Voltage-gated potassium channel antibody-associated encephalitis with basal ganglia lesions

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Antibodies reactive with neuronal voltage-gated potassium channels (VGKCs) have been reported in patients with limbic encephalitis, which affects the medial temporal lobes and is characterized by subacute onset of temporal lobe seizures, memory impairment, and personality changes.[1-5] MRI of those patients showed only abnormal signal intensity located in the medial temporal lobes.[1-5] We describe a patient with encephalitis associated with anti-VGKC antibody who had MRI abnormalities localized in the basal ganglia as well as in the medial temporal lobes that improved dramatically on corticosteroid treatment.

Case report. A previously healthy, 23-year-old woman presented with a generalized seizure and was admitted to a nearby hospital, 2 months after onset of repeated short-term fevers with cervical lymphadenopathy. She showed disorientation and memory loss. Brain CT was negative. A CSF examination showed pleocytosis (12 lymphocytes/mm^3) and a normal protein level. Viral encephalitis was diagnosed, and she was prescribed IV acyclovir. She continued to show severe memory loss and childishness. Brain MRI done 17 days after admission showed symmetric abnormal signal intensities localized in the bilateral temporal lobes and basal ganglia with scattered small lesions in the amygdala, hippocampus, thalamus, and cerebral cortex (figure, A). She was transferred to our institution on day 22 after admission.

On transfer, she was afebrile and alert, but showed disorientation to time and place. Her recent memory was severely impaired, as she was unable to recall any of three objects after 5 minutes. In contrast, her remote memory was less severely affected. Her behavior was childish: she used baby talk and was restless. There was no confabulation, paramnesia, aphasia, agnosia, or apraxia, nor was there hypermetamorphosis, oral tendencies, or change in her sexual behavior. No cranial nerve dysfunction was found despite very mild dysarthria. Tendon reflexes in her four extremities were normal, as was muscle strength. Sensory examination results were normal. There were no obvious extrapyramidal signs or symptoms and no involuntary movement was noted. Coordination and gait were intact. There was no hyperhydrosis, nor clinical evidence of neuromyotonia.

Laboratory tests showed normal blood cell counts. Blood chemistry revealed aspartate aminotransferase at 60 IU/L, alanine aminotransferase 61 IU/L, and serum C-reactive protein 1.2 mg/dL. Her serum sodium level was normal. Antibodies to the Sm, Ro, La, and RNP antigens, anti-DNA, and anti-thyroid peroxidase antibodies were negative. Virologic tests of her serum were negative for herpes simplex virus (HSV), varicella zoster virus (VZV), cytomegalovirus (CMV) antibody, human herpesvirus 6 (HHV-6), and Epstein-Barr virus (EBV).

PCR analysis of her CSF was negative for HSV, VZV, CMV, and HHV-6, and a cytologic CSF examination also was negative. EEG showed frontal intermittent rhythmic delta activity with no epileptic EEG foci. Anti-VGKC antibody, assessed retrospectively, was positive on immunoprecipitation with I^{125}-alpha-dendrotoxin (455 pM; normal < 100 pM).

Immune-mediated encephalitis was diagnosed. IV methylprednisolone was administered from the day of transfer, followed by oral prednisolone. Marked clinical improvement occurred within 2 weeks after corticosteroid treatment was begun. MRI on day 46 showed resolution of the abnormalities (figure, B), and she was discharged on day 50. Her Mini-Mental State Examination score on admission was 19/30, indicative of markedly impaired delayed recall, calculation, and orientation, but it had improved to 29/30 by the time of discharge and 30/30 at 3 months after discharge. Childishness had also improved, and she showed complete recovery, now sustained for 5 further months.

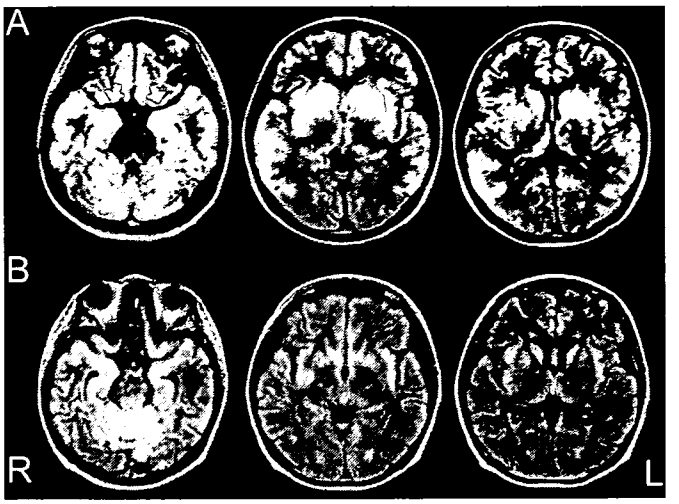

Figure. (A) Axial fluid-attenuated inversion recovery (FLAIR) MRI done 17 days after the first admission to the general hospital shows bilateral high signal intensity in the basal ganglia, as well as in the medial temporal lobes. Arrows indicate medial temporal lobes. (B) MRI after corticosteroid treatment. Axial FLAIR MRI done 45 days after the first admission shows marked resolution of the temporal lobe and basal ganglia signal abnormalities.

Discussion. The present case was characterized by subacute encephalitis with seizure, memory impairment, personality change, the presence of anti-VGKC antibody, symmetric basal ganglia lesions shown by MRI, and favorable recovery after corticosteroid treatment. In VGKC antibodies-associated encephalitis, MRI has so far shown only abnormal signal intensity located in the bilateral/unilateral medial temporal lobes, or no significant abnormalities.[1-5]

Immunohistochemistry of an acute serum sample from a patient with VGKC-antibodies and limbic encephalitis showed strong immunostaining of adult rat hippocampus, particularly the middle one-third of the molecular layer of the dentate gyrus,[1] indicating that the hippocampus is the major target of these antibodies. Serum from a patient with Morvan's syndrome with VGKC antibodies bound strongly to neuronal cells in the hippocampus and thalamus. Those antibodies also bound to neuronal cells in the striatum,[6] as do antibodies against the Kv 1.2 subtype of VGKCs.[7] Strong binding to the VGKC of hippocampus neurons may be associated with the clinical manifestations and MRI abnormalities seen in VGKC antibody-associated encephalitis. In our patient's case, anti-VGKC binding affinity to the striatum and blood-brain barrier leakiness could explain not only the hippocampal but also the bilateral basal ganglia involvement.

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References

1. Buckley C, Oger J, Clover L, et al. Potassium channel antibodies in two patients with reversible limbic encephalitis. Ann Neurol 2001;50: 73–78.
2. Schott JM, Harkness K, Barnes J, Incisa della Rocchetta A, Vincent A, Rossor MN. Amnesia, cerebral atrophy, and autoimmunity. Lancet 2003; 361:1266.
3. Pozo-Rosich P, Clover L, Saiz A, Vincent A, Graus F. Voltage-gated potassium channel antibodies in limbic encephalitis. Ann Neurol 2003; 54:530–533.
4. Thieben MJ, Lennon VA, Boeve BF, Aksamit AJ, Keegan M, Vernino S. Potentially reversible autoimmune limbic encephalitis with neuronal potassium channel antibody. Neurology 2004;62:1177–1182.
5. Vincent A, Buckley C, Schott JM, et al. Potassium channel antibody-associated encephalopathy: a potentially immunotherapy-responsive form of limbic encephalitis. Brain 2004;127:701–712.
6. Liguori R, Vincent A, Clover L, et al. Morvan's syndrome: peripheral and central nervous system and cardiac involvement with antibodies to voltage-gated potassium channels. Brain 2001;124:2417–2426.
7. Rhodes KJ, Strassle BW, Monaghan MM, Bekele-Arcuri Z, Matos MF, Trimmer JS. Association and colocalization of the Kvβ1 and Kvβ2 β-subunits with Kv1 α-subunits in mammalian brain K^+ channel complexes. J Neurosci 1997;17:8246–8258.

Pregabalin as add-on treatment to botulinum toxin in idiopathic hemifacial spasm

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Botulinum toxin (BTX) is the medical therapy of choice in hemifacial spasm (HFS). However, in some patients, its therapeutic effect is insufficient.

Case reports. Patient 1. A 64-year-old man with a 10-year history of left-sided HFS was treated with BTX over a period of 6 years with good results. However, in the last 2 years the spasms were never sufficiently relieved by BTX. The patient felt significantly impaired, but declined to undergo neurosurgical intervention. The EDB test showed a decrease in the CMAP amplitude of 60%. Without changing the BTX regime, pregabalin (initially 75 mg/day increased every 5 days by 75 mg to 150 mg twice daily) was added for a 1-month trial period in the absence of adverse effects. The HFS subsided gradually. After discontinuation of the drug at the end of the trial period, the symptoms recurred after <4 weeks. Rechallenge with pregabalin alone at a dosage of 300 mg/day over 4 weeks improved HFS but did not prevent the development of spasms. BTX therapy was therefore resumed, using the same injection points and dosage as previously, while pregabalin treatment was continued in parallel. The spasms began to disappear after approximately 1 week using the combination therapy, and subsided completely after a period of 3 months.

Patient 2. A 33-year-old woman with a 6-year history of right-sided HFS was treated with BTX over a period of 3 years. During the third year of therapy the spasms were never completely relieved by BTX. Higher doses were not tolerated. The patient declined to undergo neurosurgical intervention. The EDB test showed a decrease in the CMAP amplitude of 75%. Without changing the BTX therapy, pregabalin (initially 75 mg/day increased every 5 days by 75 mg to 150 mg in the morning and 75 mg in the evening) was added to the regime for a 1-month trial period. A higher dosage was not tolerated due to increasing fatigue. Under pregabalin dosage of 225 mg/day, the HFS subsided gradually. After discontinuation of the drug, the symptoms recurred after <4 weeks. Pregabalin alone at a dosage of 225 mg/day over 4 weeks yielded an improvement in the severity of the HFS, but did not result in the prevention of the spasms. Treatment with BTX was thus resumed and injected using the same injection points and dosage as previously, while pregabalin treatment was carried out in parallel. The spasms began to disappear after approximately 1 week using the combination therapy, and subsided completely after a period of 4 months.

The routine biochemical blood analysis under pregabalin was normal in both patients. MRI of the caudal pons with the focus at the cerebellopontine angle was normal in Patient 1, and showed a vessel loop crossing the facial nerve in Patient 2. EMG revealed synchronous bursts of repetitive high-frequency motor unit discharges in the affected orbicularis oculi and oris muscles of both patients. The blink reflex response demonstrated spreading of HFS to the perioral muscles in both patients.

Discussion. BTX is widely accepted as the method of choice in the therapy of HFS.[1] Microsurgical vascular decompression as an alternative option is curative in 70 to 95% of patients, although treatment failure, recurrences, and neurosurgical complications may occur.[2]

A number of patients do not respond to BTX injections, or have side effects that preclude the administration of an efficacious dose.[3] There is thus a need for further or alternative treatment options.

Although both patients described in the present report responded favorably to BTX as demonstrated by the EDB test,[4] the effect of increasing doses was limited by adverse effects. The decision to use pregabalin in our patients was based on previous findings, showing gabapentin to be efficacious in individual patients with HFS.[5] Gabapentin and pregabalin are known to bind to the α2δ-subunit of the voltage-dependent calcium channels, which are also present in the facial nerve,[6] but the mechanism of pregabalin in HFS is still speculative. Its pharmacology was recently reviewed.[7]

Following the administration of BTX alone, both patients continued to develop spasms. Only after the combined treatment with pregabalin and BTX was the complete and sustained cessation of HFS achieved. The therapy with pregabalin alone alleviated the spasms, but did not prevent their occurrence. Therefore, pregabalin seems to be a supplementary treatment option in those patients with facial spasms that cannot be relieved sufficiently by BTX alone. However, since pregabalin is not approved by the Food and Drug Administration for this condition, it is an individual treatment attempt (off-label use), and the long-term efficacy of pregabalin in HFS has yet to be determined.

