

Association of Nonpolio Enteroviral Infection in the Central Nervous System of Children With Febrile Seizures

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ABSTRACT. *Objective.* To clarify the relationship between enteroviral infection and febrile seizures.

Study Design. Cerebrospinal fluid (CSF), serum, throat swab, and rectal swab samples were collected for virologic examination from 67 children with febrile seizures from April 1997 to March 1999. Those samples were examined for the presence of enterovirus using cell culture and 2 polymerase chain reaction (PCR) methods.

Results. No enterovirus was isolated from cell culture of CSF, throat swab, or rectal swab samples. All samples were screened for the presence of enteroviral sequences using a sensitive PCR method (PCR-Fukushima). We obtained positive results from 14 of 67 CSF samples, 10 of 62 serum samples, 12 of 64 throat swab samples, and 13 of 64 rectal swab samples. Of 21 patients in whom febrile seizures had developed during the summer months (June through August), 13 (61.9%) had positive PCR results in the CSF. Forty-seven of the 49 samples with a positive result using PCR-Fukushima were reexamined independently for the presence of the enteroviral genome using another PCR method (PCR-Mitsubishi). PCR-Mitsubishi had slightly lower sensitivity than PCR-Fukushima but identified genotypes of enterovirus by subsequent sequence analysis of the PCR products. The presence of the enteroviral genome was confirmed in 39 of the samples (83.0%). In 8 of the 9 enteroviruses detected in the CSF and/or serum samples using PCR-Mitsubishi, the genotypes were identified as coxsackieviruses group A, which are usually difficult to isolate using cell culture methods.

Conclusions. These findings proved that the causative agents of febrile illness associated with seizures in summer were primarily enteroviruses, especially coxsackieviruses group A, and that febrile seizures might be caused by enteroviral infection in the central nervous system. *Pediatrics* 2001;107(1). URL: <http://www.pediatrics.org/cgi/content/full/107/1/e12>; *enterovirus, coxsackievirus group A, febrile seizures, polymerase chain reaction.*

ABBREVIATIONS. CNS, central nervous system; CSF, cerebrospinal fluid; PCR, polymerase chain reaction; bp, base pair.

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Enteroviruses—polioviruses, echoviruses, coxsackieviruses group A and group B, and enteroviruses—are responsible for significant and frequent human illness, including various neurologic manifestations. The most common neurologic illness is aseptic meningitis. Encephalitis and other manifestations, such as paralysis, Guillain-Barré syndrome, transverse myelitis, cerebellar ataxia, and peripheral neuritis, also occur.¹ The association of enteroviral infection in the central nervous system (CNS) with febrile seizures, however, has not been demonstrated. In general, the causative agent of the infection is not detected in the CNS of patients with febrile seizures using standard techniques.

We previously reported that the enteroviral genome was detected in cerebrospinal fluid (CSF) samples collected from some children with transient neurologic complications, such as seizures and altered states of consciousness associated with acute febrile illness.² Of the 18 patients with seizures that fulfilled diagnostic criteria for simple febrile seizures, 5 had positive results in their CSF as determined by enteroviral polymerase chain reaction (PCR). The present study was designed to clarify the relationship between enteroviral infection in the CNS and febrile seizures and to identify the enteroviruses that relate to the febrile seizures.

METHODS

Samples

From April 1997 to March 1999, CSF samples were collected from 67 children who had transient neurologic symptoms (ie, seizures and altered states of consciousness) associated with acute febrile illness and were diagnosed as having initial febrile seizures. The 67 children (28 girls and 39 boys) ranged in age from 6 months to 5.6 years (mean: 2.6 years). Of the 67 patients, 55 were diagnosed with simple febrile seizures that fulfilled 5 diagnostic criteria: occurrence in children ages 6 months to 5 years, occurrence with fever >38°C, duration of <15 minutes, occurrence during absence of CNS infection as determined using standard techniques, such as bacterial culture and viral isolation, and occurrence with absence of neurologic abnormalities after the attack. The remaining 12 patients had febrile seizures followed by status epilepticus, which is defined as a seizure or series of seizures occurring over 30 minutes. Those patients were diagnosed with complex febrile seizures. Enteroviral infection was not suspected as the cause of febrile illness, except in one patient with herpangina. Sixty-two serum, 64 throat swab, and 64 rectal swab samples were also collected from the patients at the acute febrile phase for virologic examinations. Informed consent was obtained from their parents.

Virus Isolation

HEp-2, Vero, RD-18S, HMV-II, and MDCK cells were used for the isolation of viruses, such as enteroviruses, paramyxoviruses, adenoviruses, herpes simplex viruses, and orthomyxoviruses. Confluent cell cultures were seeded in microplate wells and inoculated with 100 μ L of maintenance medium and 50 μ L of CSF, throat swab, or rectal swab samples. The cell cultures were then incubated at 34°C in 5% carbon dioxide and observed daily, except Sunday, for 7 days for the cytopathic effect. Blind passage was performed once if the cytopathic effect was not observed by the end of the observation period. Virus isolates were identified with a neutralization test or an hemagglutination inhibition test.

