Manifestation 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 hindleg 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 ($1 \times 10^8$ 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 $1 \times 10^8$ 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.

**Fig. 1.** Dengue-2 virus infection causes cytopathic effect in murine primary cell cultures. Murine primary cell cultures were infected with dengue-2 virus at an m.o.i. of 5 as described in Methods. Cytopathic effect was observed at 3 days. (A) Mock-infected control brain cells; (B) dengue-2-virus-infected brain cells; (C) mock-infected control liver cells; (D) dengue-2-virus-infected liver cells. Magnification ×175 (A, B), ×350 (C, D).
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 × 10⁶ 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 × 10⁸ 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 (Falconnar, 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
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Fig. 4. Anti-platelet antibody in dengue-2-virus-infected mice. Groups of four A/J mice were infected intravenously with $1 \times 10^8$ p.f.u. dengue-2 virus per mouse. Anti-platelet antibody was detected by FACScan as described in Methods. (A) Dilution dependence of anti-platelet antibody binding. Blood was collected at 10 days after injection. The sera were serially diluted 2-fold with PBS. Mock-infected mice were used as the control, while ‘blank’ represents the second, conjugated antibody only. (B) Kinetic analysis of anti-platelet antibody after dengue virus injection. Blood was collected at various times post-injection. Sera were diluted 25-fold with PBS. p.i., Post-infection.

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>
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<tbody>
<tr>
<td>A/J</td>
<td>a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>29/41 (71)</td>
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<tr>
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<td>d</td>
<td>-</td>
<td>±</td>
<td>+</td>
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<td>B6</td>
<td>b</td>
<td>-</td>
<td>±</td>
<td>+</td>
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<tr>
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<td>k</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>7/12 (58)</td>
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<tr>
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<td>k</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>5/30 (17)</td>
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* 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.

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
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 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\textsuperscript{dim} or CD8\textsuperscript{dim} monocytecytosis, atypical lymphocytosis, overproduction of serum IL-6 and impaired mitogenic T cell responses. Surprisingly, a transient CD4:CD8 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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