Status of Cellular Rather Than Humoral Immunity is Correlated with Clinical Outcome of Enterovirus 71

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ABSTRACT: We validated specific cellular and humoral immune response of cases of enterovirus 71 (EV71) infection and correlated immune response with clinical outcome. After obtaining informed consent, we enrolled 30 EV71 cases including 7 cases with brainstem encephalitis plus pulmonary edema, 12 cases of CNS (CNS) involvement and 11 uncomplicated cases. We measured antibodies specific to EV71, lymphocyte proliferation response and EV71-stimulated cellular response of Th1/Th2 cytokines and chemokines. The 7 EV71 cases involving brainstem encephalitis plus pulmonary edema had a significantly lower cytokine (γ-interferon) and proinflammatory cytokines. However, cases with pulmonary edema had significantly lower cytokine (γ-interferon) and proinflammatory cytokines. The laboratory method for measuring EV71 neutralizing antibodies demonstrated no difference among cases. These results suggest lower EV71-specific cellular response may be associated with immunopathogenesis of EV71-related pulmonary edema.

Most EV71 fatalities were cases of fulminant pulmonary edema (15). However, EV71 infection causes very diverse symptoms, ranging from none (about 71%) to fatality (about 0.05%) (17,18). It remains unknown why different hosts of the same EV71 infection have such a range of clinical outcomes (17,18). Perhaps this range is related to virulence or load of the virus, or particular host factors. To date no relationship has been found between EV71 genotypes and clinical outcome (19,20), and EV71 virulence factors have not been clarified. It is possible that host factors, especially host immune response, may be of ultimate importance to clinical outcome.

To clarify severe EV71 infection pathogenesis, we investigated factors of cellular versus humoral immune response and correlated this with clinical outcome.

SUBJECTS AND METHODS

Subjects. After National Taiwan University Hospital Research Ethics Committee approved this study and informed parental consent was obtained, 30 EV71 cases of different severity were enrolled. EV71 infection was confirmed by positive EV71 isolation and/or positive EV71 specific IgM at the onset of their disease.

The CNS involvement was indicated in four types of cases. Those with aseptic meningitis had headache and irritability along with cerebrospinal fluid (CSF) pleocytosis (>5 leukocytes/μL) and without an altered level of consciousness. The second type of cases involved encephalitis had altered level of consciousness plus cerebrospinal fluid (CSF) pleocytosis. Poliomyelitis-like syndrome was defined as acute limb weakness and decreased reflex and muscle strength. Finally, cases with encephalomyelitis had the occurrence of both encephalitis and poliomyelitis-like syndrome.

Laboratory studies. EV71-specific Humoral Immunity: For EV71-specific humoral immunity, EV71 neutralization antibody and IgM were measured. EV71 IgM was measured at the onset of disease and EV71 neutralization antibody was determined at the same time with the measurement of cellular immunity. The laboratory method for measuring EV71 neutralizing antibody followed the standard protocol of neutralization test in microtiter plates (21). Serum samples were inactivated at 56°C for 30 min and then serially diluted from 2- to 1,024-fold. We mixed and incubated (37°C, 2 h) 50 μL of each diluted serum with 50 μL containing one hundred 50% tissue culture infective dose (TCID 50) of EV71 strain TW/2272/98 (GenBank accession number AY193031).

Abbreviations: EV71, enterovirus 71; IFN-γ, interferon-γ; IL-6, interleukin-6; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PHI, phytohemagglutinin; RANTES, regulated on activation, normal T expressed and secreted; TCID 50, 50% tissue culture infective dose.
AF119795) in microtiter plates (19) Two replicate wells were used per serum dilution. Then microtiter plates were seeded with 100 µL of rhabdomyosarcoma cells (8 × 10^6 cells per mL) and incubated (37°C 5% CO_2 atmosphere, 2–7 d). Each test was run with cell control, serum control and virus back titration with 100–0.1 TCID_50. Cytopathic effect was observed under an inverted microscope after incubation, and seroconversion was determined when Cytopathic effect was observed in 1 TCID_50 of the virus back titration. Microtiter plates were fixed with 5% glutaraldehyde and stained with 0.1% crystal violet. Seropositivity was defined as seroconversions at 8. For EV71 IgM detection, EV71 isolate TW/2086/98 was amplified and purified as an antigen for use in /H9262—capture ELISA, whose sensitivity and specificity was 91.5% and 93.1%, respectively (22).

