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A Young Man with Anti-NMDAR Encephalitis following Guillain-Barré Syndrome

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Key Words
Anti-*N*-methyl-D-aspartate receptor encephalitis · Guillain-Barré syndrome · Parainfectious autoimmune disorder · Male gender

Abstract
A 19-year-old man developed rapidly progressive muscle weakness and dysesthesia in the extremities, and dyspnea after a flu-like episode. Nerve conduction studies showed reduced motor nerve conduction velocities with conduction block, and sensory nerve action potentials could not be evoked. The patient was diagnosed as having Guillain-Barré syndrome (GBS), and was treated with 2 cycles of intravenous immunoglobulin (IVIg) therapy and was assisted by mechanical ventilation. During the recovery course of the illness, he experienced several attacks of psychomotor agitation from the 37th hospital day, and generalized tonic convulsive seizures suddenly developed on the 42nd hospital day. Brain MRI showed high-intensity lesions in the bilateral thalamus and medial temporal lobes. The convulsions were controlled by continuous thiopental infusion (until the 50th hospital day) and mechanical ventilation (until the 84th hospital day). Intravenous methylprednisolone pulse therapy (1,000 mg/day) for 3 days followed by dexamethasone (16 mg/day) was added. After relief of convulsive seizures, prominent orolingual dyskinesia appeared, and on MRI marked atrophy of the bilateral medial temporal lobes was seen. Anti-*N*-methyl-D-aspartate receptor (NMDAR) antibodies in serum and cerebrospinal fluid were positive on the 92nd hospital day. Anti-NMDAR encephalitis usually affects young females but a small number of male cases with this disease have been reported. Our male patient was unique in having GBS, a post-infectious autoimmune disease, as a preceding disease, suggesting that anti-NMDAR encephalitis itself is caused by a parainfectious autoimmune mechanism.

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Introduction

Recently, a unique limbic encephalitis that predominantly affects young females and shows various manifestations, including initial psychosis, and subsequent central hypoventilation, intractable seizures, dysautonomia and prominent orofacial dyskinesia, has been noted [1, 2]. In patients with this disorder a new anti-neural antibody for the NR1/NR2 heteromers of the *N*-methyl-D-aspartate receptor (NMDAR) has been identified as a disease-specific hallmark [3], recently adding that the NR1 is possibly a main epitope [2]. Thus, this disorder is now called anti-NMDAR encephalitis. Although it is now widely accepted that the presence of ovarian teratoma is an important predisposing factor for the development of anti-NMDAR encephalitis [4, 5], the mechanisms that initiate the disease are still incompletely understood.

We here report on a young male patient with anti-NMDAR encephalitis which developed during the recovery course of severe Guillain-Barré syndrome (GBS), and propose that a post-infectious autoimmune mechanism may play an important role in the pathogenesis of anti-NMDAR encephalitis.

Case Report

A 19-year-old man with no history of systemic disease was hospitalized due to muscle weakness and dysesthesia in the extremities following cough and nasal discharge 2 weeks earlier. Neurological examination showed muscle weakness and sensory disturbance in the distal parts of the extremities, and all deep tendon reflexes were absent. Cerebrospinal fluid (CSF) at 5 days after onset of weakness contained a normal cell count, but the concentration of total protein was elevated (52.8 mg/dl). The patient was diagnosed as having GBS and treated with intravenous immunoglobulin (IVIg) therapy (0.4 g/kg/day) for 5 days. However, the symptoms worsened gradually, he was not able to raise his upper limbs and to walk from the 5th hospital day. Furthermore, dysarthria, dysphagia, and dyspnea appeared gradually from the 9th hospital day. He was transferred to our hospital on the 12th hospital day. On the day of transfer, nerve conduction studies showed reduced motor nerve conduction velocities (right median nerve: 11.3 m/s, normal value >55 m/s, right tibial nerve: 36.6 m/s, normal value >45 m/s) with conduction block (fig. 1), and sensory nerve action potentials could not be evoked in both nerves. The F-wave of the right tibial nerve was not evoked. There was neither anti-GM1 IgG antibody nor anti-GQ1b IgG antibody in the serum. Although mechanical ventilation was necessary from the day of transfer, muscle weakness in the extremities and respiratory function gradually improved after the second course of IVIg therapy from the 22nd hospital day for 3 days, and the patient was trained for weaning from mechanical ventilation. However, he started to experience occasional psychomotor agitation from the 37th hospital day, and generalized tonic convulsive seizures suddenly developed following paralytic ileus and tachycardia on the 42nd hospital day. Since the convulsive seizures did not respond to intravenous administration of diazepam, phenytoin, or midazolam, continuous thiopental infusion was used and mechanical ventilation was continued. CSF showed a slightly increased cell count (8/μl, mononuclear cells 7), a highly elevated level of total protein (446 mg/dl), and normal glucose concentration. Brain MRI showed high-intensity lesions in the bilateral thalamus and medial temporal lobes on diffusion and FLAIR images (fig. 2a), and the electroencephalogram showed diffuse spike and wave complexes. He was diagnosed as having some type of autoimmune encephalitis, and intravenous methylprednisolone pulse therapy (1,000 mg/day) for 3 days followed by dexamethasone (16 mg/day) was administered, and used until the 70th hospital day with a gradual dose reduction. Although the generalized convulsive seizures disappeared and the infusion of thiopental was ceased on the 52nd hospital day, orolingual dyskinesia gradually developed. On the brain MRI on the 64th hospital day, the abnormal high-intensity lesions in the bilateral thalamus were reduced, while marked atrophy of the bilateral medial temporal lobes was seen (fig. 2b). Respiratory failure and autonomic dysfunction that included disturbed bowel movement and abnormal cardiovascular responses gradually improved, and he was released from mechanical ventilation on the 84th hospital day. However, the patient remained unresponsive to verbal commands and severe orolingual dyskinesia persisted until he was transferred to a local hospital on the 193rd hospital day.

During investigation of the causes of the encephalitis, there were no laboratory data suggesting viral encephalitis, including herpes simplex, influenza, varicella zoster, cytomegalovirus, measles, and human herpes virus-6. Urological examination was normal and no detectable neoplasm was seen on either chest or abdominal CT. Two years after the onset of seizures, his condition was very similar to when he was discharged from our hospital, and repeated examinations showed no appearance of a tumor.

Immunochemical Analysis of Anti-NMDAR Antibody in Serum and CSF

Detection of anti-NMDAR antibody on the same samples was carried out as follows [6]: cDNA encoding NR1 and NR2B was ligated into the expression vectors and transfected into human embryonic kidney (HEK) 293 cells in the media containing 10 μM MK-801 using Lipofectamine (Invitrogen). Twelve hours after transfection, HEK-293 cells were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 20 min. After non-specific binding was blocked with 10% goat serum in PBS, these cells were incubated with patient sera (1:40) or CSF (1:2) overnight at 4°C and then with FITC-conjugated rabbit anti-human IgG (BD Biosciences) for 30 min at room temperature. *SlowFade* gold anti-fade reagent (Molecular Probes) was applied to the slides and the staining was observed under a fluorescence microscope.

Both serum and CSF obtained from the 92nd hospital day specifically reacted with HEK-293 cells expressing heteromers of NR1/NR2B (fig. 3).

Discussion

In our case, encephalitis was shown by abnormal intensity of the bilateral thalamus and medial temporal lobes on brain MRI at an early stage. However, the former thalamic lesions seemed to be edema due to status epilepticus, and the main lesion of this encephalitis was assumed to be in the bilateral medial temporal lobes, because the thalamic lesions rapidly resolved after treatment, whereas the bilateral medial temporal lobes showed progressive atrophy. Moreover, his clinical manifestations consisted of psychiatric symptoms, intractable seizures, dysautonomia and involuntary movements, and anti-NMDAR antibody was demonstrated in both serum and CSF. He was finally diagnosed as having anti-NMDAR encephalitis.

Anti-NMDAR encephalitis usually affects young females, and an early series of patients with this disease was found to have a high association of ovarian teratoma [3, 4]. Thus, the pathogenetic significance of ovarian teratoma in this encephalitis was investigated and it was proposed that, since the ovarian teratomas obtained from diseased patients showed mature- and immature-appearing neurons with expression of NR2B and/or NR2A [3, 4], ectopically expressed NMDARs in ovarian teratoma contribute to the production of antibodies to NMDARs [3]. Thus, early removal of ovarian teratoma has been recommended for patients with this disease [7]. However, a recent study on a large number of patients with anti-NMDAR encephalitis has shown that this ovarian tumor could not be found in about 40 to 80% of adult patients with the disease [1, 2, 8], although careful follow-up examinations on the detection of tumors are always required in these patients. Increased recognition of this unique encephalitis has also disclosed that children and adolescents also encounter it [9, 10], although the frequency of associated ovarian teratoma was much lower in children than adults. Additionally, a small number of male cases with anti-NMDAR encephalitis [11] were reported, indicating that other causes besides ovarian teratoma can produce encephalitis.

In the pathogenesis of anti-NMDAR encephalitis, the antibody immune response has been shown to be more relevant than cytotoxic T-cell mechanisms [12] and the vast majority of patients with this type of disease have a history of prodromal flu-like symptoms. It was, therefore, suggested that the preceding flu-like illness leads to the triggering of abnormal antibody production targeting NMDARs [12]. In this situation the presence of ovarian teratoma with a high expression of NMDAR epitopes may predispose or exaggerate the production of anti-NMDAR antibodies; these NMDAR antibodies then cause a specific, titer-dependent, and reversible decrease in NMDAR surface density and synaptic localization, especially in the hippocampus [13], resulting in learning, memory, and other behavioral deficits seen in patients with anti-NMDAR-encephalitis. Thus, this encephalitis seems to be causally related to a parainfectious autoimmune mechanism. Immunosuppressive therapy, including corticosteroid, plasma exchange and IVIg, has been used for the treatment of this disease [1]. Recently, rituximab, an anti-CD20 monoclonal antibody, is expected to accelerate the recovery of patients with this type of disease [14].

