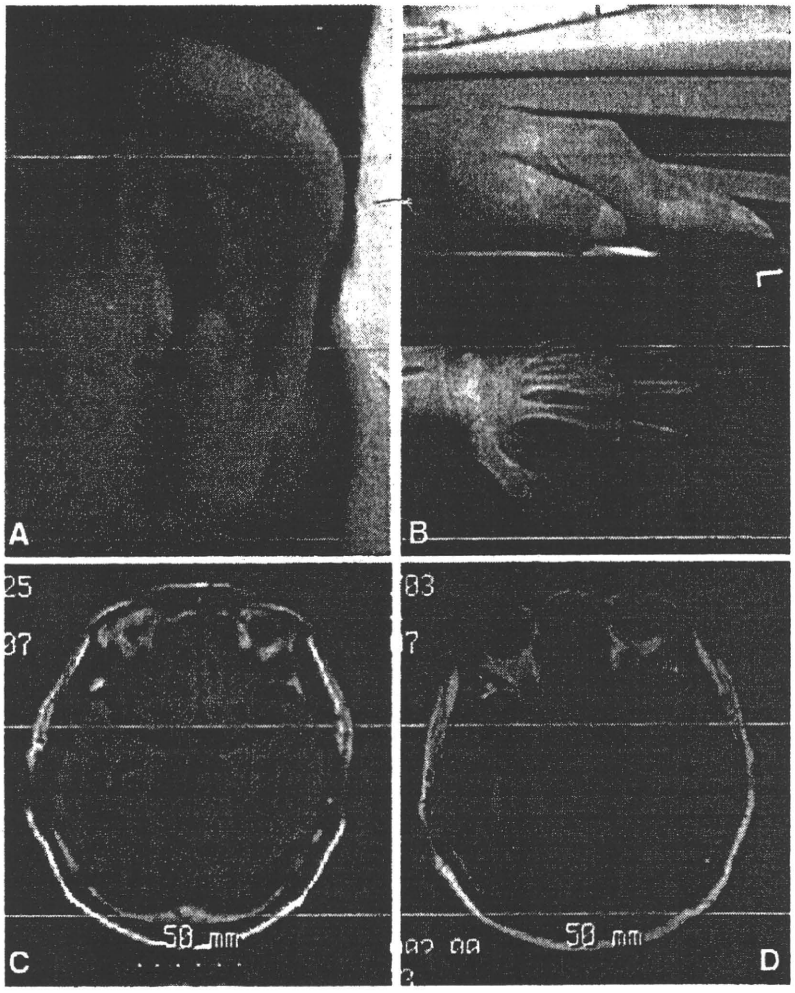

Fig. 1. The patient's clinical characteristics. A: Ear swelling, B: finger deformity, C: fluid-attenuated inversion recovery MR image of the brain at first admission showing a slightly high signal intensity lesion in the medial temporal lobe, and D: brain MR image 14 months later showing marked atrophy of the medial temporal lobe bilaterally.

encephalitis associated with relapsing polychondritis was diagnosed based on his clinical presentation of auricular signs, nasal chondritis, arthritis of the hands, previous history of conjunctivitis, systemic inflammatory reactions and the absence of any known collagen vascular disease. Intravenous injection of 500 mg/day methylprednisone 3 days a week for 3 weeks, together with phenytoin stopped his seizures, disturbed consciousness and inflammatory reaction. Once consciousness became normal, his hearing disturbance cleared up.

He was discharged on the 28th hospital day, but fever, headache and delirium recurred within a week. On readmittance his CSF showed increased protein of 64 mg/dl, an IgG level of 8.7 mg/L and a cell count of 512/mm^3 (30% lymphocytes). We found anti-GluRε2 (NR2B) IgG and IgM antibodies in his CSF and anti-GluRε2 (NR2B) IgG antibodies in his sera. No anti-GluRδ2 antibodies were detected in either fluid.

Intravenous administration of 500 mg/day methylprednisone 3 days a week for 4 weeks again was given followed by oral prednisone at 20 mg/day and tacrolimus at 3 mg/day. No fever or inflammatory reaction such as an increased white blood cell count or serum CRP level occurred thereafter, but cognitive decline continued, marked medial temporal lobe atrophy being detected 14 months after his first admission (Fig. 1D).

3. Discussion

Our patient presented with limbic encephalitis characterized by fever, headache, delirium, psychosis, seizures and dementia during the course of RP which rarely presents as limbic encephalitis [6–8] resulting in dementia [6]. Stewart et al. [5] reported extensive cerebral and systemic vasculitis to be the cause of CNS involvement in RP. In contrast, other authors have reported inflammatory changes non-specific to vasculitis in patients presenting limbic encephalitis associated with RP [7,8]. Multifocal neurological abnormalities and MRI lesions suggest that vasculitis is the cause of CNS manifestations in RP patients. Our patient's neurological symptoms and the MR findings of a medial temporal lobe lesion with progressive atrophy but no apparent asymmetrical multifocal lesion are similar to those of patients with non-herpetic limbic encephalitis rather than vasculitis. Because non-herpetic limbic encephalitis often is accompanied by anti-GluR antibodies, we tested his sera and CSF and found anti-GluRε2 (NR2B) antibodies. These antibodies have been detected in such human neurological disorders as non-herpetic, non-paraneoplastic limbic encephalitis, Rasmussen encephalitis, limbic encephalitis with ovarian teratoma, focal epilepsy, acute ischemic stroke and systemic lupus erythematosus [13–15]. The neuronal damage produced by some of these disorders may result in the release of GluR peptide [15]. In inflammatory disorders such as Rasmussen encephalitis, antigen presentation and autoantibody production could be initiated by immune cells already resident in the CNS [15]. The mechanism by which anti-GluRε2 antibodies produce limbic encephalitis remains to be clarified in our patient. Mice immunized with the GluR subtypes NR2/NR3 and gluR3 reportedly experienced CNS neuronal loss [16–18]. Gahring et al. [19] reported that GluR2 autoantibodies from a

patient with olivopontocerebellar atrophy activated α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors, a GluR subtype, in cultured mouse neurons. Dalmau [20] reported 100 patients with encephalitis-associated antibodies against N-methyl-D-aspartate receptors, another GluR subtype, and discussed the pathogenic role of these antibodies in developing encephalitis. In our patient, the release of glutamate receptor might have been caused by inflammation or ischemia of the central or peripheral nervous system associated with RP although there was no apparent ischemic change that paralleled the clinical course of CNS involvement. Alternatively, the autoantibodies could have been generated independent of the neuronal damage produced by RP. Various autoimmune diseases, including rheumatoid arthritis and Sjögren's syndrome, have been reported as being concurrent with RP [1].

The pathogenicity of anti-GluRε2 (NR2B) antibodies in limbic encephalitis has yet to be determined [15]. The anti-GluR ε2 (NR2B) antibodies generated in our patient may have caused limbic encephalopathy instead of vasculitis. This is the first report of the presence of these antibodies in RP-related CNS involvement. Our patient's case suggests that autoantibodies to the GluR ε2 (NR2B) subunit are associated with limbic encephalitis in certain patients with RP.

