IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Tonsillar Crypt Epithelium Is an Important Extra-Central Nervous System Site for Viral Replication in EV71 Encephalomyelitis

Yaoxin He,* Kien Chai Ong,1 Zifen Gao,* Xishun Zhao,* Virginia M. Anderson,‡ Michael A. McNutt,‡ Kum Thong Wong,§ and Min Lu*

From the Department of Pathology,* School of Basic Medical Sciences, Peking University, Beijing, China; the Departments of Biomedical Science1 and Pathology,§ Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; and the Department of Pathology,‡ State University of New York Health Science Center at Brooklyn, Brooklyn, New York

Accepted for publication November 18, 2013.
Address reprint requests to Min Lu, M.D., Department of Pathology, School of Basic Medical Sciences, Peking University, Beijing 100191, China; or Kum Thong Wong, M.D., Department of Pathology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. E-mail: lumin@boc.pku.edu.cn or wongkt@ummc.edu.my.

Enterovirus 71 (EV71; family Picornaviridae, species human Enterovirus A) usually causes hand, foot, and mouth disease, which may rarely be complicated by fatal encephalomyelitis. We investigated extra-central nervous system (extra-CNS) tissues capable of supporting EV71 infection and replication, and have correlated tissue infection with expression of putative viral entry receptors, scavenger receptor B2 (SCARB2), and P-selectin glycoprotein ligand-1 (PSGL-1). Formalin-fixed, paraffin-embedded CNS and extra-CNS tissues from seven autopsy cases were examined by IHC and in situ hybridization to evaluate viral antigens and RNA. Viral receptors were identified with IHC. In all seven cases, the CNS showed stereotypical distribution of inflammation and neuronal localization of viral antigens and RNA, confirming the clinical diagnosis of EV71 encephalomyelitis. In six cases in which tonsillar tissues were available, viral antigens and/or RNA were localized to squamous epithelium lining the tonsillar crypts. Tissues from the gastrointestinal tract, pancreas, mesenteric nodes, spleen, and skin were all negative for viral antigens/RNA. Our novel findings strongly suggest that tonsillar crypt squamous epithelium supports active viral replication and represents an important source of viral shedding that facilitates person-to-person transmission by both the fecal-oral or oral-oral routes. A correlation between viral infection and SCARB2 expression appears to be more significant than for PSGL-1 expression. (Am J Pathol 2014, 184: 714–720; http://dx.doi.org/10.1016/j.ajpath.2013.11.009)

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EV71 Infection of the Palatine Tonsil

decreased, there were still >500 deaths, according to the Ministry of Health of China. With a significant worldwide decrease and possible eradication of poliovirus infection, it has been speculated that EV71 may emerge as the most important enterovirus associated with neurological disease.5

The pathogenesis of severe human EV71 infection is only beginning to be understood. Most fatalities are related to neurological complications, and among these the most severe are encephalomyelitis and acute flaccid paralysis.1,2,6,7 Virus in the central nervous system (CNS) has been found to be primarily neuronotropic, and neuronal viral cytolysis may produce tissue damage and inflammation.7,8 Moreover, involvement of the medulla is believed to result in destruction of cardiorespiratory control centers and sudden collapse of the patient.7,8 The virus may enter the CNS via a retrograde peripheral motor nerve route or by a hematogenous route.5 There is evidence to suggest that peripheral sensory and autonomic nerves and the olfactory tract probably do not play significant roles in viral entry into the CNS. However, a retrograde peripheral nerve transmission route for EV71 has been demonstrated in murine models.9,10

EV71 entry into the body remains poorly understood. Based on the poliovirus infection model, it is assumed that, after viral entry and replication in the human body, viremia occurs, which may lead to neuroinvasion.11 Similar to poliovirus, EV71 may enter the body via the orodigestive tract, and the orodigestive tract itself may also be active viral replication sites. Both poliovirus and EV71 has been cultured from postmortem specimens of tonsil and intestine.6,7,12–14 However, viruses have not been directly localized in the gastrointestinal tract, pancreas, liver, or other extra-CNS tissues, such as the lung, heart, spleen, thymus, and lymph nodes, although these studies have been limited.7,8,14

