Acute Chemokine Response in the Blood and Cerebrospinal Fluid of Children with Enterovirus 71–Associated Brainstem Encephalitis

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Background. Brainstem encephalitis (BE) is a serious neurological complication of enterovirus 71 (EV71) infection. The present study was designed to determine the characteristics of the chemokine response in the blood and cerebrospinal fluid (CSF) of patients with EV71-associated BE.

Methods. Thirty-one patients with BE were studied. They consisted of 12 with uncomplicated BE, 9 with autonomic nervous system (ANS) dysregulation, and 10 with pulmonary edema (PE); 13 healthy control subjects were also studied. Plasma and CSF concentrations of various chemokines were determined by a particle-based flow cytometry immunoassay.

Results. Plasma levels of interferon (IFN)–γ–induced protein (IP)–10, monocyte chemoattractant protein (MCP)–1, monokine induced by IFN-γ (MIG), and interleukin (IL)–8 were significantly higher in patients with PE than in those with uncomplicated BE. CSF levels of MIG were significantly higher in patients with PE than in those with uncomplicated BE and ANS dysregulation. The ratios of mean CSF to plasma levels for MCP-1 and IL-8 were highest in patients with uncomplicated BE and tended to fall with increasing severity of the disease.

Conclusions. Overexpression of the chemokine cascade in the central nervous system compartment appears to play an important role in the elicitation of the immune response to EV71. The chemokine CSF to plasma ratios suggest that IL-8, IP-10, MCP-1, and possibly MIG—but not RANTES—are synthesized in the brain in response to encephalitis.

Enterovirus 71 (EV71) is an important cause of emerging infectious diseases in children worldwide. Large epidemics have been reported in Taiwan and other countries of Southeast Asia and, more recently, in the United States [1–6]. The virus was first identified in 1969 [7]. It is a member of the family Picornaviridae. EV71 infections are characterized by persistent fever, hand-foot-and-mouth disease (HFMD), or herpangina and lymphopenia [1–4, 8]. A poliomyelitis-like syndrome has also been reported [6, 9]. Cardiopulmonary complications, including pulmonary edema (PE) or hemorrhage secondary to invasion of the brainstem, pons, and medulla, are the major causes of severe or fatal cases [2–4, 6].

Lymphopenia and depletion of CD4 and CD8 T lymphocytes are associated with disease activity and adverse outcomes [8]. Overproduction of specific inflammatory cytokines, interleukin (IL)–10, IL-13, and interferon (IFN)–γ is strongly associated with PE [8]. Administration of milrinone, a phosphodiesterase inhibitor, and intravenous immunoglobulin has been shown to decrease sympathetic overactivity, decrease inflammation, and improve survival in patients with EV71-associated PE [10, 11]. Controlled clinical trials are needed to confirm this observation.

Chemokines constitute a large superfamily of small-protein (8–10 kDa) intercellular messengers that play...
multiple roles in both innate and adaptive immune responses. Chemokines are distinguished from other cytokines by their ability to act on the superfamily of G-protein–coupled serpin receptors. Chemokines are classified as constitutive (developmentally regulated) or inducible (inflammatory). Chemokine expression helps shape the immune response during viral infection. Viral subversion of the chemokine system inhibits the ability of the host to effectively control viral infections.

We explored the potential role played by selected chemokines in children with EV71–associated brainstem encephalitis (BE) because of the known increased expression of antiviral IFN-γ in Th1 diseases [12]. IFN-γ–induced protein–10 (CXCL10/IP-10), monokine induced by IFN-γ (CXCL9/MIG), and IFN-inducible T cell α chemoattractant are expressed in activated tissues [13–16]. We were further intrigued by the observation that several members of the large family of cysteine-containing (CC) chemokines (or β-chemokines) are produced by astrocytes in vitro and during the course of central nervous system (CNS) infections [17]. Viral infections have been shown to stimulate astrocyte production of monocyte chemoattractant protein–1 (CCL2/MCP-1) and regulated upon activation normal T cell–expressed and secreted (CCL5/RANTES) [18, 19].