Our young male patient with anti-NMDAR encephalitis lacked testicular or mediastinal teratoma, which was previously reported to be of paraneoplastic origin [11]. On the other hand, his clinical course was characterized by a preceding attack of GBS: this disease is a representative post-infectious peripheral demyelinating neuropathy with underlying autoimmune abnormalities [15]. Although a causative relationship between GBS and anti-NMDAR encephalitis has not been studied, it is likely that GBS-related abnormal immune reactions secondarily caused another autoimmune state with anti-NMDAR encephalitis. Although serial examinations of anti-NMDAR antibodies in both serum and CSF might be useful in clarifying the underlying immune condition of this patient, there were no available samples. This case report has provided the clinical evidence that a parainfectious autoimmune reaction is an important pathogenetic mechanism in the development of anti-NMDAR encephalitis.

Acknowledgement

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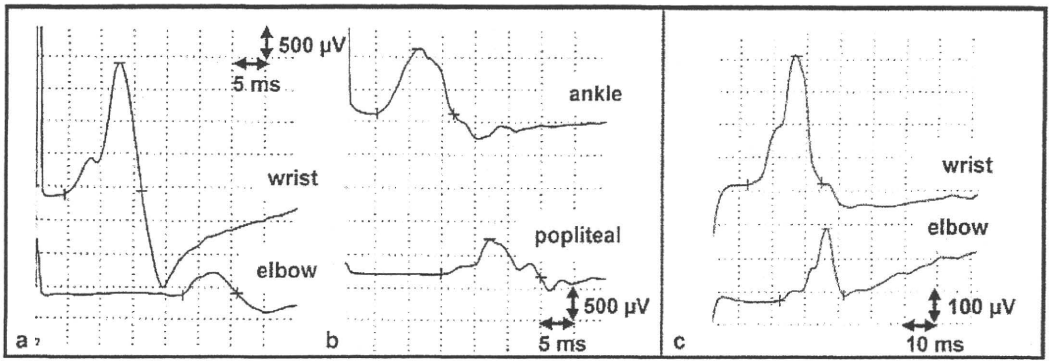

Fig. 1. Motor nerve conduction study of the right median nerve and the right tibial nerve. **a** Right median nerve on the 12th hospital day. **b** Right tibial nerve on the 12th hospital day. These studies showed reduced motor nerve conduction velocities (MCV) with conduction block (median nerve 11.3 m/s, tibial nerve 36.6 m/s). The sizes of the compound muscle action potential of the right median nerve were 2.0 mV at the wrist and 0.33 mV at the elbow, and those of the right tibial nerve were 1.0 mV at the ankle and 0.57 mV at the popliteal fossa, respectively. **c** Motor nerve conduction study of the right median nerve on the 94th hospital day, showing improvement of the MCV (23.2 m/s) and severity of the conduction block (the sizes of the compound muscle action potential were 0.404 mV at the wrist and 0.225 mV at the elbow). MCV of right tibial nerve on the 94th day was not evoked.

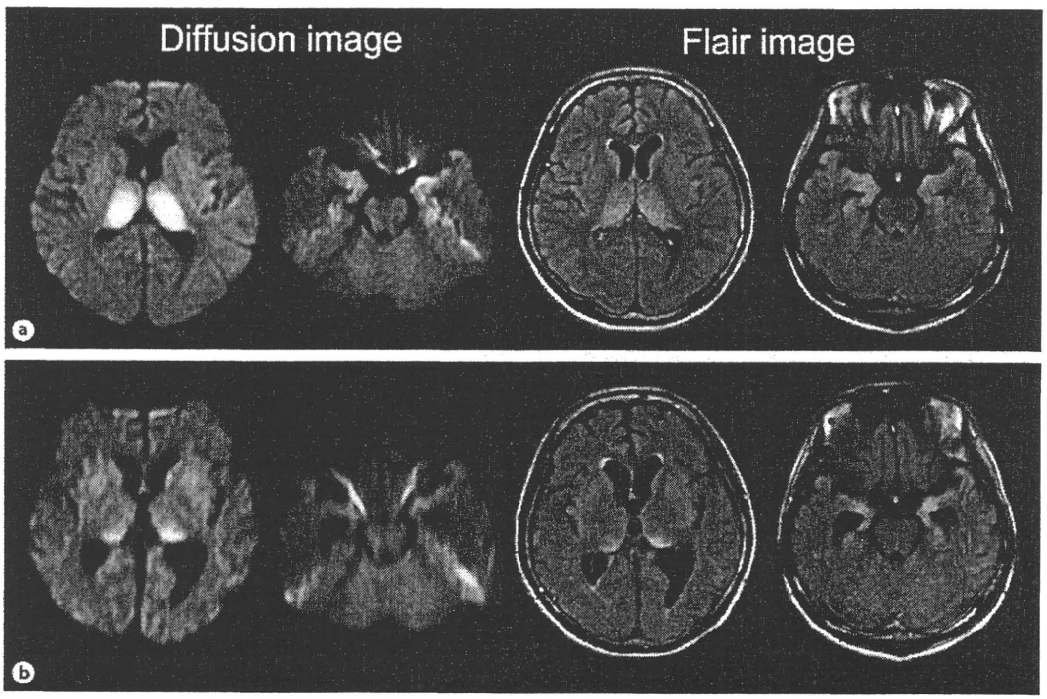

Fig. 2. Brain MRI findings. **a** Images on the 42nd hospital day showed high-intensity lesions in the bilateral thalamus and bilateral medial temporal lobes. **b** Images on the 64th hospital day revealed improved abnormal high-intensity lesions in the bilateral thalamus but progressive atrophy of the bilateral medial temporal lobes.

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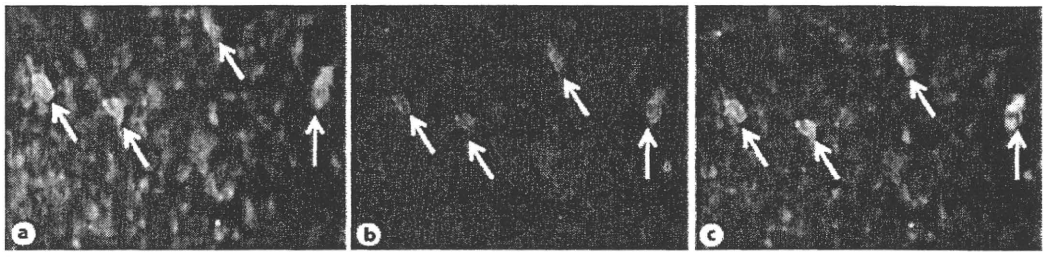

Fig. 3. Immunohistochemical demonstration of antibodies against NMDAR. **a** CSF of the patient showing positive immunoreactivity against heteromers of NR1 and NR2B subunits of NMDAR. **b** Anti-rabbit IgG showing positive immunoreactivity against NR1 subunit of NMDAR. **c** Merge image. Arrows indicate positively stained HEK cells. Immunofluorescence staining (×200).

References

1 Dalmau J, Gleichman A, Hughes EG, Rossi JE, Peng X, Lai M, Dessain S, Rosenfeld MR, Balice-Gordon R, Lynch DR: Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. Lancet Neurol 2008;7:1091–1098.

2 Irani SR, Bera K, Waters P, Zuliani L, Maxwell S, Zandi MS, Friese MA, Galea I, Kullmann DM, Beeson D, Lang B, Bien CG, Vincent A: *N*-methyl-D-aspartate antibody encephalitis: temporal progression of clinical and paraclinical observations in a predominantly non-paraneoplastic disorders of both sexes. Brain 2010;133:1655–1667.

3 Dalmau J, Tüzün E, Wu HY, Masjuan J, Rossi JE, Voloschin A, Baehring JM, Shimazaki H, Koide R, King D, Mason W, Sansing LH, Dichter MA, Rosenfeld MR, Lynch DR: Paraneoplastic *N*-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. Ann Neurol 2007;61:25–36.

4 Vitaliani R, Mason W, Ances B, Zwerdling T, Jiang Z, Dalmau J: Paraneoplastic encephalitis, psychiatric symptoms, and hypoventilation in ovarian teratoma. Ann Neurol 2005;58:594–604.

5 Iizuka T, Sakai F, Ide T, Monzen T, Yoshii S, Iigaya M, Suzuki K, Lynch DR, Suzuki N, Hara T, Dalmau J: Anti-NMDA receptor encephalitis in Japan. Long-term outcome without tumor removal. Neurology 2008;70:504–511.

6 Tachibana N, Shirakawa T, Ishii K, Takahashi Y, Tanaka K, Arima K, Yoshida T, Ikeda S: Expression of various glutamate receptors including *N*-methyl-D-aspartate receptor (NMDAR) in an ovarian teratoma removed from a young woman with anti-NMDAR encephalitis. Intern Med 2010;49:2167–2173.

7 Seki M, Suzuki S, Iizuka T, Shimizu T, Nihei Y, Suzuki N, Dalmau J: Neurological response to early removal of ovarian teratoma in anti-NMDAR encephalitis. J Neurol Neurosurg Psychiatry 2010;79:324–326.

8 Kamei S, Kuzuhara S, Ishihara M, Morita A, Taira N, Togo M, Matsui M, Ogawa M, Hisanaga K, Mizutani T, Kuno S: Nationwide survey of acute juvenile female non-herpetic encephalitis in Japan: relationship to anti-N-methyl-D-aspartate receptor encephalitis. Intern Med 2009;48:673–679.

9 Florance NR, Davis RL, Lam C, Szperka C, Zhou L, Ahmad S, Campen CJ, Moss H, Peter N, Gleichman AJ, Glaser CA, Lynch DR, Rosenfeld MR, Dalmau J: Anti-*N*-methyl-D-aspartate receptor (NMDAR) encephalitis in children and adolescents. Ann Neurol 2009;66:11–18.