The palatine tonsil has not been previously studied. Consistent with the poliovirus model, viremia has been demonstrated in 20% of patients with EV71 encephalomyelitis.15 In the mouse model, viremia was associated with CNS infection,10 whereas in the monkey model, viremia after i.v. infection clearly preceded neuroinvasion.16 Virus is presumably shed or secreted into the human orodigestive tract because person-to-person transmission is known to occur mainly by fecal-oral and oral-oral routes, and stool culture and throat swab results are frequently positive.17

Herein, we report a postmortem study of seven patients who died of EV71 infection 2 to 3 days after the onset of symptoms. To gain further insight into EV71 tissue tropism and replication sites, especially regarding the orodigestive tract, IHC, and in situ hybridization (ISH) were used to investigate viral localization in the CNS and in extra-CNS organs. We have previously shown that a combination of pathological changes identified with light microscopy showing characteristic topographic distribution of inflammation in infected brain tissues, together with demonstration of viral antigens and RNA by IHC and ISH, is sufficient evidence for a specific diagnosis of EV71 encephalomyelitis.8,18 In this study, we also correlated viral infection with two putative EV71 receptors, scavenger receptor B2 (SCARB2) and P-selectin glycoprotein ligand-1 (PSGL-1).19,20 SCARB2, alias lysosomal integral membrane protein II, is expressed ubiquitously in human tissues and, thus, may play a part in systemic EV71 infections. PSGL-1 is a sialomucin leukocyte membrane protein that is mainly expressed on hematopoietic cells.19,20 Viral receptors have essential roles in early viral infection because they could actively promote virus entry into host cells, and may, therefore, help explain some of the pathological findings and tissue tropism found in EV71 infection. No similar investigations have been previously published.

Materials and Methods

Autopsy Cases and Tissues

Materials for this study were collected from seven autopsy cases performed between May 2008 and June 2010 in the Beijing Autopsy Center (Beijing, China). One case was from Fuyang, China, where a large outbreak of EV71-associated HFMD occurred in 2008. This case was confirmed to be EV71 infection with RT-PCR using a throat swab sample and with viral isolation in the Chinese Center for Disease Control and Prevention (Beijing, China; written communication, 2008). The other six cases were autopsies requested by parents who wanted to know the cause of death because there was no antemortem diagnosis. EV71 encephalomyelitis was confirmed in all these cases using a combination of light microscopic, IHC, and ISH findings in the CNS, which demonstrated specific diagnostic features. On review, the clinical findings were also consistent with the disease.8,18 The clinical data from all seven cases are presented in Table 1.21 Consent for this research study and the publication of our findings was obtained from the next of kin for all patients, and this project was approved by the Peking University Ethical Committee (Beijing, China).

Tissue samples from the brain, spinal cord, heart, lung, kidney, liver, esophagus, small and large intestines, mesenteric lymph nodes, spleen, neck skeletal muscles, and peripheral nerves were used for study in all seven cases, and palatine tonsils were available in six cases. Skin tissue from a case (case 7) with maculopapular rash (Table 1)21 was also available for study. Tissues were fixed in 10% neutral-buffered formalin, routinely processed and embedded in paraffin. Serial tissue sections (3 μm thick) were prepared for H&E, IHC, ISH, and double-immunofluorescence (IF) stainings. H&E-stained slides were examined for evidence of viral encephalitis and other pathological changes, and the topographic distribution of inflammation in various parts of the CNS was recorded. The total number of CNS and non-CNS blocks examined is shown in Supplemental Table S1.

IHC Data

The IHC evaluation for viral antigens was performed using two primary antibodies to EV71 [polyclonal rabbit anti-human EV71, dilution 1:3000 (obtained from Dr. Hiroyuki

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Shimizu, National Institute of Infectious Diseases, Tokyo, Japan); and a monoclonal mouse anti-human EV71, dilution 1:1000 (Chemicon International, Temecula, CA). For evaluation of viral receptors in EV71-positive tissues, monoclonal mouse anti-human PSGL-1 (dilution 1:1000; Chemicon) and monoclonal mouse anti-human SCARB2 (dilution 1:5000; Sigma, London, UK) were used. Inflammatory cells were characterized by polyclonal rabbit anti-human CD3 (dilution 1:100; Sigma, London, UK) and monoclonal mouse anti-human CD68 (dilution 1:200; Dako), monoclonal mouse anti-human CD20 (dilution 1:400; Dako), human CD3 (dilution 1:100; Dako, Glostrup, Denmark), and monoclonal mouse anti-human CD68 (dilution 1:200; Dako). The IHC procedure has been previously described. Briefly, deparaffinized tissue sections were incubated with primary antibody for 2 hours at room temperature after 3% hydrogen peroxide treatment and heat-induced antigen retrieval. Secondary antibodies conjugated with horseradish peroxidase (rabbit/mouse; Dako) were incubated for 40 minutes at room temperature, followed by incubation with the substrate 3,3′-diaminobenzidine (DAB; Dako) before counterstaining with Mayer’s hematoxylin.

