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Interleukin-6: A promising disease severity index for dengue virus infection

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ABSTRACT

Objective: To study the relationship between expression of interleukin-6 (IL-6) in serum samples and dengue virus (DV) infection in Pakistani population.

Methods: This study involved three different groups, namely, DV2-infected DF patients ($n = 30$), DHF patients ($n = 30$) and healthy subjects ($n = 30$). Quantification of IL-6 expression in human serum samples among all the three groups from Pakistan was done by ELISA method. Other demographic findings, clinical parameters and symptoms were recorded and reported in the study.

Results: IL-6 expression in DHF group (mean value = 523.23 pg/mL) was significantly higher than that in DF group (mean value = 88.83 pg/mL) and healthy subjects (mean value = 6.72 pg/mL). Our data suggested that IL-6 level was considerably elevated ($P < 0.01$) with severe DV infection leading to fatal outcome in DHF patients as compared to DF and control group.

Conclusions: Our findings indicate that the up-regulation of IL-6 has a significant role towards DHF, suggesting it as a prognostic marker for severe DV infection.

1. Introduction

Dengue virus (DV) infection is an arboviral disease in humans caused by a Flaviviridae family member (DV), a single-stranded positive-sense RNA virus. It has an elevated worldwide impact on around 3.9 billion people in both tropical and sub-tropical areas. The incidence of dengue infection got a steady rise of 30-fold during the past 50 years[1,2]. To keep check on dengue disease severity markers is compelling due to lack of any suitable DV vaccine, antiviral drugs and appropriate animal study model. There are four antigenically distinct cross-reactive serotypes of DV (DV1–4) with no cross-protective immunity, and transmitted mainly by female mosquito of *Aedes aegypti*. The pattern of infection starts from a mild illness known as DF, leading to DHF characterized with plasma leakage

and hemorrhagic manifestations progressing towards dengue shock syndrome (DSS). Almost 500 000 cases of DHF and DSS have been reported annually. A mild form of DV infection progresses towards DSS through a cascade of events in terms of the shift from Th1 to Th2-type response[3,4].

DV pathogenesis is not fully explained yet, but the existing literature suggests three interacting components such as deregulation of cell-mediated immunity, antibody-dependent enhancement (ADE) and the complement system working together to produce a potentially life-threatening state during DV infection[5-7]. DV-induced Th1-type response also provokes an antiviral state in the cells[8]. IL-6, as interferon beta-2, acts as an inducer of the antiviral state in the same way. At the same time, DV encoded interferon antagonist viral proteins preventing the activation of the interferon mediated antiviral state in the host cell, thereby allowing the virus to replicate optimally. Thus an unchecked replication of virus leads to severe DV infection[9,10].

Pakistan has a temperate climate, huge agricultural area, open and artificial irrigation channels and reservoirs, massive rainfall and floods, providing enough breeding space for DV vector mosquito. DV has been endemic for many years in Pakistan with co-circulating

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cross-reactive serotypes[11-13].

A deep understanding of DV pathogenesis is necessary for its therapeutic strategies development. In this context, an early recognition marker for severe DV infection is a crucial step to reduce the disease severity in absence of a fully functional vaccine. These markers can be viral, endothelial or immunological markers. IL-6 is one of the top candidates as a prognostic marker for severe DV infection although its complete mechanism of action is not known yet[14,15].

The present study was conducted to measure IL-6 expression level in serum samples from DV2-infected patients through the course of DV infection to analyze its correlation with disease severity. Correlation of IL-6 expression to any specific DV serotype could be helpful for clinicians to determine early treatment for that specific serotype infected patients, as all four serotypes are antigenically distinct and cross-reactive. We have quantified levels of IL-6 in DV2-infected patients as well as normal healthy controls by ELISA. The aim of this study was to understand the role of IL-6 in the pathophysiology of DV infection. The elevated IL-6 expression level indicating severe DV infection will be helpful in dengue control program.

2. Materials and methods

2.1. Detection of IL-6 by ELISA

Serologically DV2 positive serum samples were collected from Jinnah Hospital, Lahore, Pakistan. This study was approved by the research committee at Institute of Public Health, Lahore and protocol was approved by Institutional Ethics Committees at National Institute of Health, Islamabad. The informed written consent was obtained from either patients or parents/guardians and the confidentiality of patients' information was preserved. Sera were separated by centrifuging the whole blood at 3500 r/min for 8 min and stored at -80°C until subjected to detection of IL-6 protein employing DRG IL-6 ELISA kit EIA-4640 (DRG Instruments GmbH, Frauenbergstr. 18D-35039, Marburg Germany) according to the manufacturer's instruction. The patients of control group ($n = 30$) were serologically negative for HCV, HBV, HCMV, EBV, JEV, HIV and DV, while DV2 positive serum samples were confirmed in DF ($n = 30$) and DHF ($n = 30$) groups.

