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Can the Mild Clinical Course of Crimean–Congo Hemorrhagic Fever in Children Be Explained by Cytokine Responses?

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Cytokines are possibly one of the factors responsible for death due to Crimean–Congo hemorrhagic fever (CCHF). This study aimed to determine the differences between the cytokine levels in children and adult patients with CCHF; the influence of cytokines; and the severity of the course of the disease, which seems to be milder in children. Thirty-four children and 36 adult patients diagnosed with CCHF between 2010 and 2011 were included in this study. Diagnosis was performed by serology or by the polymerase chain reaction for CCHF virus. Levels of IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p70, IL-13, IL-17A, and IL-22 were measured in all serum samples. Although the disease had a fatal course in three adult patients, there were no deaths in children. Statistically significant differences were not observed between the cytokine concentrations in the adults and children. No differences were detected between the serum cytokine levels in the children with moderate and those with a severe clinical course of the disease. In the adult patients with fatal outcome, significantly higher serum levels of IL-2, IL-5, IL-9, IL-12 p70, and IL-13 were detected as compared to the cytokine levels in patients who survived the infection. No differences were detected between the serum levels of IFN-γ, IL-1β, IL-17A, IL-22, IL-10, IL-6, IL-4, and TNF-α in the patients who died and those who survived. Thus, the milder clinical course in children with CCHF cannot be explained by the cytokine network alone. The incomplete maturation of the immune system and timing and scale of immune responses could change the outcome dramatically. ***J. Med. Virol. 85:1955–1959, 2013.***

KEY WORDS: Crimean–Congo hemorrhagic fever; cytokine; children; adults; severity of disease

INTRODUCTION

Crimean–Congo hemorrhagic fever (CCHF) is a potentially fatal, tick-borne, viral zoonosis with reported cases from Africa, Asia, Eastern Europe, and the Middle East [Whitehouse, 2004; Ergonul, 2006; Weber and Mirazimi, 2008; Thomas et al., 2012]. The causative agent, CCHF virus (CCHFV), a member of the genus *Nairovirus*, family Bunyaviridae, is the most extensively spread, tick-borne virus [Estrada-Pena et al., 2012]. CCHFV causes severe disease in humans, which is usually characterized by fever along with headache, myalgia, and a petechial rash, is followed frequently by a hemorrhagic state with necrotic hepatitis [Whitehouse, 2004; Ergonul, 2006, 2007; Leblebicioglu et al., 2012].

CCHF is one of the viral hemorrhagic fevers (VHFs) that have a mortality rate as high as 50% in different outbreaks in many countries including Turkey [Yilmaz et al., 2009]. Although the clinical course and outcome seem to be milder than those of other such fevers, with a mortality rate of 1.6%, in children, the data on the reasons for this milder

Conflicts of Interest: None.

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clinical course are limited [Dilber et al., 2009; Tezer et al., 2009, 2010; Gul et al., 2011; Uysal et al., 2011; Tuygun et al., 2012]. The pathogenesis of CCHF is poorly understood. However, it has been hypothesized that CCHF could be a result of the complex interplay between direct and indirect effects of the endothelium affected by viral infection. Additionally, the results of detailed histopathologic studies of visible lesions are not sufficient to explain the cause of deaths. Although the parameters of host factors have not been fully evaluated yet, recently the role of soluble mediators of the immune system, including inflammatory cytokines, are implicated in the pathogenesis of the disease [Ergonul et al., 2006a; Papa et al., 2006; Saksida et al., 2010; Connolly-Andersen et al., 2011]. This study aimed to identify the possible role of cytokines in the severity of CCHF in children.

MATERIALS AND METHODS

Patients and Sample Collection

Serum samples were collected from 34 children and 36 adult patients who were diagnosed with CCHF between 2010 and 2011, from two different cities located in Central Anatolia, and who were admitted to Cumhuriyet University and Hacettepe University Faculty of Medicine. All the patients were included in the present study. Twenty age-matched, healthy, pediatric volunteers were enrolled as controls, and their serum samples were also collected. Blood samples from patients were obtained at the time of admission. Clinical data of the patients were collected from their medical records and sent to the central laboratory for analysis. Blood samples were stored at −80°C. Clinical diagnosis of CCHFV was confirmed at the time of hospital admission by enzyme-linked immunoassay (ELISA) and/or by real-time polymerase chain reaction (RT-PCR).

