Desialylation of Plasma Proteins in Severe Dengue Infection: Possible Role of Oxidative Stress

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Abstract. Oxidative stress in dengue infection has been suggested. This study was carried out to explore the plasma protein oxidation and its sialic acid content in dengue infection. Thirty-two dengue hemorrhagic fever (DHF), 29 dengue shock syndrome (DSS), 29 dengue fever (DF), and 63 healthy controls were included in this study. The extent of desialylation, sulphydryl content, and desialylation of plasma protein was estimated in acute phase sample. Significantly higher levels of protein carbonyls and lower levels of sialic acid and sulphydryl groups were found in DHF and DSS compared with DF using one-way analysis of variance. Regression analysis showed that desialylation is dependent on protein carbonyls in DHF/DSS. This study indicates that, in dengue infection, plasma proteins undergo increased levels of desialylation, which can be attributed to the oxidative stress. Future studies on sialylation status of endothelium and platelets can show light into the pathogenesis of the dengue infection.

INTRODUCTION

Dengue is the most frequent arboviral infection, with > 100 million infections throughout the world annually, including 250,000–500,000 cases of dengue hemorrhagic fever (DHF) and 24,000 deaths.1,2 In most cases, dengue fever (DF) is self-limited. However, there is a risk of progressive development into DHF or dengue shock syndrome (DSS). DHF is a severe febrile disease characterized by abnormalities in hemostasis and increased vascular permeability, and severe progression may result in DSS. DSS is a form of hypovolemic shock that is associated clinically with hemoconcentration and that might lead to death if appropriate care is not given. Although DF is distinct from DHF/DSS by traditional classification, the various clinical manifestations after dengue virus infection show a continuum from mild to severe reactions, just as in many other viral diseases.

Sialic acid, a family of acetylated derivatives of neuraminic acid, is widely distributed in mammals. It usually occurs as a terminal component at the non-reducing end of carbohydrate chains of glycoproteins and glycolipids. In human plasma, a large quantity of sialic acid is found in orosomucoid, α₁-antitrypsin, haptoglobin, ceruloplasmin, fibrinogen, complement proteins, and transferrin.3,4 For glycoproteins, glycan moieties are important integral parts both structurally and functionally.5 Sialic acid has been found to influence the glycoprotein function and its life span.6,7 Moreover, it was reported that non-reducing terminal sialic acid residues may be a target molecule of reactive oxygen species (ROS) and ROS specifically cleave and liberate them. An apparent link has been found between oxidative stress and sialic acid content of glycoproteins.8

The mechanisms involved in the pathogenesis of dengue virus infection, especially the manifestation of DHF/DSS, remain unresolved. In secondary DHF cases, higher viremia is observed, and antibody-dependent enhancement of viral entry into phagocytic cells has been hypothesized as the causative factor.9,10 However, the mechanism by which high viremic status induces DHF is not clear. Aberrant secretion of proinflammatory cytokines has been suggested as yet another risk factor for DHF/DSS.11 In dengue viral infection, prolonged activation of T cells and their apoptosis has been proposed as a cause of delayed viral clearance.12 Although the pathogenesis of dengue fever is not well understood, oxidative stress seems to play an important role.13

After viral entry, immune system activation takes place, and phagocytic and other cells produce ROS as a defense mechanism.13 There is now much evidence that oxidants play a complex role in viral diseases, starting from influences on host cell metabolism and less desired toxic effects on host tissue.14–16 Oxidants are highly unstable and react with nucleic acid, lipids, proteins, carbohydrate, or any other molecule, causing a cascade of chain reactions resulting in cellular damage. This process might cause loss of fluidity and production of protein and lipid peroxides, which leads to cell wall destruction. Protein carbonyls (PCs) are an established marker of protein oxidation.17 The PCs have been shown to be more susceptible for cross-linking and cleavage that undergo rapid protolysis.18 Wide spectrum of antioxidant defense mechanisms inactivates and lies in balance with these oxidants. The protein-bound sulfhydryl group (PBSH) has an important role in antioxidant defense. Although mammalian erythrocytes are rich in glutathione (GSH), the concentration of GSH in extracellular content, especially in human plasma, is very low compared with the intracellular content.19 Most of the free sulfhydryl groups in human plasma are protein-bound amino acid residues, which have been suggested to contribute significantly to the antioxidant capacity of the human plasma.20

However, no information is available about the sialic acid content of plasma glycoproteins in the different clinical spectrum of dengue infection. Furthermore, the relation between bound sialic acid and oxidative changes in plasma proteins of patients with dengue has not been described.

