MAJOR ARTICLE

In Enterovirus 71 Encephalitis With Cardio-Respiratory Compromise, Elevated Interleukin 1β, Interleukin 1 Receptor Antagonist, and Granulocyte Colony-Stimulating Factor Levels Are Markers of Poor Prognosis

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Background. Enterovirus 71 (EV71) causes large outbreaks of hand, foot, and mouth disease (HFMD), with severe neurological complications and cardio-respiratory compromise, but the pathogenesis is poorly understood.

Methods. We measured levels of 30 chemokines and cytokines in serum and cerebrospinal fluid (CSF) samples from Malaysian children hospitalized with EV71 infection ($n = 88$), comprising uncomplicated HFMD ($n = 47$), meningitis ($n = 8$), acute flaccid paralysis ($n = 1$), encephalitis ($n = 21$), and encephalitis with cardio-respiratory compromise ($n = 11$). Four of the latter patients died.

Results. Both pro-inflammatory and anti-inflammatory mediator levels were elevated, with different patterns of mediator abundance in the CSF and vascular compartments. Serum concentrations of interleukin 1β (IL-1β), interleukin 1 receptor antagonist (IL-1Ra), and granulocyte colony-stimulating factor (G-CSF) were raised significantly in patients who developed cardio-respiratory compromise ($P = .013$, $P = .004$, and $P < .001$, respectively). Serum IL-1Ra and G-CSF levels were also significantly elevated in patients who died, with a serum G-CSF to interleukin 5 ratio of >100 at admission being the most accurate prognostic marker for death ($P < .001$; accuracy, 85.5%; sensitivity, 100%; specificity, 84.7%).

Conclusions. Given that IL-1β has a negative inotropic action on the heart, and that both its natural antagonist, IL-1Ra, and G-CSF are being assessed as treatments for acute cardiac impairment, the findings suggest we have identified functional markers of EV71-related cardiac dysfunction and potential treatment options.

Enterovirus 71 (EV71) is found globally, but since the mid-1990s, it has become a major public health problem in the Asia-Pacific region, causing large outbreaks of hand, foot, and mouth disease (HFMD), which may be complicated by aseptic meningitis, encephalitis, fulminant cardio-respiratory failure, and sudden death [1]. Fatal cases are characterized by neurological involvement, with myoclonus, cerebrospinal fluid (CSF) pleocytosis, brainstem lesions on magnetic resonance imaging, and cardio-respiratory compromise; this manifests as poor peripheral perfusion, reduced cardiac contractility on echocardiography, and pulmonary congestion on chest x-ray radiograph [2].

The mechanisms underlying cardio-respiratory compromise in EV71 infection remain unclear. Because it is associated with brainstem encephalitis,

Received 2 October 2011; accepted 17 February 2012; electronically published 24 July 2012.

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The Journal of Infectious Diseases 2012;206:881–92

DOI: 10.1093/infdis/jis446

neurogenic pulmonary edema has been postulated [3, 4]. As for many severe infections, dysregulation of the host immune and cytokine response has also been proposed to be a possible factor in the pathogenesis [1]. Earlier studies examining a few cytokines in relatively few cases of EV71 infection showed elevated levels of cytokines in the CSF and serum of children with HFMD and central nervous system (CNS) disease, with or without cardio-respiratory complications [5–9]. On the basis of higher concentrations of cytokines observed in the CSF compared with those in the serum, some suggested the CNS may be the source of the inflammatory cytokines detected in the serum [6, 9]. Others suggested the cardio-respiratory complications were linked to systemic pro-inflammatory responses elicited by the virus [5, 6].

There is no specific antiviral treatment or vaccine for EV71. The current treatment is largely supportive; intravenous immunoglobulin is given empirically in severe disease, and is thought to work through anti-inflammatory mechanisms [2]. A better understanding of the inflammatory response is key to understanding the pathogenesis and developing new targeted immunomodulatory treatments. We therefore studied a broad panel of cytokines and chemokines in a large series of children with HFMD. We postulated that a comparison of patterns of mediator response for different clinical presentations would give clues to the pathogenesis and point toward new treatments.

METHODS

Study Population, Period, and Setting

The study population was recruited from January through December 2006, during an epidemic of HFMD, through a long-term prospective clinical study of HFMD on the pediatric ward and intensive care unit of Sibu Hospital, Sarawak, Malaysia [10]. The study was approved by the Director of Health of Sarawak and the Ethics Committee of the Liverpool School of Tropical Medicine (UK). Informed consent was obtained from the parent or guardian of each child.

Clinical and Virological Methods

All enrolled patients had EV71 infection confirmed by virus isolation or human EV71 RNA detection. Virological and clinical methods were as described elsewhere [10].

Case Definitions

The case definition for HFMD has been described elsewhere [10]. Complicated HFMD was defined as HFMD with additional clinical symptoms and signs of CNS involvement, supported by CSF pleocytosis (leukocyte count of >5 cells/μL) with negative CSF microscopy and culture for bacteria.

Complicated HFMD patients were subclassified based on their patterns of CNS involvement. Aseptic meningitis was characterized by full consciousness, headache, meningism, and no focal neurological signs. Acute flaccid paralysis was characterized by acute onset of areflexic limb weakness. Encephalitis was characterized by impaired consciousness (including lethargy or drowsiness), seizures, or myoclonus [10].

If a patient required an inotropic agent to support peripheral perfusion, they were defined as having cardio-respiratory compromise. Marked cardio-respiratory compromise was defined by the patient requiring 2 or more inotropic agents. The initiation and administration of inotrope treatment was at the discretion of the attending pediatrician, based on the clinical assessment of capillary refill time, presence of skin mottling, temperature of the extremities, pulse volume, heart rate, and blood pressure.

Patients who were otherwise well or without CSF pleocytosis were classified as having uncomplicated HFMD.

Sample Collection

Serum samples (500 μL) were collected at hospital admission. CSF samples (500 μL) were collected at lumbar puncture, which typically occurred within 24 hours of admission. Samples were stored immediately in a −70°C freezer.

