A Potent Virus-Specific Antibody-Secreting Cell Response to Acute Enterovirus 71 Infection in Children

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Background. Enterovirus 71 (EV71) remains a leading pathogen for acute infectious diseases in children, especially in Asia. The cellular basis for establishing a virus-specific antibody response to acute EV71 infections is unclear in children.

Methods. We studied the magnitude of virus-specific antibody-secreting B cells (ASCs) and its relationship with serological response, clinical parameters, and virological parameters among children with laboratory-confirmed EV71 infection.

Results. A potent EV71 genogroup B- and virus-specific ASC response was detected in the first week of illness among genotype B5 EV71-infected children. The cross-reactive EV71-specific ASC response to genogroup C viral antigens composed about 10% of the response. The EV71-specific ASC response in children aged ≥3 years produced immunoglobulin G predominantly, but immunoglobulin M was predominant in younger children. Proliferation marker was expressed by the majority of circulating ASCs in the acute phase of EV71 infection. Virus-specific ASC responses significantly correlated with throat viral load, fever duration, and serological genogroup-specific neutralization titer.

Conclusions. The presence of a virus-specific ASC response serves an early cellular marker of an EV71-specific antibody response. Further detailed study of EV71-specific ASCs at the monoclonal level is crucial to delineate the specificity and function of antibody immunity in children.

Keywords. enterovirus 71; antibody-secreting B cells; children.

Enterovirus 71 (EV71) is generally divided into 3 distinct genogroups (A, B, and C), with genogroups B and C further divided into genotypes B1–5 and C1–5, respectively. Genotype C4 EV71 has recently been proposed as a novel genogroup D virus [1–3]. Several large-scale outbreaks of EV71 infection have caused significant mortality cases in the Asia-Pacific region [4–7]. In Taiwan, the shift to genotype C4 as the dominant circulating strain during 2010–2011 and the re-emergence of genotype B5 from 2011 to 2012 resulted in a prolonged period of EV71 endemicity and 203 cases of severe illness [3].

Symptomatic EV71 illnesses mainly present as herpangina and hand-foot-and-mouth disease, but severe illnesses with central nervous system involvement and subsequent cardiopulmonary collapse and even death could occur [8]. Most severe cases occurred in toddlers and young children [4]. Interestingly, symptomatic infections were estimated in <30% of seropositive children during the endemic [9]. The protective correlates of immunity against EV71 illnesses in children are largely unknown. Innate immunity serves as an immediate immune response against viral pathogens. The administration of anti–interferon-α/β (IFN-α/β) neutralizing antibody would exacerbate EV71 diseases and result in higher tissue viral loads, while restoration of type I IFN responses could improve the survival rate against lethal challenge in mice [10, 11]. Nevertheless, the level of proinflammatory cytokine interleukin 6 in EV71-infected patients with pulmonary edema was
found to be significantly higher than the level in those without pulmonary edema, implying that the innate immune response could be a 2-edged sword in the development of severe illnesses in humans [12]. Adaptive immune responses, including cellular and antibody-mediated responses, would be another arm of host defense system against viral pathogens and establish immunological memory against further infection. T-cell immunity has been studied in EV71-infected patients, and the relationship between cell-mediated proliferation response and clinical severity is controversial, although lower T-cell–related cytokine responses are generally noted in severe cases [13, 14].

Increasing evidences support the role of antibody-mediated protection against EV71 [15, 16]. Passive transfer of neutralizing monoclonal antibodies prevents from the development of severe illness in mice challenged with EV71 [17]. In human, enterovirus is the leading pathogen of encephalitis in patients with hypogammaglobulinemia [18–19]. Children with enteroviral infections clear virus more rapidly upon receipt of intravenous immunoglobulin containing a high titer of virus-specific neutralization antibodies [20]. Importantly, the seroepidemiology data demonstrate an inverse correlation between the preendemic age-specific seropositive rate and the incidence of severe morbidity and mortality during the endemic, which clearly indicates the importance of virus-specific neutralizing antibody against EV71 in human [9].

