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Abstract

Background

Pediatricians sometimes see patients with severe aseptic meningitis and prolonged fever or severe headache, or both. This condition generally has a good prognosis and is usually treated with supportive therapy. However, there is neither guideline nor consensus for the treatment of patients with severe aseptic meningitis. Here, we investigated the relationship between disease severity and biomarkers.

Methods

The subjects were 32 children aged 0 to 14 years, 23 of whom had aseptic meningitis and 9 of whom were meningitis-free controls. Aseptic meningitis was retrospectively categorized into two subgroups, namely mumps meningitis (MM) and viral meningitis excluding that caused by mumps (EM). We defined a novel aseptic meningitis severity score (AMSS) from the signs and symptoms of aseptic meningitis and thus evaluated disease severity. We analyzed the profiles of cytokines in the patients' cerebrospinal fluid (CSF).

Results

The AMSS in MM was significantly higher than that in EM. IL-4, IL-6, IL-8, IL-10, and G-CSF levels in MM and EM CSF were higher than those in control CSF. IFN-γ levels were higher in MM than in controls ($p<0.01$). IL-10 and IFN-γ levels in MM were higher than those in EM.

Conclusions

MM was more severe than EM. One likely reason is the higher CSF cytokine levels in MM. IFN-γ may be a potentially strong biomarker of MM severity. Our findings would help further understanding of the pathological mechanisms of severe aseptic meningitis.

Key Words: Aseptic meningitis; Cytokines; Enterovirus; Mumps virus; Viral meningitis

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Introduction

Aseptic meningitis in children is caused mostly by infection with viruses such as enterovirus or mumps virus. It is thought to be a self-limiting disease with a generally good prognosis[1-3]. However, physicians sometimes see patients with aseptic meningitis who have severe clinical symptoms such as prolonged fever, severe headache, and recurrent vomiting. There is no consensus or guideline on the treatment of patients with severe aseptic meningitis. Therefore, the management of severe cases effectively depends on the individual physician in charge. To prevent mumps meningitis (MM) and other sequelae, live vaccination against mumps is available in most advanced and developing countries; moreover, universal inoculation is being conducted in many countries[4], although there is currently no such a program in force in Japan. One of the reasons why universal inoculation has not been adopted is that MM can be a rare side effect of the mumps vaccine itself. Even if mumps vaccination were universal worldwide, there would still be concern about vaccine-related mumps meningitis. The treatment for viral meningitis is basically supportive, although severe cases including encephalitis caused by mumps virus or enterovirus are treated with intravenous steroids and gamma-globulin infusion therapy[5-7]. As yet, we have no fundamental data to guide treatment based on the pathological mechanisms of severe aseptic meningitis caused by viral infection and vaccination, especially for mumps.

We hypothesized that severe symptoms in aseptic meningitis are due to the hyper-immunological status of the central nervous system (CNS) and can be correlated with the levels of certain biomarkers. We therefore evaluated cytokine levels in samples from cerebrospinal fluid (CSF) and to find cytokines that could be used as optimal biomarkers of disease severity and therefore as indicators of pathology. Clinically, there is currently no method for evaluating the severity of meningitis, so we also tried to create a system for scoring disease severity. We assessed the severity of aseptic meningitis retrospectively from patients' clinical records and compared the scores with the levels of major cytokines as potential biomarkers.

Subjects and Methods

Subjects

All subjects had been admitted to Osaka City University Hospital, Kashiwara Municipal Hospital, or Izumi Municipal Hospital. They had undergone CSF examination between October 2010 and October 2013. To elucidate factors associated with severity of aseptic meningitis, the patients were allocated to three groups. The MM group consisted of 10 patients (male:female=5:5) with MM who were aged 4 to 11 years [6.8±2.4 (mean±standard deviation)]. The EM group (patients with viral meningitis excluding that caused by mumps) consisted of 13 patients (M:F=11:2) aged 0 to 14 years [4.6±5.3 (mean±standard deviation)]. The control group consisted of 9 patients (M:F=4:5) aged 0 to 14 years [5.3±5.6 (mean±standard deviation)] who had epilepsy (6 patients), migraine, chronic cellebelar ataxia, or genetic neuropathy and in whom infectious disease had been excluded.

This research was approved by the institutional ethics committees of Osaka City University and Kashiwara Municipal Hospital. Informed consent was obtained from all patients and their families.