This report describes our observations of selective chemokine responses in the plasma and cerebrospinal fluid (CSF) of children with EV71 BE according to disease severity. Overexpression of the chemokine cascade in the CNS compartment appears to raise the integrated immune response to EV71 and elicit the intense systemic immune reactions associated with adverse clinical outcomes.

METHODS

Subjects. The population consisted of 31 patients with BE who were admitted to a hospital in Tainan, Taiwan, during the 1998–2000 and 2001–2005 EV71 outbreaks. They consisted of 12 patients with uncomplicated BE, 9 with autonomic nervous system (ANS) dysregulation, and 10 with PE. Plasma and CSF samples were collected during the acute phase of the illness. Plasma was obtained from 13 healthy subjects for comparison.

Chemokine measurements. Blood samples were collected in tubes containing EDTA. The tubes were immersed in ice and transported immediately to the laboratory for processing. Plasma was separated by centrifugation (2000 g for 10 min) at 4°C and stored in 300-μL aliquots at –70°C until analysis. IL-8, RANTES, MCP-1, IP-10, and MIG were measured using chemokine cytometric bead array (CBA) kits (BD Pharmingen), in accordance with the manufacturer’s instructions. Briefly, 5 bead populations that had distinct fluorescence intensities and that had been coated with capturing antibodies specific for different chemokines were incubated with 50 μL of plasma. The chemokine–captured beads were then mixed with phycoerythrin-conjugated detection antibodies to form sandwich complexes. After incubation, washing, and acquisition of fluorescence data, results were generated using BD CBA software. The assay sensitivities of the 5 chemokines analyzed were 0.2 pg/mL for IL-8, 1.0 pg/mL for RANTES, 2.5 pg/mL for MIG, 2.7 pg/mL for MCP-1, and 2.8 pg/mL for IP-10.

Data analysis. CBA data were collected on a FACSCalibur instrument using CellQuest software (version 5.1) and analyzed using BD CBA software (version 1.4) (both from BD Biosciences). Flow cytometry data were acquired using CellQuest software and analyzed using FlowJo software (version 3.2; TreeStar).

Virus isolation and identification. Virus was isolated in the Clinical Virology Laboratory of National Cheng Kung University Hospital. Specimens (throat swab, stool, and CSF) were inoculated onto A549, Vero, rhabdomyosarcoma, and green monkey kidney cell cultures. Enterovirus strains were typed by neutralization tests using Lim and Benyesh-Melnick pools [20] and/or immunofluorescence tests using monoclonal antibodies. EV71 was identified in cells exhibiting viral cytopathic effect by means of 2 EV71-specific monoclonal antibodies (MAbs; 3323 and 3324; Chemicon) with immunofluorescence stains. Two MAbs were required to compensate for cross-reactions between MAb 3323 for EV71 and coxsackievirus A16, and MAb 3324 for EV71. The presence of EV71 was confirmed by neutralization test using polyclonal EV71 rabbit antibodies.

Statistical analysis. The Mann-Whitney rank sum test was used to determine significant differences because of the non-parametric distribution of data and the relatively small number of observations. All analyses were performed using the SPSS for Windows (version 11.5; SPSS). A difference with P < .05 was considered to be significant.

RESULTS

Virological experiments. Diagnosis was made on the basis of isolation of the virus from throat swab specimens (n = 7), stool specimens (n = 10), both throat swab and stool specimens (n = 10), tissue (n = 2), tracheal aspirates (n = 1), and gastric aspirates (n = 1), in addition to clinical features and/or neuroimaging. EV71 was never isolated from CSF.