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Anti-Glutamate Receptor Antibodies in Pediatric Enteroviral Encephalitis

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ABSTRACT

In order to better understand enteroviral encephalitis we investigated the clinical symptoms and several disease markers. Between 2000 and 2005 eight patients aged between 9 months and 5 years were admitted to our hospital with one case having sequela. Glutamic oxaloacetic transaminase (GOT), serum IL-6, and ferritin were elevated in most of the cases. Their IL-6 and diacron-reactive oxygen metabolites (d-ROM) in cerebral spinal fluid (CSF) were also high (86%). However, pleocytosis and high protein levels in CSF were rarely found. In viral loads of the first CSF, there were no differences between the patient with sequela and the ones without sequela. However, anti-glutamate receptor IgMδ2 was only detected in the CSF of the patient with sequela. These findings suggest that the immunological phenomenon is more closely related to the development of sequela related to enteroviral encephalitis than other disease markers, such as inflammatory cytokine, free radicals, and viral loads. Therefore, a specific therapy against immunological status might decrease the sequela; however, further research is necessary to confirm this.

KEYWORDS: anti-glutamate receptor antibodies, encephalitis, enterovirus, free radical, children, IL-6

INTRODUCTION

In 1998, there was a pandemic of encephalomyelitis accompanied with hand-foot-and-mouth disease with high mortality in Taiwan (Chang et al., 1999), and a pandemic of cerebellar ataxia accompanied with enterovirus in Japan. Recently, an increase in cases of encephalitis with enterovirus was reported in Japan. However, there are few reports about the pathophysiology and the characteristics of enterovirus-associated encephalitis. In 1999, we started to measure enterovirus RNA in cerebral spinal fluid (CSF) by using highly sensitive reverse transcription polymerase chain reaction (RT-PCR). We identified eight cases of enteroviral encephalitis. In order to choose the best treatment, we have also been measuring inflammatory cytokines, oxidative stress, viral loads, and anti-glutamate receptor antibodies in serum and CSF. In this study we investigated these disease markers and their relationship to clinical symptoms in eight patients with pediatric enteroviral encephalitis.

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METHODS

We studied eight patients who were hospitalized due to fever, convulsions, and continuous unconsciousness. All were positive for enterovirus by RT-PCR or usual viral isolation. Their EEG showed slow wave in all the cases. Brain imaging showed abnormal findings, which revealed brain edema in five out of eight cases. The serum levels of immunoglobulin of all patients were normal.

The RT-PCR target was the consensus region of the 5′ no-coding region of the enterovirus. RNA was reverse-transcribed with avian myeloblastosis virus (AMV); reverse transcriptase using an anti-sense primer and nested PCR was also done (Takami et al., 1998). The primers of the quantitative assay were selected from the same consensus region of the 5′ no-coding region. The quantitative assay was performed by using the primers and probes, which were made

according to the sequence data of nested PCR products. The two different fluorescent oligonucleotide probes, the donor and acceptor, were designed to hybridize. The fluorescent oligonucleotide was measured by LightCycler (Roche Diagnostics GmbH, Germany) (Amaha, Kawashima, Takami, Takekuma, & Hoshika, 2006).

The oxidative status was examined by measuring the hydrogen peroxide concentration in the CSF according to the method automated by the Diacron (Diacron-Reactive Oxygen Metabolites: d-ROM test, Diacron, Italy) (Cesarone et al., 1999; Cornelli, Terranova, Luca, Cornelli, & Alberti, 2001). The serum levels of the d-ROM test ranged between 250 and 300 U.CARR (Carratelli units), where 1 U.CARR corresponds to 0.8 mg/L H_2O_2. CSF negative for enterovirus as a control was obtained from patients with fever of unknown origin, urinary tract infection, influenza, pneumonia, leukemia, and febrile seizures. The CSF levels of the d-ROM in controls were below 10 U.CARR. Serum levels of IL-6 were assayed by chemoluminescence enzyme immunoassay (CLEIA) by using Lumipulse *f* (Fujirebio Diagnostics Inc, Tokyo, Japan). We also investigated white blood cells (WBC), glutamic oxaloacetic transaminase (GOT), lactate dehydrogenase (LDH), ferritin, creatine kinase (CK), c-reactive protein (CRP), and anti-glutamate receptor antibodies. The assay of anti-glutamate receptor antibodies was examined with Western blot using Takahashi methods (Takahashi et al., 2003).

RESULTS

The patients were aged between 9 months and 5 years (two males, six females). Their symptoms before convulsion were upper respiratory tract infection in all cases (100%) and digestive symptoms in five cases (62.5%). The duration of the convulsions ranged from 2 to 60 min. One-third of the patients had asymmetric convulsions. Convulsive status was seen in six cases and a series of short convulsions in two cases. One of the eight patients (12.5%) had involuntary movements and sequela (epilepsy and paraplegia). The involuntary movements of the right upper extremity appeared 7 days after admission and resolved after several months of treatment with anti-epileptic medicines. The profiles are shown in Table 1. Blood tests showed that CRP was mildly positive in most of the cases (0.4 to 4.7 mg/dl). GOT levels were high in all the cases. Serum IL-6 was high in all the patients and ferritin in 50% of the patients. IL-6 and d-ROM in CSF were both high in 86% of the patients at the time of admission. However, pleocytosis was not found in the CSF of only one patient and the protein levels were low. In the viral loads of the CSF, there were no differences between the patient with sequela and those without sequela, similar to the levels of IL-6 and d-ROM in serum and CSF.

Anti-glutamate-receptor IgM-δ2 was detected in one out of three cases in CSF and in two out of three cases in serum. Anti-glutamate-receptor IgG-δ2 was detected in one out of three cases in serum. Anti-glutamate-receptor IgG-ε2 was positive in one out of three cases (Table 2). A patient who had anti-glutamate-receptor IgMδ2 in CSF had sequela.

DISCUSSION

We investigated eight cases, aged between 9 months and 5 years with enterovirus-associated encephalitis. Only one out of eight patients had sequela. Brain damage by neuroinfection can be caused by high inflammatory cytokines, direct viral invasion, free radicals, glutamate, apoptosis, and immunological phenomenon (Lipton, 1997). We measured the viral load, IL-6, and free radicals in this study. Leukocytosis of CSF did not appear in seven out of eight cases and appears to be likely induced later by the production of IL-6. Both IL-6 and d-ROM were high in five out of seven cases during the early stage. IL-6 is a multifunctional cytokine that plays a central role in the host defense due to its wide range of immune and hematopoietic activities and its potent ability to induce an acute-phase response. Overexpression of IL-6 has been implicated in the pathology of a number of diseases including systemic inflammatory response syndrome (SIRS) and secondary multiple organ failure (Giannoudis et al., 2008).

Recently, a new test for the evaluation of oxidative status, the d-ROM test, has become available. Although there are various reports of serum or plasma d-ROM levels (Cesarone et al., 1999; Cornelli et al., 2001), CSF d-ROM levels have not been reported. We measured d-ROM in patients with meningoencephalitis with enterovirus and 88.7% cases (6 out of 7) showed high d-ROM. CSF d-ROM in influenza-associated encephalopathy and febrile seizure has been reported (Yamanaka et al., 2006). d-ROM in CSF obtained from patients with febrile seizures was below 10 U.CARR. Yang & Qin (2004) have reported the high expression of neuronal nitric oxide synthase (nNOS) in animal models with recurrent seizures. Severe cases showed high levels in CSF and were related to the protein concentration. Oxidative stress and uncontrolled stress might influence these complications. Nitrogen oxide (NO×) inhibitors, such as a free

TABLE 1 Profiles of patients

Case	1	2	3	4	5	6	7	8
Gender	F	M	F	F	F	F	M	F
Age	1 y 5 m	9 m	1 y 1 m	10 m	9 m	10 m	3 y 9 m	5 y 10 m
Fever during illness (days)	3	4	2	2	2	2	1	2
Symptoms preceding the onset of encephalitis	Coryza diarrhea	Coryza diarrhea	Coryza	Coryza diarrhea	Coryza	Moist cough xxx syndrome	Coryza OMA abdominal pain	Coryza headache vomiting
Convulsion (minutes)	55 GTC (right side dominant)	15, 60 GTC	60 GTC (upper extremities dominant)	49 GTC (right side dominant)	15, 30 GTC	6, 60 GTC	5, 10, 3 GTC	3 GTC
Others	Choreoathetosis	-	-	-	-	-	Delirium	-
Sequela (until 1 year)	Epilepsy paraplegia	-	-	-	-	-	-	-

GTC: Generalized tonic seizure.