ISH Data

Probe preparation and the ISH procedure were as previously described. Briefly, digoxigenin-labeled DNA probes were generated from a 500-bp PCR product derived from the 5′-nontranslated region of the EV71 genome. Deparaffinized tissue sections were pretreated with HCl and proteinase K (100 μg/mL, 20 minutes, 37°C). Approximately 1 ng of probe in a standard hybridization solution was layered onto tissue sections and heated to 100°C for 12 minutes, followed by incubation for 16 to 20 hours at 42°C in a moist chamber. The hybridization signal was identified using an anti-digoxigenin antibody (Roche, Mannheim, Germany) conjugated to alkaline phosphatase, followed by reaction with nitroblue tetrazolium/5-bromo-4-chloro-3-idoly phosphoric acid substrate (Roche). Slides were counterstained with Mayer’s hematoxylin before mounting.

Table 1

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (months)/Sex</th>
<th>Disease duration (days)</th>
<th>Clinical presentation</th>
<th>Organ weights (g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24/M</td>
<td>3</td>
<td>Fever, noisy breathing, cyanosis, vomiting, lethargy, rashes</td>
<td>Brain 1250; Left lung 130; Right lung 190</td>
</tr>
<tr>
<td>2</td>
<td>36/F</td>
<td>2</td>
<td>Dyspnea, coarse breathing, cyanosis, bloody oral secretions, diarrhea, hepatomegaly, lethargy</td>
<td>Brain 820; Left lung 113; Right lung 155</td>
</tr>
<tr>
<td>3</td>
<td>11/M</td>
<td>2</td>
<td>Fever, tachypnea, coarse breathing, lung crepitation, vomiting, diarrhea, hepatomegaly, lethargy</td>
<td>Brain 1160; Left lung 96; Right lung 134</td>
</tr>
<tr>
<td>4</td>
<td>48/M</td>
<td>3</td>
<td>Fever, bloody oral secretions, lethargy</td>
<td>Brain 1500; Left lung 180; Right lung 220</td>
</tr>
<tr>
<td>5</td>
<td>41/M</td>
<td>2</td>
<td>Fever, lung crepitation, vomiting, lethargy, rashes on hands</td>
<td>Brain 1400; Left lung 220; Right lung 280</td>
</tr>
<tr>
<td>6</td>
<td>13/M</td>
<td>2</td>
<td>Fever, tachypnea, lung crepitation, vomiting, lethargy</td>
<td>Brain 1157; Left lung 170; Right lung 173</td>
</tr>
<tr>
<td>7</td>
<td>36/F</td>
<td>2</td>
<td>Fever, cough, dyspnea, vomiting, lethargy, convulsions</td>
<td>Brain 1243; Left lung 167; Right lung 125</td>
</tr>
</tbody>
</table>

*Range of normal brain and lung weights (modified from Siebert) in children for selected ages: 6-month-old: brain, 660 g; right lung, 42 g; left lung, 39 g; 11- to 14-month-old: brain, 852 to 994 g; right lung, 59 to 66 g; left lung, 53 to 60 g; 24- to 48-month-old: brain, 1064 to 1191 g; right lung, 88 to 90 g; left lung, 76 to 85 g.

F, female; M, male.