Scoring of clinical severity of aseptic meningitis

We evaluated the severity of aseptic meningitis by devising an aseptic meningitis severity score (AMSS) with 0 to 18 points. The AMSS was used retrospectively to grade each patient by using data from the clinical records made by pediatricians and nurses. According to major textbooks[1-3], common

Table 1. Details of aseptic meningitis severity score (AMSS)

Score	0	1	2	3	4
A. Peak body temperature (℃)	<38.0	38.0-38.9	39.0-39.9	≧40.0	
B. Duration of fever (days)	none	≦3	4-6	≧7	
C. Duration of recurrent vomiting (days)	none	≦3	4-6	≧7	
D. Duration of headache (days)	none	≦3	4-6	≧7	
E. Neck stiffness	none	slight	obvious		
F. Time spent bedridden (days)	none	≦3	4-6	7-9	≧10

F was defined as being sufficiently severe to render the patient incapable of functioning normally.

and important findings of aseptic meningitis were sudden fever, headache, vomiting and stiff neck. Another important symptom is prolongation of the symptoms and signs. In consideration of our clinical experiences, eventually we decided that the scoring system itemized the durations of fever, headache, and vomiting; peak temperature; presence and severity of neck stiffness; and time spent bedridden, the each simple scales easy to evaluate clinically according to clinical charts retrospectively (Table 1).

Aseptic meningitis was defined from the symptoms and signs of meningitis and laboratory findings[1-3,8]. Children aged 3 or more were included in the aseptic meningitis group when they had symptoms and signs of aseptic meningitis and pleocytosis (>5 cells/mm^3 in the CSF). Children aged 2 years or less were considered to have aseptic meningitis if they had CSF pleocytosis >15 cells/mm^3 or if virus was detected in the CSF by reverse transcription-polymerase chain reaction (RT-PCR). Encephalitis was excluded from subjects. MM was further defined by the presence of specific symptoms such as parotiditis, an increase in serum mumps-specific IgM levels, and the increased level of amylase in the serum or urine. Two patients in this group had been inoculated with dried live attenuated mumps vaccine (Torii strain) 21 or 22 days before the onset of fever. They were confirmed as having vaccine-associated meningitis by sequencing of the P gene with reference to previous reports[9,10]. EM (viral meningitis excluding that caused by mumps) was defined by the absence of a history of mumps infection or mumps vaccination and a lack of serum amylase and specific IgM elevations. The control group contained patients determined not to have infectious diseases.

Detection of viral genome and sequencing

All of patients in MM were examined by RT-PCR in their CSF. Twelve patients out of 13 patients in EM were also examined by RT-PCR in their CSF. Mumps virus and enterovirus were detected by RT-PCR using a method described previously[9-14]. Viral RNA was extracted by using a Qiamp Viral RNA mini kit (Qiagen GmbH, Hilden, North Rhine-Westphalia, Germany). Complementary deoxyribonucleic acid (cDNA) was synthesized by Superscript Ⅲ reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in the presence of RNaseOUT (Invitrogen). The cDNA products were purified by using a Qiaquick Nucleotide Removal kit (Qiagen). The primers used for PCR assay of the mumps virus genome were from common regions among strains deposited in the GenBank DNA database, including the Torii vaccine strain and clinical isolates. cDNA product was subjected to nested PCR, which was carried out with EmeraldAmp PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan) according to manufacturer's protocol. PCR sequences were determined by using the ABI310 system (Applied Biosystems, Foster City, CA, USA).

Table 2. Aseptic meningitis severity score (AMSS) and cerebrospinal fluid (CSF) cytokine levels

	MM	EM	Control
number of subjects	10	13	9
age	6.8±2.4	4.6±5.3	5.3±5.6
AMSS	11.9±3.5 †	7.4±4.2	-
Cell count (/μL) in CSF	234±234 **	167±254 **	<3
IL-4	1.0±1.1 *	0.9±0.9 *	0.1±0.7
IL-6	886±1871 **	1234±1778 **	2.1±0.6
IL-8	2436±3766 **	797±862 **	12±7.5
IL-10	177±215 **††	11±11 *	1.8±1.0
G-CSF	293±519 *	688±944 **	3.0±2.3
IFN-γ	540±605 **†	63±73	3.4±4.5

MM, mumps meningitis; and EM, viral meningitis excluding that associated with mumps.
All numbers are shown as means±standard deviations. Units for all CSF cytokine levels are pg/mL.
**$p<0.01$, significantly higher than control; *$p<0.05$, significantly higher than control; ††$p<0.01$, MM significantly higher than EM; and †$p<0.05$, MM significantly higher than EM.

Cytokine analysis

To assay cytokines we used a multiple ELISA assay system, Bio-Plex Pro Human Cytokine Group 1 Panel 17-plex (BioRad Laboratories, Hercules, CA, USA). The cytokines analyzed were Interleukin (IL) -1beta (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte monocyte colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1beta (MIP-1β), and tumor necrosis factor-alfa (TNF-α). Each sample consisted of a 100-μL aliquot of CSF collected for diagnostic or therapeutic purposes. All samples were stored at −80℃ until analysis.

