Serum Levels of Trace Elements, Proinflammatory Cytokines and Nitric Oxide in NS1 Positive Cases in Acute Dengue Virus Infections

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Abstract

Background: Dengue virus (DENV) infection is characterized by severe vascular complications viz. dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) in a perceptible population justifying the need for an early marker that could reliably predict such adverse outcomes. A nonstructural protein antigen of DENV termed NS1 antigen that is detectable during acute stage of DENV infection was evaluated to find out its association with alteration of some trace element levels viz. zinc (Zn), selenium (Se), iron (Fe), copper (Cu) and magnesium (Mg), levels of nitrite (the stable product of NO), as well as levels of interferon gamma (IFN-γ), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α) in acute stage of DENV infection to find out if any alteration of these parameters associated with NS1 positivity could strengthen its predictive value for development of pathogenic consequences following acute DENV infection.

Methods: Estimation of concentrations of trace elements by flame atomic absorption spectrometry (FAAS) method, while the levels of nitrite and citrulline by Griess reaction and cytokine estimation was done by enzyme-linked immunosorbent assay (ELISA) method.

Results: There was significant depression of Cu levels in cases positive for NS1 antigen alone (n = 50) or in combination with IgM positivity (n = 15) compared to cases with IgM positivity alone (n = 50), other febrile illness (OFI) group (n = 50) and both rural and urban healthy controls (n = 50 each). Serum level of Se was increased in all the serological subgroups of acute DENV cases, in the OFI group as well as in healthy rural controls compared to urban controls. There was significant depression in nitrite level in NS1 positive cases regardless of associated IgM positivity. The reduced serum nitrite levels in NS1 positive cases, with or without associated IgM positivity, correlated positively with decrease in serum Cu level and negatively with increase in TNF-α level in the corresponding subgroups.

Conclusions: The present study revealed altered status of some markers in serum associated with NS1 positivity that may strengthen the validity of NS1 positivity as a predictive marker for development of complications thus help in timely management of DENV infection.

Keywords: NS1 antigen; DHF; DSS; Trace elements; Proinflammatory cytokines; Nitrite

Introduction

Dengue virus (DENV) infection is a rapidly emerging arboviral infection with 390 million infections in the world every year [1]. DENV, an enveloped virus with positive single-stranded RNA, is transmitted by Aedes mosquitoes that are mostly prevalent in tropics and subtropics [2]. Although majority of DENV infections in acute stage are characterized by dengue fever (DF), a perceptible proportion develops severe vascular complications viz. dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [3]. Evaluation of various laboratory and clinical parameters in acute stage of DENV infection towards prediction of severe complications has so far been proved to be of limited value [4-8]. Recently a nonstructural protein antigen of DENV termed NS1 antigen that is detectable during acute stage of DENV infection has gained importance as a useful predictive marker for the risk of developing severe DENV infection [9-11]. The first target of DENV are monocytes as evident by release of inflammatory mediators like nitric oxide (NO) and proinflammatory cytokines viz. interferon gamma (IFN-γ), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α), that are considered to be important components of innate immunity [12-14]. Several components of innate immune system required for defense against viral infections are influenced by levels of micronutrients like zinc (Zn), selenium (Se), iron (Fe), copper (Cu) and magnesium (Mg) in the body[15-16]. A study was taken up to find out serum levels of some trace elements viz.
Zn, Se, Fe, Cu, Mg and levels of nitrite (the stable product of NO), citrulline (as a surrogate marker of NO production) as well as levels of IFN-γ, IL-1β, IL-6, IL-8, IL-12 and TNF-α in acute stage of DENV infection to find out if any alteration in the levels of these parameters associated with NS1 positivity could strengthen the predictive value of NS1 antigen positivity for development of pathogenic consequences following acute DENV infection.

