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Association between proinflammatory cytokines and lipid peroxidation in patients with severe dengue disease around defervescence

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SUMMARY

Objectives: Proinflammatory cytokines and the oxidative stress response are reported to be involved in dengue viral disease. The present study investigated the correlation of proinflammatory cytokines and lipid peroxidation with dengue severity.
Methods: Clinical samples from 27 dengue fever (DF) cases, 30 dengue haemorrhagic fever (DHF) cases, and 24 dengue shock syndrome (DSS) cases were studied around defervescence, along with samples from 30 healthy controls. Plasma samples were analysed for tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) by ELISA and for malondialdehyde (MDA) by thiobarbituric acid assay.
Results: Dengue-infected individuals had significantly higher levels of TNF-α, IFN-γ, and MDA in comparison to controls. The ratio of TNF-α to IFN-γ was significantly higher in DHF and DSS than in DF. A TNF-α/IFN-γ ratio value of 5.69 around defervescence predicted DHF and DSS with moderate accuracy and thus may serve as an indicator to study dengue severity. The study observed a significant positive correlation of lipid peroxides with TNF-α levels and the TNF-α/IFN-γ ratio in severe dengue cases.
Conclusions: We propose that the oxidative stress response induced by the dengue virus may trigger the inflammatory cytokine responses in dengue severity and thereby contributes to the pathogenesis of the disease; however the interplay between the oxidative response and inflammatory activity in disease virulence needs further study.

1. Introduction

Dengue virus (DENV), a mosquito-transmitted single-strand virus, is prevalent in over 100 tropical and subtropical countries, with an estimated 96 million apparent dengue infections worldwide.[1,2] Infection with DENV may result in an asymptomatic or mild self-limiting acute febrile illness, dengue fever (DF), or life-threatening severe illnesses, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS).[3]

The pathogenesis of dengue severity remains poorly understood. Oxidative stress-induced damage has been observed in patients post-DENV infection, suggesting that this could play a role in the pathogenesis of DF or DHF/DSS.[4] Earlier studies have demonstrated that cells infected with herpes simplex virus and human immunodeficiency virus exhibit an increased generation of reactive oxygen species (ROS) with decreased antioxidant production, leading to the activation of redox-dependent transcription factors and the production of various cytokines.[5,6] However, the association of ROS and cytokine production in DENV infection has not been greatly explored. Our previous research and other studies in this area have revealed a significant pro-oxidant state in dengue viral infection and its association with thrombocytopenia.[7–9] On the other hand, cytokines are reported to play a significant role in the evolution of severe dengue infection.[10,11] The production and upregulation of cytokines in response to DENV infection are responsible for the development of complications such as cerebral oedema, increased haematocrit, and thrombocytopenia.[12] Proinflammatory cytokines such as tumour

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necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) have been found to be particularly elevated in severe dengue and have been associated with the severity of illness.[13–19]

Since no reports are available on the correlation between inflammatory and oxidative stress responses and disease pathogenesis, the present study aimed to evaluate the levels of plasma malondialdehyde (MDA), a known reactive aldehyde formed by the degradation of polyunsaturated lipids by ROS and a widely used biomarker for the oxidative stress response,[20] and the levels of proinflammatory cytokines TNF-α and IFN-γ during defervescence, in all the three clinical groups of dengue (DF, DHF, and DSS), compared to uninfected blood samples.

2. Materials and methods

2.1. Study design

Patients enrolled in the study were recruited from the Jawaharlal Institute of Postgraduate Medicine and Research (JIPMER) hospital, Pondicherry. Eighty-one dengue-infected adults between the ages of 24 and 52 years, of whom 31 were female, were selected for the study. Age- and gender-matched controls ($n = 30$) were also recruited. Written informed consent was obtained from all participants and the study was approved by the ethics review committee of JIPMER.

Infected individuals were further classified into DF ($n = 27$), DHF ($n = 30$), and DSS ($n = 24$) cases as per the World Health Organization (WHO) grading system.[21] Multiplex reverse transcriptase PCR was done for the detection and typing of DENV as described by Lanciotti et al.[22] Out of 81 cases, 15 were positive for DENV by RT-PCR, and DENV serotype 3 was identified as the aetiological agent. Virus-negative samples were further subjected to a one-step ELISA that detects the dengue-specific IgM and IgG antibodies (Novatech, Germany). Normal controls were blood donors who were negative for both anti-dengue IgM and IgG antibodies and virus.

