Determination of tumor necrosis factor-alpha levels in dengue virus infected patients by sensitive biotin-streptavidin enzyme-linked immunosorbent assay

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Abstract

A modified sandwich enzyme-linked immunosorbent assay using biotin-streptavidin system (BS-ELISA) was developed to determine levels of tumor necrosis factor-alpha (TNF-α) in serum samples of children infected with dengue virus (n = 99) and healthy controls (n = 41). The minimum detectable concentration of TNF-α by the BS-ELISA was 3.3 pg/ml. The mean TNF-α level was highest in those patients with dengue shock syndrome (DSS) or dengue hemorrhagic fever (DHF) grade III (37.44 ± 42.0 pg/ml). Lower levels were found in DHF grade I (28.44 ± 42.7 pg/ml), DHF grade II (24.21 ± 25.4 pg/ml) and dengue fever (DF) (14.10 ± 24.0 pg/ml). TNF-α in the sera of DF and DHF patients could be detected on days 2–6 after the onset of fever, the high level occurring on day 5. TNF-α was detected in 41.4% (24.01 ± 35.2 pg/ml) of dengue virus infected patients and 7.3% (4.2 ± 15.6 pg/ml) of control subjects. The sera of patients contained significantly higher levels of TNF-α than the sera of controls, P-value < 0.001. DHF patients had significantly higher levels of TNF-α than DF patients (P-value = 0.020) but no difference in the TNF-α levels from sera of DHF grades I–III patients was observed (P-value = 0.295). The results indicate that the BS-ELISA is a very sensitive method for determining TNF-α in serum samples of DF and DHF patients. The TNF-α levels might be associated with dengue virus infection and related to disease severity of DHF.

Keywords: Tumor necrosis factor-alpha; Dengue hemorrhagic fever; Dengue fever; Biotin-streptavidin ELISA

1. Introduction

Dengue virus infection is a common disease which occurs worldwide with an increasing incidence in tropical and subtropical countries. It produces a spectrum of clinical illness ranging from a mild disease as dengue fever to the more
serious dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Kautner et al., 1997). Dengue virus antigens were detected in mononuclear cells (Kittigul et al., 1997) and it was suggested that mononuclear phagocytes are the targets of dengue virus infection and contribute to homeostatic defects in DHF and DSS (Halstead, 1989). The pathogenesis of DHF is not understood fully. However, cytokines have been suggested as responsible for hemorrhagic manifestations and plasma leakage that may lead to shock in dengue virus infected patients. The pathophysiology of DHF and DSS patients is probably associated with the high levels of cytokines produced by monocytes and macrophages (Hober et al., 1993, 1996, 1998; Kurane et al., 1993). Exposure of monocytes and macrophages to dengue virus in vitro enhanced production of TNF-α (Lee et al., 1996). TNF-α in culture fluids from dengue virus-infected peripheral blood monocytes activated endothelial cells which might be a target in the pathogenesis of DHF (Anderson et al., 1997). However, similar levels of TNF-α were observed in patients with dengue fever and DHF (Laur et al., 1998). A study of TNF-α levels in relation to immunopathologic mechanisms is required to support the severity of DHF but the commercial enzyme immunoassay for this cytokine is expensive in developing countries.

The present study describes the development of a biotin-streptavidin enzyme-linked immunosorbent assay (BS-ELISA) for sensitive determination of TNF-α levels in sera of Thai patients with dengue virus infection and healthy subjects. The association between TNF-α levels and the severity of dengue diseases was examined.

2. Materials and methods

2.1. Study population

Blood specimens were collected from 99 patients admitted to hospitals and from 41 healthy control children under 15 years of age after informed consent was obtained, between June and November, 1997. The patients were diagnosed as having dengue virus infection by clinical findings and laboratory confirmation with fourfold rise in hemagglutination inhibition (HI) antibody titers in paired sera (Clarke and Casals, 1958). The sequence of dengue infection was categorized and the disease severity was graded according to World Health Organization (WHO) Guidelines (World Health Organization, 1997).