TABLE 2 The findings of peripheral blood and spinal fluid on admission

Case	1	2	3	4	5	6	7	8
Blood check								
CRP (mg/dl)	4.7	3.3	0.7	< 0.3	3.8	< 0.3	5.8	2.9
GOT (IU/L)	165	56	180	148	33	61	53	37
LDH (IU/L)	804	563	1029	619	575	667	521	553
CK (IU/L)	120	1510	118	240	98	730	92	203
Ferritin (mg/dl)	638	85.9	745.6	166	ND	ND	92.5	53.7
IL-6(pg/ml)	96.3	ND	51	ND	86.7	ND	126.2	ND
anti-Glu-R								
IgM-δ2	+		−		+			
IgG-δ2	+		−		−			
IgG-ε2	−		−		+			
CSF check								
Cell count	5	12	5	3	38	6	0	172
Protein (mg/dl)	12	27	13	5	12	22	9	9
Glucose (mg/dl)	85	65	90	78	80	66	85	73
IL-6 (<5 pg/ml	27.3	1.1	56.6	44	64.1	75	28.1	ND
d-ROM (<10 U.CARR)	19	39	71	17	39	59	3	ND
Viral load (copies)	937	ND	ND	ND	781	1275	ND	977
Anti-Glu-R			−					
IgM-δ2	+				−			

radical scavenger, edaravone, might be helpful in these cases.

Inoue (2002) reported NO and IL-6 production from microglia, which is activated by adenosine triphosphate (ATP) in mice. He showed that microglia stimulated by a low concentration of ATP rapidly releases plasminogen, which may protect neurons. However, microglia stimulated by a higher concentration of ATP releases TNF-α 2–3 hr after the stimulation and IL-6 6 hr after the stimulation. He speculated that stronger stimulation changes the function of microglia and induces apoptosis in neurons by releasing toxic factors, including NO (Inoue, 2002). Therefore, high NO might mean activation of microglia. High NO× was found in CSF obtained from severe influenza-associated encephalopathy (Kawashima et al., 2003). This oxidative stress might induce damage to CNS. Consequently, therapies against high cytokines and free radicals should be recommended during an acute phase of enteroviral encephalitis.

Anti-glutamate receptor antibodies are detected in the serum and CSF of patients with chronic progressive epilepsia partialis continua of childhood and those with Rasmussen's encephalitis (Takahashi et al., 2003). Moreover, these antibodies have been reported with encephalitis and encephalopathy, including limbic encephalitis without any deterioration (Kumakura, Miyajima, Fujii, Takahashi, & Ito, 2003; Takahashi, 2002). Interestingly, in our study anti-glutamate antibodies were detected in the CSF obtained from a patient with sequela. These findings suggest that the immunological phenomenon may result in sequela of enteroviral encephalitis than inflammatory cytokine, free radicals, and viral loads. The autoimmune mechanism might induce the pathophysiology of subsequent symptoms of enteroviral encephalitis. Therefore, these results suggest that therapies such as steroids, anti-virus agents, and free radical scavengers may help to prevent further sequela. However, further research with a larger number of patients is necessary to draw any definitive conclusions.

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INTERNAL MEDICINE

□ EDITORIAL □

Can We Predict a Prolonged Course and Intractable Cases of Herpes Simplex Encephalitis?

Hiroshi Shoji

Key words: herpes simplex encephalitis, acyclovir, prognostic factor

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(DOI: 10.2169/internalmedicine.48.1737)

In Japan, herpes simplex encephalitis (HSE) has historically been fatal in approximately 30% of all reported cases. After the induction of acyclovir (ACV), however, the mortality rate has decreased to 7.1% (1), and HSE is now regarded as a treatable disease. However, the rate of poor outcome including moderate or severe sequelae still remains at 30-40% of HSE patients, despite standard ACV treatment. It is conceivable that early detection and appropriate treatment will lead to a good prognosis for intractable HSE.

Problems in prolonged and intractable cases of HSE were taken up at the workshop held by the Japan Herpesvirus Infections Forum (JHIF) in 1996 (2) and the symposium of the Japanese Neuro-Infectious Disease Meeting in 1997 (unpublished data). At that time, a tentative definition of intractable cases of HSE was developed as follows:

1. Cases of HSE that develop to an apallic state and to fatality.

2. Prolonged cases that require more than 6 months' hospitalization.

3. Recurrent cases.

It may be that the main reasons for the development of intractable HSE are a deep consciousness disturbance, status epilepticus, and delays in starting antiviral drug therapy. Conventionally in the USA, a semicoma or coma state in patients over 30 years of age has been accepted as a predictive factor in a fatal prognosis (3).

In this issue of the journal (see also pp 89-94), Taira et al (4) analyzed variable predictors such as age, sex, Glasgo coma scale (GCS), starting date of ACV, corticosteroid administration, and cranial computed tomography (CT), as well as magnetic resonance imaging or electroencephalogram abnormalities between the prolonged group (n=8) and non-prolonged group (n=15) in 23 adult HSE patients. The prolonged group was defined as being without any neurological improvement at the time of completion of ACV treatment for 14 days, and they concluded that there are 2 significant predictors of a prolonged course of HSE; a lower GCS $\leqq$6 points at the start of antiviral treatment and a higher rate of abnormal lesion on initial CT. The 4 patients of the prolonged group had poor outcomes at 3 months after onset.

The clinical guidelines for adult HSE in Japan recommend a higher dose of ACV for severe HSE patients and alternative therapy of vidarabine in unresponsive cases to ACV treatment (5). A recent study also suggests that corticosteroid administration is a beneficial factor for HSE prognosis (6). Therefore, when HSE patients present with GCS $\leqq$6 points and CT abnormal lesion on the temporal lobe, it seems likely that we should initiate ACV treatment at a higher dosage (45-60 mg/day), or add corticosteroid administration including pulse therapy.

However, the pathophysiology for these 2 predictors should be clarified. Intractable cases with a deep consciousness disturbance or wide CT abnormality are often attributed to prolonged herpes simplex virus (HSV) infection or secondary encephalitis (postinfectious/autoimmune encephalitis). Further virologic and immunologic studies are expected to investigate the use of real-time polymerase chain reaction for HSV DNA, and changes of various cytokines in the host response.

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INTERNAL MEDICINE

CASE REPORT

Acute Encephalomyelitis Associated with Acute Viral Hepatitis Type B

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Abstract

We describe the case of a 36-year-old woman who developed acute encephalo-myelitis after acute viral hepatitis type B. She was admitted to the hospital with a history of general malaise and nausea of 5 days duration. Her serum showed high transaminase levels and positive HBs-Ag and increased IgM HBc-Ab titers. She had urinary dysfunction, myoclonus and postural tremor of her extremities. Several days later, she developed bilateral limb ataxia and alteration of consciousness. The cerebrospinal fluid examinations showed pleocytosis and increased protein. Treatment with high-dose methylprednisolone resulted in a marked improvement of the clinical and CSF examination. Magnetic resonance imaging of the brain and the spinal cord did not disclose abnormal lesions. The symptoms and clinical course were quite similar to those of acute disseminated encephalomyelitis.