10 Niehusmann P, Dalmau J, Rudlowski C, Vincent A, Elger CE, Rossi JE, Bien CG: Diagnostic value of *N*-methyl-D-aspartate receptor antibodies in women with new-onset epilepsy. Arch Neurol 2009;66:458–464.

11 Eker A, Saka E, Dalmau J, Kurne A, Bilen C, Ozen H, Ertoy D, Ogurz KK, Elibol B: Testicular teratoma and anti- *N*-methyl-D-aspartate receptor-associated encephalitis. J Neurol Neurosurg Psychiatry 2008;79:1082–1083.

12 Tzn E, Zhou L, Baehring JM, Bannykh S, Rosenfeld MR, Dalmau J: Evidence for antibody-mediated pathogenesis in anti-NMDAR encephalitis associated with ovarian teratoma. Acta Neuropathol 2009;118:737–743.

13 Hughes EG, Peng X, Gleichman AJ, Lai M, Zhou L, Tsou R, Parsons D, Lynch DR, Dalmau J, Balice-Gordon RJ: Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis. J Neurosci 2010;30:5866–5875.

14 Ishiura H, Matsuda S, Higashihara M, Hasegawa M, Hida A, Hanajima R, Yamamoto T, Shimizu J, Dalmau J, Tsuji S: Response of anti-NMDA receptor encephalitis without tumor to immunotherapy including rituximab. Neurology 2008;71:1921–1922.

15 Griffin JW, Sheikh K: The Guillain-Barré syndrome; in Peripheral Neuropathy 4th ed, Dyck PJ, Thomas PK (eds): Elssevier and Saunders, Philadelphia, 2005, pp 2197–2220.

Unusual association of diseases/symptoms

Atypical Miller Fisher syndrome associated with glutamate receptor antibodies

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Summary

The present study reports a young woman with acute ataxia, areflexia and ophthalmoplegia, accompanied by psychosis and involuntary movements (IVMs) from disease onset. Anti-GQ1b and anti-GT1a antibodies were detected allowing for a diagnosis of Miller Fisher syndrome (MFS). However, psychosis and IVMs are atypical MFS symptoms and often mimic symptoms of anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis. Interestingly, the autoantibodies against full-length glutamate receptor-ε2 (GluRε2) and glutamate NR2B- and NR2A-containing heteromers (NR1/NR2) of NMDAR were also detected in the patient serum and cerebrospinal fluid. It was concluded that psychosis and IVMs in this patient were associated with autoantibodies against various GluRs.

BACKGROUND

Miller Fisher syndrome (MFS) is an autoimmune disorder accompanied by acute progressive ophthalmoplegia, ataxia and areflexia. Bickerstaff's brainstem encephalitis (BBE) is a related syndrome in which central nervous system (CNS) features, such as altered consciousness and/or long tract signs, accompany classic triad.[1 2] Ganglioside GQ1b antibodies are often associated with both conditions, resulting in the proposal that MFS and BBE might be closely related and form a continuous disease.[3] Among the clinical features of both MFS and BBE, psychosis and involuntary movements (IVMs) are atypical.[4] The present case reports a young woman who presented with acute cerebellar ataxia, areflexia and ophthalmoplegia, which was accompanied by psychosis and IVMs from disease onset. Serum serological tests revealed high anti-GQ1b and anti-GT1a antibody titres. In addition, serum and cerebrospinal fluid (CSF) tests also detected high antibody titres against NR2B- and NR2A-containing heteromers of N-methyl-D-aspartate receptor (NMDAR) and antiglutamate receptor-ε2 (GluRε2).

CASE PRESENTATION

A 23-year-old woman presented initially with double vision and a mild, unsteady gait and was admitted to our hospital in May 2008. One week earlier, the patient experienced a respiratory infection. On the first day of illness, the patient suffered from transient double vision and dizziness. The following day, the patient was restless, agitated and cried incomprehensively. Symptoms rapidly exacerbated following hospital admission (day 2); the patient was alert, but exhibited emotional incontinence and personality changes. Extraocular movement was inhibited in all directions and conjugate eye movements were impaired with coarse nystagmus. Facial muscles were weak and the patient presented with slurred speech and dysphagia. Marked cerebellar ataxia was present in the finger-nose and heel-knee test. Muscle tone decreased and deep tendon reflexes of the upper and lower extremities were absent. The Babinski response was bilaterally negative and limb muscle strength and sensory examination were normal.

On day 3, the patient became somnolent and screamed irritably like a child. Both pupils were dilated and light reflexes were diminished. The patient developed orolingual IVMs, such as lip-licking, chewing, dyskinesia and myoclonic movements. Psychosis and IVMs mimicked features of patients with ovarian teratoma-associated encephalitis[5] (video 1). Hypersalivation was also exhibited, but hypoventilation or seizures did not develop.

INVESTIGATIONS

Routine blood analyses, including thyroid functions, were normal. Serum antibodies specific to HIV, syphilis, neurotrophic viruses, *Mycoplasma pneumoniae*, thyroid disease and autoimmune disease (antinuclear, anti-SS-A/SS-B antibodies, MPO-ANCA, PR3-ANCA and anticardiolipin antibodies) were insignificant. CSF analysis revealed a white blood cell count of 14/ml, 24 mg protein/dl and negative PCR results for herpes simplex virus genome. EEGs showed a slightly irregular basic pattern without epileptic discharge. Nerve conduction velocities and compound muscle action potentials were normal, with a significant decrease in F-wave amplitude of median and tibial nerves. Brainstem auditory-evoked potentials and cranial MRI, with or without gadolinium enhancement revealed no abnormalities. Chest, abdominal and pelvic CT were normal. ^{123}I-iodoamphetamine single photon emission CT (^{123}I-IMP SPECT), which was performed on day 13, revealed hypoperfusion in the brainstem, cerebellum, thalamus and frontotemporal cortices. Follow-up ^{123}I-IMP

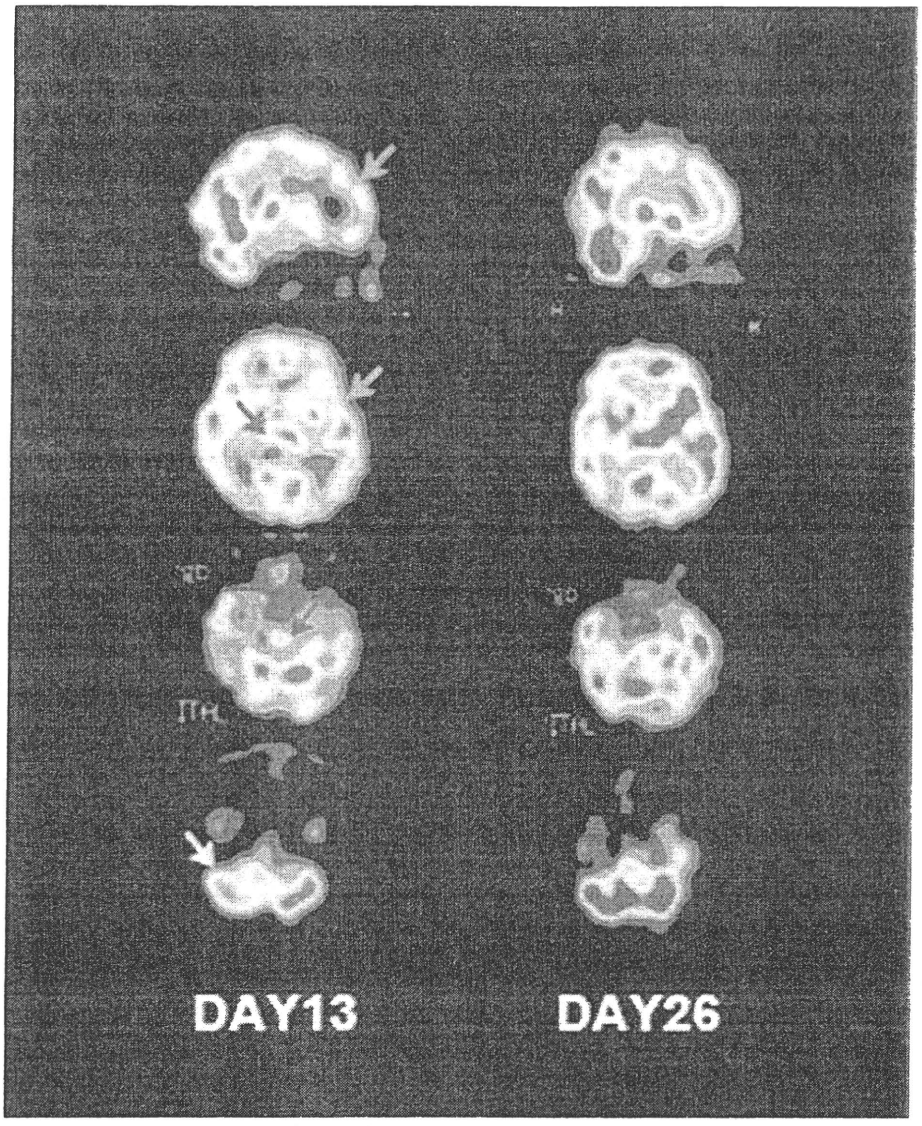

Figure 1 ^{123}I-iodoamphetamine single photon emission CT (^{123}I-IMP SPECT) imaging of the patient. On day 13, ^{123}I-IMP SPECT reveals decreased rCBF in the frontotemporal cortex (green arrows), thalamus (blue arrow), brainstem (red arrow) and cerebellum (yellow arrow) (A). Follow-up ^{123}I-IMP SPECT on day 26 shows improvement in the damaged lesion (B).

SPECT on day 26 demonstrated perfusion recovery in these lesions (figure 1).