Measurement of Cytokine and Chemokine Levels

Thirty mediators (cytokines and chemokines) were measured using Lincoplex kits according to the manufacturer's instructions. Fluorescence data were acquired by means of a Luminex 100 system with Luminex IS software (version 2.3). All samples were assayed in duplicate, and the mean concentration calculated.

Two-Way Unsupervised Sample Clustering

Samples underwent 2-way unsupervised hierarchical clustering based on the patterns of relative mediator concentrations. This approach offered an opportunity to examine the synergistic interactions of mediators and allow patients to be classified by their host mediator response rather than their clinical manifestations.

To prevent biasing of the analysis by missing data, only samples with a consistent high signal quality, whereby ≥20% of the mediators were detected among ≥80% of the patients, were included. Signal intensities for the mediators were median "centered" and normalized across the set of samples. Patients and then mediators were hierarchically clustered using the program Cluster (version 3.0), using the uncentered correlation score as the similarity metric and the average branch linkage method. Results were viewed as heat-maps using Tree View software (http://rana.lbl.gov/EisenSoftware.htm). Serum and CSF samples were analyzed separately.

Testing Association Between Mediator Concentrations and Clinical Parameters

Groups of neighboring mediators (clustered together) that exhibited consistently high or low correlations between mediator

concentrations and individual clinical parameters were identified using a dynamic programming procedure with statistical significance determined by permutation, as described elsewhere [11].

Supervised Analysis

Patients were grouped by their clinical features. Statistical comparisons were undertaken using the nonfiltered, nonscaled mediator data by means of Mann-Whitney *U* or Fisher exact tests using Stata software (version 7.0). Median values or proportions were presented.

RESULTS

Eighty-eight children admitted to hospital with confirmed EV71 infection had serum samples collected on the day of admission of sufficient volume and quality for mediator measurement. Of these children, 47 were classified with uncomplicated HFMD and 41 with complicated HFMD comprising encephalitis ($n = 32$), meningitis ($n = 8$), or acute flaccid paralysis ($n = 1$). Among the encephalitis cases, 11 had cardio-respiratory compromise, in 6 of whom it was marked. Four of the latter patients died. All 88 patients had serum samples collected, and 71 patients had CSF samples collected. Samples with a consistent high signal quality were used in the unsupervised pattern analysis. Thirty mediators were consistently detected in 85 serum samples. Ten mediators were consistently detected in 68 CSF samples. The recorded clinical parameters for the children in this study were representative of children hospitalized with HFMD. Patients with complicated HFMD exhibited a higher peak temperature, longer fever duration, and more frequent history of lethargy compared with patients with uncomplicated HFMD (Table 1), concordant with the findings for the larger HFMD cohort reported elsewhere [10].

Acute Mediator Response Within Serum Samples Distinguished Patients With Cardio-Respiratory Compromise

The mediators clustered into 2 main sets with 5 additional mediators outlying these sets. Set 1 included interleukin 1β (IL-1β), interleukin 1 receptor antagonist (IL-1Ra), interleukin 10 (IL-10), granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ); set 2 included interleukin 6 (IL-6), interleukin 8 (IL-8), and interferon-γ-inducible protein 10 (IP-10). Each set contained both pro-inflammatory and anti-inflammatory mediators; for example, set 1 included IL-1β, a prototypical pro-inflammatory mediator, and IL-1Ra, its natural antagonist (Figure 1*A*).

The patients grouped into 3 main clusters (Figure 1*A*). These clusters corresponded to 3 broad patterns of host response: pattern A, relatively high abundance of mediators composing set 1 and set 2; pattern B, relatively low abundance of mediators composing set 1 and high abundance of mediators composing set 2; and pattern C, relatively low abundance of all mediators.

Although no clinical data were incorporated into the algorithm, host response patterns corresponded with clinical features. Patients exhibiting pattern A more frequently suffered cardio-respiratory compromise compared with patients with other patterns of mediator abundance (10 [26%] of 38 patients vs 1 [2%] of 47 patients; $P = .002$). Pattern A represented an increased relative risk of 12.4 for exhibiting cardio-respiratory compromise during admission compared with the other patterns (95% confidence interval [CI], 1.8–257.8). Similarly, patients with pattern A received a significantly higher number of inotropic agents during hospital admission compared with the other patients (19 inotropic agents vs 3 inotropic agents; $P = .001$). Furthermore, a higher proportion of patients exhibiting pattern A died (3 [8%] of 38 patients vs 1 [2%] of 47 patients; $P = .32$). In contrast, host response pattern C was associated with a significantly decreased risk for cardio-respiratory compromise (relative risk [RR], 0.166 [95% CI, 0.008–1.1]; $P = .046$).

Acute Mediator Response Within CSF Samples Distinguished Patients With Complicated EV71 Illness

The mediators in the CSF clustered into 2 main sets. Set 3 included G-CSF, IL-6, IL-8, IL-1Ra, and IP-10; set 4 included IL-10, macrophage inflammatory protein 1-β MIP-1β, interleukin 1α (IL-1α), and soluble CD40 ligand (sCD40L). Each set again contained both pro-inflammatory and anti-inflammatory mediators (Figure 2*A*).

The patients grouped into 3 main clusters. These clusters corresponded to 3 patterns of host response: pattern D, relatively high abundance of mediators within set 3; pattern E, relatively high abundance of all mediators; and pattern F, relatively low abundance of all mediators (Figure 2*A*).

The patterns of host response again corresponded with clinical features. Patients exhibiting pattern D were significantly more likely to suffer complicated HFMD (14 [82%] of 17 patients vs 22 [43%] of 51 patients; $P = .005$) or encephalitis (11 [65%] of 17 patients vs 17 [33%] of 51 patients; $P = .044$); have cardio-respiratory compromise (6 [35%] of 17 patients vs 4 [8%] of 51 patients; $P = .012$); receive more doses of intravenous immunoglobulin (2.0 doses vs 0 doses; $P = .001$); or die (3 of 17 patients vs 0 of 51 patients; $P = .014$). They also exhibited significantly higher CSF total leukocyte counts (49.0 cells/mm^3 vs 3.0 cells/mm^3; $P = .008$).