Comparative analysis of chemokine concentrations according to disease severity. The mean plasma concentrations of the chemokines in control subjects and the plasma and CSF levels in patients with EV71 BE of differing severity are shown in table 1. Plasma levels of the chemokines IP-10, MIG, RANTES, and IL-8 were significantly lower (P < .05) in control subjects than in subjects with BE. Plasma levels of IP-10, MCP-1, MIG, and IL-8 were significantly higher (P < .05) in patients with PE than in patients with uncomplicated BE and in the control subjects. CSF levels of IP-10 and IL-8 in patients with EV71 BE were significantly higher than the plasma levels in the control subjects (P < .05). RANTES was the only chemokine among those studied in which the plasma levels never exceed the control values.
Table 1. Chemokine expression in plasma and cerebrospinal fluid (CSF), according to the severity of enterovirus 71 (EV71)–associated brainstem encephalitis (BE).

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Control plasma (n = 13)</th>
<th>Uncomplicated BE (n = 12)</th>
<th>ANS dysregulation (n = 9)</th>
<th>PE (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>CSF</td>
<td>Plasma</td>
<td>CSF</td>
</tr>
<tr>
<td>IP-10</td>
<td>595.2 (391.7–860.9)</td>
<td>1766.3 (503–4844.9)</td>
<td>1917.3 (378–4236.4)</td>
<td>2167.2 (503.2–2500)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>350.0 (100.8–574.9)</td>
<td>215.6 (80.3–333.4)</td>
<td>221.7 (75.8–432.5)</td>
<td>221.7 (75.8–432.5)</td>
</tr>
<tr>
<td>MIG</td>
<td>849.3 (373.8–1192.8)</td>
<td>1014.4 (443.9–2131.2)</td>
<td>567.6 (103.7–1101.5)</td>
<td>298.8 (112–481.2)</td>
</tr>
<tr>
<td>RANTES</td>
<td>14,000.6 (13,205.1–15,493.1)</td>
<td>13,037.7 (2778.1–14,593.1)</td>
<td>12,843.0 (4109–14,965.9)</td>
<td>38.7 (5.1–157.8)</td>
</tr>
<tr>
<td>IL-8</td>
<td>108.3 (19.6–416.1)</td>
<td>156.4 (21.7–352.5)</td>
<td>530.7 (17.7–1672.8)</td>
<td>1536.5 (17.7–8979.7)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean (range) concentrations of chemokines, in picograms per milliliter. ANS, autonomic nervous system; IL, interleukin; IP, interferon-γ–induced protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by interferon-γ; PE, pulmonary edema.

a P < .05, for uncomplicated BE vs. PE.
b P < .05, for uncomplicated BE vs. ANS dysregulation.
c P < .05, for ANS dysregulation vs. PE.
Table 2. Ratios of mean cerebrospinal fluid (CSF) to plasma chemokine expression, according to the severity of enterovirus 71 (EV71)–associated brainstem encephalitis (BE).

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>EV71-associated BE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP-10</td>
<td>Uncomplicated BE</td>
</tr>
<tr>
<td></td>
<td>1.11</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2.78</td>
</tr>
<tr>
<td>MIG</td>
<td>0.27</td>
</tr>
<tr>
<td>RANTES</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-8</td>
<td>3.39</td>
</tr>
</tbody>
</table>

NOTE. ANS, autonomic nervous system; IL, interleukin; IP, interferon-γ–induced protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by interferon-γ; PE, pulmonary edema.

Regardless of the severity of BE. The CSF levels of RANTES were only slightly above the detectable range for the assay.

Ratio of mean CSF to plasma chemokine concentrations according to disease severity. The ratios of mean CSF to plasma concentrations for patients with EV71 BE are shown in table 2 according to disease severity. The highest CSF to plasma ratios were noted among patients with uncomplicated BE, with the notable exception of RANTES. The CSF to plasma ratio for RANTES remained very low, at 0.001, regardless of the severity of illness. The ratio for MIG tended to increase with increasing severity of disease, whereas the ratios for the other chemokines tended to decrease with increasing severity of disease. The ratios of mean CSF to plasma concentrations for all 31 patients with EV71 BE were close to 1 for IP-10 and MCP-1 and were close to 0.5 for MIG.