Key words: acute disseminated encephalomyelitis (ADEM), acute viral hepatitis, hepatitis B virus (HBV)

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Introduction

Acute disseminated encephalomyelitis (ADEM) is an autoimmune demyelinating disease of the central nervous system that generally develops after acute viral or bacterial infection or vaccination (1-3). ADEM is usually a monophasic disease with acute onset characterized by multiple foci of central nervous system damage, predominantly in the cerebral and cerebellar white matter, although basal ganglia and gray matter may also be involved. Lesions are frequently bilateral, large, and confluent (1-3). Numerous infectious agents have been linked to ADEM. A search of the literature revealed some reports of cases associated with hepatitis A virus infection and hepatitis C virus infection (4, 5). Regarding hepatitis B virus, however, no association of hepatitis B virus infection with acute disseminated encephalomyelitis have been reported despite the fact that some cases associated with hepatitis B virus vaccination have been reported (6-8). Furthermore, the possibility that hepatitis B vaccine may cause or exacerbate multiple sclerosis (MS) stems from several reports of onset or recurrence of symptoms of CNS demyelination shortly after vaccination (9). We describe the case of a 36-year-old woman who developed acute encephalomyelitis after acute viral hepatitis type B, and discuss the pathogenesis of the disturbance.

Case Report

A 36-year-old woman was admitted to the Department of Internal Medicine, Maizuru Kyousai Hospital with a history of general malaise and nausea of 5 days duration. There was no history of toxic substance or drug ingestion. On admission, the serum transaminase (AST; 355 IU/L, ALT; 1,916 IU/L) and γ-GTP (357 IU/L) levels were elevated. WBC level (10,600/μL) in blood was slightly increased. Other routine laboratory examinations including anti-nuclear antibody (ANA), anti-DNA antibody, P-ANCA and C-ANCA were all normal or negative. Hepatitis Bs antigen (48.08) and IgM hepatitis B core antibody (IgM HBc-Ab) were positive. IgMHA-Ab,HCV-Ab, EBV VCAIgG (320), EBVVCAIgM (<10), EBNA (160) were all not significantly increased or negative. She was diagnosed as acute hepatitis B. She showed no signs of neurological deficient. Findings of elec-

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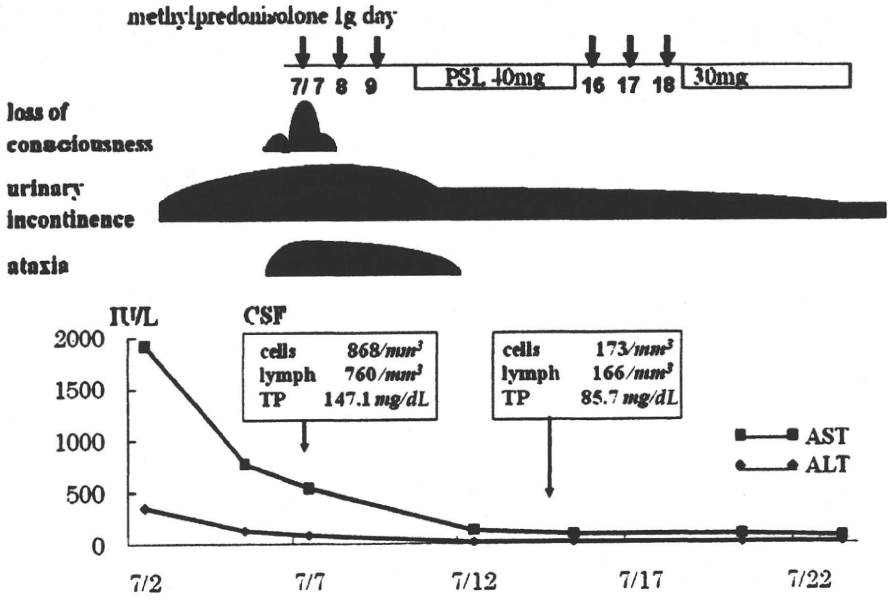

Figure 1. Clinical course of the patient.

trocardiograph and chest x-ray film examinations were normal. Ten hours later, she developed urinary dysfunction. On the second day of the admission she showed myoclonus on the inside of her thighs and postural tremor of her upper extremities. On the 10th day of admission, she developed bilateral limb ataxia and on the following day she developed alteration of consciousness. The cerebrospinal fluid examinations showed pleocytosis (868/mm^3) composed of 760/mm^3 mononuclear cells and 108/mm^3 polynuclear cells and increased total protein (147 mg/dL). Glucose in CSF was 51 mg/dL. Bacterial, mycobacterial, and fungal cultures from cerebrospinal fluid were negative. Magnetic resonance imaging (MRI) of the brain and the spinal cord revealed no abnormal findings. Gadolinium contrast enhancement MRI was not examined in this patient.

Treatment with high-dose methylprednisolone was followed by a dramatic improvement of the clinical and cerebrospinal fluid findings. The clinical course is shown in Fig. 1. Within a few months the patient recovered completely and there was no relapse during 1 year of follow-up.

Discussion

ADEM is a monophasic disease that occurs in the setting of infection or immunization. The pathological characteristics of the condition are perivascular inflammation, edema and demyelination in the central nervous system. After prodromal phase of 1-4 weeks, clinical signs including altered consciousness and multifocal neurological disturbance appear. Moderate pleocytosis in the cerebrospinal fluid is a common feature but, in contrast with multiple sclerosis, oligoclonal bands are rarely obserbed (10). MRI is considered the diagnostic tool of choice for suspected ADEM (1, 2, 3, 11). In our case, the clinical signs and symptoms combined with the serum and cerebrospinal fluid findings and the clinical marked improvement with corticosteroid therapy were strongly suggestive of ADEM, though MRI could not disclose abnormal findings in the brain or the spinal cord. The incidence of lesions on MRI is variable in ADEM and may depend on the stage of inflammation. Gadolinium enhancing lesions have been described in 30 to 100% of patients. As spinal cord involvement in ADEM has been described in 11 to 28%, spinal cord lesions could be rarely disclosed on MRI (3).

In the present case, apart from the indication of a recent hepatitis virus B infection, laboratory investigation revealed no infection or infectious agent. In recent years, several reports of new cases of central nervous system demyelination or reactivation of multiple sclerosis after hepatitis B vaccination have raised the possibility of a causal link (6-8). In addition, it was reported that hepatitis virus B polymerase shares significant amino acid similarities with the human myelin basic protein (12). However, our search of the literature revealed no previously reported case of ADEM following this infection.

There could be two possibilities, demyelination or vasculitis, for the pathogenetic mechanism of neurological involvement in this case. In general, vasculitis associated with hepatitis viral infection has occurred in the chronic stage, especially in chronic hepatitis type C (13, 14). In some patients with chronic hepatitis type C and B, complicated secondary cryoglobulinemia or polyartilitis nodosa induced damage of the blood vessels, resulting in vasculitic neuropathy (15). The CNS disorders as a complication of hepatitis rarely occur in patients with hepatitis type B, who were also in the chronic stage (15-17). In the present patient, acute onset CNS neurological deficits with CSF pleocytosis occurred shortly after the infection of hepatitis B virus, and steroid therapy showed marked effect for the disturbances. These findings suggest that the pathogenesis in this case could be

due to a demyelinating process, rather than vasculitis.

