

## Original article

# Serum and CSF levels of cytokines in acute encephalopathy following prolonged febrile seizures

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## Abstract

It is well known that an acute encephalopathy occasionally follows prolonged febrile seizures. We measured the concentrations of interferon- $\gamma$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-2 (IL-2), IL-4, IL-6, IL-10, and soluble TNF receptor 1 (sTNFR1) in serum and CSF during the acute stage in 13 children with acute encephalopathy following prolonged febrile seizures (AEPFS) and 23 with prolonged febrile seizures without encephalopathy (PFS) to investigate the pathogenesis of AEPFS. Serum IL-6, IL-10, sTNFR1, and CSF IL-6 levels were significantly higher in AEPFS and PFS compared with control subjects. CSF IL-6 levels in AEPFS were significantly higher than those in PFS, but not serum IL-6, IL-10, or sTNFR1. The CSF IL-6 levels were significantly higher than the serum levels in AEPFS, but not PFS. The serum levels of sTNFR1 and IL-10 were significantly higher than those in the CSF in AEPFS and PFS. The serum IL-10 and sTNFR1 levels in patients who did not experience a second seizure were significantly higher than those in patients who experienced a second seizure, which was characterized by clusters of complex partial seizures several days after the initial prolonged febrile seizure. Our results suggest that serum IL-6, IL-10, TNF- $\alpha$ , and CSF IL-6 are part of the regulatory system of cytokines in AEPFS.

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**Keywords:** Acute encephalopathy; Cytokine; Febrile seizure; Interleukin-6

## 1. Introduction

An acute encephalopathy sometimes follows prolonged febrile or afebrile seizures, including acute encephalopathy with febrile convulsive status epilepticus (AEFCSE) [1], acute infantile encephalopathy predominantly affecting the frontal lobes (AIEF) [2], hemiconvulsion–hemiplegia (HH) syndrome [3], and theophylline encephalopathy [4]. Our recent review dem-

onstrated that the above four encephalopathies can be regarded as the same category, acute encephalopathy caused by excitotoxicity [4]. We paid attention to acute encephalopathy following prolonged febrile seizures. A common characteristic of these encephalopathies is that they are preceded by prolonged febrile seizure, as an initial neurological symptom, and subcortical white matter lesions on diffusion-weighted MRI during the acute stage. The pathogenesis of these encephalopathies, so-called acute encephalopathy following prolonged febrile seizures (AEPFS) (named by us), remains unclear. We previously determined the serum and cerebrospinal fluid (CSF) concentrations of several cytokines in febrile sei-

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zures and prolonged febrile seizures [5,6]. However, analysis of serum and CSF cytokines in AEPFS has not been performed.

To evaluate the pathogenesis of AEPFS, we determined the serum and CSF concentrations of interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-2 (IL-2), IL-4, IL-6, IL-10, and soluble TNF receptor 1 (sTNFR1), as cytokines related to inflammation in children with AEPFS compared with those with prolonged febrile seizures without encephalopathy (PFS).

## 2. Patients and methods

Informed consent was obtained from the parents of the patients and control subjects enrolled in this study.

### 2.1. Acute encephalopathy following prolonged febrile seizures (AEPFS)

Serum and CSF samples were obtained from 13 children with AEPFS (3 males and 10 females, aged from 7 months to 2 years; median, 1.5 years) on admission to our hospital and five collaborating research hospitals in Japan, from May 1998 to July 2006 (Table 1). The criteria for the diagnosis of AEPFS were (1) a seizure or cluster of seizures longer than 30 min as an initial neurological symptom within 1 day after the onset of fever, (2) subcortical white matter lesions on diffusion-weighted MRI at day 1–5 (Fig. 1A–D), (3) continuous disturbance of consciousness level or hemiparesis after the initial prolonged seizure, (4) second seizures characterized

by clusters of complex partial seizures 1–5 days after the initial prolonged seizure in 7 out of 13 patients (Group A), and no second seizure in the remaining six patients (Group B), and (5) no bacteria or fungi on CSF culture, and excluding all other neurological, vascular, metabolic, endocrine, toxic, and drug-induced disorders.

