ULTRASTRUCTURAL ASPECTS OF MOUSE LIVER INFECTED WITH DENGUE-2 VIRUS.

ABSTRACT

Dengue hemorrhagic fever (DHF) can provoke an acute hepatitis that in some cases is similar to the lesion seen in yellow fever, but little is known about the pathogenesis of the lesion. In order to better define the morphological alterations caused by the virus in liver, a mouse model was created and characterized. BALB/c mice were inoculated with dengue-2 virus (100 TCID$_{50}$ and 10,000 TCID$_{50}$/0.2ml) by the intraperitoneal route, and sacrificed at 24, 48 and 72 hours, 14 and 35 days post-infection. Liver fragments were then fixed for light (LM) and transmission electron microscopy (TEM) using standard techniques. To further verify the impact of dengue virus on liver function, biochemical enzymatic tests were performed from the first to the seventh day of infection. The level of aspartate (AST) and alanine (ALT) transaminase enzymes increased progressively, reaching a peak at the 4th day post-infection. By TEM, the most prominent modifications consisted of multifocal endothelial injury (necrosis/disappearance) with enlargement of Disse’s space and platelet recruitment. These alterations were more evident in the higher viral load group. At the same time, the sinusoidal pole of hepatocytes showed altered microvilli, prominent superficial endocytic vacuoles as well as lipid accumulation. Exposition of hepatocytes directly to blood probably could lead to an abnormal hepatic function, an aspect demonstrated by altered blood transaminases.

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INTRODUCTION

Dengue viruses are arthropod-borne flaviviruses with four serotypes. They cause a spectrum of disease from a harmless inapparent illness (dengue virus fever) to severe and lethal manifestations (dengue hemorrhagic fever and dengue shock syndrome). However, the pathogenesis of human dengue infection remains poorly defined and neither antiviral treatment nor successful vaccine are available as yet (Eckels 1993, Rigau-Perez et al. 1998), remaining a major health problem with high morbidity and mortality in many areas of the world (Gubler 1998).

Target cells and organs for dengue virus replication in humans remain unclear. It has been demonstrated in cases of dengue hemorrhagic fever that liver cells, lymph nodes, bone marrow, lymphopoietic tissue, muscle, connective tissue and vascular endothelium (Sahaphong et al. 1980) and also brain and kidney (Hotta et al. 1981) could be the possible sites for viral replication. Furthermore, more recently, it has been demonstrated that skin dendritic cells are permissive for dengue virus replication (Wu et al. 2000) and therefore are likely to be the initial target of dengue virus infection in the arthropod-borne transmission to humans.

Moreover, dengue virus can interact with and occasionally replicate in a number of hematopoietic cells, such as monocyte/macrophagic and leucocytic populations, with bone marrow progenitors, endothelial cells as well as with Kupffer cells and human liver cell lines (Avirutnan et al. 1978, LaRusca & Innis 1995, Lin et al. 2000, Marianneau et al. 1999, Nakao et al. 1989). In vivo, hepatocytes seem to be a site of virus replication (Couvelard et al. 1999). It has been demonstrated that heparan sulphate proteoglycans could account for the initial binding of dengue viruses with hepatocytes (Hilgard & Stocker 2000).

Information about damage of the liver may be based on autopsy material obtained from cases of dengue hemorrhagic fever (Bhamarapravati et al. 1967). Centrilobular necrosis, change in lipid composition, monocytes inflammatory infiltration of the portal tract were the most common pathological findings, both in patients with dengue hemorrhagic fever and/or dengue shock syndrome. This pattern of injury is similar to that seen in early stages of yellow fever (Marianneau et al. 1998). Furthermore, it has also been demonstrated in cases of dengue hemorrhagic fever the presence of Councilman bodies and hyaline necrosis of the Kupffer cells (Burke 1968).

Although dengue viruses have not been localized in liver by electron microscopy, virus antigen has been detected in the cytoplasm of phagocytic mononuclear cells from liver, and in the sinusoidal lining of the liver (Boonpucknavig et al. 1979).

In the present investigation, ultrastructural aspects of infected mouse liver were studied. We analyzed the alterations resulting from experimental dengue-2 intraperitoneal virus infection in adult mice at 24, 48 and 72 hours, 14 and 35 days post-infection.

MATERIAL AND METHODS

Animals - Adult male BALB/c mice (2 months of age), weighing about 25g were obtained from the colony maintained in the Department of Virology, Instituto Oswaldo Cruz.