**Cellular immunity: Proliferation response, cellular Th1/Th2 cytokine and chemokine response.** To isolate peripheral blood mononuclear cells (PBMCs), blood samples were heparinized and subjected to Ficoll-Hypaque (Pharmacia Diagnostics AB, Uppsala, Sweden) gradient centrifugation. Cells at the interface were removed carefully and washed twice with PBS. The isolated PBMCs were cultured in 96-well round-bottom microplates (3 × 10^5 cells per well in 0.2 mL of culture medium) in RPMI 1640 medium supplemented with 1 mM glutamine, 1 mM sodium pyruvate, 50 µM 2-ME, 100 µM penicillin, 0.1 mg/mL streptomycin, and 2% human AB serum (Biocell Laboratories Inc., Rancho Dominguez, CA).

In addition, PBMCs were stimulated with phytohemagglutinin (PHA) (8 µg/mL) or different concentrations of EV71 whole virus antigen (1, 5, and 10 µg/mL) for 6 d of culture, 1 mCi of 3H-thymidine (Amersham, Buckinghamshire, England) was added to each well for 18 h, and the incorporated radioactivity was measured by use of Bio-Rad protein assay reagents. After 3 d of incubation, supernatant was collected and assayed for production of Th1/Th2 (T helper 1/T helper 2) cytokines (γ-interferon, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13 and TNF-α) and chemokines (eotaxin, IL-8, IP-10, MCP-1, MCP-2, MCP-3, MCP-4, MIP-1α and RANTES), which were measured with protein array kits (Fast Quant Microspot Assay, Schleicher & Scuell, Dassel, Germany). All the assays were triplicate. The detectable levels for IL (interleukin)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, γ-interferon and TNF-α (tumor necrosis factor-α) were 3–3,000, 3–3,000, 30–12,000, 100–12,000, 30–12,000, and 4–3,000 pg/mL, respectively. The detectable level for IP-10 (interferon γ-inducible protein-10) was 12.2–12,500 pg/mL and that for eotaxin, IL-8, MCP (monocyte chemotactant protein)-1, MCP-2, MCP-3, MCP-4, MIP (macrophage inflammatory protein)-α and RANTES (regulated on activation, normal T) (Biocell Laboratories Inc., Rancho Dominguez, CA) were not distributed normally, we used Kruskal-Wallis test and Mann-Whitney U-test for continuous variables. Statistical analysis was performed using StatView (SAS Institute, Cary, NC). Data are presented as medians (range) or means ± standard errors.

**RESULTS**

**Clinical syndromes and outcomes.** Immune work-up was completed with 30 EV71 cases. This included 7 cases with brainstem encephalitis plus pulmonary edema, 12 cases of CNS (CNS) involvement including 5 of septic meningitis, 5 of encephalitis, 1 of poliomyelitis-like syndrome and 1 of encephalomyelitis, and 11 uncomplicated cases of hand-foot-and-mouth disease or herpangina. In 22 cases, we isolated the virus itself, and detected positive IgM of EV71; the remaining 8 cases were negative for the virus but positive for EV71 IgM at the onset of disease. Their median (range) age at the onset of disease was 0.55 (0.16–1.6) year for the 7 cases with brainstem encephalitis plus pulmonary edema, 2.4 (1.3–4.5) years for 12 cases of CNS involvement and 1.8 (0.37–5.0) years for 11 uncomplicated cases; the age at the onset of disease was significantly younger for the group with brainstem encephalitis plus pulmonary edema than the other two groups (p = 0.007 with Kruskal-Wallis test). The male-to-female ratio was 3:4 for the 7 cases with brainstem encephalitis plus pulmonary edema, 7:5 for 12 cases with CNS involvement and 6:5 for the 11 uncomplicated cases (p = 0.80 with χ² test). The median (range) interval between their disease onset and enrollment in this study was not significantly different among the three groups: 1.9 (1.1–2.9) years for the 7 cases with brainstem encephalitis plus pulmonary edema, 2.5 (0.7–5.2) years for 12 cases with CNS involvement, and 2.6 (0.7–2.7) for 11 uncomplicated cases (p = 0.17 with Kruskal-Wallis test). Among the seven cases of brainstem encephalitis plus pulmonary edema, one recovered completely, one had sequelae of right upper-limb paralysis plus scoliosis, and five had polio-like sequelae and hyperventilation with ventilator support. All remaining 11 cases of CNS involvement as well as the 12 uncomplicated cases recovered without neurologic sequelae.

