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), 25 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 proteolysis.18 Wide spectrum of antioxidant defense mechanisms inactivates and lies in balance with these oxidants. The protein-bound sulphydryl 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 sulphydryl 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

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in September 2003. Twenty-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. 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 or other signs of plasma leakage (equivalent to WHO classification 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/mm³ and hemoconcentration as a 20% increase in hematocrit.

DHF and 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.

**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 (β). Clinical features between the different groups were compared by χ² 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 hematocrit 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, sulphydryl 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 increased in plasma protein- and high PCOs in all three clinical groups 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.33 Liver dysfunction seen in dengue viral infection might lead to reduced synthesis and release of acute phase proteins. 9 Aberrant coagulation and vascular leakage might also lead to loss of serum protein into third space.9 A recent report has shown increased oxidative stress in dengue infection and its association with disease severity.15 The altered levels of lipid peroxides and activity of antioxidant enzymes were studied to show oxidative stress.13,34 This study found increased levels of the protein carbonyls and reduced anti-oxidant capacity evaluated as sulphydryl 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.13,34 The oxidation of protein-bound sulphydryl groups is a direct indication of oxidative damage. Sulphydryl groups are known to scavenge aqueous peroxyl radicals. In this study, a significant decrease in plasma protein-bound sulphydryl groups on early

### DISCUSSION

Within recent years, there has been considerable interest and research in sialic acid and its role in health and diseases.27 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.28–32 These cytokines were found to be elevated in dengue infection, and their levels have been associated with severity of disease.11 However, C-reactive protein concentrations were not found to be different in dengue infection from controls and found to be lower than bacterial infection.32 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. The level of sialic acid residues, sulphydryl 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 increased in plasma protein- 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 observed 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 sulphydryl groups and bound sialic acid levels among DSS (r = 0.450, P = 0.024).

### Table 1

| Characteristics of patients with different clinical spectrum of dengue infection |
|-----------------------------|-----------------------------|-----------------------------|
|                            | DF                          | DHF                         | DSS                          |
| No of patients             | 29                         | 32                          | 25                          |
| 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†                 |

**Table 2**

<table>
<thead>
<tr>
<th>Mean and SD of protein-bound sialic acid, protein carbonyls, and protein-bound sulphydryl groups in dengue-infected individuals and controls</th>
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<tbody>
<tr>
<td>Groups</td>
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<tr>
<td>Dengue-infected individuals (N = 86)</td>
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<td>Controls (N = 63)</td>
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*P < 0.001 using independent Student t test compared with controls.

The detailed analysis of platelet count and hematocrit 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, sulphydryl 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 increased in plasma protein- and high PCOs in all three clinical groups 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.33 Liver dysfunction seen in dengue viral infection might lead to reduced synthesis and release of acute phase proteins. 9 Aberrant coagulation and vascular leakage might also lead to loss of serum protein into third space.9

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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. Desialyed 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

![Figure 1](attachment:image.png)

**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 ($\beta = -0.409, P < 0.001$) and (B) DSS ($\beta = -0.508, P < 0.001$).
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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