MICE LUNG EXPERIMENTALLY INFECTED WITH DENGUE-2 VIRUS: ULTRASTRUCTURAL ASPECTS.

ABSTRACT

Experimental dengue-2 virus (DENV-2) infection in lung tissue of mice, inoculated by the intraperitoneal route, was analyzed. The DEN-2 strain was isolated from a patient serum in a C6/36 mosquito cell line of Aedes albopictus. The titer of the virus was $10^{5.3}$ TCID$_{50}$/0.1ml. Adult BALB/c mice were inoculated with doses of 100 and 10,000 TCID$_{50}$/0.2ml. The animals were sacrificed by perfusion of the cardiac ventricle with paraformaldehyde and Millonig’s fixative; after 72 hours, 7, 14 and 49 days post-infection lung tissue was processed following the standard techniques for light and electron microscopy. At light microscopy we observed an interstitial pneumonia, characterized by the presence of mononuclear cells. Furthermore, an hyperplasia and hypertrophy of the bronchiolar epithelial cells was depicted by electron microscopy as well as an interseptal oedema. Damage to lung tissue was observed from 72 hours to 7 days post-infection. The course of infection was similar in the two different doses, but lesions were more severe in animals inoculated with 10,000 TCID$_{50}$. In the mouse model, DENV-2 seems to lead to a transient inflammatory process without extensive damage to the alveolar septa, although focal alterations to the blood-exchange barrier were observed. Our results show some similarities to human DENV infection and therefore provides a useful model to understand human disease pathogenesis.
INTRODUCTION

The dengue viruses (DENV) are members of the family *Flaviviridae*, which includes more than 60 members (Westaway et al. 1985). There are four serotypes, DENV-1, DENV-2, DENV-3 and DENV-4. DENV-2 viruses were first isolated in Brazil, during an outbreak in 1990 (Nogueira et al. 1990).

The clinical features of dengue hemorrhagic fever (DHF) include plasma leakage, bleeding tendency and liver involvement (Anonymous 1986). Plasma leakage is caused by a diffuse increase in capillary permeability and manifests as any combination of hemoconcentration, pleural effusion or ascites (Kalayanarooj et al. 1997).

Extensive pathological surveys have been made with tissues from fatal human cases of DHF. Gross pathological findings in cases of DHF and Dengue Shock Syndrome (DSS) include hemorrhages in the skin, subcutaneous tissues, gastrointestinal tract and heart. Significant histological changes are found in several organs including the liver, lung, spleen and cerebellum. Hemorrhagic manifestations in organs and fluid accumulation in body cavities are the prominent changes verified in vivo (Henchal & Putnak 1990).

Ultrastructural studies of human lung presenting DHF revealed that hemodynamical changes accompanied by intra-alveolar haemorrhagic foci were the characteristic picture of infection. The alveolar spaces usually contained some oedemic fluid, but no hyaline membrane. Megakaryocytes were present in the lung in larger quantity than in other tissues (Bhamarapravati et al. 1987, Bhamarapravati 1989, Burke 1968). Furthermore, an interstitial pneumonia, characterized by swelling of the alveolar septa and infiltration by mononuclear cells, was also observed.

Mononuclear phagocytes including circulating monocytes have been shown to support the replication of dengue viruses (Bhamarapravati 1990). Studies of DEN virus infection in fatal human cases, using the imunoperoxidase technique, demonstrated that DENV antigen was detected in several organs. In the lung, positivity was observed in alveolar spaces within mononuclear phagocytes (Miagostovich et al. 1997).

Studies in mice (BALB/c, B6, C3H/He, AKR and A/J) infected by DEN virus have demonstrated that DENV-2 can infect mice via a peripheral route. The mice developed transient thrombocytopenia, similar to that observed in mice infected by the intravascular route. Furthermore, they developed anti-platelet antibodies similar to DENV infection in humans. In this way, the mouse model provides a useful model to understand human disease pathogenesis, especially immune activation in DENV infection (Huang et al. 2000).

Since neither an extensive analysis of lung nor ultrastructural aspects of DENV-2 pulmonary infection in mice were available, we have studied the histopathological changes occurring in the lung of adult mice at 72 hours, 7, 14 and 49 days post-infection, inoculated with virus at concentrations of 100 and 10,000 TCID$_{50}$/0.2ml by the intraperitoneal route.