PCR for Detection of the Enteroviral Genome

All of CSF, serum, throat swab, and rectal swab samples were screened for the presence of the enteroviral genome using sensitive PCR methods. One PCR method was performed in the Department of Pediatrics, Fukushima Medical University, and named PCR-Fukushima. The procedure for nested PCR was described previously.² In brief, RNA was extracted from 250 μ L of sample. After RNA extraction, complementary DNA was synthesized from the resuspended RNA using 2.5 U of Moloney murine leukemia virus reverse transcriptase. The complementary DNA product was amplified using nested PCR. The primers were set in the 5' noncoding region, as reported by Zoll et al.³ The PCR product was run on 2% agarose gel containing ethidium bromide and photographed under ultraviolet light. A positive PCR reaction was expected to produce a 155-base pair (bp) band. The sensitivity of PCR-Fukushima, calculated from extractions of serial dilutions of titrated echovirus type 7, corresponded to 10^{-3} tissue culture infective dose.⁴ To eliminate and to detect laboratory contamination leading to false-positive PCR results, negative controls were included for each step of the assay.

To compare the amount of viral genome between CSF and serum samples, serially diluted samples from 14 patients with positive PCR results in the CSF and/or serum were tested for the presence of the viral genome using PCR-Fukushima.

PCR for Identification of Enterovirus

The original samples that had positive results using PCR-Fukushima were sent to Mitsubishi Kagaku Bio-Clinical Laboratory and were independently examined for the presence of the enteroviral genome sequences using PCR-Mitsubishi. The primers were set in the 5' noncoding region and the VP2 region as originally reported by Olive et al.⁵ PCR-Mitsubishi amplified an ~650-bp product that included part of the 5' noncoding region, complete VP4 region, and the 5' end of the VP2 region. The sensitivity of PCR-Mitsubishi corresponded to $\sim 10^{-2}$ median tissue culture infective dose.

The PCR products were gel-isolated, purified, and sequenced on an automated DNA sequencer using a fluorescent dideoxy-chain terminator. The Mitsubishi database for the complete VP4 region of all 64 human enteroviruses was constructed from the

nucleotide sequencing data of the VP4 region of 49 prototype enterovirus strains and 15 previously published enteroviral sequences obtained from GenBank (H. Ishiko et al, unpublished data). The VP4 sequences from each PCR products were phylogenetically analyzed along with those of prototypes in the Mitsubishi database by using the package program SINCA (Fujitsu Limited, Tokyo, Japan). The evolutionary distances were estimated by the Kimura 2-parameter method,⁶ and the unrooted phylogenetic trees were constructed by the neighbor-joining method.⁷ The statistical significance of phylogenies using the neighbor-joining method was estimated by bootstrap analysis with 1000 resamplings of the datasets.⁸

RESULTS

Seasonal Distribution of Febrile Seizures

A biphasic curve in the monthly incidence was observed for the 2 years that the study was conducted, with peaks in summer and winter (Fig 1). The seasonal distribution pattern was similar to that observed in the period from 1987 to 1996 at Fukushima Medical University Hospital (data not shown).

Viral Isolation

No virus was isolated from the 67 CSF samples. Influenza virus type A (H3N2) and type B were isolated from, respectively, 11 and 1 of the 64 throat swab samples. All of the samples with positive isolation for the influenza virus were collected in the winter months. Adenoviruses were isolated from 2 of 64 throat swab samples and from 2 of 64 rectal swab samples. No other viruses, including enteroviruses and paramyxoviruses, were isolated from the throat swab or rectal swab samples.

Screening for the Presence of the Enteroviral Genome

The presence of the enteroviral genome sequence in CSF, serum, throat swab, and rectal swab samples was screened using PCR-Fukushima. Positive PCR results were obtained from 14 of 67 CSF samples (20.9%), from 10 of 62 serum samples (16.1%), from 12 of 64 throat swab samples (18.8%), and from 13 of 64 rectal swab samples (20.3%; Table 1). In total, 16 of 67 patients (23.9%) showed positive PCR results in any site. Characteristics of and laboratory results for 14 children with positive PCR results in the CSF and/or serum are shown in Table 1. In 9 patients, the