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References

1. Tan NC, Chan LL, Tan EK. Hemifacial spasm and involuntary movements. Q J Med 2002;95:493–500.
2. Kondo A. Follow-up results of microvascular decompression in trigeminal neuralgia and hemifacial spasm. Neurosurgery 1997;40:46–51.
3. Brin MF, Danisi F, Blitzer A. Blepharospasm, oromandibular dystonia, Meige's syndrome and hemifacial spasm. In: Moore P, Naumann M, eds. Handbook of botulinum toxin treatment. Oxford: Blackwell, 2003;119–141.
4. Kessler KR, Benecke R. The EDB test—a clinical test for the detection of antibodies to botulinum toxin type A. Mov Disord 1997;12:95–99.
5. Daniele O, Caravaglio G, Marchini C, Mucchiut M, Capus L, Natale E. Gabapentin in the treatment of hemifacial spasm. Acta Neurol Scand 2001;104:110–112.
6. Cole RL, Lechner SM, Williams ME, et al. Differential distribution of voltage-gated calcium channel alpha-2 delta (α2δ) subunit mRNA-containing cells in the rat central nervous system and the dorsal root ganglia. J Comp Neurol 2005;491:246–269.
7. Ben-Menachem E. Pregabalin pharmacology and its relevance to clinical practice. Epilepsia 2004;45 Suppl 6:13–18.

INTERNAL MEDICINE

□ EDITORIAL □

Measles Encephalitis: Direct Viral Invasion or Autoimmune-Mediated Inflammation?

Mitsuaki Hosoya

Key words: measles, encephalitis, encephalopathy, MRI

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Encephalitis is an inflammation of the brain. The diagnosis of encephalitis can be established only by microscopic examination of brain tissue, and similarly the etiology is established only by the recovery from or the demonstration in brain tissue of an infectious agent. In clinical practice, however, the diagnosis of encephalitis frequently is based on neurologic manifestations, and the etiologic diagnosis is based on the recovery of infectious agents from other sites in the body, the serologic evidence of a specific infection, and relevant epidemiologic findings.

Encephalitis is classified as primary or as postinfectious. Primary encephalitis is an illness in which encephalitis is the major manifestation. Symptoms are caused by direct invasion and replication of an infectious agent in the central nervous system (CNS), resulting in objective clinical evidence of cerebral or cerebellar dysfunction. Postinfectious encephalitis occurs after other illness that is not CNS illness. Inflammation in CNS may be mediated immunologically. When neurologic clinical findings suggest encephalitis, but inflammation of the brain has not occurred, the condition is identified as encephalopathy.

Measles virus is known to be the cause of both the subacute form of encephalitis and the acute form of encephalitis. Subacute encephalitis is consisted from slow virus infection, i.e., subacute sclerosing panencephalitis (SSPE) and subacute measles encephalitis (SME). Altered measles virus (SSPE virus) was recovered from the brain tissue of patients with SSPE, and SSPE is considered to be a rare degenerative CNS disease caused by direct SSPE virus invasion. SME occurs by the CNS infection of wild type-measles virus in the immunocompromised host. The risk of SSPE developing in children who previously had natural measles is between 0.6 and 2.2 per 100,000 measles infections. The risk is grater in patients who acquire measles at an early age.

Clinically evident acute measles encephalitis occurs in approximately 0.5 to 1 of every 1,000 measles cases. Although both the mortality and the incidence of sequelae have varied in the available literature, the mortality rate is between 10 and 20 percent and the morbidity rate is between 20 and 40 percent of patients who recovered from measles encephalitis (1). Measles encephalitis occurs from direct viral-induced cellular damage or from an autoimmune-mediated tissue damage. Considerable controversy surrounds the mechanisms in measles encephalitis. Some investigators have recovered measles virus from the CSF and brain of affected patients (2, 3), which indicates that the virus is involved directly in the process. Other investigators have failed to isolate measles virus or to demonstrate measles virus RNA in the brain of affected patients (4). These findings have led to the belief that the illness is autoimmune.

Symptoms of acute encephalitis usually develop during the period of measles exanthema and within 8 days of the onset of illness. Occasionally, the onset of central nervous system signs and syndromes occurs during the prodromal period (5). Onset at an early phase may suggest primary viral invasion, and later onset may suggest autoimmune mechanisms. Examination of cerebrospinal fluid (CSF) in measles encephalitis usually reveals mild pleocytosis with a predominance of mononuclear cells, mildly elevated protein values, and a normal glucose level. In one study (6), 15 percent of the cases did not have pleocytosis in CSF, which may suggest the existence of encephalopathy in cases with neurologic manifestation. Myelin basic protein in CSF suggests autoimmune-mediated encephalitis. Detection of specific viral genome in CSF suggests primary viral encephalitis.

Jin et al (7) reported a case of fluminant adult-onset measles encephalitis. Acute measles encephalitis usually occurs in non-immunocompromised patients, most of whom are children and adolescents. In their reported case, neurologic manifestation was observed six days after measles onset. Pleocytosis with a predominance of mononuclear cells was observed and both oligoclonal IgG banding and myelin ba-

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sic protein were detected in CSF at neurologic onset. Such findings suggest autoimmune-mediated encephalitis rather than direct viral invasion. Brain MRI findings showed marked and diffuse cerebralatrophy during chronic phase. T2-weighted, FLAIR, and DW images demonstrated widespread hyperintense lesions around the lateral ventricles, which are consistent with marginal subpial demyelinations described as characteristic pathological findings of postinfectious encephalomyelitis. MRI findings might be useful for the differential diagnosis of autoimmune-mediated encephalitis from primary viral encephalitis.

References

1. Cherry JD. Measles virus. In: Textbook of Pediatric Infectious Diseases, 5th ed. Feigin Rd, Cherry JD, Demmler GJ, Kaolan SL, Eds. Saunders, Philadelphia, 2004: 2283-2304.
2. ter Meulen D, Kackell Y, Muller D, et al. Isolation of infectious measles virus in measles encephalitis. Lancet **2**: 1172-1175, 1972.
3. Esolen LM, Takahashi K, Johnson RT, et al. Brain endothelial cell infection in children with acute fatal measles. J Clin Invest **96**: 2478-2481, 1995.
4. Gendelman HE, Wolinsky JS, Johnson RT, et al. Measles encephalomyelitis: Lack of evidence of viral invasion of the central nervous system and quantitative study of the nature of demyelination. Ann Neurol **15**: 353-360, 1984.
5. Aarli JA. Nervous complications of measles: Clinical manifestations and prognosis. Eur Neurol **12**: 79-93, 1974.
6. LaBoccetta AC, Tornay AS. Measles encephalitis: Report of 61 cases. Am J Dis Child **107**: 247-255, 1964.
7. Jin K, Sato S, Saito R, et al. MRI findings from a case of fulminating adult-onset measles encephalitis. Intern Med **45** (12): 783-787, 2006.

ORIGINAL ARTICLE

Prognostic predictive values of serum cytochrome *c*, cytokines, and other laboratory measurements in acute encephalopathy with multiple organ failure

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Aims: To evaluate the prognostic predictive values of cytochrome *c*, cytokines, and other laboratory measurements in serum collected during neurological onset in acute encephalopathy with multiple organ failure.

Methods: In addition to general laboratory examinations, the concentrations of cytochrome *c* (apoptosis marker) and cytokines (inflammatory markers) were measured in serum samples collected at the initial phase in 29 patients with acute encephalopathy. The obtained values were evaluated as predictors for the development of severe encephalopathy.

Results: Cytochrome *c*, tumour necrosis factor α (TNF-α), interleukin 6 (IL-6), soluble TNF-receptor 1 (sTNF-R1), and aspartate aminotransferase (AST) concentrations at the initial phase were high and correlated well with patient outcome. High concentrations of serum cytochrome *c* (>45 ng/ml), sTNF-R1 (>2000 pg/ml), AST (>58 IU/dl), IL-6 (>60 pg/ml), and TNF-α (>15 pg/ml) predicted an unfavourable prognosis (sequelae and death) at 93%, 79%, 82%, 77%, and 60%, respectively. The specificity of those markers was 100%, 89%, 83%, 100%, and 100%, respectively.

Conclusions: Serum cytochrome *c* is the most sensitive and specific predictor for the development of severe encephalopathy at the initial phase. Results suggest that this marker might be used to guide decisions regarding the start of the initial treatment and further intensive care.

Central nervous system (CNS) manifestations follow various viral infections in children. Recently, the high incidence and mortality of acute encephalopathy with multiple organ failure has become a serious health problem in Japan.[1 2] This type of encephalopathy is often associated with influenza and occasionally with other viral infections mainly in children age <10 years, and develops either on the day that signs of infection, such as high fever, cough, and fatigue, appear, or on the following day. The major initial neurological signs include abrupt onset of seizure and altered consciousness or loss of consciousness. In most patients with unfavourable prognosis (sequelae and death), their neurological state deteriorates and multiple organ dysfunction (liver, renal, coagulative, and haematopoietic systems) developed. Brain oedema detected by computed tomography (CT) becomes evident on the day after neurological onset. Rates of mortality (31.8%) and disability (27.7%) are high.[2] The outcome of the disease varies and the clinical symptoms at neurological onset do not predict the prognosis.[2] A number of strategies for the treatment of the encephalopathy are proposed, but an effective therapy has not been established. A prognostic predictor in the illness is desired for deciding when to start the initial treatment and further intensive care, and for evaluation of the therapeutic efficacy.

Serum concentrations of several proinflammatory cytokines and cytokine receptors, such as tumour necrosis factor α (TNF-α), interleukin 6 (IL-6), and soluble TNF-receptor 1 (sTNF-R1) are raised in the initial phase of acute encephalopathy associated with influenza.[3–7] Serum cytochrome *c* concentrations are also high in patients with an unfavourable prognosis.[8] In the present study, we measured serum cytochrome *c* and cytokine concentrations in addition to general laboratory examinations, and those markers were evaluated as prognostic predictors in acute encephalopathy with multiple organ failure.

METHODS

From January 1997 to December 2002, 29 patients were diagnosed with acute encephalopathy in Fukushima Prefecture, Japan (table 1). In this study, acute encephalopathy was defined as an acute CNS disorder with fever characterised by altered or loss of consciousness more than 24 hours after acute onset and brain oedema detected by CT. Twenty patients had cerebrospinal fluid (CSF) examination; no pleocytosis in CSF was observed. The other nine patients had diffuse brain oedema detected by initial CT examination and did not receive CSF examination for fear of brain herniation caused by lumbar puncture. Neurological, metabolic, endocrine, toxicological, and drug induced disorders were excluded. Diffuse cerebral oedema and localised cerebral oedema were observed on CT examination in 22 and 7 patients, respectively. Of the 22 patients with diffuse cerebral oedema, two had low density areas in the brain stem and both thalami. One patient had low density areas only in both thalami. Virological examinations revealed viral infections in 17 of the 29 patients. Diagnosis of type H3N2 influenza (n = 7) was based on viral isolation from throat swabs. A diagnosis of human herpes virus 6 infection (n = 2) was based on the detection of serum IgM antibody against the virus by enzyme linked immunosorbent assay (ELISA). A diagnosis of influenza A (n = 6) and rotavirus (n = 1) was based on virus antigen detection from throat swabs by ELISA and from faeces by the latex agglutination test, respectively. Enterovirus infection (n = 1) was diagnosed based on viral genome detection from throat swab by polymerase chain reaction. The 29 patients with acute encephalopathy were

assigned to three groups according to outcome. Of 29 patients, 11 patients died (group A), 6 patients survived with sequelae (group B), and 12 patients survived without sequelae (group C). Informed consent was obtained from the parents of the patients enrolled in this study. Ethics approval for the study was obtained from our Institutional Review Board.

Blood samples were collected on day 1 (within 6 hours after neurological onset) and on day 2 (12–24 hours after the first collection). In the blood samples, we measured the following: aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic dehydrogenase (LDH), haemoglobin (Hb), and creatinine (Cr) using standard automated techniques. The remaining serum samples were stored at −80℃ until cytokine and cytochrome *c* assays were performed. TNF-α, IL-6, and sTNF-R1 concentrations were measured using commercially available ELISA kits (TNF-α and IL-6: Endogen, Inc., MA, USA; sTNF-R1: R&D Systems Inc., MN, USA). These assays were performed according to the manufacture's instructions. Sample values were determined from a standard curve. Cytochrome *c* concentrations were measured using an improved sandwich electrochemiluminescence immunoassay.[8] Briefly, microbeads (Dynabeads M-450 Epoxy, DYNAL A.S., Oslo, Norway) coated with anti-cytochrome *c* monoclonal antibody and anti- cytochrome *c* monoclonal antibody conjugated with ruthenium chelate (Ru-Ab) were used for the cytochrome *c* immunoassay. We mixed 25 μl of sample, 200 μl of dilution buffer, and 25 μl of coated microbeads. After incubation at 30℃ for 9 minutes, the microbeads were washed twice to remove non-reacted specimens and Ru-Ab was added. The microbeads were then washed twice to remove non-reacted Ru-Ab, and placed into magnet mounted flow-cell electrodes to measure the quantity of the emission. The cytochrome *c* concentration of the sample was calculated using human cytochrome *c* standard solutions (1–2000 ng/ml). All three operations were performed automatically using a Picolumi 8220 (Sanko Junyaku Co., Ltd, Tokyo, Japan), except for the dilution of the sample.

