Detection of Enterovirus by Polymerase Chain Reaction and Culture in Cerebrospinal Fluid of Children with Transient Neurologic Complications Associated with Acute Febrile Illness

Mitsuaki Hosoya, Ken Honzumi, and Hitoshi Suzuki

Cerebrospinal fluid samples collected from 23 children with neurologic symptoms, such as febrile seizures, status epilepticus, and transient altered states of consciousness, associated with acute febrile illness, were examined for infectious virus by cell culture. Enteroviruses (echovirus type 9 and coxsackievirus B3) were isolated from 2 of the cerebrospinal fluid samples. The samples were also examined for enterovirus by the polymerase chain reaction, which could detect nearly the whole human enterovirus group: Enteroviral RNA was detected in 9 of the 23 samples. The findings suggest that transient neurologic complications during the febrile phase of acute febrile illness are caused, in part, by enteroviral infection of the central nervous system.

Polymerase chain reaction (PCR) has been used to detect enteroviral RNA for a direct and rapid diagnosis of clinically important enteroviral infections [1–3]. Enteroviral RNA has been found in myocardial tissue from patients with myocarditis and cardiomyopathy [4] and in cerebrospinal fluid (CSF) of patients with aseptic meningitis [5, 6]; enteroviruses are believed to be the most common viral pathogenic agents for these diseases. Enteroviral infections, such as herpangina, hand-foot-and-mouth disease, and aseptic meningitis, have not been associated commonly with neurologic abnormalities [7]. The association of transient neurologic complications with central nervous system (CNS) infection due to enteroviruses has not been studied.

CSF samples were collected from children who were suspected of having a CNS infection because of neurologic symptoms: 23 patients were subsequently diagnosed as having febrile seizures, status epilepticus, or transient altered states of consciousness associated with acute febrile illness. CSF samples from these patients were examined for infectious virus by cell culture and for enteroviral RNA by PCR.

Note Added in Proof
We have cloned the 46-kDa protein recognized by MAb RmcB. Prototype and clinical isolates of CB3, CB4, and CB5—including some that bind to DAF and some that were not inhibited by RmcB in plaque assays—bind to and infect nonpermissive hamster cells transfected with cDNA encoding the 46-kDa receptor.
Materials and Methods

Samples. From January 1992 to December 1995, CSF samples were collected from children in Japan who developed neurologic complications (i.e., seizures or altered states of consciousness) during the febrile phase of a suspected CNS infection. Twenty-three of the children had transient symptoms and were diagnosed, on the basis of clinical and laboratory findings, as having an illness other than encephalitis or meningitis. Of the 23 patients, 18 were diagnosed with seizures, which fulfilled five diagnostic criteria for simple febrile seizures: occurrence in children ages 6 months to 5 years, occurrence with fever >38°C, duration of <15 min, occurrence during absence of CNS infection as determined by standard techniques, and occurrence with absence of neurologic abnormalities in the interictal period. Of the 5 remaining patients, 3 were diagnosed with status epilepticus, which is defined as a seizure or series of seizures occurring over >30 min. The remaining 2 patients experienced drowsiness, but the causative metabolic disorder(s) was not determined by laboratory examinations; 1 of these patients also had herpangina.

The 23 children (6 girls, 17 boys) ranged in age from 10 months to 5.2 years (mean, 2.7 years). Enteroviral infection was not suspected as the cause of febrile illness, except in the patient who developed herpangina. CSF samples from the children were examined for infectious virus by cell culture and for entroviral RNA by PCR. As positive controls for PCR, we used 6 CSF samples from persons with entroviral meningitis confirmed by virus isolation. As negative controls for PCR, we used 20 CSF samples from patients with leukemia but with no neurologic symptoms. Samples were stored at -80°C until tested for virus.

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 (HSV), and orthomyxoviruses. Confluent cell cultures were seeded in microplate wells and inoculated with 100 μL of maintenance medium and 50 μL of CSF sample. The cell cultures were then incubated at 34°C in 5% CO₂ and observed daily, except Sunday, for 7 days for cytopathic effect. Blind passage was done once if cytopathic effect was not observed by the end of the period. Virus isolates were identified by neutralization test [8].

