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Liver injury and viremia in mice infected with dengue-2 virus

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Abstract

The goal of this study was to test the feasibility of BALB/c mice as an experimental model in the study of dengue disease. BALB/c mice were intraperitoneal infected with DENV-2 obtained from a human patient. Histopathological analysis of infected animals revealed liver injury with viral antigens detection. In initial stages, the most prominent lesions were vacuolization and diffuse steatosis in hepatocytes. Serum levels of ALT and AST increased progressively, reaching the highest values 7 days p.i. and decreasing at the 14th day. Since levels of circulating virus were very low, viremia was analyzed in C6/36 cells. Virus presence was detected by ultrastructural analysis, confirmed by RT-PCR assays. Period of viremia was analyzed by flow cytometry with cells incubated with mouse-infected sera collected in different days, revealing peak virus levels at the 7th day p.i. All such data correlate to the development of the disease described in humans.

Keywords: DENV-2; Mouse; Liver; Viremia; Histopathology; Transaminases

Introduction

Dengue is an acute disease caused by the infection of dengue virus (DENV), which consists of four distinct antigenic types (DENV-1 to -4). The disease presents a wide range of clinical symptoms, including a mild self-limiting acute febrile illness (DENV fever) and hemorrhagic fever and/or shock syndrome (DHF/DSS) (Halstead, 1989). The clinical features of DHF include plasma leakage, a tendency to bleed, and liver involvement (Rothman and Ennis, 1999), which can progress to DSS and death (Henchal and Putnak, 1990). Although the liver is not a major target organ, the involvement of the liver in the pathogenesis of dengue virus infection, in particular concerning the development of DHS, was demonstrated by the abnormal liver function, tissular injury, presence of viral antigens, and RNA in human liver tissue (Bhamarapravati and Halstead, 1964; Bhamarapravati et al., 1967; Burke, 1968; Kalayanarooj et al., 1997; Kuo et al., 1992; Miagostovich et al., 1997; Nguyen et al., 1997; Rosen et al., 1989, 1999; Wang et al., 1990).

In general, most experimental studies dealt with suckling or young mice inoculated by the intracerebral route of a mouse-adapted DENV-2 (Nath et al., 1983; Raut et al., 1996). Animal responses to virus infection (clinical signs and/or degree of injury) varies according to mice strain, although these experimental models normally show that mice are susceptible for DENV infection. The full DHF/DSS manifestations, however, do not seem to occur in standard laboratory mice.

Some studies are based on other inoculation routes, such as intraperitoneal and intravenous, which resembles more to the natural infection in human population. Huang et al. (2000a, 2000b) showed that A/J mice inoculated intra-

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venously with a non-adapted DENV-2 presented a transient thrombocytopenia and anti-platelet antibodies. Such alterations were remarkable reduced when BALB/c or B6 mice were used (Huang et al., 2000a, 2000b). In nude mice, petechial and gastrointestinal hemorrhages have been observed (Raut et al., 1996). In the model of infection developed in SCID mice submitted to the transplantation of human hepatocarcinoma cell line (HepG2) foci of tissular hemorrhagy, hemoconcentration and fluid extravasation, thrombocytopenia, and prolonged partial thromboplastin time were achieved (An et al., 1999). This model takes advantage of the fact that HepG2 cells, a human hepatoma cell line, which conserved numerous characteristics of differentiated hepatocytes (Knowles et al., 1984), is permissive for DENV infection and replication. HepG2 cells transplanted into SCID mice are able to restock liver, and after viruses inoculation became the major target for their replication. Infected cells die by an apoptotic mechanism as a direct consequence of the viral infection (Marinneau et al., 1998). Moreover, these animals developed a hind-leg paralysis, liver injury, and renal dysfunction. Viremia in this model peaked at 8-day post-infection (p.i.) but viruses were already seen in 60% of mice at day 5. Viruses were detected in the hepatic tissue, brain, and blood (An et al., 1999).

The present study has been undertaken to characterize a mouse model of DENV-2 infection by intraperitoneal inoculation of a non-adapted virus isolated from a human patient. BALB/c mice developed an apparently mild infection, but with liver injury characterized by histopathological aspects and biochemical tests. Viral antigens were detected in focal areas of the damaged liver, confirming the presence DENV-2 in such tissue. Viremia was analyzed by flow cytometry with C6/36 cells incubated with mouse-infected sera collected in different times, which revealed peak virus levels at the 7th day p.i. in most animals. All such data correlate to what is observed in the human disease and therefore demonstrate that BALB/c mice strain is a susceptible animal to DENV-2 and can be used as an experimental model for the study of the pathogenesis of dengue disease.

Results

Mice infection

All mice inoculated with DENV-2 survived to the infection and did not present clinical signs, although some tissue pathological alterations could be detected. Liver of control mice, inoculated only with culture medium, did not exhibit any modification in their structure (Fig. 1a). However, hepatic injury was seen in all DENV-2-infected mice beginning from the 2nd until the 17th day p.i. (Figs. 1b–j). At the 2nd day p.i., hepatic plates maintained its architecture and were generally constituted by mononucleated hepatocytes, although some of them were binucleated. Some hepatocytes were slightly enlarged due to vacuolization while others were diminished. Hepatocyte vacuolization was observed all over the hepatic acini, but was more prominent in the zone II of the liver acini where cells were sparse. Slight variation in hepatocyte size could be observed (midzonal, Fig. 1b). At the 3rd day p.i., diffuse steatosis were noted in hepatocytes around the central vein area, mainly in sinusoidal side. Numerous hyperplastic Kupffer cells were detected in sinusoids capillary (Fig. 1c) and an increase of monocytes cells was observed in sinusoids (Fig. 1d). At the 7th day p.i., an intense area of edema was evident around the hepatocytes nucleus, causing a progressive increase of necrosis area in focal parenchyma. Sinusoid capillaries presented several monocytes and lymphocytes (Fig. 1e). Diffuse necrose of hepatocytes was also observed surrounding portal areas with increased monocyte infiltration (Fig. 1f). At the 13th day p.i., hepatocytes exhibited changes in their nuclear content and lipid-like nuclear inclusions were occasionally seen in vacuolated cells (Fig. 1g). Rare isolated or grouped inflammatory cells (mononuclear cells) were focally seen inside sinusoids at the 13th day p.i. Inflammation in hepatic lobuli was characterized by the presence of polymorphonuclear cells surrounding tumefacted hepatocytes with an intense cytoplasmic clarification (Fig. 1h). At the 17th day p.i., apoptotic cells were seen at the sinusoidal space as well as necrotic hepatocytes (Fig. 1i). Although at this time point hepatocytes seemed to be regenerated, focal rarefaction of sinusoidal cells and sinusoidal disappearance were occasionally seen at the 17th day p.i. (Fig. 1j).

