Manuscript of thrombocytopenia in dengue-2-virus-infected mice

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Dengue virus infection causes dengue fever, dengue haemorrhagic fever and dengue shock syndrome. No animal model is available that mimics these clinical manifestations. In this study, the establishment is reported of a murine model for dengue virus infection that resembles the thrombocytopenia manifestation. Dengue-2 virus (dengue virus type 2) can infect murine cells either in vitro (primary cell culture) or in vivo. Viraemia detected by RT–PCR was found transiently at 2 days after intravenous injection of dengue-2 virus. Transient thrombocytopenia developed at 10–13 days after primary or secondary infection. Anti-platelet antibody was generated after dengue-2 virus infection. There was strain variation in dengue-2 virus infection; the A/J strain was more sensitive than BALB/c or B6 mice. This dengue-2-virus-infected mouse system accompanied by thrombocytopenia and anti-platelet antibody will be a valuable model to study the pathogenicity of dengue virus infection.

Introduction

Dengue fever is an acute infectious disease caused by dengue virus. It is characterized by biphasic fever, headache, body pain, rash, lymphadenopathy and leukopenia (Halstead, 1988; Bhamarapravati, 1989; Henchal & Putnak, 1990). In most cases, dengue fever is self-limited. However, there is a risk of progression to dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS), especially when cross-infection by different serotypes occurs (the virus has four serotypes). DHF is a severe febrile disease characterized by abnormalities of haemostasis and increased vascular permeability, which in some instances results in DSS. DSS is a form of hypovolaemic shock that is associated clinically with haemoconcentration, and it can lead to death. Antibody-dependent enhancement (ADE) of infection has long been thought to play a central role in dengue virus infection (Halstead, 1970). The ADE hypothesis was formulated to explain the finding that the most severe manifestations of DHF/DSS seem to occur predominantly, although not exclusively, in children experiencing a second dengue virus infection caused by a serotype different from that which caused the first infection (Halstead & O'Rourke, 1977). However, DHF/DSS can occur after primary infection (Thein et al., 1997). Immune activation and virus virulence have also provided plausible explanations for the pathogenesis of DHF/DSS (Rothman & Ennis, 1999; Leitmeyer et al., 1999).

Dengue virus can replicate in the brains of suckling mice when the virus is inoculated intracerebrally (Meiklejohn et al., 1952). Recently, Johnson & Roehrig (1999) reported that dengue virus administered intraperitoneally can replicate in AG129 mice, which lack IFN-α/β and IFN-γ receptor genes. The mice showed neurological abnormalities, including hind-leg paralysis and blindness at 7 days, and died at 12 days after infection. Severe combined immunodeficient (SCID) mice engrafted with human K562 cells or HepG2 cells have also been used to study dengue virus infection (Lin et al., 1998; An et al., 1999). The inoculation of dengue-2 virus into the engrafted SCID mice induced neurological paralysis and death. A high titre of dengue-2 virus was also found in the brain, which correlated well with the progression of encephalopathy in the infected mice. These studies suggest that mice are a permissive host for dengue virus replication. However, no infectious model that mimics DHF/DSS has yet been reported. In this study, we report that dengue virus can infect immunocompetent mice by intravenous inoculation. The mice can manifest thrombocytopenia and generate anti-platelet antibody.

Methods

Animals. Breeder mice of BALB/c, B6, C3H/He, AKR and A/J strains were purchased from the Jackson Laboratory (Bar Harbor, ME,
USA) or Charles River Japan, Inc. They were maintained on a standard laboratory diet with water ad libitum in the animal facility of the Medical College, National Cheng Kung University, Tainan, Taiwan, ROC. The animals were raised and cared for according to the guidelines set up by the National Science Council, Republic of China. Six- to 12-week-old mice were used in all experiments.

**Dengue virus preparation.** A local isolate (PL046) of dengue-2 virus was supplied by the Institute of Preventive Medicine, Nan Kung, Taipei, Taiwan (Republic of China). The virus was propagated in the mosquito C6/36 cell line, which was incubated at 28 °C for 5 days in Eagle’s minimal essential medium containing 2% heat-inactivated FBS (Igarashi, 1978). Each virus pool contained a titre of $1 \times 10^8$ p.f.u./ml as determined by the standard method of plaque assay on BHK cells (Sukhavachana et al., 1966). The pooled virus stocks were first concentrated by ultrafiltration with a 10 kDa cut-off membrane and then ultracentrifuged at 10000 $g$ for 10 min. The supernatant was further ultracentrifuged at 100000 $g$ for 3 h to pellet the dengue virus particles. This procedure can enrich the virus stock to $1 \times 10^{11}$ p.f.u./ml.