PCR. We used a modification of the PCR method described by Zoll et al. [1]. In brief, RNA was extracted from 250 μL of whole CSF by use of an RNA extraction kit (Nippon Gene, Tokyo). After RNA extraction, cDNA was synthesized (42°C, 30 min) from the resuspended RNA by use of 2.5 U of Moloney murine leukemia virus reverse transcriptase (Toyobo, Osaka, Japan) and reaction mixture containing 50 mM KCl, 10 mM TRIS-HCl (pH 8.3), 5 mM MgCl₂, 0.2 mM each dNTP, 1 μmol of primer Fl (5’-CAA-GCACCCTGTTTCCCCGG-3’) and primer R1 (5’-ATTGTGC- ACCATAAGCAGCCA-3’), and 20 U of RNase inhibitor (Toyobo).

The cDNA product was amplified in 50 μL of reaction mixture containing 50 mM KCl, 10 mM TRIS-HCl (pH 8.3), 5 mM MgCl₂, 0.2 mM each dNTP, 0.2 μmol of primers Fl and R1, and 1.25 U of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). Thirty cycles were performed in a thermal cycler (Perkin Elmer) as follows: denaturing for 1 min at 93°C, annealing for 1 min at 45°C, and extension for 2 min at 72°C. The second PCR was done using the second primer pair—F2 (5’-TCTTCGCGCCTGAATGCG-3’) and R1—and 2 μL of the first PCR product in 30 cycles consisting of denaturing for 1 min at 93°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C. The nested PCR product was run on 2% agarose gel containing ethidium bromide and photographed under UV light. The positive PCR reaction was expected to give a 155-bp band. The primer pairs could detect 60 of 66 enterovirus serotypes, including polioviruses, coxsackieviruses A and B, echoviruses, and enteroviruses [1]. The sensitivity of the nested PCR, calculated from extractions of serial dilutions of titrated enterovirus type 7, corresponded to 10⁻³ TCID₅₀. To eliminate and detect laboratory contamination leading to false-positive PCR results, negative controls were included for each step of the assay.

Results

No CSF samples from the 23 patients with febrile illness accompanied by neurologic symptoms showed pleocytosis (>10 cells/μL). The patients’ neurologic symptoms appeared transiently, and no sequela was observed. All patients were diagnosed as having an illness other than encephalitis or meningitis (table 1). The enteroviruses echovirus type 9 and coxsackievirus B3 were isolated from 2 (8.7%) of the 23 CSF cell
Table 2. Virus isolation and PCR results for CSF samples from children with acute febrile illness and from controls.

<table>
<thead>
<tr>
<th>Subjects with neurologic complications</th>
<th>No. of samples</th>
<th>Culture</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Febrile seizures</td>
<td>18</td>
<td>2 (11)</td>
<td>5 (28)</td>
</tr>
<tr>
<td>Status epilepticus</td>
<td>3</td>
<td>0 (0)</td>
<td>2 (66)</td>
</tr>
<tr>
<td>Altered states of consciousness</td>
<td>2</td>
<td>0 (0)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Positive controls with enteroviral meningitis</td>
<td>6</td>
<td>6 (100)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>Negative controls with leukemia but no neurologic symptoms</td>
<td>20</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Discussion

PCR is proving to be a significant improvement over cell culture for diagnosis of encephalitis due to HSV and enteroviruses [7]. The PCR method we used could detect almost all prevalent enteroviruses in Japan [1], and its sensitivity was 1000 times higher than that of the virus isolation method. High sensitivity may indicate a risk of false-positive results, but all 20 control CSF samples from leukemia patients had negative PCR results; therefore, our PCR method did not appear to give false-positive results. Enteroviral RNA was detected from 39% of 23 patients who developed neurologic symptoms. Enteroviral CNS infection was confirmed by virus isolation in 2 patients with febrile seizures.

