J. Neurovirol. (2012) 18:374–387
DOI 10.1007/s13365-012-0117-y

In vivo infection by a neuroinvasive neurovirulent dengue virus

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Received: 29 March 2012 / Revised: 30 May 2012 / Accepted: 7 June 2012 / Published online: 24 July 2012

Abstract Although neurological manifestations associated with dengue infections have been reported in endemic countries, the viral or host characteristics determining the infection or alteration of nervous function have not been described. In order to investigate neurobiological conditions related to central nervous system dengue virus (DENV) infection, we established a mouse model of neuroinfection. A DENV-4 isolate was first adapted to neuroblastoma cells, later inoculated in suckling mice brain, and finally, this D4MB-6 viral variant was inoculated intraperitoneally in Balb/c mice at different postnatal days (pnd). Virus-induced fatal encephalitis in 2 and 7 pnd mice but infected at 14 and 21 pnd mice survived. The younger mice presented encephalitis at the sixth day postinfection with limb paralysis and postural instability concomitant with efficient viral replication in brain. In this mice model, we found activated microglial cells positive to viral antigen. Neurons, oligodendrocytes, and endothelial cells were also infected by the D4MB-6 virus in neonatal mice, which showed generalized and local plasma leakage with blood–brain barrier (BBB) severe damage. These results suggest that there was a viral fitness change which led to neuroinfection only in immune or neurological immature mice. Infection of neurons, endothelial, and microglial cells may be related to detrimental function or architecture found in susceptible mice. This experimental neuroinfection model could help to have a better understanding of neurological manifestations occurring during severe cases of dengue infection.

Keywords Dengue virus · Encephalitis · Neuroinfection · Neurosusceptibility · Neurovirulence

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Introduction

Dengue virus (DENV), of the genus *Flavivirus*, consists of a positive-sense, single-stranded RNA molecule that encodes three structural proteins (C, prM/M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) that are involved in the assembly and replication of the virus, respectively (Lindenbach and Rice 2001a, 2001b). There are four serotypes (DENV-1 to DENV-4) that belong to the serocomplex of dengue, which has become an emergent disease with one of the largest impacts on human health (Halstead 2002).

Clinical symptoms of DENV disease are wide and development and severity of the disease are associated with viral and host factors (Kyle and Harris 2008). Clinical symptoms in patients include high fever, cephalea (moderate to intense), retro-orbital pain, myalgia and arthralgia, skin lesions, and spontaneous bleeding (Lindenbach and Rice 2001a). In addition, between 5 and 7 % of patients exhibit encephalopathy or encephalitis, suggesting that the virus causes some kind of direct or indirect damage in the nervous system (Lum et al. 1996; Gulati and Maheswari 2007; Malavige et al. 2007). These neurological manifestations include encephalitis, encephalomyelitis, transverse myelitis,

flaccid paralysis, Guillian–Barré syndrome, and behavioral disorders that could be associated with nervous tissue infection and/or exacerbated immune response established during the infection (Chien et al. 2008; Domingues et al. 2008; Kumar et al. 2007).

There are reports of experimental DENV infections in different cell types, including human and murine neuronal lines (Imbert et al. 1994; Ramos et al. 1998), suggesting that changes in the tropism of the virus are associated with mutations, particularly in the E protein. For example, the $Glu_{126}Lys$ or $Asp_{390}His$, and $Phe_{402}Leu$ mutations in the E protein of the DENV-1, DENV-2, and DENV-4 serotypes, respectively, change the viral tropism, providing viruses with neurotropic and neurovirulent phenotypes (Kawano et al. 1993; Sánchez and Ruiz 1996; Duarte dos Santos et al. 2000; Chen et al. 2003).

In addition, when using animal models, it has been demonstrated that neuroadapted DENV induces neurological changes associated with leukocyte infiltration and neuronal death, among other morbidities (Desprès et al. 1996; 1998; Sánchez-Burgos et al. 2004; Bordignon et al. 2007). However, DENV in vivo infection models have some limitations; virus-induced disease signs are not completely replicable in experimental animals except when using genetically modified, immunocompromised animals or when the virus is adapted via successive passages in cells and tissues (Cole and Wisseman 1969; McMinn 1997; Yauch and Shresta 2008). Despite all of this, the development and establishment of infection models that reproduce the changes induced by DENV are of scientific interest, given the large impact of dengue disease in the world and because in vivo models could facilitate an understanding of the pathogenic framework of this disease or be useful tools in vaccine and antiviral development.

Although the infection and nervous tissue alteration induced by DENV are still considered an atypical pathology, currently it has an increasing frequency. According to the last WHO classification, neurological manifestations are considered a warning sign associated with disease severity (WHO 2009). Therefore, the development of new infection models describing the physiological processes associated with neuroinfection and neuropathology caused by DENV are required. The objective of this study was to establish a neuroinfection model using a neuroadapted DENV-4 strain that, when inoculated intraperitoneally into immunocompetent BALB/c mice of different ages, induced infection and efficient replication in the nervous tissues of the youngest animals and the activation of an immune response that controlled the infection in adult animals. The obtained neuroadapted virus could infect neurons and microglial cells and, to a lesser extent, endothelium and oligodendrocytes. In addition, the infection caused astrocytosis, changes in tissue architecture, alteration of vascular permeability, and leukocyte infiltration in the brains of susceptible animals.

The infection model described here suggests that DENV infection is associated with the physiological development of individual mice, which are more vulnerable when they are between 2 and 7 days old; older mice challenged with neuroadapted DENV were resistant. In addition, the neuroadapted virus, named D4MB-6, retains important characteristics, such as the capacity to change vascular and blood–brain barrier (BBB) permeability and to induce hemorrhagic events that, along with the infection of other neurons and non-neuronal cell populations, could help explain the observed neurological changes in susceptible animals.

Materials and methods

Handling and procedures applied to experimental animals in this study were previously approved by the Ethics' Committee of Universidad El Bosque (Bogotá, Colombia), which considered both global rules and Colombian norms regulating animal handling.

Cell and virus cultures

C6/36 HT mosquito cells were cultivated at 33 °C, and SH-SY5Y neuroblastoma cells (between passages 2 and 5) and LLC-MK2 cells were kept at 37 °C in DMEM supplemented with 10 % fetal bovine serum (FBS) and penicillin (100 IU/ml) and streptomycin (100 μg/ml). After reaching 80 % confluence, C6/36 HT cells were infected with a Colombian isolate DENV-4 virus. On the sixth day postinfection (dpi), cells were lysed, and the suspension was harvested and titrated using direct plaque assay; this inoculum was named Parental. For virus titration by direct plaque assay, 1×10^5 LLC-MK2 cells were seeded in 24-well plates. Cells were then inoculated with serial dilutions of each of the viral variants or homogenized tissue extracts. After 2-h incubation at 37 °C, the overlay medium (carboxymethylcellulose 1.5 %) was added; at 7 dpi, layers were fixed with 4 % paraformaldehyde (PFA) and were contrasted with crystal violet solution. Plaques were counted, and viral titer was reported as plaque-forming units per milliliter (PFU/ml).

