HISTOPATHOLOGICAL AND ULTRASTRUCTURAL STUDIES OF LUNG TISSUE OF MICE REINFECTED WITH DENGUE VIRUS SEROTYPES 1 OR 2.

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ABSTRACT

Histological and ultrastructural alterations in lung of BALB/c mice reinfected with heterologous DENV by the intravenous route with non-neuroadapted dengue viruses serotypes 1 and 2 were analyzed. The lung samples were processed following the standard techniques of photonic and transmission electron microscopy. Morphological studies showed breakdown of alveolocapilar barrier leading to alveolitis, focal zones of collapse, and intraalveolar hemorrhage. Inside alveolar septa, congested capillaries exhibited neutrophils and platelets. Alveolar capillary endothelial cells exhibited aspects of activation with exuberant phyllopodia and intracytoplasmic vesicles and vacuoles. Morphometrical analyses demonstrated an increase of the surface density of interalveolar septa in all reinfected mice while alveolar space density was decreased. All these morphometrical data corroborated the histological features emphasizing the alveolocapilar breakdown in dengue infected animals. Important is the fact that reinfection leads to intraalveolar fibrogenesis as demonstrated by histomorphometry and ultrastructural studies. DENV particles, antigens and RNA were observed 72 hours post-reinfection in mosquito cells (C6/36) inoculated with sera of the animals. The morphological alterations observed in lungs were similar to the observed in human cases of dengue fever and dengue hemorrhagic fever. The present study demonstrates that the BALB/c mice, during the secondary infection by a heterologous serotype of DENV, develop morphological alterations in lung tissue more severe than those observed in the primary infection.

INTRODUCTION

Dengue viruses (DENV), members of the Flaviviridae family, occur as four distinct serotypes (DENV-1, -2, -3, -4) that are transmitted from infected to susceptible humans mainly by Aedes aegypti mosquitoes. DENV infection is mostly asymptomatic or produces a mild self-limiting acute febrile illness, dengue fever (DF), and a life-threatening severe illness, dengue hemorrhagic fever (DHF) with minor or major bleeding from different sites (Agarwal et al. 1999). DHF has emerged as the most important arbovirus disease in man in the last three decades. It has been estimated that about 50 to 100 million cases of DF occur every year with about 50,000 to 250,000 cases of DHF (Rigau-Perez et al. 1998, WHO). It is generally accepted that DHF and the associated dengue shock syndrome (DSS) occur as a result of secondary infection by...
a heterologous DENV and that immunopathological mechanisms are involved in the pathogenesis of the condition (Halstead 1970, Thein et al. 1997). Pulmonary hemorrhage syndrome associated with DHF has been demonstrated (Sharma et al. 2007, Robert et al. 2004). Histochemical studies of human fatal cases of dengue (DEN) disease demonstrated that alveolar macrophages can be infected by DENV (Miagostovich et al. 1997). Several studies suggested that mice are a permissive host for DENV infection. In the majority of these models (Meiklejhon et al. 1952, Lin et al. 1998, Johnson & Roehrig 1999, An et al. 1999) the animals used were immunocompromised and/or inoculated by invasive routes with neuroadapted DENV in mice. Studies were carried out by our group using BALB/c mice infected with DENV-2 (non-neuroadapted) by intraperitoneal and intravenous routes. Focal alterations in the lung tissue (Barreto et al. 2002, 2007, Barth et al. 2006) were demonstrated. The virus particles were isolated in the C6/36 cell line of Aedes albopictus inoculated with the supernatant of a macerate of lung tissue from infected animals. Histopathological alterations of several mice tissues were signalized and described. Models using immunocompetent and immunocompromised mice have documented the lung involvement (Cole et al. 1973, Hotta et al. 1981). In the present study we characterized the injuries in lung tissue of BALB/c mice caused by DENV-1 and DENV-2 reinfection using photonic and electron transmission microscopy.

MATERIALS AND METHODS

DENV-1/DENV-2.