Neurologic symptoms, such as febrile seizures and bulging of the anterior fontanel, were observed in patients during the febrile phase of exanthema subitum; human herpesvirus 6 DNA was frequently detected by PCR from the CSF of those patients [9]. In contrast, enteroviral infections, such as herpangina and hand-foot-and-mouth disease, have been associated rarely with neurologic abnormalities. Our results, however, suggest that enteroviral infection of the CNS causes neurologic abnormalities without accompanying symptoms that would suggest enteroviral infections. This may be explained by the difference of the enterovirus serotypes. The mean age of patients with neurologic symptoms caused by enteroviral infection (2.9 years) is slightly higher than that of children with neurologic symptoms caused by human herpesvirus 6 infection (7.6 months) [9].

Epidemic incidence, virus surveillance, and recent molecular techniques show that enteroviruses are the most frequent causative agent of aseptic meningitis [5, 10, 11]. Although encephalitis is considered an uncommon manifestation of enteroviral infections, enteroviral encephalitis does occur epidemically and sporadically [11]. HSV, the commonest cause of severe encephalitis, is seldom isolated from CSF of patients with HSV encephalitis confirmed by brain biopsy [12], but HSV DNA is detected in CSF by PCR [13]. If these observations can be extended to other cases of viral encephalitis, our results may show that enterovirus-related mild encephalitis causes transient neurologic symptoms, such as seizures and altered states of consciousness.

In this and similar studies, it is difficult to obtain CSF samples from appropriate controls (i.e., age-matched febrile children without neurologic symptoms). However, to confirm the association between enteroviral infection of the CNS and neurologic symptoms, studies may need to investigate whether febrile children with enteroviral infection without neurologic symptoms have detectable enteroviral RNA in their CSF.

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References

Presence of Human Herpesvirus 8 Variants in the Oral Tissues of Human Immunodeficiency Virus—Infected Persons


A 210-bp DNA segment specific to the human herpesvirus 8 (HHV-8) genome was amplified by nested polymerase chain reaction from 10 of 14 archived oral biopsy samples of HIV-positive patients in London who had no evidence of oral Kaposi's sarcoma (KS). Various oral sites were represented. Oral tissues from 20 general dental patients not known to be HIV-infected were negative. When DNA sequences of these products were compared with sequences derived from 5 oral KS tissues of AIDS patients in London and 10 skin biopsies of Italian patients with Mediterranean KS (total number of positive tissues = 25), 11 were found to be unique. DNA and predicted peptide motifs of these sequences were also different from those in 28 of 36 HHV-8-positive lesions previously reported from American and African patients. HHV-8 is tropic for the oral mucosa of HIV-infected persons, and HHV-8 variants, though diverse, may be geographically restricted.

Unique DNA sequences homologous to a capsid protein gene of \( \gamma \)-herpesviruses have been identified in cutaneous Kaposi's sarcoma (KS) lesions [1–3], blood of human immunodeficiency virus (HIV)–infected patients with KS [4], effusions of patients with AIDS-related body cavity–based lymphoma [5], semen [6], prostatic and urogenital tissue [6], and lymph nodes [7]. The putative virus from which these sequences derive has been assigned to the \textit{Rhabdovirus} genus [8]. The virus has been described as KS-associated herpesvirus or, more formally, human herpesvirus 8 (HHV-8). Here, we investigate the presence of HHV-8 DNA sequences in oral tissues of HIV-infected people in London and compare these with sequences identified in cutaneous KS lesions of Italian patients and those reported from HHV-8–infected tissues of patients in the United States and Africa.

Materials and Methods

\textit{Specimens.} We examined formalin-fixed, paraffin-embedded tissues of 25 incisional oral biopsy samples taken from 23 patients with HIV infection who attended the oral medicine clinic of a genitourinary medicine department in central London. Of these, 11 had histologic features consistent with KS, 10 nonspecific ulceration, and 4 other diseases. Various sites of the oral mucosa were represented. Also studied were paraffin blocks of 16 skin biopsy samples from Italian patients with classic (Mediterranean) KS and 20 diagnostic biopsy samples from patients with various oral diseases attending general dental clinics (including 9 with oral squamous cell carcinoma). The HIV antibody status of the patients in the latter 2 groups was unknown.