Production of neuroadapted D4MB-6

The Parental virus was inoculated on SH-SY5Y human neuroblastoma cells; at 3 dpi, cells were lysed, and the suspension was harvested and titrated by plaque assay. This viral variant was adapted successively until obtaining a third passage of in vitro adaptation, which was named D4SH-3 (third passage of DENV-4 in SH-SY5Y cells) and was inoculated intracerebrally in suckling BALB/c mice. At 6 dpi, animals were sacrificed via ketamine (90 mg/kg) and xylazine (15 mg/kg) overdoses. Brains were extracted and

homogenized at 10 % in DMEM supplemented with 2 % FBS and stored at −70 °C; this variant was named D4MB-1 (first passage of DENV-4 in mouse brain). Five successive additional passages of in vivo adaptation were performed until obtaining a sixth passage variant called D4MB-6 (Fig. 1).

Viral binding assay and heparin inhibition

SH-SY5Y cells at 2×10^4 by well were cultivated for 24 h and inoculated with the virus variants Parental, D4SH-3, D4MB-1, or D4MB-6 at a multiplicity of infection (MOI) of 1 (MOI 1) at 4 °C for 30 min and then at 37 °C for 15, 30, or 60 min. After completion of the binding time, cells were washed with acid glycine, pH 3.0 to inactivate extracellular virus, and cells were then dissociated with 0.25 % trypsin and 0.5 mM EDTA; 5,000 of these cells were reseeded over a previously cultivated monolayer of LLC-MK2 cells. After 2 h of incubation, the medium was retired, and overlay medium was added; at 3 dpi, the monolayer of LLC-MK2 cells was processed with immunostaining by peroxidase to determine the number of focus-forming units (FFUs) (indirect plaque assay) (Chingsuwanrote et al. 2004).

In a separate series of experiments, each of the viral inocula was incubated with heparin at 0, 50, 100, or 200 μg/ml at 37 °C for 1 h. This suspension (heparin virus) was then added over the SH-SY5Y cells and was incubated for 2 h at 37 °C. At the end of the incubation time, the viral inoculum was retired, and cells were washed with acid glycine. The SH-SY5Y cells were then trypsinized and reseeded over LLC-MK2 cells, and overlay medium was added for three more days.

Cells were later processed to determine the number of FFUs. Briefly, monolayers of infected LLC-MK2 cells were fixed with 4 % PFA for 30 min and permeabilized with 0.3 % Triton X-100; the endogenous peroxidase was then inactivated with 50 % methanol and 5 % H_2O_2. Monolayers were incubated overnight at 4 °C with anti-*Flavivirus* antibody (MAB 8744, Chemicon) and were then incubated with biotinylated antimouse IgG (Vector) and 1 •g/ml of peroxidase-coupled streptavidin (Vector). The infectious foci were visualized with a 0.1 % diaminobenzidine solution and 0.02 % H_2O_2 and were counted under a microscope; viral titers were reported as focus-forming unit per milliliter. Adsorption and heparin inhibition assays were replicated three times in two independent cultures.

Murine in vivo infection

Twelve BALB/c animals at 2, 7, 14, and 21 postnatal days (pnd) were inoculated by intraperitoneal (ip) route with 70,000 PFU from the D4MB-6 suspension. During the incubation, animals were observed daily. At 6 dpi, the animals were processed to (i) identify the cell populations susceptible to infection through immunofluorescence; (ii) evaluate the infection and viral production in the brain, liver, and spleen; (iii) establish the infection clinical score; and (iv) evaluate

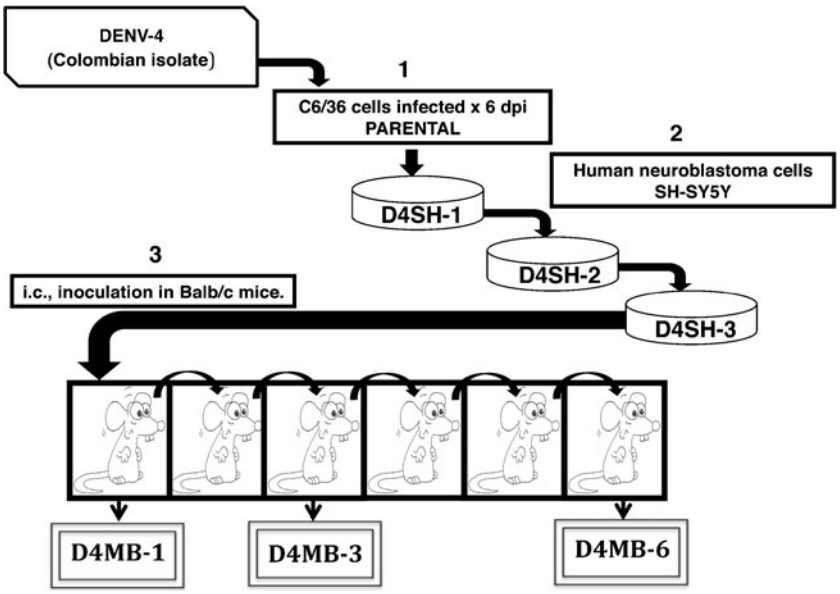

Fig. 1 Dengue virus serotype 4 adaptation scheme. *1* Parental DENV-4 was harvested from C6/36 A. albopictus cell line, inoculating a patient serum and then titrated on LLC-MK2 cells. *2* This DENV-4 virus was inoculated onto SH-SY5Y human neuroblastoma cells at MOI:1; at the third day the supernatant was collected and titrated by plaque assay. This virus variant was named Dengue virus 4, obtained in SH cells from passage 1 (D4SH-1). This viral variant was inoculated again twice on neuroblastoma cells to obtain the D4SH-2 and D4SH-3 strains. *3* This last strain was inoculated intracerebrally into suckling mice and brains were collected at the sixth day postinoculation. Brain homogenate at 10% was titrated and denominated DENV-4 obtained in mouse brain, passage 1 (D4MB-1). This virus was inoculated by serial passages in suckling mice group five times more, obtaining virus variants D4MB-2 to D4MB-6

changes in the BBB. To evaluate the virus production in the nervous tissue, two animals were sacrificed daily in each age group. In addition, at 6 dpi, infection and virus production were evaluated in the liver and spleen. In both cases, the homogenized samples from each tissue were evaluated via plaque assay. The controls for each age group and experimental treatment (n=3) included noninfected animals, animals inoculated with UV-inactivated D4MB-6 virus (n=3), and mock-inoculated animals (noninfected brain homogenate) (n=2). During the experiment, the animals of the four evaluated ages were kept under the care of their parents and were provided with water and food ad libitum.

Immunofluorescence and histological analysis

Animals were sedated and perfused with 4 % PFA in PBS. Brains were extracted, postfixed, and preserved in 30 % sucrose in PBS. Coronal sections (12 μm) were then obtained in a cryostat and were transferred to gelatin-treated slides. Immunofluorescence tests were conducted as follows: sections were hydrated, permeabilized with Triton 0.3 % X-100, and incubated overnight at 4 °C with an antibody that detects the viral antigen (MAb8744, Chemicon). Slides were then incubated with biotinylated antimouse IgG (VECTOR) and Alexa 594®-coupled streptavidin (Molecular Probes).