The DENV-1 and DENV-2 strains were isolated from patient sera in the state of Rio de Janeiro, Brazil, during the years of 2001 and 2000 respectively, in the Flavivirus Laboratory, Instituto Oswaldo Cruz, Fiocruz, and propagated in the Aedes albopictus mosquito cell line (C6/36). The sera were tested by the indirect immunofluorescence technique (Henchal et al. 1982) using type specific DENV-1 and DENV-2 monoclonal antibodies (DENV-1: 15F3, DENV-2: 3H5). The virus strains had undergone no passage in mouse brain. The titers of the viruses (DENV-1: 10^{7.5} TCID_{50}/0.1\ mL, DENV-2: 10^{6.66} TCID_{50}/0.1\ mL) were calculated by the method of Reed and Muench (1938).

Animals.

Adult male BALB/c mice, aged two months and weighing 25 g, were obtained from the Center of Animal Breeding of the Fiocruz and maintained in the Department of
Virology of the Instituto Oswaldo Cruz, Fiocruz. Mice were inoculated with DENV by the intravenous route with doses of 10,000 TCID$_{50}$/0.1 mL and euthanized 72 hours (h) post-reinfection. The animals used as control, were age- and sex-matched ones [non-infected mice and mice inoculated with Leibovitz medium (L-15 medium)], and were kept in same conditions that the infected animals. These animals were euthanized at the same day of the reinfected animals. The experiments were divided in four groups of twenty-one animals each [ten animals infected with DENV and eleven control animals (age and sex matched animals, non-infected, and inoculated with L-15 medium)]:

Group A: Mice, two months old, were infected with DENV-2, and reinjected with DENV-1 two months later.

Group B: Mice, two months old, were infected with DENV-2, and reinjected with DENV-1 four months later.

Group C: Mice, two months old, were infected with DENV-1 and reinjected with DENV-2 two months later.

Group D: Mice, two months old, were infected with DENV-1 and reinjected with DENV-2 four months later.

The experiments were previously approved by the Animal Experimentation Ethical Committee of the Institute Oswaldo Cruz, Fiocruz (license number: P0098-01).

Blood collection.

The animals were anaesthetized and blood samples were collected in the eye plexus before the first infection and 72 h post-reinfection with DENV-1 or DENV-2. Control mice samples were collected in the same time of infected animals.

Verification of corporal temperature.

The rectal temperature was verified before the first infection and 2, 6, 10 and 15 days post first infection with DENV-1 or DENV-2. Twenty animals were used for each DENV infection assay.

Verification of clinical signs.

Presence of clinical signs such as tremors, petechiae, diarrhea, irritability, and death was observed in the animals, before the first infection and 2, 6, 10 and 15 days after the first infection with DENV-1 or DENV-2. Twenty animals were used for each DENV infection assay.

Processing of tissues for photonic microscopy analysis.
The animals were euthanized and lung fragments were immediately collected from infected and non-infected mice. Samples were fixed in Millonig’s fixative, dehydrated in ethanol and paraffin-embedded. Sections (5 µm thick) were stained with haematoxilyn and eosin and Gomori’s silver impregnation for reticular fibers.

**Morphometrical analysis.**

Histomorphometry was performed using an imaging analysis system composed of a digital camera (Coolpix 990, NIKON, Japan) coupled to a light microscope (Eclipse 400, NIKON, Japan). Images were obtained with a 40x objective lens of a Zeiss-Axiophot photonic light microscope adapted to a computational system armed of the Zeiss-AxioVision 3.0 software. All the quantifications were done on captured high quality images (2048 x 1536 pixels buffer) using the Image Pro Plus 4.5.1 software (Media Cybernetics, Silver Spring, MD, USA). A single observer performed morphological measurements blindly.