Double IF Data

A double IF assay was performed on the tonsil tissue sections to confirm virus localization in epithelial cells. The antibody-to-epithelial membrane antigen (EMA) was chosen as a suitable marker for tonsillar squamous epithelium, after testing several commercially available antibodies. Briefly, the steps were as follows: deparaffinized tissue sections were incubated with a mixture of two primary antibodies [mouse anti-human EMA, dilution 1:1000 (Dako); and rabbit anti-EV71, dilution 1:2000] for 2 hours at room temperature; and tissue sections were incubated with a mixture of two secondary antibodies (goat anti-mouse IgG conjugated with Alexa Flour 488, dilution 1:1000, and goat anti-rabbit IgG conjugated with Alexa Flour 594, dilution 1:1000; both from Molecular Probes, Eugene, OR) for 1.5 hours at 37°C in the dark. The resulting positive signal for EV71 antigens was red; and for EMA, it was green. Slides were then washed in Tris-buffered saline and counterstained by incubation for 1 minute with nuclear stain (DAPI; Molecular Probes). Sections were viewed with a Nikon D-FL Epi-fluorescence microscope (Nikon Corporation, Tokyo, Japan), and then they were imaged and analyzed using the Image-Pro Plus, version 5.1.2 (Media Cybernetics, Inc., Rockville, MD).

Controls

For EV71 IHC and ISH, mouse and human tissues infected with EV71 virus were used as positive controls. The positive controls for virus receptor studies were cell lines expressing human PSGL-1 and SCARB2. Tissues from three age-matched children who died of disease unrelated to EV71 were used as negative controls. In addition, to demonstrate that there is no nonspecific EV71 staining of necrotic and/or apoptotic cells, tissues from a case of tuberculous lymphadenopathy with caseating granulomatous inflammation, a case of non-Hodgkin lymphoma, and a case of colorectal adenocarcinoma were used as additional
negative controls. Negative control assays for IHC and double IF included omission of the primary antibody and replacement with normal mouse serum, whereas negative control assays for ISH included omission of the probe and replacement with an unrelated probe.

Results

Pathological Findings and EV71 Distribution

CNS

Grossly, all seven brains showed edema, flattened gyri, and narrowed sulci associated with unilateral cerebellar tonsil herniation, but no cingulate or uncal herniation was found. All of the brains weighed more than the expected weights for age (Table 1). The entire length of the spinal cord from case 7 (Table 1) also showed edema.

Microscopically, inflammation was present predominantly in the spinal cord, medulla, pontine tegmentum, midbrain, hypothalamus, and subthalamic and dentate nuclei, representing characteristic topographic distribution of inflammation in EV71 encephalomyelitis. The involved neurons showed degeneration (Figure 1A) and necrosis with neurophagia. Perivascular cuffing by inflammatory cells was present (Figure 1D), consisting of lymphocytes (CD20⁺ and CD3⁺) and macrophages/microglia (CD68⁺). Parenchymal inflammatory cell infiltrates consisted primarily of macrophages/microglia and neutrophils in various combinations. Viral inclusions were absent.

Viral antigens (Figure 1B) and RNA (Figure 1C) were found in varying amounts in scattered neuronal cell bodies and processes. Overall, positive neurons were relatively few and scattered, even in the most intensely inflamed CNS areas, consistent with previous reports. Dorsal and ventral nerve roots, vascular endothelium, and circulating blood cells were all negative for EV71 (Figure 1E and F). The characteristic distribution of inflammation, together with IHC and ISH findings in the CNS, confirmed the diagnosis of EV71 encephalomyelitis in all seven cases. Positive and negative controls for IHC and ISH showed results as expected in both CNS and other tissues. There was no nonspecific staining in necrotic or apoptotic cells.

Orodigestive Tract

The most significant finding in the orodigestive tract was in the palatine tonsil, which showed viral localization in the tonsillar crypts. EV71 was identified in the squamous epithelium lining tonsillar crypts and in some desquamated cells within the crypts by IHC and ISH (Figure 2, B–G) in all tissue blocks from the six cases studied. The positive signals were focal but strong. Typically, in a tonsil section of approximately 1 cm² there is at least one focus of positivity, as illustrated in Figure 2, B–G. The double IF assays confirmed colocalization of EV71 antigens with EMA-positive squamous cells (Figure 2, H–J). There was no evidence of viral antigen or RNA in lymphoid cells or in squamous epithelium covering the external surface of the tonsil.

The mesenteric lymph nodes were enlarged, and prominent Peyer’s patches in the ileal mucosa showed reactive lymphoid hyperplasia. Reactive lymphoid hyperplasia in the tonsils was also noted. The esophagus, stomach, large intestine, pancreas, and liver were normal macroscopically and microscopically. IHC and ISH for virus were negative in all these tissues.