This case shows that ADEM-like CNS neurological involvement can be associated with hepatitis virus B infection. We emphasize the importance of hepatitis virus B screening in patients with acute encephalomyelitis, since many cases of hepatitis virus B infection remain anicteric or subclinical. Likewise, patients with hepatitis virus B infection should be examined carefully for central nervous system symptoms during follow-up.

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ORIGINAL ARTICLE

Differences in clinical manifestations of influenza-associated encephalopathy by age

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ABSTRACT

Data from patients in Japan was analyzed to examine the age distribution and differences by age in the clinical manifestations of influenza-associated encephalopathy. Between 1998 and 2002, 472 cases of influenza-associated encephalopathy in patients aged 15 years or younger were reported to the Collaborative Study Group on Influenza-Associated Encephalopathy. These cases were divided into two groups by age: 0–5 and 6–15 years. The differences between the groups were estimated based on the data for those aged 0–5 years, and the odds ratios and 95% confidence intervals calculated. Distribution was inversely correlated with age, with a peak at 1–2 years old. In comparison with patients aged 0–5, those aged 6–15 years had a significantly greater incidence of type B infection, lower frequency of convulsions, higher frequency of loss of consciousness and altered consciousness as the initial neurological symptom, lower serum transaminase levels, lower frequency of low-density area for brain CT upon admission, and lower incidence of sequelae. Our analysis indicates that the clinical course, laboratory data, and brain imaging findings of influenza-associated encephalopathy exhibits patterns that vary with age.

Key words age distribution, age groups, influenza-associated encephalopathy.

INTRODUCTION

Influenza-associated encephalopathy occurs worldwide, but has been reported more often in Japan than in other countries (1–4). Although this disease was previously believed to occur only in Japan, case reports from other countries have increased (5–16). Influenza-associated encephalopathy is an abrupt disorder of the nervous system that is triggered by an influenza virus infection, often leading to death or severe sequelae. There have been no nationwide data on this disease, and frontline clinicians have difficulty identifying it and determining a course of management. Therefore, in the winter of 1998, we initiated a national survey to investigate various parameters of this disease in Japan. Our first comprehensive report, which included 148 cases of influenza-associated encephalopathy in Japan, was released in 2002 (1). Here, we shed further light on the characteristics of this disease in a wider age range of victims, based on data collected over four years.

One of the noteworthy characteristics of this disease is the age distribution of its victims. Influenza viruses have a broad geographic range and affect people of all ages

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List of Abbreviations: CT, computed tomography; HI, hemagglutination inhibition.

as a consequence of antigen drift each year. The scale of the epidemic varies with the virus type. Although school-aged children play an important role in the spread of influenza, most influenza-associated encephalopathy patients are children younger than five; adults are rarely affected (1, 3, 4). We believed that the clinical manifestations of the disease in older children differed from those in patients aged 0–5 years. Therefore, we analyzed the differences in the clinical manifestations of influenza-associated encephalopathy by age.

MATERIALS AND METHODS

Data collection

Questionnaires were developed by the Collaborative Study Group on Influenza-Associated Encephalopathy, which was organized by the Japanese Ministry of Health, Labor, and Welfare. This was a cross-sectional survey of influenza cases treated at all medical facilities. Between 1998 and 2002, hospitals, clinics, and local pediatric facilities reported 585 cases of influenza-associated encephalopathy. In addition to isolation of virus, the sudden onset of high fever with respiratory signs, myalgia, and headache were used as diagnostic markers.

Case definition

The diagnosis of encephalopathy was based on clinical signs. All patients had altered consciousness (*i.e.* delirium, confusion, and senselessness) or loss of consciousness (*i.e.* deep coma, coma, semicoma, stupor, and somnolence). Patients with meningitis, myelitis, and febrile seizures without prolonged unconsciousness were excluded. Postictal unconsciousness with prompt recovery was classified as febrile convulsion.

The diagnosis of influenza infection was based on viral isolation, the viral antigen test, or RT-PCR, or a fourfold or greater increase in paired serum antibody titers (hemagglutination inhibition or complement fixation test). Patients with none of those findings were excluded from further study. In all, 472 influenza-associated encephalopathy cases in patients aged $\leq$ 15 years were analyzed. The outcomes of influenza-associated encephalopathy were defined as normal resolution, mild sequelae, severe sequelae that necessitated personal help for the activities of daily living, and death. Mild sequelae include learning disorders, mental retardation, secondary epilepsy, and mild motor and sensory paralysis.

Statistical analysis

The 472 cases were divided into two groups based on patient age: 0–5 years (n = 382, 80.9%) and 6–15 years (n = 90, 19.1%). The group of patients aged 6–15 was compared to that aged 0–5 years. Odds ratios for the data of the 0–5 age group and their 95% confidence intervals were estimated. *P*-values of dichotomous variables were calculated using the χ^2 test or Fisher's exact test as appropriate. Epi Info version 3.3.2 was used to estimate odds ratios and their confidence intervals and to calculate *P*-values.

RESULTS

Patient background

The number of patients peaked at between one and two years of age. There were only three patients under six months of age; the youngest was two months old. No remarkable differences were observed in the age distribution of patients over each of the four years of the study compared to the combined distribution for all cases (Fig. 1). However, the total number of patients in each year varied (Table 1). Over the course of the study, mortality decreased each year, but the incidence of sequelae did not.

Two antiviral drugs were used for treatment: amantadine and a neuraminidase inhibitor. The former was used frequently in the 1999–2000 season, whereas the latter was used more frequently after it became commercially available in Japan in the 2000–2001 season. The percentage of patients who took amantadine and the neuraminidase inhibitor were similar in the two age groups across the four seasons studied. Some patients received both drugs.

A history of febrile seizures was present in 54 patients; 14 of these had epilepsy. Another three patients had epilepsy with no history of febrile seizures. One patient with propionic acidemia had convulsions and loss of consciousness and died on the day of fever onset.

Differences by age

The male-to-female ratios did not differ between the age groups (data not shown). The influenza virus type was identified in 436 of the 472 cases. The other 36 cases were diagnosed using a viral antigen test that cannot distinguish between type A and type B influenza. On comparing the age groups in the 436 cases, the ratio of type B to type A was significantly higher in patients aged 6–15 than in those aged 0–5 years (odds ratio, 2.35; 95% confidence interval, 1.11–4.91). There was no significant difference in peak body temperature (Table 2).