Antibody-secreting B cells (ASCs) constitute primary cellular components of T-cell–dependent antibody response to a variety of viral pathogens [21–23]. However, little is known about EV71-specific ASC responses during acute infection in children. In adults, circulating virus-specific ASCs were believed to derive from preexisting memory B cells that underwent recent activation, differentiation, and proliferation upon repeated viral exposure [24, 25]. During early childhood, the percentages of circulating memory B-cell subsets, including marginal zone and isotype-switched B cells, are much lower than those in adolescents and adults [26, 27]. Immature B-cell responses with poor functional antibody response to bacterial and viral pathogens were generally observed in infants [28]. We speculated the physiological age-related variation would affect the magnitude and isotype use of ASC responses to acute EV71 infection in young children. In the study, we prospectively enrolled pediatric patients with suspected enteroviral infections in Taiwan during 2012, a period of EV71 endemcity. The serological and ASC responses were analyzed among children with laboratory-confirmed EV71 infections and associated with clinical and virological parameters.

Acute enteroviral infection was defined as febrile illness and the presence of oral ulcers predominantly affecting the posterior oral cavity, with or without vesicular rashes on the hands, feet, knees, and buttocks. Clinical symptoms were recorded throughout the study. Single whole-blood samples were collected within the first week of illness, and additional specimens were obtained during the convalescent stage of acute EV71 infection. Throat and/or rectal swab specimens were collected either 1 day before, on the same day as, or 1 day after the first blood sample was collected.

The study was in compliance with good clinical practice guidelines and the Declaration of Helsinki. The protocol was approved by the Research and Ethics Committee of Chang Gung Memorial Hospital.

During the enrollment period, 37 patients agreed to participate in the study, but 3 did not provide blood samples. Meanwhile, 3 healthy children (mean age ± standard deviation [SD], 4.2 ± 3.3 years; range, 1.9–8.0 years) visiting the hospital for check-ups and vaccinations were enrolled as controls. Throat swab specimens were obtained from all 3 controls and from 33 of 34 patients who provided a blood sample; the exception, patient 14, declined to undergo throat swabbing. Twelve of 34 patients provided rectal swab specimens. All subjects provided written informed consent.

**MATERIALS AND METHODS**

**Patients and Samples**

Pediatric patients (age, 0–18 years) in whom enteroviral infection was diagnosed were randomly and prospectively enrolled in Chang Gung Memorial Hospital from July to December 2012.

**Viral Culture, Reverse Transcription Polymerase Chain Reaction (RT-PCR), and EV71-Specific Immunoglobulin M (IgM) Detection**

Throat and rectal swab specimens were submitted to isolate enterovirus, respiratory syncytial virus, herpes simplex virus (HSV), influenza virus, parainfluenza virus, and cytomegalovirus by viral culture, as previously described [29]. Specimens positive for enteroviruses were further examined by type-specific monoclonal antibodies against group A coxsackievirus, group B coxsackievirus, echovirus, poliovirus, and EV71 for serotype identification in the immunofluorescence assay.

Viral RNA isolation and purification were performed on throat and/or rectal swab specimens, using a QIAamp Viral RNA Extraction Kit (Qiagen, Germany). One-step RT-PCR was then performed to amplify the VP1 region of EV71, as previously described [29]. The Formosa One Sure EV71 IgM Rapid Test kit (Formosa Biomedical Technology, Taiwan) was used to detect the EV71-specific IgM response in whole blood samples [29].

**Ex Vivo B-Cell Enzyme-Linked Immunosorbet Spot Assay (ELISpot)**

Freshly separated peripheral blood mononuclear cells (PBMCs) were used to set up the ex vivo B-cell ELISpot assay, as previously described [23]. The coated antigens included purified inactivated EV71 virions of genogroup B (B4) and C (C4b), provided by Adimmune (Taiwan). Purified recombinant influenza virus hemagglutinin was also included as controls. Each antigen was
coated in duplicate. Spot-forming cells were measured and counted using the automatic ELISpot reader with software.

**Flow Cytometry**
Fresh PBMCs were stained with a mix of antibodies specific for surface markers to examine the frequency of B-cell subsets, as previously described [23]. Fluorescein isothiocyanate conjugated to anti-Ki–67 (clone B56; BD) was used for intracellular proliferation marker detection.