Materials and Methods

Collection of sera representing acute DENV infection

During the epidemic seasons for dengue in the years 2016 and 2017, all the sera received in the Microbiology Laboratory, SGT hospital from clinically suspected dengue cases were subjected to testing by a commercial solid phase dual immuno-chromatography kit (dengue day 1 test, J. Mitra and Co, New Delhi, India) that has a provision for detection of NS1 antigen as well as anti-DENV IgM antibodies in serum. A total of 115 serum samples comprising of 50 randomly selected serum samples with positivity for NS1 antigen and equal number of age and sex matched sera with anti-DENV IgM positivity as well as all of the limited number of sera (n = 15) detected to be positive for both the markers during the study period were included as samples representing acute DENV infection [3].

Collection of sera from cases with other febrile illness (OFI) and controls

A total of 50 randomly selected serum samples from age and sex matched individuals with acute febrile illness during the study period and found to be negative for DENV infection by serology (NS1 antigen or DENV IgM antibody or DENV IgG antibody) as well as real-time PCR were included as non-dengue or OFI group [17]. In addition, 80 sera from healthy blood donors, 40 each from local rural population and urban population in the city of Delhi respectively were included as healthy controls.

Estimation of trace element levels in serum

Estimation of trace elements viz. Fe, Se, Zn, Mg and Cu in serum was carried out using flame atomic absorption spectrometry (FAAS) (Thermo Electron Corporation, UK M6 Spectro with integrated software SOLAAR AA) as described earlier [18]. Standard solutions of trace elements from commercial source (Merck Private limited, Germany; concentrations 1000 ppm) were used to prepare suitable calibration curve for calculating trace element concentrations in serum. All the working standard solutions were prepared in 10 mmol/L nitric acid. Serum samples were diluted 100 fold for Mg, 10 fold for Cu, Se and Zn in 10 mmol/L nitric acid and then aspirated directly in FAAS. A blank was used for setting of zero absorbance of spectrophotometer.

Estimation of serum levels of nitrite and citrulline

Estimation of serum levels of nitrite (the stable product of NO) was based on Griess reaction [19]. Briefly, serum sample was diluted four fold in distilled water and deproteinized by adding Zn sulfate (final concentration 15 g/L) followed by centrifugation at 10,000 g for 5 min at room temperature. Then 100 µL of supernatant as well as 100 µL of varying dilutions of nitrite standards (Sigma Chemicals, USA) were charged in flat bottom 96-well plates; 100 µL of Griess reagent (0.1% naphthalene diamine dihydrochloride, 1% sulphanilamide and 2.5% phosphoric acid) was added to each well. The plate was incubated for 10 min at room temperature in dark and was read at 550 nm in an automated plate reader (Multiskan, Lab systems). Quantity of nitrite in samples was calculated from absorbance values plotted against the standard curve obtained from the values of the serial dilutions of nitrite standards using reading of the reaction mixture without serum as blank. Each sample was run in triplicate and average value taken. The lower limit of detection of nitrite concentration in serum was 250 nmol/L.

Estimation of serum level of citrulline, as a surrogate marker for NO production through arginine pathway, was carried out by colorimetric estimation following the protocol described by Boyd and Rahmatulla based on the chemical modification of citrulline by diacetyl monoxime [20]. Briefly, serum sample was deproteinized by adding tricarboxylic acid solution (final concentration 5%) followed by centrifugation. Chromogenic reagent was prepared just before use by adding 5 mg thiosemicarbazide (Sigma Chemicals, USA) to 50 mL of 0.5% diacetyl monoxime (Sigma Chemicals, USA) solution followed by addition of 100 mL of acid-ferric solution prepared by dissolving FeCl₃ (25 mg) in 100 mL solution containing 25 mL concentrated sulfuric acid (95-98%), 20 mL concentrated phosphoric acid (85%) and 55 mL distilled water. To 100 µL of supernatant, 3 mL of chromogenic solution was added, mixed vigorously and boiled at 100 °C for 5 min. Enzyme urease (Type VII, Sigma Chemicals, USA) was included in the mixture to prevent formation of color complex by urea with diacetyl monoxime. The tubes were cooled to room temperature and absorbance was measured at 530 nm in a spectrophotometer. Quantity of citrulline in supernatant was calculated from a standard curve of absorbance values prepared by running varying dilutions of DL-citrulline standard (Sigma Chemicals, USA) simultaneously with samples. Representative samples (about 40%) were also analyzed by high performance liquid chromatography (HPLC) (Shimadzu Corporation, Japan) to validate sensitivity of the colorimetric assay (< 5% variation). The detection limit of citrulline was 2.5 µmol/L.

