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Secondary Infection With Dengue Viruses in A Murine Model : Morphological Analysis

KEYWORDS: dengue-1 virus, dengue-2 virus, BALB/c mice, lung, liver, heart, histopathology, ultrastructure

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ABSTRACT *Morphological alterations in the lungs, liver and heart of BALB/c mice reinfected intravenously with heterologous dengue virus (DENV) using non-neuroadapted DENV serotypes 1 and 2 were analysed. Tissues samples were processed using the standard techniques of photonic and transmission electron microscopy. Morphological observations of lung tissue showed breakdown of the alveolocapillary barrier leading to alveolitis, focal zones of collapse, and intraalveolar haemorrhage. Inside alveolar septa, congested capillaries contained inflammatory cells and platelets. Alveolar capillary endothelial cells exhibited aspects of activation with protruding phyllopod and intracytoplasmic vesicles and vacuoles. The liver exhibited vacuolisation of hepatocytes, inflammatory cells inside sinusoidal capillaries, enlargement of sinusoidal capillaries, foci of haemorrhage inside the interstitium, oedema in the peri-centrilobular vein space and the presence of phyllopods and pseudopod-like extensions in endothelial cells. In heart tissue, a slight loss of cytoplasm in cardiac cells, which are areas with inflammatory infiltrate, and the presence of phyllopods in endothelial cells and the disorganisation of cardiac fibres were observed. DENV particles, DENV-specific antigen and DENV RNA were observed in mosquito cell (C6/36) cultures inoculated with the sera of reinfected animals. The morphological alterations observed in lung, liver and heart tissue were similar to what has been observed in human cases of dengue and severe dengue fever. The present study demonstrates that BALB/c mice reinfected with a heterologous serotype of DENV in this model develop more severe lesions than those observed in mice during primary infection.*

INTRODUCTION

Dengue viruses (DENV), members of the Flaviviridae family, occur as four distinct serotypes (DENV-1, -2, -3, -4) that are mainly transmitted from infected to susceptible humans by *Aedes aegypti* mosquitoes. DENV infection is mostly asymptomatic or produces a mild self-limiting acute febrile illness, dengue, and a life-threatening severe illness, severe dengue fever with minor or major bleeding from different sites (Agarwal et al. 1999). Severe dengue fever has emerged as the most important arbovirus disease in man in the last three decades. It has been estimated that approximately 50 to 100 million cases of dengue occur every year with approximately 50,000 to 250,000 cases of severe dengue fever (Rigau-Perez et al. 1998, WHO 2002, Gubler 2002). It is generally accepted that severe dengue fever occur as a result of secondary infection by a heterologous DENV and that immunopathological mechanisms are involved in the pathogenesis of this condition (Halstead 1970, Thein et al. 1997). Pulmonary haemorrhage syndrome associated with severe dengue fever has been demonstrated (Sharma et al. 2007, Robert et al. 2004). Histochemical studies of fatal human cases of dengue disease have demonstrated that alveolar macrophages can be infected by DENV (Miagostovich et al. 1997). Liver involvement in the clinical presentation of severe dengue fever (Bhamarapravati 1997, Lum et al. 1993) has been corroborated by detecting DENV RNA using reverse transcription (RT)-PCR technique in archival liver samples obtained from individuals who succumbed to DENV infection (Rosen et al. 1999). These authors speculated that the liver might be the major site of DENV replication. Studies of liver samples obtained from patients with severe dengue fever revealed the presence of viral antigens in Kupffer cells, endothelium, lymphocytes and monocytes inside the lumen of blood vessels (Jessie et al. 2004, De Macedo et al. 2006). Cardiac manifestations of the disease are uncommon and are often associated with severe dengue fever. Due to the viral infection of cardiomyocytes and endothelial cells, these manifestations can lead to cases of atrioventricular conduction disorder, supraventricular arrhythmia and myocarditis. The presence of lymphocytic infiltrate and myocytolysis of cardiac cells by microscopic observation is considered a conclusive diagnosis of myocarditis. Even so, cases of myocarditis caused by DENV have proven to be mostly benign throughout the course of the disease, without long-term complications. Several studies have suggested that mice are permissive hosts for DENV infection (Meiklejhon et al. 1952, Lin et al. 1998, Johnson & Roehrig 1999, An et al. 1999). A majority of these studies have used immunocompromised mice and/or mice inoculated by invasive routes with neuroadapted DENV. Our group has carried out studies using BALB/c mice infected with DENV-2 (non-neuroadapted) by the intraperitoneal and intravenous routes. Focal alterations in the lung and hepatic tissue were demonstrated (Barreto et al. 2002, 2007, Barth et al. 2006, Paes et al. 2002, 2005). The vi-

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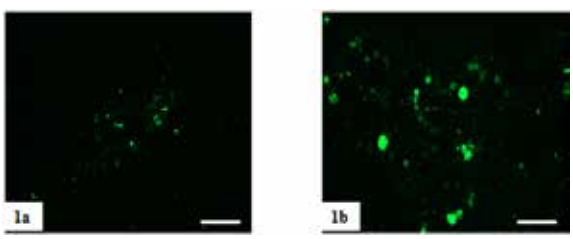
rus particles were isolated in the C6/36 cell line of *Aedes albopictus* that was inoculated with the supernatant of a macerate of lung tissue from infected animals. Focal alterations were observed in hepatic tissue. Viral antigen was detected in endothelial cells and in hepatocytes (Paes et al. 2002, 2005, Barth et al. 2006). The present study used photonic and electron transmission microscopy to characterise injuries in lung, liver and cardiac tissues of BALB/c mice caused by secondary infection with DENV-1 and/or DENV-2.

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 (IOC), Fiocruz, and propagated in the *Aedes albopictus* mosquito cell line (C6/36). The sera were tested using an indirect immunofluorescence technique (Henchal et al. 1982) with type-specific DENV-1 (fig. 1a) and DENV-2 (fig. 1b) monoclonal antibodies (DENV-1: 15F3, DENV-2: 3H5). The virus strains had undergone no passage in the mouse brain. Virus titres (DENV-1: 7.3 PFU/1mL, DENV-2: 6.5 PFU/1mL) were calculated by the method of Reed and Muench (1938).



Figures 1a-b. Detection of DENV antigen in monolayers of C6/36 cells infectec with DENV. Fig. 1a: Presence of DENV-1 antigen in cell monolayer inoculated with patient serum. Fig. 1b: Presence of DENV-2 antigen in cell monolayer inoculated with patient serum. Immunofluorescence technique (a-b: Bar= 1.25 μm).

Animals.

