Outbreak of Aseptic Meningitis due to ECHO-9 in Northern Kyushu Island in the Summer of 1997

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Summary: An outbreak of aseptic meningitis caused by echovirus type 9 (ECHO-9) occurred between June and August 1997 in the Chikugo area, Fukuoka, Japan. Clinical manifestations and laboratory data of 317 children with aseptic meningitis were analyzed. The age of the patients ranged from 1 month to 12 years with the highest incidence in 4 years old children. The male: female ratio was 2.0:1.0. Symptoms of the meningitis included fever (100%), headache (89.5%) and nausea and/or vomiting (85.6%). Skin rash was not frequent (2.2%) in contrast to previous reports of ECHO 9 infections. The number of white blood cells (WBC) in cerebrospinal fluid (CSF) ranged from 10 to 3,493 cells/ μ l (median; 412 cells/ μ l). The neutrophils were more than 50% of the WBC in CSF in one-fourth of the patients at diagnosis. Enteroviruses were identified from CSF utilizing virus culture and enterovirus-specific RT-PCR, and ECHO-9 infection was determined by antibody titer of paired sera. Finally 44 patients were diagnosed virologically or serologically as aseptic meningitis caused by ECHO-9. Sequence analysis revealed that two strains of ECHO-9 isolated from CSF in this epidemic were closely related to ECHO-9 virulent strain Barty.

Key words ECHO-9, meningitis, sequence

INTRODUCTION

Aseptic meningitis is a severe nonbacterial infection of the central nervous system. Enteroviruses are major causative agent of aseptic meningitis of children in the summer. Among them echovirus type 9 (ECHO-9) is one of the most common agents associated with aseptic meningitis [1-5]. Outbreaks of ECHO-9 meningitis were reported in the late 1950s in the United States, Canada, England and Europe [6-13]. In Japan, epidemics of ECHO-9 meningitis were reported in Kumamoto [14] in 1967, and in Kagoshima prefecture [15] in 1990.

In the summer of 1997, an epidemic of aseptic meningitis occurred in Fukuoka prefecture, Japan. Three hundred seventeen children with aseptic meningitis were admitted to the pediatric department of Kurume University and its affiliated hospitals. The present study reports the characteristics of the illness, certain clinical laboratory data, and serological and

virological studies including reverse transcriptase polymerase chain reaction (RT-PCR). The causative agents of the aseptic meningitis were identified by isolation from a specimen of cerebrospinal fluid (CSF) and by examination of serum antibody titers. Sequences of isolated ECHO-9 were determined and also compared for the first time in order to identify the ECHO-9 lineage which caused the aseptic meningitis.

PATIENTS AND METHODS

Patients

The medical records of 317 aseptic meningitis patients at the Kurume University Hospital, Kurume University Medical Center, St. Mary's Hospital, Yame General Hospital and the Hospital of Amagi-Asakura Medical Association between June and September of 1997 were reviewed. In this study,

aseptic meningitis is defined as an acute illness clinically compatible with viral meningitis associated with CSF pleocytosis of more than 10 white blood cells (WBC)/ μ l. A lumbar puncture was performed on all patients, all of whom had negative bacterial cultures of CSF.

Cells and viruses

RD-18S, Hep-2 and Vero cells were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 μ g/ml streptomycin.

Virus isolation was performed from the CSF. One hundred μ l of CSF from aseptic meningitis patients were inoculated on RD-18S, Hep-2 and Vero cells. When cytopathic effect (CPE) of the cells appeared in 2 weeks, the cells were harvested and the viruses were identified with the pool of antibodies proposed by Lim and Benyesh-Melnik [16]. The isolated viruses were also examined by neutralization with antibodies against mumpsvirus. The isolated ECHO-9 were grown on RD-18S cells until the CPE was evident in more than 90% of the cells. Then, the cells were harvested and the supernatant was used as the viral stock after three cycles of freeze and thawing. Viral RNA was extracted using Sepa Gene (Sanko Junyaku) according to the company's protocol.

Serum antibodies

Sera were taken twice from each of 24 patients, at intervals of 3-21 days, and the serum antibody titer between the two samples was compared by neutralization assay. A fourfold or greater increase in the antibody titer between the acute phase and the convalescent sera was considered necessary for the serologic diagnosis of recent infection.