As an initial neurological symptom, convulsions occurred less frequently in patients aged 6–15 than in those aged 0–5 years (Table 2). However, loss of consciousness and altered consciousness as the initial neurological symptom occurred more frequently in patients aged 6–15 than

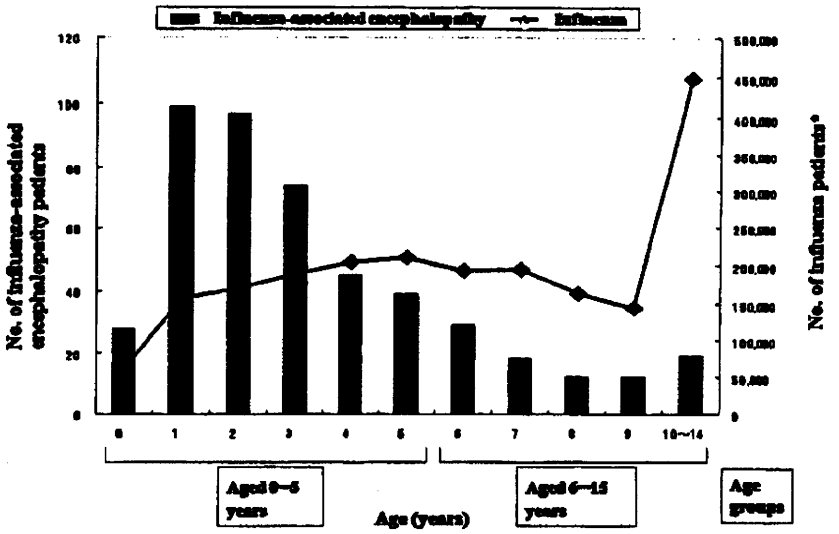

Fig. 1. Age distributions of patients with influenza-associated encephalopathy (vertical bars) and with influenza (line) in Japanese children between 1998 and 2002. *Influenza cases were reported as part of the National Epidemiological Surveillance of Infectious Diseases.

in those aged 0–5 years. The time of neurological onset (days after the onset of high-grade fever) did not differ significantly between the groups.

Concerning the relationship between age and laboratory findings on admission, patients aged 6–15 had lower serum transaminase levels than those aged 0–5 years (Table 3). Urinalysis and cerebrospinal fluid abnormalities did not differ significantly between the groups.

Brain CT was obtained on admission in 368 cases; approximately half of these were normal (Table 4). Brain edema occurred in approximately half of the cases in each age group. Low-density areas occurred less frequently in patients aged 6–15 than in those aged 0–5 years. The time from onset of neurological findings to the day of CT was no more than two days in 89% of the patients for whom both dates were reported.

Table 1 Baseline characteristics of influenza-associated encephalopathy in Japanese children between 1998 and 2002

Characteristic	1998–1999	1999–2000	2000–2001	2001–2002	Total
No. of patients	137	71	54	210	472
Age: no. (%)					
0–5 years	119 (86.9)	51 (71.8)	40 (74.1)	172 (81.9)	382 (80.9)
6–15 years	18 (13.1)	20 (28.2)	14 (25.9)	38 (18.1)	90 (19.1)
Sex: no. (%)					
Male	75 (54.7)	36 (50.7)	34 (63.0)	101 (48.1)	246 (52.1)
Female	62 (45.2)	35 (49.3)	20 (37.0)	109 (51.9)	226 (47.9)
Virus type: no./total no. (%)					
Type A	118/134 (88.1)	71/71 (100)	41/49 (83.7)	163/182 (89.6)	393/436 (90.1)
Type B	16/134 (11.9)	0/71 (0)	8/49 (16.3)	19/182 (10.4)	43/436 (9.9)
Unclassified	3	0	5	28	36
Outcome: no./total no.† (%)					
Normal resolution	54/137 (39.4)	37/71 (52.1)	28/51 (54.9)	106/183 (57.9)	225/442 (50.9)
Mild sequelae	27/137 (19.7)	11/71 (15.5)	11/51 (21.6)	27/183 (14.8)	76/442 (17.2)
Severe sequelae	12/137 (8.8)	7/71 (9.9)	5/51 (9.8)	20/183 (10.9)	44/442 (10.0)
Death	44/137 (32.1)	16/71 (22.5)	7/51 (13.7)	30/183 (16.4)	97/442 (22.0)
Medication: no./total no.‡ (%)					
Amantadine	55/133 (41.4)	52/70 (74.3)	31/39 (79.5)	91/130 (70.0)	229/372 (61.6)
Neuraminidase inhibitor	-	-	10/27 (37.0)	62/121 (51.2)	72/148 (48.6)

†Total no. consists of patients whose outcomes were ascertained.

‡Total no. consists of patients with a definite history of the medication and who took the medicine both before and after the onset of encephalopathy.

Table 2 Comparison of patient progress in the acute phase of influenza-associated encephalopathy

Parameter	No. of patients (%)		Odds ratio with respect to 0–5 years (95% CI)
	0–5 years	6–15 years	
Peak body temperature: no. of patients/total no. (%)			
≥40 °C	160/309 (51.8)	40/71 (56.3)	1.20 (0.69–2.09)
≥41 °C	25/309 (8.1)	11/71 (9.1)	2.08 (0.91–4.72)
Initial neurological symptom: no. of patients/total no. (%)			
Convulsion	270/343 (78.7)	42/76 (55.3)	0.33 (0.19–0.58)*
Loss of consciousness†	62/343 (18.1)	26/76 (34.2)	2.36 (1.31–4.22)**
Altered consciousness‡	11/343 (3.2)	8/76 (10.5)	3.55 (1.25–9.97)***
Time of neurological onset			
≥ day 3/≤ day 2 (% of ≥ day 3/total no.)	24/357 (6.3)	11/78 (12.4)	2.10 (0.92–4.71)

Total no. indicates the number of patients who responded to the question.
†Includes deep coma, coma, semicoma, stupor, and somnolence.
‡Includes delirium, confusion, and senselessness.
§Days after the onset of high-grade fever.
*: $P < 0.001$, **: $P = 0.002$, ***: $P = 0.011$ (Fisher exact).

There was no difference in mortality by age (Table 5). However, patients aged 6–15 had a significantly lower incidence of sequelae than did those aged 0–5 years. No significant differences in the severity of sequelae were observed.

DISCUSSION

Although, according to the report from the National Institute of Infectious Diseases, the age distribution of influenza-affected patients in Japan between 1998 and 2002 was generally flat from one to nine years of age, the distribution of influenza-associated encephalopathy was inversely correlated with age, with a peak at 1–2 years of age (17). The age distribution of influenza virus AH3N2 was similar to that of influenza-associated encephalopathy (data from the National Institute of Infectious Diseases, Japan [http://idsc.nih.go.jp/index.html] (17). Influenza virus AH3N2 is more likely than other types of the virus to trigger encephalopathy (1). We found that the ratio of virus type B to A among patients with encephalopathy was significantly higher in the 6–15 than in the 0–5 years group, possibly because of the prevalence rates of antibodies to each virus type. The National Institute of Infectious Diseases has reported that the antibody prevalence rate to type B virus is very low at all ages, because the scale of type B epidemics is smaller than those of other virus types (17). For example, among healthy

Table 3 Comparison of laboratory findings (blood, urine, and CSF)

	No. of patients (%)		Odds ratio with respect to 0–5 years (95% CI)
	0–5 years	6–15 years	
Blood			
AST ≥ 100 IU/L	119/313 (38.0)	13/68 (19.1)	0.39 (0.19–0.76)*
AST ≥ 500 IU/L	45/313 (14.4)	6/68 (8.8)	0.58 (0.21–1.49)
CPK ≥ 1000 IU/L	31/285 (10.9)	5/60 (8.3)	0.74 (0.24–2.13)
PT ≤ 70%	48/88 (54.5)	12/21 (57.1)	1.11 (0.39–3.23)
Platelet count ≤ $10 \times 10^4/\mu L$	55/293 (18.8)	9/65 (13.8)	0.70 (0.30–1.56)
Platelet count ≤ $5 \times 10^4/\mu L$	26/293 (8.9)	2/65 (3.1)	0.33 (0.05–1.47)
Urine			
Hematuria or proteinuria	79/240 (32.9)	13/55 (23.6)	0.63 (0.30–1.30)
CSF			
WBC count ≥ 8/μL	25/237 (10.5)	10/53 (18.9)	1.97 (0.82–4.69)
Protein level ≥ 50 mg/dl	27/230 (11.7)	10/50 (20.0)	1.88 (0.78–4.45)