Adult male BALB/c mice aged two months and weighing 25 g were obtained from the Center of Animal Breeding of Fiocruz and maintained in the Department of Virology of the IOC, Fiocruz. Mice were inoculated intravenously with doses of 10,000 $TCID_{50}$/0.1 mL DENV, and euthanised 72 hours (h) post-reinfection (p.r.). The animals used as controls [non-infected mice and mice inoculated with Leibovitz medium (L-15 medium)] and were kept in the same conditions as the infected animals. These animals were euthanised on the same day as the reinfected animals (table 1).

The experiment employed groups of twenty-one animals each [ten animals infected with DENV and eleven control animals]:

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

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

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

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

The protocols used in these experiments were previously approved by the Animal Experimentation Ethical Committee of the IOC, Fiocruz (license number: P0098-01).

Experimental Design

	Virus	Group A	Group B	Virus	Group C	Group D
		Age of mice	Age of mice		Age of mice	Age of mice
First infection	DENV-2	2 months	2 months	DENV-1	2 months	2 months
Reinfection	DENV-1	4 months	6 months	DENV-2	4 months	6 months
Euthanasia		72h p.r.	72h p.r.		72h p.r.	72h p.r.
Number of mice/ Technique		5/PM 5/ETM	5/PM 5/ETM		5/PM 5/MET	5/PM 5/ETM
	Controls	Age of mice	Age of mice	**Controls**	Age of mice	Age of mice
		4 months	6 months		4 months	6 months
Euthanasia		The same day of group A	The same day of group B		The same day of group C	The same day of group D
Number of mice/ Technique		4/PM 4/PM L-15* 3/ETM L-15*	4/PM 4/PM L-15* 3/ETM L-15*		4/PM 4/PM L-15* 3/ETM L-15*	4/PM 4/PM L-15* 3/ETM L-15*

Table 1. PM: photonic microscopy, ETM: electron transmission microscopy, L-15: Leibovitz culture medium, p.r.: post-reinfection. *: animals inoculated with L-15 medium.

Blood collection.

The animals were anaesthetised and blood samples were collected from the eye plexus before the first infection and 72 h p.r. with DENV-1 or DENV-2. Control mice samples were collected at the same time as for the reinfected animals.

Processing of tissues for photonic microscopy analysis.

The animals were euthanised and lung, liver and heart tissue 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 haematoxylin and eosin and Gomori's silver impregnation for reticular fibres and Giemsa.

Processing of tissues for transmission electron microscope analysis.

Infected animals were peritoneally anaesthetised, and the tissues were fixed by perfusion with 4% paraformaldehyde in sodium phosphate buffer (0.2 M, pH 7.2) for 30 minutes. Samples of lung, liver and heart tissue were carefully collected in that order and post-fixed by immersion in 2% glutaraldehyde in sodium cacodylate buffer (0.2 M, pH 7.2), followed by 1% buffered osmium tetroxide, dehydrated in crescent concentrations of acetone, embed-

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ded in epoxy resin and polymerised at 60°C over 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 using a Zeiss Axiophot photonic light microscope. Ultra-thin sections of 50-70 nm thickness were placed onto copper grids, stained with uranyl acetate and lead citrate (Reynolds 1963), and observed using 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 from mice 72 hours p.r. and incubated for 1 h at 28°C for virus adsorption. Monolayers were subsequently grown in L-15 medium supplemented with 1% non-essential amino acids, 10% tryptose phosphate broth, and 10% foetal bovine serum. The tubes were kept at 28°C and observed for viral cytopathic effects daily for 15 days. C6/36 normal cell monolayers were used as the negative control, while the positive control consisted of cell monolayers inoculated with DENV-1 and DENV-2. After the periods of observation, 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 using transmission electron microscopy.

RNA extraction.

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

Mice from the four experimental groups survived until 72 h p.r. with DENV-1 or DENV-2, at which time they were euthanised and examined.

Morphology.

Lung

Lung tissue of mice that were not infected and the mice that were inoculated with Leibovitz culture medium only did not exhibit any morphological alterations, which were severe in lung tissue of the reinfected animals from groups C and D.

Groups A and B.

Morphological studies via photonic microscopy of the lungs of animals from both groups A and B showed the presence of mononuclear inflammatory infiltrate in the alveolar septa, leading to a thickening of the septa (Figs. 2a, 2d, 3a-c). In the capillary lumen besides inflammatory cells (Fig. 2b) and platelets (Figs. 2a, 2c, 2d) were present.Type I and II alveolar epithelial cells were preserved (data not shown), but the endothelial cells in the alveolar capillaries exhibited signs of activation and presented numerous phyllopodia and intracytoplasmic vesicles and vacuoles (Fig. 2c).

Group C.

Group C animals exhibited an alveolitis, as was observed in group A and B animals, foci of intraalveolar haemorrhage, inflammatory infiltrate in the alveolar septa (Fig. 3d) and focal enlargement of the alveolar space. Ultrastructural analysis demonstrated the presence of mononuclear and polymorphonuclear cells inside capillaries of the alveolar septa and capillary endothelia with phyllopodia of the cell membrane (Fig. 2e).

Group D.

Group D animals presented an intense alveolitis, zones of pulmonary collapse (data not shown), foci of haemorrhage and inflammatory infiltrate (Figs. 3e-3f). Ultrastructural analysis revealed platelets inside the alveolar capillaries and an increase in the collagen fibrils in alveolar septa (Fig. 2f).