**EV71-specific neutralizing antibody titers.** EV71-specific humoral immunity is shown in Fig. 1, and neutralizing antibody titers did not differ significantly among all the three groups of different severity (p = 0.567 with Kruskal-Wallis test). Age <3 y and gender did not affect the neutralizing antibody titers significantly, either (p = 0.68 and p = 0.83 with Mann-Whitney U-test, respectively).

**Cell-mediated Immunity.** PHA-stimulation index is shown in Fig. 2. EV71 cases with pulmonary edema had significantly lower PHA-stimulated lymphocyte proliferation (median...
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Peak enterovirus 71 (EV71) stimulation index among EV71 cases with different severity (EV71 cases with pulmonary edema, EV71 cases with CNS involvement, and uncomplicated EV71 cases). All peak EV71 stimulation index were statistically identical among the three groups (p = 0.31 with Kruskal-Wallis test)

Overall only 60% (18/30) had peak EV71 SI > 2, and included 4 (57%) of pulmonary edema cases, 6 (50%) of CNS cases, and 8 (73%) of uncomplicated cases (p = 0.53 with $\chi^2$ test). Age < 3 y and gender did affect the EV71-stimulation index significantly (p = 0.11 and p = 0.47 with $\chi^2$ test, respectively), either.

**Cellular cytokine and chemokine response after EV71 stimulation.** Difference in the cytokine and chemokine response of peripheral mononuclear cells with and without EV71 antigen stimulation is shown in Table 1. In comparison with condition of absence of EV71 antigen, EV71 antigen stimulation induced a significant increase in cellular Th1 cytokine (γ-interferon) but a significant decrease in Th2 cytokine (IL-5). There were a γ-interferon median increase of 42 pg/mL and an IL-5 median decrease of 1.45 pg/mL. As well, there was an increase of the pro-inflammatory cytokines IL-6 (median increase, 500 pg/mL), IL-1β (median increase, 36 pg/mL), and TNF-α (median increase, 86 pg/mL). Overall, EV71 stimulation had a positive effect on cellular Th1 cytokine and pro-inflammatory cytokine response, but no effect or a mildly negative effect on Th2 cytokines.

**Table 1. The difference in peripheral mononuclear cellular cytokine and chemokine response with and without EV71 antigen stimulation.**