Video 1 The patient exhibits ophthalmoplegia, coarse nystagmus, sluggish papillary light reflex and involuntary movements around the eyebrows and mouth. The patient also screams irritably like a child.10.1136/bcr.08.2010.3228v1

DIFFERENTIAL DIAGNOSIS AND TREATMENT

These findings suggested the diagnosis of atypical MFS-related disorder accompanied by psychosis and IVMs. Therefore, the patient was administered intravenous immunoglobulin (Ig) (20 g/day for 5 days), followed by intravenous methylprednisolone (1 g/day for 3 days). On day 10, the patient developed rough and coarse IVMs, which affected the left arm, despite improved psychiatric behaviour. Subsequently, the patient received intravenous methylprednisolone, accompanied by steroid tapering.

ELISA revealed that serum samples from day 3 contained high IgG antibody titres to GQ1b and GT1a. Furthermore, antibodies to full-length GluRε2 (anti-GluRε2 antibodies) and glutamate NR2B- and NR2A-containing heteromers of NMDAR (anti-NR1/NR2 antibodies) were examined as described previously.[6] Anti-NR1/NR2 antibodies, as well as anti-GluRε2 IgG and IgM antibodies, were expressed in serum and CSF on day 3. On day 16, serum anti-GluRε2 IgM antibody levels were reduced, but IgG antibodies remained present.

OUTCOME AND FOLLOW-UP

Although the symptoms lasted for 2 weeks, improvement was considerable with intact neurological symptoms within 6 weeks of disease onset. After 1 year, the patient exhibited no neurological complications and pelvic MRI was normal.

DISCUSSION

Clinical findings fulfilled diagnostic criteria for MFS.[3] However, the patient also presented with atypical clinical features, such as psychosis and IVMs. In addition, anti-NR1/NR2 antibodies and anti-GluRε2 antibodies were present. ^{123}I-IMP SPECT examinations demonstrated that patient symptoms were due to impaired cortex, thalamus, cerebellum and brainstem. Interestingly, Wada *et al*, also described two BBE patients with delirium[4]; both patients fulfilled the diagnostic criteria for BBE, including positive serum titres for anti-GQ1b and -GT1a antibodies, as well as childish behaviours and emotional incontinence. The patients also exhibited rigidity and IVMs, such as tremors and hypertonia in the masseter muscles, which mimicked tetanus. SPECT analysis revealed hypoperfusion of the frontal lobe, brainstem and basal ganglia, which was similar to results from the present study. Although atypical psychosis and IVMs in these patients were possibly due to autoantibodies against various GluRs, including anti-GluRε2 antibodies and anti-NR1/NR2 antibodies, the association between these antibodies was not discussed in the paper.

Dalumau *et al* reported that limbic encephalitis is a result of anti-NR1/NR2 antibodies associated with ovarian teratoma, which has been termed anti-NMDAR encephalitis.[5] Clinical characteristics of patients with anti-NMDAR encephalitis include psychosis, seizures, IVMs, autonomic instability and central hypoventilation. Anti-GluRε2 antibody is described as an association between non-herpetic limbic encephalitis[6–8] and epilepsia partialis continua-related Rasmussen encephalitis.[9] Several studies have shown that anti-GluRε2 and anti-NR1/NR2 antibodies are detectable in patients with non-herpetic limbic encephalitis.[7 8] Kamei *et al* reported the clinical features of acute juvenile female non-herpetic encephalitis (AJFNHE) in Japan based on a nationwide questionnaire,[8] with a detection rate of autoantibodies against several GluRs of 67%. Clinical AJFNHE phenotypes closely mimic anti-NMDAR encephalitis, including psychosis and IVMs, and anti-NR1/NR2 antibodies have been detected in several patients with AJFNHE.[8] The GluRε2 subunit NR2A functions as an NMDAR component. Huerta *et al* reported that NR2 autoantibodies induce emotional abnormality in mice.[10] These studies suggested that autoantibodies against some GluR subunits could result in psychosis and IVMs in our patient.

The mechanism by which ganglioside (anti-GQ1b and anti-GT1a) and GluR antibodies were co-expressed in the patient, thereby triggering clinical features, remains unknown. There is a high prevalence of prodromal viral-like symptoms in anti-NMDAR encephalitis and AJFNHE.[5 8] Moreover, Gable *et al* reported that 4/10 patients with anti-NMDAR encephalitis exhibit serological evidence of acute Mycoplasma infection.[11] Although the number of cases in this study was very small, with a high amount of false IgM positivity results, Mycoplasma infection should be considered, as Mycoplasma could contain epitopes mimicking both GQ1b ganglioside and GluRs. The patient did not express Mycoplasma-specific IgM antibodies; however, a prodromal respiratory infection was diagnosed. Therefore, it is possible that a microorganism could trigger antibody production against these antigens, as detected in the patient.

Learning points

- Patients with MFS and related disorders rarely present with atypical psychosis and IVMs such as delirium, oral dyskinesia, tremor or myoclonus.
- CNS involvement, such as psychosis and IVMs in patients with atypical MFS-related disorders could be associated with various anti-GluR antibodies.

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Competing interests None.

Patient consent Obtained.

REFERENCES

1. **Odaka M,** Yuki N, Yamada M, *et al*. Bickerstaff's brainstem encephalitis: clinical features of 62 cases and a subgroup associated with Guillain-Barré syndrome. *Brain* 2003;**126**(Pt 10):2279–90.
2. **Overell JR,** Willison HJ. Recent developments in Miller Fisher syndrome and related disorders. *Curr Opin Neurol* 2005;**18**:562–6.
3. **Odaka M,** Yuki N, Hirata K. Anti-GQ1b IgG antibody syndrome: clinical and immunological range. *J Neurol Neurosurg Psychiatr* 2001;**70**:50–5.
4. **Wada Y,** Yanagihara C, Nishimura Y, *et al*. Delirium in two patients with Bickerstaff's brainstem encephalitis. *J Neurol Sci* 2008;**269**:184–6.
5. **Dalmau J,** Gleichman AJ, Hughes EG, *et al*. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. *Lancet Neurol* 2008;**7**:1091–8.
6. **Tachibana N,** Shirakawa T, Ishii K, *et al*. Expression of various glutamate receptors including N-methyl-D-asparate receptor (NMDAR) in an ovarian teratoma removed from a young woman with anti-NMDAR encephalitis. *Inter Med* 2010;**49**:2167–73.
7. **Takahashi Y.** Epitope of autoantibodies to N-methyl-D-aspartate receptor heteromers in paraneoplastic limbic encephalitis. *Ann Neurol* 2008;**64**:110–11; author reply 111–12.
8. **Kamei S,** Kuzuhara S, Ishihara M, *et al*. Nationwide survey of acute juvenile female non-herpetic encephalitis in Japan: relationship to anti-N-methyl-D-asparate receptor encephalitis. *Inter Med* 2009;**48**:673–9.
9. **Takahashi Y,** Mori H, Mishina M, *et al*. Autoantibodies and cell-mediated autoimmunity to NMDA-type GluRε2 in patients with Rasmussen's encephalitis and chronic progressive epilepsia partialis continua. *Epilepsia* 2005;**46**:152–8.
10. **Huerta PT,** Kowal C, DeGiorgio LA, *et al*. Immunity and behavior: antibodies alter emotion. *Proc Natl Acad Sci USA* 2006;**103**:678–83.
11. **Gable MS,** Gable S, Radner A, *et al*. Anti-NMDA receptor encephalitis: report of ten cases and comparison with viral encephalitis. *Eur J Clin Microbiol Infect Dis* 2009;**28**:1421–9.

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ORIGINAL ARTICLE

Increase of tumor necrosis factor-α in the blood induces early activation of matrix metalloproteinase-9 in the brain

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ABSTRACT

Increases of cytokine in the blood play important roles in the pathogenesis of influenza-associated encephalopathy. TNF-α was administered intravenously to wild-type mice, after which blood, CSF and brain tissue were obtained, and changes in BBB permeability, the amounts of MMP-9 and TIMP-1, and the localization of activated MMP were assessed. There was a significant increase in BBB permeability after 6 and 12 hr. MMP-9 was increased after 3 hr in the brain and cerebrospinal fluid, which was earlier than in the serum. TIMP-1 protein in the brain increased significantly after MMP-9 had increased. Activation of MMP-9 was observed in neurons in the cerebral cortex and hippocampus, and in vascular endothelial cells. These findings suggest that an increase in blood TNF-α promotes activation of MMP-9 in the brain, and may also induce an increase in permeability of the BBB. Early activation of MMP-9 in the brain may contribute to an early onset of neurological disorders and brain edema prior to multiple organ failure in those inflammatory diseases associated with highly increased concentrations of TNF-α in the blood, such as sepsis, burns, trauma and influenza-associated encephalopathy.

Key words blood-brain barrier, influenza associated encephalopathy, matrix metalloproteinase-9, tumor necrosis factor-alpha.

TNF-α is a pleiotrophic pro-inflammatory cytokine which is produced by various cells including activated monocytes, macrophages, B and T cells and fibroblasts. TNF-α plays a role in the induction of septic shock, autoimmune diseases, rheumatoid arthritis, inflammation and diabetes. TNF-α can change the permeability of the BBB where there is inflammation in the brain, as observed in meningitis (1), brain abscess (2) or brain ischemia (3).

MMP belong to a family of zinc-dependent proteolytic enzymes which are capable of degrading the components of the extracellular matrix in a variety of physiological and pathological conditions including embryogenesis, cell migration, tissue modeling, wound healing, and inflammation (4, 5). MMP are secreted as inactive pro-forms which are activated in proteolytic cascade reactions to yield active MMP. An uncontrolled increase in active MMP can result in tissue injury and persistent inflammation. MMP-9, a member of this enzyme family, is capable of degrading collagen IV, a major component of the basal membrane of the cerebral vascular endothelium, while also promoting a disruption of the BBB. MMP-9 is involved in acute brain injury such as cerebral ischemia (6–9), intracerebral hemorrhage (10, 11), neurodegenerative diseases (12, 13) and bacterial infections (14–16). In addition, MMP-9 is

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List of Abbreviations: BBB, blood-brain barrier; CSF, cerebrospinal fluid; DQ, dye-quenched; GFAP, glial fibrillary acidic protein; FITC, fluorescein isothiocyanate; IL-6, interleukin-6; IL-1β, interleukin-1β; MMP, matrix metalloproteinase; OCT, optimal cutting temperature; TIMP, tissue inhibitor of matrix metalloproteinase; TNF-α, tumor necrosis factor-alpha.

produced in the brain by several cell types, including endothelial cells, microglia, astrocytes, and neurons. MMP-9 activity is strictly regulated via gene transcription and dynamic inhibition by TIMP-1.

