Autoimmunity in Dengue Virus Infection

Chiou-Feng Lin*, Huan-Yao Lei*, Ching-Chuan Liu**, Hsiao-Sheng Liu*, Trai-Ming Yeh***, Shun-Hua Chen* and Yee-Shin Lin*

*Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan
**Department of Paediatrics, National Cheng Kung University Medical College, Tainan, Taiwan
***Department of Medical Technology, National Cheng Kung University Medical College, Tainan, Taiwan

Abstract

Dengue haemorrhagic fever (DHF) is a complicated disease associated with viral and immune pathogenesis. There is still no effective vaccine to prevent the progression of DHF because of its undefined pathogenic mechanisms. The generation of autoimmunity in dengue virus (DEN) infection has been implicated in dengue pathogenesis. Based on our previous studies showing antibodies (Abs) against DEN nonstructural protein 1 (NS1) cross-reacted with human platelets and endothelial cells, a mechanism of molecular mimicry may contribute to autoantibody (autoAb) production. Here, the generation of autoAbs against human endothelial cells in patients infected with different DEN serotypes is shown. The levels of autoAbs present in different disease stages of DHF and the induction of endothelial cell apoptosis by patient sera were also determined. The results suggest that autoimmune responses are implicated in dengue disease pathogenesis and cause concern in vaccine development.

Keywords: Dengue haemorrhagic fever, dengue virus serotype, autoimmunity, autoantibody, endothelial cells.

Introduction

Infection with dengue virus (DEN) causes dengue fever (DF) - an important arthropod-borne viral disease in terms of morbidity and mortality[1,2] and may result in severe dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS). Globally, about 2.5 billion people are at risk of the infection[3]. A recent dengue outbreak in Indonesia led to a 1.1% case-fatality rate in 58,301 cases by April 2004[4]. All four DEN serotypes were present in this outbreak. Presently, the severity of dengue disease is primarily predicted according to the effect of antibody-dependent enhancement (ADE) in different serotype cross-infections[5,6]. In order to effectively control the progression of the disease, development of an effective vaccine against DEN infection is needed. There are several vaccine candidates undergoing clinical trials[7-10]. Nevertheless, the role that antibodies (Abs) may play in increasing the severity of dengue infections[7,10] remains a matter of concern in vaccine development.

* E-mail: yslin1@mail.ncku.edu.tw
In addition to the ADE of DEN infection, autoantibody (autoAb) production may also be involved in dengue disease[11-15]. We demonstrated that the autoAbs generated in DEN infection induced endothelial cell damage[13] and inflammatory activation (in press). A mechanism of molecular mimicry in which Abs directed against DEN nonstructural protein 1 (NS1) is, at least in part, responsible for the autoimmunity. The relationships of the autoAb levels with dengue serotypes and disease severity are examined in this study.

Materials and methods

Patient sera

DEN-2 and DEN-3 patient sera were collected during the outbreaks in southern Taiwan from 1997 to January 1999[16]. DEN-4 patient sera were obtained from the Department of Dengue Hemorrhagic Fever, Children’s Hospital No. 1, Ho Chi Minh City, Viet Nam. The disease severity was based on the WHO definition[3]. Normal control sera from five healthy individuals were used as background.

Cell cultures

Human umbilical cord vein endothelial cells (HUVEC) were cultured in modified M-199 medium as described previously[13]. For experiments, 1,000 U/ml trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA) were used to detach cells.

Binding activity detection

After detachment, cells were suspended at 5×10⁵ for flow cytometry. The cells were washed briefly with phosphate-buffered saline (PBS) and fixed with 1% formaldehyde in PBS at room temperature for 10 minutes, then washed again with PBS. Patient sera were 1:25 diluted and incubated with cells at 4 °C for 1 hour. After being washed three times with PBS, the cells were incubated with 20 µl of fluorescein isothiocyanate (FITC)-conjugated anti-human IgG or IgM (PharMingen, San Diego, CA) at 4 °C for 1 hour. The binding activity of Abs to cells was analysed using flow cytometry (FACScan; BD Biosciences, San Jose, CA) with excitation set at 488 nm.

Cell death detection

For cell viability determination, cells were stained with eosin Y and counted using light microscopy. Apoptosis-induced DNA strand breaks were analysed by terminal deoxynucleotidyl transferase-mediated dUTP* nick-end-labeling (TUNEL) reaction using the ApoAlert DNA Fragmentation Assay Kit (Clontech, Palo Alto, CA). After incubation with patient sera for 24 hours, endothelial cells (1×10⁶) were fixed and stained according to the manufacturer’s instructions, and then analysed using flow cytometry.