| Cytokine or chemokine | Median (range) difference (pg/mL) | P-value with Wilcoxon signed rank test |
|------------------------|----------------------------------|---------------------------------------|
| Th1 cytokine           |                                  |                                       |
| γ-interferon           | 42 (-19–6,541)                   | <0.001                                |
| Interleukin-1β         | 1.1 (-62–138)                    | NS                                    |
| Th2 cytokine           |                                  |                                       |
| Interleukin-4          | -3.9* (-137–289)                 | NS                                    |
| Interleukin-5          | -1.45* (-14–8)                   | <0.022                                |
| Interleukin-10         | 0 (-2,713–1,123)                 | NS                                    |
| Interleukin-13         | 99 (-1,239–4,211)                | NS                                    |
| Pro-inflammatory cytokine |                                  |                                       |
| Interleukin-1β         | 36 (-73–453)                     | <0.001                                |
| Interleukin-6          | 500 (-1,088–7,170)               | <0.001                                |
| TNF-α                  | 86 (-75–605)                     | <0.001                                |
| Chemokine              |                                  |                                       |
| Interleukin-8          | -731* (-1,711–2,027)             | 0.06                                  |
| Eotaxin                | 0 (-35–9)                        | NS                                    |
| IP-10                  | 87 (0–4,885)                     | <0.02                                 |
| MCP-1                  | 8.3 (-2,038–3,373)               | NS                                    |
| MCP-2                  | 1,051 (0–5,881)                  | <0.001                                |
| MCP-3                  | 897 (-30–2,527)                  | <0.001                                |
| MCP-4                  | 7.25 (-57–59)                    | <0.05                                 |
| MIP-1α                 | 1,635 (-1,020–5,266)             | <0.001                                |
| RANTES                 | 321 (-787–3,184)                 | 0.043                                 |

The numbers in parenthesis are the ranges of the difference for peripheral mononuclear cellular cytokine and chemokine response with and without EV71 antigen stimulation.

* The “-” denotes decreased response after EV71 stimulation. P-values were measured to compare the difference of cellular cytokine/chemokine response with or without EV71 antigen stimulation by using Wilcoxon signed rank test.

NS, not significant; TNF-α, tumor necrosis factor-alpha; IP, interferon γ-inducible protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T expressed and secreted.
After EV71 stimulation, certain cellular chemokine responses significantly increased, including IP-10 (median increase, 87 pg/mL), MCP-2 (median increase, 1,051 pg/mL), MCP-3 (median increase, 897 pg/mL), MCP-4 (median increase, 7.25 pg/mL), MIP-1α (median increase, 1,635 pg/mL), and RANTES (median increase, 325 pg/mL).

After observing the significant increase in cellular response of γ-interferon, IL-1β, IL-6, TNF-α, IP-10, MCP-2, MCP-3, MCP-4, MIP-1α, and RANTES, and the significant decrease in IL-5, we compared the cellular response of these cytokines/chemokines with severity of cases. Cases with pulmonary edema were found to have significantly lower cellular γ-interferon (Fig. 3; p = 0.04), lower cellular IL-1β, lower cellular IL-6, lower cellular TNF-α response and lower cellular MIP-1A response in comparison with other EV71 cases. In cases with pulmonary edema the percentage of increase over the median level of cellular γ-interferon, IL-1β, IL-6, TNF-α and MIP-1α was lowest (Table 2). Uncomplicated EV71 cases had highest percentages of increase over the median level of cellular γ-interferon, IL-1β, IL-6, and TNF-α (Table 2). Age and gender did not affect their cellular cytokine response significantly. After age and gender adjustment, the percentage of increased cellular cytokines was still significantly different among the three groups with different clinical severity (p = 0.02 for γ-interferon, IL-1β, IL-6, and TNF-α) (Fig. 4).

In contrast, 67% (8/12) cases with CNS involvement and 64% (7/11) uncomplicated cases had decrease over the median level of cellular IL-5 response after EV71 stimulation whereas none of the pulmonary edema cases had decrease of cellular IL-5 response (p = 0.01). In general, EV71 antigen enhanced cellular Th1 cytokine, pro-inflammatory cytokine and chemokine response, and reduced cellular Th2 cytokine (IL-5) response in most cases of CNS involvement and uncomplicated cases. However, EV71 antigen had little effect on cellular cytokine and chemokine response in most cases with brain-stem encephalitis plus pulmonary edema.