**Dengue-2 virus infection in mice.** Infection of murine primary hepatocytes or brain cells with dengue-2 virus was manifested in vitro or in vivo. For in vitro study, the livers or brains of neonatal BALB/c mice were dispersed and cultured in DMEM containing 20% FBS. The confluent cells were subcultured every 4 or 5 days. The primary mixed cells of hepatocytes or brain cells were used within 10 passages. The cells were infected with dengue-2 virus at an m.o.i. of 5 and the cytopathic effect was observed under the microscope. For infection in vivo, groups of four to six mice were inoculated intravenously with dengue-2 virus ($1 \times 10^6$ p.f.u. per mouse). The mice were monitored daily for clinical signs of paraplegia. The genome of dengue-2 virus was detected by RT–PCR analysis with dengue-virus-specific primers. RNA was extracted from blood with the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. Oligonucleotides AD3 (5’ CTGATTTCCATCCCGTA 3’) and AD4 (5’ GATATGGGTTATTGGATAGA 3’) were used as the antisense and sense primers, respectively (Liu et al., 1995, 1997). At 2 or 3 weeks post-infection, the mice showed symptoms ranging from mild paraplegia to death.

**Induction of thrombocytopenia by dengue-2 virus.** Groups of four to six mice were injected intravenously with $1 \times 10^6$ p.f.u. dengue-2 virus. At intervals after injection, blood was collected by cardiac puncture into EDTA tubes (Monoject, Sherwood Medical). Circulating platelets, red blood cells and leukocytes were counted by using a particle counter model PC-608 (Erma Inc., Tokyo, Japan). In some experiments, the sensitized mice were re-infected intravenously with the same dose of dengue-2 virus.
Detection of anti-platelet antibody. Human peripheral blood collected in sodium EDTA (2 mg/ml) was centrifuged at 100 g for 10 min at room temperature. The upper layer of platelet-rich plasma was removed to a 15 ml tube, mixed with 0.34% EDTA in PBS and centrifuged at 1000 g for 15 min. The pellets were washed three times with 0.34% EDTA–PBS and fixed in 10 ml 1% formaldehyde in PBS at room temperature for 10 min. The fixed platelet suspension was centrifuged at 1000 g for 15 min. The pellets were washed twice in PBS and resuspended in 2 ml PBS. The platelet count was determined by using a haemocytometer. Anti-platelet antibody binding was determined by flow cytometry analysis as described previously (Schwarz et al., 1999). Platelets (2.5 x 10^6 in 0.1 ml) were incubated with various dilutions of serum for 60 min on ice and then washed twice with PBS. The secondary FITC-conjugated goat anti-mouse IgG antibody (Cappel, Organon Teknika) was added and the mixture was incubated for 40 min on ice. After washing twice with PBS, the platelets were suspended in PBS and analysed by FACScan (Becton-Dickinson) with excitation set at 488 nm.

Results

Dengue-2 virus can infect mice and induce transient thrombocytopenia

Dengue-2 virus can infect the murine liver or brain primary cells and cause cytopathic effect in these cells (Fig. 1). The replicative intermediate of negative-stranded dengue virus RNA and envelope protein could be detected by RT–PCR and immunofluorescent staining, respectively (data not shown). Infection in vivo was demonstrated in mice. Intravenous injection of 1 x 10^6 p.f.u. dengue-2 virus into A/J mice induced paraplegia at 2–3 weeks, while the mock-infected controls were normal (data not shown). The dengue-2 virus RNA genome was detected in blood at 2 days but at no other time after injection (Fig. 2). This suggests that dengue-2 virus can infect mice in vitro and in vivo. Although A/J mice developed paraplegia after dengue-2 virus infection, they recovered after 1 month. However, there was transient thrombocytopenia at 10–13 days after injection. When the mice were re-infected with the same dengue-2 virus 2 months later, thrombocytopenia was manifested again at 10 days after infection (Fig. 3). This suggests that dengue-2 virus infection, whether primary or secondary, can cause transient thrombocytopenia.