The semiquantitative analysis demonstrated that in general at the 2nd day p.i., hepatocytes did not change their diameter in relation to control mice ($P > 0.05$). However, at the 13th day p.i., their diameter was significantly increased ($P < 0.05$) (Fig. 2a). Sinusoidal capillaries had a diminished diameter ($P < 0.05$) at the 2nd day p.i. and became significantly enlarged later on ($P < 0.05$) (Fig. 2b). Analysis of sinusoidal cells revealed a significantly decrease of the number such cells in all time points ($P < 0.05$) (Fig. 2c).

Detection of viral antigen in the liver of infected mice

In order to confirm the presence of DENV-2 in liver tissue, immunohistochemistry assays were performed using a monkey polyclonal anti-DENV-2 antibody. At the 2nd day p.i., viral antigens were detected in focal hepatocytes (Fig. 3c) and in the capillar endothelium of the central lobular vein (Fig. 3d). The antigens were also detected in the hepatocytes around the portal space (Fig. 3e). At the 13th day p.i., virus antigens were also detected in focal zones (Fig. 3g), although in a large extension as compared to the 2nd day p.i. Antigens were observed in plates of hepatocytes, in the same areas that exhibiting hepatic injury (Fig.

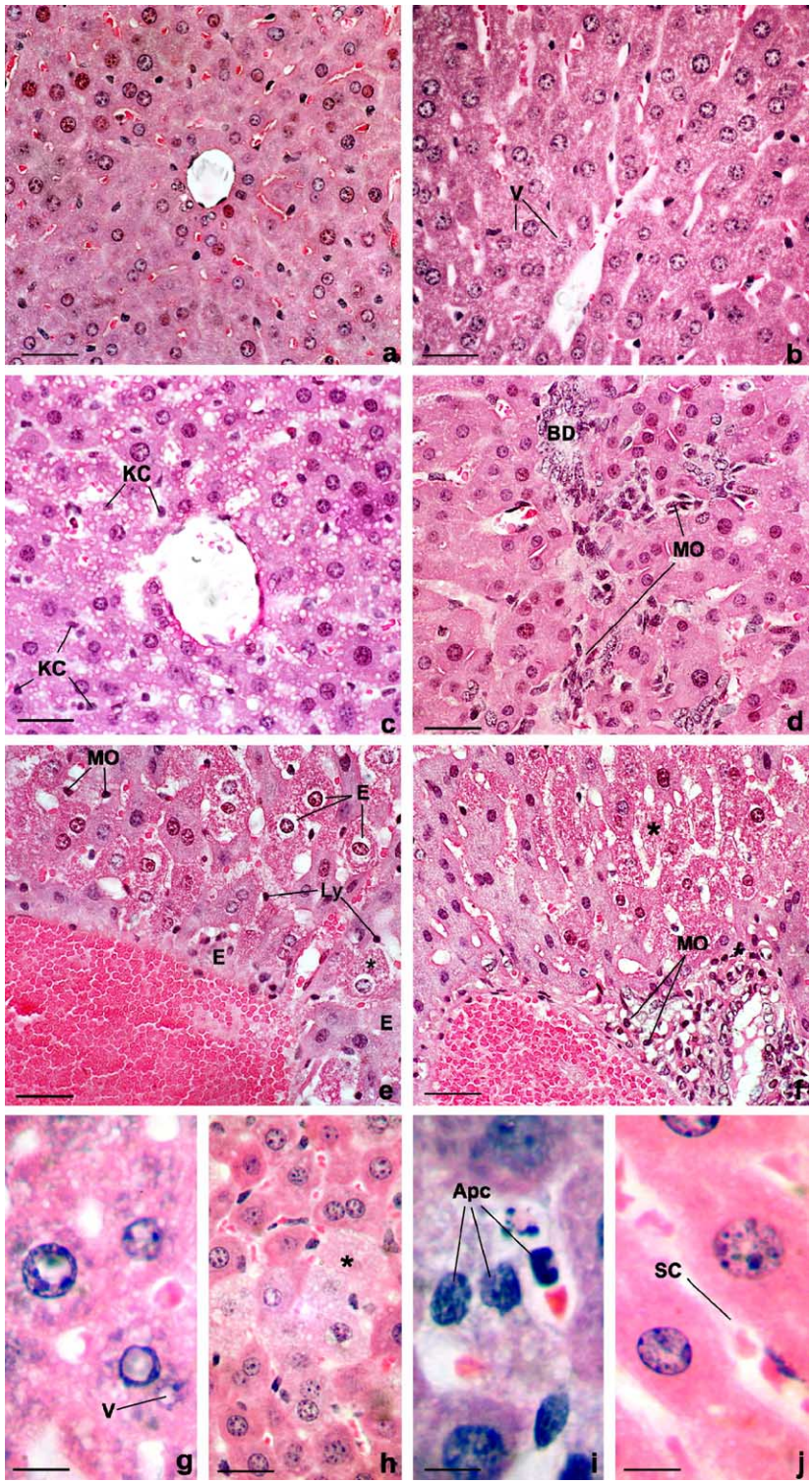


Fig. 1. Histological analysis of liver sections of control and DENV-2-infected mice, HE staining. (a) Liver section of a mouse inoculated with L-15 medium, presenting hepatocytes, sinusoid capillars, and a central vein without alterations (scale bar = 30 μm); (b) liver of a mouse at the 2nd day p.i., showing vacuolization (V) of hepatocytes and dilatation of sinusoid capillars around the central vein (scale bar = 20 μm); (c and d) liver of a mouse at the 3rd day p.i. with diffuse steatosis at the sinusoidal side of hepatocytes and increase of the number of Kupffer cells (KC), observed around the central vein (c), and microvesicular steatosis noted in the portal space and monocyte cells (MO) observed inside the sinusoids (d) (scale bars = 30 μm); (e and f) liver of a mouse at the 7th day p.i. with necrosis of hepatocytes and tissue edema (E) and an increase of the number of monocytes (MO) and lymphocytes (LY) inside the sinusoid capillaries (e), and hepatocyte necrosis (asterisk) with the presence of monocytes (MO) observed in portal areas (f) (scale bars = 30 μm); (g and h) liver section of a mouse at the 13th day p.i. in which lipid-like nuclear inclusions may be observed in vacuolated (V) and tumefacted (asterisk) hepatocytes (scale bar = 8 μm and 15 μm in panels g and h, respectively); (i and j) liver section of a mouse at the 17th day p.i. with apoptotic cells (Apc) occurring inside the sinusoidal capillar space (i) and the disappearance of sinusoidal cells (SC) (j) (scale bars = 6 μm).