MATERIALS AND METHODS

**Virus** - The viruses used in our experiments were isolated from a patient serum during an epidemic of DENV-2 in the state of Rio de Janeiro in 1995 and propagated in a *Aedes albopictus* mosquito cell line (C6/36). The serum was tested by the indirect
immunofluorescence technique using a type-specific serotype 2 monoclonal (3H5, DEN-2) antibody. The virus had not undergone passage in mouse brain. The titer of the virus was (10^{6.3} TCID_{50}/0.1ml) calculated by the method of Reed & Muench (Reed & Muench 1938).

**Animals** - Adult male BALB/c mice, aged 2 months and weighing 25 g were used. The animals were obtained from the mouse colony maintained in the Department of Virology. The mice were intraperitoneally inoculated with doses of 100 TCID_{50} and 10,000 TCID_{50}/0.2ml. Non-infected mice and mice inoculated with medium L-15 were used as controls.

**Morphology** - Lung fragments were collected after 72 hours, 7, 14 and 49 days post-infection. The animals were anaesthetized intraperitoneally with 4% chloral hydrate (0.4 ml/25g of animal). Perfusion in situ was carried out through the heart left ventricle using physiological saline (NaCl 0.9%, pH 7.2) for 2 minutes to remove blood cells, followed by 4% buffered paraformaldehyde perfusion. Lung tissue fragments were fixed for light microscopy using Millonig's fixative, dehydrated in ethanol and paraffin-embedded. Sections 5mm tick were stained with haematoxilin and eosin. For electron microscopy, fragments were immersed in 2% glutaraldehyde in cacodylate buffer (0.2 M, pH 7.2), dehydrated in acetone, post-fixed with 1% buffered osmium tetroxide, embedded in epoxy resin and polymerized at 60°C during three days. Semi-thin sections of 0.5mm thickness were obtained using a diamond knife (Diatome) adapted to a Reichert-Jung Ultracut E microtome. Sections stuck on glass slides were stained with a methylene blue and azure II solution (Humphrey & Pittman 1974) and observed using a Zeiss Axiophot light microscope.

Ultra-thin sections of 50-70nm thickness were obtained using a diamond knife as described. The sections 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.

**In vitro culture of peritoneal macrophages of infected mice** - Male BALB/c mice were peritoneally inoculated with DENV-2 (100 TCID_{50}/0.2ml) for the culture of macrophages. After 72 hours of infection, 5ml of the culture medium 199 was inoculated into the peritoneum. The peritoneal exudate was collected with a syringe under aseptic conditions and grown for 48 hours in multiwell plates incubated at 37°C in a 5% CO_2 atmosphere. The cells were then fixed in 1% buffered glutaraldehyde, dehydrated and embedded in epoxy resin as described above for observation by transmission electron microscopy.

**Isolation of DENV-2 virus in the C6/36 cell line starting with a supernatant of lung tissue macerate** - Male BALB/c mice were peritoneally inoculated with DENV-2 virus (10,000 TCID_{50}/0.2ml). After 48 hours of infection they were anaesthetized with 4% chloral hydrate (0.4ml), the lung 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 was collected. 100ml of the supernatant was inoculated in a C6/36 monolayer culture and the cells incubated for one hour at 28°C for virus adsorption. Thereafter, the monolayer was grown in L-15 medium supplemented with 1% non-essential amino acids, 10% tryptose phosphate broth and 10% fetal bovine serum. The tubes were kept at 28°C and observed daily for viral cytopathic effects for fifteen days. After this period of observation, the monolayer was divided into two parts, the first was tested using the indirect immunofluorescence technique with type-specific
monoclonal antibodies for 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 DENV-2 infected mice groups showed neither mortality nor symptoms of the disease. Observations light (paraffin and semi-thin sections) and transmission electron microscopy of the infected lung tissue demonstrated the presence of an interstitial pneumonia, which was similar for both doses of intraperitoneal inoculation.