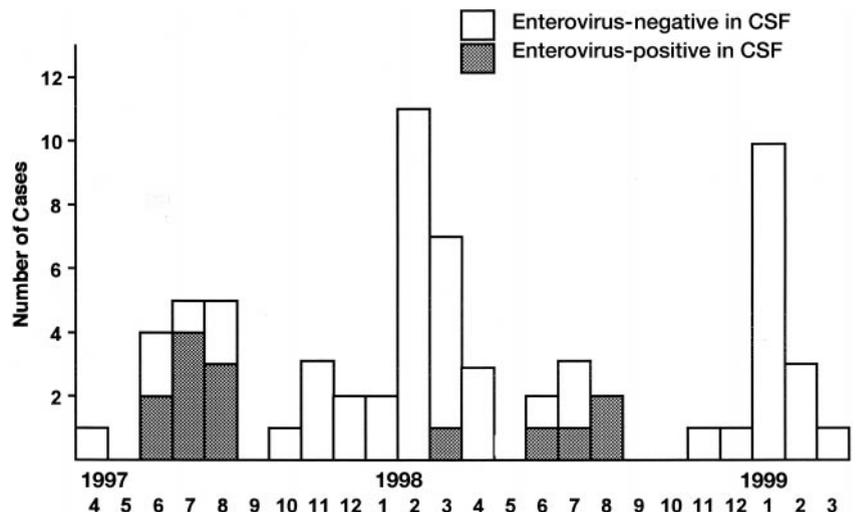


Fig 1. Seasonal distribution of febrile seizures. A biphasic curve of the monthly incidence of febrile seizures was seen. The causative agent associated with seizures in summer months was primarily attributed to enteroviruses.

TABLE 1. Characteristics of and Laboratory Results for 14 Children With Positive PCR Results

Subject	Date	Age	Gender	Clinical Diagnosis	Degree of Fever (°C)	CSF Findings			PCR Results							
						Cell Counts (Cells/mL)	Protein (mg/dL)	Glucose (mg/dL)	PCR-Fukushima				PCR-Mitsubishi			
									CSF	Serum	TS	RS	CSF	Serum	TS	RS
1	1997.6	1.08	M	RI, FS	39.6	0	32	65	+	-	-	-	-	NT	NT	NT
2	1997.6	3.00	M	RI, FS	40.6	1	21	104	+	+	+	+	A	A	A	A
3	1997.7	1.67	M	RI, FS	40.0	5	20	61	+	-	+	+	-	NT	E30	-
4	1997.7	2.67	M	RI, FS, SE	39.6	2	25	83	+	+	+	+	CA4	CA4	CA4	CA4
5	1997.7	1.33	M	RI, FS	39.8	2	16	75	+	+	+	+	CA4	CA4	CA4	CA4
6	1997.7	1.42	M	Hr, FS	39.3	0	28	55	+	+	+	+	CA4	CA4	CA4	CA4
7	1997.8	3.67	F	RI, FS	39.2	0	71	87	+	-	-	-	-	NT	NT	NT
8	1997.8	1.17	M	RI, FS	38.8	2	18	63	+	+	+	+	B	B	B	B
9	1997.8	2.58	M	RI, FS	38.7	1	30	83	+	+	+	+	CA4	CA4	CA4	CA4
10	1998.3	.92	M	RI, FS	39.3	1	22	120	+	-	-	+	NT	NT	NT	E18
11	1998.6	4.50	M	RI, FS	40.0	2	17	53	+	+	+	-	-	-	CB5	NT
12	1998.7	1.50	M	RI, FS	40.4	2	14	90	+	+	+	+	NT	CA5	CA5	CA5
13	1998.8	2.00	F	RI, FS	40.4	2	24	59	+	+	+	+	-	A	A	A
14	1998.8	1.00	F	RI, FS	39.3	1	18	74	+	+	+	+	-	A	A	A

TS indicates throat swab; RS, rectal swab; RI, respiratory infection; FS, febrile seizures; SE, status epilepticus; Hr, herpangina; NT, not tested; A, A cluster of enteroviruses (coxsackievirus group A-like genotype); B, B cluster of enteroviruses (coxsackievirus group B or echovirus-like genotype); E30, echovirus 30; CA4, coxsackievirus A4; E18, echovirus 18; CB5, coxsackievirus B5; CA5 coxsackievirus A5.

enteroviral genome was detected in all samples taken from 4 sites, ie, throat, feces, blood, and CSF. Of 21 patients in whom febrile seizures developed in the summer months (June through August), 13 (61.9%) had positive PCR results in the CSF (Fig 1).

Comparison of the Amount of the Enteroviral Genome Between CSF and Serum Samples

Serially diluted CSF and serum samples from the 14 patients with positive PCR results in the CSF and/or serum samples were tested for the presence of the enteroviral genome using PCR-Fukushima. The maximal dilution of the sample with positive results was compared among CSF and serum samples. Double serial dilution higher was considered to constitute a significant difference. Of the 14 patients, the amount of the viral genome in the CSF was significantly lower in 7, but was significantly higher in 2, than that in the serum. There was no significant difference of the amount of the viral genome between CSF and serum samples in 5 patients.