Statistical analysis

The statistical difference was analysed using unpaired Student’s t-tests in SigmaPlot version 4.0 for Windows (Cytel Software Corporation, Cambridge, MA).

Results

Generation of autoAbs in dengue patients infected with different serotypes and at different disease stages

Our previous studies demonstrated the presence of anti-platelet and anti-endothelial cell autoAbs in dengue patient sera[12,13]. The levels of these autoAbs were

1 deoxyuridine triphosphate
higher in DHF/DSS than in DF patient sera. The dysfunction of platelets and endothelial cells caused by the autoAbs was also shown. The cross-reactivity of patient sera with endothelial cells was the highest in the acute stage (3-7 days after fever onset) and subsequently decreased in the convalescent (1-3 weeks after acute phase) and later (8-9 months) stages. In our previous study, patient sera were collected from an outbreak of DEN-3 infection. In this study, we further examined the autoAb levels produced by patients infected with different DEN serotypes and the relationship between the autoAb levels and disease severity. The results showed that the levels of anti-endothelial cell Abs, as determined by both the percentages of endothelial cells reactive with patient sera IgM or IgG and the mean fluorescence intensity, were similar in patients infected with DEN-2, 3 or 4 (Table 1). There was no significant difference between different serotype infections. The levels of autoAbs were higher in DHF/DSS than in DF patient sera. In addition, the levels of IgM isotype of autoAbs were higher than those of IgG. The DEN-1 serotype was not tested because we had no DEN-1-infected patient sera. We next investigated the endothelial cell cross-reactivity of DHF patient sera at different disease grades. DHF patient sera collected from Grades I to IV with DEN-4 infection, according to the WHO definition, were tested. There was no significant difference between the four grades of DHF in both anti-endothelial cell IgM and IgG (Table 1). Due to our limited sample sizes of patient sera, especially of Grades I and IV, we were unable to determine whether there was any correlation of autoAbs with disease severity.

### Table 1. Anti-endothelial cell IgM/IgG levels in the sera of dengue patients infected with different dengue serotypes and at different disease grades

<table>
<thead>
<tr>
<th></th>
<th>% of endothelial cells reactive with patient sera</th>
<th>Mean fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM Mean (SD) IgG Mean (SD)</td>
<td>IgM Mean (SD) IgG Mean (SD)</td>
</tr>
<tr>
<td>Normal (n=5)</td>
<td>4.7 (0.5) 2.6 (0.6)</td>
<td>12.8 (1.5) 6.6 (0.8)</td>
</tr>
<tr>
<td>DEN-2 infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (n=5)</td>
<td>38.6 (1.4)*** 14.8 (5.1)*</td>
<td>62.5 (4.1)*** 14.1 (2.1)*</td>
</tr>
<tr>
<td>DEN-3 infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (n=6)</td>
<td>35.1 (3.7)*** 12.7 (2.1)*</td>
<td>65.7 (5.7)*** 15.7 (1.7)*</td>
</tr>
<tr>
<td>DHF/DSS (n=5)</td>
<td>54.6 (4.4)*** 23.7 (1.9)**</td>
<td>74.9 (4.6)*** 24.6 (3.6)**</td>
</tr>
<tr>
<td>DEN-4 infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (n=5)</td>
<td>35.9 (1.6)*** 15.1 (3.5)*</td>
<td>66.6 (6.3)*** 10.0 (2.3)*</td>
</tr>
<tr>
<td>DHF (n=36)</td>
<td>50.5 (9.5)*** 24.9 (8.3)**</td>
<td>72.1 (13.9)*** 11.1 (4.4)*</td>
</tr>
<tr>
<td>Grade I (n=1)</td>
<td>66.9 17.8</td>
<td>83.4 8.8</td>
</tr>
<tr>
<td>Grade II (n=26)</td>
<td>51.7 (9.0)*** 25.5 (7.7)*</td>
<td>73.6 (10.4)*** 11.4 (4.3)*</td>
</tr>
<tr>
<td>Grade III (n=8)</td>
<td>45.4 (8.6)*** 24.7 (10.6)*</td>
<td>64.7 (7.0)*** 13.9 (6.4)*</td>
</tr>
<tr>
<td>Grade IV (n=1)</td>
<td>42.2 17.9</td>
<td>63.0 11.6</td>
</tr>
</tbody>
</table>

Student’s t-tests: *P < 0.05 vs Normal; **P < 0.01 vs Normal; ***P < 0.001 vs Normal.
Induction of endothelial cell apoptosis by sera of dengue patients infected with different serotypes

Anti-endothelial cell autoAbs caused cell damage which was characterized by apoptosis\[^{13}\]. The ability of patient sera with different DEN serotype infections to induce endothelial cell apoptosis was tested. HUVEC were treated with a 1:25 dilution of dengue patient or healthy-control sera for 24 hours, and cell apoptosis was measured using TUNEL reaction followed by flow cytometric analysis. The histogram and the percentages of apoptotic cells from one set of duplicate cultures are shown in the Figure below. The results indicated that cell apoptosis was induced by all patient sera and the cells underwent a higher percentage of apoptosis when induced by DHF patient sera than by DF patient sera. Healthy-control sera showed only the background level. Cell viability detected using eosin Y staining showed an inverse relationship with the percentages of apoptosis (Table 2). There was no significant difference in endothelial cell apoptosis induced by patient sera with different serotype infections.