Differences in the laboratory measurements between day 1 and day 2 were assessed using a paired *t* test and those between the three outcome groups at day 1 were assessed using the Mann-Whitney rank sum test. A value of 0.05 or less was considered significant. Statistical analysis was performed on a Macintosh computer with a software package for statistical analysis (Stat View, Abacus Concepts, Berkeley, USA). Receiver operating characteristic (ROC) curves[9] and their areas under the curve (AUC) were calculated by use of StatFlex, version 5.0, for Windows (Artec Co., Ltd, Osaka, Japan). In the ROC curve, true positive is plotted on the vertical axis and false positive on the horizontal axis at any cut-off level. Thus, the ROC curve visually represents diagnostic sensitivity and specificity. The advantages of this curve are as follows: diagnostic accuracy can be compared by AUC, the significance of the reference interval in diagnosis can be evaluated, and the most discriminative cut-off (threshold) level can be determined on the basis of the value in the top left corner of the ROC curve. Using the threshold level, we calculated sensitivity and specificity.

RESULTS

Comparison of laboratory measurements

We compared serum AST, ALT, LDH, Hb, Cr, cytochrome *c*, TNF-α, sTNF-R1, and IL-6 concentrations between day 1 and day 2. AST, ALT, and LDH levels generally increased on day 2 compared to day 1. Among groups A, B, and C, the levels significantly increased in group A, but not in groups B and C. Changes in Cr, Hb, cytochrome *c*, TNF-α, sTNF-R1, and IL-6 levels varied between day 1 and day 2. Differences in the laboratory measurements between the three outcome groups at day 1 were assessed. AST, LDH, Cr, Hb, cytochrome *c*, TNF-α, sTNF-R1, and IL-6 levels at the early stage were significantly higher in group A than in group C.

Prognostic predictive values of laboratory measurements

Prognostic predictive values of the nine laboratory measurements at the initial phase (day 1) were compared using AUC

Table 1 Summary of 29 patients with acute encephalopathy

Group	Outcome	Gender	Age	CT findings	Pathogen
A	Dead	F	1y 11m	Diffuse CE	Unknown
	Dead	M	4y 9m	Diffuse CE	Influenza AH3
	Dead	M	8y 0m	Diffuse CE	Influenza AH3
	Dead	M	3y 5m	Diffuse CE	Influenza AH3
	Dead	M	2y 1m	Diffuse CE + brain stem LD	Unknown
	Dead	F	7y 5m	Diffuse CE	Unknown
	Dead	F	7y 5m	Diffuse CE	Unknown
	Dead	F	3y 4m	Diffuse CE	Unknown
	Dead	F	7y 0m	Diffuse CE + brain stem LD	Influenza AH3
	Dead	M	3y 10m	Diffuse CE	Influenza A
	Dead	M	3y 5m	Diffuse CE	Influenza A
B	Alive with sequelae	M	0y 11m	Left CE	HHV-6
	Alive with sequelae	F	0y 9m	Right CE	HHV-6
	Alive with sequelae	M	3y 10m	Diffuse CE	Influenza A
	Alive with sequelae	F	1y 10m	Right CE	Influenza AH3
	Alive with sequelae	M	1y 4m	Diffuse CE	Unknown
	Alive with sequelae	F	1y 3m	Right CE	Influenza AH3
C	Alive without sequelae	M	0y 9m	Diffuse CE	Rotavirus
	Alive without sequelae	M	1y 3m	Bilateral talami LD	Unknown
	Alive without sequelae	M	12y 6m	Diffuse CE	Unknown
	Alive without sequelae	M	5y 2m	Diffuse CE	Enterovirus
	Alive without sequelae	M	0y 11m	Right CE	Influenza AH3
	Alive without sequelae	M	4y 11m	Diffuse CE	Unknown
	Alive without sequelae	F	0y 11m	Diffuse CE	Unknown
	Alive without sequelae	F	2y 4m	Diffuse CE	Unknown
	Alive without sequelae	F	3y 3m	Diffuse CE	Influenza A
	Alive without sequelae	M	9y 11m	Bilateral parietal CE	Influenza A
	Alive without sequelae	F	1y 11m	Right CE	Unknown
	Alive without sequelae	F	4y 0m	Diffuse CE	Influenza A

CE, cerebral oedema; LD, low density.

determined by ROC curve analysis (see fig 1). Serum cytochrome *c*, TNF-α, IL-6, sTNF-R1, and AST were better predictive markers for an unfavourable prognosis (sequelae or death) than the others (fig 1A). When we evaluated those markers as predictors for a poor prognosis (death), Hb, Cr, and sTNF-R1 were better than the others (fig 1B).

Sensitivity and specificity of laboratory measurements

When the threshold was set at 45 ng/ml for cytochrome *c*, 2000 pg/ml for sTNF-R1, 58 IU/dl for AST, 60 pg/ml for IL-6, and 15 pg/ml for TNF-α, the sensitivity of these measurements as a predictor for an unfavourable prognosis was calculated to be 93%, 79%, 82%, 77%, and 60%, respectively. The specificity of these was 100%, 89%, 83%, 100%, and 100%, respectively. High concentrations of serum Hb (>13.0 g/dl), Cr (>0.65 mg/dl), and sTNF-R1 (>2000 pg/ml) predicted the patients with a poor prognosis at 91%, 73%, and 90%, respectively. The specificity of Hb, Cr, and sTNF-R1 was 83%, 94%, and 77%, respectively.

DISCUSSION

In acute encephalopathy associated with influenza, there are raised serum concentrations of several cytokines, such as TNF-α, sTNF-R1, IL-1β, and IL-6.[3-7] Hypercytokinaemia, therefore, is suggested to have an important role in the pathogenesis of acute encephalopathy and multiple organ dysfunction.[9] From the suspected pathogenesis, anti-inflammatory therapies (methylprednisolone pulse therapy, high dose gamma globulin therapy, and plasma exchange therapy) are proposed for the treatment of the illness. We initiated the anti-inflammatory therapies for the treatment of our patients when brain oedema was detected by CT and multiple organ dysfunction was evident at the exacerbation phase. These therapies, however, seemed to have no effect on the clinical course of the patients with a poor prognosis. We believe that therapy should be initiated at an earlier time point to be effective, and therefore a predictive marker identifying high risk patients is necessary. Serum IL-6,[3,4] sTNF-R1,[5,6] and cytochrome *c*[8] might be predictive for the development of severe illness before massive brain oedema and multiple organ failure develop. We thus compared the values of specific markers for apoptosis (cytochrome *c*) and inflammation (TNF-α, sTNF-R1, and IL-6) and general measurements for organ dysfunctions (AST, ALT, LDH, Cr, and Hb) as predictors for the prognosis in acute encephalopathy.

ROC analysis indicated that high concentrations of cytochrome *c* and cytokines on day 1 were predictive for the patients with an unfavourable outcome (sequelae or death); cytochrome *c* seemed to be the most sensitive and specific predictor for the development of severe encephalopathy. High concentrations of Cr and Hb on day 1 were predictive for the patients with a poor prognosis (death).

Why do high cytochrome *c* and cytokine levels predict an unfavourable prognosis at an early stage? Cytochrome *c* is an intramitochondrial protein normally residing in the intermembrane spaces. It triggers the execution phase of apoptosis by massive translocation into the cytoplasm, leading to Apaf-1 mediated caspase activation.[10] Our results suggested that apoptosis as well as inflammation[3-7] in vascular vessels was involved in the development of severe encephalopathy. Findings from pathological examination suggest that direct viral invasion and inflammation in CNS are not likely to cause this disease.[2,3] Vascular damage with subsequent leakage of plasma and intravascular formation of thrombi is observed in systemic organs as well as in the brain.[2,11] TNF-α is a major apoptosis inducing factor. We hypothesise that inflammatory cytokines might be closely involved in the development of severe encephalopathy and multiple organ dysfunction through the induction of apoptosis in the vascular endothelium[2] in CNS and other systemic organs. High blood levels of Hb and Cr at the initial phase might directly represent haemoconcentration and decreased renal flow by massive leakage of plasma, and thus suggest a poor prognosis. High blood levels of AST, ALT, and LDH might represent the results of systemic organ injury, and thus those values increase on day 2.

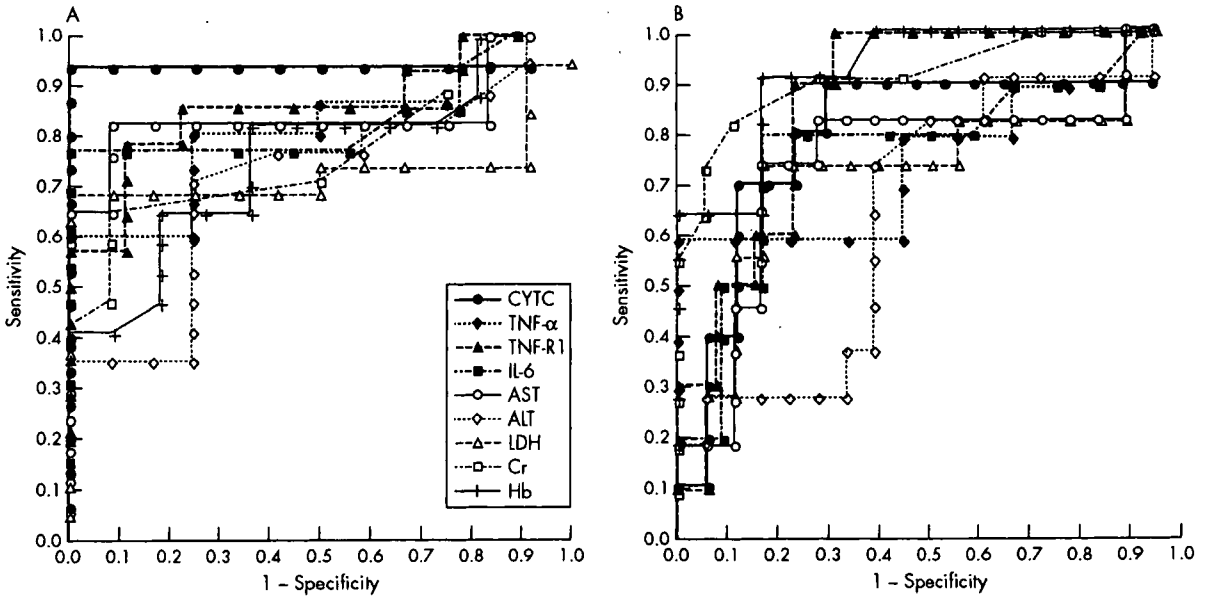

Figure 1 Prognostic predictive values at the neurological onset. Prognostic predictive values of the nine laboratory measurements were evaluated using ROC curve analysis. (A) At the initial phase, cytochrome *c*, TNF-α, sTNF-R1, IL-6, and AST levels were sensitive predictive markers for an unfavourable prognosis (sequelae or death). (B) Hb, Cr, and sTNF-R1 were sensitive predictive markers for a poor prognosis (death).

What is already known on this topic

- In the initial phase of acute encephalopathy associated with influenza, serum concentrations of several pro-inflammatory cytokines and cytokine receptors, such as TNF-α, IL-6, and sTNF-R1 are raised
- Serum cytochrome *c* concentrations are also high in patients with an unfavourable prognosis

What this study adds

- Serum cytochrome *c* is the most sensitive and specific predictor for the development of severe encephalopathy at the initial phase
- This marker might be used to guide decisions regarding the start of the initial treatment and further intensive care in acute encephalopathy with multiple organ failure

In conclusion, serum cytochrome *c* level was the most sensitive and specific predictor for an unfavourable prognosis at the initial phase, as intravascular apoptosis might have a significant role in the development of severe encephalopathy. Real time monitoring of serum cytochrome *c* might be useful for deciding when to start intensive care in acute encephalopathy. This needs confirmation in another large patient cohort.

