Status of circulating immune complexes, IL8 titers and cryoglobulins in patients with dengue infection

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Dengue, a serious viral infection caused by the mosquito vector, Aedes aegyptii, affects about 390 million people annually from more than 125 countries across the globe. However, until now, there is no reliable clinical or laboratory indicator to accurately predict the development of dengue severity. Here, we explored critical pathophysiological determinants like IL8, circulating immune complex (CIC) and cryoglobulin in dengue-infected patients for identification of novel dengue severity biomarker(s). Totally, 100 clinically suspected dengue cases were tested by NS1 ELISA and MAC ELISA for dengue virus aetiology. For control, 49 healthy volunteers were included. Blood profiling (complete hemogram and liver function test) of patient population were done using automated cell counter and standard auto analyzer based biochemical analysis. Serum CIC was quantified by PEG precipitation. Serum cryoglobulins were estimated by Folin assay. Levels of serum IL-8 were assessed by standard sandwich ELISA kits. Patient CIC were further characterized by SDS Gel electrophoresis. Forty per cent of the cases tested positive, of which 11 patients had severe clinical manifestation. The mean ±SEM of cryoglobulin concentration for DHF, DF, and HC were 1.30±0.31, 0.59±0.08 and 0.143±0.009 μg/μl, respectively. Thus, DHF and DF patients have shown 9- and 2.2-fold increase in cryoglobulin levels; and 18- and 5-fold increased CIC, respectively compared to HC patients. The mean ±SEM of CIC-PEG index for DHF, DF and HC were 491±41.22, 146±14.19 and 27.98±2.56, respectively. Raised levels of IL8 titers were also found in all 11 DHF patients. Peak levels of CIC, cryoglobulin and IL8 titers were associated with thrombocytopenia. SDS PAGE analysis of CIC from DHF revealed the presence of at least six protein bands that were not observed in samples from DF and HC. Prediction efficacy of IL8, CIC and cryoglobulin for DHF was determined using the receiver operator characteristic curve (ROC). The area under the curve was 1.00 for IL8, 0.99 for CIC and 0.74 for cryoglobulins. Overall, the results suggest that CIC, IL-8 and cryoglobulins may serve as important laboratory parameters to monitor dengue infection progression.

Keywords: Aedes mosquitoes, CIC, Cryoglobulinemia, Dengue hemorrhagic fever (DHF), Dengue Shock Syndrome (DSS), Immune Complexes (ICs),

Dengue, a flavivirus transmitted by the Aedes mosquitoes, are a cause of great concern to public health all over the world. Currently, over 50% of the world’s population live in areas where they are at risk of this arboviral infection, and approximately 50% live in dengue endemic countries1,2. More than 125 countries of the world are known to be dengue endemic3. In India too, dengue outbreaks in the last two decades are not uncommon4. Recent study on the global distribution and burden of dengue using cartographic approaches observed that about 390 million dengue infections occur per year, of which, 96 million manifests apparently5.

Dengue virus infection in humans can lead to a wide range of clinical manifestations, from mild fever to potentially fatal dengue shock syndrome (DSS)6. The clinical presentation of acute dengue infection is non-specific but 5-10% of patients progress to severe dengue hemorrhagic fever (DHF)/DSS, which can result in death if it is not, managed appropriately6. During the acute phase of illness, it is difficult to distinguish the severe DHF from dengue fever (DF) and other illnesses found in tropical areas. The differential diagnoses during the acute phase of illness include influenza, leptospirosis, malaria, measles, rubella typhoid and other viral hemorrhagic fevers7. There is no pathognomonic sign or symptom for DHF during the acute stage. Currently, there is no early marker for DHF, which could predict dengue severe cases. As there is no specific antiviral therapy3, only

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Abbreviation— ALT, alanine transaminase; AST, aspartate transaminase; BBS, borate buffered saline; CIC, circulating immune complex; CI, confidence interval; DF, dengue fever; DHF, dengue haemorrhagic fever; DSS, dengue shock syndrome; HC, healthy controls; OR, odds ratio; PBS, phosphate buffered saline; ROC, receiver operative characteristic curve.
prompt detection of severe cases and appropriate clinical management may help in reducing fatality in dengue infection. In search of such novel severity biomarkers, we systematically analyzed critical pathophysiological factors namely, IL8, serum circulating immune complexes (CICs) and cryoglobulins in patients with dengue infection. The dengue virus is known to cause immune dysregulation by altering the chemokine profile. High titers of Serum IL8 have been reported in severe dengue patients from northwestern India and Columbia, and may thus, serve as severity markers. Cryoglobulinemia has been associated with thrombo-hemorrhagic phenomena and is defined as the presence of cold precipitable proteins in the plasma. Cryoglobulinemia has been reported in auto immune disorder, renal dysfunction, malaria, etc. However, its status in dengue patients remains unexplored.