**Real-Time RT-PCR**
Viral load in throat swab specimens that were positive for EV71 was examined by real-time RT-PCR according to the method described by Monpoeho et al [30]. Briefly, EV71 RNA was extracted from throat swab medium and eluted in RNase-free water. Reverse transcription was performed with a first-strand complementary DNA (cDNA) synthesis kit (ReverTraAce-α, Toyobo, Japan). Real-time RT-PCR for EV71 was performed with the primers 5′-GATTGTCCATATAAGCAGC-3′ and 5′-CCCCTGATTCGGCTTACTC-3′ and the probe 5′-FAM-CGGAACC GACTACTTTGGGGTGTCCGT-TAMRA-Phosphor-3′. Positive controls of EV71 cDNA and negative controls from clinical samples and cell lines free of enterovirus were included.

**Neutralization Test**
Serological responses against enteroviruses were evaluated by the standard protocol of a plaque reduction neutralization test, as previously described [9]. All serum samples were examined in duplicate. Neutralization titers less than 1:8 were allocated a value of 1:4 (Supplementary Table 1).

**Statistical Analysis**
Graphs were created using GraphPad Prism software, and statistical analyses were done by GraphPad Prism and SPSS. A P value of <.05 was considered statistically significant.

**RESULTS**

**Identification of Pediatric Patients With Acute EV71 Infections**
Among 34 pediatric patients with the initial diagnosis of acute enteroviral infections enrolled in the study, 28 had laboratory-confirmed EV71 infections, 4 were infected with HSV-1, 1 was infected with group A10 coxsackievirus, and 1 was infected with group B3 coxsackievirus. All HSV-1–infected and coxsackievirus-infected patients had negative results of EV71-specific RT-PCR and IgM tests. Acute EV71 infections were confirmed by virus isolation, positive RT-PCR results, and/or positive IgM test results (Table 1). All EV71 strains isolated in the study belonged to genogroup B (genotype B5), as determined by VP1 sequence analysis (Supplementary Figure 1).

Among EV71-infected patients, 14 (50%) were male, and the mean age (±SD) was 4.0 ± 2.6 years (range, 0.6–14.1 years). All enrolled patients were previously healthy, were not receiving immunosuppressive medications, and had no immunocompromised conditions. During acute EV71 infections, 26 (93%) had fever, and 10 (36%) developed neurological manifestations (Table 1). One patient (patient 3) experienced cardiac failure and pulmonary hemorrhage and was rescued with extracorporeal membrane oxygenation. There was no fatality in the study. The majority of individuals with EV71 infection recovered completely; 1 subject developed neurological sequelae.

**Detection of Virus-Specific ASC Response Induced by Acute EV71 Infection**
We detected a robust induction of genogroup B EV71–specific ASCs within the first week of illness onset (Figure 1A). The sampling day occurred a mean duration (±SD) of 4.7 ± 1.9 days after onset. There was a mean (±SD) of 491 ± 569 immunoglobulin G (IgG)–producing genotype B EV71–specific ASCs per million PBMCs, 472 ± 566 IgM–producing genotype B EV71–specific ASCs per million PBMCs, and 187 ± 261 immunoglobulin A (IgA)–producing genotype B EV71–specific ASCs per million PBMCs among EV71-infected patients (Figure 1B). Genogroup B EV71–specific ASCs accounted for a mean (±SD) of 26.2% ± 16.7% of the total circulating antibody-secreting cells. The frequency of EV71-specific ASCs varied with the sampling day, was highest on days 4–7 after onset, followed by days 1–3 and days 8–11 after onset (mean frequency [±SD], 1539 ± 980, 343 ± 412, and 80 ± 99, respectively; P = .0010, by analysis of variance [ANOVA]; Figure 1C). The EV71-specific ASCs disappeared in the peripheral blood at the convalescent stage, with a mean duration (±SD) of 20.3 ± 1.5 days after onset (Figure 1C). No EV71-specific ASCs were detected by ex vivo ELISpot among pediatric patients infected with HSV-1, group A10 coxsackievirus, and group B3 coxsackievirus and healthy controls (Figure 1A). In addition, there was no bystander ASC response to influenza virus antigens among enrolled patients and healthy controls.