Infected animals exhibited swollen alveolar septa in which, at the initial stage, a mononuclear inflammatory infiltrate could be observed, when compared to the controls (Figures 1, 2). From 72 hours to 7 days post-infection inflammation was also depicted in a peribronchiolar localization (bronchiolitis), admixed with platelets (Figures 3, 4, 5). Recruitment of this cell type was still evident later, at 14 and 49 days of infection, but decreasing progressively, with platelets persisting inside blood vessels (Figures 6, 7). Polymorphonuclear cells were recruited at a later stage, up to 14 days post-infection. Up to 72 hours post-infection, a hyperplasia of bronchiolar epithelia was observed (Figure 8). Foci of subepithelial oedema were depicted at 7 days post-infection, but rare pneumocytes showed evidence of injury at this stage. They exhibited a dense cytoplasm with swollen mitochondria, whereas in the alveolar septa interstitial fibroblasts containing lipid droplets became evident (Figure 9). The endothelial cells maintained their structure without signs of reversible injury or necrosis.

Virus particles were detected inside membrane bound cytoplasmic vesicles of peritoneal macrophages obtained from infected mice and maintained in culture for 48 hours (Figure 10).

The DENV-2 virus could be isolated in a C6/36 mosquito cell culture inoculated with a supernatant of lung tissue macerate (Figure 12). The syncytial cytopathic effect was visible around thirteen days after inoculation. Virus particles were detected inside membrane bound cytoplasmic vesicles, as observed with the in vitro infected macrophages.

DISCUSSION

Our results have demonstrated that lung damage in BALB/c mice infected by DENV-2 virus consisted of a multifocal interstitial pneumonia associated with capillary congestion and hemorrhagic foci, peaking at 72 hours and vanishing at 49 days post-infection. Therefore, our results are similar to those shown in other animal studies (Bhamarapravati 1989, Hotta et al. 1981). It must be stressed however, that in mice, lung damage is not so severe as that observed in human beings.

Necropsies of fatal human cases of DEN exhibited in lungs an interstitial pneumonia with a predominant lymphocytic infiltrate similar to DEN virus infected mouse lungs. Focal alveolar hemorrhages, alveolar capillaries congestion and oedema were also present. Dengue virus antigen has been localized in alveolar macrophages (Miagostovich et al. 1997). Moreover, by immunofluorescence, it has been shown that DEN virus is present in lung of athymic nude mice (Hotta et al. 1981).

Until now, pathogenesis of DENV infection is poorly known. The main features of DHF (increased capillary permeability leading
to extensive plasma leakage, increased haematocrit, thrombocytopenia, altered number and functions of leukocytes) associated with the lack of important structural damage, and also with the remarkable rapid recovery of children with DHF/DSS, suggest the involvement of a soluble mediator (Innis 1995). A high level of a unique cytokine, produced by CD4+ cells was isolated from sera of patients with DHF (Agarwal et al. 1998, Mukerjee et al. 1997). The mouse cytotoxic factor and its homologue in man are capable of reproducing DHF-like pathological lesions, such as increased capillary permeability and cerebral oedema (Chaturvedi et al. 1991, Dhawan et al. 1990, Khanna et al. 1990). The permeability of capillaries depends upon the ultrastructure of their endothelial cells and their junctions. Transport across the capillary wall is mediated either by passage of molecules through opened cell junctions, by micropinocytosis or through the fenestrated channels across the cell cytoplasm. An increased capillary permeability could be due to damage to capillary endothelial cells or to the secretion of various vasoactive mediators. Our ultrastructural study does not support the former hypothesis. Different vasoactive mediators are produced in the plasma, namely fibrin-split products, kinins and complement components C3a and C5a, or by cells, specially circulating platelets. Tissue mast cells probably are also involved in dengue lung injury, since they are usually found in normal and abnormal alveolar wall (Fox et al. 1981). They produce a series of vasoactive mediators, including histamine and histamine-like products.

In our experiments, we could only detect minor lung modifications, that were present at the air-blood barrier site. Both alveolar epithelial and endothelial cells maintained their structure without signs of either reversible injury or necrosis. Furthermore, endothelial cell junctions were preserved, but the presence of a larger number of endocytic vacuoles and filopodia could favour the possibility of these cells being activated (Feroze 1997). It seems that endothelia comprise one of the central cells in the pathogenesis of DENV injury. They can support viral replication and they secrete various inflammatory mediators including a selective up-regulation of IL-8 and RANTES (Avirutnan et al. 1998, Juffrie et al. 2000). These substances are able to recruit neutrophils and indirectly also lead to an increase of vascular permeability. In our model, we could also verify that polymorphonuclear cells were recruited at a late stage, up to 14 days post-infection. Their involvement is therefore probably related to the release of IL-8 and RANTES, which occurred after endothelial activation by DENV.