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REFERENCES

1 **Kasai T,** Togashi T, Morishima T. Encephalopathy associated with influenza epidemics. *Lancet* 2000;**355**:1558–9.
2 **Morishima T,** Togashi T, Yokota S, *et al.* Encephalitis and encephalopathy associated with an influenza epidemic in Japan. *Clin Infect Dis* 2002;**35**:512–17.
3 **Ito Y,** Ichiyama T, Kimura H, *et al.* Detection of influenza virus RNA by reverse transcription-PCR and proinflammatory cytokines in influenza-virus-associated encephalopathy. *J Med Virol* 1999;**58**:420–5.
4 **Aiba H,** Mochizuki M, Kimura M, *et al.* Predictive value of serum interleukin-6 level in influenza virus-associated encephalopathy. *Neurology* 2001;**57**:295–9.
5 **Ichiyama T,** Nishikawa M, Yoshitomi T, *et al.* Tumor necrosis factor-α, interleukin-1β, and interleukin-6 in cerebrospinal fluid from children with prolonged febrile seizures: comparison with acute encephalitis/encephalopathy. *Neurology* 1998;**50**:407–11.
6 **Ichiyama T,** Morishima T, Isumi H, *et al.* Analysis of cytokine levels and NF-kappaB activation in peripheral blood mononuclear cells in influenza virus-associated encephalopathy. *Cytokine* 2004;**27**:31–7.
7 **Kawada J,** Kimura H, Ito, et al. Systemic cytokine responses in patients with influenza-associated encephalopathy. *J Infect Dis* 2003;**188**:690–8.
8 **Hosoya M,** Nunoi H, Aoyama M, *et al.* Cytochrome c and TNF-α levels in serum and CSF of patients with influenza-associated encephalopathy. *Pediatr Infect Dis J* 2005;**24**:467–70.
9 **Swets JA.** Measuring the accuracy of diagnostic systems. *Science* 1988;**240**:1285–93.
10 **Li P,** Najhawan D, Budihardjo I, *et al.* Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997;**91**:479–89.
11 **Takahashi M,** Yamada T, Nakashita Y, *et al.* Influenza virus-induced encephalopathy: clinicopathologic study of an autopsied case. *Pediatr Int* 2000;**42**:204–14.

Genetic Diversity of Enterovirus 71 Associated With Hand, Foot and Mouth Disease Epidemics in Japan from 1983 to 2003

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Background: Enterovirus 71 (EV71) is one of the major etiologic agents of hand, foot and mouth disease (HFMD). The surveillance data indicate that EV71 infection follows an epidemic mode of transmission, causing large outbreaks and then becoming quiescent for a few years.
Methods: We investigated the genetic diversity of a total of 121 EV71 strains isolated from patients with HFMD in Fukushima, Japan, from 1983 to 2003 and compared their genetic relation with the 164 EV71 strains isolated in the world using phylogenetic analysis based on the VP4 sequence.
Results: We observed EV71-related HFMD outbreaks in Fukushima in 1984, 1987, 1990, 1993, 1997, 2000 and 2003. Phylogenetic reconstruction of EV71 strains isolated in Fukushima demonstrated 8 genetically distinct clusters, including 6 subgroups previously designated as B-1, B-2 and 3, B-4, C-1, C-2, and C-3 and 2 subgroups newly designated as B-5 and C-4. Additional 2 indistinct clusters belonged to genogroup C and were named C-U1 and C-U2. Of those subgroups, B-1, C-U1, C-U2, C-2, B4, and C-4 and B-5 dominantly related to epidemics that occurred in the years 1984, 1987 and 1990, 1993, 1997, 2000 and 2003, respectively. EV71 strains derived from each outbreak in Fukushima formed a single cluster with those isolated during almost the same time period in other area of Japan and in other countries.
Conclusions: Our results suggested that the repeated EV71 outbreaks might be the result of the worldwide transmission of the newly introduced genetically divergent EV71 strains.

Key Words: enterovirus 71, genetic diversity, phylogenetic analysis

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Human enterovirus 71 (EV71) is one of the major etiologic agents of hand, foot and mouth disease (HFMD). The surveillance data indicate that EV71 infection follows an epidemic mode of transmission, causing large outbreaks and then becoming quiescent for a few years.

Molecular epidemiology of EV71 strains isolated in the United States and 5 other countries had been described by Brown et al.[1] In their study, the prototype strain, BrCr-CA-70, isolated in California in 1970, was the sole member of genogroup A. Strains isolated in the United States and Australia from 1972 to 1988 was all members of genogroup B, and the group has not been isolated in the United States from 1988. Genogroup C was isolated in 1985 or later in the United States, Canada, Australia and the Republic of China. The study showed that EV71 was a genetically evolving virus.

EV71 is also associated with cases of acute neurologic diseases, including poliomyelitis-like paralysis, encephalitis and aseptic meningitis. In 1997, deaths associated with epidemics of EV71-associated HFMD in Sarawak, Malaysia, followed by outbreaks with high mortality in Taiwan in 1998 and 2000, have raised considerable public concern about the virulence of this virus.[2] Several groups[3,4] have attempted to describe the molecular epidemiology of recent EV71 isolates in the Asia-Pacific region. In Sarawak, in 1997, all isolates from fatal and nonfatal cases belonged to subgroup B3. Viruses belonging to B3 were also isolated in Singapore in 1998 and in Perth in 1999. Some EV71 strains isolated from children with severe neurologic disease during Perth epidemics in 1999 belonged to subgroup C2. Another recently described subgroup B cluster (B4) was identified in Singapore in 1997 and continued to circulate there in 2000 through 2002. Viruses from B4 were also identified as the primary cause of a large outbreak in Sarawak in 2000. These studies suggested that dominant EV71 strains causing recent epidemics were genetically changed and the divergent strains were transmitted in the Asia-Pacific region.

However, the relationship of repeated HFMD outbreaks caused by EV71 with the genetic diversity of the virus strains has not been fully described. We attempted to provide a more complete picture of the relationship between the longitudinal EV71 epidemics for more than 20 years in a restricted area, that is, Fukushima Prefecture, Japan, and the genetic diversity of the EV71 strains, and the transversal genetic relationship between the EV71 strains isolated in Fukushima and those isolated in the world using phylogenetic analysis constructed using the neighbor-joining method on the basis of the VP4 sequence.

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MATERIALS AND METHODS

Pharyngeal swab and/or rectal swab samples were collected from patients with HFMD in Fukushima Prefecture for virus surveillance and transferred to Fukushima Institute of Public Health for virus isolation. HEp-2, Vero and RD-18 cells were used for the isolation of enteroviruses. Confluent cell cultures were seeded in microplate wells and inoculated with 100 μL of maintenance medium and 50 μL of pharyngeal swab samples. The cell culture were then incubated at 34°C in 5% CO_2/95% air and observed for 7 days to check for cytopathic effects. Virus isolates were identified by a neutralization test using anti-EV71 polyclonal antibodies provided from National Institute of Infectious Diseases in Japan. A total of 186 EV71 strains were isolated and identified from 1983 to 2003. Those isolates were stored at −80°C.

Selected isolates (35 of 100 strains) from 1983 to 1998 and all isolates (86 strains) from 1999 to 2003 were used for further genetic analysis. All strains were isolated from patients with HFMD. There was no case with fatal outcome or severe neurologic complications. All strains were isolated from pharyngeal swab, except 3 strains isolated from rectal swabs (Fukushima/1448–2/96, Fukushima/9990/03 and Fukushima/10029/03).

The methods of molecular diagnosis of enteroviruses by nested reverse transcription (RT)–polymerase chain reaction (PCR) and phylogeny-based classification using the VP4 sequences are described elsewhere.[5] Briefly, viral RNA was directly extracted from 100 μL of the stock virus samples using the Smitest R kit (Genome Science Laboratories) according to the manufacturer's instructions. The RNA was dissolved with 10 μL of RNase-free distilled water containing 40 U of ribonuclease inhibitor (RNasin; Promega) and 50 pmol of a reverse primer, OL68-1 (nt 1178–1197, 5′-GGTAA(C/T)TTCCACCACCA[A/G/C/T]CC-3′). The positions of the primers for RT-PCR were numbered according to the complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain.[6] The RNA was subjected to heat denaturation for 15 seconds at 100°C followed by snap-cooling in an ice-water bath. To each RNA sample, we added 10 μL of a reaction mixture, 200 U of Moloney murine leukemia virus reverse transcription (Life Technology), 2.5 mM dNTPs and 40 U of RNasin (Promega). cDNA synthesis was performed for 1 hour at 37°C. In total, 5 μL of the cDNA reaction mixture was added to 45 μL of 1× *Taq* buffer containing 12.5 pmol of a forward primer, MD91 (nt 444–468, 5′-′CCTCCGGCCCCTGAATGCGGCTAAT-3′) and 2.5 U of *Taq*DNA polymerase (Roche Diagnostic Systems). Seminested PCR was performed using 5 μL of the PCR product with a pair of primers, EVP4 (nt 541–560, 5′-CTACTTTGGGTGTCCGTGTT-3′) and OL68-1. After initial denaturation at 94°C for 5 minutes, 40 cycles of amplification were performed using the GeneAmp PCR System 9600 (PE-Applied Biosystems). Each cycle consisted of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds and an extension reaction at 72°C for 1 minute followed by a final extension at 72°C for 7 minutes. The PCR products, including entire VP4 sequences, were separated in 1% agarose gels and purified with a QIA quick-gel extraction kit (Qiagen). The nucleotide sequence was determined with a 373A DNA autosequencer (PE-Applied Biosystems) with fluorescent dideoxy chain terminations (PE-Applied Biosystems) and EVP4 and OL68-1 primers.

The VP4 nucleotide sequences of the 121 EV71 strains isolated in Fukushima were used for phylogeny-based analysis along with those of 64 prototype enterovirus strains (Table 1 [online only]). We estimated the evolutionary distances using the Kimura 2-parameter method[7] and constructed unrooted phylogenetic trees with the neighbor-joining method.[8] Bootstrap analysis was performed by resampling the data sets 1000 times. Bootstrap values greater than 70% were considered to be statistically significant for the grouping. The VP4 sequences of representative 43 EV71 strains isolated in Fukushima were also compared with those of 65 strains from other regions in Japan and 99 strains from other countries, including Taiwan, Malaysia, Korea, China, Australia, the United Kingdom and the United States taken from international databases (Genbank) using phylogenetic analysis (Table 2 [online only]). Available clinical information on those cases was obtained from the literature, and the cases with fatal prognosis or severe illness such as encephalitis, paralysis and Guillain-Barré syndrome were indicated in figures.

RESULTS

We observed HFMD outbreaks in Fukushima in 1984, 1987, 1990, 1993, 1997 and 2000, and a more recent, larger outbreak in 2003 (Fig. 1). During 21 epidemics, we observed no HFMD case with encephalitis or fatal prognosis in Fukushima Prefecture. A nested RT-PCR assay was performed for the detection of enteroviral genome sequences and a positive PCR result was obtained in all 121 samples. Detected enterovirus strains were identified as EV71 using phylogeny-based analysis. Phylogenetic reconstruction of EV71 strains isolated in Fukushima from 1983 to 2001 demonstrated 6 genetically distinct subgroups, which were previously designated as B-1, B-2 and 3, B-4, C-1, C-2, and C-3 and other 2 indistinct subgroups, which belonged to genogroup C but were not classified into previously designated subgroups. The later 2 subgroups were named as C-U1 and C-U2, because their independence was not supported by bootstrap values. Of those subgroups, B-1, C-U1, C-U2, C-2 and B4 dominantly related to epidemics that occurred in the years 1984, 1987 and 1990, 1993, 1997 and 2000, respectively (Fig. 2 [online only]).

EV71 strains isolated from 2002 to 2003 in Fukushima were grouped to 4 distinct clusters. Of those subgroups, 2 were classified into subgroups C-1 and B-4, and the other 2 belonged to group B and group C but were distinct from the previously designated subgroups. The later 2 subgroups were very recently designated as B-5[9] and C-4,[10] respectively. Subgroups B5 and C4 were dominant in 2003.