The characteristics of children were assessed according to the severity criteria defined by Swanepoel et al. [1989]. However, none of the children fulfilled these criteria completely, and therefore, more suitable criteria were needed to classify the severe cases in children. The criteria defined by Swanepoel et al. [1989] were modified as follows: children were defined as having "severe" disease when they had melana/hematemesis, somnolence, a white blood cell (WBC) count $>10{,}000/mm^3$ or $<4{,}000/mm^3$ (instead of $\geq 10{,}000/mm^3$), platelet count (PC) $\leq 50{,}000/mm^3$ (instead of $\leq 20{,}000/mm^3$), aspartate transferase (AST) level $\geq$135 U/L (instead of $\geq$200 U/L), alanine transferase (ALT) level $\geq$90 U/L (instead of $\geq$150 U/L), activated partial thromboplastin time (aPTT) $\geq$44 sec (instead of $\geq$60 sec), and fibrinogen level $\leq$150 mg/dl (instead of $\leq$110 mg/dl). Children fulfilling three of the above criteria were defined as having severe CCHF. The children were then divided into two groups according to the modified severity criteria used in this study: mild/moderate and severe. In addition, the adult patients were divided into two groups: fatal or nonfatal.

The study was approved by Turkish Ministry of Health, Refik Saydam National Public Health Agency (RSNPHA) Scientific Committee (B.10.1. RSH.0.05.00.00-605-38), and approved by the Ethical Committee of the University of Ankara (number: B.30.2.ANK.0.20.05.04).

Laboratory Diagnosis of CCHF

Patients who met the criteria for suspected CCHF and whose blood samples were positive for serum immunoglobulin (Ig) M antibody by ELISA to CCHFV and/or viral RNA by RT-PCR were confirmed to have CCHF. Samples were analyzed in the Virology Laboratory of Turkish Ministry of Health RSNPHA, Ankara, Turkey.

Cytokine Measurement

Levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p70, IL-13, IL-17A, and IL-22 were determined in each patient's serum sample. These cytokine levels were measured suing a commercially available fluorescent bead immunoassay, Human Th1/Th2/Th9/Th17/Th22 13plex Flow Cytomix Multiplex kit (eBioscience, Vienna, Austria) according to the manufacturer's instructions. Briefly, fluorescent beads coated by monoclonal antibodies specific to distinct cytokines, together with cytokine-specific, biotin-conjugated, monoclonal antibodies were suspended in serum samples or serially diluted. After 2-hr incubation, the beads were washed twice and incubated with streptavidin–phycoerythrin. Forward and side scatter voltages were adjusted with assay specific setup beads; FL2 detectors (575 nm) were used for bead quantitation, and an FL4 detector (675 nm) was used for bead differentiation. Standard curves were determined for each cytokine using the following ranges: 0–80,000 pg/ml for IL-22; 0–20,000 pg/ml for IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p70, IL-13; 0–10,000 pg/ml for IL-17A; and 0–2,000 pg/ml for IL-9. Samples were then analyzed using a Beckman Coulter Cytomics FC500 flow cytometre. The concentrations were measured using Flow Cytomix Pro 2.3 software (eBioscience). For each analysis, 5000 beads were collected.

Statistical Analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS), version 15.0 (SPSS, Chicago, IL). Continuous variables are presented as means ± standard deviation and median (min–max). Categorical variables are presented as per frequencies and in percentages and were compared using the Chi-square test. Differences in continuous variables between the groups were

tested by using the Kruskal–Wallis test and Mann–Whitney *U*-test. The correction method proposed by Machado [2007] was used to adjust statistical confidence measures. The adjusted significance threshold, α, was 0.0184. Values of $P \leq 0.0184$ were considered statistically significant.

RESULTS

Thirty-four children and 36 adult patients were confirmed to have acute CCHF infection. On the basis of the case records, there were 33 surviving adult patients and three adult patients with fatal outcome. Six children had severe disease and 28 children had moderate/mild disease according to the modified criteria. There was no fatal outcome in children. The mean age of the adults was 43.3 ± 15.5 years (range, 19–71 years) and of the children was 12.4 ± 4.4 years (range, 0.5–18 years). The male to female ratio was 0.8 (16 men, 20 women) for adults and 1.6 (21 boys, 13 girls) for children.

There were no significant differences in the cytokine levels in the children and adult cases (Table I). In addition, no differences were observed between the cytokine levels in the children with moderate and severe clinical course of the disease and the controls (Table II). When the parameters of adult patients with fatal outcomes of CCHF and adult patients who survived during the infection were compared, significantly higher serum levels of IL-2, IL-5, IL-9, IL-12 p70, and IL-13 were detected in the former group, but there were no differences in the serum IFN-γ, IL-1β, IL-17A, IL-22, IL-10, IL-6, IL-4, and TNF-α levels of the two groups (Table III).