Virus - Dengue-2 virus was isolated from a patient serum of dengue outbreak in 1995 in Rio de Janeiro in the Aedes albopictus mosquito C6/36 cell line. The serum was tested by the indirect immunofluorescence technique using a type specific serotype 2 monoclonal (3H5 dengue-2) antibody.
Infection - Mice were intraperitoneally inoculated with dengue-2 virus. The titre of the virus (10^{6.3} TCID_{50}/0.1ml) was calculated by the method of Reed & Muench (1938) and inoculated as 100 TCID_{50} or 10,000 TCID_{50}/0.2ml in two groups of mice. Animals were sacrificed at 24, 48 and 72 hours, as well as 14 and 35 days after infection. Non-infected mice and mice inoculated with medium L-15 were used as controls.

Liver tissue obtention - Mice were anaesthetized with 4% chlortal hydrate (0.4 ml/25g of animal). A cannula was introduced into the left ventricle and perfusion was performed using 4% paraformaldehyde in 0.2M sodium phosphate buffer, pH 7.4 for 30 minutes, after blood washing with saline (NaCl 0.9% pH 7.2). Fragments of liver were fixed in 2.0% buffered glutaraldehyde solution (0.2M sodium cacodylate buffer pH 7.2) for transmission electron microscopy. The tissue was then washed with the same buffer, post-fixed with 1% buffered osmium tetroxide, washed again with the same buffer and distilled water, dehydrated by increasing acetone concentrations, embedded in epoxy resin and polymerized at 60°C during three days. Semi-thin sections were obtained and stained with a methylene blue azure II solution (Humphrey & Pittman 1974); when adequate sampling of modified zones was visualized, the material was submitted to ultra-thin sectioning. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed with a Zeiss EM-900 transmission electron microscope.

Biochemical analysis of hepatic enzymes - Mice were anaesthetized with as formerly described and sacrificed by cardiac puncture. The blood of intraperitoneally injected mice was collected from the first to the seventh day post-infection. The level of aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) were verified by the UV optimized (IFCC) method (Karmen 1955).

Culture of macrophages of adult BALB/c mice infected with dengue-2 virus -

Mice were intraperitoneally inoculated with dengue-2 (100 TCID_{50}/0.2ml), and anaesthetized 72 hours post-inoculation. Peritoneal washing was done with 5ml of the culture medium 199, which was collected and placed in cell culture wells (35mm microplates). The cell cultures were incubated at 37°C in a 5% CO_{2} atmosphere. After 48 hours of culture, macrophages were fixed in 1% buffered glutaraldehyde and embedded in epoxy resin for subsequent observations by transmission electron microscopy. As a control, non-infected adult BALB/c mice macrophages were recovered and submitted to the same procedures.

Isolation of dengue-2 viruses of mouse liver in C6/36 mosquito cell culture - Adult BALB/c mice were peritoneally infected with dengue-2 virus (10,000 TCID_{50}/0.2ml). After 48 hours of infection they were anaesthetized with 4% chlortal hydrate (0.4ml), the liver tissue was collected and macerated in Leibovitz medium (L-15), supplemented with antibiotics. The suspension was incubated for one hour for antibiotic action, centrifuged at 1,400g for five minutes and the supernatant collected. 100ml of the supernatant were inoculated into monolayers of the C6/36 cell line and the cells were incubated for one hour at 28°C for virus adsorption. Thereafter these monolayers were grown in L-15 medium supplemented with 1% non-essensial aminoacids, 10% trypose phosphate broth and 10% fetal bovine serum. The tubes were kept at 28°C and observed daily for viral cytopathic effect for fifteen days. After this period of observation, the monolayers were divided into two parts; the first was tested by the indirect immunofluorescence technique with a type specific monoclonal antibody for dengue serotype 2 (3H5) and the second was fixed in 1% buffered glutaraldehyde, dehydrated and embedded in epoxy resin as described above for observation by transmission electron microscopy.
RESULTS

Both mice groups inoculated with dengue-2 virus (100 and 10,000 TCID₅₀/0.2ml) presented no clinical signs of the disease. Furthermore, no mortality occurred during the period of observation, up to the 35th day post-infection.

Hepatocytes in normal liver presented several mitochondria and glycogen, granules, as well as rough and smooth endoplasmic reticulum profiles. Short finger-like cytoplasmic extensions were observed in the sinusoidal border (Figures 1 and 7).

At the beginning of infection (24 hours post-infection, 100TCID₅₀/0.2 ml) an abnormal hepatic organization was present, with modified cellular adhesions that allowed inflammatory cells penetration between the hepatocytes (Figure 2). In localized areas focal zones, endothelial cell denudation, platelets in sinusoidal lumen and also prominent stellate cells containing lipid-droplets (Figures 2, 3) were often observed in the liver tissue. In the later period of infection (7 and 14 days post-infection, 100 TCID₅₀/0.2 ml), polymorphonuclear cells were seen adherent to the endothelium. Hepatocyte cells showed vacuoles in the cytoplasm, and increased amounts of glycogen and lipid droplets (Figure 5).