Respiratory Tract

Macroscopically in all seven cases, the lungs were congested and focally hemorrhagic, but not consolidated. All of the lungs were heavier than the expected weights for age (Table 1). Microscopic examination confirmed pulmonary congestion, edema, and focal hemorrhage, and mild secondary bacterial infection with neutrophils and lymphocytes was observed in two cases. Neither viral antigens nor RNA was found in epithelial cells of the alveoli and tracheobronchial tree, endothelium, or alveolar macrophages. There was no evidence of severe acute bronchopneumonia, interstitial pneumonitis, fibrosis, thromboembolization, infarction, or diffuse alveolar damage.

Figure 1 Immediately adjacent sections of infected spinal cord neurons (arrows; A) stained for viral antigens (arrows; B) and viral RNA (arrows; C), as shown by IHC and in situ hybridization (ISH), respectively. Immediately adjacent sections of medulla visualized for perivascular cuffing (D) of inflammatory cells, but viral antigens (E) and viral RNA (F) could not be detected in these cells or blood vessel wall. A and D: H&E stains. B and E: IHC with DAB chromogen and hematoxylin counterstains. C and F: ISH with NBT/BCIP substrate and Mayer’s hematoxylin counterstains. Original magnification, ×40 (A–F).
Other Organs and Tissues

The heart, kidney, and spleen were grossly normal and had normal weights. Congestion was identified, but no other remarkable changes were observed microscopically. IHC and ISH were negative for EV71 in these organs, as well as in skeletal muscles, peripheral nerves, and adipose tissues from around the trachea and neck. The skin sample showing maculopapular rash from the left foot showed only mild lymphocytic infiltration around small dermal vessels, with no other abnormalities, and was also IHC and ISH negative.

SCARB2 and PSGL-1 Expression in EV71-Positive Tissues

PSGL-1 was identified in some lymphoid cells and vascular endothelium but not in squamous epithelium (Figure 3A). In contrast, in the tonsils, diffuse SCARB2 staining was only found in the cytoplasm of crypt squamous epithelial cells, but not in the surface squamous epithelium covering the tonsil, lymphoid, or other cells (Figure 3B). In the CNS, PSGL-1 IHC was negative in neurons but positive in macrophages/microglia, neutrophils, vascular endothelium, and perivascular lymphocytes (Figure 3C). SCARB2 was identified in the cytoplasm of neuronal cell bodies and processes (Figure 3D). Some macrophages/microglia and neutrophils were also SCARB2 positive, but vascular endothelium and perivascular lymphocytes were uniformly negative.

Discussion

This new study of seven fatal cases of EV71 infection with confirmed diagnoses of encephalomyelitis demonstrated that the palatine tonsil is an important site of viral infection and replication, as evidenced by the detection of viral antigens and RNA in tonsillar crypt squamous epithelium by IHC and ISH staining, respectively, in six patients. We believe this novel finding will have significant impact on our understanding of EV71 transmission and pathogenesis in recognizing that the tonsil plays a pivotal role in viral shedding and, possibly, in viral entry into the body.

Because transmission of EV71 occurs by fecal-oral and oral-oral routes, and in view of the importance of the tonsil as a site-harboring virus, we suggest that once virus gets into the oral cavity, it may first infect and undergo primary replication and propagation in the tonsillar crypt squamous epithelium. Subsequently, there is direct shedding from the tonsil into the lumen of the orodigestive tract, potentiating viral transmission via oral secretions and feces. Throat...
swabs have been reported to have a higher viral diagnostic yield than rectal swabs, consistent with the relative importance of the upper orodigestive tract, including the tonsil, as a primary viral replication site. Apart from the tonsil, the oropharyngeal mucosa itself may be an alternative site for viral infection and replication because oropharyngeal vesicles are part of HFMD and herpangina. However, thus far, there is no direct evidence for this. Neither is there any evidence that the lower orodigestive tract, including gastrointestinal tract epithelium and pancreas, support viral replication or facilitate virus shedding based on our findings. Nonetheless, it is still possible that low concentrations of viruses in these tissues may not be detectable by IHC or ISH. Further studies are needed to confirm these findings.