Mapping Mediator Abundance to Clinical Parameters Demonstrated Mediators in Set 1 Correlated With Inotrope Use

The dynamic programming algorithm (which controlled for multiple comparisons) demonstrated a significant positive correlation between the pattern of serum abundance for

Table 1. Clinical Features and Serum Mediator Concentrations for 88 Patients With Enterovirus 71 Infection

	Patient Groups and Comparisons												
Clinical Parameters	Uncomplicated HFMD	Complicated HFMD	*P*	Nonfatal	Fatal	*P*	No CRC	CRC	*P*	Marked CRC	*P*[a]	Encephalitis	ASM
No. (%) of patients	47 (53)	41 (47)	…	84 (95)	4 (4)	…	77 (88)	11 (12)	…	6 (7)	…	26 (29)	8 (9)
Age, months	25.7	19.7	NS	22.99	11	NS	22.9	21.2	NS	17.9	NS	19.6	17.1
Peak hospital temperature, °C	38	38.8	<.001	38.4	39.9	.001	38.3	39.6	<.001	39.9	<.001	38.8	38.8
Total duration of fever, d	1.5	3.5	<.001	2.5	3.8	NS	2.5	4	.003	4	.013	3	3.8
No. (%) of patients with history of lethargy	8 (17)	16 (39)	.03	20 (24)	4 (100)	.005	18 (23)	6 (55)	NS	4 (67)	.04	10 (38)	2 (25)
Heart rate, bpm[b]	138	148	.007	143	155	NS	142	155	.017	151	NS	162	143
Respiratory rate, bpm[c]	36	35	NS	36	39	NS	36	35	NS	39	NS	34.5	41
Duration of fever at home, d	1	1	NS	1	3	.033	1	3	.024	3	.024	1	1.5
Total leukocyte count, $\times 10^9$ cells/L	13.4	12.5	NS	12.7	15.8	NS	12.7	13.8	NS	17.3	NS	12.0	12.0
Lymphocyte count, %	27.6	30	NS	30	31	NS	30	28.5	NS	29	NS	30	38.5
CSF leukocyte count, cells/mm^3	1.5	48	<.001	4	97	.016	4	80	<.001	97	.003	23.5	39
CSF lymphocyte count, %	100	97	<.001	100	91	.039	100	95	<.001	91	.012	97.5	99
CSF protein level, g/dL	0.27	0.36	.001	0.3	0.62	.001	0.28	0.47	<.001	0.56	<.001	0.34	0.36
CSF glucose level, mmol/L	3.4	3.9	NS	3.4	5.1	.017	3.4	4.3	.045	5.1	.002	3.4	3.3
Plasma glucose level, mmol/L	4.9	5.1	NS	5	7.1	NS	5	6.0	NS	7.4	NS	5	4.8
CSF/plasma glucose ratio	0.68	0.69	NS	0.68	0.72	NS	0.69	0.69	NS	0.69	NS	0.69	0.68
Serum IL-1Ra level, pg/mL[d]	1753.5	1408.9	NS	1478	10 000	.043	1443.9	10 000	.004	10 000	.04	1376.2	1610.6
Serum G-CSF level, pg/mL	235.1	391.8	NS	259.1	2524.3	.004	235	987.2	<.001	1590.9	.004	361.2	192
Serum IL-10 level, pg/mL	111.9	97.7	NS	104.6	563.2	NS	94.3	288.5	.002	563.2	.025	95.8	88.4
Serum IL-1β level, pg/mL	8.1	7.3	NS	7.3	17.7	NS	6.7	27.4	.013	17.7	NS	4.8	13.3
Serum IL-15 level, pg/mL	19.7	19.9	NS	19.6	69.4	NS	16.8	118.9	.003	69.4	NS	14.3	38.6

Data are medians unless otherwise indicated. *P* values indicate significant differences in parameters between patients with uncomplicated and complicated HFMD; nonfatal and fatal cases; and no CRC and CRC.

Abbreviations: ASM, aseptic meningitis; CRC, cardio-respiratory compromise; CSF, cerebrospinal fluid; G-CSF, granulocyte colony-stimulating factor; HFMD, hand, foot, and mouth disease; IL-1β, interleukin 1β; IL-1Ra, interleukin 1 receptor antagonist; IL-10, interleukin receptor 10; IL-15, interleukin 15; NS, not significant.

[a] Between patients with marked CRC and all other patients. Significance tested via Mann-Whitney *U* test or Fisher exact test.

[b] Beats per minute.

[c] Breaths per minute.

[d] Concentration above detection limit in multiple samples (>10 000 pg/mL).

