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Heart Compromise and Detection of Dengue Virus-Like Particles in Cardiac Tissue of Experimentally Infected Murine Model

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Abstract: *The involvement of the myocardium in human cases of dengue has been reported, but the mechanism leading to myocarditis, one of the most commonly observed pathologies, remains unclear. In this study, BALB/c mice were infected with different strains of non-neuroadapted dengue virus serotype 2 and morphological analysis of heart were performed by transmission electron microscopy. For detection and quantification of the Viral RNA, Real-Time Reverse Transcriptase PCR assay was performed. Our analyses showed involvement of heart in DENV infection. DENV-like particles were observed inside endothelial cells and cardiomyocytes. DENV RNA was detected in 15 heart and four serum samples. In three samples, we observed titers higher than that of the inocula.*

Keywords: *Endothelial cells, dengue virus, heart, BALB/c mice.*

1. INTRODUCTION

With more than one-third of the world's population living in areas at risk for infection, dengue is a leading cause of illness and death in the tropics and subtropics. Around 400 million people are infected yearly [1]. It is caused by the Dengue virus (DENV), a group four serologically distinct RNA viruses (DENV-1, DENV-2, DENV-3 and DENV-4) which are transmitted to humans by the bite of the vector mosquitoes *Aedes aegypti* (*Ae. aegypti*) and *Aedes albopictus (Ae. albopictus)*. Asymptomatic and undifferentiated febrile illness is common. Subsequent infections with different serotypes of DENV are common, as the neutralizing antibodies against the primary infectious virus does not effectively protect from other serotypes [2]. Secondary infections and primary infections in elder children and adults are associated with more severe manifestations. According to the World Health Organization [3], DENV infections are divided into two categories: dengue, subdivided into dengue without warning signs and with warning signs and severe dengue (SD). The main characteristics of SD are increasing of vascular permeability without morphological damage to the capillary endothelium, thrombocytopenia, altered number and function of leucocytes, altered haemostasis and liver injury [4]. It is not yet clear which cell type or tissues are involved in the replication of DENV. DENV antigen was observed in different tissue and cell samples from SD patients, including myocardial endothelium and cardiomyocytes, Kupffer and sinusoidal endothelial cells of the liver, macrophages and vascular endothelium in the lung, spleen, lymph node, thymus and kidney tubules [5-9]. Cardiac involvement in dengue has been reported in few studies, usually resulting in a benign and self-limited disease [8, 10, 11]. Immunohistochemistry of heart from fatal cases of dengue showed distinct perinuclear staining in endothelial cells and cardiomyocytes as small granular deposits within the cytoplasm[9]. Although cases of a more severe disease with progression to cardiogenic shock and death have been increasingly described, [9, 11-14], the pathogenesis of myocardial lesions has not been elucidated. Herein, we aim to present evidence of heart involvement during DENV infection by using immunocompetent BALB/c mice inoculated with a non-neuroadapted DENV-2.

2. MATERIAL AND METHODS

2.1. Ethical Statement

The procedures performed in this study were approved by the Animal Ethic Committee (protocol LW-50/11) and the Human Research Ethic Committee (protocol 247/05) of Oswaldo Cruz Foundation (FIOCRUZ).

2.2. Viral Strais

DENV-2 strains BR/RJ66985/2000 and BR/RJ0337/2008, isolated from patient sera at the Flavivirus Laboratory, Oswaldo Cruz Institute (IOC), FIOCRUZ, during the epidemics of 2000 and 2008 respectively, are representative of two distinct Lineages: Lineage I and Lineage II [15]. Serotype was confirmed by indirect immunofluorescence using DENV-2 specific monoclonal antibody (3H5) and RT- PCR [16]. For production of viral stock, 100µL of each strain were inoculated into cell culture bottles containing mosquito *Ae. albopictus* C6/36 cell line at a concentration of $5x10^5$ cells/mL. Titers of both strains [BR/RJ66985/2000: $10^{6.66}TCID_{50}$/1mL ($7,0x10^5PFU$) and BR/RJ0337/2008: 10^9TCID_{50}/1mL ($7,0x10^9PFU$)] were calculated by the Reed & Muench method [17]. The viruses did not undergo any passages through mice brain for neuroadaptation.