Hence this work was carried out on dengue infection to look for 1) the sialic acid content of serum glycoproteins and 2) its relationship with markers of plasma protein oxidation.

MATERIALS AND METHODS

Study design, patients, and controls. In Pondicherry town, South India, a dengue outbreak was reported for the first time
in September 2003.\textsuperscript{21} Eighty-six dengue-infected adults (age range, 26–54 years; 48 men and 38 women) participated in the study. Sixty-three healthy subjects (age range, 23–55 years; 42 men and 21 women) were included as controls. Infected individuals were further classified into 29 DF, 32 DHF, and 25 DSS cases per the WHO grading system explained below. Normal controls were blood donors who were negative for both anti-dengue IgM and IgG antibodies. Written informed consent was obtained from all participants, and the study was approved by the ethical review committee of the institution.

**Clinical definitions and dengue virus testing.** A complete clinical history, laboratory tests, and other parameters pertaining to the diagnosis of dengue infection such as the tourniquet test, hematocrit, and platelet count (Sysmex-k-100 automated cell counter) carried out at local hospitals were recorded. Cases were classified according to the WHO grading system.\textsuperscript{22} Dengue infection was divided into classic DF, DHF, and DSS. DF was a mild self-limiting acute febrile illness. DHF was defined as fever with hemorrhagic manifestations, thrombocytopenia, and hemoconcentration as a 20% increase in hematocrit. Thrombocytopenia was defined as a platelet count < 100,000/\textsuperscript{\textmu}L. DHF Grades I and II. DSS was defined as DHF criteria plus hypotension for the age or narrow pulse pressure (> 20 mm of Hg) or the clinical signs of shock, e.g., slow capillary filling, cold clammy skin (equivalent to DHF Grades III and IV). Thrombocytopenia was defined as a platelet count < 100,000/\textsuperscript{\textmu}L and hemoconcentration as a 20% increase in hematocrit above normal for age and sex. Serologic testing of blood samples for both IgM and IgG antidengue antibodies was undertaken using an ELISA kit (Novatech, Dietzenbach, Germany), as per the procedure described by the manufacturer. Multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out, and the dengue 3 serotype was found to be responsible for this outbreak.\textsuperscript{23}

**Estimation of plasma sialic acid.** Sialic acid content of plasma protein was measured by using the following modifications of Aminoff’s method.\textsuperscript{24} Briefly, 50 \mu L plasma was taken and diluted in distilled water (1:4). Serum proteins were precipitated by centrifuging with 125 \mu L of 5% TCA at 2,000 rpm for 5 minutes. Supernatant was discarded, and the sialic acid moieties of the protein pellet were hydrolyzed with 0.5 mL of 0.1 mol/L \textsubscript{H}2\textsubscript{SO}_4 at 80°C for 60 minutes. To the hydrolysates, 250 \mu L of periodate reagent (25 mmol/L periodic acid in 0.125 mol/L \textsubscript{H}2\textsubscript{SO}_4, pH 1.2) was added and incubated in a 37°C water bath for 30 minutes. Excess of periodate was reduced by 0.2 mL sodium arsenite (2% solution of sodium arsenite in 0.5 mol/L HCl). As soon as yellow color of liberated iodine started fading off, 2 mL of thiobarbituric acid (0.1 mol/L solution of 2-thiobarbituric acid in water, pH adjusted to 9.0 with NaOH) was added, and with a stopper, the tubes were incubated in a boiling water bath for 8 minutes. The tubes were cooled in ice water and extracted with 5 mL of acid butanol mixture (butan-1-ol containing 5% vol/vol in HCl); the separation of two phases was done by a short rapid centrifugation, and color intensity of butanol phase was measured spectrophotometrically at 549 nm. The sialic acid level was calculated using molar extinction coefficient (70.7 103 L/mol cm) and expressed in micrograms per milligram protein considering the molecular weight of sialic acid as 309.3. Total serum protein was measured by the Biuret method using a Ciba Corning 550 Express autoanalyzer (Ciba Corning Diagnostics, Oberlin, OH).

**Assay of carbonylation of plasma protein.** The assay was done according to the method of Levine and others.\textsuperscript{25} The millimolar extinction coefficient of the colored product is 22.01 mmol/cm at 366 nm. The carbonylated protein reacts with 2,4-dinitrophenyldrazine (DNPH) to form a colored adduct of 2,4-dinitrophenylhydradrazone. Plasma total protein was estimated by the biuret method using fully automated auto-analyzer 550 express plus (Ciba Corning Diagnostics).