Although infection of the palatine tonsils by other picornaviruses, such as human rhinovirus, porcine teschovirus, foot and mouth disease virus, and poliovirus, in various animal models and in natural human infections has been previously reported, EV71 infection of the tonsil has not been reported. In children with tonsillar rhinovirus infection, viral RNA has been shown to localize to epithelial and lymphoid cells. In porcine teschovirus-infected pigs, viral antigens have been demonstrated in epithelial cells of the tonsil, whereas in foot and mouth disease virus, viral RNA was demonstrated within lymphoid follicles of infected bovine tonsils. In poliovirus-infected chimpanzees, virus can be isolated from tonsils, but it is not known whether tonsillar squamous epithelium supports viral replication because there has been no attempt to directly visualize virus in this tissue. A few nonpicornaviruses, such as HIV, human papillomavirus, Epstein-Barr virus, and WU polyomavirus, have been detected, isolated, or shown to replicate in tonsillar tissue. The tonsil has been reported as a viral entry site in foot and mouth disease virus and HIV. We speculate that the tonsil and oropharyngeal mucosa are sites for EV71 entry into the systemic circulation, leading to viremia.

Although, in this study, we did not localize virus in one available skin sample, it has previously been shown that virus can be cultured from swabs of skin vesicles. The absence of viral antigens in the single skin specimen may be related to the late stage of infection. It is possible that skin could be a primary viral replication site after infection via direct contact with shed virus, or the skin may become secondarily infected after viremia and serve as a secondary replication site. Our results suggest that leukocytes and lymphoid cells in the tonsil, spleen, ileal Peyer’s patches, mesenteric lymph nodes, and other tissues may not support viral replication. However, further studies are needed to confirm this.

EV71 could invade the CNS via peripheral motor nerves, but how virus enters the nerve and results in retrograde spread into the CNS is unknown. Circulating virus may gain access via the neuromuscular junction through adjacent skeletal muscle infection, or directly into the junction or even the peripheral motor nerve itself. However, our current study and published data showed no evidence of viral localization in neck skeletal muscle or peripheral nerves.

SCARB2 expression was more consistently observed in neurons and tonsillar crypt squamous epithelium, which are tissues found to support viral replication in this study. Specifically, SCARB2 localized to cytoplasm of cell bodies and processes of neurons. However, SCARB2 was also expressed in cells (macrophages and neutrophils) that were not found to be infected by EV71, suggesting that perhaps other unknown factors may be involved in cellular permissivity to EV71 infection. Conversely, absence of SCARB2 expression in the tonsillar surface squamous epithelium, vascular endothelium, and lymphocytes correlates well with the absence of EV71 infection. Further studies are needed to further characterize the distribution of SCARB2 expression and its relationship to EV71 infection.

On the other hand, PSGL-1 was found mainly in leukocytes in various locations, and these cells did not show any apparent evidence of viral infection. Recently, it has been demonstrated that the PSGL-1 receptor lacks the ability to initiate EV71 uncoating, and this may be the reason why transgenic mice expressing human PSGL-1 receptors did not appear to be susceptible to infection. In contrast, transgenic mice expressing human SCARB2 receptors were highly susceptible to viruses derived directly from clinical isolates, showing skin and CNS involvement, features that are comparable to human infection. These results are consistent with the notion that SCARB2 may play a more important role than PSGL-1 in human infection, but further investigations are warranted.

In conclusion, apart from the CNS, we believe the tonsillar crypt epithelium is an important viral replication site, likely to play a significant part in viral shedding and transmission. Further understanding of the possible role of the tonsil in viral entry and immune response could have a significant impact on our understanding of EV71 pathogenesis. Possible involvement of skin and lack of involvement of lymphoid tissues as replication sites suggest that EV71 infection is significantly different from the poliovirus infection model.

Acknowledgments

We thank Dr. Hiroyuki Shimizu (National Institute of Infectious Diseases, Tokyo, Japan) for providing EV71 primary antibody and PSGL-1 cells for IHC, Dr. Satoshi Koike (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan) for providing SCARB2 cells for IHC, and Dr. Yanfeng Zhong, Dr. Jianying Liu, Dr. Hua Wang, Dr. Yang Yu, Dr. Encong Gong, and Dr. Jie Zheng for performing some of the autopsies included in this study.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.11.009.
References