Of the patients enrolled in the study, 15% had primary dengue virus infection and 85% had secondary antibody response. There were 37 patients classified as DF, 34 as DHF grade I, 12 as grade II, and 16 as grade III or DSS.

2.2. Serum samples

A blood sample (3–5 ml) was obtained from each patient by venepuncture within a day of hospital admission. It was allowed to clot at room temperature and the serum was separated and then stored frozen at –70°C until use. Details of the patients’ case histories, physical examinations and laboratory findings were collected.

2.3. Assay for TNF-α

TNF-α levels in human sera were measured using a BS-ELISA. Assay conditions were optimised by checkerboard titration of recombinant human TNF-α standard (rh TNF-α). The BS-ELISA method was modified from the procedure described essentially by Kittigul et al. (1998) and Pisa et al. (1990). Briefly, a microtiter plate (Nunc Inter Med, Roskilde, Denmark) was coated at 2–4°C overnight with 100 µl of mouse monoclonal antibody anti-TNF-α (Genzyme Diagnostics, Cambridge, MA) at a concentration of 5 µg/ml in 0.05 M carbonate bicarbonate buffer, pH 9.6. After washing the plate with 0.15 M phosphate-buffered saline (PBS), nonspecific binding sites were blocked by adding 2% bovine serum albumin (BSA)–PBS (250 µl) and incubated at 37°C for 1 h. Then, the plate was washed six times with PBS-Tween 20 (PBST). Following the last wash, rh TNF-α or serum sample diluted 1:10 in 1% BSA-PBST was added (100 µl/well) and the plate was incubated at 37°C for 1 h. After washing, 100 µl of chicken IgY anti-TNF-α polyclonal antibody (2 µg/ml) (Promega Corporation,
Madison, WI) was added to each well, incubated at 37°C for 1 h and washed again. Biotin conjugated anti-chicken Ig (Promega Corporation, Madison, WI) at a concentration of 0.5 μg/ml (100 μl/well) was added to each well. The plate was incubated at 37°C for 1 h and washed again. Then, 100 μl of streptavidin-horseradish peroxidase (Vector laboratory, Burlingame, CA) diluted 1:3000 was added to each well and incubated at 37°C for 1 h. Finally, the plate was washed and the color was developed by adding the substrate buffer solution containing tetramethylbenzidine (TMB) and hydrogen peroxide (100 μl/well). The color reaction was stopped after 10 min incubation by the addition of 0.46 M sulfuric acid (50 μl/well). Optical densities (ODs) were read at 450 nm in an ELISA plate reader (Biotek, Winooski, VT). Minimal detectable concentration was defined by the standard deviation of dose measurement at zero dose or background (Larsson et al., 1997). The levels of TNF-α in serum samples were interpolated from the rh TNF-α standard calibration curve.

2.4. Statistical analysis

The difference of TNF-α levels in dengue virus infected patients and healthy controls was evaluated by the Mann–Whitney U-test. The Kruskal–Wallis test was used when more than two groups were compared. Differences giving P-value < 0.05 were considered significant.

3. Results

3.1. Optimization of the assay conditions

The strongest signal of ELISA-OD in determination of rh TNF-α was achieved with the use of 5 μg/ml monoclonal mouse anti-human TNF-α and 2 μg/ml anti-human TNF-α polyclonal antibody. Biotin conjugated anti-chicken IgY at the concentration of 0.5 μg/ml and streptavidin-horseradish peroxidase diluted 1:3000 gave a better signal-to background ratio than other concentrations tested. The pooled negative control sera diluted 1:10 gave the lowest background with the highest difference in OD value of rh TNF-α and was thus chosen to be the standard diluent in the BS-ELISA. Standard curves of rh TNF-α were plotted with known amounts of rh TNF-α versus their OD values. A linear fit was obtained at a concentration range up to 64 pg/ml with r² of 0.9999 as shown in Fig. 1.