The diagnoses of influenza ( $n = 3$ ) were based on a fourfold increase in the antibody titer determined by the hemagglutination inhibition test ( $n = 1$ , Patient 8), virus antigen detection in the throat with the latex agglutination test ( $n = 1$ , Patient 2), and the detection of influenza virus RNA in the throat by polymerase chain reaction ( $n = 1$ , Patient 5). The diagnosis of human herpesvirus 6 (HHV6) ( $n = 1$ ) was based on the detection of HHV6 DNA in the CSF by polymerase chain reaction. The diagnosis of respiratory syncytial virus infection ( $n = 1$ ) was based on virus antigen detection in the throat with the latex agglutination test. The diagnosis of rotavirus ( $n = 3$ ) was based on virus antigen detection from feces by latex agglutination test. The diagnosis of acute pyelonephritis by *Escherichia coli* ( $n = 1$ ) was based on urine cultures. The day of onset of the initial prolonged febrile seizure was considered the first day of illness. CSF and serum samples were obtained from children with AEPFS on day  $3.2 \pm 1.7$  (range, 1–5) of the illness. The samples were obtained before immunosuppressive therapies, including steroid. Samples were stored at  $-70^\circ\text{C}$ . Moreover, the clinical records of the patients were also analyzed. Patient 10 died and another 12 patients had neurological sequelae (Table 1). Patients 9, 12, and 13 had severe tetraplegia. Therefore, these

Table 1  
Clinical characteristics of 13 patients with acute encephalopathy following prolonged febrile seizure

Patient no./age/sex	Causative agents	Neurological symptoms after the initial prolonged febrile seizure	Second seizures (Day)	MRI (Day)	Sampling day	CSF cell count (/ $\mu\text{L}$ )	CSF protein (mg/dL)	Prognosis
<i>Group A</i>								
1/18 months/M	Unknown	Somnolence	4	4	5	10	31	MeR, Epi
2/18 months/F	Influenza A	Somnolence, right hemiparesis	6	5	5	1	14	MoP, MeR, Epi
3/7 months/F	Unknown	Left hemiparesis	4	4	4	41	31	MoP, MeR
4/2 years/M	Unknown	Disorientation	5	5	5	4	19	MeR
5/2 years/F	Influenza H3N2	Somnolence	2	5	5	8	24	MeR
6/21 months/F	<i>Escherichia coli</i>	Somnolence	4	5	4	2	22	MoP, MeR, Epi
7/10 months/F	HHV6	Left hemiparesis	4	5	4	1	9	MoP
<i>Group B</i>								
8/9 months/F	Influenza H3N2	Semicoma	–	2	2	1	14	Epi
9/10 months/F	Unknown	Coma	–	3	1	1	14	MoP, MeR
10/17 months/F	Rotavirus	Coma	–	4	2	4	23	Deceased
11/18 months/M	RSV	Somnolence, left hemiparesis	–	1	1	0	19	MoP, MeR
12/12 months/F	Rotavirus	Coma	–	1	1	2	11	MoP, MeR, Epi
13/21 months/F	Rotavirus	Semicoma	–	5	2	2	20	MoP, MeR, Epi

Group A, patients who experienced a second seizure; Group B, patients who did not experience a second seizure; M, male; F, female; HHV6, human herpesvirus 6; RSV, respiratory syncytial virus; MoP, motor paralysis; MeR, mental retardation; Epi, epilepsy.