AST, aspartate transaminase; CPK, creatinine phosphokinase; PT, prothrombin time; CSF, cerebrospinal fluid; WBC, white blood cells.
*: $P = 0.005$

Table 4 Comparison of brain CT findings (on admission)

Finding	No. of patients/total no. (%)		Odds ratio with respect to 0–5 years (95% CI)
	0–5 years	6–15 years	
Normal	120/306 (39.2)	32/62 (51.6)	1.65 (0.92–2.96)
Edema	154/306 (50.3)	25/62 (40.3)	0.67 (0.37–1.20)
Low-density area	40/306 (13.1)	2/62 (3.2)	0.22 (0.04–0.97)*
Hemorrhage	4/306 (1.3)	1/62 (1.6)	1.24 (0.02–12.78)

*: $P = 0.045$

individuals stratified by age groups, the 2002 rates of HI titers ≥ 40 to virus type B/Shandong/7/97 (Victoria lineage) were < 10% in all age groups other than the 20–29 years group. However, the rates of HI titers of ≥ 40 to A/New Caledonia/20/99(H1N1) were 40–50% in the 5–19 years age group and approximately 20% in the 0–4 years age group. Finally, the rates of HI titers of ≥ 40 to A/Panama/2007/99(H3N2) were slightly less than 70% in patients aged 5–9 years, 55–65% in teens, and 25% in patients aged 0–4 years. Therefore, older children appear to be more susceptible to the type B virus.

Most of the influenza-associated encephalopathy patients had convulsions as an initial neurological sign (74.5% of all cases). However, older children were more likely to experience loss of consciousness or altered consciousness. This may represent information bias, as non-differential misclassification could occur as a result of underestimation of minor neurological signs like altered consciousness, especially in younger children. The age distribution of influenza-associated encephalopathy is similar to that of febrile seizures. Difficulty in distinguishing these two diseases could cause non-differential misclassification. However, differential misclassification might be low because there was almost no information about influenza-associated encephalopathy when we conducted our study, and our selection biases might be very low because almost every case in Japan was reported. There have been some reports that the incidence of febrile seizures is higher in Asia than in Western Europe and the USA (18). In turn, more cases of influenza-associated encephalopathy have been reported in Japan than in Western Europe and the USA (1, 2, 4, 6, 19). Cases of acute necrotizing encephalopathy, a type of acute encephalopathy, have also accumulated in East Asia (20), although reports from other areas have increased recently (9, 11, 12, 21, 22). Based on this data, a genetic background might be involved in the pathogenesis of these diseases.

Patients with influenza-associated encephalopathy often die from multiple organ failure, which is thought to be caused by mitochondria-mediated apoptosis (23). Hosoya *et al.* and Nunoi *et al.* have reported that cytochrome c, a mitochondrial protein found in the intermembrane space, is a good marker for evaluating the clinical severity of influenza-associated encephalopathy (24, 25). Our comparison showed that, upon admission, patients aged 6–15 had a lower incidence of liver dysfunction, and of low-density areas with brain CT, than did those in the group aged 0–5 years. Other studies have also shown that the prevalence of neuroimaging abnormalities is higher in younger than in older children (16, 26). A smaller extent of apoptosis may have caused these findings, which correlate with the low frequency of sequelae in the group aged 6–15 years. Clarke *et al.* have reported that young age is associated with a poor outcome, such as death or severe sequelae, in childhood encephalopathy (27). We suspect that age is a prognostic factor for the sequelae of influenza-associated encephalopathy, although it is not a prognostic factor for death (28). In summary, we

Table 5 Comparison of outcomes

Outcome	No. of patients (%)		Odds ratio with respect to 0–5 years (95% CI)
	0–5 years	6–15 years	
Mortality: no./total no.† (%)	80/359 (22.3)	17/83 (20.5)	0.90 (0.48–1.67)
Existence of sequelae: no./no. alive (%)	105/279 (37.6)	15/66 (22.7)	0.49 (0.25–0.95)*
Severity of sequelae			
Severe sequelae‡/mild sequelae (% of total no. of sequelae)	38/67 (36.2)	6/9 (40.0)	1.18 (0.34–3.99)

†Number of patients whose outcomes were reported.

‡Require personal help for activities of daily living.

*: $P = 0.032$

found that influenza-associated encephalopathy exhibits patterns that vary with age.

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High prevalence of autoantibodies against phosphoglycerate mutase 1 in patients with autoimmune central nervous system diseases

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ABSTRACT

We identified the autoantibody against phosphoglycerate mutase 1 (PGAM1), which is a glycolytic enzyme, in sera from multiple sclerosis (MS) patients by proteomics-based analysis. We further searched this autoantibody in sera from patients with other neurological diseases. The prevalence of the anti-PGAM1 antibody is much higher in patients with MS and neuromyelitis optica (NMO) than in those with other neurological diseases and in healthy controls. It was reported that the anti-PGAM1 antibody is frequently detected in patients with autoimmune hepatitis (AIH). Results of our study suggest that the anti-PGAM1 antibody is not only a marker of AIH but also a nonspecific marker of central nervous system autoimmune diseases.

1. Introduction

Over the past few years, compelling data on the roles of B cells as sensors, coordinators and regulators of the immune response have strengthened the view that B cells and autoantibodies are fundamental factors for activating T cells and/or mediating tissue injury in several autoimmune-mediated diseases of the central nervous system (CNS) (Dalakas, 2008; Hasler and Zouali, 2006). In this study, we identified the autoantibody against phosphoglycerate mutase 1 (PGAM1) in sera from multiple sclerosis (MS) patients by proteomics-based analysis. 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, there are two types of phosphoglycerate mutase: type M (also known as PGAM2) in muscles and type B (also known as PGAM1) in other tissues (Omenn and Cheung, 1974; Zhang et al., 2001). However, there are few reports on the anti-PGAM1 antibody (Lu et al., 2008; Zephir et al., 2006) and the specificity of this autoantibody is not clearly understood. To evaluate the specificity of this autoantibody, we assessed the prevalence of this autoantibody in sera from patients with various neurological diseases and healthy controls.

2. Patients and methods

2.1. Patients

Serum samples were obtained from patients with MS [$n=21$; male:female = 9:12; age range, 31–75; mean age, 49], neuromyelitis optica (NMO) [$n=13$; male:female = 2:11; age range, 26–79; mean age, 49], multiple cerebral infarctions (MCI) [$n=20$; male:female = 9:11; age range, 53–83; mean age, 71], infectious meningoencephalitis (IME) [$n=19$; male:female = 14:5; age range, 15–71; mean age, 45], and Parkinson's disease (PD) [$n=21$; male:female = 11:10; age range, 50–85; mean age, 68], and from healthy controls [$n=17$; male:female = 7:10; age range, 25–74; mean age, 44]. All the MS patients were diagnosed with clinically definite MS according to the criteria of Poser et al. (1983). All the NMO patients satisfied the 2006 revision to the Wingerchuk diagnostic criteria (Wingerchuk et al., 2006).