In summary, EV71 cases had highest percentages of increase over the median level of cellular γ-interferon, IL-1β, IL-6, and TNF-α (Table 2). Age and gender did not affect their cellular cytokine response significantly. After age and gender adjustment, the percentage of increased cellular cytokines was still significantly different among the three groups with different clinical severity (p = 0.02 for γ-interferon, IL-1β, IL-6, and TNF-α) (Fig. 4). In contrast, 67% (8/12) cases with CNS involvement and 64% (7/11) uncomplicated cases had decrease over the median level of cellular IL-5 response after EV71 stimulation whereas none of the pulmonary edema cases had decrease of cellular IL-5 response (p = 0.01). In general, EV71 antigen enhanced cellular Th1 cytokine, pro-inflammatory cytokine and chemokine response, and reduced cellular Th2 cytokine (IL-5) response in most cases of CNS involvement and uncomplicated cases. However, EV71 antigen had little effect on cellular cytokine and chemokine response in most cases with brain-stem encephalitis plus pulmonary edema.

Table 2. The percentage of cases with a response above the median level after EV71 stimulation among EV71 cases with different severity

| Cytokine/Chemokine | Pulmonary edema | CNS involvement | Uncomplicated cases | P-value |
|--------------------|----------------|-----------------|---------------------|---------|
| Th1 Cytokine       |                |                 |                     |         |
| γ-interferon >42 pg/mL | 1 (14%) | 5 (42%) | 8 (73%) | 0.04 |
| Th2 Cytokine       |                |                 |                     |         |
| Interleukin-5 < -1.45 pg/mL* | 0 | 8 (67%) | 7 (64%) | 0.01 |
| Pro-inflammatory cytokine |               |                 |                     |         |
| Interleukin-1β >36 pg/mL | 1 (14%) | 5 (42%) | 8 (73%) | 0.04 |
| Interleukin-6 >500 pg/mL | 1 (14%) | 5 (42%) | 8 (73%) | 0.04 |
| TNF-α >86 g/mL | 1 (14%) | 5 (42%) | 8 (73%) | 0.04 |
| Chemokine |                |                 |                     |         |
| IP-10 >87 pg/mL | 1 (14%) | 7 (58%) | 4 (36%) | 0.14 |
| MCP-2 >1051 pg/mL | 1 (14%) | 6 (50%) | 5 (45%) | 0.24 |
| MCP-3 >897 pg/mL | 1 (14%) | 6 (50%) | 5 (45%) | 0.24 |
| MCP-4 >7.25 pg/mL | 1 (14%) | 6 (50%) | 5 (45%) | 0.24 |
| MIP-1α >1635 pg/mL | 1 (14%) | 8 (67%) | 3 (27%) | 0.04 |
| RANTES >321 pg/mL | 2 (29%) | 6 (50%) | 4 (36%) | 0.62 |

* The "-" denotes decreased response after EV71 stimulation. P-value was measured to compare the percentages of cases with a response above the median level by using likelihood ratio χ² test among the three groups.

DISCUSSION

This study suggests that cellular immunity rather than humoral immunity may be associated with the clinical outcome of EV71 infections. Age and gender may influence the immunity, however we demonstrate cellular immunity (PHA stimulation index) and cellular cytokine (γ-interferon, IL-1β, IL-6, and TNF-α) response were significantly weaker in EV71 cases with pulmonary edema than the other EV71 cases after age and gender adjustment. On the contrary, the humoral immunity (EV71-specific neutralizing antibody) did not differ significantly among cases with different clinical severity. This study was an exploratory study with the small number of the cases and was not investigated prospectively, so the results

Figure 4. Cellular interferon-γ response after EV71 stimulation at the concentration of 10 μg/mL among EV71 cases with different severity (EV71 cases with pulmonary edema, EV71 cases with CNS involvement, and uncomplicated EV71 cases). EV71 cases with pulmonary edema had significantly lower interferon-γ response than the other EV71 cases (p = 0.04, measured to compare the percentages of a response over the median level of increase of all the EV71 cases by using likelihood ratio χ² test).
must be validated by further prospective immunologic studies in EV71 cases or host immune genetic studies.