Dengue infection accelerates the formation of antibody in the circulation. These antibodies form antigen-antibody complexes. Circulating immune complexes (CIC) play an important role in the pathogenesis of some diseases, such as chronic hepatitis, measles, malaria, as well as in DHF. CIC in dengue hemorrhagic fever (DHF) have been found to be localized in tissue and blood elements. In light of the important role played by CIC, cryoglobulin and IL8 in disease pathogenesis, here, we investigated their status in DF and DHF as compared to normal healthy controls (HC). Additionally, characterization of serum CIC was also performed to check differential banding patterns between DF and DHF, if any.

Materials and Methods

Study population—The study population consisted of 100 clinically suspect dengue patients. The study was carried out in Calcutta School of Tropical Medicine, India during the period November 2013 to September 2014. The study was approved by the Institutional Ethics Committee and was initiated subsequently. Out of 100 dengue patients, 40 patients were confirmed by NS1 and IgM ELISA. Based on WHO-Regional guideline SEARO (2011), New Delhi, among the 40 confirmed patients, 29 were categorized as dengue fever (DF) and 11 as dengue hemorrhagic fever (DHF, Grade 1). Additionally, 49 healthy control (HC) subjects were also enrolled for this study. Sera from the entire study subject were obtained and stored at −20°C until further use.

Laboratory methods—Dengue diagnosis was performed by NS1 detection kit and Dengue IgM detection kit (Panbio, Australia). In addition, other physiological parameters like complete hemogram, liver function tests, and enzymes alanine transaminase (ALT) and aspartate transaminase (AST) were done by standard biochemical assays.

Quantification of circulating immune complexes (CIC)—Extraction of patient or control sera CIC was accomplished by polyethylene glycol (PEG) precipitation method of Creighton as done in Chattopodhyay and Sen. Briefly, sera were diluted in the ratio of 1:3 with 0.1 M borate buffer, pH 8.4, and mixed with 2 ml of 4.16% PEG 6000 in borate buffer [4.16 g PEG MW-6000 dissolved in 100 ml borate buffered saline (BBS)] and incubated at 4°C for 1 h. Absorbance was measured at 450 nm to determine the turbidity due to precipitation of CIC in PEG containing tubes, which was matched with the absorbance of the control containing borate buffer.

The results were expressed as PEG index derived by the following formula

\[
\text{PEG Index} = \frac{\text{Absorbance with PEG}}{\text{Absorbance with BBS}} \times 1000
\]

All the tests were performed in triplicates.

Cryoglobulin quantification—For isolation of cryoglobulins, serum samples (40 μl) were incubated in glass tubes for 96 h at 4°C. The incubated samples were centrifuged for 20 min at 1700 rpm, and cryoprecipitate was washed with phosphate NaCl buffer at 4°C, and quantified by folin phenol reagent. All the tests were performed in triplicates.

Quantification of IL8 titers—The quantification of IL8 was carried out using ELISA kits (Ray Biotech, USA). The tests were carried out as per manufacturer’s instruction. All samples were tested in duplicate and the mean value taken for analysis. The standard curve was constructed for each assay based on the standards provided in the kit and cytokine concentration was calculated by extrapolation from the standard curve.

Characterization of CICs by SDS PAGE analysis—Circulating immune complexes were isolated from serum by incubating it with 8% PEG 6000 overnight at 4°C. The supernatants were removed and the precipitates were washed and re-suspended in 3% PEG in PBS (pH 7.2), and again centrifuged at 13000 rpm
for 20 min at 4°C. The supernatants were then removed, precipitates dissolved in equal amounts of PBS and incubated at 37°C for 1 h. Following the incubation period, the complexes were dissociated and analyzed by 10 % SDS-PAGE. The isolated patient CIC was subjected to 70 V constant voltage for 3.5 h. The gel was stained with coomassie for 20 min, and subsequently destained and photographed. The relative mobility of the protein standards were calculated and standard graph generated. The molecular weight of the unknown patient proteins CIC were derived from the standard graph generated.

Statistical analysis— Results were presented as mean ± SEM by measuring three individual replicates. Statistical analysis was performed using the Graph-Pad Prism statistics software (Graph-Pad Software Inc., San Diego, CA, USA), online MedCalc calculator, and between-group comparisons were performed by student’s t-tests. Differences with P values <0.05 were considered to be statistically significant.