VP4 sequences detected from representative 43 EV71 strains isolated in Fukushima were compared with those from international databases (Genbank), which included 65 strains isolated in other parts of Japan, 68 strains in Asia-Pacific region, 17 strains in the United States and 14 strains in Europe (Fig. 3 A, B [online only]). Subgroup B-1 included 7 EV71 strains isolated in Fukushima from 1983 to 1985, 12 strains in other parts of Japan from 1970 to 1984, 4 strains in

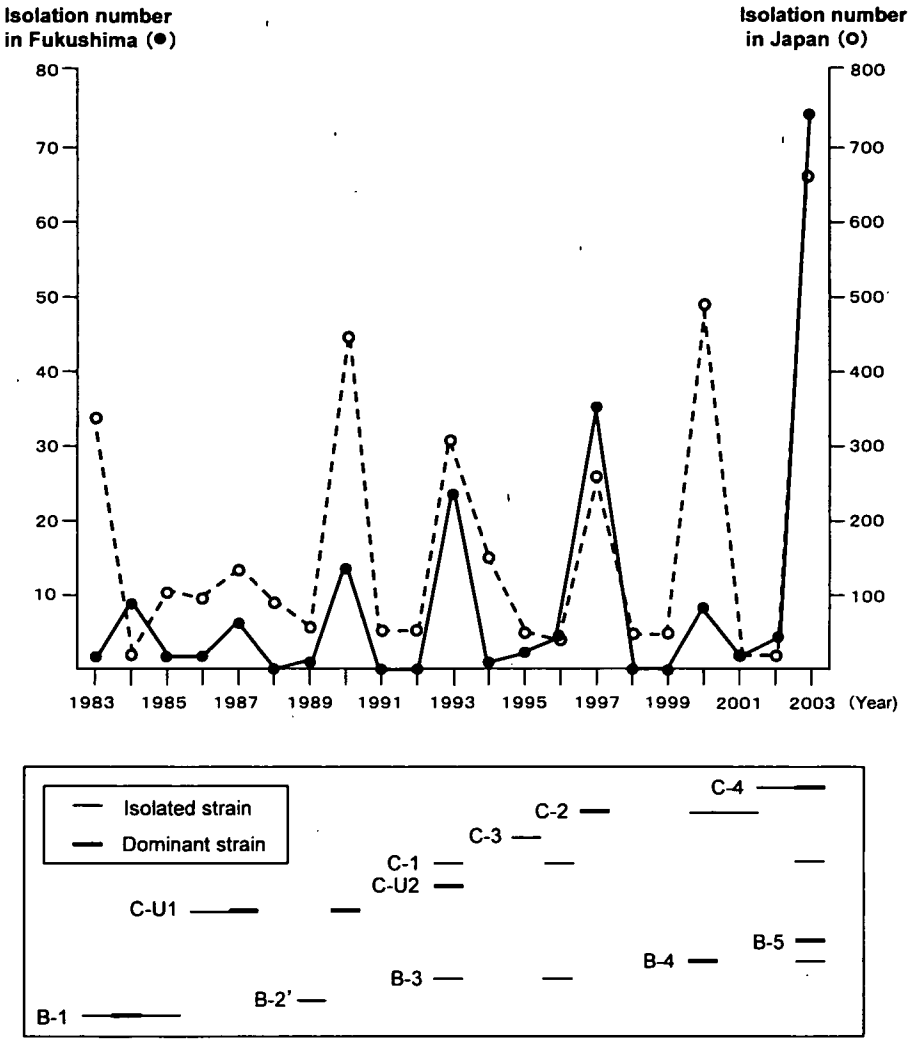

FIGURE 1. Numbers of enterovirus 71 (EV71) isolates in Fukushima Prefecture and in Japan between 1983 and 2003. Numbers of EV71 isolates in Fukushima (closed circle) and in Japan (open circle) are expressed as reported to the Infectious Disease Surveillance Center in Japan by prefectural and municipal public health institutes through the Japanese infectious agents' surveillance program.

Asia from 1973 to 1986, 6 strains in the United States from1976 to 1987 and 2 strains in Europe from 1975 to 1978 (Fig. 3A online). Subgroup C-2 included 13 EV71 strains isolated in Fukushima from 1997 to 2001, 10 strains in other parts of Japan from 1997 to 2000, 12 strains in Taiwan in 1998, 5 strains in the United Kingdom from 1996 to 1999 and 3 strains in Australia in 1999 (Fig. 3B [online only]). Newly designated subgroup B-5 included 22 EV71 strains isolated in Fukushima in 2003, 3 strains in other parts of Japan in 2003 and one strain in Malaysia in 2003.[9] Subgroup C-4 included 49 EV71 strains isolated in Fukushima from 2002 to 2003 and 15 strains in China from 1998 to 2005. In general, EV71 isolates derived from each outbreak occurred in Fukushima made a single cluster with those isolated during almost the same time period in another area of Japan and in other countries.

Available clinical information on the cases was obtained from the literature. The cases with fatal prognoses or neurologic complications were indicated in Figures 3A and 3B. We could not find any association of serious cases with certain subgenogroups in the cluster analysis.

DISCUSSION

EV71 causes large outbreaks of HFMD worldwide. Enterovirus surveillance data in Fukushima Prefecture from 1983 to 2003 indicate that the annual proportion of EV71 isolates relative to total enterovirus isolates fluctuates widely, from 0% in 1988, 1991, 1992, 1998 and 1999 to 29.0% in 2003. Peaks of EV71 isolations from HFMD occurred in the years 1984, 1987, 1990, 1993, 1997, 2000 and 2003. This epidemic pattern is very similar to that observed in Japanese surveillance data (Fig. 1).[11] Those observations indicate that EV71 follows an epidemic mode of transmission, causing large outbreaks and then becoming quiescent for a few years. Quiescence between outbreaks is probably the result of the development of population immunity that occurs during a high-infection-rate epidemic.

Phylogeny-based classification by use of the VP4 sequence is useful for the identification of human enteroviruses.[12,13] The method takes advantage of the detection of the divergence in VP4 sequences both between and within serotypes, and thus is also of use for global epidemiologic studies

of enteroviruses.[4,5] We investigated the genetic diversity of EV71 associated with HFMD outbreaks in Fukushima Prefecture, Japan, from 1983 to 2003 and compared their genetic relation with those isolated in other regions of Japan and in other countries using the same method. The VP1 dendrograms provide great confidence by high bootstrap values when elucidating new genogroups.[1] In the present study, EV71 genogroups A, B and C were designated based on the VP4 sequences by differing at 12.1 to 24.2% at the nucleotide level, and the differing was similar to that based on the VP1 gene analysis.

Phylogenetic reconstruction of EV71 strains isolated in Fukushima from 1983 to 2003 demonstrated at least 8 genetically distinct clusters, which included 6 subgroups previously designated as B-1, B-2 and 3, B-4, C-1, C-2 and C-3, and 2 subgroups newly designated as B-5 and C-4. Other 2 indistinct clusters belonged to genogroup C and were named C-U1 and C-U2. Of those subgroups, B-1, C-U1, C-U2, C-2, B4, and B-5 and C-4 dominantly related to epidemics that occurred in the years 1984, 1987 and 1990, 1993, 1997, 2000 and 2003, respectively. Our results showed that EV71 strains causing HFMD epidemics in Fukushima, Japan, had been genetically changed, and the repeated large outbreaks might be caused by the introduction of genetically divergent EV71 strains. EV71 isolates derived from each outbreak in Fukushima made a single cluster with those isolated during almost the same time period in another area of Japan and in other countries. Those results demonstrated that the genetically divergent EV71 strains might be transmitted from other regions in the world to Japan, be predominant for a few years and disappear.

In clinical viewpoints, HFMD cases with neurologic complications or fatal prognosis were genetically, chronologically and geographically widely distributed. This indicates that the virulent EV71 genogroups might not relate to the cases with severe illness.

In conclusion, our results suggest that the large repeated EV71-related HFMD outbreaks in the world might be the result of worldwide transmission of newly introduced genetically divergent EV71 strains as well as a large cohort of nonimmune individuals. To confirm the aspect, a worldwide surveillance system for HFMD and genetic analysis of isolated EV71 strains is necessary.

REFERENCES

1. Brown BA, Oberste MS, Alexander JP Jr, Kennett ML, Pallansch MA. Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. *J Virol.* 1999;73:9969–9975.
2. Chang LY, Huang YC, Lin TY. Fulminant neurogenic pulmonary oedema with hand, foot and mouth disease. *Lancet.* 1998;352:367–368.
3. McMinn P, Lindsay K, Perera D, et al. Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia. *J Virol.* 2001;75:7732–7738.
4. Cardosa MJ, Perera D, Brown BA, et al. Molecular epidemiology of human enterovirus 71 strains and recent outbreaks in the Asia-Pacific region: comparative analysis of the VP1 and VP4 genes. *Emrge Infect Dis.* 2003;9:461–468.
5. Ishiko H, Shimada Y, Yonaha M, et al. Molecular diagnosis of human enteroviruses by phyloneny-based classification by use of the VP4 sequence. *J Infect Dis.* 2002;185:744–754.
6. Nomoto A, Omata T, Toyoda H, et al. Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. *Proc Natl Acad Sci U S A.* 1982;79:5793–5797.
7. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol.* 1980;16:111–120.
8. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol.* 1987;4:406–425.
9. Mizuta K, Abiko C, Murata T, et al. Frequent importation of enterovirus 71 from surrounding countries into the local community of Yamagata, Japan, between 1998 and 2003. *J Clin Microbiol.* 2005;43:6171–6175.
10. Lin KH, Hwang KP, Ke GM, et al. Evolution of EV71 genogroup in Taiwan from 1998 to 2005: an emerging of subgenogroup C4 of EV71. *J Med Virol.* 2006;78:254–262.
11. Infectious Agents Surveillance Report. *Enterovirus Isolation From Aseptic Meningitis Cases, 1982–2003* [in Japanese]. Tokyo, Japan: Infectious Disease Surveillance Center; 2004.
12. Hosoya M, Sato M, Honzumi K, et al. Association of nonpolio enteroviral infection in the central nervous system of children with febrile seizures. *Pediatrics.* 2001;107:E12.
13. Hosoya M, Ishiko H, Shimada Y, et al. Diagnosis of group A coxsackieviral infection using polymerase chain reaction. *Arch Dis Child.* 2002;87:316–319.

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Serum levels of matrix metalloproteinase-9 and its tissue inhibitor (TIMP-1) in acute disseminated encephalomyelitis

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Abstract

In multiple sclerosis, there have been many reports on matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). However, MMPs and TIMPs have not been reported in acute disseminated encephalomyelitis (ADEM). We determined the relationship between the serum concentrations of MMP-9 and TIMP-1 and activity of lesions on MRI in 14 patients with ADEM to investigate the roles of MMP-9 and TIMP-1 in the pathogenesis of ADEM. Serum MMP-9 and TIMP-1 levels, measured by ELISA and gadolinium-enhanced (Gd+) brain MRI, were analyzed. Serum MMP-9 and TIMP-1 levels at the acute stage were higher than controls, and the serum MMP-9 levels at the acute stage were higher than those at the convalescent stage in ADEM. In seven patients with Gd+ lesions on brain MRI, serum MMP-9 levels and the MMP-9/TIMP-1 ratio at the acute stage were higher than those at the convalescent stage, and serum TIMP1 levels at the acute stage were lower than those at the convalescent stage. In seven patients without Gd+ lesions on brain MRI, serum TIMP-1 levels at the acute stage were higher than those at the convalescent stage. We speculated that MMP-9 is related to lesion formation at the early stage in ADEM and that TIMP-1 is induced to modulate MMP-9 activity. These findings suggest that MMP-9 and TIMP-1 secondarily play some roles in the inflammatory cascade of ADEM.

Keywords: Acute disseminated encephalomyelitis; MMP-9; MRI; TIMP-1

1. Introduction

Matrix metalloproteinases (MMPs) constitute a family of enzymes that mediate the degradation of extracellular matrix proteins (Chandler et al., 1997). MMP-9 is a member of this family which is capable of degrading collagen IV, a major component of the basement membrane of the cerebral epithelium and also responsible for the integrity of the blood–brain-barrier (BBB) (Lukes et al., 1999). The activity of MMPs is further controlled by specific tissue inhibitors (TIMPs) (Murphy and Knäuper, 1997). TIMP-1 has a high avidity for MMP-9 (Lacraz et al., 1995).