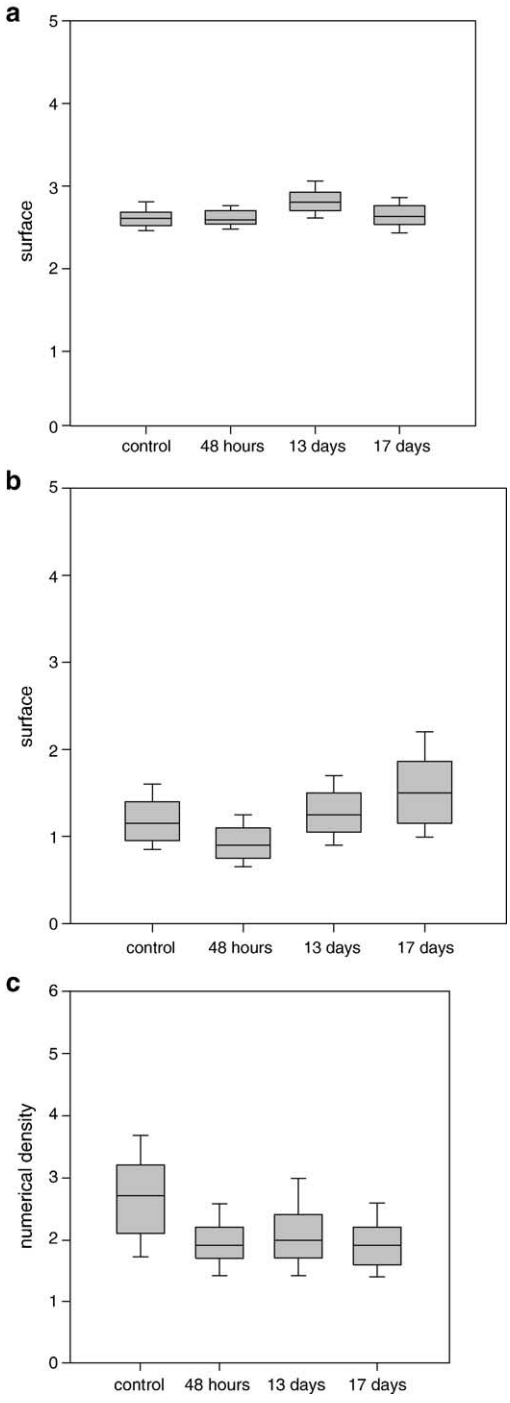


Fig. 2. Semiquantitative analysis of hepatocyte (a), sinusoidal capillars (b), and sinusoidal cells (c). (a) Hepatocytes in liver injury did not change their diameters in relation to control mice ($P > 0.05$), at the 2nd day p.i., while their diameters were significantly increased ($P < 0.05$) at the 13th day p.i.; (b) sinusoidal capillar had a diminished diameter ($P < 0.05$) at the 2nd day p.i. and became enlarged significantly ($P < 0.05$) in late infection; (c) the number of sinusoidal cells diminished at the 2nd day p.i. and increase in late infection ($P < 0.05$).

3g). As expected, negative control, constituted of liver of non-infected mice reacting with anti-DENV-2 antibodies, did not present any virus antigen (Fig. 3a). Furthermore, livers of infected mice at the 2nd and 13th days p.i. also did not present any reaction with the anti-human IgG conjugate (Figs. 3b and f, respectively).

Transaminase level quantifications in serum samples of DENV-2-infected mice

In order to investigate whether the hepatic injury detected in infected mice correlates to alterations in transaminase levels, semiquantitative analyses of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were performed. In general, at the 7th and 14th days p.i., levels of ALT and AST increased in most of the tested serum samples as compared to control sera (Fig. 4). Apparently, both transaminases presented peak level at the 7th day p.i. The statistical analysis of the data, however, showed no significance with the $P = 0.051$ at the 7th day p.i. The lack of significance was probably due to a considerable inter-individual variation.

Detection of DENV-2 in the C6/36 cell line inoculated with sera from infected BALB/c mice

Since levels of circulating virus in infected mice were very low (data not shown), for further analysis, C6/36 cells were used in order to amplify the amount of DENV-2 particles obtained from serum samples collected from these animals. Cells inoculated with mouse sera from the 2nd and the 11th days p.i., examined 15 days after incubation, presented viral particles inside the cisternae of the rough endoplasmic reticulum (Figs. 5c and d, respectively). Vacuolization was also observed in these cells, similar to those detected in positive control cells, infected with DENV-2 (Fig. 5b).

Detection of DENV-2 viral genome by RT-PCR amplification of the NS1 gene sequence

One DNA fragment of 1.1 kb, corresponding to the NS1 gene sequence, was detected in extracts of cells inoculated with mouse sera collected at the 2nd and the 11th days p.i. No amplification was observed in control samples cultivated with non-inoculated mouse sera (Fig. 6). These results confirmed the presence of DENV-2 in serum samples of infected mice and indicated that such virus is still able to infect other cells.