**Determination of protein-bound sulphydryl group content.** Protein-bound sulphydryl group contents were determined according to the method of Sedlak and Lindsay,\textsuperscript{26} with 5, 5’-dithiobis-2-nitrobenzoic acid (DTNB) with minor modifications. Briefly, 200 \mu L plasma was taken and diluted in distilled water (1:4). Plasma proteins were precipitated by centrifuging with 500 \mu L of 5% TCA at 15,000 rpm for 3 minutes. Supernatant was discarded, and the protein pellet was mixed with 2 mL of 0.2 mol/L Tris-HCl buffer (pH 8.2)–20 mmol/L EDTA and 20 \mu L of 10 mmol/L DTNB and 0.15 mL of 5% SDS. Finally, absorbance was measured at 412 nm against a sample blank (without DTNB) and also corrected for a reagent blank (without sample). The absorbance readings were made 15 minutes after adding DTNB to test samples. The experimentally determined molar extinction coefficient was 13.1/mm/cm. Values are expressed in nmoles per milligram protein.

**Statistical analysis.** Data were analyzed using the statistical program SPSS for windows, version 13 (SPSS, Chicago, IL). Results are given as mean ± SD. The independent Student t test was used to determine the statistically significant difference between cases and controls. One-way ANOVA followed by a post hoc test Tukey HSD was used to find difference in the parameters studied within the clinical groups of dengue infection. The relationship between PCOs and bound sialic acid content was evaluated using linear regression. Results are expressed as regression coefficient (\beta). Clinical features between the different groups were compared by \chi^2 test for binary variables and Student t test or Mann-Whitney U test where appropriate. P < 0.05 was considered statistically significant.

Patients were bled between 3 and 5 days of onset of symptoms around defervescence. Blood samples were collected in a heparinized bottle, and separated plasma was stored at −70°C until analysis.

**RESULTS**

Table 1 shows the clinical characteristics of dengue-infected patients included in this study. All patients presented with fever (100%). The mean (SD) duration of fever was 4.2 (1.1) days (range, 1–9 days). There were no significant differences in the duration of fever between the different clinical groups (P = 0.07). Duration of hospitalization was more in the DHF and DSS groups compared with the DF group (P < 0.001). Headache, arthralgia, and petechia were significantly more common in DHF in comparison to DF (P < 0.001), whereas hepatomegaly and clinical bleeding were significantly more frequent in DSS (P = 0.002). During hospitalization, hematocrit levels of the patients increased, and patients with shock (DSS) had significantly higher peaks of hematocrit compared with the DF and DHF groups (P < 0.001). The hematocrit values of DHF patients were higher than in DF patients (P <
0.001). The overall mean time (days) to reach the maximum hemocrit level after onset of fever was higher in DHF (4.9) and DSS (5.6) compared with DF (4.2) patients.

The level of sialic acid residues, sulphhydril groups, and carbonyl content of plasma proteins in dengue-infected individuals and controls are shown in the Table 2. The levels of sialic acid residues and PBSH were found to be significantly (P < 0.001) decreased in dengue infection, whereas PCOs were significantly (P < 0.001) increased in these cases in comparison to control subjects. One-way ANOVA followed by a post hoc Tukey HSD test showed that a significant difference was observed with low bound sialic acid content and PBSH and high PCOs in all three clinical groups compared with controls (Table 3). Similar results were found in DHF and DSS cases compared with DF cases (P < 0.05). Between DHF and DSS, a significant difference was found in bound sialic acid (P = 0.033) and PCOs levels (P < 0.001); however, no difference was found in PBSH content (P = 0.456). Linear regression analysis showed a significant negative association between protein carbonyls and bound sialic acid levels in DHF (β = −0.409, P < 0.001) and DSS (β = −0.508, P < 0.001; Figure 1). A positive correlation existed between sulphhydril groups and bound sialic acid levels among DSS (r = 0.450, P = 0.024).