Acute encephalopathy, a severe complication of influenza infection in children, often results in severe brain edema and a grave prognosis (17). Several studies have demonstrated that concentrations of inflammatory cytokines, such as TNF-α, interleukin-6 and interleukin-1β, are increased in the serum or CSF of these patients (18–20). Interestingly, although neurological symptoms can occur at an early stage and a severe prognosis can easily follow, no influenza virus is found in the brain. Although these facts suggest that inflammatory cytokines are involved in the development of neurological injury in influenza-associated encephalopathy, the mechanism is still unclear. In the acute phase, serum concentrations of MMP-9 are much greater in influenza-associated encephalopathy than in influenza infection with no neurologic complications (21).

Although *in vitro* TNF-α can induce MMP-9 activation in various cells, such as macrophages, granulocytes, astrocytes, neurons, microglial cells, brain endothelial cells and choroid plexus epithelial cells, there have so far been no reports describing whether, *in vivo*, MMP-9 is actually activated in the brain in response to an increase of serum TNF-α. The current study evaluated time-dependent changes in the amount of MMP-9 in brain tissue, serum and CSF to determine the relationships between increases in serum TNF-α and activation of MMP-9 in the brain. In addition, under conditions of experimental hypercytokinemia with TNF-α *in vivo*, this study also showed changes in the brain in both TIMP-1 protein and localization of increased MMP.

MATERIALS AND METHODS

Animals

Eight-week-old male C57BL/6 mice weighing 25–30 g were obtained from Japan SLC, Shizuoka, Japan. The animals were housed in an air-conditioned room with a 12-hr light/dark cycle and free access to food and water. All procedures involving the animals were conducted in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center.

Treatment of animals and tissue preparation

One hundred μg/kg of murine recombinant TNF-α, IL-6 or IL-1β (Pepro Tech EC, London, UK) was injected intravenously into mice. Some mice were treated with saline as a vehicle control using the same volume and time schedule as for the cytokine treatments. The mice were deeply anesthetized with 1% halothane in 30% oxygen and 70% nitrous oxide using a face mask at 0, 3, 6, 12, 24 and 48 hr after the intravenous injection, and both blood and CSF quickly collected. They were then perfused transcardially with ice-cold PBS, after which their brains were quickly removed, divided into hemispheres, immediately frozen in liquid nitrogen, and stored at −80°C. After complete coagulation the blood samples were centrifuged at 3000 *g* for 10 min. The supernatants were collected and stored at −80°C. CSF samples were directly stored at −80°C.

Quantitative evaluation of extravasation of Evans blue

Vascular permeability was quantitatively evaluated using Evans blue dye. 2% Evans blue (Wako, Osaka, Japan) in saline was injected intravenously (4 ml/kg) as a BBB permeability tracer 1 hr prior to the termination of each time point ($n = 5$ per time point). The mice were killed one hr after this injection and then transcardially perfused with 100 ml of ice-cold PBS to remove the intravascular dye. The brains were removed, homogenized in 1 ml of 50% trichloroacetic acid, and then centrifuged (10 000 *g*, 20 min). A spectrophotometer was used to quantify the dye concentrations at 605 nm. The calculations were based on external standards (50–1000 μg/ml) dissolved in the same solvent. The amount of extravasated Evans blue was quantified as one microgram per gram of brain.

MMP inhibition *in vivo*

Intraperitoneal GM6001 (Calibiochem, San Diego, CA, USA) at 65 mg/kg or vehicle (1.5 ml of 10% dimethyl sulfoxide) was injected 1 hr before administration of TNF-α. Six hours after the administration of TNF-α, 2% Evans blue dye was injected intravenously and the brains dissected out 1 hr later.

Protein extraction

All procedures were carried out at 4°C. The brains were homogenized in a Polytron homogenizer (Paterson, NJ, USA) in 2 ml of working buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 1% Triton X-100, 0.05% BRIJ-35) and centrifuged, then the supernatants were stored at −80°C for further analysis. The total protein concentration was measured using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The total protein concentration of the serum samples was also measured using the same method.

ELISA

ELISA was used to measure the amounts of MMP-3, MMP-9 and TIMP-1 in order to detect the proteins that were expressed in the brain ($n = 5$ per time point). The MMP-9 assay recognizes both the pro- and active forms of MMP-9. Each capture antibody, the biotinylated detection antibodies, protein standards, and detection reagents for the ELISA were obtained from R&D Systems (Minneapolis, MN, USA).

Gelatin zymography

Equal amounts of prepared protein samples were diluted in twice the volume of sample buffer (125 mM Tris-HCl, 20% glycerol, 4% SDS, 0.003% bromophenol blue) without β-mercaptoethanol and were then loaded onto 10% SDS-PAGE gels containing 1% gelatin under non-reducing conditions. The gels were separated by electrophoresis at 4°C. After electrophoresis, the gels were washed twice for 20 min each in renaturing buffer containing 2.5% Triton X-100 at room temperature to remove any SDS, and were then incubated for 24 hr in developing buffer (10 mM CaCl2, 50 mM Tris, 50 mM NaCl) at 37°C. After incubation, the gel was stained for 1 hr with 0.5% Coomassie blue G-250 in 40% methanol and 10% acetic acid and was then destained in the same buffer without dye.

In situ gelatin zymography

In situ gelatin zymography was performed with DQ-gelatin-FITC (Molecular Probes, Eugene, OR, USA) on frozen fresh brain slices obtained from the mice. The brains were dissected out, immediately embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen on dry ice. Ten μm thick coronal sections were cut using a cryostat, and then air-dried for 30 min at room temperature, re-hydrated in PBS and incubated overnight at 37°C with 40 μg/ml of DQ-gelatin-FITC in PBS. Excess fluorogenic substrate was removed by washing three times in PBS. The brain slices were incubated with DQ-gelatin-FITC including the MMP inhibitor GM6001 (Calibiochem) to determine whether the gelatinolytic activity was due to matrix metalloproteinases. After *in situ* gelatin zymography had been done, immunohistochemistry was performed on the same brain sections to assess the cellular localization of gelatinases. Neurons were stained with NeuroTrace fluorescent Nissl stain solution (1:100; Molecular Probes), astrocytes with anti-GFAP antibody (1:200; Molecular Probes) and cerebral vascular endothelial cells with anti-claudin-5 H-52 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After blocking with 2% BSA the sections were incubated for 20 min at room temperature with NeuroTrace fluorescent Nissl stain solution to stain the neurons. After washing in PBS, the sections were incubated for 2 hr at room temperature. The sections were treated with primary antibodies for astrocytes or vascular endothelial cells for 2 hr at 37°C after blocking. The sections were then washed in PBS, and incubated with secondary antibody solutions (anti-rabbit Alexa Fluor 594, 1:200; Molecular Probes) for 30 min at 37°C. Finally, the slides were washed in PBS and mounted on Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). The tissue sections were photographed by fluorescence microscopy (Keyence BZ-9000) using the Keyence BZ-2 system (Keyence, Osaka, Japan).

Statistical analysis

All analyses were performed using the SPSS for Windows software package (SPSS, Chicago, IL, USA). Quantitative data were expressed as the mean ± d standard deviation. Statistical comparisons were conducted using ANOVA followed by the Tukey–Kramer tests for multiple comparisons. Differences with a P value of < 0.05 were considered to be statistically significant.

RESULTS

Quantitative evaluation of Evans blue extravasation

Changes in vascular permeability of the BBB were observed by measuring the amount of Evans blue leaking into the brain from the blood. Intravenous murine TNF-α induced a significant increase in Evans blue leaking outside the blood vessels 6, 12 and 24 hr later, in comparison to that found at the time of TNF-α injection ($P < 0.05$; Fig. 1). Intravenous saline as control produced no change in the amount of extravasated Evans blue up until 48 hr later. In comparison to the controls, significant increases in Evans blue extravasation were also seen both 6 and 12 hr after TNF-α injection ($P < 0.05$). Murine recombinant IL-6 and IL-1β administered in the same way had no effect on the amount of extravasated Evans blue into the brain 24 and 48 hr later (Fig. 2a, b).

Effect of MMP inhibition on Evans blue extravasation

GM6001 is a potent broad-spectrum inhibitor of matrix metalloproteinases and has been used in a number of animal models of disease where matrix metalloproteinases are thought to be involved. GM6001 solution was administered intraperitoneally prior to intravenous injection of TNF-α. Extravasated dye in the brain was measured 6 hr after injection of TNF-α. The amount of extravasated

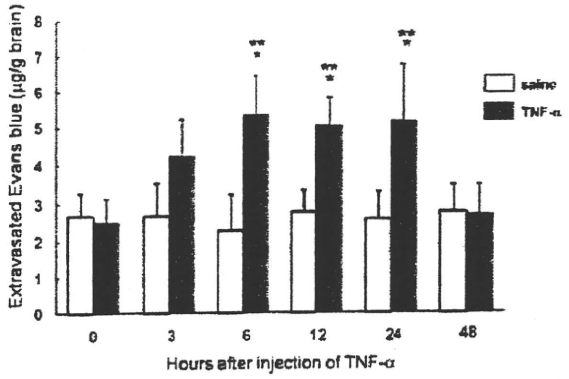

Fig. 1. Quantitative evaluation of Evans blue extravasation in the brain after intravenous injection of TNF-α (100 μg/kg). Extravasated Evans blue is expressed as μg/g of brain tissue. The values represent the mean ± standard deviation ($n = 5$ per time point). *$P < 0.05$, in comparison to 0 hr after TNF-α administration; **$P < 0.05$, in comparison to saline administration at the each time point.

