalescent stages were analysed using the Wilcoxon matched-

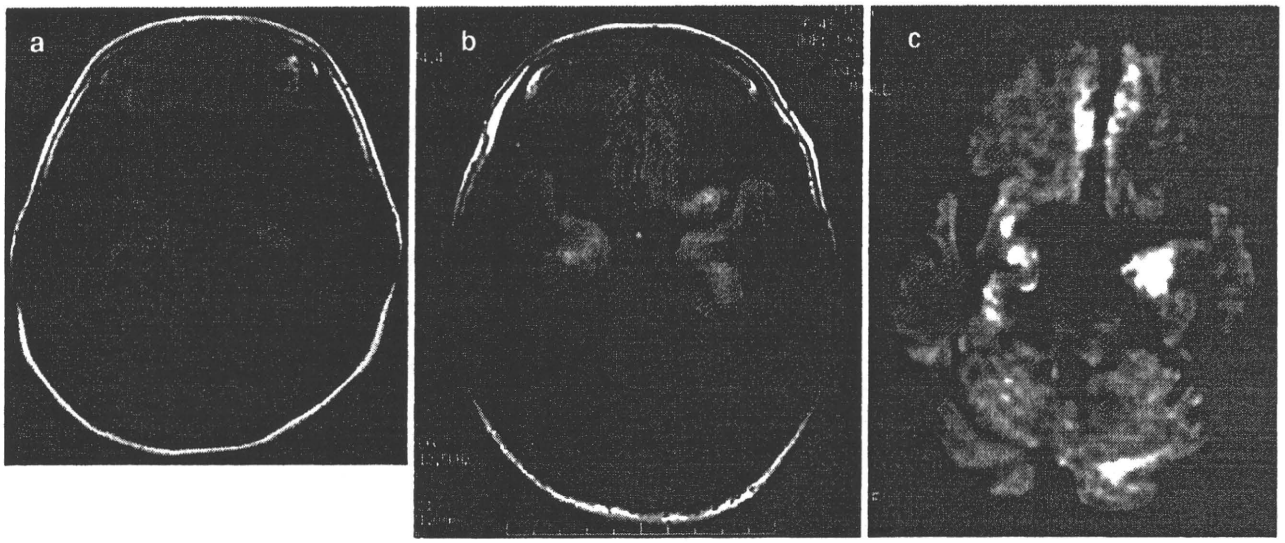

Fig. 1 **a** Fast fluid-attenuated inversion-recovery (FLAIR) MRI of a 42-year-old female patient revealing a high-signal-intensity lesion in both temporal lobes. **b** FLAIR MRI of a 43-year-old female patient showing a high-signal-intensity lesion in both temporal lobes. **c** Diffusion-weighted MRI of a 45-year-old female patient showing bilateral high-signal-intensity lesions in the temporal and frontal lobes

pairs rank-sum test. P values less than 0.05 were considered significant. Analyses and calculations were performed using SPSS-12.0 (SPSS, Chicago, IL, USA).

Results

Serum MMP-9 and TIMP-1 concentrations

The serum MMP-9 concentrations in the control subjects were 6.3–231.8 ng/mL (median 27.5 ng/mL); the TIMP-1 concentrations were 69.3–206.6 ng/mL (median 120.4 ng/mL); and the MMP-9/TIMP-1 ratios were 0.05–2.15 (median 0.22) (Fig. 2). Serum MMP-9 concentrations and the serum MMP-9/TIMP-1 ratio were significantly higher in patients with NHALE in the acute and convalescent stages than in the control subjects ($P < 0.001$). Further, these values were significantly higher in patients with NHALE in the acute stage than in patients in the convalescent stage ($P = 0.004$ and $P = 0.014$, respectively). In contrast, serum TIMP-1 concentrations were significantly lower in patients with NHALE in the acute and convalescent stages than in the control subjects ($P < 0.001$); further, these concentrations did not differ between patients with NHALE in the acute and convalescent stages.

Serum MMP-9 concentrations were not significantly correlated with serum TIMP-1 concentrations in patients with NHALE in the acute and convalescent stages (data not shown). Further, serum MMP-9 and TIMP-1 concentrations in the acute stage were not correlated with the corresponding concentrations in the convalescent stage in patients with NHALE (data not shown).