2.3. Mouse Model for Experimental Infection

Two months old male BALB/c mice, obtained from the FIOCRUZ Center of Animal Breeding, were kept under controlled temperature, photoperiod, nutrition and hydration conditions during the experiments.

2.4. Study Design

For the infection with the DENV-2 BR/RJ66985/2000 strain, ten mice were inoculated by the intravenous (IV) route and ten, by the intraperitoneal (IP) route. The same procedure was used to infect mice with the DENV-2 BR/RJ0337/2008 strain. Inocula volume was 100µL and viral concentration was 10,000 $TCID_{50}$. 72 hours (h) after infection, the mice were anesthetized, the sera was collected by cardiac puncture, and euthanized. Ten non-infected mice were used as controls. Hearts were divided into two. Half the organ was fixed in glutaraldehyde for ultrastructural studies. The other half and sera samples, were kept at -80^0C for viral RNA extraction, Fig. 1.

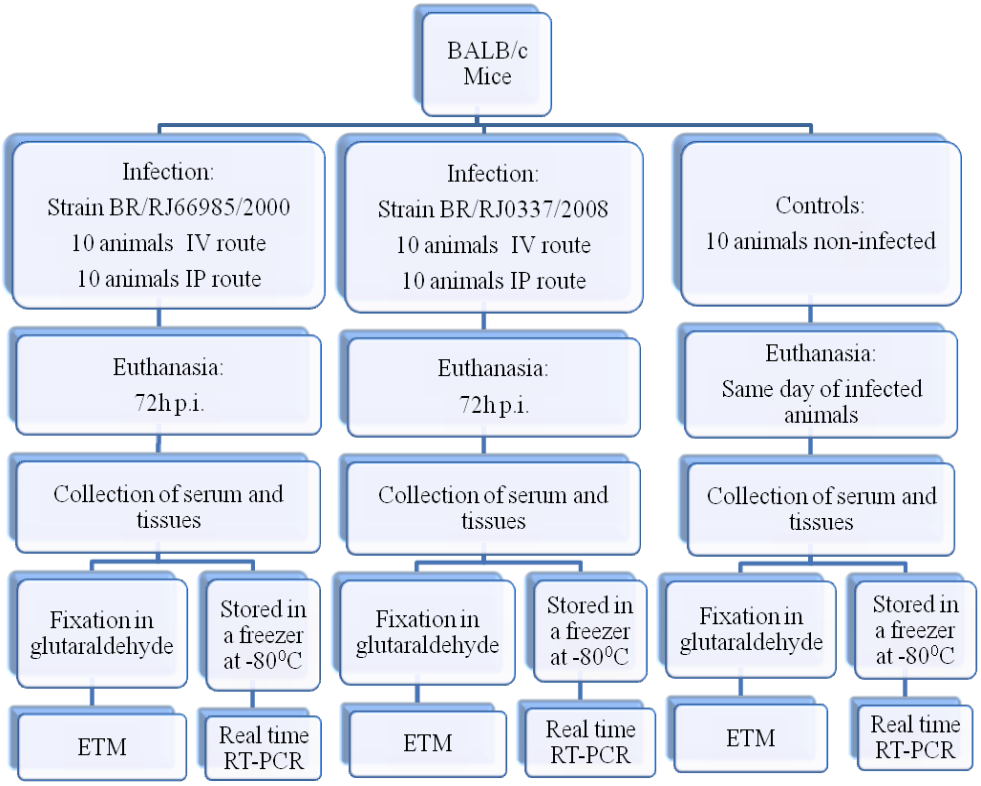

Figure1. *Flowchart of the study design. IV: intravenous; IP: intraperitoneal, p.i.: post-infection, TEM: Transmission electron microscopy.*

2.5. RNA Extraction

For viral RNA extraction, samples of infected and control mice were macerated using 500µL of Leibovitz medium (Sigma) supplemented with 1% fungizone and 2% penicillin-streptomycin (Gibco). RNA was extracted from 140µL of supernatant of macerated heart, serum and inocula samples by using QIAmp Viral RNA mini kit (Qiagen) as described by manufacturer's protocol.