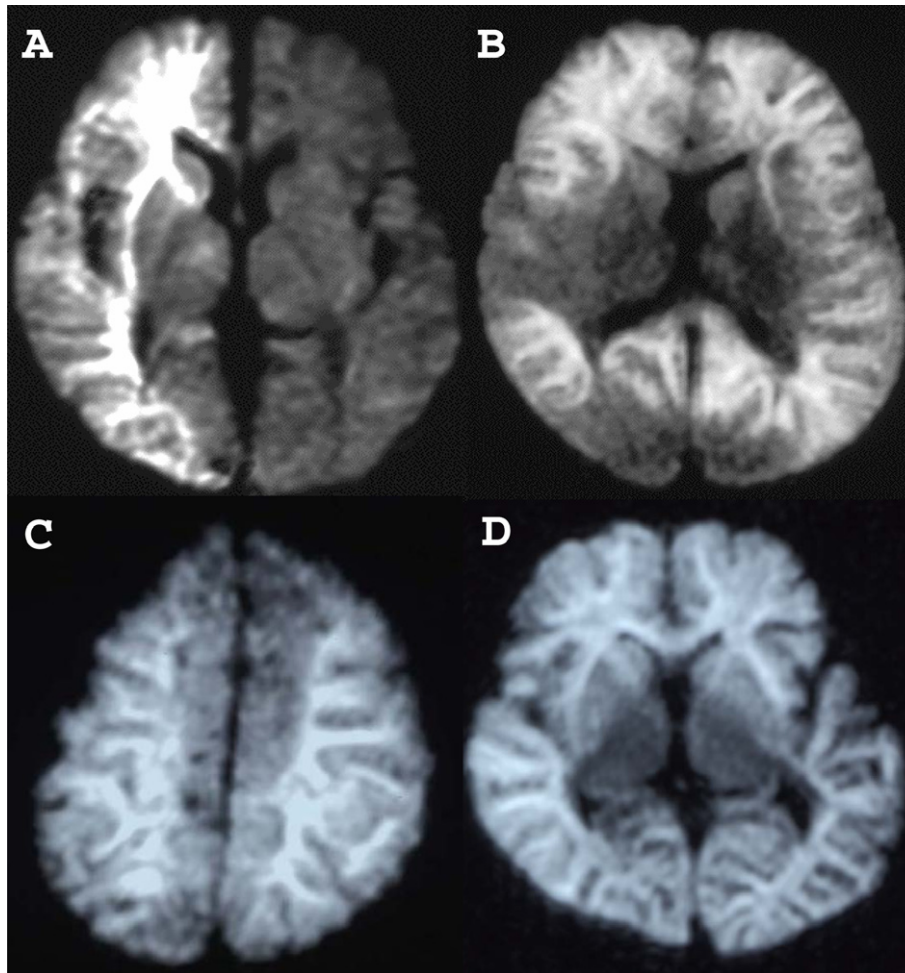


Fig. 1. Diffusion-weighted imaging for Patient 3 (A), Patient 4 (B), Patient 6 (C), and Patient 9 (D) demonstrated high signal lesions in the subcortical white matter.

four patients (Patients 9, 10, 12, and 13) had serious poor prognosis.

### 2.2. Prolonged febrile seizures without encephalopathy (PFS)

Serum and CSF samples were obtained from 23 children with PFS (15 males and 8 females, aged from 8 months to 8 years; median, 2.5 years) on admission to our hospital and a collaborating research hospital in Japan, from June 2002 to March 2007. Febrile seizures were defined as seizures provoked by fever (temperature greater than 38.0 °C). PFS were defined as seizures lasting for more than 30 min or recurrent seizures lasting a total of more than 30 min without the child fully regaining consciousness and with impaired consciousness lasting less than 24 h without neurological sequelae. The causative agents of the patients included influenza A ( $n = 6$ ), HHV6 ( $n = 3$ ), and influenza B ( $n = 1$ ); those of other 13 patients were unknown.

### 2.3. Control subjects

The control subjects for the serum cytokine levels were 73 healthy 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 non-infectious children with neurological disorders, such as psychomotor delay, epilepsy, etc. (12 males and 9 females, aged from 3 months to 15 years; median, 5.1 years). CSF samples were obtained from these subjects on routine analysis and they all exhibited normal CSF cell counts.

### 2.4. Determination of cytokine concentrations

The concentrations of serum and CSF IFN- $\gamma$ , TNF- $\alpha$ , 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 instructions, as described previously [7–9]. Data analysis was performed using GraphPad Prism software (Graph-

Pad 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 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 for transformation and analysis. Starting with standard dilutions, the software performed log transformation of the data, and then fitted a curve to 10 discrete points using a four-parameter logistic model. A calibration curve generated for each cytokine was used to determine the cytokine concentrations of the samples. The lower detection limits for IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-6, and IL-10 were 7.1, 2.8, 2.6, 2.6, 2.5, and 2.8 pg/mL, respectively.