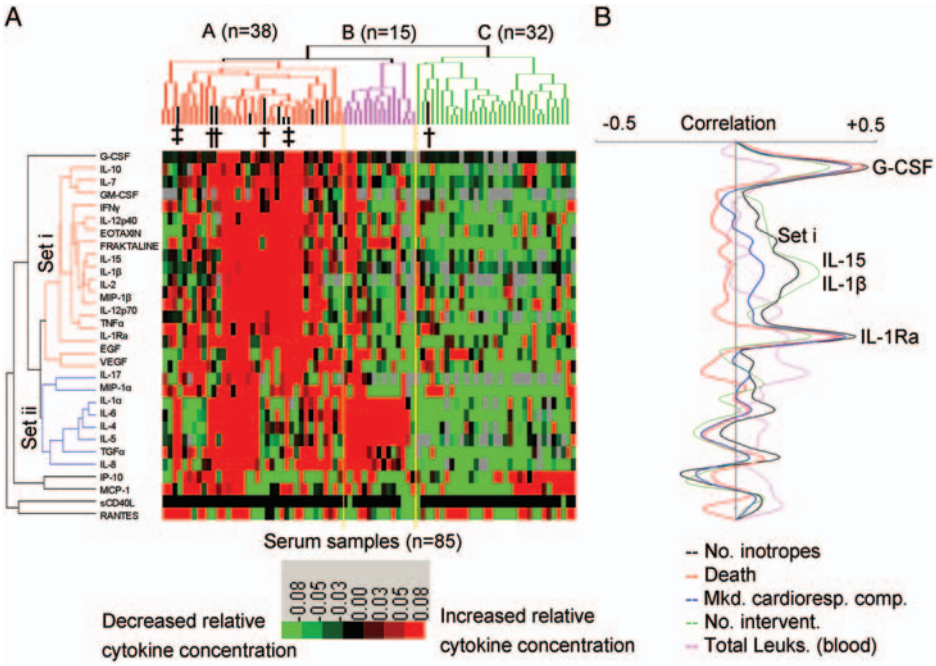

Figure 1. Patterns of mediator concentration in serum samples at hospital admission showing correspondence with inotrope use and cardio-respiratory compromise. *A,* Heat-map displaying the relative concentration of 30 cytokines in 85 serum samples from patients hospitalized with acute enterovirus 71 infection: 11 patients with cardio-respiratory compromise (black terminal branches within sample tree) and 74 patients without cardio-respiratory compromise. Samples and mediators were organized via a 2-way unsupervised hierarchical clustering algorithm along the horizontal and vertical axes, respectively. Patients who later died or developed marked cardio-respiratory compromise are labeled on the x-axis with †or ‡ symbols, respectively. Tile color indicates relative mediator concentration: *red,* increased; *green,* decreased; *black,* at median concentration (for the samples); *gray,* concentration below detection threshold in that sample. On the vertical axis, the mediators segregated into 2 main sets (set 1 and set 2). On the horizontal axis, the samples segregated into 3 main host response patterns: pattern A, high abundance among mediators within sets 1 and 2; pattern B, low abundance within mediator set 1 and high abundance within mediator set 2; pattern C, low abundance within mediator sets 1 and 2. A higher proportion of patients exhibiting pattern A had cardio-respiratory compromise compared with patients exhibiting the other patterns (10 [26%] of 38 patients vs 1 [2%] of 47 patients; $P = .002$). Significance was calculated by the Fisher exact test. *B,* Correlation plot between individual clinical parameters and mediator concentration among the samples. The Pearson correlation score (R) is presented on the x-axis for each mediator (y-axis). Five clinical parameters were examined: number of inotropic agents received during admission (*black line*); death (+1, died; −1, alive) (*blue line*); marked cardio-respiratory compromise (+1, present; −1, absent) (*red line*); number of clinical interventions during admission (ie, a lumbar puncture, provision of a single course of intravenous immunoglobulin (IVIG), or provision of a single inotropic agent counted as 1 intervention) (*green line*); total leukocyte count (*brown line*). Neighboring or individual cytokines whose corresponding concentration significantly correlates with a clinical parameter are labeled on the plot. Statistical significance was calculated by permutation (see Methods).

16 neighboring mediators within set 1 (G-CSF to IL-1Ra) and the number of inotropic agents received ($P = .03$). Correlation was strongest for G-CSF and IL-1Ra (Figure 1*B*). One pair of mediators from set 1, IL-1β and IL-15, also showed a significant positive association with overall number of clinical interventions undertaken during hospitalization ($P = .049$). G-CSF also exhibited a significant positive association with marked cardio-respiratory compromise and death ($P = .01$ and $P = .02$, respectively). There were no significant correlations between these parameters and mediator abundance within set 2.

Among the CSF samples, there was a significant correlation between the pattern of abundance within mediator set 3 and several clinical parameters linked to complicated EV71 illness: number of inotropic agents received by the patient, mortality, marked cardio-respiratory compromise, and CSF leukocyte count ($P < .01$ for all). Correlation with cardio-respiratory compromise and death was particularly strong for G-CSF and IL-1Ra (Figure 2*B*).

Distinct Patterns of Mediator Abundance Between the CSF and Serum Samples

Given the results of the unsupervised analysis for the CSF samples, the corresponding serum samples were reorganized to match the sample and cytokine order within the CSF. The resulting heat-maps exhibited distinct patterns of relative mediator abundance among CSF and serum samples (Figure 3).

The CSF-to-serum mediator abundance ratio was also measured across all paired samples from patients with CNS involvement ($n = 41$). The majority of the mediators exhibited a higher median concentration in the serum samples, including