2.6. Real Time RT-PCR

For viral RNA quantification, the protocol described by Poersch [18] was performed. Primers and probe utilized were DEN2-R (5-ACCATAGGAACGACACATTTCC-3) and DEN2-F (5-CAACGCATTGTCATTGAAGGA-3) and (FAM-5-AGGGCCTTGATTTTCATCTTACTGACAGC-3-TAMRA). The kit used for amplification reaction was TaqMan Fast Virus One-step Master Mix (Applied Biosystems). Five microliters of extracted RNA and a mix containing 12,5µL of reaction mix (Invitrogen), 1µL of DEN2-F and DEN2-R primers (Sigma), 0,75µL of DEN2-P probe (Sigma), 3,65µL of nuclease free water (Gibco), 1µL of $MgSO_4$ (Invitrogen) and 0,5µL of Super Script III Platinum One-Step Quantitative RT-PCR (Invitrogen) were applied to a 96 well microplate. The assay was performed in 7500 FAST platform (Applied Biosystems) and the thermal cycling parameters were: reverse transcription at 50°C, 15 minutes (min), 1 cycle for enzyme activation at 95°C, 2 min, 1 cycle of denaturation at 95°C, 15 seconds, 40 cycles and annealing/elongation at 60°C, 1 min, 40 cycles.

2.7. Sample Processing for Transmission Electron Microscopy (TEM)

Samples were processed as described by Barreto-Vieira [19]. Briefly, tissue was fixed by immersion in 2% glutaraldehyde diluted in sodium cacodylate buffer (0,2M, pH 7,2), cut into smaller fragments (~1mm^3), post-fixed in osmium 1% tetroxide and dehydrated in increasing concentrations of acetone (10%, 15%, 30%, 50%, 70% in uranyl acetate, 90% and 100% in copper sulfate). Subsequently, samples were embedded in Epoxy resin. The ultrathin sections were stained with uranyl acetate and lead citrate and analyzed in a Zeiss EM-900 transmission electron microscope.

3. RESULTS

3.1. Detection and Quantification of Viral Genome from Heart and Serum Samples by Real Time RT-PCR

Eight out of 10 hearts of mice infected with the DENV-2 strain 66985/2000, representative of Lineage I, tested positive for viral RNA detection, five of them from mice inoculated by the IV route. When inoculated with the DENV-2 strain 0337/2008, representative of Lineage II, seven animals were positive, five of them infected by IV route and two by IP route (Table 1).

Table1. *DENV-2 genome detection in heart and sera of experimentally infected Balb/c mice.*

	66985/2000 Lineage I				**0337/2008 Lineage II**			
Route of infection	Titer (copies/mL)							
	Intravenous		Intraperitoneal		Intravenous		Intraperitoneal	
Sample/ Mice	Heart	Serum	Heart	Serum	Heart	Serum	Heart	Serum
1	ND	ND	ND	ND	$6,8X10^3$	ND	ND	ND
2	ND	$1.20x10^3$	$1,43X10^6$	ND	$3,61X10^6$	ND	$7,49X10^2$	ND
3	ND	ND	ND	ND	ND	$1,17x10^3$	ND	ND
4	ND	ND	ND	ND	ND	ND	ND	ND
5	ND	ND	$6,49X10^8$	ND	ND	ND	ND	ND
6	$1,28X10^5$	ND	ND	ND	ND	ND	ND	ND
7	$3,11X10^5$	ND	ND	ND	$1,73X10^{15}$	ND	ND	ND
8	$8,63X10^7$	$3,96x10^3$	$1,72X10^9$	ND	$4,62X10^{14}$	ND	ND	ND
9	$3,63X10^7$	ND	ND	ND	$2,39X10^5$	ND	$4,59X10^1$	ND
10	$1,94X10^6$	$1,0x10^{13}$	ND	ND	ND	ND	ND	ND

ND: non-detected.

In sera samples, DENV genome was detected in three mice inoculated with Lineage I, by the IV route. When infected with Lineage II, one animal, inoculated by the IV route, was positive.

The inocula titers used for infection with DENV-2 66985/2000 and 0337/2008 strains were $1{,}09x10^{7}$ and $7{,}49x10^{8}$ RNA copies/mL, respectively. Two out of the 15 positive heart samples, both of mice infected by the IV route with the 0337/2008 strain, and one out of 4 positive serum samples, from one mouse infected by the IV route with the 66985/2000 strain, presented titers higher than the inocula titers, $4{,}62x10^{14}$, $1{,}73x10^{15}$ and $1{,}0x10^{13}$ (copies/mL), respectively.