In the most severe EV71 cases with pulmonary edema there is lower cellular Th1 cytokine coupled with a lower lymphocyte proliferation response. Higher incidence of most severe EV71 diseases were found in children under 3 y of age (15,16), and this might be explained by their weaker cellular immunity. We hypothesize that lower cellular immunity may delay viral killing or clearance, thus resulting in viral dissemination, sustained systemic inflammatory response and subsequent pulmonary edema. Host genetic factors may also play an important role and will require further investigation. Yang KD et al. reported that patients with EV 71 meningoencephalitis had a higher frequency of G/G genotype with a polymorphism of the cytotoxic T lymphocyte antigen-4 at position 49 of exon 1 than did control subjects without meningoencephalitis. In these cases there was no difference in specific EV 71 neutralizing antibody titers in the 2 groups (23). In our study, humoral immunity did not affect the clinical outcome of EV71 infection, either. Results from the two studies suggest that younger children with genetics involving decreased cellular rather than humoral response may be linked to severe EV 71 infection.

In this study control for differences in the ages of patients was tried in each of three presentations, but truly valid conclusions regarding differences in cell-mediated immune response in the brainstem encephalitis plus pulmonary edema group can only be established using age matched controls in the CNS involvement and uncomplicated disease groups. Another query is whether maternal EV71 could have been important in later cellular immune response antibody making the lower cellular EV71 immune response of the youngest subjects, as maternal antibody might be after administration of live viral vaccines. However, we had a seroepidemiology study, done in 1999 (18), which showed that 94% (177/189) of 3- to 6-month-old children and 92% (265/287) of 7- to 12-month-old children did not have EV71 neutralizing antibody. The 8% of 7- to 12-month-old children might get their antibody through natural EV71 infection rather than maternal antibody. The results suggested that even the youngest group of EV71 cases seldom had maternal EV71 antibody, so their lower cellular immunity might be related to other causes rather than maternal antibody.

Our previous studies reported that patients with pulmonary edema had dramatically high blood values of IL-1β, IL-6, tumor necrosis factor-α, white blood cell counts, and glucose levels (24,25). These findings suggest that a combination of CNS and systemic inflammatory response may trigger EV71-related cardiopulmonary collapse (24). Another study of patients with pulmonary edema showed significant immunomodulator elevation as well as lower circulating CD4+ T-cells, CD8+ T-cells, and natural killer cells (26). They also suggested that the combination of an extensive systemic and CNS inflammatory response and lymphocyte depletion appears to be responsible for the pathogenesis of EV71-associated pulmonary edema (26).

Suggested treatments have varied. Since those cases with pulmonary edema had lower cellular immunity, a rational treatment may involve regimens to enhance cellular immunity. In the case of poliomyelitis, IFN-γ treatment of age-dependent poliomyelitis-susceptible mice protected them from paralytic disease (27). Recent studies have also shown that interferon-γ synergizes with IFN-α/β to inhibit the replication of both RNA and DNA viruses. Scagnolari et al. investigated the effects of IFNs on the replication of severe acute respiratory syndrome-associated coronavirus (SARS-CoV), finding that although SARS-CoV is only moderately sensitive to IFN-β and weakly sensitive to IFN-α and IFN-γ, in combination they have a strong synergistic effect on virus replication (28). These two studies imply treatments for severe EV71 infection, but further animal or clinical trial is mandatory to prove the efficacy of immunomodulators. In addition, use of an antiviral agent to decrease replication may also be beneficial (29). Animal studies are ongoing on this topic. In the Liu et al. study, an early administration of recombinant mouse interferon-α protected the mice against EV71 infection and in vitro analysis of virus-induced death showed that human type I interferons exerted a direct protective effect on EV71, so interferons may play an important role in controlling EV71 infection and replication (30).

Currently there is no vaccine for EV71. Since the major cytokine response of mononuclear cells after EV71 challenge is Th1 cytokines, induction of Th1 cellular response may be critical to vaccine development for prevention of severe EV71 disease. Therefore, an ideal vaccine should trigger adequate immunity in both cellular and humoral systems.

In conclusion, we found that cellular immunity rather than humoral immunity may be related to the clinical outcome of EV71 infections. EV71 cases with decreased cellular immunity plus lower cellular γ-interferon and other cytokine/chemokine response may be prone to disseminated EV71 infection and subsequent pulmonary edema.

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