At 35 days post-infection (100TCID₅₀/0.2ml) a recovery of the endothelial lining could be seen, platelets already being present in the lumen of sinusoidal capillaries. On the other hand, hepatocytes showed the same morphological aspects as seen at 7 or 14 days of infection (Figure 6).

In the groups inoculated with 10,000TCID₅₀/0.2ml we could observe 24 hours post-infection prominent modifications in the hepatic structure. Hepatic sinusoids were dilated, presenting hyperplastic Kupffer cells and inflammatory cells causing damage to sinusoid capillaries. Focal disorganization of the endothelial lining was observed. Hepatocyte cells showed disorganization of microvilli presenting peripheral vacuoles (Figure 4).

After 72 hours post-infection (10,000TCID₅₀/0.2ml), intensive vacuolization of the cytoplasm in hepatocyte cells was observed. In sinusoid capillaries a change in vascular permeability in the form of denudation of endothelial cells could be seen (Figure 8).

At a later stage of infection (14 days post-infection, 10,000TCID₅₀/0.2ml) hepatocytes were vacuolated and the number of microvilli reduced at the sinusoidal border (Figure 9).

In both concentrations (100TCID₅₀ and 10,000TCID₅₀/0.2ml) of virus, the level of transaminases (AST and ALT) was at its highest peak by the 4th day post-infection (Table 1).

Table 1: Biochemical analysis of hepatic enzymes in mice controls and infected with dengue-2 virus expressed as standard units of enzymes/ml of serum.

<table>
<thead>
<tr>
<th>ENZYMES</th>
<th>Controls</th>
<th>Days post-infection with dengue-2 virus</th>
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<tbody>
<tr>
<td></td>
<td>NC</td>
<td>MC</td>
</tr>
<tr>
<td>AST a)</td>
<td>1224</td>
<td>1418</td>
</tr>
<tr>
<td>ALT b)</td>
<td>820</td>
<td>1013</td>
</tr>
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AST - aspartate aminotransferase, ALT - alanine aminotransferase. a) and b): values resulting from infection of mice with dengue-2 virus doses of 100TCID₅₀/0.2ml (first line) and 10,000TCID₅₀/0.2ml (second line). NC - normal control mice. MC - control mice inoculated with medium L-15.
Peritoneal macrophages infected with dengue-2 virus showed several viral particles inside membrane bound vesicles (Figure 10).

Immunofluorescence assay showed an intracellular positive reaction after 15 days of incubation when the supernatant of macerated liver tissue infected with dengue-2 virus was inoculated into C6/36 mosquito cell culture (Figure 11).

Transmission electron microscopy showed several viral particles in C6/36 mosquito cells infected with dengue-2 virus present in the macerated mouse liver (Figure 12).

**DISCUSSION**

The results of the present study show that although the liver is not considered to be the major target organ affected by dengue virus, damage to hepatic tissue must be considered as important and it may be a source of viral replication (Huerre et al. 2001, Rosen et al. 1989, Rosen et al. 1999) and organ dysfunction (Nguyen et al. 1997).

Virus particles have already been detected in liver isolates by immunofluorescence (Rosen et al. 1989) or by RT-PCR (Rosen et al. 1999), and even viral antigens have been demonstrated in hepatocytes of fatal dengue fever (Huerre et al. 2001). In liver biopsies from fatal cases of dengue or dengue hemorrhagic fever, the characteristic pattern is of a mild or moderate acute hepatitis with a varying amount of necrosis and inflammatory infiltrate. This may be accompanied by steatosis and apoptotic cell death (Marianneau et al. 1998). In our ultrastructural study we never observed hepatocyte necrosis or irreversible injury, but only some increase in the number of endocytic vesicles, associated with few lipid droplets. It is possible that the absence of a significant degree of cell injury was related to the areas sampled, since the hepatic fragments contained randomly chosen zones of the lobule.

The most prominent observation was on the transiently diminished or absent endothelial cell extensions, suggesting that sinusoidal endothelial cells are probably affected by viruses. The same pattern of lesion, with decrease in the number of sinusoidal endothelial fenestrae was previously detected in murine type 3 hepatitis (Steffan et al. 1995).