RT-PCR and sequence

RT-PCR was performed using Ready-to-Go RT-PCR kit (Pharmacia). Briefly, cDNA was synthesized at 37 °C for 10 min and at 42 °C for 30 min from 25 ng of viral RNA using 1 ng/ml of random 9 mer (Takara) as a primer. After cDNA was synthesized, ECHO-9 sequences encoding a part of VP1 and 2A (903 bp) were PCR amplified using a primer pair of 5'-GGTAGGGTTGCTGACACAATACG-3' (nucleotide position 2481-2503 of ECHO-9 strain Barty) and 5'-CACATACACAGCTCCAGATTCTTGG-3' (nucleotide position 3349-3383 of ECHO-9 strain Barty) for 30 cycles under the conditions of

denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min, followed by final extension at 72 °C for 5 min. Amplified fragments were purified through 1% agarose gel electrophoresis and ligated into pT7Blue-T vector (Novagen). The cloned plasmids were transformed in *E.coli* JM109 (Toyobo) and the plasmid DNA was purified using QIAprep spin (Qiagen).

Sequence was determined using Big-Dye cycle sequence kit (Perkin Elmer) from both directions by ABI PRISM 310 DNA sequencer (Perkin Elmer), and analyzed using Gene Works program (Intelli Genitics).

Fig. 1. Distribution of number of patients of aseptic meningitis according to week of onset of symptoms. Number of patients with aseptic meningitis between June and August 1997 was summarized weekly.

Fig. 2. Age distribution of 317 children with aseptic meningitis. Age and sex distribution of aseptic meningitis patients is indicated. Female is lightly shaded and male is dark shaded.

RESULTS

Epidemiology of aseptic meningitis

Three hundred seventeen cases of aseptic meningitis in Chikugo area, Southern region of Fukuoka Prefecture, were diagnosed between June and August, 1997 (Fig. 1). The distribution of patients by age and sex is shown in Fig. 2. The age of the patients ranged from 1 month to 12 years and the highest incidence was in the 4-year-old children. The male: female ratio was 2.0:1.

Clinical signs and symptoms

All of the patients were febrile on admission, and 33.4% of them had a fever with two peaks (Table 1). The mean duration of fever was 7.6 days (standard deviation; ± 3.5). Headache was present in 89.5% of the patients who were 3 years old and older (184 of 230 patients), and nausea and/or vomiting occurred in 85.6%. Neck stiffness and Kernig's sign were observed in 58.9% and 10.9%, respectively. Only 2.2% of the patients had skin rash. Febrile seizures occurred in 7 patients but there were no neurologic abnormalities present.

Laboratory findings

The mean peripheral WBC count was 11,162 cells/ μ l (Table 2). Leukocytosis of $\geq 15,000$ cells/ μ l was found in 35 (11.3%) of 311 patients examined. The mean value of C-reactive protein (CRP) was 0.74 mg/dl and 5.8% of the patients had more than 3.0 mg/dl. The CSF findings are summarized in Table 3. The WBC in CSF (cWBC) ranged between 10 and 3,493 cells/ μ l and the mean cWBC was 574 cells/ μ l. The median of cWBC counts among ages were not significantly different (data not shown). The differential cell count in CSF revealed that 75.5% of the patients had lymphocytosis of more than 50% at diagnosis (median; 5 days of illness), while predominance of neutrophils was seen in one-fourth of the patients (median, 2 days of illness).

The glucose concentration in CSF was <40 mg/dl in 7 (2.2%) of 313 patients examined, but was still more than half of the blood glucose concentration. The protein concentration in CSF was elevated over 100 mg/dl in 5 (1.7%) of all the patients, and the highest was 173 mg/dl.

Paired sera from 24 patients were examined. The antibody titer of 9 cases had more than fourfold increase of antibodies against ECHO-9, and one of them also had those against coxsackievirus B3. In

TABLE 1.
Clinical signs and symptoms of the patients on admission

	cases	%
fever:	317	100
(duration of fever 7.6 ± 3.5 days)		
two-peak fever	106	33.4
nausea and/or vomiting:	269	85.6
headache (over 3 years old):	214	89.5
neck stiffness:	181	58.9
Kernig's sign:	34	10.9
rash:	7	2.2
complication: febrile seizure	7	2.2