Genogroup B EV71–specific ASC responses differed with regard to isotype use among cases (Figure 1B). In the first week of illness, a virus-specific IgG-producing ASC response could be detected, while some responses were accompanied with a nearly equivalent IgM-producing ASC response. Isotype use by the EV71-specific ASC response did not differ significantly within days 1–3 (mean [±SD], 44 ± 45 for IgG-producing ASCs, 235 ± 416 for IgM-producing ASCs, and 64 ± 77 for IgA-producing ASCs, respectively; P = .2027, by ANOVA) and days 4–7 (mean [±SD], 700 ± 584 for IgG-producing ASCs, 593 ± 604 for IgM-producing ASCs, and 246 ± 299 for IgA-producing ASCs, respectively; P = .1804, by ANOVA) after the onset of illness. Nevertheless, among pediatric patients <3 years old, the EV71-specific IgM-producing ASC response was predominant in the peripheral blood, while IgG-producing and IgA-producing ASC responses were barely detectable (P = .0038, by ANOVA). In contrast, among individuals aged...
≥3 years, the EV71-specific IgG-producing ASC response was higher in comparison to IgM-producing and IgA-producing ASC responses (P = .0286, by ANOVA; Figure 1C).

In the present study, the mean frequencies (±SD) of cross-reactive genogroup C EV71–specific ASC responses were 28 ± 34 for IgG-producing cells, 44 ± 55 for IgM-producing cells, and 31 ± 48 for IgA-producing cells, each of which was significantly lower than genogroup B EV71–specific ASC responses (P < .001 for each isotype comparison; Figure 1B). The mean frequency (±SD) of the total genogroup B EV71–specific ASC response was 10-fold higher than that against genogroup C viral antigens (1151 ± 1007 vs 105 ± 81; P < .0001).

By flow cytometry, we observed that the ASC population in the peripheral blood accounted for a mean (±SD) of 0.2% ± 0.2% of the total lymphocyte population during the acute phase of EV71 infection (Figure 1D). Proliferation marker Ki-67 was expressed in the majority of ASCs in the first week of illness. The frequency of circulating ASCs detected by fluorescence-activating cell sorting analysis significantly correlated with the magnitude of the genogroup B EV71–specific ASC response measured by ELISpot (Figure 1D). The ASC population was significantly decreased during the convalescent stage, with a frequency similar to that among healthy controls (P = .6982). The frequency of ASCs detected in the acute

Table 1. Characteristics of Pediatric Patients With Laboratory-Confirmed Enterovirus 71 (EV71) Infection