Blood samples were obtained from the patients at the time of defervescence; 3 ml of blood was collected into a heparinized bottle and centrifuged at 2500 *g* for 5 min to separate the plasma, which was stored at −70 °C until analysis.

2.2. Assay for proinflammatory cytokines and lipid peroxidation

Plasma levels of TNF-α and IFN-γ were estimated using the BD OptEIA ELISA Kit (BD Biosciences, San Jose, CA, USA). Cytokine concentrations were interpolated from the calibration curve as per the manufacturer's instructions. Plasma MDA was estimated by Satoh method[23] in which 0.5 ml of plasma was added to 20% trichloroacetic acid (TCA) and centrifuged at 3500 rpm for 10 min. The precipitate was washed with 0.05 M H_2SO_4 and resuspended in 0.05 M H_2SO_4 and 0.22% thiobarbituric acid (TBA). The mixture was further heated at 100 °C for 30 min and extracted with *n*-butanol. The colour that developed was measured spectrophotometrically at 532 nm and the amount of MDA was determined from the standard curve, as described previously.[23] In this reaction MDA reacts with TBA under acidic conditions to give a final pink-coloured product; the molar extinction coefficient of the final coloured product of the assay was $1.56 \times 10^5\ M^{-1}cm^{-1}$.

2.3. Statistical analysis

Data were analyzed using the statistical program SPSS for Windows, version 13 (SPSS Inc., Chicago, IL, USA). Results are given as the mean ± standard deviation (SD). Platelet counts are expressed as the median (range). The independent Student's *t*-test was used for comparisons of unpaired data between groups. The Mann–Whitney *U*-test was used for comparisons of platelet counts (non-parametric data). One-way analysis of variance (ANOVA) followed by the post-hoc Tukey HSD test and Kruskal–Wallis tests were used to determine differences in MDA, IFN-γ, and TNF-α between different clinical groups of dengue infection, for parametric and non-parametric data, respectively. Relationships between MDA, IFN-γ, and TNF-α levels were evaluated using Pearson correlation for normally distributed data and Spearman correlation for non-normally distributed data, and the results expressed with the correlation coefficient (*r*). Receiver operating characteristic (ROC) curve analysis was performed to study the proposed parameters for their predictive potential for severe dengue disease. The Youden index (sensitivity + specificity − 1) was used as a measure of accuracy. A *p*-value of less than 0.05 was considered statistically significant.[24]

3. Results

3.1. Clinical characteristics of the study group

Clinical and haematological characteristics of dengue-infected patients recruited into the study are shown in Table 1. Most of the patients presented signs of defervescence on day 4 or 5 post-onset of fever. DHF patients presented symptoms of headache, arthralgia, and petechiae more frequently than DF patients ($p < 0.001$), whereas both DHF and DSS cases had more frequent presentation of hepatomegaly and clinical bleeding than DF cases. Higher peaks of haematocrit were found in patients with DSS than patients with DF and DHF ($p < 0.001$), and a similar difference was observed in DHF compared to DF cases. DSS patients had a significantly lower

Table 1
Characteristics of the study patients with the different clinical presentations of dengue infection[a]

	DF	DHF	DSS
Number of patients	27	30	24
Sex, male/female	0.92/1.0	1.2/1.0	1.07/1.0
Age, years, median (range)	22 (16–67)	35 (20–59)	24 (16–65)
Symptoms and signs, *n* (%)			
Cough	10 (37)	10 (33.3)	4 (16.7)
Headache	7 (25.9)	14 (46.7)[b]	6 (25)
Arthralgia	10 (37)	21 (70)[b]	5 (20.8)
Hepatomegaly	6 (22.2)	23 (76.7)[b]	10 (41.7)[b]
Petechiae	11 (40.7)	16 (53.3)[b]	8 (33.3)
Clinical bleeding	3 (11.1)	23 (76.7)[b]	12 (50)[b]
Maximum haematocrit, %, mean (SD)	33.1 (3.4)	36.3 (4.5)[b]	39.5 (6.3)[b,c]
Minimum platelet count, $\times 10^9$/l, median (range)	105.6 (11.0–290.0)	55.0 (12.5–98.00)[b]	22.0 (10.0–82.0)[b,c]

DF, dengue fever; DHF, dengue haemorrhagic fever; DSS, dengue shock syndrome; SD, standard deviation.

[a] *p*-Values were calculated by Chi-square test for binary variables and independent Student's *t*-test for continuous variables; the *p*-value was calculated by Mann–Whitney *U*-test for platelet count.

[b] [c] $p < 0.05$ when compared with DF and DHF cases, respectively.