Estimation of serum levels of cytokines

Measurement of levels of proinflammatory cytokines viz. IL-1β, IL-6, IL-8, IL-12 p70 (the bioactive form of IL-12) and TNF-α in serum was carried out employing commercial reagents and kits (Quantikine, R&D Systems, Minneapolis, USA) with sensitivity levels as 1 pg/mL, 0.70 pg/mL, 1.5 pg/mL, 1.7 pg/mL and 0.38 pg/mL respectively. The co-efficient of vari-
Evaluation of serum trace element levels revealed significant depression of Cu levels in cases positive for NS1 antigen alone or in combination with IgM positivity compared to cases with IgM positivity alone, OFI group and both rural and urban healthy controls. Level of Se were increased in all the subgroups of acute DENV phase sera i.e. cases positive for NS1 antigen alone or in combination with IgM positivity and cases with IgM positivity alone, the OFI group as well as healthy rural controls were comparable to urban controls. The levels of other trace elements viz. Fe, Zn and Mg were unaltered in various subgroups of acute DENV phase sera with levels comparable to both rural and urban controls (Table 1).

Levels of all the six proinflammatory cytokines studied viz. IFN-γ, IL-1β, IL-6, IL-8, IL-12 p70 and TNF-α were elevated in all three subgroups of acute DENV infections i.e. NS1 positive alone cases, NS1 positive cases associated with IgM positivity and cases with only IgM positivity alone compared to controls. However, while elevations of all the cytokines were of similar degree, increase in level of TNF-α was more marked in NS1 positive cases, regardless of its association with IgM positivity compared to cases with IgM positivity alone. The OFI group also showed elevation of all the proinflammatory cytokines in sera comparable to sera from subgroups of acute DENV infection with the exception that unlike NS1 positive cases elevation in TNF-α was not marked and was rather comparable to anti-IgM group (Table 2).

There was significant depression in serum NO level, expressed as nitrite level, in NS1 positive cases regardless of associated IgM positivity while cases with IgM positivity alone and to those from OFI group showed levels comparable to controls (Table 2). The reduced serum nitrite levels in NS1 positive cases with or without associated IgM positivity correlated negatively with increase in TNF-α level and positively with decrease in serum Cu level in the corresponding subgroups (Fig. 1a, b and Fig. 2a, b). Citrulline levels in all the subgroups of acute DENV cases elevation in TNF-α was not marked and was rather comparable to anti-IgM group (Table 2). Statistical comparisons (P values): (i) Fe, Zn, Mg: Overall = NS; Between various subgroups = NS. (ii) Se: Overall = 0.001; Between each of subgroups I, II, III, OFI, Cont (R) vs. Cont (U) < 0.001; Between subgroups I, II, III, OFI, Cont (U) = NS. (iii) Cu: Overall < 0.001: Subgroups I/II vs. Cont (R)/Cont (U) < 0.001, < 0.001; Subgroup III vs. Cont (R)/Cont (U) = NS (0.65); NS (0.10); OFI vs. Cont (R), Cont (U) = NS (0.53), NS (0.15).

Results

In the present study, the age range and male to female ratio among NS1 positive cases, combined NS1 and IgM positive cases and IgM positive cases were similar (age ranges between 9 and 62 years, 5 to 60 years and 8 to 56 years with male to female ratio as 1:0.72, 1: 0.78 and 1:0.63 in the three groups respectively).