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

### 2.5. Statistical analysis

Statistical analysis was performed using the Wilcoxon matched paired test, with a  $p$ -value of less than 0.05 being considered significant. The differences in the results between groups were analyzed by means of the Mann–Whitney  $U$  test. Correlations were analyzed using Spearman's rank correlation coefficient test.

### 3. Results

The cytokine concentrations of the controls are presented as the means  $\pm$  2 SD. The serum IFN- $\gamma$ , TNF- $\alpha$ , 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 CSF IFN- $\gamma$ , TNF- $\alpha$ , 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 serum IL-6, IL-10, sTNFR1, and CSF IL-6 levels in AEPFS and PFS were significantly higher than those in control subjects ( $p < 0.0001$ , respectively, for each cytokine). The CSF IL-6 levels in AEPFS were significantly higher than those in PFS ( $p = 0.0016$ ), but not serum IL-6, IL-10, sTNFR1, CSF IL-10, or sTNFR1 (Fig. 2). The CSF IL-6 levels were significantly higher than the serum levels in AEPFS ( $p = 0.0414$ ), but not in PFS. The serum levels of IL-10 and sTNFR1 were significantly higher than in the CSF in AEPFS and PFS (AEPFS,  $p = 0.0121$ , and  $p = 0.0022$ , respectively; PFS,  $p < 0.0001$ , and  $p = 0.0002$ , respectively).

The serum IL-10 and sTNFR1 levels in Group B were significantly higher than those in Group A ( $p = 0.0161$ , and  $p = 0.0104$ , respectively), but not serum IL-6, CSF IL-6, IL-10, or sTNFR1. The serum IL-10 levels in patients with serious poor prognosis (Patients 9, 10, 12, and 13) were significantly higher than those with non-serious poor prognosis ( $p = 0.0270$ ), but not serum IL-6, sTNFR1, CSF IL-6, IL-10, or sTNFR1. There were no correlations between serum and CSF IL-6, IL-10 or sTNFR1 levels. Serum IFN- $\gamma$ , TNF- $\alpha$ , IL-2,

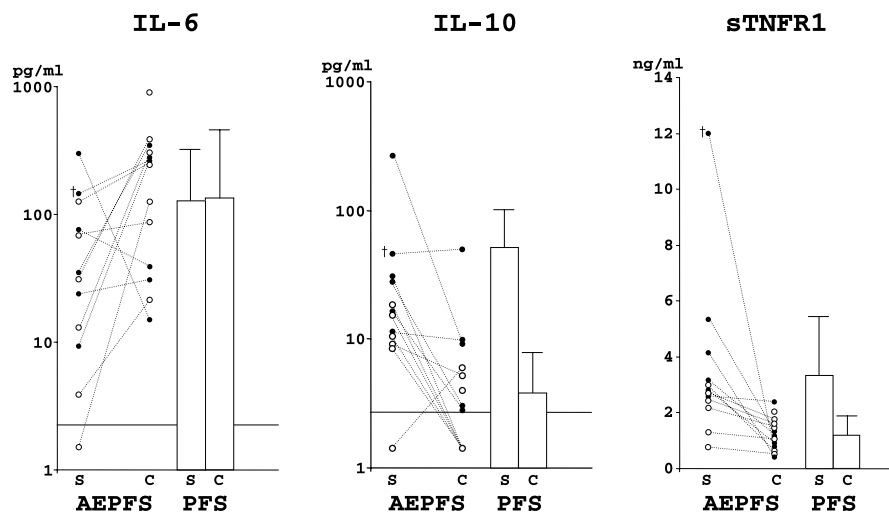


Fig. 2. Serum (S) and CSF (C) concentrations of IL-6, IL-10, and sTNFR1 in children with acute encephalopathy following prolonged febrile seizures (AEPFS) and prolonged febrile seizures without encephalopathy (PFS). Horizontal lines denote the detection limits. Dotted lines indicate samples from the same children on the same day. Open circles, Group A (patients who experienced a second seizure); closed circles, Group B (patients who did not experience a second seizure); †deceased case. Data of the patients with PFS are presented as means  $\pm$  1 SD.



IL-4, CSF IFN- $\gamma$ , TNF- $\alpha$ , IL-2, or IL-4 levels were not elevated in AEPFS and PFS.