Detection of the period of viremia in serum of BALB/c mice infected with DENV-2 and inoculated in C6/36 cells (flow cytometry)

In an attempt to quantify the degree of infection in the present mouse model, flow cytometry techniques were applied. A DENV-2 hyperimmune mouse ascitic fluid was used for the quantification of the virus in the cultures treated with sera of infected animals. The serum was purchased in three different time periods after infection (3, 7, and 14

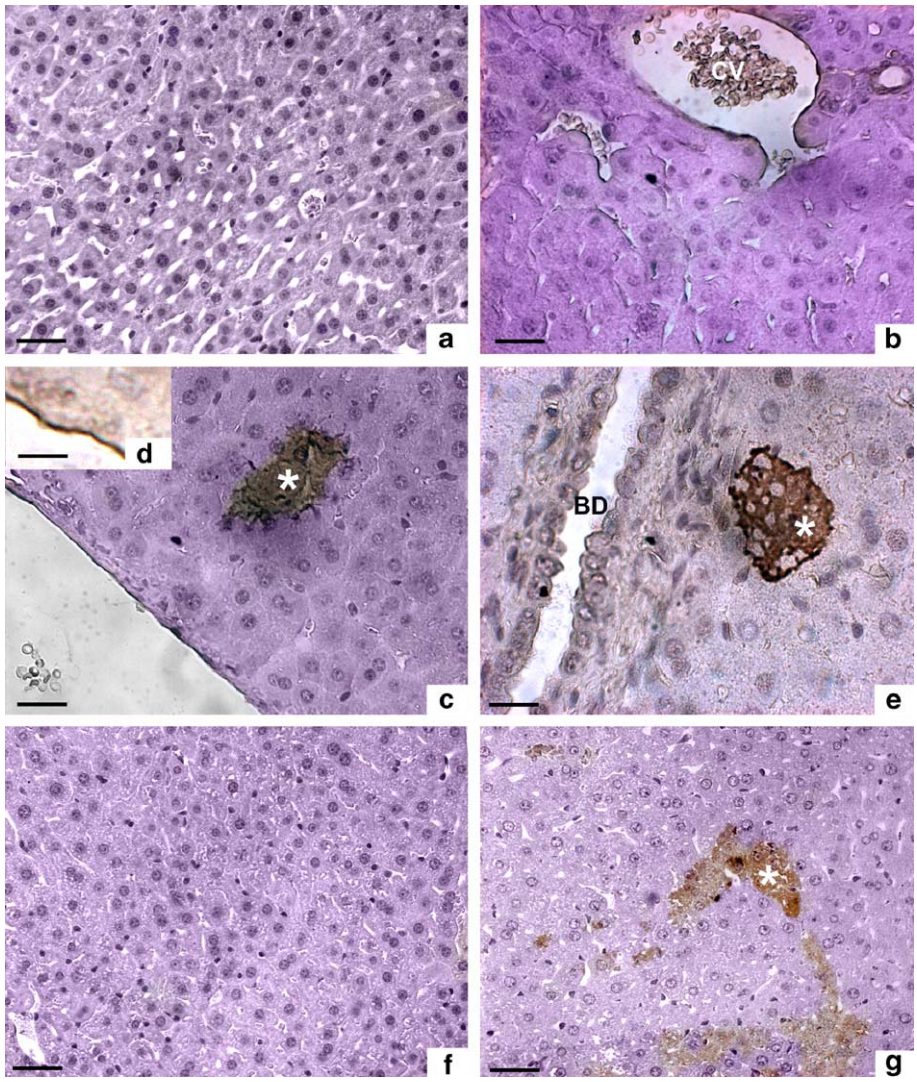


Fig. 3. Detection of DENV-2 antigens in liver of infected mice. (a) Negative control obtained with liver of a non-infected mouse (scale bar = 10 μm); (b) liver of an infected mouse at the 2nd day p.i., incubated only with anti-human IgG-horseradish peroxidase conjugate, central vein (scale bar = 10 μm); (c–e) liver of an infected mouse at the 2nd day p.i., incubated with anti-DENV-2 antibodies and anti-human IgG-horseradish peroxidase conjugate. Note the positive reaction in hepatocytes in focal midzonal (c) and portal space (e) areas (asterisk) (scale bar = 5 μm), and in endothelium capillar (d) (scale bar = 1 μm), biliar duct (BD); (f and g) liver of an infected mouse at the 13th day p.i., incubated only with the secondary antibody (f) or with primary and secondary antibodies (g) (scale bars = 10 μm). Note the positive reaction in several hepatocytes (asterisk).

days). Fig. 7a shows original histograms of one sample at the three different time periods illustrating the clear reaction of this antibody. Anti-DENV-2 was significantly positive in cells incubated with serum collected 7 and 14 days after infection, where a clear shift to the right side of the graph was observed. The peak of viremia was detected using the flow cytometer, revealing a ten-fold increase in the percentage of positive cells for the anti-DENV-2 antibodies 7 days after infection, followed by a subsequent decline of viremia at day 14th p.i. (Figs. 7a and b). A considerable individual variation, however, was determined. Six out of the 8 animals studied were positive to anti-DENV-2 (Figs. 7b and c). Four positive animals achieved the peak of viremia at day the 7th day p.i., whereas two animals behaved differently, one reaching its highest level of infection at the 14th day p.i. and the other at the 3rd day p.i. (Figs. 7b and c).

Discussion

Dengue is an intriguing disease and the mechanisms involved in the pathogenesis of DHF/DSS are yet poorly understood. In an attempt to understand the disease and/or to test new drugs or vaccines, several studies suggested the use of murine models (Atrasheuskaya et al., 2003; Huang et al., 2000a, 2000b; Shresta et al., 2004), although none of them reproduce the exactly symptoms observed in humans. In the present report, we evaluated the use of BALB/c as an experimental mouse model for DENV-2 infection. A

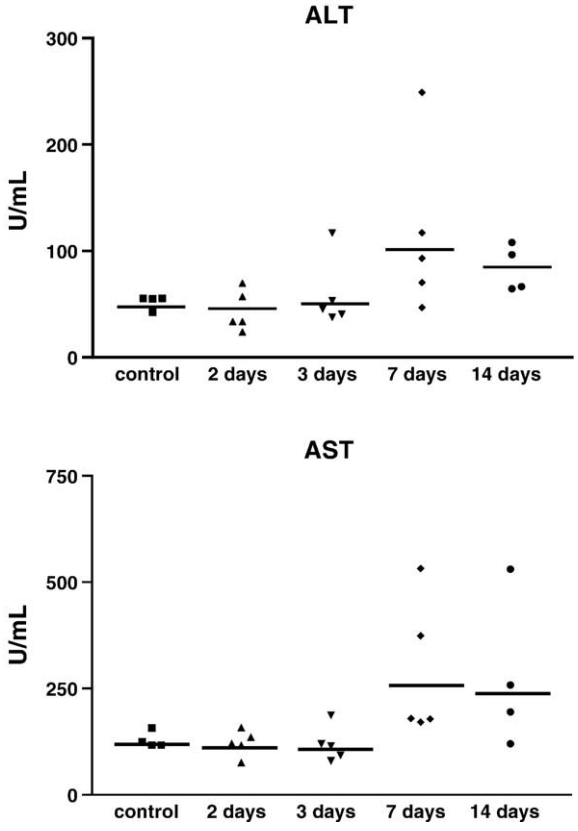


Fig. 4. This serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice experimentally infected with DENV-2, at the 2nd, 3rd, 7th, and 14th days p.i.