**Surface density of alveolar septa and alveolar spaces.**

The surface density of the alveolar septa and alveolar spaces was obtained from histological sections of lung tissue from mice 72 h post-reinfection stained with hematoxilyn and eosin. One hundred and thirty six images of the five non-infected animals four and six months old, and one hundred and thirty six images of parenchyma of the five infected animals were captured.

**Surface density of reticular fibers.**

The surface density of reticular fibers was obtained from histological sections of lung fragments from mice 72 h post-reinfection stained with Gomori’s method for reticular fibres (Bancroft & Stevens 1996). One hundred and thirty six images of the five non-infected animals of four and six months old and one hundred and thirty six images of parenchyma of the five infected animals were obtained and reticular fibers were quantified.

**Statistical analysis.**

Results are given as means ± standard deviation. Comparison between groups was done by one-way analysis of variance (ANOVA) or Kruskal–Wallis, and post-tested by Tukey’s test. A p value of less than 0.05 was considered significant.

**Processing of tissues for transmission electron microscope analysis.**

The infected animals were peritoneally anaesthetized and the lung tissue fixed by perfusion with 4% paraformaldeyde in sodium phosphate buffer (0.2M, pH 7.2) by 30 minutes. In sequence the tissue samples were carefully collected, post-fixed by
immersion in 2% glutaraldehyde in sodium cacodylate buffer (0.2M, pH 7.2), followed by 1% buffered osmium tetroxide, dehydrated in crescent concentrations of acetone, embedded in epoxy resin and polymerized at 60°C during three days. Semi-thin sections of 0.5 µm were obtained using a diamond knife (Diatome) adapted to a Reichert-Jung Ultracut E microtome. The sections were stained with methylene blue and azure II solution (Humprey & Pittman 1974) and observed in a Zeiss Axiophot photonic light microscope. Ultra-thin sections of 50-70 nm thickness were picked up onto copper grids and stained with uranyl acetate and lead citrate (Reynolds 1963), and observed in a Zeiss EM-900 transmission electron microscope.

Isolation of DENV in the C6/36 cell line inoculated with sera from reinfected BALB/c mice.

Cell monolayers were inoculated with 100 µL of serum of mice 72 hours post-reinfection and incubated for 1 h at 28°C for virus adsorption. Subsequently monolayers were grown in L-15 medium supplemented with 1% non-essential aminoacids, 10% tryptose phosphate broth, and 10% fetal bovine serum. The tubes were kept at 28°C and observed daily for viral cytopathic effects for 15 days. C6/36 normal cell monolayers were used as negative control, while the positive control consisted of cell monolayers inoculated with DENV-1 and DENV-2. After the periods of observation, the monolayers were divided into two groups; the first was tested using the indirect immunofluorescence technique (Henchal et al. 1982) with a type-specific monoclonal antibody for dengue (DENV-1: 15F3, DENV-2: 3H5), and the second was fixed in 1% buffered glutaraldehyde, dehydrated and embedded in epoxy resin as described above for analysis in a transmission electron microscope.

RNA extraction.

The RNA was extracted from strains of C6/36 cells inoculated with positive sera by the indirect immunofluorescence technique using a QIAmp Viral RNA Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol.

Reverse transcription (RT-PCR).

Detection and typing of DENV in C6/36 cell culture fluid was carried out according to Lanciotti et al. (1992). This protocol detects the four serotypes of DENV simultaneously in a semi-nested procedure, generating amplified products (amplicons) of specific sizes (in bp) for each serotype of the DENV.
RESULTS

All mice of the four experimental groups survived until 72 h post-reinfection with DENV-1 or DENV-2, when they were euthanized, and examined.

Analyses of clinical signs.

Some animals of the DENV-1 first infected groups presented clinical signs (Table 1) as tremors (three animals in the tenth day post-infection), irritability (five animals in the tenth day post-infection), diarrhea (two animals, one in the second day post-infection and the other, in the tenth day post-infection). No clinical sign was observed in animals infected with DENV-2 only and in non-infected animals.

Table 1. Clinical signs of BALB/c mice infected with DENV.