Result

Demography of the study population— The study population included 100 suspected dengue patients among whom 40 (40%) were NS1 and Dengue IgM positive. An additional 49 healthy subjects served as HC. Among the suspected cases, 72.5% had dengue fever (DF) and 27.5% dengue hemorrhagic fever (DHF). In the dengue infected patient group 57.5% were male and 42.5% were female (Table 1).

Blood profile— DHF and DF patients had about 3.75- and 1.23- fold decreased platelet count as compared to HC. AST levels (IU/L) for DHF, DF and HC were found to be (mean ± SEM) 185.73 ± 31.8, 71.3 ± 14.96 and 25.14 ± 2.7, respectively. Whereas, ALT levels (IU/L) were 165.14 ± 42.18, 41.6 ± 5.7 and 25.84 ± 2.98, respectively (Table 1).

Clinical profile— All the patients observed (DF and DHF respectively) had fever along with myalgia (86.20% and 72.72%), rash (75.86% and 90.90), headache (34.38% and 36.36%), arthralgia (44.82% and 45.45%) and itching (72.41 % and 81.81%) (Table 2).

PEG index— The mean ±SEM of PEG index for DHF, DF and HC were 491 ± 41.22, 146 ± 14.19 and 27.98 ± 2.56, respectively. Thus, DHF and DF patients showed 18- and 5-fold increased CIC, respectively compared to HC. ANOVA analysis among the groups revealed a P value of <0.0001, which was statistically significant (Fig. 1A). Additionally, high measure of associations were obtained for DHF (OR 3.98, 95% CI = 0.82-19.37 and DF (OR 4.60, 95% CI = 1.30-16.21).

Cryoglobulin concentration— The mean ±SEM of cryoglobulin concentration for DHF, DF, HC were 1.30 ± 0.31 μg/μl, 0.5599 ± 0.08 μg/μl and 0.143 ± 0.009, respectively. Thus, DF and DHF patients showed 2.2 - and 9 - fold increased cryoglobulins, respectively compared to HC (Fig. 1B). ANOVA analysis showed a P value <0.0001, statistically significant. High associations of cryoglobulins were obtained for DHF (OR 1.20, 95% CI = 0.26-5.46, and DF (OR 2.06, 95% CI = 0.82-5.17).

IL8 concentration— The mean ± SEM of IL8 concentration for DHF, DF, HC were 393.7 ± 16.20 pg/ml, 147 ± 16.10 pg/ml and 23.45 ± 1.56 pg/ml, respectively. Thus, 6- and 17- fold increased IL8 titers were obtained for DF and DHF, respectively as compared to HC. ANOVA analysis depicted a statistically significant P value <0.0001 (Fig. 1C). High associations of IL8 titers were obtained for DHF (OR 1.99, 95% CI = 0.57-6.89) and DF (OR 2.27, 95% CI = 0.96-5.38).

Table 1—Comparison of certain blood parameters in Dengue fever (DF), Dengue hemorrhagic fever (DHF) patient’s vs. Healthy controls (HC). Values are Mean ± SEM; n=3

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of population</td>
<td>29 11 49</td>
</tr>
<tr>
<td>Males:Females</td>
<td>16:13</td>
</tr>
<tr>
<td>Liver enzymes (IU/L)</td>
<td></td>
</tr>
<tr>
<td>AST 71.3±14.96</td>
<td>185.73±31.8</td>
</tr>
<tr>
<td>ALT 41.6±5.7</td>
<td>165.14±42.18</td>
</tr>
<tr>
<td>Platelet count (X10^3/µl)</td>
<td>158.7±5 52.3±6.93, 195.4±4.2</td>
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</tbody>
</table>

Table 2—Clinical signs and symptoms of the study population

<table>
<thead>
<tr>
<th>Signs and Symptoms</th>
<th>No of cases in each group</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>DF (n=29)</td>
</tr>
<tr>
<td></td>
<td>DHF (n=11)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>25 8</td>
</tr>
<tr>
<td>Rash</td>
<td>22 10</td>
</tr>
<tr>
<td>Headache</td>
<td>10 4</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>13 5</td>
</tr>
<tr>
<td>Itching</td>
<td>21 9</td>
</tr>
</tbody>
</table>
SDS PAGE analysis—CICs isolated from patients and controls by PEG precipitation were submitted to one dimensional SDS PAGE. They were subsequently processed for coomassie stain, the gel showed lot of protein bands, both major and minor. Analysis revealed that the CIC of DHF had six protein bands i.e., 12, 19, 22, 26, 29 and 31 kDa which were totally absent in DF and HC (Fig. 2). Thus, characteristic differential banding pattern was obtained in CIC of DHF compared to DF patients and HC.