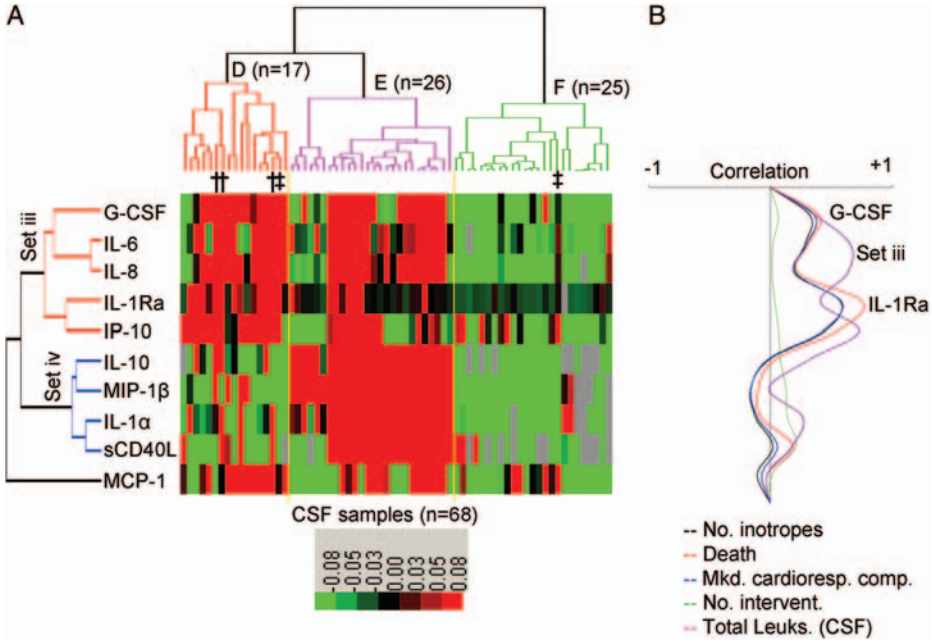

Figure 2. Patterns of mediator concentration in cerebrospinal fluid (CSF) samples at hospital admission showing correspondence with clinical central nervous system (CNS) involvement among patients with enterovirus 71 infection. *A,* Heat-map displaying the relative concentration of 10 cytokines within 68 CSF samples from patients hospitalized with acute EV71 infection: 36 patients with CNS complications (encephalitis, meningitis, or acute flaccid paralysis) and 32 patients with uncomplicated hand, foot, and mouth disease (HFMD). Samples and mediators were organized via an unsupervised hierarchical clustering algorithm along the horizontal and vertical axes, respectively. Patients who later died or developed marked cardio-respiratory compromise are labeled on the x-axis with †or ‡ symbols, respectively. Tile color indicates relative mediator concentration: *red,* increased; *green,* decreased; *black,* at median concentration (for the samples); *gray,* concentration below detection threshold in that sample. On the vertical axis, the samples segregated into 2 main sets (set 3 and set 4). On the horizontal axis, the samples segregated into 3 main host response patterns: pattern D, high abundance among mediators within set 3; pattern E, high abundance within mediator set 4; pattern F, low abundance within mediator sets 3 and 4. A higher proportion of patients exhibiting pattern D had complicated HFMD compared with patients exhibiting the other patterns (14 [82%] of 17 patients vs 22 [43%] of 51 patients; $P = .006$). Significance was calculated via the Fisher exact test. *B,* Correlation plot between individual clinical parameters and mediator concentration among the samples. The Pearson correlation score (R) is presented on the x-axis for each mediator (y-axis). Five clinical parameters were examined: number of inotropic agents received during admission (*black line*); death (+1, died; −1, alive) (*blue line*); marked cardio-respiratory compromise (+1, present; −1, absent) (*red line*); number of clinical interventions during admission (ie, a lumbar puncture, provision of a single course of IVIG, or provision of a single inotropic agent counted as 1 intervention) (*green line*); total leukocyte count (*brown line*). Neighboring or individual cytokines whose corresponding concentration significantly correlates with a clinical parameter are labeled on the plot. Statistical significance was calculated by permutation (see Methods).

IL-1Ra, G-CSF, and IL-1β. Only 4 mediators showed a higher concentration in the CSF samples: IL-6, IL-8, monocyte chemotactic protein 1 (MCP-1), and interleukin 13 (Figure 4*A*). Concentrations in CSF samples of G-CSF, IL-6, IL-8, IL-1Ra, and IP-10 showed a significant positive correlation with CSF leukocyte count ($P = .0014$ for G-CSF and $P < .001$ for the others) (Figure 4*B* and *C*).

Confirmation of Findings by Supervised Analysis

We sought to confirm the results of the unsupervised sample clustering through comparison against the findings from supervised analysis. All serum samples ($n = 88$) were manually organized into 4 clinical groups of patients: fatal ($n = 4$), marked cardio-respiratory compromise ($n = 6$), cardio-respiratory compromise ($n = 11$), and noncompromised ($n = 77$). As in the unsupervised analysis, serum levels for several mediators, including G-CSF, IL-1Ra, IL-10, and IL-1β, were significantly higher in patients with cardio-respiratory compromise compared with those in noncompromised patients ($P < .0001$, $P = .004$, $P = .002$, and $P = .013$, respectively) (Table 1). Levels of G-CSF, IL-1Ra, and IL-10 were also higher among patients with marked cardio-respiratory compromise ($P = .006$, $P = .04$, and $P = .025$, respectively) and those who died ($P = .005$, $P = .043$, and $P = .07$, respectively).

Furthermore, a serum G-CSF to interleukin 5 (IL-5) ratio of >100 was significantly associated with fatal cases compared with nonfatal cases (4 [100%] of 4 cases vs 10 [15%] of 66 cases; $P < .001$; accuracy, 85.7%; sensitivity, 100%; specificity, 84.8%). Combining a serum G-CSF/IL-5 ratio of >100 with clinical features of a raised heart rate (>140 beats per minute) and peak temperature (>38.5°C) during admission provided a highly accurate prognostic indicator for death (4 [100%] of 4

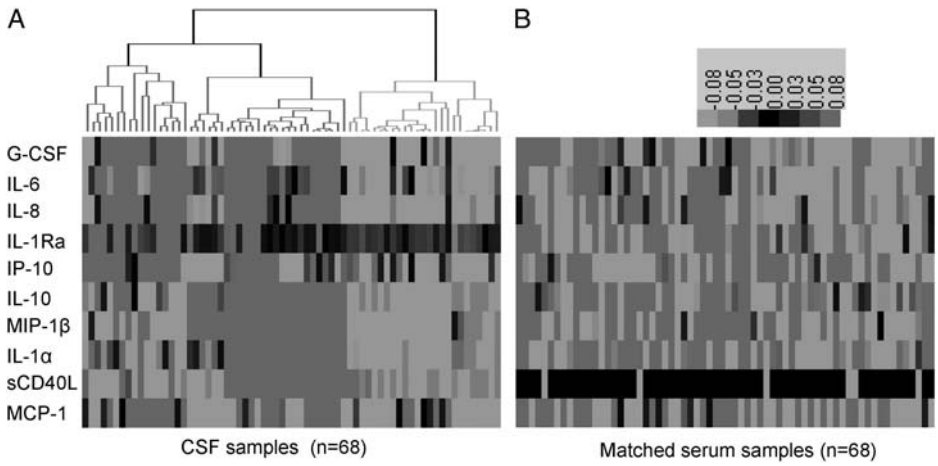

Figure 3. Divergent patterns of relative mediator concentrations demonstrated in cerebrospinal fluid (CSF) and serum samples, with heat-maps displaying relative mediator concentrations for CSF samples (*A*) and serum samples (*B*). CSF samples and mediators were organized via an unsupervised hierarchical clustering algorithm along the horizontal and vertical axes, respectively. The corresponding serum samples were then organized in matching mediator and sample order. The heat-maps display divergent patterns of relative mediator concentration (tile color) for corresponding CSF and serum samples.

fatal cases vs 3 [4%] of 66 nonfatal cases; $P < .001$; accuracy, 95.7%; sensitivity, 100%; specificity, 95.5%; positive predictive value, 57.1%; negative predictive value, 100%).