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>CLINICAL SIGNS</th>
<th>B.I.(*)</th>
<th>2D. P.I.(*)</th>
<th>6D. P.I.(*)</th>
<th>10D. P.I.(*)</th>
<th>15D. P.I.(*)</th>
</tr>
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<tbody>
<tr>
<td>DENV-1</td>
<td>Obit</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>1/20(*)</td>
<td>0/20</td>
</tr>
<tr>
<td></td>
<td>Petechial</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>n= 20</td>
<td>Tremors</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>3/20</td>
<td>0/20</td>
</tr>
<tr>
<td></td>
<td>Irritability</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>5/20</td>
<td>0/20</td>
</tr>
<tr>
<td></td>
<td>Diarrhea</td>
<td>0/20</td>
<td>1/20</td>
<td>0/20</td>
<td>1/20</td>
<td>0/20</td>
</tr>
<tr>
<td></td>
<td>Fever</td>
<td>2/20</td>
<td>4/20</td>
<td>1/20</td>
<td>1/20</td>
<td>0/20</td>
</tr>
<tr>
<td>DENV-2</td>
<td>Obit</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
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<tr>
<td></td>
<td>Petechial</td>
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<td>0/20</td>
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<tr>
<td>n= 20</td>
<td>Tremors</td>
<td>0/20</td>
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<td>Irritability</td>
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<td>Diarrhea</td>
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<td>0/20</td>
<td>0/20</td>
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<tr>
<td></td>
<td>Fever</td>
<td>7/20(*)</td>
<td>16/20</td>
<td>14/20</td>
<td>6/20</td>
<td>0/20</td>
</tr>
</tbody>
</table>

n= total number of animals
B.I.: before infection
D.: days
P.I.: post-infection
(*) : obit due technical proceeding
(2) : The temperature of the three of these animals was 37.1\(^{\circ}\)C.
*: animals with clinical signs of dengue virus infection/ total number of animals analyzed

An increase of corporal temperature in animals during the first infection with DENV-1 or DENV-2 (Table 1) was verified. A large number of animals with temperature above 37\(^{\circ}\)C (4 animals) in the second day post-infection as well as in the group infected with DENV-2 (16 animals) in the group of animals infected with DENV-1 was observed. High temperatures were observed in the sixth and tenth day post-infection with DENV-1 or DENV-2. Alteration of corporal temperature was recorded in
nine animals before infection with DENV-1 or DENV-2; the temperature of three of these animals reached 37.1°C.

**Morphology.**

Lung of non-infected and the inoculated mice with Leibovitz culture medium only, did not exhibit any morphological alterations, being severe in lung tissue of the reinfected animals of groups C and D (Table 2).

**Table 2.** Morphological alterations in lungs of BALB/c mice reinfected with DENV-1 and DENV-2.

<table>
<thead>
<tr>
<th>Alterations</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory infiltrate in interstitio</td>
<td>XX</td>
<td>XX</td>
<td>XXX</td>
<td>XXXXX</td>
</tr>
<tr>
<td>Inflammatory infiltrate in peribronchiolar space</td>
<td>X</td>
<td>X</td>
<td>XXXXX</td>
<td>XXXXX</td>
</tr>
<tr>
<td>Inflammatory infiltrate in lumens of bronchiole</td>
<td>-</td>
<td>-</td>
<td>XXX</td>
<td>XXX</td>
</tr>
<tr>
<td>Diminished alveolar space</td>
<td>XX</td>
<td>XX</td>
<td>X</td>
<td>XXX</td>
</tr>
<tr>
<td>Erythrocytes inside alveolar space</td>
<td>X</td>
<td>X</td>
<td>XXX</td>
<td>XXX</td>
</tr>
<tr>
<td>Foci of hemorrhage</td>
<td>-</td>
<td>X</td>
<td>XXX</td>
<td>XXX</td>
</tr>
<tr>
<td>Swelling of interalveolar septa</td>
<td>XX</td>
<td>XX</td>
<td>XX</td>
<td>XXX</td>
</tr>
<tr>
<td>Oedema</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