In addition, cellular antigens to detect neurons (NeuN, Chemicon), astrocytes (glial fibrillary acidic protein, GFAP, DAKO), microglia (CD11b, e-Biosciences), endothelium (Isolectin IB4, Vector), and oligodendrocytes (O4, Chemicon) were incubated overnight at 4 °C and then with the respective secondary antibodies coupled to FITC (Vector). Finally, nuclei were contrasted with Hoechst 33342 (Sigma), and sections were mounted with VectaShield (Vector) for confocal microscopic observation using a Leica TCS-SP5 microscope. For the histological analysis, infected and noninfected animals of each age group were fixed through perfusion, and their tissues (brain, liver, and spleen) were postfixed in 4 % PFA, dehydrated, paraffin-embedded, and hematoxylin–eosin stained.

Clinical score and blood–brain barrier evaluation

D4MB-6-infected and noninfected animals were observed and weighed daily over the entire course of the infection. To establish the clinical score, a 0 value was assigned to the absence of neurological signs, 1 to the onset of those signs, 2 to moderate signs, and 3 to severe signs. In addition, at 6 dpi, three infected and three noninfected animals were injected via ip with 300 μl of 0.5 % Evans Blue in PBS; after 1 h, the animals were sedated and perfused with PBS, and their brains were extracted for evaluation.

GFAP transcript quantification

Three infected, three noninfected, and three mock-inoculated animals from independent litters were sacrificed at 6 dpi by anesthetic overdose. Brains were dissected and homogenized in Trizol® (Invitrogen) for total RNA extraction. The reverse transcription was performed using the M-MLV (Invitrogen) enzyme and random primers. To quantify the GFAP transcript with real-time PCR, the primers (sense 5′-*CAACCTCCAGATCCGAGAAA*-3′ antisense 5′-*TCACATCACCACGTCCTTGT*-3′) and SYBR Green (Finnzymes) were used with Chromo4 (BioRad®) equipment. Data normalization was conducted by geNorm® software, with GAPDH and β-actin as housekeeping genes.

Results

When inoculated intracerebrally or intraperitoneally into animals at the four evaluated ages, the parental virus did not induce any symptoms. Therefore, the adaptation process was initiated by inoculating the parental virus over SH-SY5Y culture cells, obtaining a viral variant named D4SH-1 with a 2.7×10^5 PFU/ml viral titer. Afterwards, two successive passages were conducted on the same cells, obtaining the viral variants D4SH-2 and D4SH-3, with titers of 2.6×10^5 and 1.6×10^6 PFU/ml, respectively. The D4SH-3 virus was then inoculated intracerebrally in BALB/c suckling mice, resulting in the viral variant D4MB-1, which was adapted to mouse brain and achieved a 2.4×10^4 PFU/ml viral titer. This first passage was inoculated into a second litter of animals, resulting in the D4MB-2 variant with a 3.6×10^6 PFU/ml viral titer. Likewise, the viral variants D4MB-3 through D4MB-6 were obtained, all of which had similar viral titers (Table 1). Finally,

Table 1 Viral titers of the in vitro and in vivo neuroadapted variants. The parental virus (DENV4) was inoculated in neuroblastoma cells SH-SY5Y and then the third cell adaptation passage in SH-SY5Y cells (D4SH-3) was inoculated i.c., in Balb/c mice of 2 pnd; this last procedure was repeated five more times until obtaining in vivo adapted variant D4MB-6

Adaptation	Name	PFU/ml
Inoculum	Parental	1.8×10^6
In vitro	D4SH-1	2.7×10^5
	D4SH-2	2.6×10^5
	D4SH-3	1.6×10^6
In vivo	D4MB-1	2.4×10^4
	D4MB-2	3.6×10^6
	D4MB-3	2.4×10^6
	D4MB-4	1.5×10^6
	D4MB-5	1.7×10^6
	D4MB-6	3.6×10^6

the sixth adaptation passage variant (D4MB-6) was inoculated in different aged mice intraperitoneally or intracerebrally; in both cases, it induced neurological changes in animals of 2 and 7 pnd. Therefore, this variant was used in the subsequent experiments with ip inoculation.

Neuroadaptation induced fitness changes in DENV-4

To characterize the different viral variants obtained in the in vitro and in vivo adaptation passages, viral binding and heparin infection inhibition were evaluated. Viral adsorption was evaluated using the indirect plaque assay method reported by Chingsuwanrote et al. (2004) with some modifications. In this study, human neuroblastoma SH-SY5Y cells were incubated with the parental virus and the neuroadapted D4SH-3, D4MB-1, and D4MB-6 variants for 30 min at 4 °C. Cells were then incubated at 37 °C for 15, 30, or 60 min to allow entry and were then washed with acid glycine and trypsinized. Neuroblastoma cells were then recultivated on LLC-MK2 cell monolayers; after 3 days, the formation of foci and the number of infected LLC-MK2 cells were evaluated using a DENV antibody.

In this way, it was observed that the parental virus infected few SH-SY5Y cells, generating between one and two foci over the LLC-MK2 cells at the three evaluated times, thus confirming the weak DENV neurotropism. Because of the very low index infection of Parental virus, its integrity was confirmed, tested by plaque formation ability of an aliquot of virus. In contrast, DVSH-3 infected a larger number of SH-SY5Y cells in the three evaluated binding