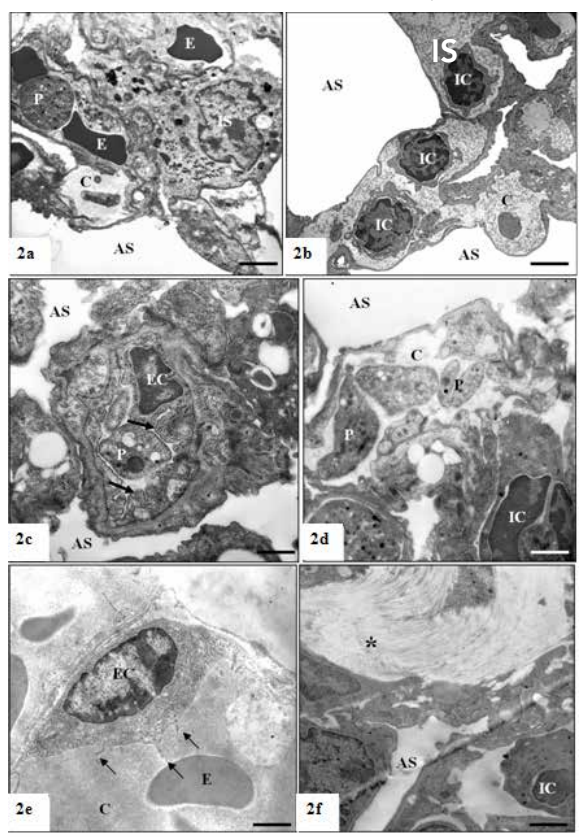

Figure 2. Ultra-thin sections of lung tissue from BALB/c mice reinfected with DENV. Fig. 2a: Group A mouse lung. Note interalveolar septa swelling. Bar= 2,27μm. Fig. 2b: Group A mouse lung showing mononuclear inflammatory cells inside a capillary. Bar= 3,33μm. Fig. 2c: Endothelial cell from a capillary of a group A mouse lung showing phyllopodia in the cell membrane (arrows). Bar= 0,83μm. Figure 2d: Capillary from a group A mouse lung containing platelets and inflammatory cells. Bar= 1,36μm. Fig. 2e: Capillary from a mouse lung (group C) showing an endothelial cell containing phyllopodia in the cell membrane (arrows). Bar= 1,27μm. Figure 2f: Group D mouse lung displaying increased production of elastin (*). Bar= μm. Alveolar space (AS), capillary (C), endothelial cell (EC), erythrocyte (E), inflammatory cells (IC), interalveolar septa (IS), platelet (P). Bar= 1,14μm.

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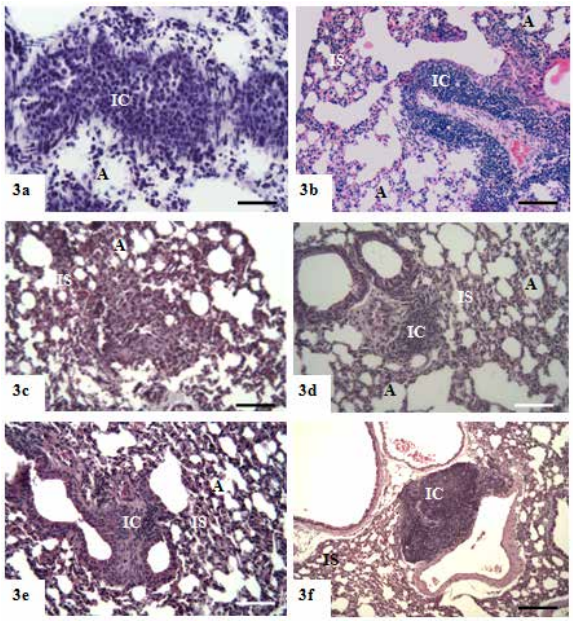

Figure 3. Sections of lung tissue from BALB/c mice reinfected with DENV stained with haematoxylin and eosin and Giemsa. Note presence of interstitial inflammatory infiltrate (IC) and interalveolar septa (IS) swelling. Alveolar space (AS). Fig. 3a: Section of a group A mouse lung. Bar= 40μm. Fig. 3b: Section of a group A mouse lung. Bar= 90μm. Fig. 3c: Sections of a group B mouse. Bar= 90μm. Fig. 3D: Sections of a group C mouse. Bar= 90μm. Fig. 3e-f: Section of a group D mouse. Bar= 90μm and 100μm, respectively.

LIVER

Livers from mice that were not infected and mice that were inoculated with Leibovitz culture medium only did not exhibit any modifications of their morphology.

The alterations observed in hepatic tissue were more severe in group A and B animals.

Group A

Histopathology of liver tissue revealed oedema, inflammatory infiltrate and dilated lymphatic vessel in the portal space (Figs. 3a-b), a portal vein containing dilated sinusoidal capillary (Fig. 4b), some erythrocytes presenting morphological alterations (Fig. 4c), inflammatory infiltrate in the interstitium, focus of haemorrhage and an increased reticulin network (Fig. 4d). Ultrastructural analysis showed enlargement of bile canaliculi and hepatocytes with dilation of the rough endoplasmic reticulum and lipid inclusions inside the cytoplasm.

Group B.

The livers of these animals displayed hepatocytes with microvesicular steatosis and glycogen particles inside the cytoplasm (Fig. 5a); some cells showed vacuolisation and rarefied cytoplasm (Fig. 5b). Multivesicular stellate cells presenting electron-dense and clear lipid vesicles in the space of Disse and synthesising collagen were observed (data not shown). Endothelial cells in the sinusoidal capillaries showed altered phyllopod membranes that had pseudopod-like extensions (Fig. 5c).

Group C.

These animals showed discrete vacuolisation of hepatocytes (Fig. 4e) with inflammatory infiltrate in the interstitial space (Fig. 4f) and the peri-portal space. Ultrastructural analysis of hepatocytes showed degranulation and dilation of the rough endoplasmic reticulum, lipid inclusions (Fig. 5e) and dilation of the bile canaliculi presenting electron-dense filamentous material (data not shown).

Group D.

These animals presented inflammatory infiltrate in the peri-portal space. Endothelial cells of the sinusoidal capillaries presenting phyllopods and electron-dense material inside the cytoplasm, enlargement of bile canaliculi, some hepatocytes with dilatation and alteration of mitochondria cristae were also observed (data not shown). Hepatocytes showed degranulation and dilation of the rough endoplasmic reticulum and lipid inclusions inside the cytoplasm (Fig 5f). The morphological alterations observed in this group were less severe than in group C.

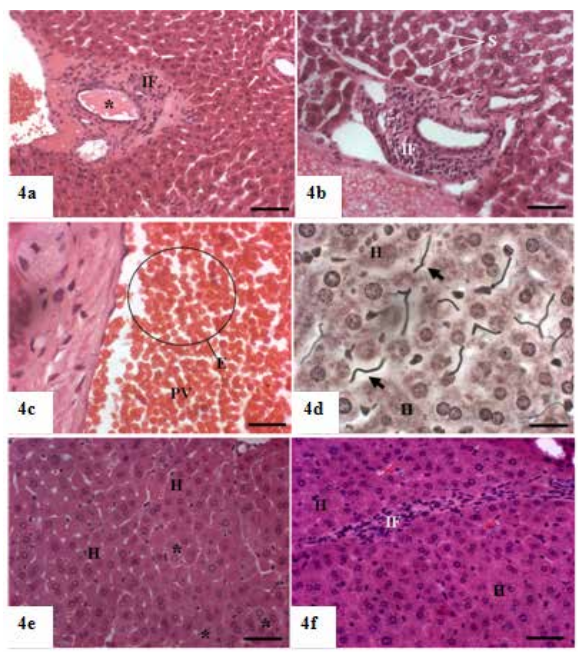

Figure 4. Sections of liver tissue from BALB/c mice reinfected with DENV stained with haematoxylin and eosin (4a, b, c, e, f) and reticulin (4d). **Figs. 4a-d:** Section of a group A mouse liver. Note oedema (*), inflammatory infiltrate and dilated sinusoidal capillaries, portal vein containing deformed erythrocytes, groups of reticulin fibres (arrow) inside the sinusoidal capillaries. **Fig. 4e-f:** Hepatic tissue from a group C animal showing some hepatocytes with light vacuolisation (*) and inflammatory infiltrate in the interstitial space. Erythrocytes (E), hepatocytes (H), inflammatory infiltrate (IF), portal vein (PV), sinusoidal capillaries (S). **a:** bar=90μm, **b:** bar= 90μm, **c:** bar= 40μm, **d:**bar= 40μm, **e:** bar= 90μm, **f:** bar= 90μm.