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Neurological Symptom/Sign</th>
<th>Viral Isolation Resulta</th>
<th>EV71-Specific IgM Result</th>
<th>EV71-Specific RT-PCR Resultb</th>
<th>Sample Collection, Day After Illness Onset</th>
<th>EV71-Specific ASCs c</th>
<th>EV71-Specific Titerd</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>Myoclonic jerk</td>
<td>EV71 Positive</td>
<td>Positive</td>
<td>5</td>
<td>1490</td>
<td>8192</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.6</td>
<td>Seizure, limb weakness</td>
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<td>Positive</td>
<td>6</td>
<td>1535</td>
<td>2048</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6.2</td>
<td>...</td>
<td>No growth</td>
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<td>Not done</td>
<td>580</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.3</td>
<td>Myoclonic jerk, ataxia</td>
<td>CPE Positive</td>
<td>Positive</td>
<td>Not done</td>
<td>2935</td>
<td>8192</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4.7</td>
<td>...</td>
<td>EV71 Negative</td>
<td>Positive</td>
<td>1</td>
<td>60</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>9</td>
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<td>...</td>
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<td>Positive</td>
<td>3</td>
<td>1270</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>10</td>
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<td>...</td>
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<td>3</td>
<td>175</td>
<td>64</td>
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<tr>
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<td>Positive</td>
<td>2</td>
<td>20</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2.1</td>
<td>...</td>
<td>EV71 Positive</td>
<td>Positive</td>
<td>4</td>
<td>75</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>3.6</td>
<td>...</td>
<td>Not done</td>
<td>Positive</td>
<td>Not done</td>
<td>150</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.7</td>
<td>Myoclonic jerk</td>
<td>EV71 Positive</td>
<td>Positive</td>
<td>5</td>
<td>1420</td>
<td>1024</td>
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</tr>
<tr>
<td>16</td>
<td>14.1</td>
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<td>Positive</td>
<td>5</td>
<td>1780</td>
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<td></td>
</tr>
<tr>
<td>17</td>
<td>4.9</td>
<td>...</td>
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<td>Positive</td>
<td>6</td>
<td>2345</td>
<td>4096</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>5.4</td>
<td>...</td>
<td>EV71 Positive</td>
<td>Positive</td>
<td>5</td>
<td>430</td>
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<td></td>
</tr>
<tr>
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<td>...</td>
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<td>4</td>
<td>15</td>
<td>4</td>
<td></td>
</tr>
<tr>
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<td>6</td>
<td>...</td>
<td>No growth</td>
<td>Positive</td>
<td>Not done</td>
<td>210</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>5.6</td>
<td>...</td>
<td>EV71 Negative</td>
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<td>280</td>
<td>512</td>
<td></td>
</tr>
<tr>
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<td>1.6</td>
<td>...</td>
<td>EV71 Positive</td>
<td>Positive</td>
<td>5</td>
<td>1450</td>
<td>2048</td>
<td></td>
</tr>
<tr>
<td>24</td>
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<td>Myoclonic jerk, ataxia</td>
<td>No growth</td>
<td>Positive</td>
<td>5</td>
<td>3900</td>
<td>8192</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>4.6</td>
<td>...</td>
<td>No growth</td>
<td>Positive</td>
<td>Negative</td>
<td>7</td>
<td>2050</td>
<td>16384</td>
</tr>
<tr>
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<td>Unsteady gait</td>
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<td>Negative</td>
<td>9</td>
<td>225</td>
<td>8192</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>3.1</td>
<td>Myoclonic jerk</td>
<td>EV71 Positive</td>
<td>Positive</td>
<td>6</td>
<td>2710</td>
<td>256</td>
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</tr>
<tr>
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<td>...</td>
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<td>7</td>
<td>1530</td>
<td>8192</td>
</tr>
<tr>
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<td>...</td>
<td>No growth</td>
<td>Positive</td>
<td>Not done</td>
<td>7</td>
<td>1085</td>
<td>4096</td>
</tr>
<tr>
<td>36</td>
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<td>...</td>
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<td>Positive</td>
<td>Not done</td>
<td>4</td>
<td>520</td>
<td>2048</td>
</tr>
<tr>
<td>37</td>
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<td>...</td>
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<td>Positive</td>
<td>5</td>
<td>1625</td>
<td>16384</td>
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</tr>
<tr>
<td>38</td>
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<td>Myoclonic jerk</td>
<td>EV71 Positive</td>
<td>Positive</td>
<td>6</td>
<td>870</td>
<td>2048</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>5.3</td>
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<td>No growth</td>
<td>Positive</td>
<td>Not done</td>
<td>5</td>
<td>1475</td>
<td>2048</td>
</tr>
</tbody>
</table>

Abbreviations: ASCs, antibody-secreting cells; CPE, cytopathic effect; IgM, immunoglobulin M; RT-PCR, reverse transcription polymerase chain reaction.

a Data are results of virus isolation tests on throat swab specimens. Patient 14 declined to undergo throat/rectal swab sampling, and virus isolation was therefore not performed. Patients 1, 3, 11, 12, 15, 16, 20, 24, 28, 29, and 38 also provided rectal swab specimens, and 4 (patients 1, 3, 11, and 16) were positive for EV71 in rectal swabs.

b EV71-specific RT-PCR was not performed for patients 5, 6, 21, 33, 36, and 40, owing to insufficient sample. Patient 14 declined to undergo throat/rectal swab sampling, and EV71-specific RT-PCR was therefore not performed.