DENV-2 isolated from a serum sample obtained from one patient, followed by cultivation in mosquito cells, was directly inoculated in mice by the intraperitoneal route. Animals did not present clinical signs of the infection, significant hepatic alterations were observed. Histopathological analysis revealed focal injury in lobular parenchyma in hepatocytes. These results correlate to what is observed in humans, in which liver seems to be one target organ. The biology and clinical aspects of hepatitis is a well-known feature of human dengue infection (Innis, 1995; Rosen et al., 1989), although DENV can lead to a mild degree of hepatic involvement. However, it has been reported in recent years a severe involvement of the liver, including cases of fulminant hepatitis with high mortality (Lum et al., 1993).

The hepatic injury observed in the present mouse model began 2 days p.i., and at the 3rd day p.i. hepatocytes presented diffuse steatosis in midzonal areas. Necrosis of hepatocytes and a strong flux of edema was observed at 7 days p.i. The descriptions of liver histopathology in humans infected with DENV is yet scarce. The study of a dengue fatal case in human also showed diffuse hepatitis with midzonal necrosis and steatosis recruitment of inflammatory cells into hepatic parenchyma (Huerre et al., 2001). The data reported in this study correlated with our results. The observed hepatic injury was similar to that of early stages of yellow fever infection. An increase of plasma was reported when infection occurred through the intraperitoneal route. Simultaneously an increase in the transaminase levels, fatty changes in hepatocytes, Kupffer cell hyperplasia, and centrilobular and midzonal necrosis was observed (Innis, 1995; Kuo et al., 1992). The inflammatory infiltrate presented monocytes and lymphocytes in sinusoids of focal areas of the portal space. In accordance to this observations, in our model, mice infected with DENV-2 also presented numerous hyperplastic Kupffer and monocytes cells into sinusoids capillar and portal space, respectively. Some reports have shown that endothelial and Kupffer cells were susceptible for DENV replication (Hall et al., 1991; Innis, 1995) suffering, therefore, viral cytopathic effects, which may explain the hyperplasia observed. In fact, in the present work, viral antigens were detected in capillar endothelium of the central lobular vein confirming its susceptibility to virus replication, as well as in several hepatocytes. However, such antigens were not verified in Kupffer cells. In humans, antigens of DENV-2 have been detected in liver tissue, either in Kupffer's cells or in hepatocytes, spleen, lymph nodes, pulmonary alveolae, blood mononuclear cells, peripheral B cells, and thymic cortex (Couvelard et al., 1999; Hall et al., 1991; Huerre et al., 2001; Innis, 1995; Kuo et al., 1992; Monath, 1986; Nguyen et al., 1997; Rosen et al., 1999; Scott et al., 1980; Waterman et al., 1985). Further studies will be necessary in order to analyze or evaluate the extension of injury in other organs in mice, with the presence of viral antigens.

Apoptosis is a major feature of viral hepatitis (Galle et al., 1995) and also participates of the DENV pathological spectrum. In our work we could note the presence of apoptotic cells, mainly at the sinusoidal space, in later stage of infection, at the 17th day p.i. This apoptosis is probably related to focal rarefaction of sinusoidal cells and sinusoidal endothelial. In vitro, various cell lines susceptible for DENV, such as HepG2 and a mouse neuroblastoma cell line (Neuro2a), exhibited apoptotic cell death when infected with DENV (Desprès et al., 1996; Marianneau et al., 1997). In humans, apoptosis could also occur in endothelial and Kupffer cells, therefore leading to the focal sinusoidal denudation demonstrated herein. Probably the sinusoidal denudation seen in DENV-2 infection may cause the microvascular barrier derangement and may be pivotal in the development of parenchymal damage, contributing to the microvascular disruption caused by cytokines secreted by activated cells (Dhawan et al., 1990; Khanna et al., 1990).

The degree of hepatocytic viral injury induced in DENV infection could also be observed in the transient liver dysfunction depicted by enzyme levels. Serum levels of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) can be significantly higher in humans infected with any of the DENV serotypes. In general, DF is usually associated with mild to moderate elevations of such enzymes, while in patients with DHF/DSS ALT and AST