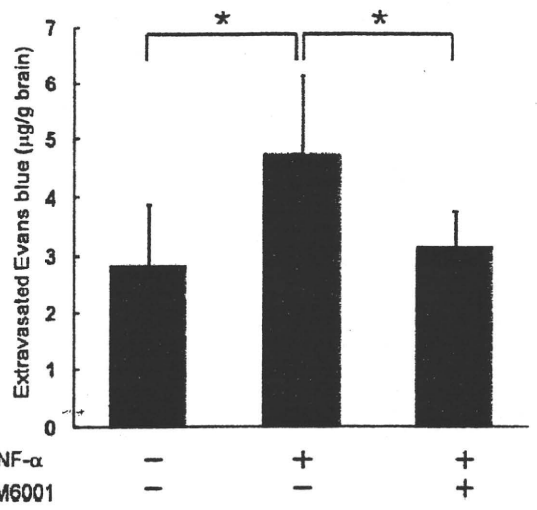

Fig. 3. Inhibition of the increase in Evans blue extravasation after intravenous injection of TNF-α (100 μg/kg) by intraperitoneal administration of an MMP inhibitor (GM6001). The values represent the mean ± standard deviation ($n = 8$ per time point). *$P < 0.05$.

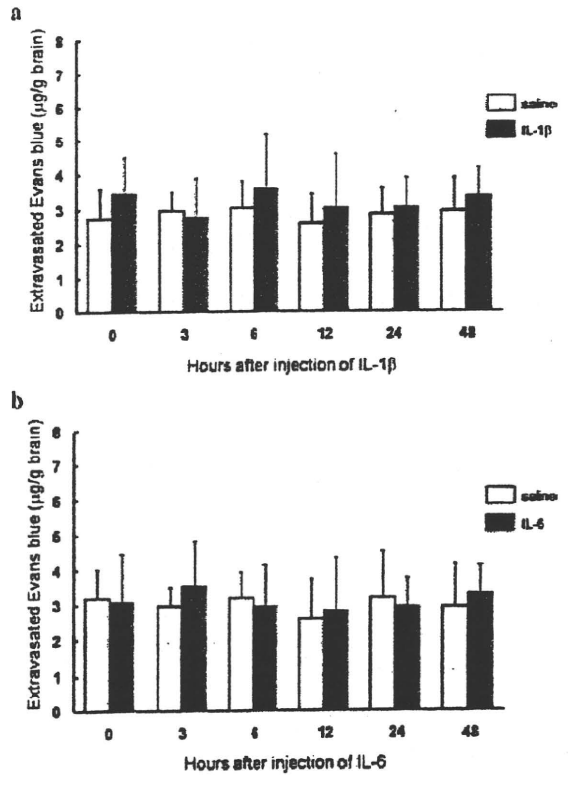

Fig. 2. Quantitative evaluation of Evans blue extravasation after intravenous injection of (a) IL-6 (100 μg/kg) and (b) IL-1β (100 μg/kg). The amount of extravasated Evans blue dye had not changed by 48 hr after the injection ($n = 5$ per time point).

dye was found to be significantly less in the brains of mice treated with GM6001 solution than in mice treated with solvent solution only ($P < 0.05$; Fig. 3).

MMP-3, MMP-9 and TIMP-1 protein in the brain

Time-dependent changes in MMP-9 and TIMP-1 proteins in the brain after TNF-α injection were evaluated using ELISA. The assay for MMP-9 was designed to measure total mouse MMP-9 (pro-MMP-9, active MMP-9 and TIMP-complexed MMP-9). MMP-9 protein in the brain was increased significantly at 3 hr post injection ($P < 0.01$), and gradually decreased thereafter (Fig. 4a). TIMP-1 protein in the brain was significantly increased at 12 hr post injection ($P < 0.01$), but had decreased significantly by 24 hr post injection (Fig. 4b). Time-dependent changes in MMP-3 protein in the brain were also evaluated using ELISA. MMP-3 protein was not detected in the brain (Fig. 4c). MMP-3 protein was detected from 0 to 48 hr in the serum, but was not significantly increased.

MMP-2 and MMP-9 in the brain, serum and CSF

After electrophoresis under non-reducing conditions, gelatin zymography classically produces two bands for murine MMP-2 (proenzyme and active forms; 72 and 65 kDa, respectively) and murine MMP-9 (proenzyme and active forms; 105 and 97 kDa, respectively) on the basis of molecular weight. Time-dependent changes in MMP-9 in the brain, serum and CSF were evaluated using

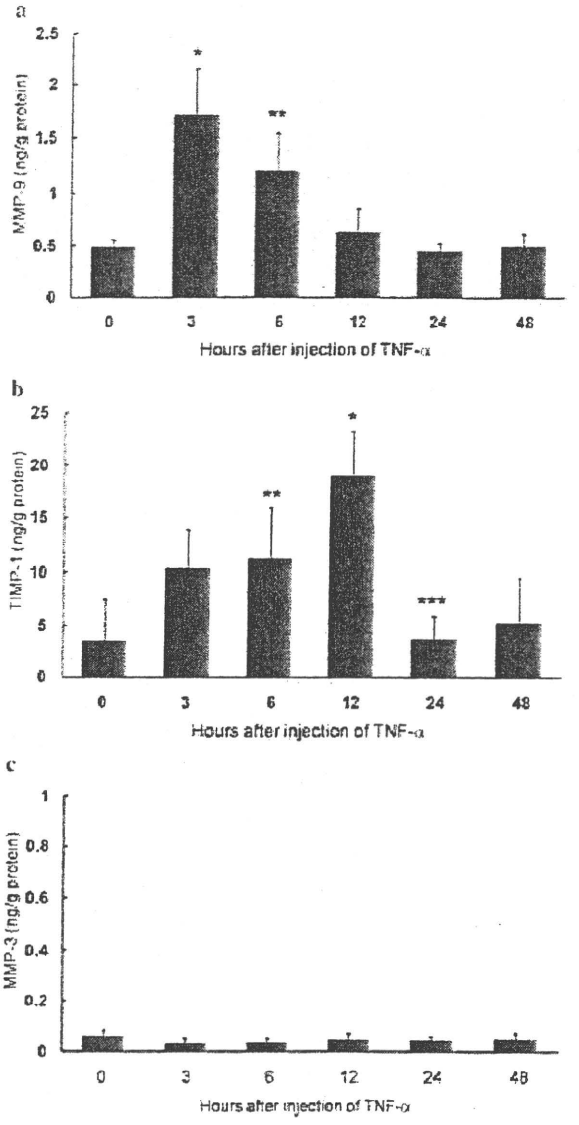

Fig. 4. Time-dependent changes in the amounts of (a) MMP-9, (b) TIMP-1 and (c) MMP-3 protein in the brain after intravenous injection of TNF-α as quantified by ELISA. The amount of each protein is expressed as ng/g of brain tissue. The values represent the mean ± standard deviation ($n = 5$ per time point). *$P < 0.01$, in comparison to at the time of TNF-α administration; **$P < 0.05$, in comparison to at the time of TNF-α administration; ***$P < 0.01$, in comparison to 12 hr after TNF-α administration.

gelatin zymography. MMP-9 increased predominantly in the active form in the brain, serum and CSF. The amounts of active MMP-9 in the brain and CSF peaked 3 hr after injection (Fig. 5). In comparison, the amount of active-MMP-9 in the serum was increased at 6 hr after injection. MMP-2 degrades gelatin as well as MMP-9. Active MMP-2 was continuously detectable in the brain and CSF from

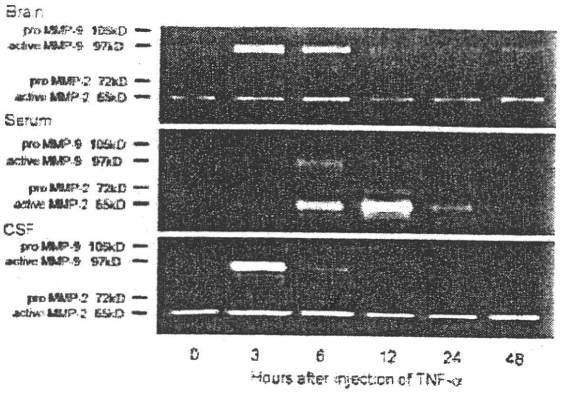

Fig. 5. Gelatin zymography after intravenous injection of TNF-α (100 μg/kg). Active MMP-9 was detectable in the brain, serum and CSF. MMP-9 increased transiently after the injection, and active MMP-9 in the brain and CSF were detected earlier than in the serum. MMP-2 was continuously detectable and demonstrated no significant change in the brain and CSF until 48 hr later, but MMP-2 increased in the serum at 6 and 12 hr post injection.

0 to 48 hr and did not seem to change significantly after administration of TNF-α. In contrast, both forms of MMP-2 were strongly detectable in the serum at 12 hr post injection. No other bands were detected by gelatin zymography.

Localization of gelatinases in the brain

MMP-9 can degrade not only collagen VI, but also gelatin. *In situ* gelatin zymography was used to determine in which parts of the brain gelatinases increased after TNF-α injection. After the brain had been put on gelatin labeled with FITC and incubated, the sites where there were activated gelatinases could be detected by the presence of digestion of the gelatin. We found that gelatinolytic activity was increased in the brain 3 hr later. In addition, *in situ* gelatin zymography with MMP inhibitor showed a decrease in most FITC signals (Fig. 6). Double staining with Nissl dye demonstrated that gelatinolytic activation was localized to the neurons of the cerebral cortex and hippocampus (Fig. 7). Activation was also detected in vascular endothelial cells by double staining with claudin-5. Astrocytes were found to come into contact with, or be surrounded by, cells that were positive for gelatinase activity, rather than being colocalized with this activity.