In multiple sclerosis (MS), which is a chronic inflammatory demyelinating disease of the central nervous system (CNS), MMP-9, TIMP-1 and the balance between MMP-9 and TIMP-1 have been investigated (Waubant et al., 1999; Avolio et al., 2003; Dubois et al., 2003; Yushchenko et al., 2003; Waubant et al., 2003; Blanco et al., 2004; Karabudak et al., 2004; Mirowska et al., 2004; Sastre-Garriga et al., 2004; Abraham et al., 2005; Rosenberg, 2005).

Acute disseminated encephalomyelitis (ADEM) is an acute inflammatory demyelinating disorder of the central nervous system (CNS) commonly seen in children and young adults. ADEM is clinically characterized by the acute onset of neurological symptoms including alternation of consciousness, paresis, ataxia, seizures, behavioral changes and urinary incontinence after a viral infection or immunization (Alvord, 1985). Magnetic resonance imaging (MRI) reveals increased signal intensity in T2-

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weighted images representing multiple white matter lesions, and gadolinium-enhanced (Gd+) lesions indicate more active lesions (Caldemeyer et al., 1994). Experimental autoimmune encephalomyelitis (EAE), a model of MS, has histopathological resemblance with ADEM (Ben-Nun et al., 1984; Kuchroo et al., 1991; Linington et al., 1992). Therefore, ADEM may be an autoimmune disorder. However, the pathogenesis of ADEM is not well understood. MMPs and TIMPs have not been reported in ADEM. To investigate the role of MMP-9 and TIMP-1 in the pathogenesis of ADEM, we determined the relationship between serum concentrations of MMP-9 and TIMP-1 and activity of lesions on MRI in ADEM patients.

2. Materials and methods

2.1. Acute disseminated encephalomyelitis

Informed consent was obtained from the parents of the patients enrolled in this study. Serum samples were obtained from 14 patients with ADEM on admission to our hospital from June 1997 to February 2005. The diagnosis of ADEM was based on characteristic clinical symptoms such as acute-onset of various neurological symptoms following infection or immunization (sometimes idiopathic) and MRI findings consistent with a disseminated demyelinating process with/without an elevated myelin basic protein (MBP) level in CSF (normal level: less than 4.0 ng/ml). For a diagnosis of ADEM, T2 signal hyperintensities were present in at least two locations. The clinical features of the affected patients are shown in Table 1. Subjects comprised eight males and six females, aged from 2 years to 15 years (median, 5.5 years). Moreover, all patients were evaluated on Gd+ MRI at the acute stage. We divided the children with ADEM into two groups: those who had Gd+ lesions on MRI at the acute stage ($n=7$), and those who did not have Gd+ lesions on MRI at the acute stage ($n=7$). The day of onset of neurological symptoms was considered as the first day of illness. Serum samples were taken from the children with ADEM on days 2 to 7 (median, 3.5 days) of illness before treatment and the convalescent stage (median, 7.0 days (range, 6 to 20 days) after the first serum sampling). The patients with ADEM were treated with high-dose intravenous methylprednisolone and recovered without neurological sequela. Gd+ MRI on the second sampling day (the convalescent stage) revealed no Gd+ lesions in all patients. All patients had already finished steroid therapy before the second sampling day. The second sampling day was 3 to 17 days (median, 4 days) after finishing steroid therapy. The patients were followed up for 8 months to 8 years (median, 4.8 years) and none had recurrent neurological symptoms.

2.2. Control subjects

The control subjects for the serum levels of MMP-9 and TIMP-1 were 33 healthy children (15 males and 18 females, aged from 2 to 15 years: median, 5.8 years).

2.3. Determination of MMP-9 and TIMP-1 concentrations

The serum concentrations of MMP-9 and TIMP-1 were determined with sandwich-type ELISA kits (Amersham, Buckinghamshire, England). Assays were performed following the instructions of the manufacturer. The detection limits were 2.5 ng/ml for MMP-9 and 2.4 ng/ml for TIMP-1. The assay of MMP-9 recognizes the pro and active forms of MMP-9. Both pro and active MMP-9/TIMP-1 complexes as well as active MMP-9/TIMP-2 complexes had showed a degree of cross-reactivity in the assay.

Table 1
Clinical characteristics of the 14 patients with acute disseminated encephalomyelitis

Patient no./age/sex	Proceeding illness	Main symptoms on admission	T2 high intensity lesions on MRI
Patients with Gd+ lesions on MRI at acute stage			
1/2 years/M	mumps 5 days pta	fever, vomiting, somnolence	CWM (multiple)
2/9 years/M	fever 2 weeks pta	semicoma, paresis of lower extremities, urinary incontinence	CWM (multiple), cerebellum
3/10 years/F	–	behavioral changes, ataxia	CWM (multiple), cerebellum
4/9 years/F	GE 4 days pta	ataxia	CWM (multiple), cerebellar peduncle
5/12 years/M	URI 1 week pta	ataxia, facial nerve palsy	CWM (multiple), cerebellum
6/2 years/M	URI 2 weeks pta	somnolence, vomiting	CWM (multiple), thalamus
7/10 years/M	GE 10 days pta	sensory disturbance, unstable gait	CWM (multiple)
Patients without Gd+ lesions on MRI at acute stage			
8/5 years/F	URI 2 weeks pta	fever, nuchal rigidity, tremor	CWM (multiple), basal ganglia
9/5 years/M	–	vertigo, vomiting, gait disturbance	CWM (multiple), cerebellum, brainstem
10/5 years/F	GE 1 week pta	somnolence, ataxia, urinary incontinence	CWM (multiple), cerebellum
11/2 years/F	URI 1 week pta	fever, vomiting, gait disturbance	CWM (multiple)
12/3 years/M	fever 6 days pta	gait disturbance, strabismus	CWM (multiple), thalamus, cerebellar peduncle
13/3 years/F	pneumonia 2 weeks pta	hemiballism	CWM (multiple), thalamus, mesencephalon
14/15 years/M	influenza 1 week pta	tetraplegia, semicoma	CWM (multiple), brainstem, spinal cord

M=male, F=female, pta=prior to admission, GE=gastroenteritis, URI=upper respiratory infection, CWM=cerebral white matter.

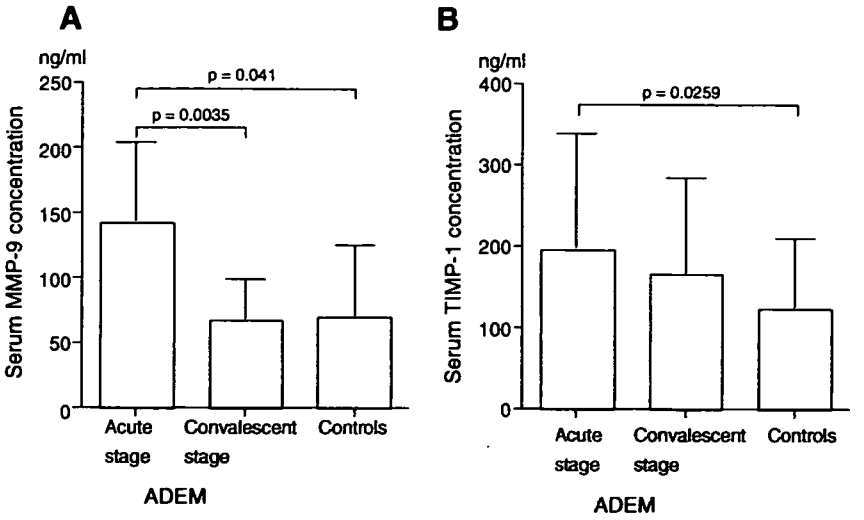

Fig. 1. Serum concentrations of MMP-9 (A) and TIMP-1 (B) in patients with ADEM at the acute stage and the convalescent stage, and controls. Data are presented as medians+1 S.D.

2.4. Statistical analysis

All values are medians±S.D. The differences in the results between groups were analyzed by the Mann–Whitney *U* test. The differences in the results between the acute stage and the convalescent stage were analyzed by the Wilcoxon matched paired test.

3. Results

The serum MMP-9, TIMP-1 and MMP-9/TIMP-1 levels of controls were 70.0±54.9 ng/ml, 124.9±85.4 ng/ml and 0.51±0.61, respectively. Serum MMP-9 and TIMP-1 levels of patients with ADEM at the acute stage were significantly higher than controls (142.2±60.5 ng/ml, $p=0.041$, and 195.1±143.6 ng/ml, $p=0.0259$, respectively), and the serum MMP-9 levels at the acute stage were significantly higher than those at the convalescent stage in ADEM (142.2±60.5 ng/ml vs. 66.8±30.8 ng/ml, $p=0.0035$) (Fig. 1A and B). There were no significant differences among the MMP-9/TIMP-1 ratios of patients with ADEM at the acute stage, those at the convalescent stage, and controls. In seven ADEM patients with Gd+ lesions on brain MRI, serum MMP-9 levels and the MMP-9/TIMP-1 ratio at the acute stage were significantly higher than those at the convalescent stage (159.3±47.6 ng/ml vs. 67.0±20.1 ng/ml, $p=0.018$, and 0.95±0.51 vs. 0.34±0.16, $p=0.028$, respectively), and serum TIMP-1 levels at the acute stage were significantly lower than those at the convalescent stage (159.1±53.0 ng/ml vs. 260.8±132.0 ng/ml, $p=0.0464$) (Fig. 2A, B and C). In the seven ADEM patients without Gd+ lesions on brain MRI, serum TIMP-1 levels at the acute stage were significantly higher than those at the convalescent stage (338.4±141.8 ng/ml vs. 123.6±50.1 ng/ml, $p=0.018$).

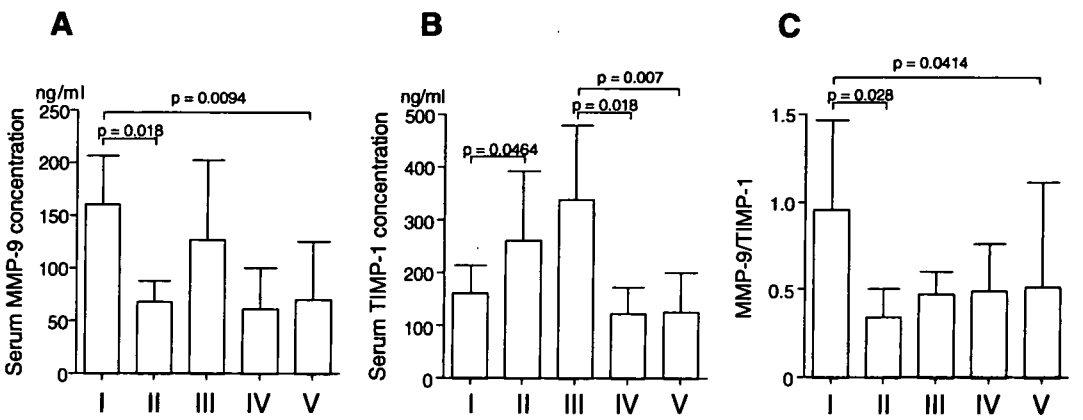

Fig. 2. Serum concentrations of MMP-9 (A), TIMP-1 (B) and MMP-9/TIMP-1 (C) in patients with ADEM at the acute stage and the convalescent stage, and controls. Group I, ADEM patients with Gd+ lesions on MRI at the acute stage; group II, ADEM patients with Gd+ lesions on MRI at the convalescent stage; group III, ADEM patients without Gd+ lesions on MRI at the acute stage; group IV, ADEM patients without Gd+ lesions on MRI at the convalescent stage; group V, controls. Data are presented as medians+1 S.D.

4. Discussion

Several ADEM studies have reported on cytokines (Yoshitomi et al., 2000; Ichiyama et al., 2002; Dale and Morovat, 2003; Leake et al., 2004). We newly analyzed serum MMP-9 and TIMP-1 levels in ADEM. MMPs are produced by a wide variety of cells such as monocytes/macrophages, T cells, neutrophils, endothelial cells, microglia, astrocytes and oligodendrocytes (Welgus et al., 1990; Chandler et al., 1997). It is likely that proinflammatory cytokines and MMPs facilitate the migration of T cells and macrophages from the intravascular compartment into the CNS in MS (Rosenberg et al., 1996; Leppert et al., 1995). Moreover, it is believed that MMPs induce myelin breakdown, proinflammatory cytokine production and axonal damage in MS (Chandler et al., 1995; Hartung and Kieseier, 2000; Newman et al., 2001).