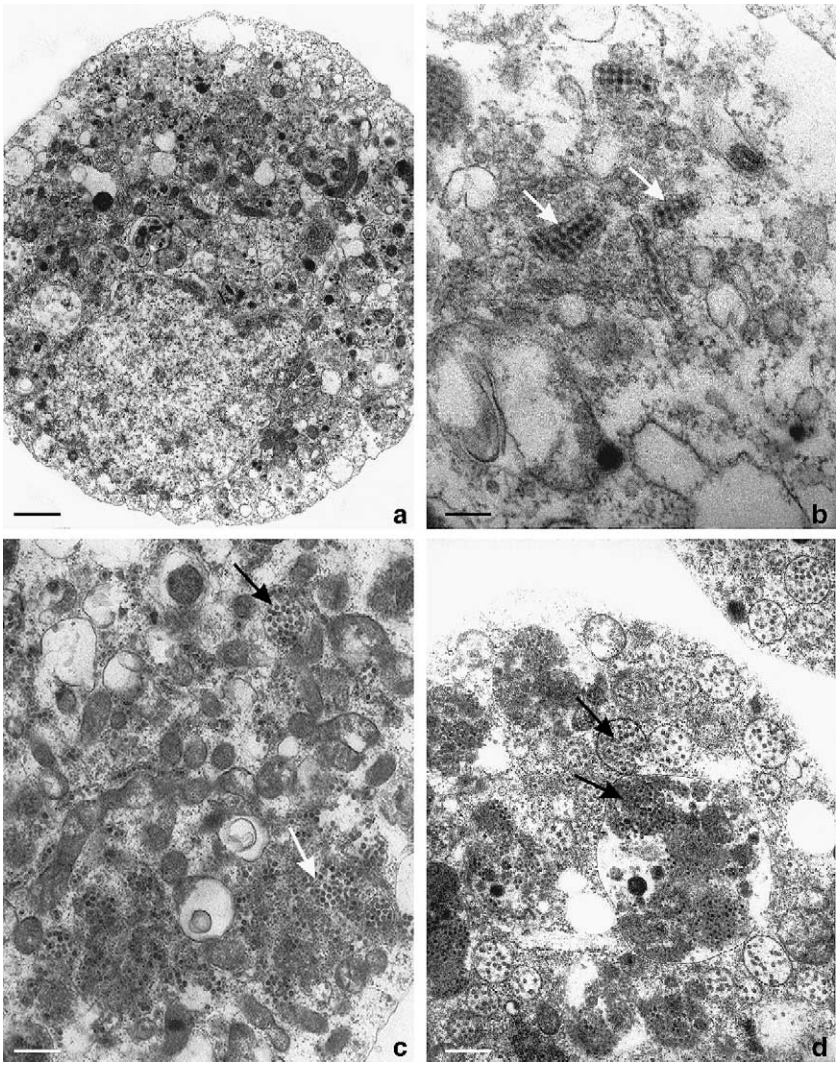


Fig. 5. Electron microscopy of C6/36 cells incubated with sera from DENV-2-infected mice. (a) Negative control of C6/36 cell (scale bar = 1 μm); (b) positive control of C6/36 cell infected with DENV-2. Note the virus particles (arrow) (scale bar = 0.2 μm); (c and d) C6/36 culture cells inoculated with sera from mice collected at the 2nd (c) and 11th days p.i. (d). Note the virus particles inside vesicles and vacuoles (arrows) (scale bar = 0.5 μm).

levels became remarkable higher (Kuo et al., 1992; Souza et al., 2004). Peak levels of the transaminases normally occurred at the 7th day p.i. and decreased in the following 2 weeks. Similar results were observed in the experimental mouse model here presented, in which levels of both hepatic transaminases (ALT and AST) increased in most of the tested serum samples, with peak levels at the 7th day p.i. Moreover, AST levels in mice were higher than ALT, which is also verified in humans (Nguyen et al., 1997).

Another parameter for evaluation of DENV infection is the viremia, which correlates to transaminase levels and other hepatic damage. In most experimental mouse models, viremia could not be characterized, probably due to the fact that circulating DENV is found in a very low level in such animals, which stands below the sensibility assays for normal virus titrage and/or isolation (Bhamarapravati and Halstead, 1964; Boonpucknavig et al., 1981; Nath et al., 1983; Shresta et al., 2004). In order to amplify the amount of DENV-2 particles obtained from mouse sera, we inoculated C6/36 cells with serum samples collected in different time points during the animal infection. Viral particles could be detected inside those cells by ultrastructure analysis and its presence was confirmed by PCR assays. Such results indicated that in fact DENV-2 particles were circulating in blood samples of infected mice and that such virus maintains its capacity to replicate in different cells.

In an attempt to detect the period of viremia in these animals, C6/36 cells were incubated with mouse serum samples purchased in different times and analyzed by flow cytometry. Peak of viremia was observed at the 7th day p.i. in most animals followed by a subsequent decline at the 14th day p.i. In humans, the peak of viremia is also verified around the 7th day p.i. with a decrease after 1 week (Kuo et al., 1992; Mohan et al., 2000). Huang et al. (2000a, 2000b) also detected viremia in blood samples of A/J mice by RT-PCR but not in BALB/c mice. However, virus could only be detected 2 days after inoculation by the intravenous route

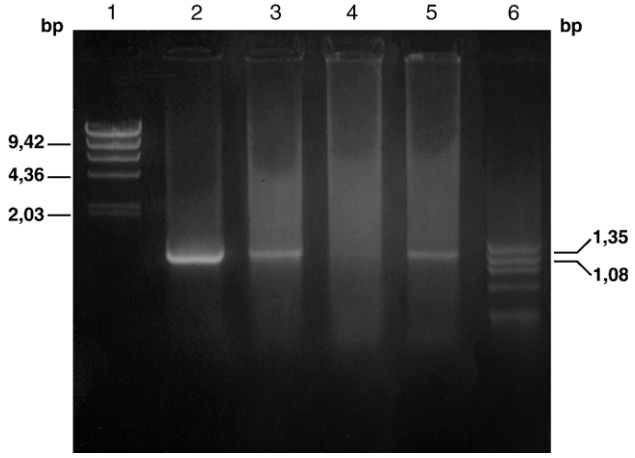


Fig. 6. The DENV-2 NS1 gene amplified by PCR and electrophoresed in 1% agarose gel stained with ethidium bromide. Serum samples collected from mice infected with DENV-2 were added to C6/36 cells and PCR reactions were performed using cDNAs made from extracts of such cells. Lanes: (1) standard marker of λ DNA digested with *Hin*dIII; (2) extract of cells cultivated with mouse sera collected 2 days following virus infection; (3) extract of cells cultivated with mouse sera collected 11 days following virus infection; (4) negative control with extract of cells cultivated with non-infected mouse sera; (5) positive control with extract of cells infected with DENV-2; and (6) standard marker of $\phi \times 174$ DNA digested with *Hae*III.

and it disappeared in the following days. Similar results were described by Atrasheuskaya et al. (2003), using young BALB/c mice inoculated with DENV-2 by the intraperitoneal route, in which viremia was detected by PCR after the 2nd day p.i. These experiments, nevertheless, were conducted with a previous mouse brain adapted. In our work, assays were performed with a DENV-2 isolated from a patient serum, cultivated in mosquito cells, and directly infected in mice by the intraperitoneal route.

In our mouse model we found: hepatic alterations, including liver histopathological injury with the presence of viral antigens, elevated levels of transaminases, mainly at the 7th day p.i., and a correlation between the peak of viremia, and ALT and AST levels. Our results revealed the ability of a DENV-2 circulating in a human population to infect BALB/c mice and are therefore a relevant physiological approach. Moreover, in conclusion, they demonstrated that this mouse model is feasible for the study of DENV disease. Furthermore, studies of experimental drugs or vaccines might be performed using the BALB/c mice.