--; alterations not observed; X: focal alterations; XX: light alterations; XXX: moderate alterations; XXXX: strong alterations

**Groups A and B.**

Morphological studies in photonic light microscopy of lungs of animals of both groups A and B showed presence of inflammatory infiltrate in the alveolar septa leading to a thickening of the septa (data not shown). Ultrastructural analyses showed that alveolar epithelial cells type I and II were preserved (Fig 2a) but in the alveolar capillaries the endothelial cells exhibited signs of activation presenting numerous phylopodia and intracitoplasmic vesicles and vacuoles (Fig 2e). In the capillary lumen besides polymorphonuclear cells, platelets were present. The only differential aspect between both groups was the increase of the reticular network in alveolar septa in group B (Graphic 3) aspect also seen by electron microscopy (data not shown).
**Group C.**

Animals of group C showed an alveolitis as animals of the A and B foci of intraalveolar hemorrhage, and focal enlargement of alveolar space (Fig 1a). Ultrastructural analysis demonstrated the presence of mononuclear and polymorphonuclear cells inside capillaries of the alveolar septa (Fig 2b) and capillary endothelia with phylopodia of the cell membrane (Figs 2c-d).

**Group D.**

Animals of group D presented an intense alveolitis and important zones of pulmonary collapse (Fig 1b). Ultrastructural analysis disclosed platelets inside alveolar capillaries and increase of the collagen fibrils in alveolar septa (Fig 2f).

**Figure 1a:** Section of mouse lung of group C. Note enlargement of alveolar space (AS). Interalveolar septa (S). Haematoxilyn and eosin staining. Bar= 50 µm.

**Figure 1b:** Section of mouse lung of group D showing swelling of interalveolar septa (S) with presence of inflammatory cells (circle). Alveolar space (AS). Haematoxilyn and eosin staining. Bar= 50 µm.

**Morphometry.**

**Average of surface density of interalveolar septa.**

All control animals (4 and 6 months old) did not exhibit differences between their interalveolar septa surface density. In this way they were represented together. A significant increase of surface density of alveolar septa (p<0,05) was observed in all groups of reinfected mice in relation to the group control (Graphic 1). In the group D (two months old animals infected with DENV-1 and reinfected with DENV-2 four months later) the increase of the surface density of interalveolar septa was stronger than in the other groups.

**Average of surface density of alveolar spaces.**

All control animals (4 and 6 months old) did not exhibit differences between their surface density of alveolar spaces. A decrease of surface density of the alveolar
space in all groups of reinfected mice in relation to the group control (Graphic 2) was observed. In the group D (two months old animals infected with DENV-1 and reinfected with DENV-2 four months later) the decrease was stronger than in the others groups.

*Figure 2a:* Mouse lung of group A. Note interalveolar septa swelling. Pneumocyte type II (PII), alveolar space (AS), capillary (C), erythrocyte (E), inflammatory cell (IF). Ultra-thin section. Bar= 1.8 µm.
**Figure 2b**: Mouse lung of group C showing interalveolar septum swelling with presence of mononuclear and polymorphonuclear (*) inflammatory cells inside a capillary. Alveolar space (AS). Ultra-thin section. Bar= 1.8 µm.

**Figure 2c**: Endothelial cell of a capillary (EC) of lung of mouse (group C) showing phyllopodia of the cell in cell membrane (arrows). Erythrocyte (E), alveolar space (AS). Ultra-thin section. Bar= 1.0 µm.

**Figure 2d**: Phyllopodium (arrow) of the cell membrane of an endothelial cell (EC) of mouse of group C. Capillary (C). Ultra-thin section. Bar= 0.5 µm.