Figure. Dengue patient sera induced endothelial cell apoptosis
**Table 2.** Endothelial cell apoptosis induced by sera of dengue patients infected with different dengue serotypes

<table>
<thead>
<tr>
<th></th>
<th>% of cell viability</th>
<th>% of endothelial cell apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n=5)</td>
<td>94.1 (3.5)</td>
<td>6.9 (2.8)</td>
</tr>
<tr>
<td><strong>DEN-2 infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (n=5)</td>
<td>75.2 (5.4)**</td>
<td>21.9 (5.6)**</td>
</tr>
<tr>
<td><strong>DEN-3 infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (n=6)</td>
<td>81.7 (5.1)**</td>
<td>19.5 (7.1)**</td>
</tr>
<tr>
<td>DHF/DSS (n=5)</td>
<td>66.6 (10.8)****</td>
<td>29.7 (9.3)****</td>
</tr>
<tr>
<td><strong>DEN-4 infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (n=5)</td>
<td>72.0 (9.5)**</td>
<td>22.0 (5.6)**</td>
</tr>
<tr>
<td>DHF (n=5)</td>
<td>63.3 (15.1)****</td>
<td>37.2 (5.5)****</td>
</tr>
</tbody>
</table>

Student’s t-test: **P<0.01 vs Normal; ***P<0.001 vs Normal.

**Discussion**

DHF is a life-threatening disease with poorly defined pathogenic mechanisms[1,2,5,10]. An ADE effect in different serotype cross-infection is frequent[2,5,6]. Patients may develop severe complications of progressive DHF. Vascular leakage and haemorrhagic diathesis are the hallmarks in DHF patients. A number of studies have demonstrated abnormal immune responses caused by DEN infection, including cytokine and chemokine production, complement activation and immune cell activation[10,11,17-20]. In addition, autoimmune responses may be involved in DHF pathogenesis[12-13,22]. Dengue patients produced Abs which cross-reacted with human platelets and endothelial cells[12,13]. Anti-NS1 produced after DEN infection may at least in part, account for the cross-reactivity of patient sera with endothelial cells. In this study, we further showed that the levels of anti-endothelial cell Abs were similar in patients infected with different DEN serotypes. The percentages of endothelial cells reactive with DHF/DSS patient sera were higher than those with DF patient sera. However, there was no difference in anti-endothelial cell Ab levels at different DHF disease grades. The sample size of patient sera needs to be increased to gain an insight into the role of anti-endothelial cell Abs in DHF pathogenesis. These autoAbs exerted similar effects in the induction of endothelial cell apoptosis of patients infected with different DEN serotypes.

In dengue pathology, various cytokines and chemokines including TNF-α, IL-6, IL-8, and RANTES[17,18,20] have been detected in patient sera with DHF/DSS and in DEN-infected endothelial cell culture supernatants[17,18,20]. Our recent studies also demonstrate that anti-NS1 Abs can stimulate cytokine and chemokine production (in press). Therefore,
both immune activation and apoptosis occur in endothelial cells after stimulation by autoAbs.

There are no dengue vaccines available. Yet, several potential vaccines, including life-attenuated whole DEN and DNA vaccines, are undergoing clinical trials\(^7\)-\(^\text{10}\). It is hoped that a fusion or a chimera dengue vaccine will be developed to provide protection against all serotypes of DEN infection. In addition, DEN NS1 protein used as a vaccine candidate in mice showed resistance to fatal DEN encephalitis\(^2\). Passive administration of anti-NS1 Abs also conferred protection in mice when challenged with lethal doses of DEN\(^3\). However, these previous studies only monitored the survival rates of mice but did not examine the potential histopathological effects. Studies by Falconar\(^2\) showed the cross-reactivity of anti-NS1 to host antigens and cells and a haemorrhage-like hallmark in mice. This, taken together with our findings, suggests that a potential pathogenic effect of DEN NS1 vaccine should be taken into consideration. The possible approaches include gene modifications of DEN NS1 to truncate or mutate the epitopes that may cause the pathogenic effects.

Acknowledgement

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References


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