DISCUSSION

An increase in the vascular permeability of the BBB was induced by intravenous administration of murine recombinant TNF-α *in vivo*. Intracranial administration of TNF-α increases the permeability of the BBB *in vivo* (22). In

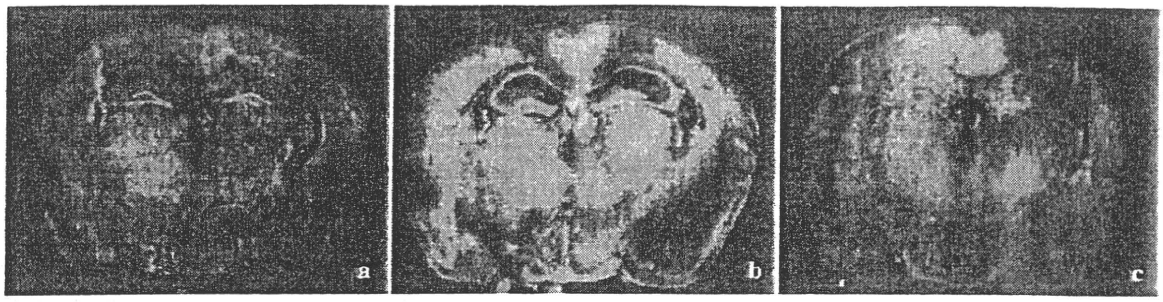

Fig. 6. *In situ* gelatin zymography of the brains after intravenous injection of TNF-α. *In situ* gelatin zymography was performed (a) at the time of injection, (b) 3 hr after injection and (c) with MMP inhibitor at 3 hr after the injection.

addition, an increase in BBB permeability was visualized by electron microscopy after intravenous administration of TNF-α (23). Our results are consistent with those of previous reports, suggesting that increased serum TNF-α can affect the permeability of the BBB.

An increase in MMP-9 protein, but not other MMP, in the brain was observed after intravenous administration of TNF-α, while MMP-9 gradually decreased after this injection. In addition, TIMP-1 protein, which can deactivate the gelatinase activity of MMP-9, increased in the brain after intravenous administration of TNF-α, the increase in TIMP-1 occurring later than that in MMP-9. TIMP-1 is produced in macrophages, brain endothelial cells, astrocytes and microglia *in vitro*, and TIMP-1 protein has been reported to be increased in the brain in studies of bacterial meningitis model mice (24) and herpes simplex virus encephalitis (25). The current results are consistent with those of previous reports, supporting the view that TIMP-1 protein is produced in the brain in response to a prior increase in MMP-9 protein.

An increase in serum TNF-α in promoted a transient increase in active MMP-9 in the brain, serum and CSF, and this active MMP-9 increased earlier in the brain and CSF than in the blood. Intracranial injection of TNF-α or the TNF superfamily can promote an increase in MMP-9 in the brain *in vivo* (22, 26). The current study suggests that active MMP-9 can increase in the brain in response to TNF-α transferred from blood vessels across the BBB. The time-lag of the increase in MMP-9 between brain and serum has not previously been estimated. This result may be due to differences between the cell types in response to stimulation by TNF-α. It is known that neurological symptoms can occur rapidly prior to multiple organ failure in influenza-associated encephalopathy. An early increase in active MMP-9 in the brain might therefore contribute to the induction of early neurological symptoms prior to the development of systemic inflammation.

Gelatinases were activated in neurons of the cerebral cortex and hippocampus and in vascular endothelium after administration of TNF-α. A decrease in gelatinolytic activity was observed after incubation with an MMP inhibitor; MMP are probably the dominant gelatinases activated in neurons and endothelial cells. Although both MMP-2 and MMP-9 contribute to *in situ* gelatin zymography, whereas MMP-2 was detected in the brain, there was no change in the amount of MMP-2 until 48 hr according to gelatin zymography. The results suggest that the increase in FITC signals in the brain at 3 hr post injection in comparison to that at the time of injection is probably due to activation of MMP-9. MMP-9 is activated in the walls of blood vessels and in neurons and astrocytes of the hippocampus in a mice model of cerebral ischemia (27), and in intracerebral hemorrhage (28), herpes simplex virus encephalitis (25) and bacterial meningitis (14). It is not clear whether activation of MMP-9 in the neurons led directly to digestion of the basal lamina of the BBB in the current study. Strong activation of MMP-9 in neurons may contribute to neuronal dysfunction rather than breakage of the basal lamina.

In summary, an increase in serum TNF-α can promote early activation of MMP-9 in the brain. This report supports the hypothesis that an increase in serum TNF-α can alter the permeability of the BBB by activating MMP-9 in the brain. The acute onset of neurological symptoms, such as influenza-associated encephalopathy, can often be seen in human patients. Although the influenza virus can result in inflammation in the lung separately from that in the brain, severe neurological symptoms can subsequently be observed in the absence of direct viral invasion of the brain. Our results have led us to believe that an increase in serum TNF-α can contribute to the development of neurological symptoms in influenza encephalopathy.

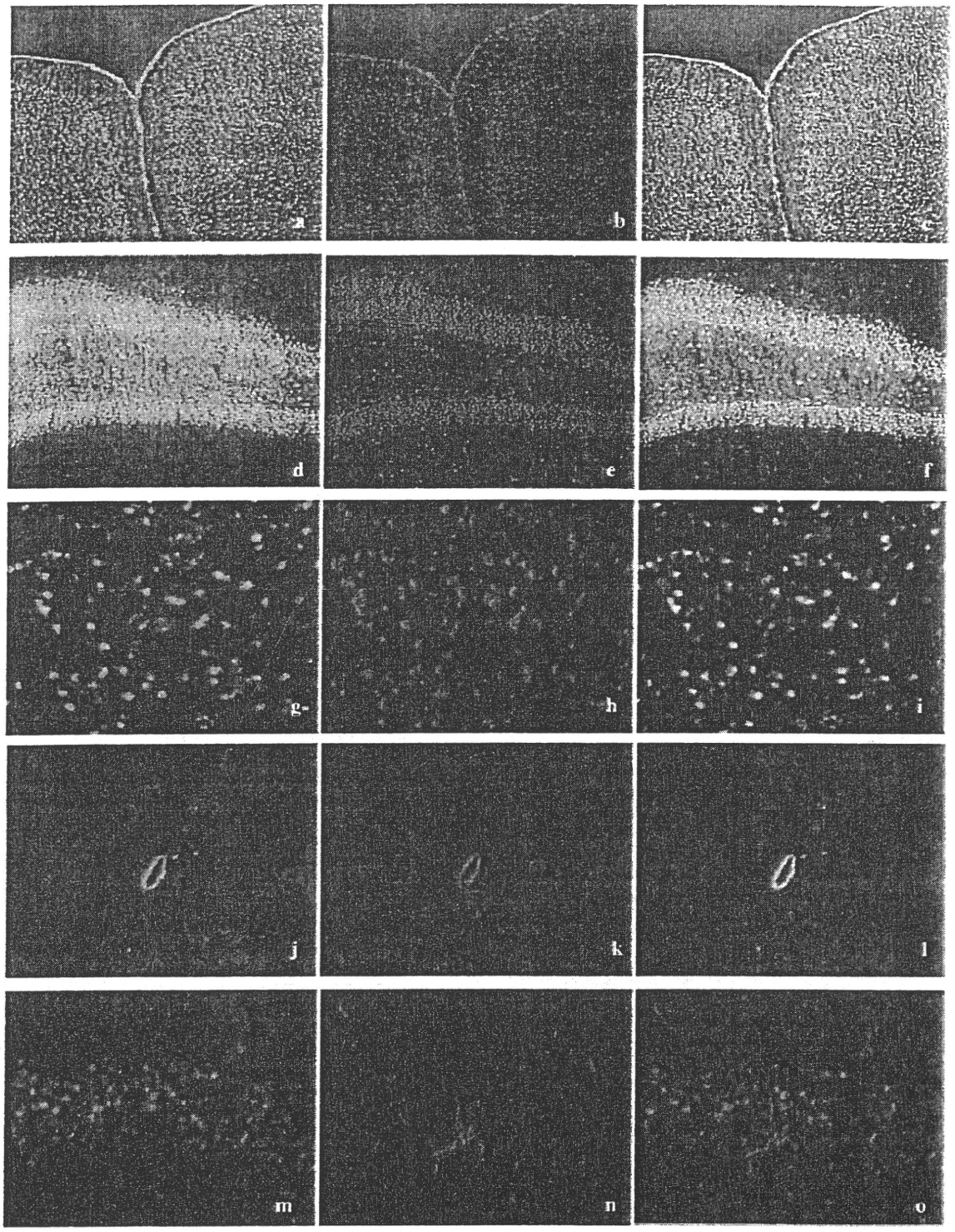

Fig. 7. (a, d, g, j, m) *In situ* gelatin zymography of the brains 3 hr after injection. (b, e, h) Doublestained with fluorescent Nissl stain, (k) with claudin-5 antibody and (n) with GFAP antibody. (c, f, i, l, o) Merged imaging. Co-localization of gelatinolytic activation in Nissl-stained neurons was observed (a–c) in the cerebral cortex and (d–f) hippocampus. Panels g–i show high magnifications of panels d–f respectively. Gelatinolytic activation was also recognized (j–l) in vascular endothelial cells, but (p–r) no co-localization was observed with GFAP-positive astrocytes.