Discussion

EV71 is an important global viral pathogen. It may lead to life-threatening complications such as PE and cardiopulmonary collapse [2, 5, 6, 8]. The progression of the disease occurs in 3 distinct phases: BE, BE with ANS dysregulation, and BE with PE [8]. The intensity of the host response to viral infections plays an important role in determining the outcome of the disease [21]. We previously reported that cytokines released into the systemic compartment trigger changes in pulmonary vascular permeability, resulting in PE [8].

The present study demonstrates that the levels of several important chemokines are elevated in the plasma of children with EV71 BE and tend to increase with the severity of the disease. The major exception is RANTES, which was not elevated either in the plasma or CSF regardless of severity. The finding that CSF levels of IP-10, MCP-1, and IL-8 were either the same or exceeded plasma levels in children with uncomplicated BE, together with the observation that CSF levels of IP-10 and IL-8 were significantly higher than plasma levels in healthy control subjects, strongly suggests that these small proteins originate in the CNS.

It is also reasonable to suppose that MIG originated in CSF because of its very high concentration in patients with PE. The decrease in CSF to plasma concentration ratios for MCP-1 and IL-8 as the severity of disease increased can be accounted for by the relatively greater increase in plasma levels as the disease progressed. It is doubtful that the high levels of chemokines in CSF could have been due to passive diffusion from the blood because of their relatively large size (8–10 kDa). The present findings are in accordance with previous findings by our group and others that cytokines and chemokines play an important role in the pathogenesis EV71 BE [8].

IP-10 expression can be up-regulated by the Th1 cytokine IFN-γ during acute lung inflammation. This is consistent with our findings that both circulating IFN-γ [8] and IP-10 levels were increased in patients with EV71 PE. IP-10 is prominently expressed within the CNS of mice after infection with viral encephalitis [22]. Early expression of IP-10 within the CNS after virus infection is important in initiating and maintaining a protective Th1 immune response. This is characterized by high-level production of the antiviral cytokine IFN-γ [22, 23]. Early expression of IP-10 appears to be beneficial by attracting Th1 T lymphocytes into the CNS, which participate in viral clearance.

A broader role for MCP-1 in the systemic inflammatory response has been suggested [24]. The concentration of MCP-1 has been shown to increase in the plasma of patients with persistent acute respiratory distress syndrome [24]. Levels of MCP-1 have also been shown to be increased in patients with sepsis and shock and correlate with increased survival [25]. MCP-1 is expressed by astrocytes within hours of mechanical injury to the CNS, before leukocyte accumulation occurs, and is thought to mediate recruitment and activation of mononuclear phagocytes [26]. This may provide an explanation for the decrease in MCP levels in CSF when the disease progresses from uncomplicated BE to ANS dysregulation and PE.

MIG plays a role in host defense after viral infection [27, 28]. Salazar-Mather et al. [28] have demonstrated that MIG expression is important in viral clearance in mice infected with murine cytomegalovirus, presumably through the recruitment of antiviral effector cells to sites of infection. We found levels of MIG to be significantly more elevated in patients with EV71 PE than in those with EV71 ANS dysregulation and uncomplicated BE. These findings support the notion that MIG contributes to host defense by promoting a protective Th1 response against viral infection of the CNS.

In conclusion, specific chemokines are associated with the systemic and CNS inflammatory response to EV71 infection. The initial chemokine responses appear to be protective but exacerbate the disease process in patients with CNS involvement. A better understanding of the pathogenetic role chemokines play in affecting the course and severity of illness and their interplay with other inflammatory mediators may help in the develop-
ment of novel therapeutic and preventive strategies to control the inflammatory response and the progression of the disease.

Acknowledgments

We thank Dr. Calvin M. Kunin for providing invaluable suggestions and critically reviewing the manuscript. We thank Ms. Chao-Ping Liao for the collection of clinical specimens.

References