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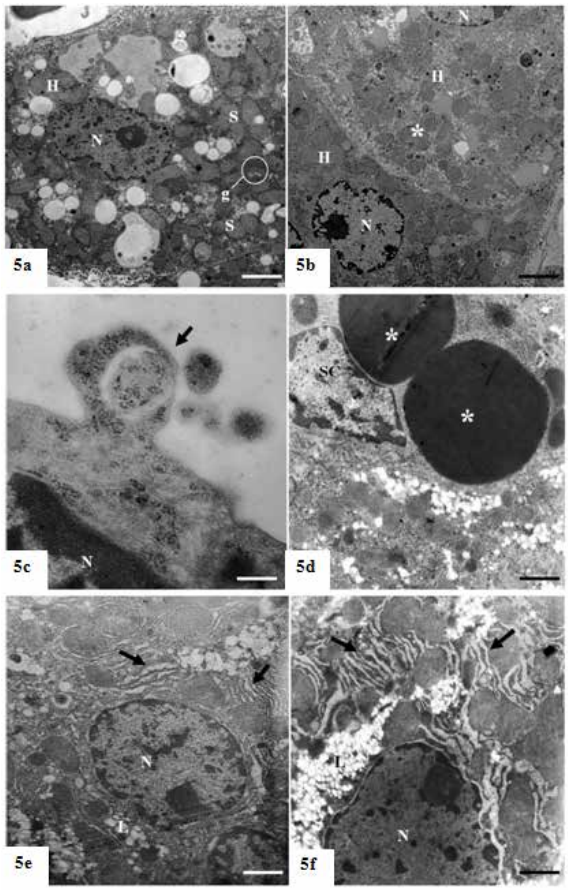
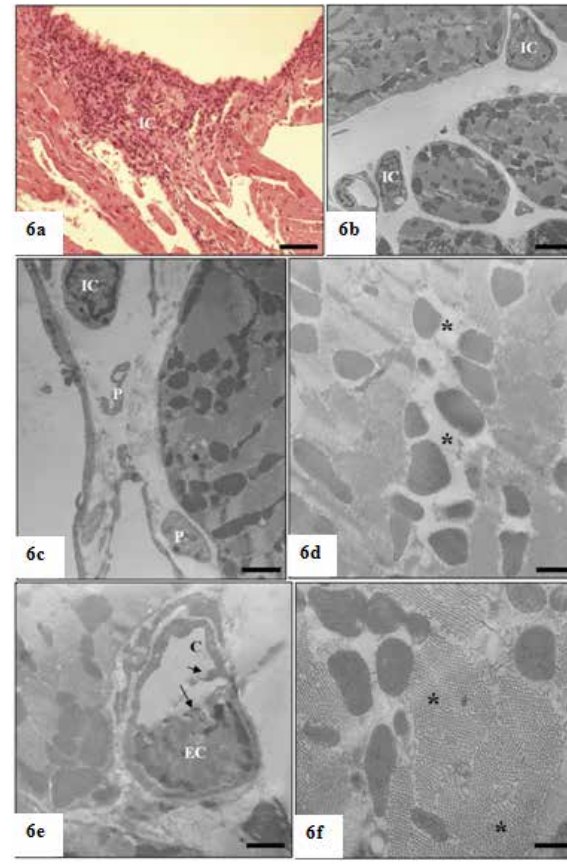


Figure 5. Ultra-thin sections of liver tissue from BALB/c mice reinfected with DENV. Fig. 5a: A hepatocyte from a group B mouse presenting microvesicular steatosis and glycogen particles (circle) inside the cytoplasm. Bar= 2,3μm. Fig. 5b: Two hepatocytes from a group B mouse showing rarefied cytoplasm (*) in one of them. Nucleus. Bar= 2,3μm. Fig. 5c: Endothelial cell from a group B mouse presenting membrane alterations (arrow) with pseudopod-like extensions. Bar= 0,25μm. Fig. 5d: Hepatic tissue from a group B mouse presenting multivesicular stellate cells with some electron-dense (*) vesicles. Bar= 1,19μm. Figs. 5e-f: Hepatocytes from group C and group D mice, respectively. Note the degranulation and dilation (arrow) of the rough-surfaced endoplasmic reticulum and lipid inclusions. Glycogen (g), hepatocyte (H), lipid inclusions (L), nucleus (N), stellate cells (SC), steatosis (S). e: bar= 1,07μm, f: bar= 1,07μm.

HEART

Histopathological and ultrastructural analysis of cardiac tissue showed similar alterations in all groups. The main observations were areas with inflammatory infiltrate (myocarditis) (Figs. 6a-c), loss of cytoplasm in cardiomyocytes (Fig. 6d), endothelial cells presenting phyllopodia in their membranes (Fig. 6e) and disorganisation of cardiac fibres (Fig. 6f). Morphological changes were not observed in cardiac tissue from non-infected animals.



Figures 6a-f: Sections of cardiac tissue of BALB/c mice reinfected with DENV of group B. Note inflammatory cells. a: bar= μm, b: bar= 2,5μm, c: bar= 1,4μm. Fig. 6d: Cardiomyocytes with rarefied cytoplasm (*). Bar= 0,6μm. Fig. 6e: Capillary with endothelial cell having extending in its cytoplasmic membrane (arrow). Bar= 0,8μm. Fig. 6f: Presence of areas of disorganization of cardiac fibres (*). Bar= 0,6μm. Capillary (C), endothelial cell (EC), Inflammatory cells (IC), platelets (P).

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

The syncytial cytopathic effect began to be visible around the 13th day post-infection in C6/36 cell monolayers of the positive control and in cell cultures inoculated with sera from reinfected animals. At the 15th day p.i., the cell monolayer from the negative control showed no morphological alterations, and exhibited neither DENV antigen nor virus particles. In cell cultures of the positive control and in cell monolayers inoculated with sera from group A and B animals, the DENV-1 antigen, and a syncytial cytopathic effect was observed using the indirect immunofluorescence technique in all monolayers (Figs. 7a-b). Ultrastructural observations of cell cultures from the positive control and cell monolayers inoculated with mice sera, showed virus particles inside cytoplasmic vesicles (Fig. 7c) and in cisterns of the rough endoplasmic reticulum (Fig 7d). All cell monolayers inoculated with sera from group C and D animals were contaminated, which hampered virus isolation.