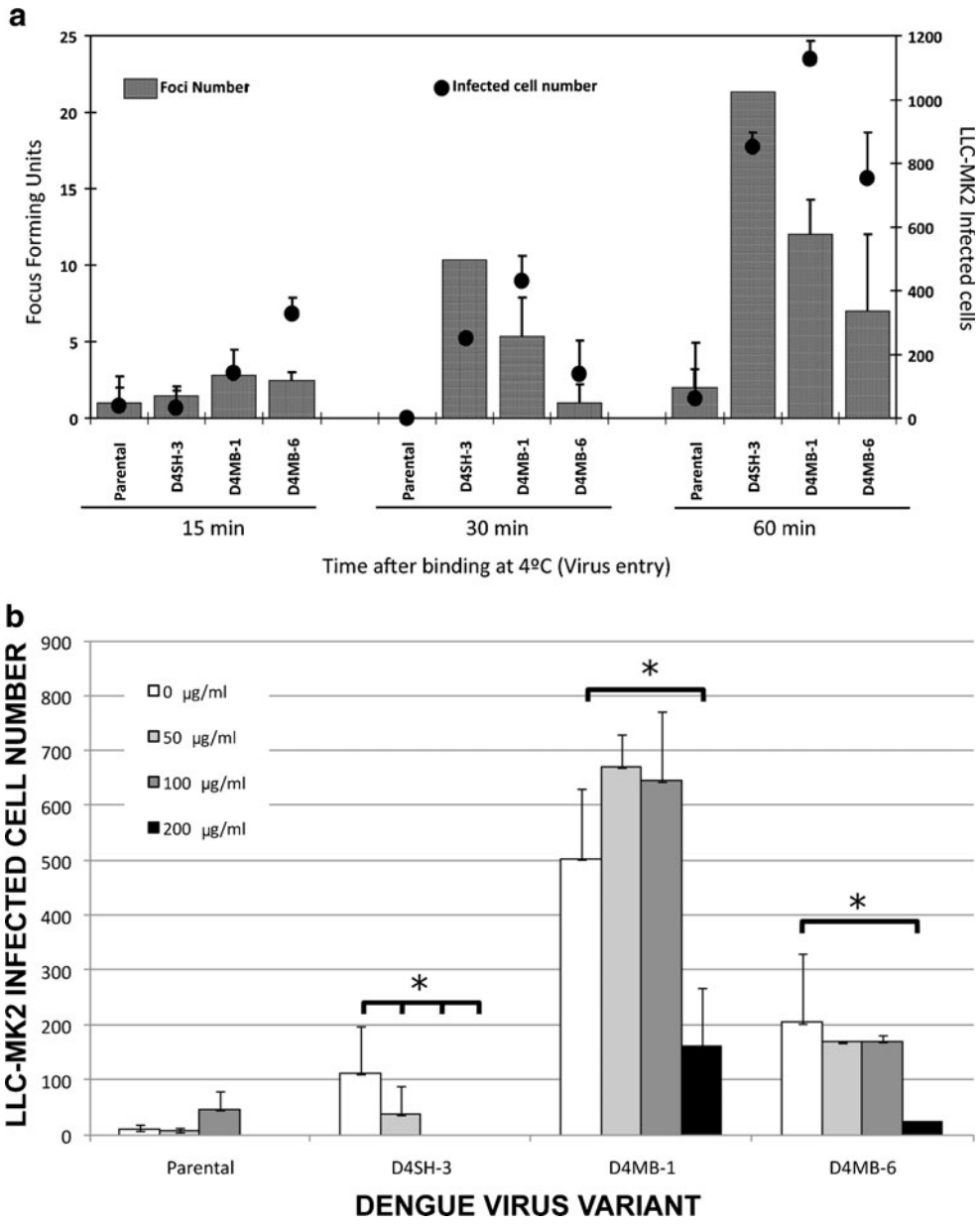

Fig. 2 **a** Viral binding tests. Parental and variants virus were inoculated 30 min on SH-SY5Y cells at 4 °C and then switched to 37 °C for 15, 30, or 60 min, trypsinized and re-seeded on LLC-MK2 monolayer, covered by overlay medium and maintained for 3 days. Infectious foci were detected by immunoperoxidase (*gray bars*) and infected cells counted (*dots and lines*). Data are mean (±SD) from triplicates of two independent experiments. **b** Heparin inhibition of dengue virus infection assays. Each viral variant was incubated with heparin by 1 h and then they were inoculated on SH-SY5Y cells for 2 h at 37 °C, trypsinized, and re-seeded on LLC-MK2 cells, covered with overlay medium and processed for immunostaining at the third day. Data are mean (±SD) of infected LLC-MK2 cells in each condition. *Asterisk* point out significant differences comparing heparin untreated virus and each heparin concentration (ANOVA and Least Significant Difference posthoc test, $p < 0.05$)

times, as reflected in the increase in the number of foci formed, with averages of 35, 253, and 853 LLC-MK2 cells infected in each of the binding times, respectively; this suggests that this viral variant acquired a specific *fitness* to infect this type of cells during the in vitro adaptation passages. Variants D4MB-1 and D4MB-6 formed a lower number of foci in the LLC-MK2 cell monolayers compared to those induced by the D4SH-3 variant. However, the infectious foci of the D4MB-1 and D4MB-6 variants had larger numbers of infected cells at 15, 30, and 60-min entry times (140, 428, and 1,125 for D4MB-1, and 326, 133, and 753 for D4MB-6, mean number of positive cells per focus), suggesting that these variants have higher spreading capacity in LLC-MK2 cells (Fig. 2a).

The heparin infection inhibition results showed that Parental virus does not bind to neuroblastoma cells (with or without heparin). Despite heparin 200 •g/ml treatment reducing infected cells and plaques when we use D4MB-1 and D4MB-6 strains, treatment to these strains with heparin 50 or 100 •g/ml do not affect cell binding, suggesting that these strains do not depend on glycosaminoglycans (GAGs) to infect SH-SY5Y neuroblastoma cells (Fig. 2b). Interestingly, heparin treatment abolished neuroblastoma binding of D4SH-3, pointed out that the cell culture adaptation selects a virus strain that depend on GAGs to bind, and infected neuroblastoma cells.

The D4MB-6 variant inoculated intraperitoneally induced encephalitis, paralysis, and blood–brain barrier damage in suckling mice

The D4MB-6 variant, which was inoculated intraperitoneally in mice, induced neurological changes only in animals of 2 and 7 pnd. In these animals, the virus was detected in the brain at 3 dpi and was characterized by disease signs, such as inactivity, prostration, and spasmodic movements (Table 2 and Fig. 3). At 3 and 5 dpi, an increase in virus production was observed in infected animals. Neurological signs were more evident and included incoordination, loss of balance, kyphoscoliosis, and partial paralysis of posterior limbs. A slight delay in growth and development was also observed. Finally, at 6 dpi, the viral titer in the brain increased to 2 to 3 logs, and the neurological alterations, such as posture instability, ataxia, involuntary contractions, spasmodic movements, and limb paralysis, appeared. At this time, no virus or histological changes were detected in the liver or spleen (Table 3).

Infection of 2 and 7 pnd mice with D4MB-6 induced Evans Blue leakage into the interstitial compartment, demonstrating increased vascular and BBB permeability, suggesting that infection with this variant induced an alteration in endothelial function in the youngest animals (Fig. 4). These physiological alteration signs, including changes in the BBB, were not observed in 14–21-day-old infected mice or in control animals in the four ages examined. This demonstrates that D4MB-6 is a neurotropic, neuroinvasive, and neurovirulent virus in developing individuals.

D4MB-6 altered brain architecture and infected mainly neurons, microglial cells, and some oligodendrocytes and endothelial cells

In 2 and 7 pnd animals infected with D4MB-6, changes in tissue architecture were observed, such as disorganization of the cortex and dilation of vessels and capillaries present in the cortex, hippocampus, and brainstem (Figs. 5–6). In addition, numerous infiltrated leukocytes (i.e., lymphocytes and macrophages) were observed to be associated with the subventricular zones and capillaries; in these same areas, numerous hemorrhage were evident. This, along with the Evans Blue leakage, explains the alteration in vascular permeability during the infection with the D4MB-6 viral variant. These signs were not observed in animals infected with the D4MB-6 on postnatal days 14 and 21, suggesting that the neurological and immune immaturity of the animals determines neuropathology and neuroinfection by D4MB-6.

Table 2 Description of the neurological signs and clinical score in the D4MB-6-infected animal at 6 dpi. The Balb/c mice of 2 and 7 d.p.n. were inoculated i.p., with D4MB-6 and were observed and evaluated everyday during 6 days. The data were collected from at least 25 animals and the value of 0 suggests the absence of neurological alterations; 1, apparition of the signs; 2, moderate signs; and 3, severe signs

	2 pnd				7 pnd			
Hours postinfection / Clinical Rating	72 h	96 h	120 h	144 h	72 h	96 h	120 h	144 h
Inactivity	2	2	3	3	1	2	3	3
Weigth loss	2	2	3	3	1	2	3	3
Retarded growth								
Lack of coordination Kyphoscoliosis	1	2	3	3	1	1	2	3
Postural instability Paralysis	1	2	3	3	0	1	2	3
Distonia	0	2	2	3	0	0	2	3
Ataxia								
Postration	0	1	2	3	0	0	2	3
Score	8	14	19	21	4	6	17	21