TABLE 2.
Laboratory results of the patients with aseptic meningitis on admission

Range	cases	%	Mean $\pm$ SD*
WBC (cells/dL)			
<10000	146	46.9	11162.0 $\pm$ 6371.8
10000- <15000	130	41.8	
≥ 15000	35	11.3	
CRP (mg/dL)			
<1.0	238	76.0	0.74 $\pm$ 1.11
1.0- <3.0	57	18.2	
≥ 3.0	18	5.8	

* standard deviation

TABLE 3.
Findings in cerebrospinal fluid on admission

Range	cases	%	Median	Mean $\pm$ SD*
Cell count (cells/ μ L)				
<50	45	14.2	412	573.9 $\pm$ 598.8
50- <1000	212	67.1		
≥ 1000	59	18.7		
Glucose (mg/dL)				
<40	7	2.2	61.0	62.9 $\pm$ 14.6
≥ 40	306	97.8		
Protein (mg/dL)				
<45	225	71.8	36.0	38.7 $\pm$ 20.6
45- <100	83	26.5		
≥ 100	5	1.7		

* standard deviation

length as strain Barty in the PCR amplified region where most of capsid protein, VP1 (genome positions 2481-3365 and amino acid positions 581-875), and part of 2A protein (genome positions 3366-3383 and amino acid positions 876-881) are encoded. There is only one nucleotide difference at the genome position 2693 between No. 66 and 106. The deduced amino acid sequences are identical between the two. There are 14.8% of nucleotide difference between No. 66 and strain Barty, and 15.0% nucleotide difference between No. 106 and strain Barty, respectively. There are 3.7% amino acid differences among No. 66, No. 106 and strain Barty.

There were 30 nucleotides loss in strain Hill (genome positions 3318-3347 of strain Barty), resulting loss of 10 amino acids in the same frame (amino acid positions 860-869 of strain Barty). There were four nucleotide gaps at the positions 2701, 2714, 2718 and two nucleotides insertion at the position between 2842 and 2843, and 2869 and 2870 in strain Hill, resulting in frame shift of amino acid at positions 654-660 and 700-710.

Figure 5 shows the dendrogram constructed from nucleotide (A) and amino acid sequences (B) by UPGMA method [19].

Strain Barty	GRVADTIRSG	PSNSEAVPAL	TAAETGHTSQ	VVPSDTMQTR	HVKNYHSRSE	630
No.66						50
No.106						50
Strain Hill						630
Strain Barty	STIENFLCRS	ACVRMAKYE	RGDPESTDRF	DAWEISVRDM	VQMRKCEMF	680
No.66	A			I...	..L.....	100
No.106	A			I...	..L.....	100
Strain Hill			...LKALT-L		..L.....	679
Strain Barty	TYLRFDVEVT	FVITSYQHOG	SINQDMPPM-	THQIMYIPPG	GPFPKKVDGY	729
No.66			T.....-		..I.....	149
No.106			T.....-		..I.....	149
Strain Hill		R..	TSSIQIC.YD	A.....	..I.....	729
Strain Barty	EWQTSTNPSI	FWTEGNAPPR	MSIPFISIGN	AYSSFYDGWS	HFDSKGAYGF	779
No.66						199
No.106						199
Strain Hill						779
Strain Barty	NTLNKMGHIY	CRHVNKETPA	EVTSYIRIYF	KPKHIRAWVP	RPPRLCQYKN	829
No.66						249
No.106						249
Strain Hill		T	K.....	...V.....	M.	829
Strain Barty	KANVNFEATA	FTETRDTINT	VPVSNHGSGR	RGDLAALSTH	GAFGQESGAV	879
No.66	D...	V....G....	D.	N..	.V.....	299
No.106	D...	V....G....	D.	N..	.V.....	299
Strain Hill		V.D.....	..L.T..VS.	-----	..Y.HQ...R	869
Strain Barty	YV	881				
No.66	..	301				
No.106	..	301				
Strain Hill	..	871				

Fig. 4. Amino acid sequences of ECHO-9. Amino acid sequences of ECHO-9 are presented by one-letter code. Only differences from the reference strain, Barty, are indicated. Dots indicates the same amino acid sequence as that of strain Barty. A dash indicates a deletion of amino acids. The position of VP1 and 2A protein is indicated in the figure.