Autoantibodies against GluRε2

Autoantibodies against GluRε2 in the serum and/or CSF were detected in 18 of 22 patients with NHALE (82%) in the acute stage and in 17 of 22 patients (77%) in the convalescent stage. In the acute stage, IgG and IgM autoantibodies against GluRε2 were detected in the serum of 10 and 14 of 22 patients with NHALE, respectively, and in the CSF of 7 and 5 patients, respectively (Table 2). In the convalescent stage of NHALE, IgG and IgM autoantibodies against GluRε2 were present in the serum of 9 of 21 patients, and in the CSF of 2 of 15 patients, respectively (Table 2). There were no significant differences in serum levels of MMP-9 and TIMP-1 or in the MMP-9/TIMP-1 ratios between patients with and without autoantibodies in the serum or CSF in the acute or convalescent stage (data not shown).

Discussion

The lesions observed in NHALE were primarily located in both the temporal lobes, particularly in the hippocampus and amygdala, and these lesions were similar to those observed in HSE. However, neither HSV DNA nor anti-HSV antibodies were detected in the CSF of patients with NHALE. In previously conducted autopsies on patients with NHALE, HSV-1 and HSV-2 were not detected in the brain [20, 23]. Therefore, NHALE is regarded as a novel type of encephalitis, especially in Japan [1, 17, 23, 26]. Several autoantibodies, including those against the *N*-methyl-D-aspartate GluR and voltage-gated potassium channels, were detected

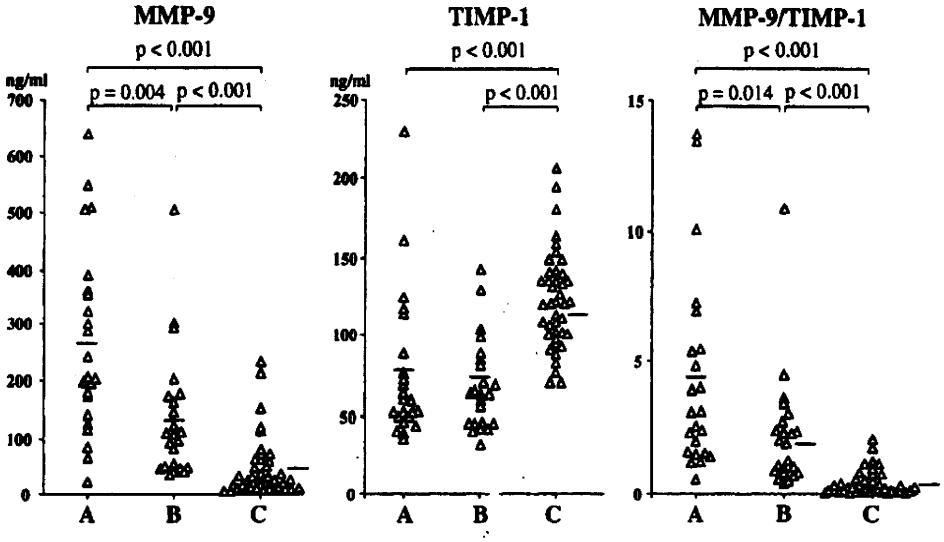

Fig. 2 Serum concentrations of MMP-9 and TIMP-1 and the MMP-9/TIMP-1 ratio in patients with NHALE ($n = 23$) and in controls ($n = 41$). **a** Patients with NHALE in the acute stage, **b** patients with NHALE in the convalescent stage, and **c** controls. The *horizontal lines* indicate median values

Table 2 The presence of autoantibodies against glutamate receptor ε2 subunit in patients with NHALE

	Serum		CSF	
	IgG	IgM	IgG	IgM
Acute stage	10/22 (45%)	14/22 (64%)	7/22 (32%)	5/22 (23%)
Convalescent stage	9/21 (43%)	9/21 (43%)	2/15 (13%)	2/15 (13%)

in patients with NHALE [4, 15, 16, 23, 31]. In addition, some cases of limbic encephalitis associated with autoimmune disease, including Hashimoto's disease, Sjögren's syndrome, and systemic lupus erythematosus, have been reported [14, 22, 27]. Moreover, we recently demonstrated that the CSF concentrations of interferon-γ (IFN-γ) were not elevated in patients with NHALE [11]. We previously demonstrated that CSF IFN-γ levels were elevated in central nervous system (CNS) disorders caused by direct viral invasion, for example viral meningitis and HSE [1, 6, 21], but not in immune-mediated CNS disorders, for example acute disseminated encephalomyelitis, influenza-associated encephalopathy, acute encephalopathy following prolonged febrile seizures, and haemolytic uremic syndrome with encephalopathy [8, 10, 13, 25]. Taking these findings into consideration, because NHALE was not associated with elevated CSF IFN-γ levels in this study, it can be concluded that NHALE is not caused by direct viral infection. The results of the above-mentioned studies suggest that NHALE is an immune-mediated form of encephalitis.