DISCUSSION

Cytokines are possibly one of the factors responsible for death in cases of fatal VHFs [Andersson et al., 2006; Andersson et al., 2008; Ergonul, 2012]. The release of proinflammatory cytokines including IL-1, IL-6, TNF-α, and IFN-γ during CCHF has been suggested to be related to the severity of disease course [Papa et al., 2006; Bente et al., 2010; Saksida et al., 2010; Connolly-Andersen et al., 2011]. In this study, there were no significant differences in the serum levels of IL-10, TNF-α, IFN-γ, IL-1, and IL-6; however, IL-12 and IL-2 levels were significantly higher in patients with fatal outcome than in surviving adult patients. In another study, the serum levels of IL-6 and TNF-α were higher in fatal cases than in nonfatal cases of CCHF, but there were no significant differences between the regulatory cytokine levels of IL-10 in these groups [Ergonul et al., 2006a]. In another study, high TNF levels correlated with the severity of CCHF, and the results suggested that the IL-6 level was high in both mild and severe cases of CCHF [Papa et al., 2006]. Saksida et al. [2010] reported that serum IL-10, IFN-γ, and TNF-α level were significantly higher in patients with fatal outcome than in those who survived the infection. Additionally, no differences were detected between the serum IL-12 levels of the patients who died and those who survived the infection. These results are in contrast with the findings of the present study. Although the severity of CCHF is linked to hypercytokinemia in adults as mentioned above, the causal relationship between pathogenicity and cytokine response remains unclear, according to the results of the present study.

TABLE I. Cytokine Levels of the Children and Adult Cases With Crimean–Congo Hemorrhagic Fever

Cytokine levels (pg/dl)	Children (n = 34), median (min–max)	Adult cases (n = 36), median (min–max)	*P*-value
IL-12p70	2.4 (1.7–50.2)	2.4 (1.6–31.2)	0.11
IFN-γ	5.5 (1.0–706.2)	5.5 (2.7–303.3)	0.91
IL-17A	11.1 (1.01–728.2)	11.2 (5.7–505.2)	0.91
IL-2	15.6 (9.6–7493.7)	14.9 (7.5–417.4)	0.32
IL-10	4.7 (3.3–223.3)	5.1 (2.5–444.1)	0.27
IL-9	12.6 (5.7–836.7)	12.3 (4.9–466.5)	0.11
IL-22	6.7 (1.0–225.4)	6.9 (4.0–123.1)	0.91
IL-6	2.7 (1.0–504.6)	2.7 (1.6–310.3)	0.91
IL-13	3.2 (2.4–672.5)	3.1 (2.1–48.7)	0.32
IL-4	10.9 (8.1–204.9)	11.8 (6.3–339.8)	0.27
IL-5	5.0 (2.6–287.6)	4.9 (2.3–160.5)	0.11
IL-1β	2.8 (1.0–184.4)	2.9 (1.8–85.4)	0.91
TNF-α	7.2 (1.0–1857.7)	7.3 (3.2–1141.4)	0.91

Few studies have been published on the relationship of the cytokine response with the course of the disease in children. Tezer et al. [2011] reported that a low level of IL-6 might be associated with mild disease course. Nevertheless, proinflammatory cytokine levels including those of IL-6 in children and adults were not significantly different in this study. Also, cytokine levels in some children were as high as those in adults with fatal outcome as result of CCHF. No mortality was observed in the children. On the basis of these results, it was concluded that although cytokines are central drivers and controllers of immune-mediated virus elimination, the milder clinical course in children with CCHF could not be explained by the cytokine network alone. The difference in these proinflammatory and anti-inflammatory cytokine networks in adult and children may be due to the variation of host-dependent factors including genetic diversity or immunological response. Children who do not have an intact immune system will require repeated antigenic stimulation and sequential changes in the functional capacity of lymphocytes to achieve a complex maturation stage immunologically. The mild clinical course of CCHF in children may possibly be attributed to their developing immune system.

Another hypothesis on the cause of mild clinical course of CCHF in children is related to the timing of the cytokine response. Due to the retrospective nature of this study, study of the samples from different stages of disease was not possible. Most

TABLE II. Comparisons of the Cytokine Levels of the Children According to the Severity

Cytokine levels (pg/dl)	Severe children cases (n = 6), median (min–max)	Mild/Moderate children cases (n = 28), median (min–max)	Controls (n = 20), median (min–max)	*P*-value
IL-12p70	2.3 (2.3–50.2)	2.4 (1.7–38.4)	2.4 (1.9–4.1)	0.85
IFN-γ	5.1 (1.0–11.9)	5.6 (4.0–706.2)	5.8 (4.0–14.9)	0.37
IL-17A	12.9 (1.0–19.8)	11.1 (8.0–728.2)	11.3 (8.2–33.9)	0.86
IL-2	16.4 (12.9–7493.7)	15.6 (9.6–24.3)	17.2 (12.1–26.8)	0.68
IL-10	4.6 (3.6–223.3)	4.7 (3.3–16.9)	4.5 (3.3–5.9)	0.50
IL-9	12.0 (11.6–836.7)	12.9 (5.7–602.4)	13.2 (8.0–31.7)	0.85
IL-22	6.5 (1.0–12.2)	7.1 (5.4–225.4)	7.2 (5.5–14.4)	0.37
IL-6	3.1 (1.0–4.9)	2.7 (2.1–504.6)	2.8 (2.1–9.2)	0.86
IL-13	3.3 (2.8–672.5)	3.2 (2.4–4.4)	3.4 (2.7–4.7)	0.68
IL-4	10.9 (8.6–204.9)	10.9 (8.1–30.2)	10.5 (7.3–13.3)	0.50
IL-5	4.8 (4.6–287.6)	5.1 (2.6–207.1)	5.2 (3.4–11.5)	0.85
IL-1β	2.6 (1.0–5.0)	2.8 (2.2–184.4)	2.9 (2.3–6.0)	0.37
TNF-α	8.8 (1.0–15.3)	7.2 (4.9–1857.7)	7.5 (5.0–31.1)	0.86