3.2. Morphological Studies on Heart of DENV-2 Infected Mice

Real Time RT-PCR positive samples were processed for TEM analysis. Morphological studies of heart showed similar alterations, regardless of the chosen infection route or DENV-2 strain used. Alterations observed were focal. Samples showed areas of unaltered tissue.

Ultrastructural alterations observed were: platelets (Fig. 2A), edema (Fig. 2B) and mononuclear inflammatory cells in capillaries (Fig. 2C) and in tissue interstice (Fig. 2D) and small granular perinuclear deposits in cardiomyocytes (Fig. 3A). Swelling of cytoplasm and a large number of electron-lucid vesicles (Fig. 3B) and cytoplasmic membrane extensions were observed in endothelial cells (Fig. 3C). Neither necrosis nor changes in the interaction between endothelial cells were observed. Cardiac muscle fibers were well preserved. Structures similar to DENV particles measuring approximately 60nm were observed inside the cytoplasm of endothelial cells and cardiomyocytes (Fig. 3D).

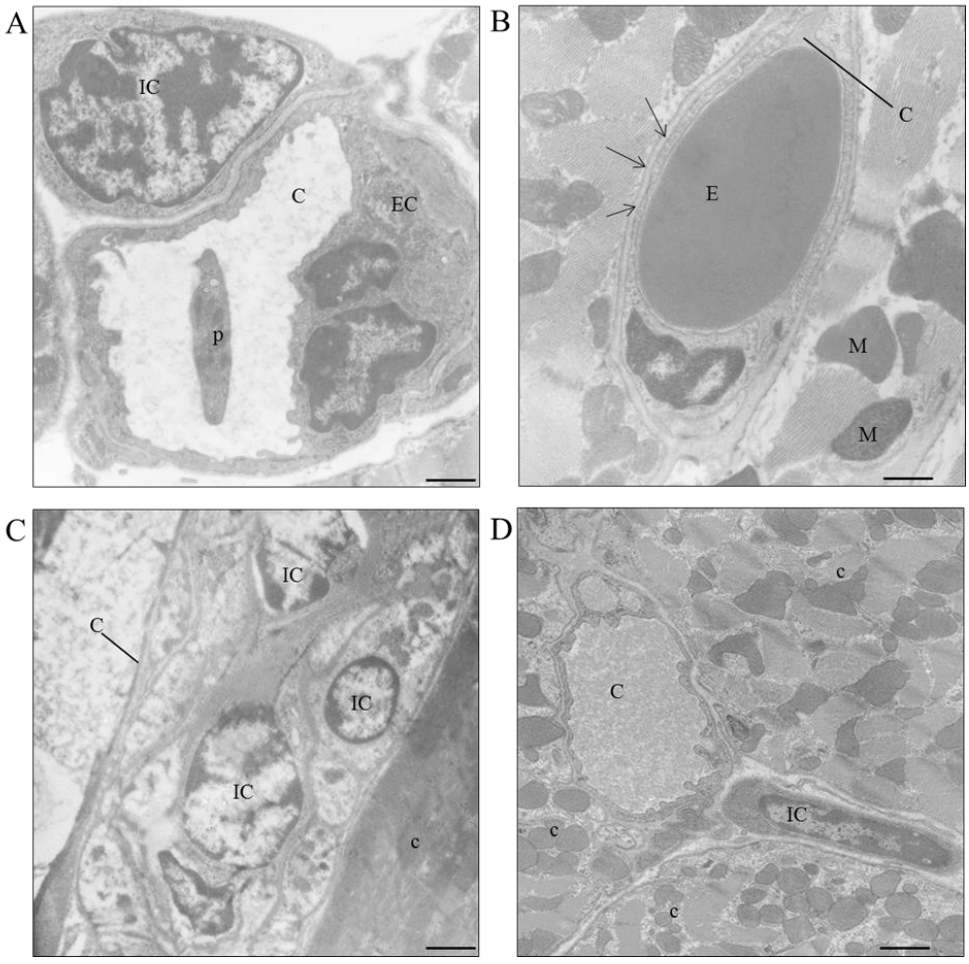