Despite the fact that no virus particles were found in the lung of infected animals by electron microscopy, they were detected in cytoplasmic vesicles of peritoneal macrophages of infected BALB/c mice. Monocytes have been reported to be the most permissive cell population in peripheral blood mononuclear cells (PBMC) for dengue virus infection in vitro (Halstead & O'Rourke 1977). Similarly, in PBMC obtained from patients with dengue infection, DENV was detected frequently and at higher level in the adherent PBMC population than in the nonadherent cell population (Scott et al. 1980). Based upon these findings, monocytes and tissue macrophages were thought to be predominant cell targets for DENV infection in vivo (Rothman & Ennis 1999). Furthermore, the presence of viral antigens in C6/36 mosquito cell cultures inoculated with supernatant of lung tissue is also another indirect proof of the DENV infection, and confirms that BALB/c mice are a permissive host for DENV replication. The cell and tissue damages observed during the infection were similar using the 100 TCID₅₀ and 10,000 TCID₅₀/0.2ml, but tissue damage to the lung was stronger in the last case.
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REFERENCES


Fig. 1 - Control mouse lung showing a bronchiole (B). Note epithelium (Ep). Around the bronchiole, alveolar spaces (A), alveolar septa (S), alveolar duct (AD) and alveolar sac (AS) were observed. Light microscopy. Bar = 80 mm. Fig. 2 - 72 hours post-infection, 10,000 TCID₅₀. Note the vascular congestion (VC) and swollen alveolar septa (S). Alveolar space (A). Light microscopy. Bar = 40 mm. Fig. 3 - 7 days post-infection, 100 TCID₅₀. Note a bronchiole (B) with hyperplastic epithelium (Ep). Inflammatory cells (IC) in peribronchiolar space (pBS), vascular congestion (VC) and interstitial oedema (IO). Alveolar space (A). Light microscopy. Bar = 40 mm. Fig. 4 - 7 days post-infection, 100 TCID₅₀. Note a bronchiole (B) with hyperplastic epithelium (Ep), presence of inflammatory cells (IC) in peribronchiolar space (pBS), swollen alveolar septa (S) with congestion and erythrocytes (E) in the alveolar space (A). Light microscopy. Bar = 40 mm. Fig. 5 - 14 days post-infection, 100 TCID₅₀. Note a bronchiole (B) with hyperplastic epithelium (Ep), vascular congestion (VC) and swollen alveolar septa (S). Inflammatory cells (IC), Alveolar sac (AS), alveolar space (A). Light microscopy. Bar = 40 mm. Fig. 6 - 49 days post-infection, 100 TCID₅₀. Note alveolar septum (S) with capillary (C). Inside the capillary there are some platelets (P) and erythrocytes (E). Pneumocyte type II (Pll), alveolar space (A). Transmission electron microscopy. Bar = 3.5 mm.
Fig. 7 - 49 days post-infection, 10,000 TCID\textsubscript{50}. Note capillaries (C) with inflammatory cell (IC), platelets (P) and erythrocytes (E) inside. Endothelial cell (EC), alveolar space (A). Transmission electron microscopy. Bar= 3.2mm. Fig. 8 - 72 hours post-infection, 10,000 TCID\textsubscript{50}. Note a bronchiole (B) with hyperplastic epithelium (Ep) and swollen alveolar septa (S). Alveolar space (A). Light microscopy. Bar= 40mm.

Fig. 9 - 7 days post-infection, 100 TCID\textsubscript{50}. Note the diffuse damage of the epithelium of a bronchiole, hyperplasia of the ciliated cell (cc) and Clara cells (Cc). Subadjacent lamina propria muscular cells (MC) are observed. Nuclei (n), mitochondrium (M). Transmission electron microscopy. Bar= 3.2mm. Fig. 10 - Peritoneal macrophage, 100 TCID\textsubscript{50}. Macrophages showed several viral particles (V) inside membrane bound vesicles. Transmission electron microscopy. Bar= 20mm. Fig. 11 - Control of virus in monolayer of C6/36 mosquito cell line, 15 days post-infection. Note syncytial cytopathic effect (Sc). Indirect immunofluorescence technique. Bar= 50mm. Fig. 12 - Dengue virus antigen (Da) detected in C6/36 mosquito cell culture inoculated with supernatant of lung macerate. Indirect immunofluorescence technique. Bar= 50mm.