viruses including CCHFV interfere with multiple stages of the IFN response [Versteeg and Garcia-Sastre, 2010; Devhare et al., 2013]. Although early IFN synthesis is responsible for efficient host protection, successful viruses frequently encode factors that suppress production of IFN [Haller et al., 2006]. A correlation has been reported between the release time of IFN and the disease course of VHF in some animal studies [Bowick et al., 2012]. Later onset of the IFN response is found to be associated with more severe disease, disseminated intravascular coagulation, microangiopathic hemolytic anemia, and intravascular deposition of fibrin thrombi [Cosgriff et al., 1989; Morrill et al., 1990; Bowick et al., 2012]. These data illustrates the difficulty in understanding the role of cytokines to explain the immunopathogenesis of the disease, since cytokine levels may be critically dependent on the stage of immune priming or recall.

The need to categorize the patients as having "severe" infection is important for early and accurate management of the disease. The characteristics in children in the present study were assessed according to the severity criteria defined by Swanepoel et al. [1989]. However, none of the children fulfilled these criteria completely. Most of the previous published data are based on extensive clinical features of CCHF in adults [Bakir et al., 2005; Ergonul, 2006; Ergonul et al., 2006b], but the presenting signs and symptoms of CCHF in children have been addressed only in a limited number of studies [Sharifi-Mood et al., 2008; Dilber et al., 2009; Tezer et al., 2010; Tuygun et al., 2012]. Therefore, the reasons of the inconsistency of children to the severity criteria may be attributed to that the criteria have been devised to assess adults. Considering the differences in the acute phase responses of children, due to their immature immune system, and of adults, it is obvious that new criteria are needed to determine the treatment plan for children. However, according to the modified criteria in this study, there were no differences in the cytokine levels in children with severe and mild/moderate disease.

TABLE III. Cytokine Levels of the Adult Cases With Crimean–Congo Hemorrhagic Fever

Cytokine levels (pg/dl)	Fatal adult cases (n = 3), median (min–max)	Nonfatal adult cases (n = 33), median (min–max)	*P*-value
IL-12p70	4.9 (3.0–31.2)	2.3 (1.6–2.9)	0.00*
IFN-γ	34.3 (6.9–303.3)	5.3 (2.8–22.1)	0.02
IL-17A	83.9 (9.9–505.2)	11.1 (5.7–66.0)	0.16
IL-2	28.1 (17.3–417.4)	14.3 (7.5–51.9)	0.01*
IL-10	15.9 (4.1–444.1)	5.0 (2.6–19.3)	0.22
IL-9	41.6 (19.5–466.5)	11.9 (4.9–17.5)	0.00*
IL-22	26.0 (8.2–123.1)	6.8 (4.0–19.0)	0.02
IL-6	29.0 (2.4–310.3)	2.7 (1.6–21.2)	0.16
IL-13	4.9 (3.5–48.7)	3.0 (2.1–8.0)	0.01*
IL-4	28.8 (9.8–339.8)	11.7 (6.3–33.4)	0.22
IL-5	14.9 (7.3–160.5)	4.7 (2.3–6.6)	0.00*
IL-1β	12.2 (3.3–85.4)	2.7 (1.8–8.4)	0.02
TNF-α	104.1 (6.3–1141.4)	7.2 (3.2–75.5)	0.16

*Significant difference.

This study has several limitations. First, it was performed in a specific setting and only a relatively small number of children who were admitted to hospital were enrolled. This prevents further generalization of the findings. This may also pose a bias in selection of patients. Second, serum sampling of the children with CCHF was not based on the timing of different phases of the course of the infection. Thus, there may also be an assessment bias that may affect the reliability of the data.

In conclusion, understanding the complex cytokine interactions in immune responses to CCHF is still not possible. Investigation of the role of other soluble mediators including chemokine and determination of multiple host-dependent mechanisms in the course of the disease are of great importance for not only understanding the underlying mechanism of the infection but also management and treatment of the disease.

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