Material and methods

Virus

DENV-2 was isolated from a patient serum during an outbreak of the virus in the state of Rio de Janeiro, in 1995 (Nogueira et al., 1995), kindly provided by the Flaviviruses Laboratory of the Department of Virology, Fiocruz, RJ. Virus had not undergone any passage in mouse brain. It was propagated in *Aedes albopictus* mosquito cell line monolayers (C6/36), in 10 ml tubes, with L-15 medium (Sigma, USA) supplemented with 1% non-essential amino acids, 10% tryptose phosphate broth and 10% fetal bovine serum, and maintained at 28 °C for 15 days.

Animals and experimental protocol

All experiments with mice were conducted in compliance with Ethical Principles in Animal Experimentation stated in the Brazilian College of Animal Experimentation and approved by the Institute's Animal Use Ethical Committee. Adult male BALB/c mice (age of 2 months), weighing 25 g, obtained from the mouse colony maintained in the Department of Virology of the Instituto Oswaldo Cruz, were submitted to an intraperitoneal inoculation with DENV-2 (10^4 $TCID_{50}$/0.2 ml). For histopathological analysis and biochemical tests, mice, anesthesiated with 4% chloral hydrate (0.4 ml/25 g of animal), were sacrificed by total cardiac puncture, while for viremia detections blood samples were collected several times by partial cardiac puncture. Control animals consisted of non-infected or L-15 medium inoculated mice, sacrificed as described above.

Histological study of liver (light microscopy)

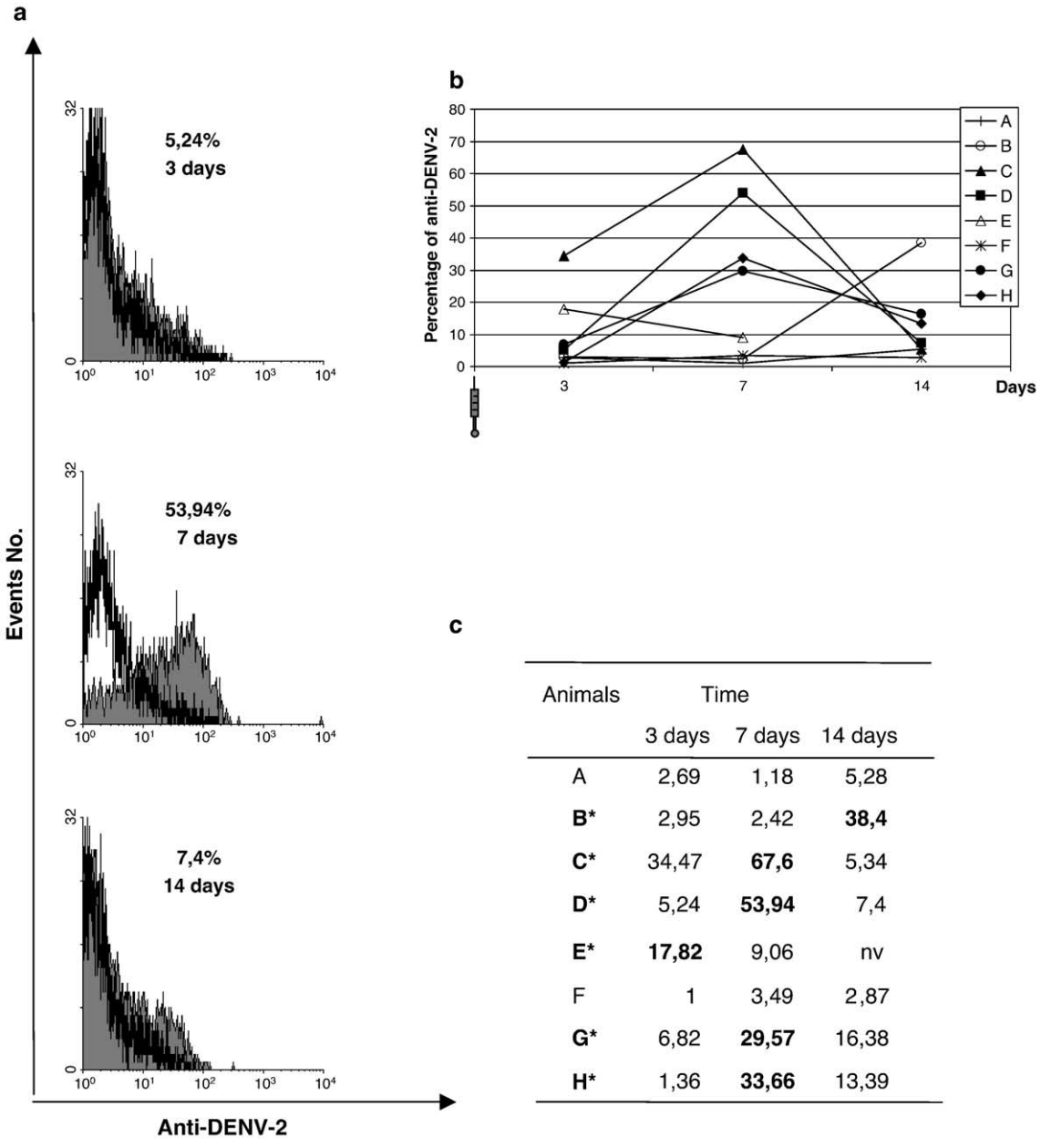
Five DENV-2 inoculated animals were sacrificed at the 2nd, 3rd, 7th, 13th, and 17th days p.i. Liver slices were fixed in Millonig's fixative, dehydrated in ethanol, and paraffin embedded. Sections (5 μm thickness) were stained with hematoxylin and eosin (HE).

Morphometrical analysis

Hepatocyte, sinusoidal density surface, and sinusoidal cells numerical density were obtained in HE stained slides, from animals at the 2nd, 13th, and 17th days p.i. A hundred and thirty images of liver parenchyma avoiding portal spaces were obtained in a NIKON Eclipse 104 light microscope (40× objective camera) and digitalized by a NIKON Coolpix 990 digital camera. From each images, diameters of 20 hepatocytes and 10 sinusoidal spaces, as well as sinusoidal cells counting, were achieved using a public software Scion (ScionCorp, USA). Data were submitted to statistical analysis using *t* test or Mann–Whitney rank sum test considering significance at $P < 0.05$.