Statistical analysis

The non-parametric Mann-Whitney *U*-test was used for statistical analysis ; p values were two-tailed and $p<0.05$ was considered statistically significant. Statistical analysis was carried out with SPSS ver. 11.0 (SPSS Inc., Chicago, IL, USA).

Results

The MM group was found to include 2 patients with meningitis associated with mumps vaccination and three patients with mumps infection of wild-type strain, as genetically identified by RT-PCR and the sequencing from their CSF. In the EM group enterovirus genome were detected from CSF in 6 patients by RT-PCR. We used our new AMSS to evaluate disease severity (Table 2). AMSS was significantly higher in the MM group [11.9±3.5 (mean±standard deviation)] than in the EM group [7.4±4.2 (mean±standard deviation)]. Thus patients with MM were more severe than EM. All MM and EM patients had fever over 38.0℃. Vomiting was observed in all MM patients and in 6 of the 7 EM patients excluding younger children aged 2 years or under. In contrast, those patients aged 2 years or under (all of them were infants) in the EM group did not present with vomiting. The average length of recurrent vomiting was 4.2 days for MM patients; this was significantly longer than that in the case of EM patients (1.6 days) ($p=0.016$). Five of 10 MM patients had obvious neck stiffness. Six of the 7 non-infant EM patients had obvious neck stiffness. On the other hand, there was no significant difference between the CSF cell counts of MM [234±234/μL (mean±standard deviation)] and EM [167±254/μL (mean±standard deviation)] patients. In our analysis of 17 kinds of

Table 3. Comparison between severity of aseptic meningitis and cerebrospinal fluid (CSF) cytokine levels

	MM		EM	
	Moderate	Severe	Moderate	Severe
number of subjests	5	5	10	3
IL-4	0.5±0.4	1.4±1.4	0.8±1.0	1.3±0.6
IL-6	147±127	1625±2549	1114±1949	1653±1435
IL-8	1198±1540	3674±5071	702±967	1131±201
IL-10	89±90	264±278	10±11	14±13
G-CSF	85±71	501±701	547±871	1181±1395
IFN-γ	332±346	749±771	59±83	78±18

MM, mumps meningitis; and EM, viral meningitis excluding that associated with mumps.

All numbers are means±standard deviations. Units for all cytokines are pg/mL. We used the total AMSS (aseptic meningitis severity score) values to allocate patients to either of two disease subgroups, namely moderate and severe. Moderate was defined as an AMSS value of 10 or less, and severe was defined as 11 or more.

cytokine, IL-4, IL-6, IL-8, IL-10, G-CSF, and IFN-γ levels were significantly higher in MM than in the controls (Table 2; $p<0.05$ or 0.01). (Data for the other cytokines are not shown, because there were no significant differences). Levels of all of these cytokines except for IFN-γ were also significantly higher in EM than in the controls (Table 2; $p<0.05$ or 0.01). Levels of IL-10 ($p<0.01$) and IFN-γ ($p<0.05$) were significantly higher in MM than in EM.

We then investigated the relationships between AMSS and the levels of these cytokines (Table 3). We used the total AMSS values to allocate the patients to two disease subgroups, namely moderate and severe. Moderate was defined as an AMSS value of 10 or less, whereas severe was defined as a value of 11 or more. In both MM and EM, the levels of all of cytokines (IL-4, IL-6, IL-8, IL-10, G-CSF, and IFN-γ) were higher in severe disease than in moderate disease. IL-6 levels were higher in severe patients (MM and EM combined) than in the moderates ($p<0.05$; data not shown).

Discussion

To manage severe aseptic meningitis, it is important to understand the pathogenesis of the disease and to use CSF analysis and observations of signs and symptoms to estimate disease severity. Hypercytokine status of the central nervous system has been revealed by CSF studies in meningitis. However, most of this research has focused on comparisons between bacterial meningitis and aseptic meningitis or encephalitis[15-21]. Aseptic meningitis is usually milder than bacterial meningitis, so once viral meningitis is diagnosed the decision is usually made to offer only supportive therapy[1-3]. However, some patients with viral meningitis suffer complications such as meningoencephalitis, prolonged fever, deafness, severe and prolonged headache, recurrent vomiting, or systemic sequelae[3,4]. Although we sometimes see such patients with severe aseptic meningitis, there has been little investigation of the pathological mechanism and the optimum treatment, and there are no guidelines. Moreover, to our knowledge there have been no reports on evaluation of the severity of aseptic meningitis and the correlation between severity and cytokine levels. We therefore tried to evaluate the severity of aseptic meningitis and find biomarkers of severe disease that could be used to decide on treatment. Here, our retrospectively assessed and calculated AMSS values revealed that MM was more severe than EM, most of which is considered to result from enterovirus infection[1,2].