**Figure 2e**: Capillary (C) of mouse lung (group A) showing the endothelial cell (EC) containing numerous vesicles (arrow) and two platelets (P). Ultra-thin section. Bar= 0.48 µm.

**Figure 2f**: Mouse lung of group D showing increased production of elastine (*) and a capillary (C) with an inflammatory cell (IF) inside. Alveolar space (AS). Ultra-thin section. Bar= 1.0 µm.

**Graphic 1**: Average percentage of surface density of interalveolar septa of BALB/c mice infected and reinfected with DENV serotypes 1 and 2. N= 136 images per group. C: control (average of percentage of surface density of animals with four and six months of age), GA: group A, GB: group B, GC: group C, GD: group D, 1: first infection of 2 months old mice and second infection 2 months later, 2: first infection of 2 months old mice and second infection 4 months later. The percentages were expressed in µm².
Average percentage of surface density of alveolar space

**Graphic 2:** Average percentage of surface density of alveolar spaces of BALB/c mice infected and reinfected with DENV serotypes 1 and 2. N= 136 images per group.

Average percentage of surface density of reticular fiber network

**Graphic 3:** Average percentage of surface density of elastic fibers of lungs of BALB/c mice infected and reinfected with DENV serotypes 1 and 2. N= 136 images per group.

*Average of surface density of reticular fiber network of lung.*

All control animals (4 and 6 months old) did not exhibit differences between surface density of the reticular network in alveolar spaces. In this way they were represented together. Significant increase of surface density of elastic fibers (p<0.05) was present in groups B and D in relation to the control group (Graphic 2). Group B animals (animals infected with DENV-2 with two months of age and reinfected when of DENV-1 with six months of age) presented the greatest increase of the reticular network. A little decrease in relation to the control group was observed in the group A.
Detection of DENV in the C6/36 the cell line inoculated with sera from reinfected BALB/c mice.

The syncytial cytopathic effect started to be visible around the 13\textsuperscript{th} day post-infection in C6/36 cell monolayers of the positive control and in cell cultures inoculated with sera of reinfected animals. At the 15\textsuperscript{th} day p.i., the cell monolayer of the negative control showed no morphological alterations, exhibited neither DENV antigen and nor virus particles. In cell cultures of the positive control and in cell monolayers inoculated with the sera of group A and B animals, the DENV-1 antigen, and a syncytial cytopathic effect was observed by the indirect immunofluorescence technique in all monolayers (Figs 3a-b). Ultrastructural observations of cell cultures of the positive control and cell monolayers inoculated with mice sera, showed virus particles inside cytoplasmic vesicles (Fig. 3c) and in cisterns of the rough endoplasmic reticulum (Fig 3d). All cell monolayers inoculated with the sera of group C and D animals contaminated hampering viruses isolation.

Detection of DENV viral genome by RT-PCR.

The genome of DENV-1 was detected in extracts of C6/36 cells inoculated with mouse sera of A and B groups collected at 72 hours post-reinfection (Fig 4). No virus genome was observed in the cell monolayer of negative control. These results confirmed the presence of DENV-1 in serum samples of reinfected mice and indicated that the virus is still able to infect other cells.
Figure 3 a-d: Detection of DENV antigen and virus particles in monolayers of C6/36 cells. a-b: Presence of DENV-1 antigen in cell monolayer inoculated with serum of animals of the group A. Immunofluorescence technique (a/b: 1.25 µm); c: Presence of DENV-1 particles inside a cytoplasmic vesicle of a C6/36 cell culture inoculated with serum of mouse of group A (Bar= 0.3 µm); d: presence of incomplete DENV-1 particles inside lumen of the rough endoplasmic reticulum of a C6/36 cell culture inoculated with serum of mouse of group B (Bar= 0.1 µm). Transmission electron microscopy technique.