Anti-platelet antibody in dengue-2-virus-infected mice

Mouse anti-NS1 antibodies can cross-react with platelets (Falcoran, 1997). Activity of immune serum from dengue-2-virus-infected mice against platelets was detected by flow cytometry analysis. As shown in Fig. 4(A), serum from A/J mice infected with dengue-2 virus contained anti-platelet antibody and the anti-platelet binding was dilution-dependent. Serum from mock-infected control mice contained no anti-platelet antibody. Kinetic analysis of serum collected from A/J mice infected with dengue-2 virus showed that the anti-platelet antibody appeared as early as 4 days after infection and the titre was maintained at a similar level for 2 or 3 weeks (Fig. 4B).

Strain susceptibility to dengue-2 virus infection

Several strains of mice were screened for their susceptibility to dengue-2 virus. The parameters used included viraemia at 2
A day after infection (detected with RT–PCR), thrombocytopenia at 10–13 days after infection, anti-platelet antibody in serum and manifestation of paraplegia at 2–3 weeks after infection. As shown in Table 1, strain A/J was the most susceptible. However, in strains BALB/c and B6, fewer than 10% of mice showed paraplegia after dengue-2 virus infection. The manifestation of transient thrombocytopenia after primary dengue-2 virus infection varied in BALB/c and B6 mice. However, the serum from dengue-2-virus-infected BALB/c and B6 mice did contain anti-platelet antibody. Although we inoculated a large quantity of dengue-2 virus (1 × 10⁸ p.f.u. per mouse) intravenously, the dengue-2 virus genome could only be detected by RT–PCR in blood collected from A/J mice at 2 days after injection.

**Discussion**

Primary infections of dengue virus can develop into DHF or DSS (Thein et al., 1997). The mechanisms involved in the pathogenesis of DHF/DSS remain poorly understood, although the ADE hypothesis was proposed to explain DHF/DSS in secondary infections with different serotypes. ADE has been observed *in vitro* when a subneutralizing amount of antibody is present with virus in the culture. The virus might attach to the cells via the Fc receptor and the number of target cells is increased (Halstead & O'Rourke, 1977). This concept of DHF/DSS pathogenicity has been dominant for two decades, but other viewpoints have recently been expressed (Bielefeldt-Ohmann, 1997). Immune activation and virus virulence have also provided plausible explanations for the development of DHF/DSS (Rothman & Ennis, 1999; Leitmeyer et al., 1999). In this study, we have demonstrated that dengue-2 virus can infect mice via a peripheral route. The mice developed transient thrombocytopenia and contained anti-platelet antibody upon dengue virus infection. This

**Table 1. Strain variation of dengue-2 virus infection**

<table>
<thead>
<tr>
<th>Strain</th>
<th>H-2</th>
<th>RT–PCR*</th>
<th>Thrombocytopenia†</th>
<th>Anti-plt Ab‡</th>
<th>Paraplegia (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>29/41 (71)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>d</td>
<td>–</td>
<td>±</td>
<td>+</td>
<td>2/29 (7)</td>
</tr>
<tr>
<td>B6</td>
<td>b</td>
<td>–</td>
<td>±</td>
<td>+</td>
<td>2/20 (10)</td>
</tr>
<tr>
<td>AKR</td>
<td>k</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>7/12 (58)</td>
</tr>
<tr>
<td>C3H/He</td>
<td>k</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>5/30 (17)</td>
</tr>
</tbody>
</table>

* Blood collected from 1–3 days after injection was examined for dengue virus genome by RT–PCR.
† Platelet numbers were counted at 10–13 days after dengue virus infection.
‡ Anti-platelet (plt) antibody was detected by flow cytometry analysis.
§ Paraplegia was observed at 2–3 weeks after dengue virus infection. Numbers of paralysed mice of total mice tested are indicated, with percentages in parentheses.
ND, Not determined.
provides a mouse model to study pathogenesis, especially immune activation in dengue virus infection.