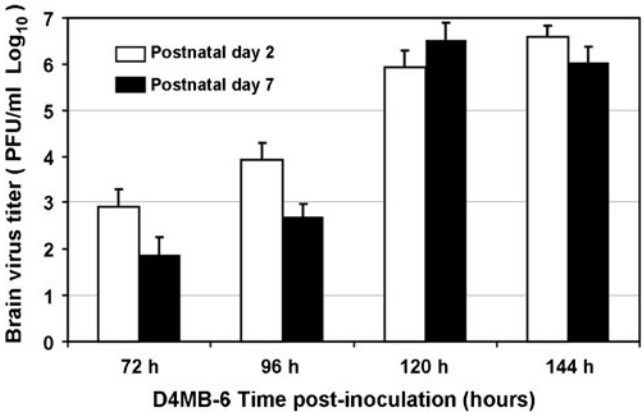

Fig. 3 Dengue virus titers in brain homogenates of 2 and 7 pnd-infected mice with D4MB-6 variant inoculated intraperitoneally. Infected mice were evaluated daily and brains from two individuals were collected each time, homogenated, and titrated by plaque assay. Data are mean from two independent experiments

Additionally, when evaluating the presence of the viral antigen in different cell populations in infected 2- and 7-pnd mice, it was observed that those neurons (NeuN+) located in the cortex, hippocampus, and brainstem were positive to viral antigen. In addition, DENV was detected in microglial cells, which exhibited phenotypic changes associated with the expression of the CD11b marker and cytoplasmic extensions, thus suggesting an activation of these cells, as has been reported in humans (Ramos et al. 1998). Viral antigen was also observed in some endothelial cells and oligodendrocytes located in the cerebral cortex, hippocampus, and brainstem (Fig. 7).

These markers were also evaluated in brain sections from infected 14- and 21-pnd mice; neither virus- nor CD11b-positive cells were found, confirming the resistance to infection and the nonactivation of microglia in animals of this age. Astrocytes were resistant to the infection, but 2, 7, and 14 pnd mice showed an increase in GFAP marker intensity and obvious changes in the number, size, and shape of these cells, suggesting their activation during D4MB-6 infection (Fig. 8a). This increase in GFAP reactivity was confirmed through qPCR by observing that infected animals of those ages presented an increase in the relative expression of GFAP transcript compared to their respective controls, suggesting that the infection induced tissue astrocytosis as part of the local immune response (Fig. 8b). Finally, in the mock or uninfected control groups of animals at each examined age, there were no signs of neurological changes, and viral antigen was not detected in the areas and cell populations examined.

Table 3 3 Production of virus titrated from brain, liver, and spleen of D4MB-6-infected mice at 6 dpi

	Tissues	Animal 1	Animal 2	Animal 3
2 pnd	Brain	4.8×10^5	4.9×10^5	4.4×10^5
	Spleen	ND	3×10^2	ND
	Liver	ND	ND	ND
7 pnd	Brain	2.7×10^4	3.3×10^5	2.7×10^5
	Spleen	ND	ND	ND
	Liver	ND	ND	ND

Discussion

Several reports indicate that, in different countries, the clinical profile caused by DENV is changing (Miagostovich et al. 1997; Thisyakorn et al. 1999; Jackson et al. 2008; Méndez and González 2003, 2006), and clinical symptoms, such as encephalitis, meningitis, myelitis (Malheiros et al. 1993; Kunishige et al. 2004), encephalopathy (Kumar et al. 2008), disseminated acute encephalomyelitis (Gera and George 2010), transverse myelitis (Leyo et al. 2002), neuromyelitis, optical neuritis (Bacsal et al. 2007; Miranda et al. 2007), and Guillain–Barré syndrome (Esack et al. 1999), are present more frequently during or after the infection. Yet, the viral and host factors that could be involved are still unknown.

For these reasons, it is important to establish in vivo infection models that reproduce those neurological signs that are associated with DENV infection and that would allow the identification of host and viral factors associated with the onset of those signs. This study developed a neuroinfection model in mice inoculated ip with a neuroadapted DENV-4 strain, D4MB-6, which efficiently infected the brain and induced neurological signs in infected mice.

Acquisition of neurotropic and neuroinvasive characteristics in the D4MB-6 strain

Given the weak neurotropism present in DENV, an adaptation process for nervous tissue was required (Lindenbach and Rice 2001a). However, after a short adaptation process on SH-SY5Y cells (three passages), this virus increased its infection and replication capabilities (i.e., *fitness*) on these cells. D4SH-3, inoculated i.c., in suckling mice produced the first viral variant adapted to nervous tissue (D4MB-1), which still infected and easily dispersed in SH-SY5Y cells but had low replication capacity in nervous tissue. This suggests this variant possesses neurotropic characteristics, but it is not yet neurovirulent in animals. The successive in vivo passages yielded a viral variant, D4MB-6. This variant, in addition to acquiring a neurotropic phenotype, was also neuroinvasive and neurovirulent when inoculated extraneurally in animals of 2 and 7 pnd.

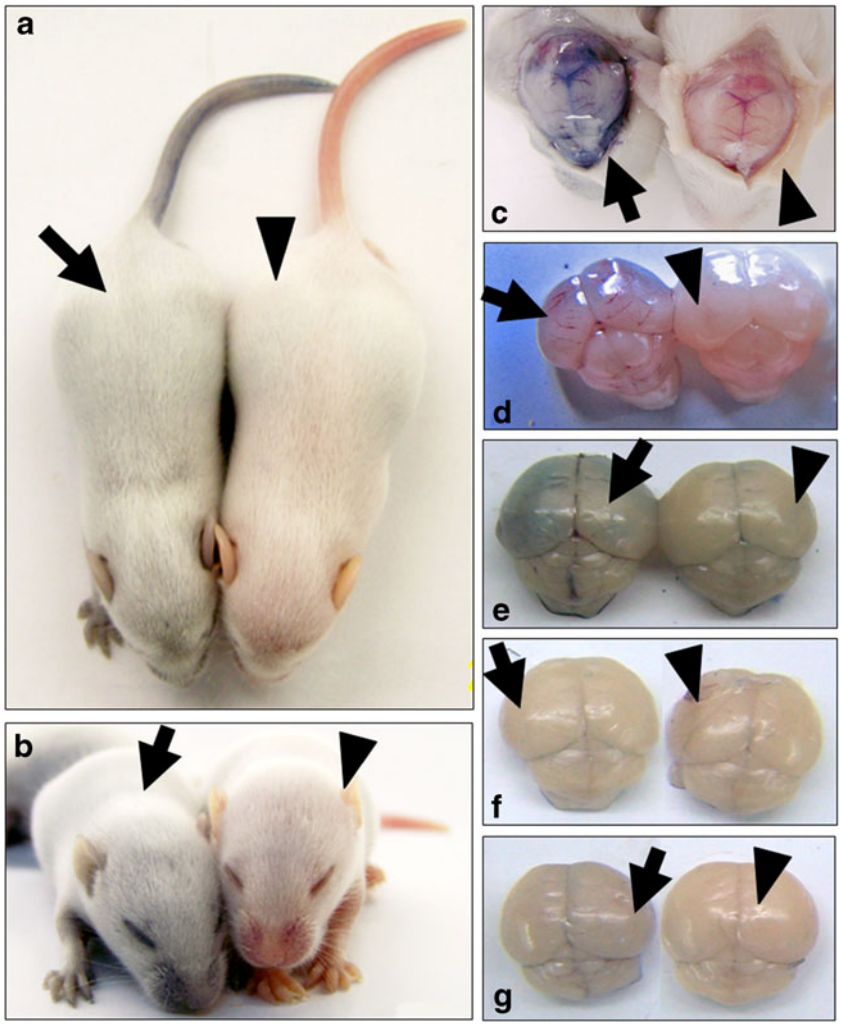

Fig. 4 Viral variant D4MB-6 altered vascular permeability and blood–brain barrier integrity in the youngest mice. Virus was inoculated intraperitoneally and evaluated at 6 days pi injecting Evans blue solution. Stain leakage was observed in all tissues of infected mice, like (**a**) tail and feet or (**b**) nose and ears. **c** Aspect of perfused mice after Evans blue inoculation infection (*arrows*) and mock infection (*arrow head*). Dissected brains from 2 postnatal days (pnd) mice (**d**), 7 pnd (**e**), 14 pnd (**f**), and 21 pnd (**g**)