Figure2. *Ultrathin sections of heart of BALB/c mice infected with two strains DENV-2.* ***A*** *(66985-2000/IP): Platelet within capillary (p), endothelial cell (EC), cardiomyocytes (c). BAR = 0,8µm.* ***B*** *(66985-2000/IP): Disorganized cardiac muscle fibers (*) and endothelial cell presenting transport vesicles (arrow) and edema (E) within capillary (C). BAR = 0,4µm.* ***C*** *(0337-2008/IV): mononuclear inflammatory cells (IC) within capillary (C), cardiomyocyte (c). BAR = 1,5 µm.* ***D*** *(0337-2008/IV): mononuclear inflammatory cells (IC) within the interstice. BAR = 1,3µm. BAR = 2,2µm.*

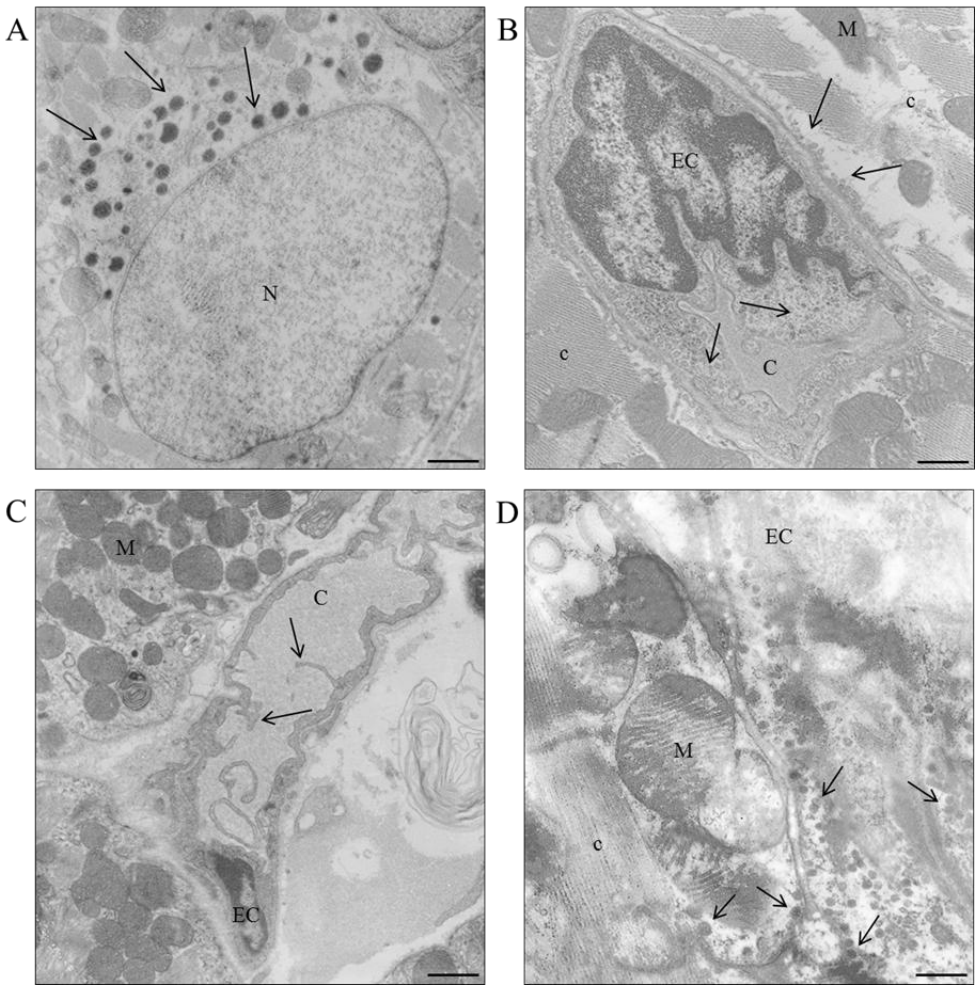

Figure3. *Ultrathin sections of heart of BALB/c mice infected with two DENV-2 strains.* ***A*** *(0337-2008/IV): Granular cytoplasmic perinuclear inclusions (arrow) in cardiomyocyte. BAR =0,6µm.* ***B*** *(66985-2000/IP): Reduced capillary lumen (C), endothelial cell (EC) showing thickened cytoplasm with presence of transport vesicles (arrow). BAR = 1,9µm.* ***C*** *(0337-2008/IV): Activated endothelial cell (EC) showing filopodia (arrow). BAR = 0,9µm.* ***D*** *(66985-2000/IV): DENV-like particles (arrow) within endothelial cell (EC) and cardiomyocyte (c) cytoplasm. Mitochondria (M), cardiomyocyte (c), erythrocytes (e). BAR = 0,2µm.*