#### 4. Discussion

In AEPFS, IL-6 levels were elevated in the CSF, and IL-6, IL-10, and sTNFR1 levels were elevated in the serum. The CSF IL-6 levels in AEPFS were significantly higher than those in PFS. Moreover, CSF IL-6 levels were significantly higher than those in serum, and serum IL-10 and sTNFR1 levels were significantly higher than those in CSF. The cytokine profiles of serum and CSF were different in AEPFS. These findings suggested that the immunological response of the CSF was different from that of serum in AEPFS. IL-10, as an anti-inflammatory cytokine, decreases the production of IL-1, IL-6, and TNF- $\alpha$ , induced by endotoxins or bacteria [10,11]. It is likely that IL-10 is induced to inhibit the production of pro-inflammatory cytokines. Previous studies demonstrated that sTNFR is the natural homeostatic regulator of the actions of TNF- $\alpha$ , and that its level, rather than that of TNF- $\alpha$ , reflects the true biological activity of TNF- $\alpha$  [12–14]. Therefore, elevated IL-6, IL-10, and sTNFR1 levels in the serum likely indicate the existence of inflammation in the blood of AEPFS and PFS. Serum IL-10 and sTNFR1 levels in the patients in Group B, who did not experience second seizures after the initial prolonged febrile seizure, were significantly higher than those of Group A, who did experience a second seizure. Differences of the sampling day may be responsible for these differences in the serum IL-10 and sTNFR1 levels, as the sampling days for Group B (mean, Day 1.5) were earlier than those for Group A (mean, Day 4.7). However, the neurological symptoms after the initial prolonged febrile seizure in Group B were severe, including coma in three patients and semicoma in two patients, compared to Group A. Serum IL-10 and sTNFR1 levels may be related to the severity of neurological symptoms after the initial prolonged febrile seizure. However, the question remains: what do elevated IL-6 levels without elevated IL-10 or sTNFR1 levels in the CSF mean in AEPFS?

Several previous studies reported that a convulsive status induced ischemic changes in the brain [15–17]. The brain requires increased oxygen and glucose during the convulsive states, and thus cerebral blood flow increases [18]. However, a mismatch occurs between the sustained increased oxygen and glucose utilization and a fall in cerebral blood flow after 30 min [18]. Therefore, convulsive states lasting more than 30 min induce dysfunction and damage of the brain [18]. Recent studies revealed that white matter has a lower threshold for infarction than gray matter for cerebral

blood flow and cerebral blood volume [19,20]. Cerebral ischemia induces IL-6 in the brain [21–23], which subsequently acts to protect neurons [24,25]. Administration of an anti-IL-6 receptor antibody increased the infarct size in mice, 24 h after middle cerebral artery occlusion [26]. The prolonged seizures also bring excitotoxic damage to the brain. Takanashi et al. demonstrated that a glutamine/glutamate complex was increased in the subcortical white matter of a patient with AEFCSE, and they suggested that excitotoxic damage plays a role in the pathogenesis of AEFCSE [1]. IL-6 protected glutamate-induced excitotoxic damage of the neurons [27,28]. IL-6 increased neuronal survival following *N*-methyl-D-aspartic acid (NMDA) treatment [29]. Ischemia-induced interleukin-6 exerted neuroprotective effects on NMDA receptor-mediated excitotoxicity in the brain [25]. Therefore, we suggest that prolonged febrile seizures result in ischemic and excitotoxic damages in the brain, and that the subcortical white matter lesions on diffusion-weighted MRI in AEPFS indicate damaged lesions as a consequence of the prolonged febrile seizures. It is likely that the elevated IL-6 levels, without elevated IL-10 or sTNFR1 in the CSF, played a role in protecting against the ischemic and excitotoxic damaged lesions because the CSF IL-6 levels in AEPFS were significantly higher than those in PFS. However, our hypothesis needs further investigation.

In summary, the cytokine profiles of serum and CSF were different in AEPFS. We suggest that elevated serum levels of IL-6, IL-10, and sTNFR1 indicated inflammation in the blood in AEPFS. Taking the previous reports into consideration, it is our speculation that IL-6 is induced in the central nervous system to protect the ischemic and excitotoxic damaged brain following prolonged febrile seizure and results in elevated IL-6 levels in the CSF in AEPFS.

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