Figure 4: RT-PCR of extracts of C6/36 cells inoculated with mouse sera of the group A and group B. Lines 1/10: 100-bp, ladder (Gibco). Lines- 2 and 4: C6/36 cells inoculated with strain of serum of BALB/c mice of the B group; 3 and 5: C6/36 cells inoculated with strain of serum of BALB/c mice of the A group; 6: DENV-1 positive control; 7: DENV-2 positive control; 8: DENV-3 positive control; 9: Negative control (H2O).
DISCUSSION

The lack of an appropriate animal model developing an infection similar to human cases of DHF and DSS is a handicap to the development of vaccine candidates against DENV. Studies demonstrated that immunodeficient mice developed clinical signs of DF, DHF and DSS as in humans (fever, rash and thrombocytopenia) (Marchette et al. 1973, Lucia & Kangwanpong 1994, Déprès et al. 1998, Na et al. 2003, Bente et al. 2005). In these animal models the mice were inoculated by invasive routes with neuroadapted mouse DENV strains, contrasting with our model (Barreto et al. 2002, 2004, 2007, Barth et al. 2006, Paes et al. 2002, 2005).

Our studies with immunocompetent BALB/c mice showed a large number of animals with temperature above 37°C in the second day post the first infection with DENV-1 or DENV-2. The animals developed stronger lung damage during the reinfection with heterologous serotypes (non-neuroadapted strains of DENV-1 and DENV-2) by the intravenous route including severe alveolitis, foci of pulmonary collapse, and intraalveolar hemorrhage, similarly with human cases of DHF and DSS. Cases of pulmonary hemorrhage syndrome associated with DHF have been demonstrated in humans (Robert et al. 2004, Sharma et al. 2007). These patients developed pulmonary hemorrhage, haemoptysis and anemia, requiring blood transfusion. Our analyses of reinfected animals showed that they have thickened alveolar septa (surface density of alveolar septa) indicating that all animals developed an alveolitis and that all of them had zones of collapse (decrease in surface density of alveolar spaces) compared with control mice. Furthermore, in group A and C there were no changes in their collagen network as observed in groups B and D. In both groups there was an increase in the reticulin network which could represent the beginning of the remodeling process due to the lung injury with activation of interstitial fibroblasts and subsequent collagen deposition.

Focal alterations of capillary endothelial cells were verified in our studies. These cells exhibited phyllopodia of the cell membrane and numerous cytoplasmic vesicles, but necrosis was not observed. According to Feroze (1997), the presence of a great number of endocytic vacuoles and phyllopodia in endothelial cells can be indicative of cell activation. It was suggested that endothelial cells can support DENV replication and liberation of several inflammatory mediators including an interleukin 8 (IL-8) and RANTES (Avirutnan et al. 1998, Juffrie et al. 2000). These substances are capable to recruit neutrophils and promote vascular permeability increase. In the present work the
recruitment of mononuclear and polymorphonuclear cells was verified. The recruitment of polymorphonuclear cells probably is due to the release of IL-8 and RANTES by activated endothelial cells.

The infection of BALB/c mice inoculated with neuroadapted DENV by intraperitoneal and intravenous route was demonstrated formerly (Atrasheukaya et al. 2003, Huang et al. 2000). In our studies, using no previously adapted DENV and a less aggressive route of virus inoculation into mice, virus particles, viral antigen and RNA of DENV-1 was detected in C6/36 cell cultures inoculated with sera of BALB/c mice of groups A and B, 72 hours post-reinfection. Ultrastructural studies showed the presence of DENV particles inside cytoplasmic vesicles and immature particles in cisterns of the rough endoplasmic reticulum. These findings and the clinical signs observed are a demonstration of DENV reinfection in BALB/c mice.

All morphological alterations observed in the present studies were more severe than that observed in our previous experiments (Barreto et al. 2002, 2004, 2007, Barth et al. 2006, Paes et al. 2002, 2005), in which the animals were infected only with one serotype of DENV only. Pre-existing heterotypic dengue antibodies (Chatuverdi et al. 2005) are a risk factor for increasing of the disease severity, and that it was confirmed in our animal model.

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