In our analysis of cytokines in patients with meningitis, levels of IL-4, the inflammatory cytokines

IL-6, IL-8, G-CSF, and IFN-γ in the CSF were generally higher than those of other cytokines, but the levels of IL-10, which is a strong anti-inflammatory cytokine, were also elevated.

IL-4 is produced by T helper (Th) 2 cells, CD8T cells, mast cells, basophils, and natural killer (NK) T cells[22]; it accelerates the production of IgG and IgE. Those absolute values of IL-4 were not high. It is known that IL-4 acts as suppressor for Th1activity[23] such as IFN-γ, so IL-4 can be induced by much increased IFN-γ. IL-6 is produced by monocytes and macrophages stimulated by IL-1 and by endothelial cells and fibroblasts; it accelerates the proliferation of plasma cells and the production of IgG, IgM, and IgA, it also reported the increase in meningitis and encephalitis[17,22-29]. IL-8 is produced by monocytes and macrophages and endothelial cells and has a chemotactic effect on neutrophils, it was also reported the increase caused by meningitis and encephalitis[18,19,22,30]. Levels of the chemokine G-CSF were also increased in both MM and EM; G-CSF is associated with the stimulation of neutrophil function and thought to accelerate inflammatory response[31,32].

IL-10 and IFN-γ levels were much higher in MM than in EM. IFN-γ is produced by Th1 cells, cytotoxic T cells, and NK T cells; it targets various cells, including macrophages, activated B cells, and Th2 cells, and its levels can increase in various CNS infections[22,33-38]. In our subjects, the CSF cell counts did not differ significantly between MM and EM (Table 2). IL-10 is produced by Th2 cells and target macrophages. Increased production of IL-10 as an anti-inflammatory mediator in MM patients may represent an immunological defense response against the excessive spread of intracranial inflammation as a result of the large increase in IFN-γ production[33-38]. Moreover, some reports have suggested that IL-10 production is associated with some types of encephalitis[25-27,39]. In influenza-associated encephalopathy, which is a hyper-cytokine disorder, elevated levels of both anti-inflammatory and inflammatory cytokines can reflect severe symptoms[25,39].

We considered that IL-4, IL-6, IL-8, IL-10, G-CSF, and IFN-γ needed further examination because their levels were significantly higher in MM (and in EM, with the exception of IFN-γ) than in the controls. One purpose of this study was to clarify the relationship between cytokine levels and the severity of meningitis; we therefore compared the levels of these cytokines with the AMSS values. IL-4, IL-6, IL-8, IL-10, G-CSF, and IFN-γ levels were all non-significantly higher in severe disease than in moderate disease in both MM and EM. However, only the level of IL-6 levels were higher in severe patients (MM and EM combined) than in the moderates.

These results suggest that IL-4, IL-6, IL-8, IL-10, G-CSF, and IFN-γ levels in the CSF have strong potential as biomarkers for assessing the severity of aseptic meningitis.

MM tends to be more severe than other viral meningitis, and IL-10 and IFN-γ levels are worthy of further study as biomarkers of this severity. Because of the particularly large difference in its levels between MM and the controls, IFN-γ especially has the potential to be a strong biomarker of severity in MM.

Aseptic meningitis is diagnosed from clinical symptoms and CSF pleocytosis. However, it is sometimes difficult to diagnose if pleocytosis is absent and requires virus detection by isolation or PCR. It has recently been reported that pleocytosis does not always occur in aseptic meningitis[40-43]. Clinically, physicians do not always try to obtain CSF for a diagnosis, except in young infants and neonates, because of children's generally good prognosis and the risk of complications of pediatric lumbar puncture, including headache, dizziness, prolonged lumbar pain, traumatic bleeding, and secondary infection due to bacterial contamination, and also more staff are usually needed for the procedure than in adults. Although the levels of some cytokines in the CNS reflect duration from the

onset of viral or bacterial infection[23,44], once a child is diagnosed as aseptic meningitis the procedure is unlikely to be repeated. Some patients with aseptic meningitis do not come to hospital until 3 or 4 days after fever onset. This is likely one of the reasons why it has been difficult for clinicians to gain a full understanding of the etiology of severe aseptic meningitis.

The AMSS is a retrospective assessment of disease severity that is obtained from patient charts, so in its present state it cannot be used in the real-time assessment of patients with viral meningitis. Further studies of cytokine levels with large patient numbers will be needed. We have to be able to assess cytokine levels in CSF sampled at any time during the clinical course of the disease. Although we recognize the need to increase the number of study patients and to perform a prospective study, we considered it important to start with this study to investigate the mechanism behind severe pediatric aseptic meningitis.

To our knowledge, this is the first report to address the severity of aseptic meningitis and to investigate the relationship between disease severity and biomarkers of inflammation in the intracranial space. Our findings would help to elucidate the pathological mechanism of aseptic meningitis.

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