When the CSF samples were manually organized into clinical groups, the findings reflected those of the unsupervised analysis (Figure 5). All mediators in set 3 demonstrated

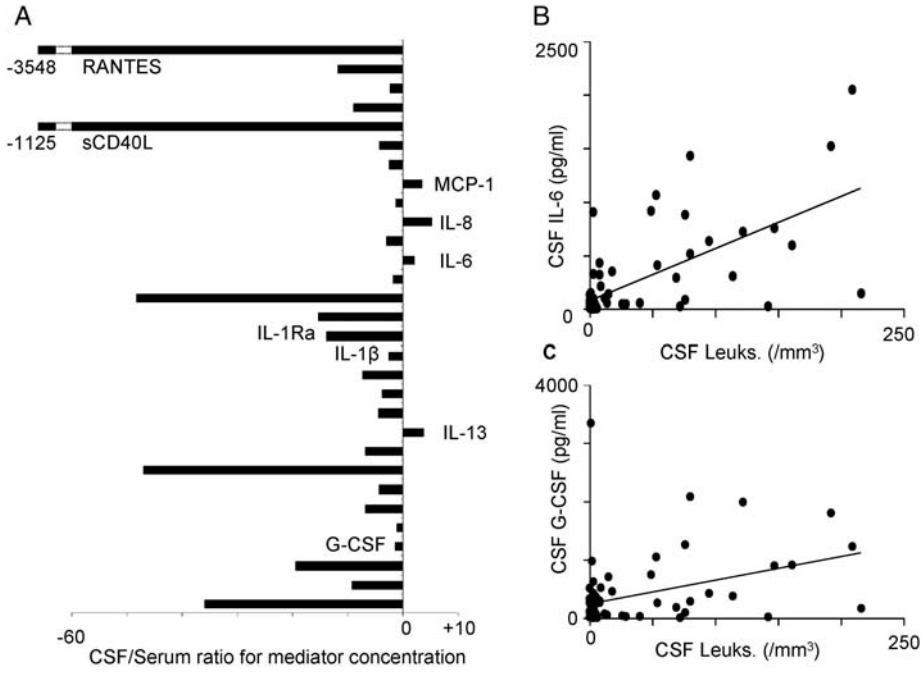

Figure 4. Majority of mediators exhibiting higher concentrations in the serum samples than in the cerebrospinal fluid (CSF) samples. *A,* Plot of the CSF/serum ratio for each mediator, based on the median concentration in CSF and serum samples in each patient with hand, foot, and mouth disease with central nervous system involvement ($n = 41$). CSF/serum ratio is presented on the x-axis (range, −60 to +10). The mediators ($n = 30$) are presented on the y-axis. The majority of the mediators (26 of 30) exhibit a negative ratio (ie, have a higher mediator concentration in the serum). The ratios for RANTES and sCD40L extend beyond the x-axis (actual values are presented on the plot). Also shown are plots of CSF IL-6 (*B*) and G-CSF (*C*) concentration against CSF total leukocyte count for each patient ($n = 71$). CSF IL-6 and G-CSF concentrations demonstrated significant positive linear correlations with total CSF leukocyte count ($r^2 = 0.43$, $P < .001$; $r^2 = 0.14$, $P = .0014$; respectively). Significance was tested via linear regression using Stata.

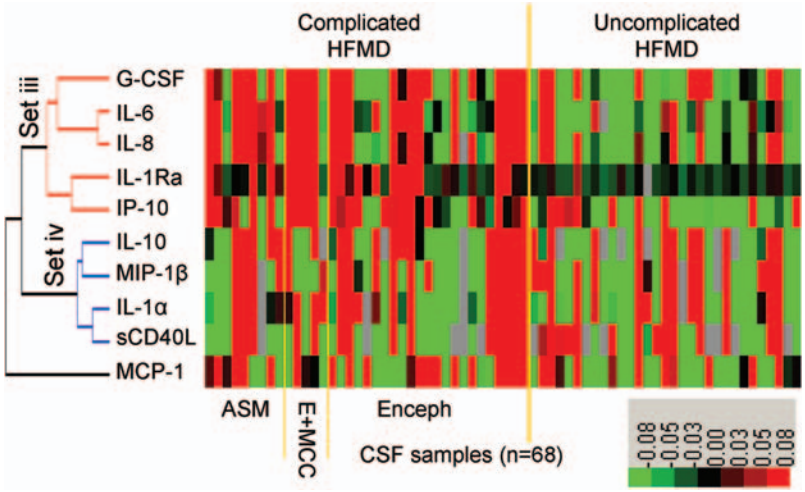

Figure 5. Mediator concentrations in cerebrospinal fluid (CSF) samples exhibiting correspondence with clinical groups. CSF samples ($n = 68$) were manually organized into 2 main groups: complicated hand, foot, and mouth disease (HFMD) and uncomplicated HFMD. The patients with complicated HFMD were organized into 3 clinical subgroups: ASM, aseptic meningitis; E + MCC, encephalitis and marked cardio-respiratory compromise; Enceph, encephalitis. The mediators were organized via an unsupervised hierarchical clustering algorithm. The heat-maps exhibit higher relative concentration for mediators composing set 3, among complicated compared with uncomplicated HFMD patients.

significantly higher CSF concentration among complicated and fatal cases compared with uncomplicated and nonfatal cases, respectively. IL-1Ra, IL-6, and IP-10 also showed significantly higher levels in cardio-respiratory compromised patients compared with noncompromised patients. G-CSF, IL-6, IL-8, and IP-10 exhibited higher median CSF concentrations in aseptic meningitis compared with encephalitis (Table 2).

DISCUSSION

Enterovirus 71 is now recognized as a major public health problem across the Asia-Pacific region [2]. Although the majority of children develop mild HFMD, a significant proportion of children suffer CNS complications, with or without cardio-respiratory compromise, which may be fatal. The pathogenesis of the cardio-respiratory compromise is uncertain. Early autopsy studies of patients with EV71 brainstem encephalitis and pulmonary edema found no histopathological evidence of cardiac injury or myocarditis [4], although elevation of troponin I (a specific marker for cardiac damage) is predictive of cardiac failure [12]. Our data suggest that cardio-respiratory compromise is associated with a widespread elevation of both pro-inflammatory and anti-inflammatory mediators in the serum and CSF. Serum IL-1Ra and G-CSF levels were significantly elevated in patients who died, with a serum G-CSF/IL-5 ratio of >100 being the most accurate prognostic marker. Although correlation with viral load would have been interesting, EV71 is poorly detected in the CSF and serum [4, 13].