Discussion

Currently there is no effective treatment or vaccine against DENV infection. As DENV infection may present as nonspecific viral illness in the early phase of infection followed by complications like DHS and DSS in a proportion of cases, an early diagnosis of DENV infection is crucial for timely management of complications. Detection of DENV infection by
Correlations between TNF-α (pg/mL) and NO$_2$ (µmol/L) levels in serum in cases positive for (a) NS1 and (b) NS1 with anti-DENV IgM (indicated as IgM) positivity.

![Figure 1](image1)

**Figure 1.** Correlations between TNF-α (pg/mL) and NO$_2$ (µmol/L) levels in serum in cases positive for (a) NS1 and (b) NS1 with anti-DENV IgM (indicated as IgM) positivity.

Correlations between Cu (µg/dL) and NO$_2$ (µmol/L) levels in serum in cases positive for (a) NS1 and (b) NS1 with anti-DENV IgM (indicated as IgM) positivity.

![Figure 2](image2)

**Figure 2.** Correlations between Cu (µg/dL) and NO$_2$ (µmol/L) levels in serum in cases positive for (a) NS1 and (b) NS1 with anti-DENV IgM (indicated as IgM) positivity.
However comparable elevations in levels of IL-1β, IL-6, IL-8 and IL-12 in NS1 positive cases with or without IgM positivity and incases with IgM positivity alone as observed in the present study indicate lack of any association of such elevations with NS1 positivity in acute dengue cases. Disproportionate elevation of IL-12 in OFI cases compared to other subgroups of acute DENV infection could be due to possible inclusion of other viral infections viz. chikungunya virus and parasitic infections viz. malaria in the OFI group that also peak in this part of the country during the period covered in the present study [38]. On the other hand, level of TNF-α, another monocytes-induced proinflammatory cytokine, was markedly elevated in NS1 positive cases with or without associated IgM positivity compared to the group with IgM positivity alone in the present study. It has been proposed that TNF-α induces endothelial cell production of reactive nitrogen and oxygen species leading to apoptotic cell death and hemorrhage [39, 40]. A strong correlation between high concentration of TNF-α in blood and the severity of DHF has been reported in several studies [41-42].

The level of nitrite, an indirect indicator of NO production, was depressed in our study in cases with NS1 positivity compared to other groups. NO, produced by inducible nitric oxide synthase (iNOS) is considered to be an important agent for macrophage-mediated defense against many viral infections apart from DENV viz. SARS coronavirus, hantavirus, members of genus Flaviviridae, including Japanese encephalitis [43-45]. Positive correlations between levels of nitrite and that of citrulline in all the subgroups i.e. acute DENV cases, OFI group and controls strengthened specificity of observed NOx production through arginine pathway [46]. It is known that most of the beneficial antiviral effect of NO molecule is mediated through cytotoxicity induced by NO which in turn is attributable to production of peroxynitrite, generated through interaction between NO and another free radical, the superoxide anion [47-49]. These reports also support elevation of nitrite level in the OFI group recorded in our study since the period coincided with the season for many other viral infections in this part of the country. On the other hand significantly lower level of NO has been reported in Asiatic children with DF and DHF [50], which is in agreement with the finding of the present study. In murine model increasing levels of Cu consistently elevated nitrite production in macrophage cell line RAW264.7 and macrophages; whereas chelating Cu with tetraethyl-pentamine (TEPA) decreased significantly nitrite production in the same in vitro model [51]. This report may possibly explain the correlation between decrease in Cu level and that in nitrite level recorded in the present study. NO can protect cells from apoptosis induced by TNF-α by exerting its inhibitory effect on TNF-α release from human peripheral blood monocytes [52, 53]. These reports support the observed negative correlation between elevated levels of TNF-α with reduced levels of nitrite seemingly induced by low levels of Cu. Alternatively elevation in TNF-α level could be due to reduced Cu level as shown in mouse model with experimental coxsackie B virus infection [54]. The present study revealed altered status of some markers in serum associated with NS1 positivity that may strengthen the validity of NS1 positivity as a predictive marker for development of complications thus help in timely management of DENV infection.

References