c Data are genogroup B EV71–specific ASCs (immunoglobulin G, IgM, and immunoglobulin A producing) per million peripheral blood mononuclear cells.

d Data are neutralization titer against genogroup B EV71 strain TW-96015-2012.
Analysis of virus-specific antibody-secreting B-cell (ASC) responses to acute enterovirus 71 (EV71) infection in children. A, EV71 genogroup B–specific ASCs detected by an ex vivo enzyme-linked immunosorbent spot (ELISpot) assay from 3 EV71 genotype B5–infected children, 1 herpes simplex virus type 1 (HSV-1)–infected child, 1 group A10 coxsackievirus–infected child, and 1 healthy child. The ratio of EV71–specific immunoglobulin G (IgG)–producing, immunoglobulin M (IgM)–producing, immunoglobulin A (IgA)–producing, and total IgG–producing ASCs to incubated peripheral blood mononuclear cells (PBMCs) per well is shown in the figure. B, The magnitudes of EV71-specific ASC responses against genogroup B and genogroup C EV71 vaccine antigens
producing cells (mean [±SD], 5.2 ± 1.8 vs 2.2 ± 1.3 days; P = .0002).}

**DISCUSSION**

This study characterized the magnitude of the virus-specific ASC responses to acute EV71 infection in children and its correlation with the antibody titer and virological and clinical parameters. We detected genogroup B and virus-specific ASCs in the peripheral blood as early as the first day of illness onset, while the response became prominent on days 4–7 after onset. On the basis of an incubation period of at least 3 days [31, 32], EV71-specific ASC responses seemed to be generated as quickly as 4 days after infection, were strongest 7 days after infection, and remained detectable in the peripheral blood within 12 days after infection. This phenomenon is comparable to peripheral ASC responses to other acute respiratory viral infections and vaccinations in adults [21–25, 33]. In children, few studies demonstrated virus-specific ASC responses to acute dengue virus infection and live-attenuated influenza immunization [21, 34]. The magnitude of virus-specific ASC responses could vary from 1% to 30% of peripheral B cells. In the present study, an average of around 2% of CD19+ B cells were genogroup B EV71–specific ASCs in the acute phase of infection. While the kinetics of the EV71-specific ASC response is an interesting question, the only time points available in the study for assessing responses in the blood were after infection, and the majority of enrolled patients provided a 1-time sample within the first week of illness. The ASC response clearly disappeared in the peripheral blood among those who completely recovered and provided repeated samples in the convalescent stage.

A strong IgG-producing EV71-specific ASC response was induced in the pediatric patients, especially those aged ≥3 years. They had recently proliferated and were circulating in the peripheral blood. This indicates the recall of immune memory established by previous exposure, which is similar to the B-cell response to repeated viral infections in adults [22, 23, 33].

Interestingly, we noted that a dominant IgM-producing ASC response was generated quickly after acute EV71 infection in younger children who were exclusively <3 years old. The origin of these EV71-specific IgM ASCs remains undetermined. In view of their rapid elicitation within the first week of illness, 2 types of B cells might be responsible for this ASC response. One is isotype-switched IgM-producing memory B cells, and the other is the circulating equivalent of splenic marginal zone B cells. T cell–independent activation either through Toll-like receptors or by B-cell receptor–dependent bacterial antigens typically leads to an induction of short-lived antigen-specific IgM-producing B cells [35, 36]. Other studies demonstrated a
robust and rapid marginal zone B-cell response to the first exposure of viral antigens, and both virus-specific IgM and IgG responses were observed, which indicates the plasticity of marginal zone B-cell activation and the potential of isotype switching in certain settings [37, 38]. While the T-cell–independent immune response was generally underdeveloped in the first 2 years of life in humans, T-cell–dependent immune responses could develop early after birth [28, 39]. In young children, isotype-switched memory B cells might be generated in the germinal center during the previous infection and may quickly differentiate into ASCs when antigens are encountered again [40, 41]. Assessment of the preinfection immune status in terms of preexisting memory B cells and antibody levels would be helpful in elucidating whether the IgM-producing cellular response represents a primary or recalled immune response, but this information was unavailable in this study. A long-term and

Figure 2. Correlation of the enterovirus 71 (EV71)–specific antibody-secreting B-cell (ASC) frequency and the serological neutralization titer (A), the fever duration (B), and the viral load (C) in EV71-infected children. Linear regression was performed to examine their association. Abbreviations: IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; PBMCs, peripheral blood mononuclear cells.
prospective study following up young children before and after natural infection or a well-designed human challenge study might be conducted to tackle the question.