Identification of the Enteroviral Genotypes

Of 49 samples that had positive results using PCR-Fukushima, 47 were sent to Mitsubishi Kagaku Bio-Clinical Laboratory and reexamined independently for the presence of the enteroviral genome using PCR-Mitsubishi. We obtained positive results from 6 of 12 CSF samples, 9 of 10 serum samples, 12 of 12 throat swab samples, and 12 of 13 rectal swab samples. Thus, the presence of the enteroviral genome was confirmed in 39 of 47 tested samples (83.0%; Table 1). Phylogenetic analysis using the VP4 protein-coding region (209 bp) of 64 enterovirus strains from the Mitsubishi database produced 4 major clusters, which is compatible with the results of phylogenetic analysis using the VP1 sequences by Oberste et al⁹ (H. Ishiko et al, unpublished observations). Analyzing the sequences of the PCR products allowed us to identify the genotypes of enteroviruses (Fig 2). Genomic sequences detected from different sites of a patient were completely identical. Eight of

9 enterovirus genotypes detected in CSF and/or serum samples were found to group within the A cluster of enteroviruses (coxsackievirus group A-like genotype; Table 1). The other genotype was localized within the B cluster (coxsackievirus group B or echovirus-like genotype; Table 1). The genotypes of 5 enteroviruses detected by PCR-Mitsubishi in throat swab and/or rectal swab samples, but not in CSF or serum samples, belonged to the B cluster (echovirus 30 and 18, and coxsackievirus B5-like; Table 1) and the C cluster (poliovirus 3-like) of enteroviruses. Poliovirus 3-like genotype, detected in 1 throat swab and 2 rectal swab samples taken from 2 patients, was not likely to relate to febrile seizures. Those patients had a recent history of polio vaccination, and no enteroviral genome was detected in the CSF or serum samples using PCR-Fukushima. Those 2 patients were omitted from children with positive PCR results (Table 1).

DISCUSSION

A biphasic curve of the monthly incidence of febrile seizures has been reported,⁷ with peaks in summer and winter. The causative agents of febrile illness associated with seizures in the winter months are primarily attributed to influenza viruses and respiratory syncytial virus. A possible relationship of the summer-month peaks to enteroviruses has been mentioned, but enteroviruses such as echoviruses and coxsackieviruses are occasionally isolated from throat swab and fecal samples and are rarely isolated from CSF samples of patients with febrile seizures.^{10,11} In the present study, there was a similar biphasic curve in the monthly incidence of febrile seizures. The relationship of the winter-month peaks to influenza virus infections was confirmed by virus isolation, but enteroviruses were not isolated from any samples of the patients with febrile seizures. Cells used for enteroviral isolation were HEp-2, Vero, and RD-18S cells. These cells were sensitive for many enteroviruses, such as echoviruses, coxsackieviruses group B, and some group A (ie, A9, A10, and

zures during the summer months. The causative agent of febrile seizures in summer is attributed primarily to enteroviruses.

Eight of 9 enterovirus genotypes detected in the CSF and/or serum samples using PCR-Mitsubishi belonged to the A cluster of enteroviruses (coxsackievirus group A-like genotype), which are usually difficult to isolate from samples using cell culture methods. The remaining enterovirus was localized within the B cluster (coxsackievirus group B- or echovirus-like genotype). CNS infection by these viruses (participants 2, 4, 5, 6, 8, 9, 12, 13, and 14) probably caused the febrile seizures. Echo virus 30 and 18 and coxsackievirus B5-like genotypes detected in throat swab and/or rectal swab samples, but not in CSF or serum samples using PCR-Mitsubishi (participants 3, 10, and 11) might also relate to the febrile seizures. Those 3 patients had a detectable enteroviral genome in the CSF using PCR-Fukushima.

The present results confirmed that enteroviruses, especially coxsackieviruses group A, often infiltrated into the CNS of the patients with febrile seizures. When the amount of the enteroviral genome was compared between the CSF samples and serum samples, the amount in the serum was not always higher than that in the CSF. These results suggest that the presence of the enteroviral genome in CSF is not the result of a leak of the virus to the CSF from crossing the unaffected blood-brain barrier. Neurologic symptoms, such as seizures and bulging of the anterior fontanel, were observed in some patients during the febrile phase of exanthema subitum; human herpesvirus 6 DNA was frequently detected by PCR from the CSF of those patients.¹² The finding of viral genome in the CSF of the patients with febrile seizures strongly suggested that viral infection in the CNS might be the cause of the seizures.

Pathologic proof is required to confirm the enteroviral infection in the CNS of patients with febrile

seizures. Histologic examination, however, is quite difficult in a benign illness, such as febrile seizures. Additional studies are needed to investigate how the viral infection affects the CNS and why it results in the transient neurologic complication, such as febrile seizures, by biochemical and/or biophysical examinations.

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