REFERENCES

1. Kieseier B.C., Paul R., Koedel U., Seifert T., Clements J.M., Gearing A.J., Pfister H.W., Hartung H.P. (1999) Differential expression of matrix metalloproteinases in bacterial meningitis. *Brain* **122:** 1579–87.
2. Baldwin A.C., Kielian T. (2004) Persistent immune activation associated with a mouse model of Staphylococcus aureus-induced experimental brain abscess. *J Neuroimmunol* **151:** 24–32.
3. Yang G.Y., Gong C., Qin Z., Liu X.H., Lorris Betz A. (1999) Tumor necrosis factor alpha expression produces increased blood-brain barrier permeability following temporary focal cerebral ischemia in mice. *Brain Res Mol Brain Res* **69:** 135–43.
4. Yong V.W., Power C., Forsyth P., Edwards D.R. (2001) Metalloproteinases in biology and pathology of the nervous system. *Nat Rev Neurosci* **2:** 502–11.
5. Rosenberg G.A. (2002) Matrix metalloproteinases in neuroinflammation. *Glia* **39:** 279–91.
6. Romanic A.M., White R.F., Arleth A.J., Ohlstein E.H., Barone F.C. (1998) Matrix metalloproteinase expression increases after cerebral focal ischemia in rats: inhibition of matrix metalloproteinase-9 reduces infarct size. *Stroke* **29:** 1020–30.
7. Fujimura M., Gasche Y., Morita-Fujimura Y., Massengale J., Kawase M., Chan P.H. (1999) Early appearance of activated matrix metalloproteinase-9 and blood-brain barrier disruption in mice after focal cerebral ischemia and reperfusion. *Brain Res* **842:** 92–100.
8. Lee S.R., Tsuji K., Lee S.R., Lo E.H. (2004) Role of matrix metalloproteinases in delayed neuronal damage after transient global cerebral ischemia. *J Neurosci* **24:** 671–8.
9. Lee H., Park J.W., Kim S.P., Lo E.H., Lee S.R. (2009) Doxycycline inhibits matrix metalloproteinase-9 and laminin degradation after transient global cerebral ischemia. *Neurobiol Dis* **34:** 189–98.
10. Rosenberg G.A., Navratil M. (1997) Metalloproteinase inhibition blocks edema in intracerebral hemorrhage in the rat. *Neurology* **48:** 921–6.
11. Horstmann S., Su Y., Koziol J., Meyding-Lamadé U., Nagel S., Wagner S. (2006) MMP-2 and MMP-9 levels in peripheral blood after subarachnoid hemorrhage. *J Neurol Sci* **251:** 82–6.
12. Lorenzl S., Albers D.S., Narr S., Chirichigno J., Beal M.F. (2002) Expression of MMP-2, MMP-9, and MMP-1 and their endogenous counterregulators TIMP-1 and TIMP-2 in postmortem brain tissue of Parkinson's disease. *Exp Neurol* **178:** 13–20.
13. Kiaei M., Kipiani K., Calingasan N.Y., Wille E., Chen J., Heissig B., Rafii S., Lorenzl S., Beal M.F. (2007) Matrix metalloproteinase-9 regulates TNF-alpha and FasL expression in neuronal, glial cells and its absence extends life in a transgenic mouse model of amyotrophic lateral sclerosis. *Exp Neurol* **205:** 74–81.
14. Leib S.L., Leppert D., Clements J., Täuber M.G. (2000) Matrix metalloproteinases contribute to brain damage in experimental pneumococcal meningitis. *Infect Immun* **68:** 615–20.
15. Leib S.L., Clements J.M., Lindberg R.L., Heimgartner C., Loeffler J.M., Pfister L.A., Täuber M.G., Leppert D. (2001) Inhibition of matrix metalloproteinases and tumour necrosis factor alpha converting enzyme as adjuvant therapy in pneumococcal meningitis. *Brain* **124:** 1734–42.
16. Lee K.Y., Kim E.H., Yang W.S., Ryu H., Cho S.N., Lee B.I., Heo J.H. (2004) Persistent increase of matrix metalloproteinases in cerebrospinal fluid of tuberculous meningitis. *J Neurol Sci* **220:** 73–8.
17. Morishima T., Togashi T., Yokota S., Okuno Y., Miyazaki C., Tashiro M., Okabe N.; Collaborative Study Group on Influenza-Associated Encephalopathy in Japan. (2002) Encephalitis and encephalopathy associated with an influenza epidemic in Japan. *Clin Infect Dis* **35:** 512–7.
18. Kawada J., Kimura H., Ito Y., Hara S., Iriyama M., Yoshikawa T., Morishima T. (2003) Systemic cytokine responses in patients with influenza-associated encephalopathy. *J Infect Dis* **188:** 690–8.
19. Ichiyama T., Isumi H., Ozawa H., Matsubara T., Morishima T., Furukawa S. (2003) Cerebrospinal fluid and serum levels of cytokines and soluble tumor necrosis factor receptor in influenza virus-associated encephalopathy. *Scand J Infect Dis* **35:** 59–61.
20. Ichiyama T., Morishima T., Isumi H., Matsufuji H., Matsubara T., Furukawa S. (2004) Analysis of cytokine levels and NF-kappaB activation in peripheral blood mononuclear cells in influenza virus-associated encephalopathy. *Cytokine* **27:** 31–7.
21. Ichiyama T., Morishima T., Kajimoto M., Matsushige T., Matsubara T., Furukawa S. (2007) Matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases 1 in influenza-associated encephalopathy. *Pediatr Infect Dis J* **26:** 542–4.
22. Rosenberg G.A., Estrada E.Y., Dencoff J.E., Stetler-Stevenson W.G. (1995) Tumor necrosis factor-alpha-induced gelatinase B causes delayed opening of the blood-brain barrier: an expanded therapeutic window. *Brain Res* **703:** 151–5.
23. Abraham C.S., Deli M.A., Joo F., Megyeri P., Torpier G. (1996) Intracarotid tumor necrosis factor-alpha administration increases the blood-brain barrier permeability in cerebral cortex of the newborn pig: quantitative aspects of double-labelling studies and confocal laser scanning analysis. *Neurosci Lett* **208:** 85–8.
24. Sellner J., Leib S.L. (2006) In bacterial meningitis cortical brain damage is associated with changes in parenchymal MMP-9/TIMP-1 ratio and increased collagen type IV degradation. *Neurobiol Dis* **21:** 647–56.
25. Sellner J., Simon F., Meyding-Lamade U., Leib S.L. (2006) Herpes-simplex virus encephalitis is characterized by an early MMP-9 increase and collagen type IV degradation. *Brain Res* **1125:** 155–62.
26. Polavarapu R., Gongora M.C., Winkles J.A., Yepes M. (2005) Tumor necrosis factor-like weak inducer of apoptosis increases the permeability of the neurovascular unit through nuclear factor-kappa B pathway activation. *J Neurosci* **25:** 10094–100.
27. Asahi M., Wang X., Mori T., Sumii T., Jung J.C., Moskowitz M.A., Fini M.E., Lo E.H. (2001) Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. *J Neurosci* **21:** 7724–32.
28. Copin J.C., Merlani P., Sugawara T., Chan P.H., Gasche Y. (2008) Delayed matrix metalloproteinase inhibition reduces intracerebral hemorrhage after embolic stroke in rats. *Exp Neurol* **213:** 196–201.

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Comparison of the Levels of Human Herpesvirus 6 (HHV-6) DNA and Cytokines in the Cerebrospinal Fluid and Serum of Children With HHV-6 Encephalopathy

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Primary human herpesvirus-6 (HHV-6) infection is a common cause of acute sporadic encephalopathy in Japanese children. Occasionally, HHV-6 is not detected in the cerebrospinal fluid (CSF) of patients with encephalopathy, for example, in those with focal viral encephalitis, such as herpes simplex viral encephalitis. This indicates that HHV-6 encephalopathy is caused by an indirect mechanism, although this is not fully understood. HHV-6 DNA, cytokines (interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12 p70, tumor necrosis factor-α, interferon-γ), and matrix metalloproteinase-9 were quantitated in both the CSF and serum of 13 patients with HHV-6 encephalopathy during the acute phase of the disease. HHV-6 DNA was detected in the CSF of seven patients with HHV-6 encephalopathy. The viral DNA concentration was significantly higher in serum than in CSF (mean 1.64×10^4 vs. 5.70×10^1 copies/ml; $P = 0.003$). The lack or low level of viral DNA in the CSF samples suggests that direct invasion of the central nervous system by HHV-6 is not the main cause of encephalopathy. Additionally, the IL-10 concentration was significantly higher in serum than in CSF ($P < 0.001$), whereas there was no significant difference in IL-6 levels between the CSF and serum samples. Interestingly, the IL-8 concentration was significantly higher in CSF than in serum ($P = 0.038$). The distribution of these cytokines differed between CSF and serum. The high CSF concentration of IL-8 could play an important role in the pathogenesis of encephalopathy. ***J. Med. Virol. 82:1410–1415, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: human herpesvirus 6; encephalopathy; viral invasion; interleukin-8

INTRODUCTION

Human herpesvirus-6 (HHV-6) infects the majority of human populations worldwide and causes roseola (exanthem subitum), undifferentiated febrile illness without rash, and other acute febrile illnesses. The fever is characteristically high and persists for several days. In roseola, an erythematous maculopapular rash, lasting for a short period, is noted once the fever has resolved. Occasionally, primary HHV-6 infection has accompanying neurological complications, including febrile seizures and acute encephalitis/encephalopathy [Yamanishi et al., 2007; American Academy of Pediatrics, 2009]. In Japan, HHV-6 is the second most common pathogen causing acute sporadic encephalitis/encephalopathy in children (National Survey in 2003–2004, published exclusively in Japanese). The cause of this central nervous system (CNS) disorder remains controversial; several reports have suggested that the encephalitis/encephalopathy is caused by a direct virus-invasion mechanism [Yoshikawa et al., 1992; Kamei et al., 1997; Yoshikawa and Asano, 2000], while others have suggested indirect immune-mediated mechanisms [Enoki et al., 2006; Kubo et al., 2006; Ichiyama et al., 2009]. The presence of virus DNA in cerebrospinal fluid (CSF) samples may support a direct

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