Probably consequent to the extensive loss of the fenestrations, hepatocytes become functionally abnormal, which could explain the enzymatic alterations observed in our study. Such abnormal levels of transaminases were already demonstrated, but generally with a low or moderate increase, reflecting at least a moderate degree of liver damage (Sahaphong et al. 1980).

A study of the endothelial cell in skin blood vessels of human cases of dengue hemorrhagic fever has demonstrated that endothelial cells become swollen, with bleb formation. Regeneration of the endothelial lining was also demonstrated but instead of virus presence, tubuloreticular structures were evident (Sahaphong et al. 1980). This is probably related to the high amount of cytokines released by activated monocytic/macrophagic and Kupffer cells (Lee et al. 1996, Marianneau et al. 1999), as verified in interferon gamma treated hepatitis C patients (Watanabe et al. 1995), and that is indicative of immune mediated injury. Taken all together, hepatic injury in the mouse model seems to be more related to the systemic activation of blood system elements with secretion/liberation of cytokines and growth factors and endothelial cell death than to a direct hepatocyte injury.

Mice inoculated with a dose of 10,000TCID₅₀/0.2ml produced a higher level of transaminases (AST and ALT) when compared with the levels observed in the animals inoculated with 100 TCID₅₀/0.2ml (Table 1). The
cell and tissue damage that occurred during infection was similar using 100 TCID$_{50}$ and 10,000 TCID$_{50}$/0.2ml. However, liver damage was more pronounced with the higher titer of virus.


Fig. 1: Control liver hepatocyte at the sinusoidal regular structured border with short finger-like cytoplasmic extensions (arrows). The arrowhead shows a fenestrated endothelium. Disse's space limits (D). Ultra-thin section. Bar=2.5mm. Fig. 2: Control of hepatocyte cells (H) showing the normal cytoplasm and a regular sinusoid capillary (SC). Disse's space limits (D). Ultra-thin section. Bar=2.5mm. Fig. 3: Disorganization of liver sinusoid with slight degree of modified cellular adhesion and inflammation cells penetration (IC). Platelet (PL). (24 hours post-infection, 100TCID₅₀/0.2ml of virus concentration). Ultra-thin section. Bar=1.6mm. Fig. 4: Initial predominant injury consisting of focal zones of sinusoidal endothelial denudation (star). Vacuoles (V). (24 hours post-infection, 100TCID₅₀/0.2ml of virus concentration). Ultra-thin section. Bar=1mm. Fig. 5: A polymorphonuclear cell (PMN) is adherent to the endothelium. (V) vacuoles. Lipids (L). Glycogen (G). (14 days post-infection, 100TCID₅₀/0.2ml of virus concentration). Ultra-thin section. Bar=1mm. Fig. 6: Platelets (PL) are still present in liver sinusoid and focal endothelial (E) lining is starting. Note glycogen (G) inside hepatocyte and lipid droplets accumulation (L). (35 days post-infection, 100TCID₅₀/0.2ml of virus concentration). Ultra-thin section. Bar=0.2mm.
Fig. 7 - Dilated lumen of hepatic sinusoid containing an inflammatory cell (IC), showing focal disorganization of endothelial lining (arrow) and disorganized microvilli (star) of an hepatocyte with peripherally disposed vacuoles (V). (24 hours post-infection, 10,000 TCID₉₀/0.2ml of virus concentration). Ultra-thin section. Bar=1.1mm.

Fig. 8 - Sinusoidal cells (stars) present alterations with intensive vacuolization (V) of the cytoplasm. Note increased change of vascular permeability, shown by derudation of endothelial cells (arrow). (72 hours post-infection, 10,000 TCID₉₀/0.2ml of virus concentration). Ultra-thin section. Bar=1.8mm.

Fig. 9 - Hepatocytes showing vacuolization (V) and reduced microvilli (stars) around the sinusoidal border. Endothelial cell (ENC). (14 days post-infection, 10,000 TCID₉₀/0.2ml of virus concentration). Ultra-thin section. Bar=1.5mm

Fig. 10 - Peritoneal macrophage of mouse infected with dengue-2 virus. Note several viral particles (arrows) inside a membrane bound vesicle. Ultra-thin section. Bar=0.3mm.

Fig. 11 - Detection of viral antigen (arrows) by indirect immunofluorescence in cultured C6/36 mosquito cells incubated with liver extract from an infected mouse (48 hours post-infection, 10,000 TCID₉₀/0.2ml). Bar=60mm.

Fig. 12 - C6/36 mosquito cell from a culture infected with a liver extract from an infected mouse (48 hours post-infection, 10,000 TCID₉₀/0.2ml). Note the virus particles inside vesicles (arrows). Bar=0.5mm.