We have previously investigated serum MMP-9 and TIMP-1 levels in other neurological diseases [5, 7, 9, 12, 25, 28, 29], and our findings suggest that imbalances in the MMP-9/TIMP-1 ratio could affect the pathogenesis of each neurological disorder and/or be related to the outcome. In this study, serum MMP-9 levels and MMP-9/TIMP-1 ratios were high and serum TIMP-1 levels were low in patients with NHALE in both the acute and convalescent stages. We believe that high MMP-9 levels and low TIMP-1 levels tend to injure the BBB. Prolonged injury to the BBB may promote invasion of the CNS by leukocytes from the peripheral blood, production of autoantibodies against the CNS, and/or continued inflammation in the CNS. It is likely that MMP-9 and TIMP-1 may cause secondary modifications in the pathogenesis and clinical course of NHALE. Moreover, we have demonstrated that most patients with NHALE exhibit autoantibodies against GluRε2 in the serum and/or CSF during the illness. The autoantibodies may play roles in the pathogenesis of NHALE. However, it is still unclear whether these autoantibodies affect MMP-9 and TIMP-1 production and BBB function. A further large-scale study on the relationship between the presence of autoantibodies and serum MMP-9 and TIMP-1 levels is required to clarify this point.

In conclusion, we have shown that serum MMP-9 levels and MMP-9/TIMP-1 ratios were high and serum TIMP-1 levels were low in patients with NHALE at the acute and convalescent stages. Therefore, the imbalance of MMP-9 and TIMP-1 may affect the inflammatory process and clinical course of NHALE.

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ELSEVIER

Exanthem Subitum-Associated Encephalitis: Nationwide Survey in Japan

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We sought to clarify clinical features of exanthem subitum associated-encephalitis/encephalopathy, generally caused by primary human herpesvirus-6 infection in Japan. A two-part questionnaire was sent to hospitals between January 2003-December 2004. Of 3357 questionnaires, 2357 (70.2%) were returned, and 2293 (68.3%) were eligible for analysis. Eighty-six cases of exanthem subitum-associated encephalitis/encephalopathy were reported. Seventy-seven (89.5%) of 86 patients were diagnosed with human herpesvirus-6 infection by virologic examination. Although 41 (50.6%) of 81 patients had no sequelae, 38 (46.9%) had neurologic sequelae. Moreover, two fatal cases (2.5%) were reported. Pleocytosis was evident in only 4 (7.5%) of 53 patients, and cerebrospinal fluid protein levels were within normal range (23.4 ± 14.6 mg/dL S.D.) in all patients. Human herpesvirus-6 DNA was detected in 21 (53.8%) of 39 patients. Abnormal computed tomography findings were a predictor of neurologic sequelae ($P = 0.0097$). As a consequence of this survey, we estimate that 61.9 cases of exanthem subitum-associated encephalitis occur every year. The disease prognosis was unexpectedly poor.

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Introduction

Primary human herpesvirus-6 infection can cause exanthem subitum in infants and young children [1]. Although the disease is generally a benign, febrile illness with a self-limiting clinical course [2], several severe manifestations, particularly in the central nervous system, can occur [3-13]. Exanthem subitum is associated with febrile seizures [14]. Moreover, we found that the incidence of severe forms of febrile seizures, e.g., hemiconvulsions, prolonged seizures, and repeated seizures, was high in cases of exanthem subitum-associated febrile seizures [15]. After human herpesvirus-6 was identified as an etiologic agent of exanthem subitum, human herpesvirus-6 encephalitis/encephalopathy was reported by several investigators [3-13]. Some studies reported on patients who recovered completely, whereas others manifested severe neurologic sequelae, including several cases with fatal outcomes [4,8,16]. Human herpesvirus-6 DNA was detected in the cerebrospinal fluid of several patients via polymerase chain reaction [5,6], suggesting direct viral invasion of the central nervous system. Saito et al. [17] detected viral antigens and DNA in postmortem brain tissues obtained from AIDS patients, which supports the concept of direct invasion of the virus into the central nervous system. Moreover, it was suggested that human herpesvirus-6 can infect not only neurologic cell lines [18-20] but also fetal astrocytes [21], and can alter cytokine synthesis in infected cells [22]. Thus, human herpesvirus-6 is recognized as a neuro-pathogen. Although a recent study from the United Kingdom indicated that human herpesvirus-6 and human herpesvirus-7 are associated with encephalitis or severe forms of febrile seizure [23], details of the clinical features and frequency of human herpesvirus-6 encephalitis/encephalopathy remain unclear. Therefore, we performed a nationwide survey to determine the frequency and clinical features of exanthem subitum-associated encephalitis/encephalopathy in Japan.

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