Patients with relatively high serum abundance of a subset of mediators (set 1) were significantly more likely to require inotropic agents. These mediators included both pro-inflammatory agents (eg, IL-1β and TNF-α) anti-inflammatory agents (eg, IL-1Ra and IL-10) and the stem-cell-mobilizing factor G-CSF. Evidence from other diseases supports the putative importance of an elevated pro-inflammatory response in the periphery contributing to EV71-associated cardiac failure. For example, elevated IL-1β level in the lungs is linked to acute respiratory distress syndrome [14]. Similarly, the acute cardiac compromise occasionally seen in children with severe burns is thought to be driven by the adverse effect of pro-inflammatory mediators, particularly IL-1β and TNF-α, on the heart [15]. Both IL-1β and TNF-α have a negative inotropic effect on heart contractility [16], and they stimulate cardiomyocyte apoptosis in experimental models of burn injury [17]. Interestingly, a recent ultrastructural study of cardiac tissue recovered from patients ($n = 7$) with EV71-associated cardiac failure showed evidence of both myofibrillar degeneration and cardiomyocyte apoptosis [18]; a similar pattern of ultrastructural damage to the heart has been observed following burn injury [17]. As in severe burns, in EV71 illness, the pro-inflammatory response may influence the development of acute cardio-respiratory compromise and death, but leave limited evidence of cardiac damage at autopsy.

Although serum IL-1β concentrations were higher among fatal cases compared with noncompromised cases, this finding was not significant, probably reflecting the relatively small number of patients who died. A previous study also showed no significant association of IL-1β level with death, but it did not examine the relationship with inotrope use or other markers of cardiac failure [6].

Table 2. Clinical Features and Cerebrospinal Fluid Mediator Concentrations for 71 Patients With Enterovirus 71 Infection

	Patient Groups and Comparisons												
Clinical Parameters	Uncomplicated HFMD	Complicated HFMD	*P*	Nonfatal	Fatal	*P*	No CRC	CRC	*P*	Marked CRC	*P* [a]	Encephalitis	ASM
No. (%) of patients	35 (49)	36 (51)	…	68 (96)	3 (4)	…	61 (86)	10 (14)	…	5 (7)	…	22 (31)	8 (11)
Age, months	25.7	20.2	NS	22.99	13.6	NS	21	24.5	NS	21.2	NS	20	17.1
Peak hospital temperature, °C	38.2	39	.002	38.5	39.7	.016	38.5	39.4	.004	39.7	.002	38.8	38.8
Total duration of fever, d	2	3.5	<.001	2.8	4	NS	2.5	4	.015	4	.023	3	3.75
Heart rate, bpm[b]	142	146	NS	144	155	NS	143	158.5	.028	147	NS	153.5	142.5
Respiratory rate, bpm[c]	36	35	NS	36	39	NS	36	35	NS	39	NS	33	41
Duration of fever at home, d	1	1	NS	1	3	NS	1	2.5	NS	3	.037	1	1.5
Total leukocyte count, $\times 10^9$ cells/L	13.3	12.7	NS	12.7	15.2	NS	12.7	13.1	NS	17.7	.045	12.1	12.0
CSF leukocyte count, cells/mm^3	2	44.5	<.001	4	114	.011	3	87.5	<.001	114	.001	23.5	39
CSF lymphocyte count, %	100	97.5	<.001	100	97	NS	100	96	<.001	97	.018	97.5	99
CSF protein level, g/dL	0.27	0.36	.008	0.3	0.54	.016	0.28	0.44	<.001	0.54	.003	0.31	0.36
CSF glucose level, mmol/L	3.4	3.4	NS	3.4	5.1	.012	3.4	4.3	.028	5.1	.001	3.3	3.3
Plasma glucose level, mmol/L	4.9	5	NS	4.9	7.1	NS	4.9	6.0	NS	7.4	NS	5	4.8
CSF/plasma glucose ratio	0.67	0.67	NS	0.66	0.72	NS	0.67	0.69	NS	0.69	NS	0.6	0.68
CSF IL-1Ra level, pg/mL	37.7	102.5	<.001	51.1	5490.6	<.001	49.8	283.1	.002	990.2	.031	95.4	82.4
CSF G-CSF level, pg/mL	105.8	294.0	.016	139.0	1986.8	.01	148.0	401.7	NS	1258.3	NS	275.5	310.4
CSF IL-6 level, pg/mL	27.0	251.5	<.001	50.6	720.7	.02	54.4	468.2	.047	720.7	.037	118.1	305.1
CSF IL-8 level, pg/mL	33.7	179.2	<.001	69.1	443.2	.018	70.2	316.0	NS	443.2	NS	101.0	179.2
CSF IP-10 level, pg/mL	349.9	832.0	.002	592.6	8374.1	.001	585.7	3569.6	.04	6438.8	.029	764.5	910.0

Seventy-one of the 88 patients in the study underwent lumbar puncture. Data are medians unless otherwise indicated. *P* values indicate significant differences in parameters between patients with uncomplicated and complicated HFMD; nonfatal and fatal cases; and no CRC and CRC.

Abbreviations: ASM, aseptic meningitis; CRC, cardio-respiratory compromise; CSF, cerebrospinal fluid; G-CSF, granulocyte colony-stimulating factor; HFMD, hand, foot, and mouth disease; IL-1Ra, interleukin 1 receptor antagonist; IL-6, interleukin receptor 6; IL-8, interleukin 8; NS, not significant.

[a] Between patients with marked CRC and all other patients. Significance tested via Mann-Whitney *U* test or Fisher exact test.

[b] Beats per minute.

[c] Breaths per minute.