It is also likely that MMP-9 and TIMP-1 are related to the inflammatory cascade of ADEM because their serum levels were elevated in patients with ADEM at the acute stage. We investigated the relationships between serum MMP-9 and TIMP-1 levels and Gd+ lesions on MRI. In patients with Gd+ lesions on brain MRI, serum MMP-9 levels and the MMP-9/TIMP-1 ratio were higher at the acute stage compared with the convalescent stage, and serum TIMP-1 levels were higher at the convalescent stage compared with the acute stage. These findings suggest that MMP-9 is related with the formation of the active lesions as Gd+ lesions on MRI, and TIMP-1 is induced to modulate MMP-9 activity and the lesions at a later stage. Interestingly, serum TIMP-1 levels were higher at the acute stage compared with the convalescent stage in patients without Gd+ lesions on brain MRI. Large amounts of TIMP-1 induced quickly may inhibit MMP-9 activity and prevent the formation of the active lesions as Gd+ lesions on MRI. Another hypothesis is that the inspection time for patients without Gd+ lesions on brain MRI passed the peak of the disease, so the serum TIMP-1 levels of these patients were already elevated. However, data collected at the convalescent stage might be affected by steroids because the sampling day at the convalescent stage was a median of 4 days (range, 3 to 17 days) after finishing steroid therapy. Methylprednisolone reduced CSF MMPs levels (Rosenberg et al., 1996). Dexamethasone partially inhibited the cytokine-induced upregulation of MMP-9 (Harkness et al., 2000).

In relapsing-remitting MS, serum MMP-9 but not TIMP-1 levels were elevated, and high MMP-9 and low TIMP-1 levels preceded the appearance of new Gd+ lesions (Waubant et al., 1999). However, another study demonstrated that pre-treatment TIMP-1 but not MMP-9 levels were elevated (Karabudak et al., 2004). In secondary progressive MS, a high ratio of MMP-9/TIMP-1 predicted new Gd+ lesions (Waubant et al., 2003). In primary progressive MS, pre-treatment MMP-9 but not TIMP-1 levels were elevated (Yushchenko et al., 2003). However, another study revealed that serum MMP-9 levels were decreased (Sastre-Garriga et al., 2004). In MS, patterns of MMP-9 and TIMP-1 levels are debated. However, the relationship between a high ratio of MMP-9/TIMP-1 and new Gd+ lesions in MS is similar to our data in ADEM.

In summary, serum MMP-9 and TIMP-1 levels were elevated in ADEM at the acute stage, and it is likely that MMP-9 and TIMP-1 secondarily play some roles in the inflammatory cascade of ADEM.

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References

Abraham, M., Shapiro, S., Karni, A., Weiner, H.L., Miller, A., 2005. Gelatinases (MMP-2 and MMP-9) are preferentially expressed by Th1 vs. Th2 cells. J. Neuroimmunol. 163, 157–164.

Alvord Jr., E.C., 1985. Disseminated encephalomyelitis: its variations in form and their relationships to other diseases of the nervous system. In: Vinken, J., Bruyn, G.W., Klawans, H.L. (Eds.), Handbook of Clinical Neurology vol. 47, Series 3. Elsevier Science, Amsterdam, pp. 450–467.

Avolio, C., Ruggieri, M., Giuliani, F., Liuzzi, G.M., Leante, R., Riccio, P., Livrea, P., Trojano, M., 2003. Serum MMP-2 and MMP-9 are elevated in different multiple sclerosis subtypes. J. Neuroimmunol. 136, 46–53.

Ben-Nun, A., Wekerle, H., Cohen, J.R., 1984. The rapid isolation of clonable antigen specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. Eur. J. Immunol. 11, 195–199.

Blanco, Y., Saiz, A., Carreras, E., Graus, F., 2004. Changes of matrix metalloproteinase-9 and its tissue inhibitor (TIMP-1) after autologous hematopoietic stem cell transplantation in multiple sclerosis. J. Neuroimmunol. 153, 190–194.

Caldemeyer, K.S., Smith, R.R., Harris, T.M., Edwards, M.K., 1994. MRI in acute disseminated encephalomyelitis. Neuroradiology 36, 216–220.

Chandler, S., Coates, R., Gearing, A., Lury, J., Wells, G., Bone, E., 1995. Matrix metalloproteinases degrade myelin basic protein. Neurosci. Lett. 201, 223–226.

Chandler, S., Miller, K.M., Clements, J.M., Lury, J., Corkill, D., Anthony, D.C., Adams, S.E., Gearing, A.J., 1997. Matrix metalloproteinases, tumor necrosis factor and multiple sclerosis: an overview. J. Neuroimmunol. 72, 155–161.

Dale, R.C., Morovat, A., 2003. Interleukin-6 and oligoclonal IgG synthesis in children with acute disseminated encephalomyelitis. Neuropediatrics 34, 141–145.

Dubois, B., Leary, S.M., Nelissen, I., Opdenakker, G., Giovannoni, G., Thompson, A.J., 2003. Serum gelatinase B/MMP-9 in primary progressive multiple sclerosis patients treated with interferon-beta-1a. J. Neurol. 250, 1037–1043.

Harkness, K.A., Adamson, P., Sussman, J.D., Davies-Jones, G.A., Greenwood, J., Woodroofe, M.N., 2000. Dexamethasone regulation of matrix metalloproteinase expression in CNS vascular endothelium. Brain 123, 698–709.

Hartung, H.P., Kieseier, B.C., 2000. The role of matrix metalloproteinases in autoimmune damage to the central and peripheral nervous system. J. Neuroimmunol. 107, 140–147.

Ichiyama, T., Shoji, H., Kato, M., Sawaishi, Y., Ozawa, H., Matsubara, T., Furukawa, S., 2002. Cerebrospinal fluid levels of cytokines and soluble

tumour necrosis factor receptor in acute disseminated encephalomyelitis. Eur. J. Pediatr. 161, 133–137.

Karabudak, R., Kurne, A., Guc, D., Sengelen, M., Canpinar, H., Kansu, E., 2004. Effect of interferon beta-1a on serum matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase (TIMP-1) in relapsing remitting multiple sclerosis patients. One year follow-up results. J. Neurol. 251, 279–283.

Kuchroo, V.K., Sobel, R.A., Yamamura, T., Greenfield, E., Dorf, M.E., Lees, M.B., 1991. Induction of experimental allergic encephalomyelitis by myelin proteolipid-protein-specific T cell clones and synthetic peptides. Pathobiology 59, 305–312.

Lacraz, S., Nicod, L.P., Chicheportiche, R., Welgus, H.G., Dayer, J.M., 1995. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. J. Clin. Invest. 96, 2304–2310.

Leake, J.A., Albani, S., Kao, A.S., Senac, M.O., Billman, G.F., Nespeca, M.P., Paulino, A.D., Quintela, E.R., Sawyer, M.H., Bradley, J.S., 2004. Acute disseminated encephalomyelitis in childhood: epidemiologic, clinical and laboratory features. Pediatr. Infect. Dis. J. 23, 756–764.

Leppert, D., Waubant, E., Galardy, R., Bunnett, N.W., Hauser, S.L., 1995. T cell gelatinases mediate basement membrane transmigration in vitro. J. Immunol. 154, 4379–4389.

Linington, C., Engelhardt, B., Kapocs, G., Lassman, H., 1992. Induction of persistently demyelinated lesions in the rat following the repeated adoptive transfer of encephalitogenic T cells and demyelinating antibody. J. Neuroimmunol. 40, 219–224.

Lukes, A., Mun-Bryce, S., Lukes, M., Rosenberg, G.A., 1999. Extracellular matrix degradation by metalloproteinases and central nervous system diseases. Mol. Neurobiol. 19, 267–284.

Mirowska, D., Wicha, W., Czlonkowski, A., Czlonkowska, A., Weber, F., 2004. Increase of matrix metalloproteinase-9 in peripheral blood of multiple sclerosis patients treated with high doses of methylprednisolone. J. Neuroimmunol. 146, 171–175.

Murphy, G., Knäuper, V., 1997. Relating matrix metalloproteinase structure to function: why the "hemopexin" domain? Matrix Biol. 15, 511–518.

Newman, T.A., Woolley, S.T., Hughes, P.M., Sibson, N.R., Anthony, D.C., Perry, V.H., 2001. T-cell- and macrophage-mediated axon damage in the absence of a CNS-specific immune response: involvement of metalloproteinases. Brain 124, 2203–2214.

Rosenberg, G.A., 2005. Matrix metalloproteinases biomarkers in multiple sclerosis. Lancet 365, 1291–1293.

Rosenberg, G.A., Dencoff, J.E., Correa Jr., N., Reiners, M., Ford, C.C., 1996. Effect of steroids on CSF matrix metalloproteinases in multiple sclerosis: relation to blood–brain barrier injury. Neurology 46, 1626–1632.

Sastre-Garriga, J., Comabella, M., Brieva, L., Rovira, A., Tintore, M., Montalban, X., 2004. Decreased MMP-9 production in primary progressive multiple sclerosis patients. Mult. Scler. 10, 376–380.

Waubant, E., Goodkin, D.E., Gee, L., Bacchetti, P., Sloan, R., Stewart, T., Andersson, P.B., Stabler, G., Miller, K., 1999. Serum MMP-9 and TIMP-1 levels are related to MRI activity in relapsing multiple sclerosis. Neurology 53, 1397–1401.

Waubant, E., Goodkin, D., Bostrom, A., Bacchetti, P., Hietpas, J., Lindberg, R., Leppert, D., 2003. IFNβ lowers MMP-9/TIMP-1 ratio, which predicts new enhancing lesions in patients with SPMS. Neurology 60, 52–57.

Welgus, H.G., Campbell, E.J., Cury, J.D., Eisen, A.Z., Senior, R.M., Wilhelm, S.M., Goldberg, G.I., 1990. Neutral metalloproteinases produced by human mononuclear phagocytes. Enzyme profile, regulation, and expression during cellular development. J. Clin. Invest. 186, 1496–1502.

Yoshitomi, T., Matsubara, T., Nishikawa, M., Katayama, K., Ichiyama, T., Hayashi, T., Furukawa, S., 2000. Increased peripheral blood interferon gamma-producing T cells in acute disseminated encephalomyelitis. J. Neuroimmunol. 111, 224–228.

Yushchenko, M., Mäder, M., Elitok, E., Bitsch, A., Dressel, A., Tumani, H., Bogumil, T., Kitze, B., Poser, S., Weber, F., 2003. Interferon-β-1b decreased matrix metalloproteinase-9 serum levels in primary progressive multiple sclerosis. J. Neurol. 250, 1224–1228.

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Analysis of serum and cerebrospinal fluid cytokine levels in subacute sclerosing panencephalitis in Papua New Guinea

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Abstract

Background: Subacute sclerosing panencephalitis (SSPE) is a rare progressive inflammatory disease characterized by the persistent infection of the brain by the measles virus. However, the immunological pathophysiology of SSPE is still unclear.
Methods: We measured the concentrations of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-6, IL-10, and soluble TNF receptor 1 (sTNFR1) in the serum and cerebrospinal fluid (CSF) of 23 patients with SSPE in Papua New Guinea (PNG), a country with a high incidence of SSPE, and Japanese controls by cytometric bead array or ELISA.
Results: The serum IL-6 and IL-10 levels of SSPE patients were significantly higher than those of controls ($p = 0.0075$, and $p = 0.0019$, respectively). The serum IL-6 and IL-10 levels of SSPE patients with fever were significantly higher than those without fever ($p = 0.0107$, and $p = 0.0006$, respectively). The CSF IL-6 levels of SSPE patients were significantly higher than those of controls ($p = 0.0218$). The CSF IL-6 levels of SSPE patients with myoclonic jerks were significantly higher than those without myoclonic jerks ($p = 0.0189$). There were no differences in serum IFN-γ, TNF-α, IL-2, IL-4, and sTNFR1, or CSF IFN-γ, TNF-α, IL-2, IL-4, IL-10, and sTNFR1 levels between the affected patients and controls.
Conclusion: Our present study suggests that serum IL-6 and IL-10 levels are related to fever, and the CSF IL-6 level, myoclonic jerks, in SSPE patients in PNG.