2.2. Preparation of tissue proteins

Under ether anesthesia, adult Wistar rats were sacrificed. Their cerebrums were immediately removed and frozen in dry-ice powder. The frozen brain tissue was homogenized in lysis buffer (7 M urea, 2 M thiourea, 0.4% CHAPS, 0.1% DTT, 0.5% Triton X-100, and 0.2% SDS) and centrifuged at 100,000 × *g* for 40 min. The obtained supernatant was used in all experiments.

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2.3. Screening for autoantibodies against protein sample in sera from MS patients

We examined autoantibodies against a prepared protein sample in sera from five MS patients and five healthy controls by one-dimensional electrophoresis (1DE) and immunoblotting. The extracted proteins were applied at 20 μg/well to 4–20% polyacrylamide gel for western blotting. The proteins were separated by SDS-PAGE and separated proteins were blotted onto polyvinylidine difluoride (PVDF) membranes at 0.8 mA/cm^2 for 1 h using a semidry blotting apparatus (Trans-Blot SD semidry transfer cell, Bio-Rad Laboratories). Subsequently, the membranes were incubated in blocking solution overnight in a cold room, and then reacted with the sera from MS patients and healthy controls (diluted at 1:1500) for 1 h at room temperature, followed by washing. Then the membranes were incubated with HRP-conjugated anti-human Ig (A + G + M) antibodies (Zymed) (diluted at 1:2000) for 1 h at room temperature and reacted with the ECL-Plus Western blotting detection system (GE Healthcare).

2.4. Two-dimensional electrophoresis (2DE) and immunoblotting

A sample was loaded onto an immobilized and rehydrated dry strip (pH 3–10, nonlinear 18 cm long, GE Healthcare). Up to 100 μg of extracted proteins was applied to the dry strip for western blotting. Isoelectric focusing was carried out at 20 °C for 85,000 Vh at a maximum of 8000 V using a horizontal electrophoresis system (Coolphorestar IPG-IEF Type-PX, Anatech). This IPG strip was transferred to 12.5% polyacrylamide gel. The second-dimension run was carried out vertically using an electrophoresis apparatus (Coolphorestar SDS-PAGE Dual-200 K, Anatech) at 30 mA/gel. After the electrophoresis, the SDS-PAGE gels were stained with SyproRuby (Bio-Rad Laboratories) or used for protein transfer onto PVDF membranes (Toda and Kimura, 1997). Separated proteins were electrophoretically transferred to a PVDF membrane at a constant voltage of 32 V for 3 h using a buffer transfer tank with cool equipment (Toda et al., 2000). Subsequently, this membrane was incubated in a blocking solution (5% skim milk in 1× TBST and 1× TBS containing 0.1% Tween 20) overnight in a cold room, and then reacted with serum from a patient diluted (1:1500) with 1% skim milk in 1× TBST for 1 h at room temperature. The PVDF membrane was washed five times with 1× TBST and reacted with peroxidase-conjugated goat anti-human Ig (A + G + M) antibodies (Zymed) diluted (1:2000) with 1% skim milk in 1× TBST for 1 h at room temperature. After six washes, the membrane was incubated with the WB detection reagent (ECL-Plus, GE Healthcare) for 5 min and then scanned using a variable-mode imager (Typhoon 9400, GE Healthcare). The antibody-reactive protein spots were matched with the protein spots stained with SyproRuby (Bio-Rad Laboratories) using image analysis software (Adobe Photoshop 6.0).

2.5. In-gel digestion and mass spectrometry

Proteins were detected by staining with SyproRuby (Bio-Rad Laboratories). For mass spectrometric identification, the target protein spot on the SyproRuby-stained 2D electrophoresis gel was excised using FluoroPhoreStar 3000 (Anatech). In-gel digestion was performed in according with a standard protocol (Toda and Kimura, 1997) with minor modifications. Briefly, gel pieces were dehydrated and the dried gel pieces were rehydrated in 5 μl of 100 mM ammonium bicarbonate containing 10 μg/ml trypsin (Promega) for 3 h at 37 °C. After digestion, tryptic peptides were extracted twice with 50 μl of 66% acetonitrile in 0.1% trifluoroacetic acid (TFA) in a sonicator. The extracted peptides were dried, redissolved in 0.1% TFA, and injected onto a MonoCap 0.1 mm × 250 mm monolithic C18 column (Kyoto Monotech) with Prominence Nano (Shimadzu). The column eluent was spotted every 15 s onto a μFocus MALDI plate (Shimadzu GLC) with α-cyano-4-hydroxycinnamic acid (Sigma Aldrich) as a matrix using AccuSpot (Shimadzu). Buffer A consisted of 5% acetonitrile and 0.1% (v/v) TFA and buffer B consisted of 90% acetonitrile and 0.1% (v/v) TFA. The separation gradient was 5–60% buffer B over 30 min at a flow rate of 1 μl/min. The digests were

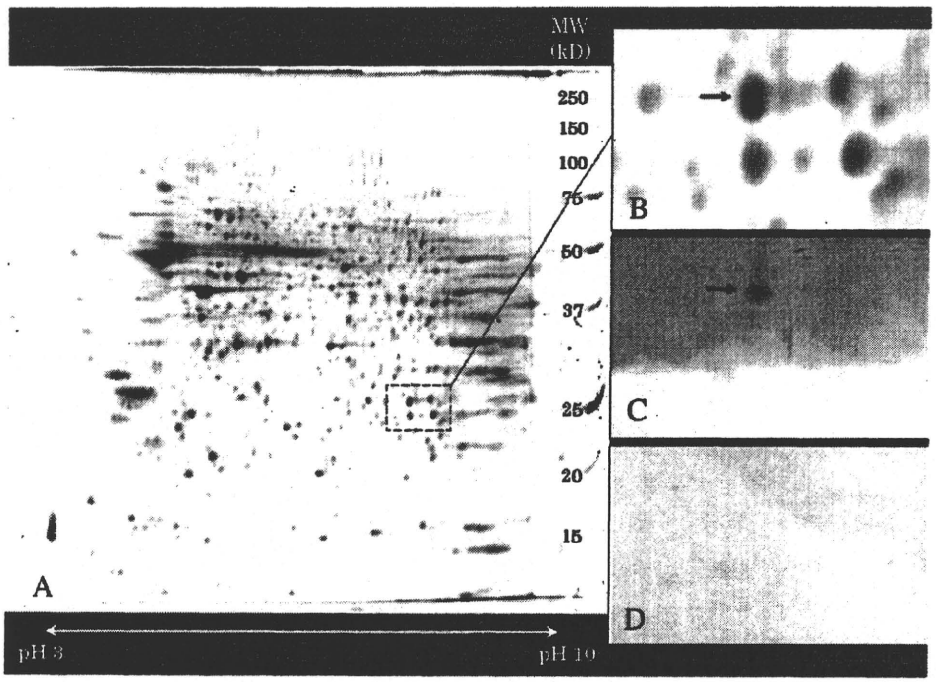

Fig. 1. Autoantibodies visualized by two-dimensional electrophoresis (2DE) and immunoblotting in sera from patients with multiple sclerosis (MS) and from healthy controls. (A, B) Total protein extracts of homogenized rat brain tissue were separated by 2DE, followed by SyproRuby staining. Arrow indicates the protein spot matching the immunoreactive spot detected by western blotting of MS patients' sera. Subsequently, this spot was identified as phosphoglycerate mutase 1 (PGAM1) by MALDI TOF-MS; (C) 1:1500-diluted MS patient's sera; arrow indicates the protein spot (PGAM1) recognized in sera from MS patients; and (D) 1:1500-diluted sera from healthy controls.