The principle of the kit was to use the monoclonal antibodies (MAbs) directed against distinct epitopes of IL-6. Samples react with the capture monoclonal antibody (MAB 1) coated on microtiter well and with a monoclonal antibody (MAB 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated MAB1-human IL-6-MAB2-HRP, the microtiter plate was washed to remove unbound enzyme-labeled antibody. The bound enzyme-labeled antibody was measured through a chromogenic reaction. Chromogenic solution (TMB) was added and incubated. The reaction was stopped with the addition of stop solution and the microtiter plate was then read at the appropriate wavelength. The amount of substrate turnover was determined colorimetrically by measuring the absorbance, which was proportional to the IL-6 concentration. To check the accuracy and authenticity of experiment, negative and positive controls were employed with ELISA kit.

2.2. Statistical analysis

Statistical analysis was performed using Student unpaired *t*-test and difference was statistically significant when $P < 0.01$.

3. Results

3.1. Demographics/clinical findings and frequently associated symptoms

The demographics/clinical findings of collected samples (Table 1) along with a few frequently associated symptoms were analyzed for DF and DHF groups (Figure 1). Mean age of DF and DHF group patients was 18 and 29.6 years, respectively. In DF group, 23.3% were male and 76.7% female, while in DHF group, 63.3% were male and 36.7% female. Hematocrit, TLC (total leukocyte count), ALT and AST levels were comparatively higher in DHF group (Table 1).

Table 1

Demographics and clinical parameters recorded from DF and DHF patients.

Demographic/clinical parameters	DF	DHF
Mean age (years)	18	29.6
Male	7	19
Female	23	11
TLC count ^a (cells $\times 10^3$)	4.0 \pm 2.1	4.9 \pm 2.8
Hematocrit ^a (%)	36.4 \pm 4.1	39.2 \pm 5.2
ALP levels ^a (IU/L)	236.5 \pm 228	260.4 \pm 222.1
ALT levels ^a (IU/L)	96.0 \pm 87.1	170.2 \pm 229.8
AST levels ^a (IU/L)	109.1 \pm 107.2	180.0 \pm 173.2
Bilirubin levels ^a (mg%)	0.83 \pm 0.46	13.2 \pm 61.9

^a: Results were expressed as mean \pm SD. TLC: Total leukocyte count; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

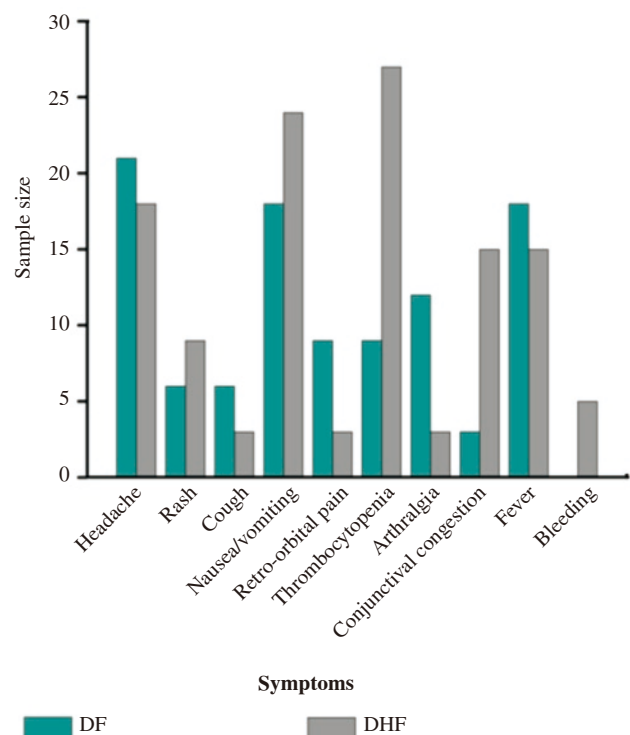


Figure 1. Frequently associated symptoms in DF and DHF groups.

The symptoms of headache, rash, cough, nausea and fever were found almost equally associated to both DF and DHF groups, while thrombocytopenia, conjunctival congestion and bleeding were highly associated to DHF group (Figure 1).

3.2. IL-6 expression level

Serologically DV2 positive serum samples in groups of DF and DHF showed differentially elevated IL-6 expression. Control group

indicated a mean level of IL-6 expression in healthy subjects as 6.72 pg/mL, while in DF and DHF groups mean level of IL-6 expression was increased up to 150 and 591.59 pg/mL, respectively (Figure 2). The level of IL-6 expression in DF group was significantly lower than that in DHF group ($P < 0.01$).