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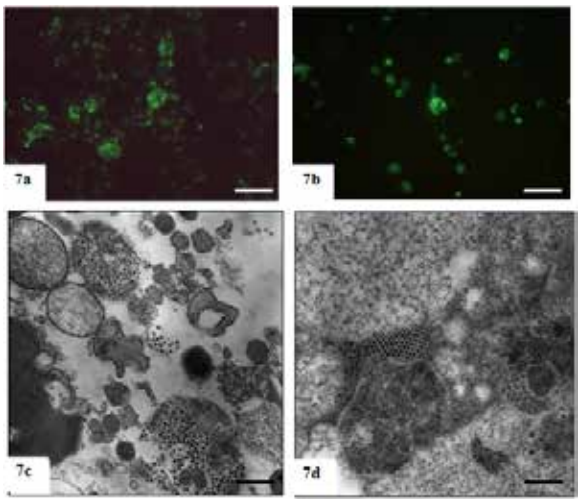

Figure 7a-d: Detection of DENV antigen and virus particles in monolayers of C6/36 cells using indirect immunofluorescence technique and transmission electron microscopy. a-b: Presence of DENV-1 antigen in cell monolayer inoculated with serum of animals of the group A. (a-b: Bar= 1,25 μm); c: Presence of DENV-1 particles inside a cytoplasmic vesicle of a C6/36 cell culture inoculated with serum from a group A mouse (Bar= 0,3μm); d: presence of incomplete DENV-1 particles inside the lumen of the rough endoplasmic reticulum of a C6/36 cell culture inoculated with serum from a group B mouse (Bar= 0,3μm).

Detection of the DENV viral genome by RT-PCR.
The genome of DENV-1 was detected in extracts of C6/36 cells inoculated with mouse sera from groups A and B collected at 72 h p.r. (Fig. 8). No virus genome was observed in the cell monolayer from the negative control. These results confirmed the presence of DENV-1 in serum samples from reinfected mice and indicated that the virus was still able to infect other cells.

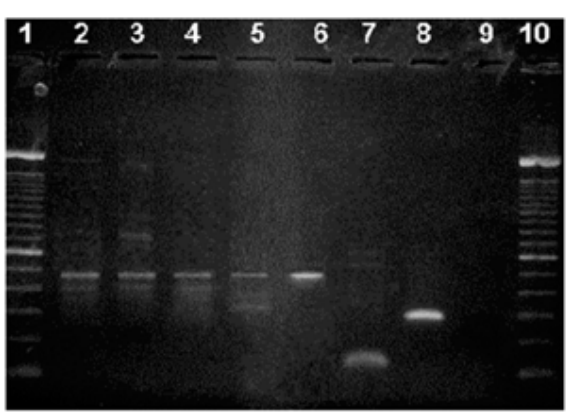

Figure 8: RT-PCR of extracts from C6/36 cells inoculated with group A and B mouse sera. Lanes 1 and 10: 100-bp, ladder (Gibco). Lanes 2 and 4: C6/36 cells inoculated with a strain of serum from group B BALB/c mice; Lanes 3 and 5: C6/36 cells inoculated with a strain of serum from group A BALB/c mice; Lane 6: DENV-1 positive control; Lane 7: DENV-2 positive control; Lane 8: DENV-3 positive control; Lane 9: Negative control (H_2O).

DISCUSSION

The lack of an appropriate animal model developing an infection similar to human cases of severe dengue fever is a handicap to the development of vaccine candidates against DENV. Studies have demonstrated that immunodeficient mice develop clinical signs of dengue and severe dengue fever as in humans (fever, rash and thrombocytopenia) (Marchette et al. 1973, Lucia & Kangwanpong 1994, Deprès et al. 1998, Na et al. 2003, Bente et al. 2005). In these animal models, mice were inoculated by invasive routes with neuroadapted mouse DENV strains. This is in contrast with our model, in which animals are infected intravenously with non-neuroadapted DENV (Barreto et al. 2002, 2004, 2007, Barth et al. 2006, Paes et al. 2002, 2005). The objective of the present study was to present a murine model that better resembles human severe dengue fever disease. Animals intravenously reinfected with heterologous serotypes (non-neuroadapted strains of DENV-1 and DENV-2) presented alterations in the lung and hepatic tissue similar to human cases of severe dengue fever. The morphological alterations that were observed were more severe than those observed in our previous studies (Barreto et al. 2002, 2004, 2007, Barth et al. 2006), in which the animals were only infected with one serotype of DENV.

In this study, severe alveolitis, foci of pulmonary collapse, and intraalveolar haemorrhage, similarly to human cases of severe dengue fever were observed in lung tissue. Cases of pulmonary haemorrhage syndrome associated with severe dengue fever have been demonstrated in humans (Robert et al. 2004, Sharma et al. 2007). These patients developed pulmonary haemorrhage, haemoptysis and anaemia, requiring blood transfusion. Our analyses of reinfected animals demonstrated that they have thickened 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) when compared with control mice.

Hepatic involvement in DENV infections has been documented (Havens 1954). The dengue antigen has been identified within Kupffer cells, the endothelium, lymphocytes and monocytes inside the lumen of the sinusoidal capillaries in individuals presenting dengue or severe dengue fever (Guzman et al. 2002, Jessie et al. 2004). Studies have reported fatal hepatic failure in an adult arising as a complication of severe dengue fever (Lawn et al. 2003, Ling et al. 2007). Liver histology revealed marked steatosis and a florid hepatitis with necrosis. Similar alterations were observed in the present study. Hepatocyte alterations, microvesicular steatosis, infiltrate of inflammatory cells and multivesicular stellate cells storing electron-dense and clear lipid vesicles and secreting collagen into the Disse space, were observed in all groups of reinfected animals, being the most prominent in groups A and B. Current knowledge ascribes this to liver-specific pericytes, conferring a major role to hepatic stellate cells in extracellular matrix production and re-modelling (Pinzani 1995, Gressner & Bachem 1995, Gressner 1996, Gressner & Weiskirchen 2006). Their dominant role in fibrogenesis is based on their ability to change their phenotype when hepatic stellate cells are rechallenged by necro-inflammatory stimuli from retinoid-storing, resting cells to contractile, smooth-muscle α-actin positive, vitamin A-depleted myofibroblasts with a strongly developed endoplasmic reticulum and Golgi apparatus (Bataller & Brenner 2005, Friedman 2004).