**DISCUSSION**

Within recent years, there has been considerable interest and research in sialic acid and its role in health and diseases. Serum sialic acid is a marker of the acute-phase response. Acute-phase glycoproteins with sialic acid as a component of the oligosaccharide side chain being produced by the liver, stimulated by proinflammatory cytokines such as interleukin-6, interleukin-6 and tumor necrosis factor-α. These cytokines were found to be elevated in dengue infection, and their levels have been associated with severity of disease. However, C-reactive protein concentrations were not found to be different in dengue infection from controls and found to be lower than bacterial infection. Our research indicates that there is a significant decrease (P < 0.001) in plasma protein-bound sialic acid content in dengue patients compared with controls. This finding was also observed when patients with DF, DHF, and DSS were compared with controls. It was noted that the extent of desialylation was maximal in DSS, followed by DHF, and its levels were minimal in DF. There are several possible explanations for the decrease in sialic acid concentrations. It has been reported that ROS may specifically cleave terminal sialic acid residues. Liver dysfunction seen in dengue viral infection might lead to reduced synthesis and release of acute phase proteins. Aberrant coagulation and vascular leakage might also lead to loss of serum protein into third space.

A recent report has shown increased oxidative stress in dengue infection and its association with disease severity. The altered levels of lipid peroxides and activity of antioxidant enzymes were studied to show oxidative stress. This study found increased levels of the protein carbonyls and reduced antioxidant capacity evaluated as sulphhydril group content of plasma proteins during early phase of dengue viral infection. This finding is consistent with another report of increased lipid peroxidation and lowered antioxidative serum capacity associated with dengue viral illness. The oxidation of protein-bound sulphhydril groups is a direct indication of oxidative damage. Sulphhydril groups are known to scavenge aqueous peroxyl radicals. In this study, a significant decrease in plasma protein-bound sulphhydril groups on early COVID-19 infection was observed, which is consistent with the findings of previous studies. This suggests that oxidative stress plays a significant role in the pathogenesis of COVID-19 infection.

**Table 1**

| Characteristics of patients with different clinical spectrum of dengue infection |
|---------------------------------|-----------------|-----------------|
| No of patients                  | DF              | DHF             | DSS             |
| Sex (male:female)               | 0.95:1.0        | 1.3:1.0         | 1.05:1.0        |
| Age, median (range) (years)     | 23 (15–66)      | 37 (21–58)      | 25 (16–64)      |
| Days of hospitalization, mean (SD) (days) | 5.7 ± 0.78 | 11.5 ± 1.2† | 12.6 ± 2.2‡ |
| Symptoms and signs [N (%)]      |                 |                 |
| Cough                           | 9 (33.3)        | 11 (34.4)       | 3 (14.3)        |
| Headache                        | 8 (29.6)        | 15 (46.9)†      | 6 (28.6)        |
| Arthralgia                      | 11 (40.7)       | 22 (68.8)†      | 4 (19.1)        |
| Hepatomegaly                    | 5 (18.5)        | 25 (78.1)†      | 9 (42.9)‡       |
| Petechiae                       | 12 (44.4)       | 18 (56.3)†      | 9 (42.9)        |
| Clinical bleeding               | 4 (14.8)        | 25 (78.1)†      | 11 (52.4)‡      |
| Tourniquet test                 | 13 (48.2)       | 19 (59.4)‡      | 10 (47.6)       |
| Maximum hemocrit, mean (SD) %   | 32.3 (3.7)      | 36.2 (4.9)‡     | 39.5 (6.3)‡‡    |
| Day of maximum hemocrit, mean (SD) (days)* | 4.2 (1.3) | 4.9 (1.2)      | 5.6 (1.3)       |
| Minimum platelet count, median (range)/mm³ | 106,500 (11,500–280,000) | 52,000 (12,000–98,000)† | 23,000 (9,000–86,000)‡‡ |
| Day of minimum platelet count, mean (SD) (days)* | 4.2 (1.3) | 4.8 (1.2)     | 5.4 (1.3)       |

* P < 0.001 using independent Student t test for continuous variables. Mann Whitney U test for analysis of platelet count. † P < 0.05 compared with DF and DHF cases, respectively.

**Table 2**

Mean and SD of protein-bound sialic acid, protein carbonyls, and protein-bound sulphhydril groups in dengue-infected individuals and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sialic acid content of serum protein (μg/mg of protein)</th>
<th>PCOs (nmol/mg of protein)</th>
<th>PBSH (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dengue-infected individuals (N = 86)</td>
<td>4.9 ± 0.97*</td>
<td>5.85 ± 0.91*</td>
<td>4.88 ± 0.74*</td>
</tr>
<tr>
<td>Controls (N = 63)</td>
<td>6.58 ± 0.85</td>
<td>1.97 ± 0.56</td>
<td>6.91 ± 0.89</td>
</tr>
</tbody>
</table>

* P < 0.001 using independent Student t test compared with controls.
days of illness showed that they may be one of the early antioxidant defenses to combat the peroxyl radical attack in dengue infection. However, the rest of the total antioxidant status needs to be studied to corroborate this result. The alteration of the redox markers indicates that these changes probably started before the onset of clinical symptoms, suggesting an early compromise of the oxidant/antioxidant balance.