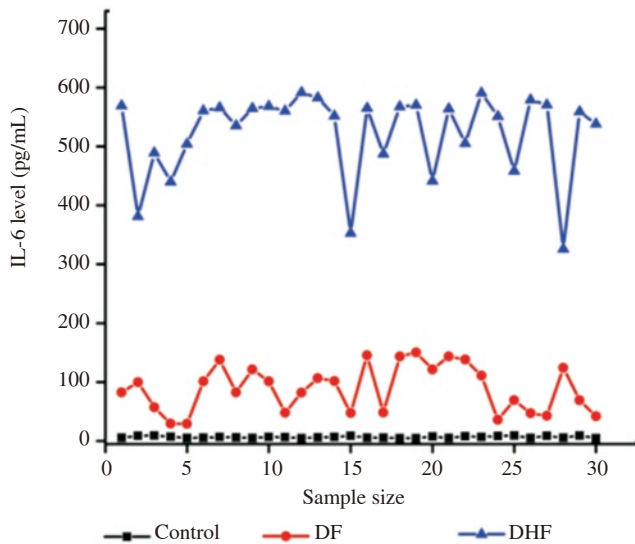


Figure 2. IL-6 expression level of control, DF and DHF groups.

4. Discussion

In Pakistan, the first DV attack was observed in Lahore in 1982 with later on outbreaks in three other provinces. No proper practices were implemented for prospective investigation and control program of dengue disease. In 2011, an outbreak occurred again in Punjab Province confined to Lahore District, while in 2013, Swat and Mansehra, two cities of Khyber Pakhtunkhwa Province, underwent dengue outbreak[11,16].

Through this study we have tried to find out the factors causing severe dengue infections, so as to reduce the disease burden and fatal outcomes in future. The analysis of demographic features *e.g.* age and gender for differentially elevated level of IL-6 among DF and DHF patients didn't clearly indicate the role of these parameters in serum cytokine level. Plasma leakage or bleeding was the key pathophysiological characteristic differentiating DHF from DF which results from abnormal haemostasis and increased vascular permeability[17,18].

The aim of this study was to show the role of a pro-inflammatory marker (IL-6) in the pathogenesis of DV infection. IL-6 is a multifunctional cytokine that acts on different target cells to induce a variety of biological responses involving immune regulation and signal transduction[19]. Results of this study indicate a close connection between sequential changes of serum IL-6 level and clinical progression of the disease from DF to DHF. Our data showed that IL-6 expression level in DHF patients was significantly higher than that in DF patients and healthy controls, which is completely in agreement with the findings of other report[20]. A number of clinical studies demonstrated higher level of pro-inflammatory cytokines most importantly IL-6 in sera from either DF or DHF patients in comparison with healthy subjects[21-24].

A close connection between IL-6 up-regulation and severe dengue infection can be established because high plasma level of IL-6 is not

only working as activation marker of coagulation and fibrinolysis in severe DV infections, but also involved in the onset and regulation of hemostasis[25,26]. Severe dengue infection also holds the virulence of DV as a devastating phenomenon for hemorrhage being a virologic factor. In addition, synergistic effects of mediators such as IL-2, IL-6, TNF, IFN- γ , PAF, complement C3a, C5a and histamine also provoke increased vascular permeability, plasma leakage, breakdown of the coagulation system and shock leading to hemorrhage[14,27].

Recently, available evidences indicate that the cytokines such as IL-6 and IL-10, IFN- γ , macrophage migration inhibitory factor (MIF) and CCL4 can serve as a potential predictor for severe DV infection. Several studies have emphasized on correlation of high level of IL-6 in both non-specific and specific immune responses with severe dengue infection[28-31].

Moreover, it has also been reported that dengue patients with early hemorrhagic manifestations lose coordinate expression of IL-6 and IL-8 with anti-inflammatory cytokine IL-10[32]. Similarly, synergistic activities of other cytokines like IL-12p70, TNF- α and IL-8 with IL-6 modulate the severity of DV infection[20,33]. Such synergistic activities and loss of coordinate expression sort of interplay among IL-6 and other cytokines, which are worth studying to explain DHF pathogenesis.

Our data with elevated level of IL-6 in DHF patients comparable to that of DF patients and healthy controls supporting above mentioned clinical findings can be helpful to disclose the intricate nature of severe dengue infection in future.

As a result, we propose that IL-6 secreted during the course of DV infection is essentially involved in disease progression towards DHF. Prediction and reduction of mortality rate among DHF patients need careful monitoring for considerably high IL-6 serum level. It will also help clinicians to isolate the 'at risk' patients in early hours.

It is concluded that serum of DV2-infected patients showed up-regulation of IL-6 both in DF and DHF groups, and this may create an immunosuppressive system, helping affected cells to avoid immune attack. Elevated levels of IL-6 correlate with disease progression and poor clinical outcomes. Further studies are needed to confirm whether it is an independent factor to cause disease severity or working synergistically with other cytokines and to envisage the mechanism to down-regulate its secretion. It could be either done through designing its receptor's antagonist or mutating the IL-6 gene. Moreover, the analysis of the infecting serotype is also required to understand the induction of cytokines. A better perception to IL-6 cytokine structure, function and its role in DV infection will facilitate new treatment programs and measures to reduce severe DV infection.

Conflict of interest statement

We declare that we have no conflict of interest.

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