Our results suggest that during the neuroadaptation process, the dominance of a viral subpopulation present in the initial inoculum was favored and increased its fitness towards nervous tissue cells during the in vivo and in vitro adaptation processes. It was confirmed that the low neurotropism of this Parental DENV-4 isolate and its independence of GAG interaction to infect neurons lead to conclude that other than matrix, molecules could be used by DENV to infect target cells (Bielefeldt-Ohmann et al 2001). Neuroblastoma cell-adapted virus D4SH-3 acquired during passages depended on GAGs to bind its cell substrate, but this characteristic was lost in in vivo-adapted D4MB-1 and D4MB-6 strains, which do not change their binding pattern upon treatment with heparin and retaining its independence of GAGs, pointing out the remarkable plasticity of RNA virus.

These results suggest that in vitro and in vivo DENV-4 adaptation induces phenotypic or genotypic changes that should be maintained to improve the neurotropic and neuroinvasive behavior of neuroadapted virus (Añez et al 2009). This virus–GAG interaction independence could favor binding and infection of different cells in nervous tissue, inducing neurological alterations in younger mice, and at the same time, allowing replication without infecting or stimulating immune- or antigen-presenting cells as has been reported previously (Prestwood et al 2008; Ip and Liao 2010). Nucleotide sequence changes responsible of modification in the binding and infection pattern has been found mainly in dengue E protein and perhaps other viral proteins (Duarte dos Santos et al. 2000; Bordignon et al. 2007). Currently, we do not know the sequence variations that took place in the viral variants obtained in this study; therefore, genome sequencing is currently underway.

Neuroinfection and neurological alterations caused by D4MB-6

The neurotropic, neuroinvasive, and neurovirulent characteristics of the D4MB-6 viral variant were evident when inoculating the virus intraperitoneally. No infectious virus or evident tissue damage were apparent in extraneural

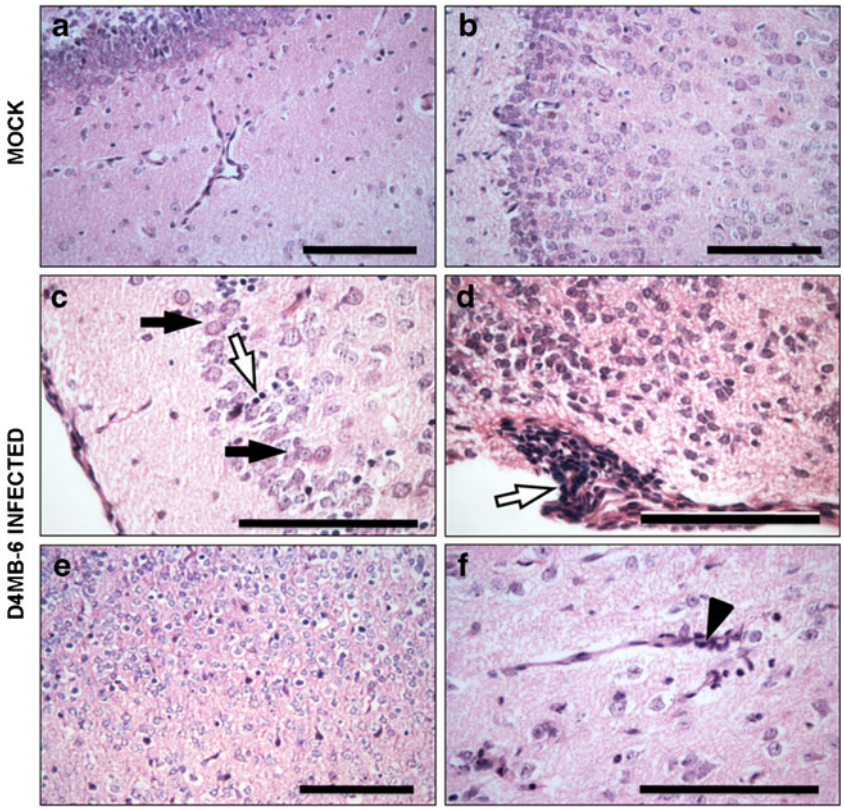

Fig. 5 D4MB-6 variant induced histological alteration in 2 pnd mice. H&E staining of brain sections of mock-infected mice (**a** and **b**) or infected mice (**c**–**f**). Note the organized normal aspect of neurons in **b** and abnormal architecture in infected mice (**c**) (*black arrows*). It was found that a lymphocyte infiltration in brain parenchyma (*white arrow*) in **c** and **e**, associated with subventricular spaces (**d**) or near to blood vessels (*arrowhead* in **f**). *Bar* corresponds to 10 μm

tissue, such as the liver and spleen. These variant-induced neurological changes associated with damage to the brain architecture, leukocyte infiltration, and BBB alteration. In addition, it induced the activation of microglial cells and astrocytes and infected neurons, microglial cells, endothelium, and oligodendrocytes only in animals of 2 and 7 pnd.