3.2. Sensitivity of BS-ELISA

Background absorbance was measured using pooled negative sera diluted 1:10 and gave values ranging from 0.385 to 0.527 with the average of 0.47 ± 0.0655 (mean ± S.D.) in five experiments. The absorbance values of background and three lowest standard concentrations were plotted and are shown in Fig. 2. By calculation, the sensitivity of BS-ELISA for detecting TNF-α in human serum was approximately 3.3 pg/ml.

3.3. TNF-α levels in the sera of dengue fever or dengue hemorrhagic fever patients

The serum samples from patients with dengue fever or DHF were examined for levels of TNF-α. It was found that TNF-α was detected in 29.7% (11 of 37) of DF (mean ± S.D.; 14.10 ± 24.0 pg/ml) and 48.3% (30 of 62) of DHF patients (29.95 ± 39.5 pg/ml). Among 62 DHF patients, the TNF-α level was highest in DHF grade III (37.44 ± 42.0 pg/ml) followed by DHF grade I (28.44 ± 42.7 pg/ml) and DHF grade II (24.21 ± 25.4 pg/ml), as shown in Table 1.
Fig. 2. Determination of the detection limit of the BS-ELISA. Zero dose (background) and absorbance values of the 3 lowest standard concentrations of recombinant human TNF-α were in a linear regression. The detection limit was given by the S.D. of background divided by the slope of the regression line.

Based on the time after the onset of fever in individuals, TNF-α could be detected in patients with dengue fever whose sera were collected on days 2–6, the high value occurring on day 5 (Fig. 3). Similarly, TNF-α levels were found in DHF sera collected on days 2–5 with the high value also on day 5 after onset of fever (Fig. 4).

3.4. TNF-α levels during shock syndrome

Table 2 shows the individual levels of TNF-α in serum samples from DSS patients (nine of 16 cases). A very high TNF-α concentration was observed on the day the patient went into shock (126 pg/ml). The average TNF-α levels were highest in the sera collected one day before shock (44.75 ± 51.8 pg/ml), followed by those collected on the day of shock (38.5 ± 45.4 pg/ml), and 2 days before shock (24.30 ± 24.0 pg/ml).

3.5. Comparison of the TNF-α levels among the studied groups

Table 1

<table>
<thead>
<tr>
<th>Clinical syndrome</th>
<th>No. of patients</th>
<th>Positive (%)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Total</td>
<td></td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>DF</td>
<td>37</td>
<td>11 (29.7)</td>
<td>14.10 ± 24.0</td>
</tr>
<tr>
<td></td>
<td>DHF I</td>
<td>34</td>
<td>14 (41.2)</td>
</tr>
<tr>
<td></td>
<td>DHF II</td>
<td>12</td>
<td>7 (58.3)</td>
</tr>
<tr>
<td></td>
<td>DHF III</td>
<td>16</td>
<td>9 (56.3)</td>
</tr>
<tr>
<td></td>
<td>Total DHF</td>
<td>62</td>
<td>30 (48.3)</td>
</tr>
<tr>
<td></td>
<td>Total (DF+DHF)</td>
<td>99</td>
<td>41 (41.4%)</td>
</tr>
</tbody>
</table>

Table 1

Levels of TNF-α in sera from dengue virus infected patients

Fig. 3. TNF-α levels in the sera of DF patients on day after the onset of fever. The symbol represents the TNF-α value obtained from each patient on the indicated day. Blood samples were obtained within 24 h of admission, allowed to clot and the sera were separated. The day post onset of fever in the patients was derived from interviews.

TNF-α levels in the sera of dengue virus infected patients (41.4%) were compared with those of healthy subjects (7.3%) and are shown in Table 3. The presence of TNF-α in patients with dengue virus infection (24.01 ± 35.2 pg/ml) were signifi-
Fig. 4. TNF-α levels in the sera of DHF patients on day after the onset of fever. The symbol represents the TNF-α value obtained from individual patients. Blood samples were obtained within 24 h of admission, allowed to clot and the sera were separated. The day post onset of fever in the patients was derived from interviews.