The finding of dominant IgM ASC response in younger children could be linked to observations from a previous study focusing on the relationship of T-cell response and clinical severity in EV71-infected children [13]. Yang et al found a significantly decreased expression of the CD40 ligand of T cells in young children with severe illnesses whose average age was 2.5 years. The T-cell CD40 ligand is important to the interaction between B cells, dendritic cells, and T cells and, thus, the activation and isotype switching from IgM to IgG among antigen-specific B cells in human. Although no significant differences in both EV71-specific IgG and IgM ASC frequencies between patients with and those without neurological manifestations were found in the study, the impact of the bias of isotype use in the acute-stage ASC responses on clinical severity warrants further investigations.

In the study, infection with genogroup B (B5) EV71 induced a strong genogroup B EV71–specific but weak genogroup C (C4b)–specific ASC response in children. The neutralization titer against genogroup B viruses significantly correlated with genogroup B EV71–specific ASC responses but not with genogroup C EV71–specific ASC responses. EV71 is recognized as single serotype. Between genogroup B and C viruses, the amino acid homology in the P1 capsid region is as much as 97%–98% [42]. Cross-reactive antibody responses to EV71 of different genogroups or genotypes could be expected but in fact varied in immunized individuals. Previous studies noted that exposure to genogroup C EV71 elicited cross-reactive antibody responses to both genogroup B and C viruses [43, 44]. Nevertheless, genogroup B EV71 exposure preferentially induced antibodies against strains of the same genogroup, rather than against several genotype C strains, especially genotype C4b viruses, in animal models and human vaccine trials [45, 46]. This might partially explain the weak cross-reactive ASC response to C4b antigens and the poor correlation between the C4b EV71–specific ASC response and the serological titer against genogroup C EV71 in genotype B5 EV71–infected children, although the underlined mechanisms of the relationship between the EV71–specific ASC response and the serological neutralization titer against viruses of either the same or different genogroup remain largely unclear. Nevertheless, Huang et al pointed out that antigenic variations might not have a clear pattern between genogroup B and C EV71 in their analysis of the specificity and breadth of antibody response at serological level in infected individuals [42]. Cross-reactive neutralizing monoclonal antibodies against linear and conformational epitopes on the viral capsid have been recently reported [17, 47], although these antibodies were all derived from mice. The EV71 capsid protein epitopes relevant for cross-reactivity and neutralization by human antibodies still remain unknown at the clonal level.

The correlation between the ASC responses and throat viral load and fever duration brings about the question whether these cells involve the pathogenesis of acute illness. In dengue virus infections, the magnitude of virus-specific ASC responses was related to the development of immunopathology in patients with severe disease [34]. Nonneutralizing antibodies might facilitate the entry of dengue virus into host cells. However, the ASC frequency did not differ significantly between patients with and those without neurological manifestations in the study. Besides, during natural infection, the inoculum of infectious virus, preinfection immune status, and genetic susceptibility might vary among individuals, and these factors would also probably interfere with the severity of illnesses in susceptible hosts [9, 48]. Since rectal swab specimens were unavailable in 61% (17 of 28) of laboratory-confirmed EV71 cases and a small number of positive rectal swab specimens were noted in the study, the viral load in 4 positive rectal swab specimens was not determined, and the information about the relationship between the rectal viral load and EV71–specific ASC response was lacking.

The present study provided the cellular basis for the EV71–specific antibody response established during the acute stage of natural infection in children. Advanced techniques have been developed to facilitate the generation of human monoclonal antibodies from virus-specific B cells [24]. Human monoclonal antibodies that resemble natural antibodies that are present in the serum of healthy individuals have the potential to be used as novel agents against viral infectious diseases when no other therapy is available.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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