Fig. 5. Evolutionary trees of ECHO-9. Evolutionary trees of ECHO-9 No. 66, No. 106, strain Barty and Hill were constructed by nucleotide (A) and amino acid (B) sequences using UPGMA [19]. Number indicates the calculated genetic distance of each branch.

DISCUSSION

The epidemics of aseptic meningitis in the summer are caused mainly by enteroviruses. A prospective study in Baltimore, in the United States, confirmed that more than 90% of community-acquired cases of viral meningitis were caused by group B coxsackieviruses; coxsackievirus serotype B2 and B5, and echoviruses; echovirus serotype 4, 6, 9, 11, 16, and 30 [20]. Three hundred seventeen cases of aseptic meningitis of children in the southern region of Fukuoka prefecture, Japan, were examined in the summer of 1997 and ECHO-9 was identified in 44 cases serologically or by virus isolation. Epidemics of aseptic meningitis caused by ECHO-9 in Europe and North America were reported in the late 1950s [6-13]. In Japan, epidemics of aseptic meningitis due to ECHO-9 in Kumamoto (the neighboring prefecture of Fukuoka) and Kagoshima (the neighboring prefecture of Kumamoto) prefectures were reported in 1967 and 1990, respectively [14,15]. In the present study, frequencies of neck stiffness (58.9%), Kernig's sign (10.9%), and skin rash (2.2%) were lower than in the previous reports [3,5,6,8,11,15]. It has been reported that one-third to one-half of the patients with ECHO-9 meningitis had

skin rash [3,21,22].

Although some reports have suggested a moderate risk of long-term neurologic and cognitive sequelae for infants who suffered viral meningitis at an early age [23,24], recent prospective studies of more patients have demonstrated virtually no detectable effects [25,26], including the cases with neurologic findings in the acute stage [27]. In this study, all the patients, including young infants, recovered spontaneously without any complications. Several epidemics of enteroviral meningitis were reported with very different age-group susceptibility [3,4,28,29]. Such variations in the epidemiology of enterovirus infections are not well understood. A sero-epidemiologic study of an outbreak of ECHO-9 meningitis in Japan in 1990 indicated that accumulation of susceptible infants and young children may have predisposed to the epidemic [15]. Such strain variation and the increase in newly susceptible young children may contribute to the spread of specific enterovirus infection among human populations. Intensive analysis of outbreaks of viral meningitis should be continued in order to confirm the clinical characteristics of ECHO-9 infection.

It was reported previously that the predominance of neutrophils in the CSF lasted no longer than 24

hours [30] and that cell count in the CSF decreased with patient age [15,31]. In this study the period of the shift of WBC to lymphocyte predominance was observed on the second day of illness or later, and the median of WBC count in CSF did not increase in any age groups. In early stage of enterovirus meningitis, the reason of predominance of neutrophils in CSF is not well known. A study on the measurement of granulocyte colony-stimulating factor (GCSF) in CSF revealed that the concentration of GCSF in CSF of enterovirus meningitis was significantly higher than in CSF of mumps meningitis [32]. Some cytokines may play an important role in the percentage of neutrophils in CSF.

Sequences of the isolated ECHO-9 were determined in order to find out the characteristic genome structure which would explain the unique clinical feature of the aseptic meningitis in this outbreak. Sequence analysis revealed that two strains of ECHO-9, No. 66 and 106, isolated from the recent meningitis patients in Japan were closely related to echovirus-9 virulent strain Barty which was also isolated from a meningitis patient (Figs 5 and 6) [17]. No. 66 and No. 106 were almost identical in their genome sequence of VP1 region. Their amino acid differences from strain Barty were seen between conserved amino acids; 640 S to A, 667 V to I, 671 M to L, 701 S to T, 722 F to I, 836 E to D, 840 F to V, 845 D to G, 858 G to D, 867 S to N, and 871 A to V.

A virulent strain Hill which was isolated from a healthy child had a 10 amino acid deletion in the C-terminal region of VP1 protein, where one of two RGD sequences of VP1 is localized [33,34]. RGD sequences of VP1 protein are the binding sites of echoviruses to the host cell receptor, VLA-2, which is a member of the integrin family. The lack of a second RGD sequence is the major difference between virulent and avirulent strains of ECHO-9.

In this study, unique genome structure and genetic lineage of ECHO-9 which caused aseptic meningitis were identified from the limited data. However, gene structure which may be related with infrequency of skin rash remains to be elucidated.

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