Significantly higher than those in healthy subjects (4.2 ± 15.6 pg/ml), P-value < 0.001.

The sera of DHF patients had higher TNF-α levels than that of dengue fever patients (29.95 ± 39.5 versus 14.10 ± 24.0 pg/ml). This was statistically significant, P-value = 0.020. Although the highest value of TNF-α was found in the sera of DHF grade III patients, there was no significant difference in DHF grade I–III (P-value = 0.295).

Multivariable technique was used to determine how well several independent variables, both separately and together, explained the variation in TNF-α levels. By multiple classification analysis (MCA), there was no effect caused by the age and sex of the studied subjects on TNF-α levels; age:

Table 2
Levels of TNF-α in serum samples from patients with shock syndrome

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Day of collection (before shock)*</th>
<th>Time-post onset of fever</th>
<th>TNF-α level (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
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<td>2</td>
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<td>5</td>
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</tr>
<tr>
<td>8</td>
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<td>4</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>3</td>
<td>76</td>
</tr>
</tbody>
</table>

* The sera were collected on the day of the patient’s admission.

$P$-value of 0.242, sex: $P$-value of 0.731. The TNF-α levels in dengue virus infected patients were significantly different from healthy controls ($P$-value, 0.001). If the time after the onset of fever was controlled, the TNF-α levels in the serum samples of DHF and DF patients were significantly different ($P$-value, 0.030).

4. Discussion

In this study, TNF-α levels were determined in the sera of patients with dengue fever and dengue hemorrhagic fever by a highly sensitive BS-ELISA. Mouse monoclonal anti-human TNF-α antibody, specifically reactive with human TNF-α and lacking cross-reactivity was used as the capture reagent in the assay. Anti-human TNF-α
polyclonal antibody was produced in immunized chicken and purified from egg yolk by resin chromatography. This antibody specifically reacts with human TNF-α, has no cross reactivity with mammalian IgG (Ambrosius and Hadge, 1987), and does not bind bacterial or mammalian Fc receptor (Larsson and Sjoguist, 1990). To increase the sensitivity, biotin-conjugate anti-chicken IgY and streptavidin-horseradish peroxidase were included in the assay. Excellent linearity of the standard curve was obtained. The BS-ELISA method had a high sensitivity and specificity. It was simple to perform and was quantitative, and the cost of the method was less than that of the commercial kit. Thus, the BS-ELISA might be useful for facilitating the expanded application of clinical TNF-α data.

TNF-α could affect both endothelial cells (Anderson et al., 1997) and participate in some manifestations of DHF and DSS such as thrombocytopenia, hemorrhage and extravasation of fluid from the vascular compartment progressing to hypovolemic shock (Tracey et al., 1986). Although half of dengue virus infected patients were negative for TNF-α, elevated levels in the patients with dengue diseases were observed when compared with that in healthy controls. The elevated levels of TNF-α in dengue fever and DHF patients indicate the activation of TNF-α production during dengue virus infection. In addition, TNF-α levels in DHF patients were significantly higher than that in patients with dengue fever and the highest level was observed in the severe form of dengue diseases (DHF grade III or DSS). These support the belief that TNF-α production is related to immunopathologic mechanisms in DHF and DSS.

The TNF-α levels could be detected in the sera of dengue patients as early as day 2 with the high value on day 5 after onset of fever and the day that the patients went into shock. Unfortunately, serum samples from patients after the day of shock were not obtained since only a single serum was collected on the day of patient’s admission. Therefore, the duration of immune activation after shock syndrome was not examined.

The modified sandwich BS-ELISA described above has been shown to be of value to determine TNF-α in serum samples. This method will be particularly useful for studies on the pathogenesis of disease caused by dengue viruses in those countries where DHF is still a public health problem.

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References