IL-1Ra is a natural antagonist against IL-1 cytokines. It binds to IL-1 receptors and blocks the binding of IL-1α and IL-1β. Descriptive studies have shown plasma IL-1Ra levels to correlate with the extent of cardiomyocyte loss and the severity of hemodynamic and clinical impairment among patients with acute myocardial infarction [19, 20]. However, intervention studies using anakinra, a synthetic pharmacological derivative of IL-1Ra, suggest IL-1Ra may also offer potential therapy against cardiac damage [21]. Anakinra improves cardiac function in animal models of acute cardiac injury [22] and rheumatoid patients with cardiac impairment [23]. It is currently undergoing clinical trials in acute coronary syndromes [24, 25]. Based on these intervention studies, elevation of IL-1Ra levels during EV71 illness may reflect a "restorative" host response to minimize inflammation and tissue damage during EV71 illness.

G-CSF is a potent stem cell chemo-attractant, which in cardiac ischemia is thought to promote the migration of stem cells to areas of cardiac damage [26]. In clinical studies it improves cardiac function following acute myocardial infarction [27]. IL-10 is an anti-inflammatory mediator known to down-regulate IL-1β, IL-6, TNF-α, and INF-γ production [28]. In experimental models of cardiac impairment, elevated IL-10 level is associated with improved function [29]. Furthermore, provision of IL-10 has been shown to mitigate cardiac dysfunction in models of viral infection of the brain and heart [30]. IL-10 has been used to attenuate the pro-inflammatory response associated with psoriasis [31]. Like IL-1Ra, the paired elevation of G-CSF and IL-10 may again reflect a restorative host response to minimize inflammation and tissue damage during EV71 infection.

Elevated IL-1Ra and G-CSF levels in the CSF samples were again associated with cardio-respiratory compromise and death. However, levels of these mediators were elevated in close association with those of IL-6, IL-8, and IP-10. IL-1β was not consistently detected in the CSF, concordant with previous studies of brain injury suggesting CNS IL-1β concentration increases are early and transient [32].

A relatively narrower range of mediators was consistently detected in the CSF than in the serum. Few studies have measured a broad panel of mediators in CNS and vascular compartments. One study has demonstrated lower concentrations of several mediators in brain microdialysate than in plasma among traumatic brain injury patients [32]. The results may reflect the limited number and/or diversity of immune cells in the CNS compartment [33, 34].

The differences between CSF and serum responses were especially well demonstrated for patients with matched samples (Figure 3). It has previously been proposed that the CNS may be the source of the inflammatory cytokines detected in the serum of patients with EV71-associated cardiac dysfunction [9], whereas others have suggested a combination of CNS and systemic inflammatory responses occur [6]. The data from our study support distinct inflammatory responses occurring in the CNS and peripheral compartments. This could imply that the inflammatory responses are occurring completely independently in these compartments or that the CNS inflammatory response stimulates a peripheral immune response. Stroke studies have observed rapid and dramatic activation of the peripheral immune system following brain insult [35, 36], with the peripheral responses exhibiting cytokine patterns which are distinct from those occurring in the CNS [36, 37]. The mechanisms underlying the induction of the peripheral immune responses following brain insult are unclear. Sympathetic neural signals activating leukocytes in the spleen and lymph nodes has been postulated [36, 38].

CSF IL-1Ra levels are elevated following acute subarachnoid hemorrhage, with higher levels in those with a more complicated clinical course [39]. Again, intervention studies show IL-1Ra protects against ischemic brain damage in animal models, and it is being assessed as a clinical neuroprotective agent in stroke [40]. G-CSF treatment has also been shown to protect against ischemic brain damage in animal models, with reduced infarct volumes and improved functional outcome. [41, 42]. G-CSF is being assessed as an adjunctive neuroprotective agent for stroke in clinical trial [43, 44].

Intravenous immunoglobulin reduces systemic levels of pro-inflammatory mediators during EV71 illness [45] and has been reported to reduce morbidity [46]. The use of more targeted host modifying agents, such as IL-1Ra or G-CSF, might provide further benefit. However, because levels of pro-inflammatory mediators, such as IL-1β, can be abruptly elevated, assessment of the optimum time frame for delivering these agents may be needed to ensure success.

IL-6 and IL-8 CSF levels are elevated in a broad range of CNS inflammatory disorders, including viral encephalitis, meningitis, stroke, subarachnoid hemorrhage, and traumatic brain injury [47–50]. CSF IL-6 levels have previously been reported to be higher among EV71-infected patients with encephalitis and cardio-respiratory compromise than among those with encephalitis [8].

Higher CSF IL-6 levels in meningitis than in encephalitis have been reported elsewhere [8]. We found CSF IL-6, IL-8, G-CSF, and IP-10 levels were nonsignificantly higher in meningitis cases than in encephalitis cases without cardio-respiratory compromise. However, these mediators demonstrated a significant positive correlation with total CSF leukocyte count. The higher CSF mediator concentrations in meningitis compared with encephalitis and among fatal compared with nonfatal cases may reflect the higher CSF leukocyte counts among the corresponding patient groups. These findings are consistent with the suggestion that during CNS inflammation CSF mediators are secreted by activated leukocytes that migrate into the CSF compartment [33, 34].

The pro-inflammatory and anti-inflammatory responses associated with different clinical syndromes during EV71 illness underscore the complex interplay of mediators during this infection. The separate patterns of mediator response observed in the CNS and vascular compartments suggest distinct immune responses occurring in each compartment, although these responses may still be interlinked. Because IL-1β impairs heart function and both IL-1Ra and G-CSF are currently being assessed as treatments for acute cardiac impairment, the findings suggest we have identified both functional markers of EV71-related cardiac dysfunction and potential avenues for treatment of this often fatal complication.

Notes

Acknowledgments. We thank all the medical staff at Sibu Hospital for their support.

Financial support. This work was supported by the Wellcome Trust (Clinical Training Fellowship 076330/B/05/Z to M. H. O. and International Collaborative Health Research Grant WT071588MA to M. J. C.); the Medical Research Council (Senior Clinical Fellowship G116/194 to T. S.); and the National Institute for Health Research (Clinical Lectureship to M. J. G.).

Potential conflicts of interest. M. J. C. is chief scientific officer to Sentinext Therapeutics, Penang, Malaysia. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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