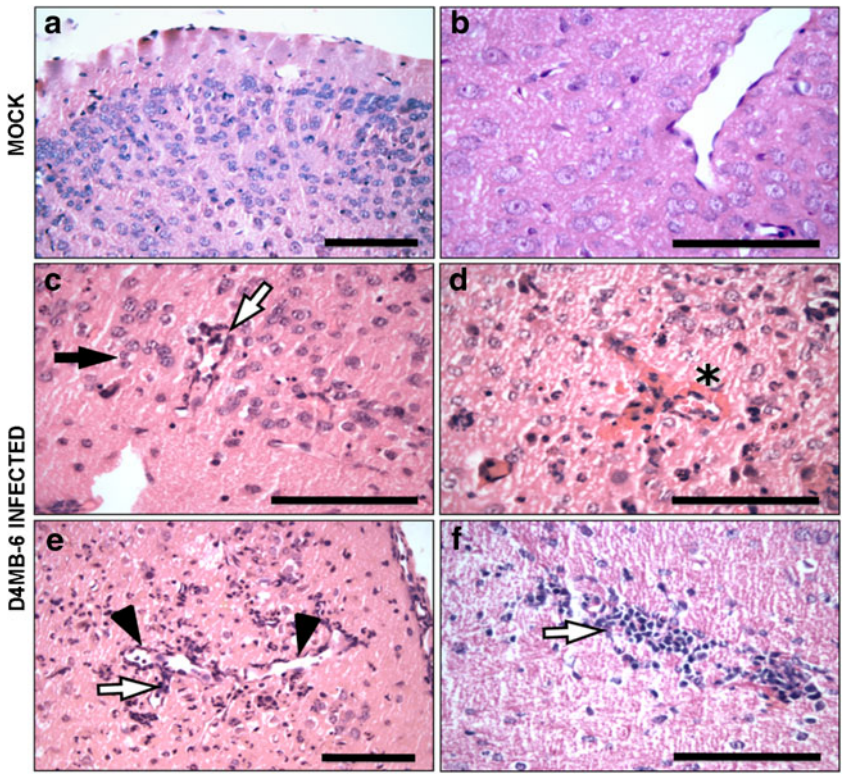

Fig. 6 H&E histology sections of 7 pnd brains. Mock-infected mice with normal aspect of cortical (**a**) and brainstem (**b**) regions. **c** Loss of organization of cortical region in D4MB-6-infected mice (*black arrow*) with lymphocyte infiltrate in brain parenchyma (*white arrow* in **c** and **e**). **d** Dilated and hemorrhagic blood vessels (*asterisk*). **f** Lymphocyte infiltrate near the subventricular space. *Bar* corresponds 10 μm

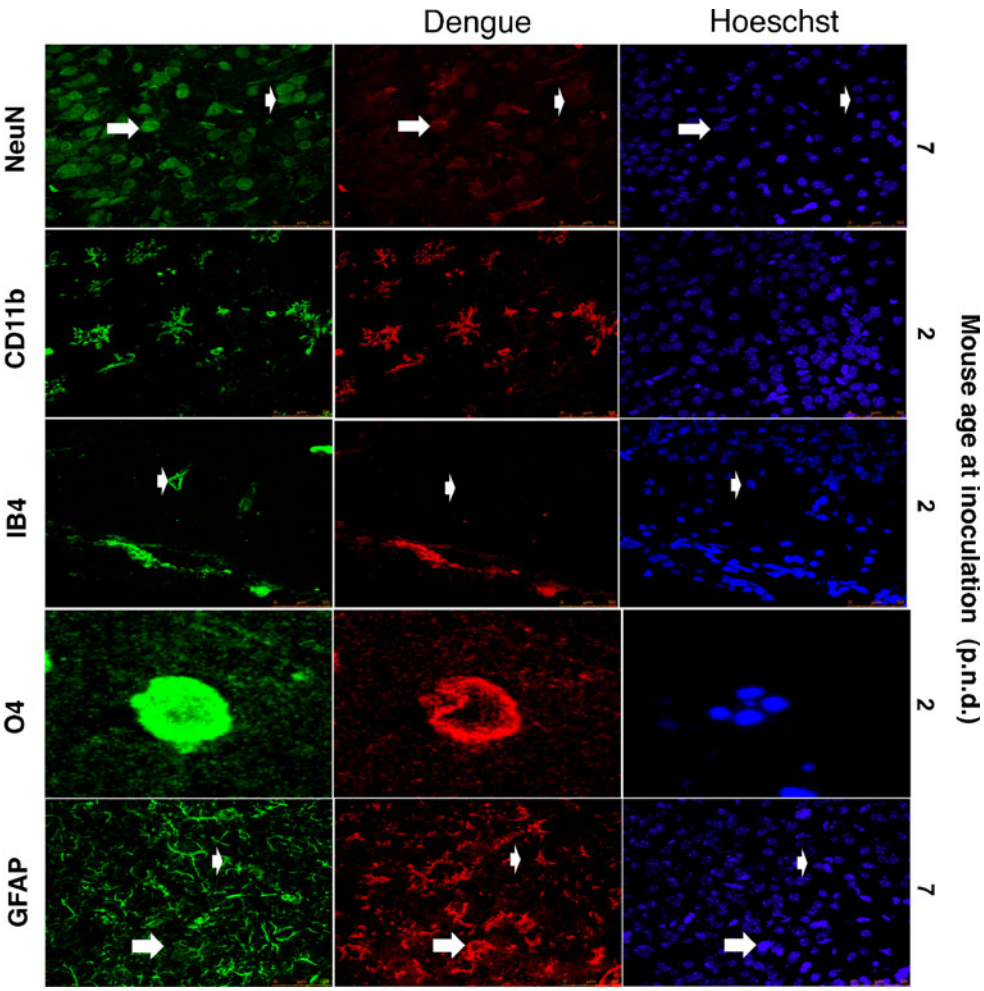

Fig. 7 D4MB-6 viral variant infects different cell subpopulations in 2 and 7 pnd mice. Confocal microscopy of brain sections of infected mice, stained to different cell markers (*in green*) and viral antigen (*in red color*). *NeuN* neuronal marker, *CD11b* activated microglia, *IB4* endothelial cells, *O4* oligodendrocytes, *GFAP* astrocytes. *Long white arrows* point to infected cells and *short white arrows* point to noninfected cells and cell marker positive cells. *Bar* corresponds to 50 μm

The observed BBB damage in animals infected by neuroadapted DENV has not been described in any previously reported neuroinfection models (Sánchez-Burgos et al. 2004; Chaturvedi et al. 1991). Therefore, our model suggests that the D4MB-6 viral variant has the capacity to infect and change the physiology of the different cell types present in the nervous tissue, including the cerebrovascular endothelium. These functional changes in the BBB have been observed in the nervous system injury caused by WNV (Wang et al. 2003) and other neurotropic flaviviruses, such as JEV (Mishra et al. 2009). Therefore, changes in the BBB may be a characteristic trademark of the neuroinfection and neuropathology caused by flaviviruses, including DENV.

Endothelial dysfunction and plasma leakage are among the most important signs of dengue disease (Basu and Chaturvedi 2008); they apparently depend on the activation of the endothelium, changes in the interendothelial joints, and cell apoptosis (Lin et al. 2008; Avirutnan et al. 1998; Chen et al. 2007). Along with endothelial damage, our model suggests that D4MB-6 infection and the activation of microglial cells, previously reported in other models (Barth et al 2006; Barreto et al. 2007; Huang et al. 2000) and human biopsies (Ramos et al. 1998), along with the activation of astrocytes contribute with the dysfunction of endothelia and favoring the passage of leukocytes, soluble mediators, and perhaps the virus, which could be responsible for the changes in tissue architecture and function, as has been reported previously during JEV infection (Chen et al. 2009).