Keywords: Cerebrospinal fluid; Cytokine; Interleukin-6; Serum; Subacute sclerosing panencephalitis

1. Introduction

Subacute sclerosing panencephalitis (SSPE) is a rare progressive inflammatory disease of the brain caused by persistent infection by the measles virus. However, the immunological pathophysiology of SSPE is still unclear.

A high incidence of SSPE has been previously reported in Papua New Guinea (PNG) [1]. The annual incidence of SSPE in the Eastern Highlands Province (EHP) of PNG in 1997–1998 was 98 per million of population under 20 years of age, the highest ever reported [2]. The incidence of SSPE was reported to range from 0.1 to 6 cases per million of population in other places [3–5]. Therefore, the incidence of SSPE in the EHP of PNG was more than ten times higher [2].

To evaluate the immunological pathogenesis of SSPE in PNG, we determined the serum and cerebrospinal fluid (CSF) concentrations of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-6, IL-10, and soluble TNF receptor 1 (sTNFR1) as cytokines related to inflammation in patients with SSPE in PNG.

2. Patients and methods

Informed consent was obtained from the parents of the patients and controls enrolled in this study. The protocol was

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approved by the Medical Research Advisory Committee of PNG (MRAC No. 04/01).

2.1. Subacute sclerosing panencephalitis (SSPE)

Serum and CSF samples were obtained from 23 children with SSPE (11 males and 12 females, aged from 4 to 14 years; median, 6.8 years) at Goroka Base General Hospital, from October 1997 to April 1999. The criteria for the diagnosis of SSPE were (1) progressive neurological disorder, particularly mental or motor deterioration, associated with a positive history or the presence of myoclonic jerks, (2) positive CSF measles antibody titer determined by enzyme-linked immunosorbent assaying (EIA), (3) high serum EIA values to an extent comparable to those of cases that fulfill criteria 1 and 2, and (4) periodic synchronous discharges (PSDs) in the EEG ($n = 20$). Samples were stored at −70 °C. Moreover, the clinical records of the patients were analyzed.

2.2. Control subjects

Informed consent was obtained from the parents of the subjects enrolled in the study. The control subjects for the serum levels of the cytokines were 73 healthy Japanese children (41 males and 32 females, aged from 3 months to 15 years; median, 6.7 years). The control subjects for the CSF levels of the cytokines were 21 afebrile and noninfectious Japanese children with neurological disorders, such as psychomotor delay, epilepsy, etc. (12 males and nine females, aged from 3 months to 15 years; median, 5.1 years). CSF samples were obtained from them on routine analysis and they all had normal CSF cell counts.

2.3. Determination of cytokine concentrations

The concentrations of serum and CSF IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 were measured with a cytometric bead array (CBA) kit (BD PharMingen, San Diego, CA) according to the manufacturer's manual, as previously described [6–8], with modification of the data analysis using GraphPad Prism software (GraphPad Prism Software, San Diego, CA). Briefly, a CBA comprises a series of beads exhibiting discrete fluorescence intensities at 670 nm. Each series of beads is coated with a monoclonal antibody against a single cytokine, and a mixture of the six series of beads can detect six cytokines in one sample. A secondary phycoerythrin (PE)-conjugated monoclonal antibody stains the beads proportionally to the amount of bound cytokine. After fluorescence intensity calibration and electronic color compensation procedures, standard and test samples were analyzed with a FACScan flow cytometer equipped with CellQuest software (BD PharMingen). Data were transferred to GraphPad Prism. Starting with standard dilutions, the software performed log transformation of the data, and then fitted a curve to 10 discrete points using a four-parameter logistic model. The calibration curve created for each cytokine was used to determine the cytokine concentrations of the samples. The lower detection limits for IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 were 7.1 pg/ml, 2.8 pg/ml, 2.6 pg/ml, 2.6 pg/ml, 2.5 pg/ml, and 2.8 pg/ml, respectively.

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

2.4. Statistical analysis

Differences in the results were analyzed by means of the Mann–Whitney U-test and χ^2 test, with a p value of less than 0.05 being taken as significant. Correlations were analyzed using Spearman's rank correlation coefficient test.

3. Results

The cytokine concentrations of the controls are shown as the value of the means ± 2 SD. The serum IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, and sTNFR1 concentrations of the controls were <42.9 pg/ml, <11.1 pg/ml, <4.5 pg/ml, <15.0 pg/ml, <19.9 pg/ml, <14.2 pg/ml, and 0.1–2.1 ng/ml, respectively. The serum IL-6 and IL-10 levels of patients with SSPE were significantly higher than those of controls ($p = 0.0075$, and $p = 0.0019$, respectively) (Table 1). The serum IL-6 and IL-10 levels of SSPE patients with fever were significantly higher than those without fever ($p = 0.0107$, and $p = 0.0006$, respectively) (Table 2). There were no differences in the serum IFN-γ, TNF-α, IL-2, IL-4, or sTNFR1 levels between the affected patients and controls. There were no correlations between serum cytokine levels and the serum measles antibody titer.

The CSF IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, and sTNFR1 concentrations of the controls were <46.6 pg/ml, <6.2 pg/ml, <4.6 pg/ml, <11.6 pg/ml, <9.7 pg/ml, <6.1 pg/ml, and <1.9 ng/ml, respectively. The CSF IL-6 levels of patients with SSPE were significantly higher than those of the controls ($p = 0.0218$) (Table 1). The CSF IL-6 levels of SSPE patients with myoclonic jerks were significantly higher than those without myoclonic jerks ($p = 0.0189$) (Table 3). There were no differences in the CSF IFN-γ, TNF-α, IL-2, IL-4, IL-10, or sTNFR1 levels between the affected patients and controls. There were no correlations between CSF cytokine levels and the CSF measles antibody titer or CSF protein concentrations.

The number of SSPE patients with both elevated serum and CSF IL-6 levels was one. All five patients with elevated

Table 1
Numbr of SSPE patients who had elevated serum and CSF cytokine levels

	Serum	CSF
IFN-γ	1 (46.6)	0
TNF-α	2 (60.9, 16.7–105)	0
IL-2	0	0
IL-4	0	1 (19.5)
IL-6	7 (23.4, 21.4–83.9)**	9 (18.0, 14.7–30.4)*
IL-10	5 (21.9, 12.2–308)*	2 (7.4, 7.0–7.7)
sTNFR1	2 (2.95, 2.44–3.46)	0

IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10, pg/ml; STNFR1, ng/ml (median, range). **$p < 0.01$ and *$p < 0.05$ compared to controls.

Table 2
The relationship between the number of SSPE patients with fever and those who had elevated serum cytokine levels

	Fever + ($n = 7$)	Fever − ($n = 16$)
IL-6	5*	2
IL-10	5**	0
TNF-α	2	0
sTNFR1	2	0
IFN-γ	1	0

**$p < 0.01$ and *$p < 0.05$ compared to patients without fever.

serum IL-10 levels had elevated serum IL-6 levels, and the two patients with elevated CSF IL-10 levels had elevated CSF IL-6 levels.

4. Discussion

Previous immunohistochemical studies revealed that the cytokines mediating inflammation are expressed in SSPE brain lesions [9–11]. They demonstrated the presence of IL-1β, IL-2, IL-6, TNF-α, and IFN-γ in the lesions [9–11]. These data suggested that these cytokines were produced in SSPE lesions and played an important role in the immunopathogenesis of SSPE.

We previously reported on a 9-year-old boy with SSPE who had an elevated CSF sTNFR1 level at the terminal stage of the disease [12]. In the present study, we analyzed serum and CSF cytokine levels in 23 SSPE patients in the EHP of PNG. Until now, there has never been a report on the analysis of the cytokine profiles of a large number of SSPE patients. However, several studies have reported on the serum and CSF cytokine levels of a few SSPE patients [13–16]. It was reported that CSF IL-1β and soluble intercellular adhesion molecule-1 levels were elevated in SSPE patients, CSF and plasma TNF-α levels were elevated in SSPE patients with a rapidly progressive course, CSF and serum IL-1β and IL-1 receptor antagonist levels were not elevated in SSPE patients, and that CSF IL-10 levels were elevated in SSPE patients but CSF IL-4 or IFN-γ levels were not [13–16]. We demonstrated that serum IL-6 levels were elevated in 30% of the affected patients in PNG, CSF IL-6 levels in 39%, serum IL-10 levels in 17%, and CSF IL-10 levels in 9%, respectively. However, 71% of the patients with fever had elevated serum IL-6 and IL-10 levels, but only 13% and 0% of the patients without fever had elevated serum IL-6 and IL-10 levels, respectively. Moreover, 56% of the patients with myoclonic jerks had elevated CSF IL-6 levels, but none of the patients without myoclonic jerks had elevated CSF IL-6 levels.

Table 3
The relationship between the number of SSPE patients with myoclonic jerks and those who had elevated CSF cytokine levels

	Myoclonic jerks + ($n = 16$)	Myoclonic jerks − ($n = 7$)
IL-6	9*	0
IL-10	2	0
IL-4	0	1

*$p < 0.05$ compared to patients without myoclonic jerks.

IL-6 is a cytokine well known to play an important role in inflammatory responses. It is recognized as a primary mediator in the pathogenesis of inflammation [17,18]. Previous studies have shown that CSF IL-6 is often elevated in patients with inflammatory disorders of the CNS [19–22]. An elevated CSF IL-6 level would demonstrate the existence of CNS inflammation. In our present study, we newly revealed that some SSPE patients in PNG had elevated IL-6 levels in serum and/or CSF, and that it is likely that serum IL-6 and IL-10 values are related to fever, and CSF IL-6, myoclonic jerks. Fever may not be always due to SSPE, but due to other infections. It is unclear whether these findings are characteristic of all SSPE patients or only those SSPE patients in PNG, an area with a high incidence of SSPE.

In conclusion, the fever of SSPE patients in PNG may be related to elevated serum IL-6 and IL-10 levels, and myoclonic jerks, an elevated CSF IL-6 level.

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References

[1] Lucas KM, Sanders RC, Rongap A, Rongap T, Pinai S, Alpers MP. Subacute sclerosing panencephalitis (SSPE) in Papua New Guinea: a high incidence in young children. Epidemiol Infect 1992;108:547–53.
[2] Takasu T, Mgone JM, Mgone CS, Miki K, Komase K, Namae H, et al. A continuing high incidence of subacute sclerosing panencephalitis (SSPE) in the Eastern Highlands of Papua New Guinea. Epidemiol Infect 2003;131:887–98.
[3] CDC. Subacute sclerosing panencephalitis surveillance – United States. MMWR Morb Mortal Wkly Rep 1982;31:585–8.
[4] Dyken PR. Subacute sclerosing panencephalitis. Current status. Neurol Clin 1985;3:179–96.
[5] Editorial. SSPE in the developing world. Lancet 1990;336:600.
[6] Chen R, Lowe L, Wilson JD, Crowther E, Tzeggai K, Bishop JE, et al. Simultaneous quantification of six human cytokines in a single sample using microparticle-based flow cytometric technology. Clin Chem 1999;45:1693–4.
[7] Cook EB, Stahl JL, Lowe L, Chen R, Morgan E, Wilson J, et al. Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics. J Immunol Methods 2001;254:109–18.
[8] Metelitsa LS, Naidenko OV, Kant A, Wu HW, Loza MJ, Perussia B, et al. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. J Immunol 2001;167:3114–22.
[9] Hofman FM, Hinton DR, Baemayr J, Weil M, Merrill JE. Lymphokines and immunoregulatory molecules in subacute sclerosing panencephalitis. Clin Immunol Immunopathol 1991;58:331–42.
[10] Anlar B, Söylemezoğlu F, Aysun S, Köse G, Belen D, Yalaz K. Tissue inflammatory response in subacute sclerosing panencephalitis (SSPE). J Child Neurol 2001;16:895–900.
[11] Nagano I, Nakamura S, Yoshioka M, Onodera J, Kogure K, Itoyama Y. Expression of cytokines in brain lesions in subacute sclerosing panencephalitis. Neurology 1994;44:710–5.