The endoplasmic reticulum is an important site of protein synthesis and transport, and it also contains enzymes

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involved in drug and steroid metabolism. Several endogenous imbalances in cells, such as massive protein production, loss of calcium homeostasis, inhibition of N-linked glycosylation, and accumulation of mutant protein, often contribute to its malfunction (Yu et al. 2006). One of the major morphological changes in Flavivirus-infected cells is the proliferation and hypertrophy of endoplasmic reticulum membranes where virus particles accumulate inside (Barth 2000, Burke & Monath 2001, Hase et al. 1992). We observed hepatocytes with degranulation and dilation of the rough endoplasmic reticulum. Lipid inclusions were also observed inside these cells.

Because the endothelium forms the primary barrier of the circulatory system, dysfunction of endothelial cells during acute diseases can broadly affect vascular permeability and cause plasma leakage (Lee et al. 2006). In our mouse model, the endothelial cells of the liver and lung capillaries exhibited phyllopodia, alterations of cytoplasm membranes (blebs) and vacuoles of secretion. Necrosis of this cell type 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. Researchers have suggested that endothelial cells can support DENV replication and the liberation of several inflammatory mediators, including interleukin 8 (IL-8) and RANTES (Avirutnan et al. 1998, Juffrie et al. 2000). These substances are capable of enlisting neutrophils and promoting an increase in vascular permeability. Foci of haemorrhage and oedema were observed in the present experiments, most likely due to the release of IL-8 and RANTES by activated endothelial cells.

The bile canaliculi showed dilation, the presence of electron-dense filamentous material inside and a loss of microvilli in the present ultrastructural analysis. The loss of microvilli, formation of surface membrane blebs and disorganisation of the pericanalicular actin filament web are common features in many forms of cholestasis (Scheuer & Lefkowitch 2000). For example, in the cholestasis related to preservation injury after liver transplantation, ischemia and reperfusion injury result in canalicular dilatation, loss of microvilli and compaction of actin filaments (Cutrin et al. 1996).

Cardiac complications in patients with dengue are not uncommon, although most cases are clinically mild heart complications (Ravindral et al. 2007, Promphan et al. 2004, Khongphatthanayothin et al. 2007, Pesaro et al. 2007, Lee et al. 2009). More severe myocardial dysfunction has been reported in patients who progressed to more severe DSS, compared to those with dengue or severe dengue fever. In cases of infection by DENV in which there is impairment of cardiac tissue, myocarditis is usually also present (Obeyesekere et al. 1972, Obeyesekere et al. 1973, Nagaratnam et al. 1973, Wali et al. 1998, Kularatne et al. 2005, Salgado et al. 2010, Lee et al. 2010, Sam et al. 2013). Interstitial oedema with inflammatory infiltrate and necrosis of myocardial cells can also be observed in these cases (Marques et al. 2013, Weerakook et al. 2011) and are closely associated with myocarditis (Cooper 2003). The pathophysiology of myocardial cell injury is still not fully understood. The tissue involvement mentioned above can be directly related to replication of DENV in heart muscle cells and/or a cytokine-mediated immune response (Hober et al. 1993, Hober et al. 1996). The present study revealed morphological alterations in the cardiac tissue of BALB/c during secondary infection. The main alterations were myocarditis, disorganisation of cardiac fibres, cardiomyocytes with rarefied cytoplasm and endothelial cells emitting membrane extensions. These changes are similar to those observed in human cases of dengue. There is evidence that the target cells for DENV include dendritic cells, monocytes, lymphocytes, hepatocytes, and vascular endothelial cells. Viral replication appears to occur in dendritic cells, circulating monocytes and possibly lymphoid cells (Leong et al. 2007). In immunohistochemical analysis of a heart tissue from a fatal case of severe dengue fever, DENV-specific antigen was observed in cardiomyocytes, myocardial interstitial cells and endothelial cells (Salted et al. 2010).

There are several studies utilising mice as an experimental model for DENV infection. The susceptibility of BALB/c mice inoculated intraperitoneally or intravenously with neuroadapted DENV in a primary infection has been demonstrated (Atrasheukaya et al. 2003, Huang et al. 2000). In the present study, DENV-1 was ultrastructurally identified and immunolocalised, and its RNA was detected in extracts of C6/36 cell cultures inoculated with the serum of BALB/c mice 72 h p.r. Ultrastructural studies showed the presence of DENV particles inside cytoplasmic vesicles and immature particles in cisterns of the rough endoplasmic reticulum.

These findings confirm that BALB/c mice are susceptible to infection and re-infection by DENV-1 or DENV-2 and that they are capable of developing morphological alterations similar to those observed in human cases of dengue haemorrhagic fever.