4. DISCUSSION

The lack of an appropriate animal model that reproduces an infection similar to human cases of SD represents a challenge regarding the development of vaccine candidates against DENV. Studies suggested that mice are permissive hosts for DENV infection [20, 21]. In these models, neuroadapted strains were inoculated by invasive routes. Our group has carried out studies using BALB/c mice infected with non-neuroadapted DENV-2 by the IP and IV routes. Focal alterations in the lung, liver, cerebellum and kidney were demonstrated [22-25]. Virus particles and DENV specific antigen were observed in *Ae. albopictus* C6/36 cell line culture inoculated with the supernatant of macerated infected mice heart. Viral antigen was detected in endothelial cells of the liver and in hepatocytes [23, 25].

The Southeast Asian/American genotype of DENV-2 was introduced in Brazil in the 90's causing the first DHF/DSS cases and hospitalizations [26]. In 2007-2008, this serotype reemerged causing one of the most severe epidemics reported. Children were severely affected and a distinct DENV-2 Lineage was identified [15, 27]. However, the role of those Lineages in the disease severity was never addressed. In the present study, we described ultrastructural alterations and viral titers observed in heart and sera of BALB/c mice infected with two different Lineages of non-neuroadapted DENV-2 strains.

Platelets, edema and mononuclear inflammatory cells in capillaries and in tissue interstice and signs of endothelium activation, characterized by cytoplasm swelling and presence of electron-translucent vesicles and cytoplasmic membrane extensions were observed. Neither necrosis nor any changes in

the interaction between endothelial cells were observed. Cardiac muscle fibers were mostly preserved. These changes corroborate with Rasinhas' findings (unpublished data) on heart of BALB/c mice infected with non-neuroadapted DENV-1, -2 or -3 by the IV route. Cardiac function abnormalities in SD patients have been reported [28]. Studies demonstrated that primary and secondary dengue patients developed severe cardiac dysfunction including hypotension and arrhythmias, although cardiac involvement was significantly more pronounced during secondary infection [29]. The most commonly observed alterations are myocarditis and pericarditis [12, 13, 30-32]. The main histological findings were interstitial edema, inflammatory cell infiltration and necrosis of myocardial fibers [10]. The mechanism of myocardial involvement in dengue infection is not clearly understood. Viruses may invade the myocardium directly, damaging the muscle fibers or give rise to a hypersensitively or autoimmune reaction [13]. Multiple factors such as cytokines, tumor necrosis factor alpha and oxygen radicals are released during acute viral infections, which may play an important role in the pathogenesis of viral myocarditis [33-36]. It is becoming increasingly apparent that idiopathic dilated cardiomyopathy probably results from an acute viral myocarditis [35, 37, 38].

In our analyses, DENV-like particles were observed in the cytoplasm endothelial cells and cardiomyocytes of mice infected with two different DENV-2 strains. The DENV RNA was detected by Real time RT-PCR technique in those same samples and the virus titers observed were higher than those of the inoculum (in some cases) suggesting viral replication. These results corroborate with findings on autopsy samples and in murine models [7, 9, 39-41]. This suggests that DENV infected endothelial cells may contribute directly to pathogenesis by increasing viremia, secreting cytokines, modulating complement pathways, or transforming the endothelium into an immunologic target of cellular and humoral immune responses. Leakage of the vascular endothelium is a central component of dengue and studies suggest that the DENV infected endothelium may contribute to pathogenic immune responses and immune targeting of the endothelium.

5. CONCLUSION

Morphological alterations observed in heart of mice infected with DENV-2 were similar to those observed in human cases and confirm the susceptibility of BALB/c mice to non-neuroadapted DENV-2 strains. These data are important for the understanding of the involvement of heart during dengue infection in humans as well as of target cells for viral replication.

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