**CICs, cryoglobulins and IL8 as diagnostic markers for DHF evaluated by ROC curve**—As PEG-CICs, cryoglobulins and IL8 were related to the development of DHF, an attempt was made to differentiate DHF from DF by these markers. ROC curves for PEG-CICs, cryoglobulins and IL8 are shown in Fig. 3. The calculated area under the ROC curve was 0.99 for PEG-CICs (---), 0.74 for cryoglobulins (----) and 1.0 for IL8 (-).

**Discussion**

Dengue is one of the most underreported tropical diseases, though the estimated disease burden is very high\(^2\). Application of repellants such as diethyl-phenylacetamide (DEPA) in the water bodies near households has been suggested as a preventive measure in the integrated management of the vectors\(^2\). Further, a recent study on the genetic variants of dengue virus in Kerala highlighted the need for a wider geographic analysis of evolutionary adaptation of the dengue serotypes for development of...
an effective vaccine\textsuperscript{26}. Lack of specific laboratory test and typical clinical symptoms that contribute to the high mortality rate of the disease stress the need for identification of markers that could be useful in early diagnosis of the severe cases\textsuperscript{27}.

Many factors contribute to the pathogenesis of dengue virus infection. One of the hypothesis suggests an autoimmune response to be the underlying mechanism in the pathogenesis of dengue infection\textsuperscript{28-32}. In this hypothesis, CIC formed by autoantibodies and human proteins are the main feature resulting in severity of the disease. Autoimmune disorders have also been reported in viral diseases like hepatitis C virus infection\textsuperscript{33}. The HCV virus is known to trigger formation of cryoglobulins, another autoantibody that can cause immune complex deposition. The consequence of cryoglobulinemia can result in vasculitis-related skin ulcer and immune complex related glomerulonephropathy\textsuperscript{33}. The most common cause of tissue injury in patients with HCV-related cryoglobulinemia is immune complex-mediated vasculitis. Circulating cryoglobulins can combine with other immunoglobulins and complements to form large immune complexes, and deposition of immune complexes may result in vascular inflammation and damage. Earlier studies have also reported that cryoglobulins and CIC stimulate the production of inflammatory cytokines like IL8\textsuperscript{34,35}. Taken together, all these factors add up to the severity of diseases. Considering the important role of CIC, cryoglobulin and IL8 in virus induced pathogenesis, their status also needs to be evaluated in severe dengue patients vs. non severe patients. The present study, thus addresses these unknown facets of dengue pathogenesis with the aim of identifying novel markers for the early diagnosis of severe dengue cases. In another recent study, we have reported differential banding pattern of CICs in patients of visceral leishmaniasis and post kala azar dermal leishmaniasis and thereby revealed the scope for developing a novel differential diagnostic assay\textsuperscript{36}.

Accordingly, this study has characterized critical pathophysiological determinants like IL8, circulating immune complex (CIC) and cryoglobulin in dengue-infected patients (DF and DHF). As observed in this study, myalgia, arthralgia and rash were the most common clinical manifestation of DF/DHF patients (Table 2). Based on the clinical manifestation it was difficult to distinguish DHF from DF. However, investigations of the CICs titers in both the groups revealed 3.36 higher PEG index in DHF cases as compared to DF (Fig. 1A). Further, student’s t-test analysis between DF vs. DHF revealed a significant ($P <0.0001$) value. The DHF patients also had significantly ($P <0.008$) higher levels of cryoglobulins (Fig. 1B). Similarly, the chemokine IL8 titers also measured a 2.67-fold increase in DHF patients as compared to DF ($P <0.0001$) (Fig. 1C). All the studied parameters like CIC, IL8 and cryoglobulin had good correlation with platelet count of dengue patients revealing their importance as clinical parameters (Fig. 4). Further, all these parameters had significant Odds ratio for the different categories of dengue patients (DF/DHF). Thus, these parameters could serve as important tool for differentiating DF from DHF cases. Additionally, investigation of the serum CIC revealed the presence of six distinct protein bands only in DHF patients. These bands were
completely absent in healthy controls and DF patients (Fig. 2). Further, in this study, prediction efficacy of IL8, CIC and cryoglobulin for DHF was also employed using the receiver operator characteristic curve (ROC). Impressive values for area under the curve were obtained for all the studied parameters. Taken together, the present study illustrated that the combined monitoring of IL8 titers, CIC levels and cryoglobulins along with CIC SDS PAGE analysis could be helpful for differentiating DHF from DF cases and also identification of possible novel markers for early detection of DHF cases.

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Conflict of interest
The authors declare that they have no conflict of interest.

References