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REFERENCE

Agarwal R, Kapoor S, Nagar R, Misra A, Tandon R, Mathur A 1999. A clinical study of the patients with dengue hemorrhagic fever during the epidemic of 1996 at Lucknow, India. Southeast Asian J. Trop. Med. Public Health 30: 735-740. | | An J, Kimura-Kuroda J, Hirabayashi Y, Yasui K 1999. Development of a novel mouse model for dengue virus infection. Virology 263: 70-77. | | Avirutnan P, Malasit P, Seliger B, Bhakdi S, Husman M 1998. Dengue virus infection in human endothelial cells leads to chemokine production, complement activation and apoptosis. J. Immunol. 161: 6338-6346. | | Barreto DF, Paes MV, Takiya CM, Pinhão AT, Côrtes LMC, Majerowicz S, Barth OM 2002. Mice lung experimentally infected with dengue-2 virus: ultrastructural aspects. Virus Reviews & Research 7(2): 47-55. | | Barreto DF, Takiya CM, Paes MV, Farias-Filho J, Pinhão AT, Alves AMB, Costa SM, Barth OM 2004. Histopathological aspects of dengue-2 virus infected mice tissues and complementary virus isolation. J. Submicrosc. Cytol. Pathol. 36: 121-130 | | Barreto DF, Takiya CM, Schatzmayr HG, Nogueira RMR, Farias-Filho J, Barth OM 2007. Histopathological and ultrastructural aspects of mice lung experimentally infected with dengue virus serotype 2. Mem. Inst. Oswaldo Cruz 102: 175-182. | | Barreto-Vieira DF, Schatzmayr HG, Takiya CM, Jácome FC, Silva MEV, Faria NRC, Nogueira RMR, Barth OM 2010. Morphological studies of hepatic tissue of mice reinfected with dengue virus serotypes 1 or 2. Virus Reviews and Research 15(2): 1-27. | | Barreto-Vieira DF, Schatzmayr HG, Takiya CM, Jácome FC, Silva MEV, Faria NRC, Nogueira RMR, Barth OM 2009. Histopathological and ultrastructural studies of lung tissue of mice reinfected with dengue virus serotypes 1 or 2. Virus Reviews and Research 14(2): 30-49. | | Barth OM, Barreto DF, Pinhão AT, Takiya CM, Paes MV, Shatzmayr HG 2006. Morphological studies in a model for dengue-2 virus infection in mice. Mem. Inst. Oswaldo Cruz 101: 905-915. | | Bataller R, Brenner DA 2005. Liver fibrosis. J. Clin. Invest. 115: 209-218. | | Bente DA, Melkus MW, Garcia JV, Rico-Hesse R 2005. Dengue fever in humanized NOD/Scid Mice. J. Virol. 79: 13797-13799. | | Bhamarapravati N 1997. Pathology of dengue infections. In: DJ Gubler, G Kuno (eds.). Dengue and dengue hemorrhagic fever. CAB International, New York, p. 115-132. | | Burke DS, Monath TP 2001. Flaviviruses. In: DM Knipe and PM Howley (eds.). Fields Virology, 4th ed., vol. 1. Lippincott-Williams & Wilkins, Philadelphia, Pa, p. 1043-1125. | | Cooper JR, Leslie T 2003. Myocarditis: bench to bedside. New Jersey: Humana Press, 621 p. | | CY, YW, Liao CL, Lin YL 2006. Flavivirus infection activates the XBP1 pathway of the unfolded protein response to cope with endoplasmic reticulum stress. J. Virol. 80: 11868-11880. | | Cutrin JC, Cantino D, Biasi F 1996. Reperfusion damage to the bile canaliculi in transplanted human liver. Hepatology 24: 1053-1057. | | De Macedo FC, Nicol AF, Cooper LD, Yearsley M, Pires AR, Nuovo GJ 2006. Histologic, viral, and molecular correlates of dengue fever infection of the liver using highly sensitive immunohistochemistry. Diagn. Mol. Pathol. 15: 223-228. | | Deprès P, Frenkiel M-P, Ceccaldi P-E, Dos Santos CD, Deubel V 1998. Apoptosis in the mouse central nervous system in the response to infection with mouse-neurovirulent dengue viruses. J. Virol. 72: 823-829. | | Feroze NG 1997. Ultrastructural pathology on the cell and matrix. Fourth edition, (2): 619-1414. | | Friedman SL 2004. Mechanisms of disease: mechanisms of hepatic fibrosis and therapeutic implications. Nat. Clin. Pract. Gastr. 1: 98–105. | | Gressner AM 1996. Transdifferentiation of hepatic stellate cells (Ito cells) to myofibroblasts: a key event in hepatic fibrogenesis. Kidney Int. 49: S 39–45. | | Gressner AM, Bachem MG 1995. Molecular mechanisms of liver fibrogenesis - a homage to the role of activated fat-storing cells. Digestion 56: 335–346. | | Gressner AM, Weiskirchen R 2006. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. J. Cell Mol. Med. 10: 76–99. | | Gubler DJ 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol. 10:100–103 | | Guzman MG, Kouri G 2002. Dengue: an update. Lancet Infect. Dis. 2: 33-42. | | Halstead SB 1970. Observations related to pathogenesis of dengue haemorrhagic fever. VI. Hypotheses and discussion. Yale J. Bio. Med. 42: 350. | | Hase T, Summers PL, Ray P, Asafo-Adjei E 1992. Cytopathology of PC12 cells infected with japonese encephalitis virus. Virchows Arch. B. 63: 25-36. | | Havens Jr WP 1954. Hepatitis, yellow fever, and dengue. Annu. Rev. Microbiol. 8: 289-310. | Henchal EA, Gentry MK, McCown JM, Brandt WE 1982. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am. J. Trop. Med. Hyg. 31: 830-836. | Hober D, Delannoy AS, Benyoucef S, DE Groote D, Wattré P 1996. High levels of S TNFR p75 and TNF alpha in dengue-infected patients. Microbial. Immunol. 40: 59-573. | | Hober D, Poli L, Roblin B, Gestas P, Chungue G, Granic G 1993. Serum levels of tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 beta) in dengue infected patients. Am. J. Trop. Med. Hyg. 48: 324-333. | | Humprey CD, Pittman EE 1974. A simple methylene blue-azure-II basic fuchsin for epoxy-embedded tissue sections. Stain Technol. 49: 9. | | Jessie K, Fong MY, Devi S, Lam SK, Wong KT 2004. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J. Infect. Dis. 189: 1411-1418. | | Johnson AJ, Roehrig JT 1999. New mouse model for degue virus vaccine testing. J. Virol. 73: 783-786. | | Juffrie M, Meer DM, Hack CE, Haasnoot K, Sutaryo, Veerman AJ, Thijs LG 2000. Inflammatory mediators in dengue virus infection in children: interleukin-8 and its relationship to neutrophil degranulation. Infect. Immun. 68: 702-707. | | Khongphatthanayothin P, Lertsapcharoen P, Supachokchaiwattana V, Ia-orkhun V, Khumtonvong A, Boonlarptaveechoke C, Pancharoen C 2007. Myocardial depression in dengue hemorrhagic fever: prevalence and clinical description. Pediatr. Crit. Care Med. 8: 524-529. | | Kularatne SAM, Pathirage MMK, Kumarasiri PVR, Gunasena S, Mahundawanse SI 2007. Cardiac complications of a dengue fever outbreak in Sri Lanka, 2005. Trans R Soc. Trop. Med. Hyg. 101: 804-808. | | Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam V 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J. Clin. Microbiol. 