From this study, oxidative stress–induced changes in plasma proteins were found to be more in severe dengue infection (DHF/DSS) compared with milder infection (DF). Also, the observations of the significant negative correlation between carbonyl and sialic acid content of plasma proteins among DHF/DSS cases probably indicated that oxidative stress was involved in enhanced desialylation of proteins in blood in dengue infection. The previous studies have shown that oxidative stress can cause desialylation of glycoproteins of serum protein, platelets, and low-density lipoprotein (LDL) particles. However, the exact mechanism is not clearly understood. Desialysed plasma proteins produced by oxidative damage are taken up by the liver, because it is a generalized mechanism of removal of asialoglycoproteins. In DHF/DSS, increased vascular permeability is suggested because of malfunction of endothelium rather than structural destruction. When endothelial cells are stimulated by cytokines, ROS generation can be detected, and this process has been reported in dengue infection. Several studies on the relationship between ROS and vascular dysfunction have been reported. These results suggest a higher severity of oxidative stress and desialylation of plasma proteins in DHF/DSS patients compared with mild dengue infection. Research studies have also indicated that vascular permeability is regulated by sialic acid moieties. The vascular endothelium carries a high concentration of sialic acid, and hence, extensive endothelial damage associated with severe dengue infection could account for its shedding into the circulation. Therefore, it raises the possibility of oxidative stress and associated glycoprotein changes in inducing endothelial dysfunction and vascular leakage, the pathognomonic features of severe dengue infection.

It has been shown that thrombocytopenia in dengue

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**TABLE 3**

Mean and SD of protein-bound sialic acid, protein carbonyls, and protein-bound SH group levels in DF, DHF, DSS, and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sialic acid content of serum protein (µg/mg of protein)</th>
<th>PCOs (nmol/mg of protein)</th>
<th>PBSH (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF (N = 29)</td>
<td>5.50 ± 0.93*</td>
<td>5.04 ± 0.47*</td>
<td>5.31 ± 0.63*</td>
</tr>
<tr>
<td>DHF (N = 32)</td>
<td>4.84 ± 0.63†</td>
<td>5.99 ± 0.61†</td>
<td>4.83 ± 0.62†</td>
</tr>
<tr>
<td>DSS (N = 25)</td>
<td>4.22 ± 0.94†‡</td>
<td>6.68 ± 0.70†‡</td>
<td>4.51 ± 0.77†</td>
</tr>
<tr>
<td>Controls (N = 63)</td>
<td>6.38 ± 0.85</td>
<td>1.95 ± 0.54</td>
<td>6.94 ± 0.82</td>
</tr>
</tbody>
</table>

* P < 0.001 compared with controls.
†‡ P < 0.05 in comparison to DF and DHF, respectively, calculated by one-way ANOVA by post hoc Tukey HSD.

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**FIGURE 1.** Association of protein carbonyls and protein-bound sialic acid levels in patients with DHF and DSS. Plasma protein carbonyls are plotted against protein-bound sialic acid levels for all patients with (A) DHF (β = –0.409, P < 0.001) and (B) DSS (β = –0.508, P < 0.001).
evolved from the increase in the destruction rate of peripheral platelets rather than their insufficient formation. Although the cause and mechanisms of peripheral platelet destruction are not very clear, it is suggested that this destruction is a result of some immunologic events depending on antibody activities. A study on malaria showed a negative relation between the thrombocyte counts and plasma lipid peroxidation and also with platelet malendialdehyde levels. Hence, it is worth studying whether glycosylation of the platelet membrane is reduced in dengue viral infection and oxidative stress and its relative contribution toward sialic acid content of platelets in explaining the pathogenesis of thrombocytopenia in dengue viral infection.

This study concluded that desialylation of plasma protein was found in dengue viral infection. The extent of desialylation was maximal in DSS, followed by DHF, and its severity was minimal in DF. Also the degree of desialylation was found to be associated with the extent of protein oxidation in severe dengue disease. Hence, future studies on the sialylation status of endothelial cells and platelets may shed some light into the pathogenesis of dengue viral infection.

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