Immunoperoxidase technique for the detection of viral antigen in mice liver

Paraffin-embedded sections of the liver of infected and control animals were deparaffinized with three washes in



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Animals	Time		
	3 days	7 days	14 days
A	2,69	1,18	5,28
B*	2,95	2,42	**38,4**
C*	34,47	**67,6**	5,34
D*	5,24	**53,94**	7,4
E*	**17,82**	9,06	nv
F	1	3,49	2,87
G*	6,82	**29,57**	16,38
H*	1,36	**33,66**	13,39

Fig. 7. FACs analysis of C6/36 cells inoculated with sera collected from DENV-2-infected mice at different time points. (a) Original FACs histograms showing the time-dependent positivity for the anti-DENV-2 antibody in C6/36 cells treated with sera of one infected animal. Anti-DENV-2 = gray color/histogram, 2nd antibody = transparent overlaid histogram. Percentage of dengue virus and time of the analysis are shown at the right top of each histogram. (b) Graph showing the kinetic of infection for all animals studied as percentage of anti-DENV-2-positive cells. Each line represents one animal and each point represents the day of analysis. Day 1 of infection = . (c) Table containing the values for anti-DENV-2-positive cells for all infected animals in all studied days. Asterisk/bold* letters showing that in the majority of the animals studied the infection was detected using this experimental model (nv = no value).

xylol and serial washes in 100%, 90%, and 70% ethanol followed by a final wash in PBS. For detection of DENV antigens, samples of liver from 2 and 13 days p.i. were incubated over night at 4 °C with an anti-DENV-2 serum raised in monkey (gently provided by Dr. R. Galler, Laboratory of Molecular Biology of Flavivirus, Department of Biochemistry and Molecular Biology, Fiocruz, RJ). After washing with Tris–HCl (0.05 M pH 7.6), the sections were further incubated with rabbit anti-human IgG-horseradish peroxidase conjugate (Sigma) for 30 min at 37 °C. The slices were revelated with DAB (Sigma) and were counter-stained with Mayer's hematoxylin. Controls were stained with the secondary antibody only.

Biochemical analysis of serum hepatic enzyme

Five infected mice were bleed at the 2nd, 3rd, 7th, until the 14th days p.i. and serum samples were obtained after centrifugation at 400 rpm for 5 min and stored at −70 °C. Levels of alanine aminotransferase (ALT) and aspartate

aminotransferase (AST) were detected by the UV optimized (IFCC) method (Karmen, 1955), using commercial kits (Clm, Brazil).

Statistical analysis

Experimental results on biochemical tests were analyzed for their statistical significance by *t* Student and Kruskal–Wallis test $P = 0.051$.

Detection by transmission electron microscopy of DENV-2 in C6/36 cell line inoculated with sera from infected BALB/c mice

Serum samples (100 μl) collected from DENV-2-infected mice at the 2nd and 11th days p.i. were added to monolayers of C6/36 cells, grown as described above. Cells were daily observed for viral cytopathic effects for 15 days. Positive and negative controls consisted of DENV-2 (10^2 $TCID_{50}$/0.1 ml) infected and non-infected C6/36 monolayers, respectively. Cells were fixed in 1% buffered glutaraldehyde, dehydrated and embedded in epoxy resin for electron microscopy observations. Ultra-thin sections of 50–70 nm thickness were obtained using a diamond knife, double stained with uranyl acetate and lead citrate, and observed in a Zeiss EM-900 transmission electron microscope.

Detection of the NS1 DENV-2 gene by PCR amplification in C6/36 cell line inoculated with sera from infected BALB/c mice

C6/36 cells were used in order to increase virus titer. The cells were incubated with mice sera collected at the 2nd and 11th days p.i., as described above, and were used for the detection of the DENV-2 non-structural protein 1 (NS1). PCR reactions were performed using cDNAs made from extracts of such cells, and total RNAs were then extracted with Trizol (Invitrogen, USA), according to the manufacturer protocol. The RNA was used as template for the synthesis of a cDNA, carried out with the oligonucleotide antisense primer 5′-CAT AAG CTT ACA GAG GTT CCC CCA TG-3′, which hybridize between nucleotides 1422 and 1438 in the NS3 gene. The cDNA was then used for amplification of the NS1 gene sequence by Nested PCR. The first PCR reaction was made with two oligonucleotide primers (sense 5′-G GGG GAT ATC ATG CTG TCT GTG TCA CTA G-3′ and antisense 5′-G GGG CTC GAG TTA CCC TGT GAT CAA TG-3′), which anneals between nucleotides 1425 and 1441 and between nucleotides 125 and 141 in the E and NS2A protein genes, respectively. The oligonucleotide primers sense 5′-GGG GGA TAT CGA TAG TGG TTG CGT TG-3′ and antisense 5′-GGG GCT CGA GTT AGG CTG TGA CCA AG-3′ were then used for the amplification of the NS1 gene sequence in the second PCR reaction was. The two PCR reactions were programmed as follows: 94 °C for 2 min, 40 cycles of 92 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and an extension step at 72 °C for 5 min at the end of the cycle. The amplified products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and photographed with Polaroid film.

Viremia detection by flow cytometry

Eight BALB/c mice were inoculated with DENV-2 as described above and serum samples of each animal were collected at the 3rd, 7th, and 14th days post-infection and stored at −70 °C. C6/36 cells were incubated with the serum and maintained for 10 days at 28 °C. Infected and non-infected cells were analyzed by a FACScalibur (Becton Dickinson). Performance control of the flow cytometer was accomplished on a regular basis using Calibrite Bead (Becton Dickinson). All analyses were performed with intracellular labeling techniques using a PBS/Saponin solution (0,05%) with preceding cell fixation (fixing reagents Paraformaldehyde 1%). Primary antibody used was a DENV-2 hyperimmune mouse ascitic fluid (ATCC, USA) and the second antibody was a fluorescein-conjugated goat anti-mouse IgG (Southern Biotechnology, USA). The standard incubation time with the first antibody was 1 h at 4 °C and samples underwent a supplementary 30-min incubation step with the second antibody.

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