30: 545-551. | | Lawn SD, Tilley R, Lloyd G, Finlayson C, Tolley H, Newmann P, Rice P, Harrison TS 2003. Dengue hemorrhagic fever with fulminant hepatic failure in an immigrant returning to Bangladesh. Clin. Infect. Dis. 37: 1-4. | | Lee CH, Teo C, Low AF 2009. Fulminant dengue myocarditis masquerading as acute myocardial infarction. Int. J. Cardiol. 136: e69-71. | | Lee I, Lee W, Liu J, Yang KD 2010. Acute myocarditis in dengue hemorrhagic fever: a case report and review of cardiac complications in dengue-affected patients. Int. J. Infect. Dis. 14:e919-922. | | Lee YR, Liu MT, Lei HY, Liu CC, Wu JM, Tung YC, Lin YS, Yeh TM, Chen SH, Liu HS 2006. MCP-1, a highly expressed chemokine in dengue haemorrhagic fever/dengue shock syndrome patients, may cause permeability change, possibly through reduced tight junctions of vascular endothelium cells. J. Gen. Virol. 87: 3623-3630. | | Leong AS, Wong KT, Tan PH, Wannakrairot P 2007. The pathology of dengue hemorrhagic fever. Semin. Diagn. Pathol. 24(4): 227-36. | | Ling LM, Wilder-Smith A, Leo YS 2007. Fulminant hepatitis in dengue haemorrhagic fever. J. Clin. Virol. 38: 265-268. | | Lin Y, Liao CL, Chen LK, Yeh CT, Liu CI, Ma SH, Huang YY, Huang YL, Kao CL, King CC 1998. Study of dengue virus infection in SCID mice engrafted with human K562 cells. J. Virol. 72: 9729-9737. | | Lucia HL, Kangwanpong D 1994. Identification of dengue virus-infected cells in paraffin-embedded tissue using in situ polymerase chain reaction and DNA hybridization. J. Virol. Methods 48: 1-8. | | Lum LCS, Lam SK, George R, Devi S 1993. Fulminant hepatitis in dengue infection. Southeast Asian J. Trop. Med. Public. Hlth. 24: 467-471. | | Marchette NJ, Halstead SB, Falkler WA Jr, Stenhouse A, Nash D 1973. Studies on the pathogenesis of dengue infection in monkeys. 3. Sequential distribuition of the virus in primary and heterologous infection. J. Infect. Dis. 128: 23-30. | | Marques N, Gan VC, Leo YS 2013. Dengue myocarditis in Singapore: two case reports. Infection, 41:709-714, 2013. | | Meiklejohn G, England B, Lennette EH 1952. Adaptation of dengue virus strains in unweaned mice. Am. J. Trop. Med. Hyg. 1: 51-58. | | Miagostovich MP, Ramos RG, Nicol AF, Nogueira RMR, Cruzzi-Maya T, Oliveira AV, Marchevsky RS, Mesquita RP, Schatzmayr HG 1997. Retrospective study on dengue fatal cases. Clin. Neuropathol. 16: 204-208. | | Nagaratnam N, Siripala K, DE Silva N 1973. Arbovirus (dengue type) as a cause of acute myocarditis and pericarditis. Br. Heart J. 35: 204-206. | | Na J, Zhou DS, Kawasaki K, Yasui K 2003. The pathogenesis of spinal cord involvement in dengue virus infection. Virchows Arch. 442: 472-481. | | Obeyesekere I, Hermon Y 1973. Arbovirus heart disease: Myocarditis and cardiomyopathy following dengue and chikungunya fever – A follow-up study. Am. Heart J. 85(2): 186-194. | | Obeyesekere I, Hermon Y 1972. Myocarditis and cardiomyopathy after arbovirus infections (dengue and chikungunya fever). Br. Heart J. 34: 821-827. | | Paes MV, Barreto DF, Takiya CM, Pinhão AT, Côrtes LMC, Majerowicz S, Barth OM 2002. Ultrastructural aspects of mouse liver infected with dengue-2 virus. Virus Reviews & Research 7(2): 38-46. | | Paes MV, Pinhão AT, Barreto DF, Costa SM, Oliveira MP, Nogueira AC, Takiya CM, Farias-Filho JC, Schatzmayr HG, Alves AMB, Barth OM 2005. Liver injury and viremia in mice infected with dengue-2 virus. Virology 338: 236-246. | | Pesaro AE, D'amico E, Aranha LF 2007. Dengue: cardiac manifestations and implications in antithrombotic treatment. Arq. Bras. Cardiol. 89: e12-e15. | | Pinzani M 1995. Hepatic stellate (Ito) cells: expanding roles for a liver-specific pericyte. J. Hepatol. 22: 700-706. | | Promphan W, Sopontammarak S, Pruekprasert P, Kajornwattanakul W, Kongpattanayothin A 2004; Dengue myocarditis. Southeast Asian J. Trop. Med. Public Health, 35: 611-613. | | Ravindral S, Kanagasinham A, Neomali A, Amerasena A, Uditha B, Deshu VS 2007. Asymptomatic myocardial involvement in acute dengue virus infection in a cohort of adult Sri Lankans admitted to a tertiary referral centre. Br. J. Cardiol. 14: 171-173. | | Reed LJ, Muench H 1938. A simple method of stimating fifty percents endpoints. The Am. J. Hyg. 27: 493-497. | | Reynolds 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17: 208-212. | | Rigau-Perez JG, Clark GG, Gubler DJ, Reiter P, Sanders EJ, Vorndem AV 1998. Dengue and dengue hemorrhagic fever. Lancet 352: 971-977. | | Robert FMAJ, Daniel MDCOL, Julia DOCOL, David MDLTC, Brian KMAJ, Michelle KCPT, Bret MDMAJ 2004. Pulmonary hemorrhage syndrome associated with an autochthonous case of dengue hemorrhagic fever. Southern Med. J. 97: 7 | | Rosen L, Drouet MT, Deubel V 1999. Detection of dengue virus RNA by reverse transcription-polymerase chain reation in the liver and lymphoid organs but not in brain in fatal human infection. Am. J. Trop. Med. Hyg. 61: 720-724. | | Salgado DM, Eltit JM, Mansfield K, Panqueba C, Castro D, Vega MR, Xhaja K, Schmidt D, Martin KJ, Allen PD, Rodriguez JA, Dinsmore JH, López JR, Bosch I 2010. Heart and Skeletal Muscle Are Targets of Dengue Virus Infection. Pediatr. Infect. Dis. J. 29(3): 238–242. | | SAM, S.; OMAR, S. F. S.; TEOH, B.; ABD-JAMIL, J.; ABUBAKAR, S. Review of Dengue Hemorrhagic Fever Fatal Cases Seen Among Adults: A Retrospective Study. PLoS Negl. Trop. Dis. 7(5) e2194, 2013. | | Scheuer PJ, Lefkowitch JH 2000. Liver biopsy interpretation, 4th ed. Ed. Harcourt, Health Sciences. Chapter 18, Electron microscopy and other techniques, p. 345-364. | | Sharma SK, Gupta BS, Devpura G, Agarwal A, Anand S 2007. Pulmonary haemorrhage syndrome associated with dengue haemorrhagic fever. J. Assoc. Physicians India 55: 729-730. | | Thein S, Aung MM, Shwe TN, Aye M, Zaw A, Aye K, Aye KM, Aaskov J 1997. Risck factors in dengue shock syndrome. Am. J. Trop. Med. Hyg. 56: 566-572. | | Wali JP, Biswas A, Chandra S, Malhotra A, Aggarwal P, Handa R, Wig N, Bahl VK 1998. Cardiac involvement in dengue haemorrhagic fever. Int. J. Cardiol. 64: 31–36. | | Weerakoon KGAD, Kularatne SAM, Edussuriya DH, Kodikara SKA, Gunatilake L PG, Pinto VG, Seneviratne AB, Gunasena S 2011. Histopathological diagnosis of myocarditis in a dengue outbreak in Sri Lanka, 2009. BMC Research Notes, 4: 268. | Wiwanitkit V 2008. Dengue cardiac infection, a brief review. Acta Cardiol Sin.;24:226. | | World Health Organization, Fact sheet 117, 2002. |