Currently, there are no animal models that completely reproduce all signs of dengue disease. Models that partially develop some hemorrhagic signs of DENV infection had been obtained from immunocompromised SCID mice transplanted with K562-, HepG2-, or CD34-infected human cells (An et al. 1999; Lin et al. 1998; Bente et al. 2005; Mota and Rico-Hesse 2009); AG129 mice lacking the genes for IFN type I and IFN type II receptor (Willianms et al., 2009; Johnson and Roehring 1999; Yauch and Shresta 2008; Tan et al. 2010); and knockout mice to CXCL-10 (Ip and Liao 2010) or immunocompetent mice, such as BALB/c, A/J, and C57BL/6 (Shresta et al. 2004; Huang et al. 2000; Barreto et al. 2007; Wu-Hsieh et al. 2009). In some of these models, dengue manifestations, such as hemorrhage, thrombocytopenia, and production of cytokines and chemokines, have been successfully induced when inoculating high dosages of

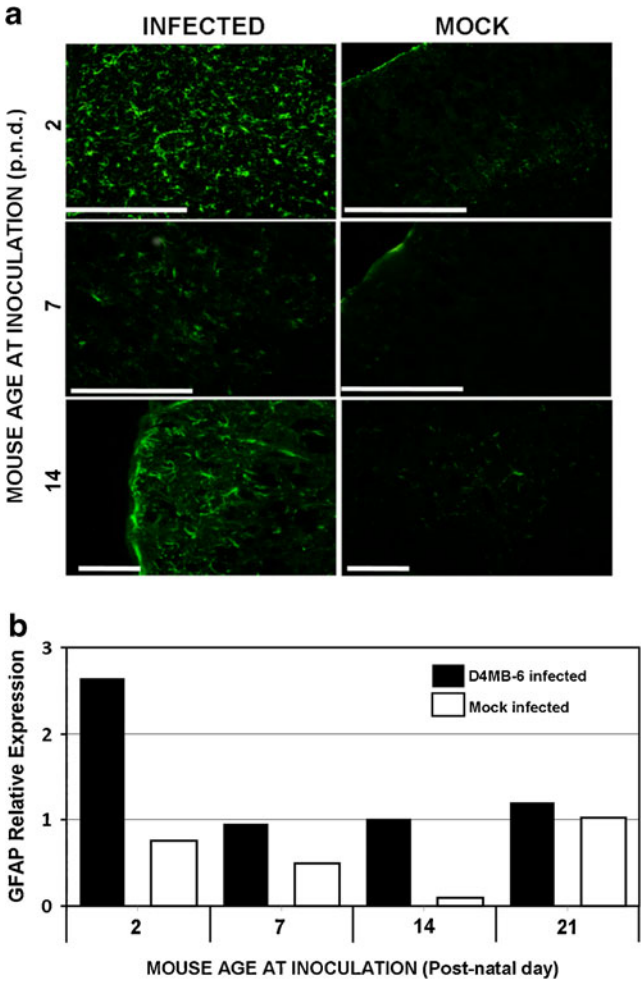

Fig. 8 Neuroadapted dengue virus (D4MB-6) induced astrocytosis in infected mice. **a** GFAP immunofluorescence of brain sections of infected mice with D4MB-6 (*left panel*) and mock-infected mice (*right panel*) inoculated at different ages. There was a huge increase in GFAP-positive astrocytes even in 14 pnd mice which do not show neurologic symptoms nor was viral antigen detected. *Bar* corresponds to 40 μm. **b** Data of quantitative PCR to evaluate GFAP transcript in infected mouse brain normalized with normal noninfected mice of the same age. Data are the average of six infected, three mock infected, and three normal noninfected mice

virus intracerebrally or intravenously. In these models, neurological changes have been reported, such as hind limb paralysis, associated mainly with generalized infection inducing damage and changes in the physiology of the nervous system (i.e., encephalopathies) (Chen et al. 2007). However, neuroinfection and neurological alterations induced by DENV reported in some infection models and in patients are still considered atypical, though they are associated with higher severity and lethality (Gulati and Maheshwari 2007; Murthy 2010). Therefore, a DENV infection model is needed that allows detailed in vivo study of the causes and consequences associated with the neuroinfection and neuropathology caused by the virus.

The previously reported DENV neuroinfection models have been obtained by using adapted viruses that infect and cause damage to extraneural tissues, such as the liver and spleen, and that infect and replicate with low efficacy in the CNS (Atrasheuskaya et al. 2003; Souza et al. 2009). Other models report neurological alterations, such as limb paralysis, associated with neuronal death in animals of 2 pnd, inoculated i.c. with virus (Després et al. 1996, 1998; Bordignon et al. 2007). However, these models do not provide detailed studies of some of the aspects associated with neuroinfection, such as the ages of the individuals and the nature of the inoculum. Regarding our model, we sped up DENV adaptation by initially harvesting the virus on neuroblastoma cells, which induced the early acquisition of nervous tissue-specific fitness. In this way, the number of in vivo viral adaptation passages was reduced.

Finally, in our model, we observed that the neurological and immunological maturity of individuals determined the susceptibility to neuroinfection and neuropathology caused by DENV. Thus, it was found that infected 2- and 7-pnd animals were highly susceptible to the infection and succumbed to it, given their neuronal and immune immaturity; in contrast, in the animals of 21 pnd, which are physiologically mature, infection and neurological damage were avoided. However, it is interesting to note an intermediate point between susceptibility and control of the infection in the 14-pnd animals; local defense mechanisms were established, such as the activation of astrocytes and systemic mechanisms, such as the increased production of interferon-gamma (data not shown), which allow for control of the infection and avoid changes in nervous system function. Therefore, our model allows for the suggestion that the neuroinfection and neuropathology caused by DENV in mice, and possibly in humans, may depend on the physiological stages and ages of individuals. These findings coincide with those reported previously for infection models with neurotropic flaviviruses, such as JEV (Ogata et al. 1991), and reports about children and adolescents infected with DENV (Guzmán et al. 2002). However, our model is the first to report these differences, and more experimental approaches will be required to learn more about them. It is possible that variations in the structural and nonstructural proteins that might have arisen during the adaptation of D4MB-6 are responsible for this change in viral phenotype. These variations may include those that favor the infection, not only of neurons but also of other cell populations, and may have increased the replication and dispersion capacities of the virus and thus the physiological alterations of the tissue. It seems that any viral serotype can be related to the induction of neurological alterations in animals and humans (Sánchez-Burgos et al. 2004; Després et al. 1996, 1998; Bordignon et al. 2007; Ramos et al. 1998).

In summary, we can suggest that D4MB-6, inoculated intraperitoneally, interacts with proteoglycans to infect

neuronal cells. This feature, along with the possible variations in the sequence, can confer the neurotropic, neuroinvasive, and neuropathological character to this viral variant in immunocompetent animals of 2 and 7 pnd. This viral variant mainly infected neurons and microglial cells and, to a lesser extent, endothelium and oligodendrocytes. In addition, it stimulated the local immune response, inducing morphological and phenotypic changes in glial cells that, as a group, could induce changes in the BBB and in vascular permeability, thus enhancing the infection and increasing the infiltration of leukocytes and the alteration of neurological function. However, in animals of 14 pnd, this viral variant stimulated an immune response that avoided infection and tissue damage. With these features, our neuroinfection model will allow further descriptions of other aspects of DENV neuroinfection and the immune response established by the tissue against the virus.

Acknowledgments

The authors thank Dr. Rosa María del Ángel and Dr. Juan Ernesto Lúder of Departamento de Infectómica Patogénesis Molecular, and Iván Galván-Mendoza of Departamento de Servicios Generales to CINVESTAV-IPN México for technical assistance in confocal microscopy. We also acknowledge Jeanette Prada-Arismendy for technical assistance in virus titrations. This work was supported by División de Investigaciones—Universidad El Bosque and Colciencias-Colombia (Project 1308-489-25267) and grants from the Fundación para la Promoción de la Investigación y la Tecnología del Banco de la Republica (Project 2515-2008). ML Velandia had a scholarship from Colciencias, Programa de Doctorados Nacionales.

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