The mouse is not the natural host of dengue virus. However, it is well known that dengue virus can replicate in the brains of suckling mice (Meiklejohn et al., 1952) and that there is strain variation of susceptibility to dengue virus infection (Raut et al., 1996) when the virus is inoculated intracerebrally. AG129 mice, which lack IFN-α/β and IFN-γ receptor genes, are permissible for replication of mouse-adapted dengue-2 virus New Guinea C strain (Johnson & Roehrig, 1999). We showed further that normal mice, such as strain A/J, can host dengue-2 virus. The dengue-2 virus strain used in our study is the same clinical isolate (PL046) as used by Lin et al. (1998) in their study of infection in K562-grafted SCID mice. An et al. (1999) also used HepG2-engrafted SCID mice to demonstrate dengue-2 virus infection in liver and brain. The dengue-2-virus-infected mice showed signs of neurological paralysis and died 2 weeks after injection. Our study confirmed that dengue-2 virus can indeed infect mice. This model has several distinct features. Firstly, we had to inoculate with a large quantity of dengue-2 virus; a dose of less than 1 × 10⁸ p.f.u. per mouse was not effective in causing paraplegia (unpublished observation). There was strain variation with regard to susceptibility to dengue virus infection: strains A/J and AKR were more sensitive than strains BALB/c, B6 or C3H/He. A/J mice are reported to have significantly lower lymphoid, granuloid, monocytoid and NK cell counts than B6 mice (Miller & Kearney, 1998; Whyte & Miller, 1998). Depletion of IFN-γ increases the susceptibility of A/J mice to mouse hepatitis virus infection (Lucchiarì et al., 1992). Secondly, viraemia was low and transient in immunocompetent mice compared with that in SCID or IFN-deficient AG129 mice. We could not isolate dengue-2 virus from the blood of infected mice. It could only be detected by sensitive RT–PCR in A/J mice. Immune responses, especially IFN, play a very important role in the clearance of dengue virus. However, dengue virus will reappear in the brain, spinal cord and spleen in paralysed mice at 2 or 3 weeks after infection (unpublished observation). Where dengue virus ‘hides’ is currently under investigation. Thirdly, transient thrombocytopenia was manifested at 10–13 days after infection. A/J mice showed a more consistent pattern of thrombocytopenia than did BALB/c or B6 mice, probably reflecting the greater sensitivity of A/J mice to dengue virus infection. Finally, anti-platelet antibody was detected in serum of dengue-2-virus-infected mice and was associated with the development of thrombocytopenia. The anti-platelet antibody was IgG class; its titre was higher in A/J mice than in BALB/c or B6 mice. Although clinical symptoms, such as thrombocytopenia, prolonged partial thromboplastin time and increased haematocrit, were reported in paralysed dengue-2-virus-infected, HepG2-grafted SCID mice, they are not related to immune responses to dengue virus infection.

Thrombocytopenia is common in dengue fever and is constant in DHF/DSS (Bhamarapravati, 1989; Henchal & Putnak, 1990). The pathogenesis of thrombocytopenia is poorly understood. La Russa & Innis (1995) suggested that dengue virus-induced bone marrow suppression depressed platelet synthesis and resulted in thrombocytopenia. Wang et al. (1995) found that dengue-2 virus can bind to human platelets in the presence of virus-specific antibody and proposed that the immune-mediated clearance of platelets was involved in the pathogenesis of thrombocytopenia in DHF/DSS. Parvovirus infection is associated with childhood idiopathic thrombocytopenic purpura (Yoto et al., 1993; Heegaard et al., 1999). To our knowledge, this study is the first to report the generation of anti-platelet antibody after dengue-2 virus infection and to describe the association between anti-dengue virus immune response and its cross-reactivity to platelets. The anti-platelet antibody will lyse platelets in the presence of complement. It also interferes with thrombin-induced platelet aggregation (unpublished observation). Recently, we also found the presence of anti-platelet auto-antibody in the sera of dengue patients, and its level was higher in DHF/DSS patients than in dengue fever patients (unpublished data). Further studies are needed to clarify the relationship between dengue virus infection and autoimmunity.

Dengue virus infection produces biphasic fever and DHF and DSS usually occur at the late stage of infection. There was an outbreak of dengue infection in southern Taiwan in October–December 1998. We took the opportunity to study the immune parameters in dengue virus-infected patients. We found that patients had immune aberrations, such as immature neutrophil, CD4⁺/CD8⁺ cell ratio inversion occurred in acute infection (6–14 days after onset of fever) (unpublished data). Immune deviation is not related to disease severity per se, but might initiate the consequent development of DHF/DSS. We think that immune deviation induced by dengue virus infection, not ADE, is more appropriate to explain the pathogenesis of dengue infection. This murine model of dengue-2 virus infection that mimics the clinical thrombocytopenia symptoms in humans should be useful in studying the immunopathogenesis of dengue virus infection. We are currently investigating the combination of infection/re-infection with different serotypes of dengue virus. This model will help to elucidate the mechanism of DHF/DSS pathogenicity, especially the significance of ADE in vivo.

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References


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