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Neotropical primary bat cell lines show restricted dengue virus replication

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ABSTRACT

Dengue is the most widespread arboviral disease affecting humans. Bats are recognized carriers of emerging viral zoonoses and have been proposed as dengue reservoirs, since RNA/NS1 and/or antiviral antibodies have been detected. Yet, experimental inoculation of *Artibeus* bats failed to show virus replication. This conflicting results prevent drawing further conclusions of whether bats sustain dengue infection. To test bat cellular permissivity to dengue infection, we established primary bat embryonic cells from diverse organs and tissues of *Artibeus jamaicensis*, *Molossus sinaloae*, and *Desmodus rotundus*. We observed a limited serotype-, organ-, and bat species- specific dengue susceptibility. Only some *Molossus*-derived primary cells sustained poorly initial Dengue serotype-1 replication, though it was latter absent. To elucidate if *Molossus* bats may play a role in dengue replication, ecological or in vivo experiments must be performed. Taken together our results show that Dengue did not replicate efficiently in cell lines derived from Neotropical bat species.

1. Introduction

Dengue is the most widespread arboviral disease that affects humans worldwide and a major cause of disease in tropical endemic countries [1]. Dengue virus (DENV) is divided into four distinct but genetically related serotypes [2]. Dengue causes a wide disease spectra in humans, which can go from a clinically unapparent infection to hemorrhagic fever and death [1]. The World Health Organization (WHO) estimates around 50–100 million human cases per year [2]. DENV in the old world is transmitted and maintained between two distinct sylvatic and urban cycles. The sylvatic cycle is well documented in Africa and Asia, where the virus is maintained between old world primates and *Aedes* mosquitoes [3,4]. Even though a definite animal host has not yet been identified in America, several research groups found evidence of infection in different mammals [5–7]. Within mammals, bats have been proposed as carriers of emerging viral zoonoses [8,9]. Furthermore, several groups have shown presence of viral nucleic acid and antibodies against dengue [5–7,10–14] suggesting a putative role as DENV reservoirs or hosts. Additionally, experimental infection studies in controlled laboratory settings showed no evidence of pathology or infection [15,16]. In one study, 35% of *Artibeus intermedius* bats infected with an excess of DENV-2 displayed seroconversion (even at low level) and one bat kidney as well as many sera were DENV RNA positive [16]. Nevertheless these results may be due to an excessively high viral dose for infection, suggesting that these bats are not suitable hosts for DENV-2 [16]. Similar results were found in systematic experimental inoculation of DENV serotypes 1 and 4 in *Artibeus jamaicensis* [15]. Other more recent study, suggested that American bats may not be reservoirs or amplification hosts for DENV infection since they were not able to detect virus RNA in liver or spleen tissue [17]. Overall results so far are conflicting and no conclusions can be withdrawn regarding the role of bats in DENV transmission and epidemiology. In vitro, one study using a bat cell line TB.1 Lu (derived from *Tadarida brasiliensis*) showed only DENV-1 infection by RT- PCR [18]. Another study in India failed to detect DENV-2 replication in a *Pipistrellus* embryonic cell line [19]. Most of these studies face limitations due to the bats species and organs used for generating cell lines. Therefore, studies about the susceptibility to in vitro infection by 4 DENV serotypes in different bat cells derived from several species are needed in order to assess if bat cells from different bat species or organs may be permissive to viral entry and be able to produce viral progeny in vitro. Here, we report on the susceptibility of different Neotropical primary bat cell cultures to the 4 DENV serotypes infection.

Abbreviations: DENV, dengue virus; MOI, multiplicity of infection; FBS, fetal bovine serum; RT-PCR, retro transcription-polymerase chain reaction.

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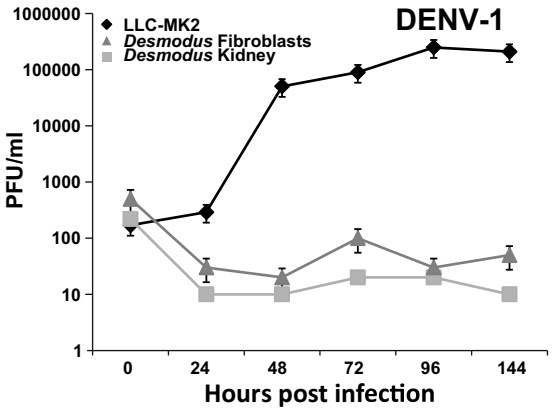

Fig 1. Viral growth kinetics of DENV serotype 1 in 2 *Desmodus* derived bat primary embryonic cells and LLC-MK2 cells. Cells were infected with DENV-1 at low MOI (0.1), absorbed at 37 °C during 1 h and incubated at 37 °C 5% CO_2. Supernatants were collected every 24 h. Titers of infectious virus were determined by a standard plaque assay in LLC-MK2 cells. One representative experiment of many is shown. Each value depicted represents the mean of triplicates (n = 3).

2. Materials and methods

2.1. Bats capturing and processing

Pregnant individuals of three bat species presenting different trophic guilds (*Artibeus jamaicensis*-frugivorous, *Molossus sinaloae*-insectivorous, and *Desmodus rotundus*-hematophagous) were captured. Permission for bat capturing and processing was obtained from the Institutional Committee of Care and Use of Animals of the University of Costa Rica (CICUA-124-12) according to international animal welfare standards and by University of Costa Rica project B4-656. The individuals were captured using mist nets and immediately transported to the laboratory. Bats were euthanized using a combination of Ketamine (1 mg/ml) and Xylazine (1 mg/ml).

2.2. Embryonic primary cell cultures

The excision of fetal tissues and isolation of primary bat cells were made as described elsewhere [20]. Briefly, after a 1- or 2-h trypsinization of fetal tissues (mesenchymal fibroblasts of the whole embryonic body without internal organs or the head) or whole dissected organs, cells were allowed to adhere and grow in a 5% CO_2 atmosphere in D-MEM supplemented with 10% FBS (All from Gibco, USA). We therefore established primary embryonic bat cultures resulting from different organs (kidney, liver, intestine) disaggregation and mesenchymal fibroblasts from the three bat species. Cultures derived from bat organs may include a mixture of different cell types consisting of epithelial, endothelial and organ characteristic (such as hepatocytes for the liver) cells. Primary bat cultures were screened for *Mycoplasma* sp. and Rabies virus by PCR (negative results, data not shown). No primary embryonic bat culture was used over passage 4 after sub-culturing to lessen cell differentiation or loss and to heighten culture homogeneity.

2.3. Detection of DENV virus infection through plaque quantification and immunofluorescence assays

Dengue reference viruses DENV-1 Angola (D1/AO/XX/1988), DENV-2 Jamaica (D2/JM/1409/1983), DENV-3 Nicaragua (D3/NI/30-94/1994), and DENV-4 Dominica (D4/DM/814669/1981) stocks were produced in C6/36 cells and titrated in LLC-MK2 using standard plaque assays.

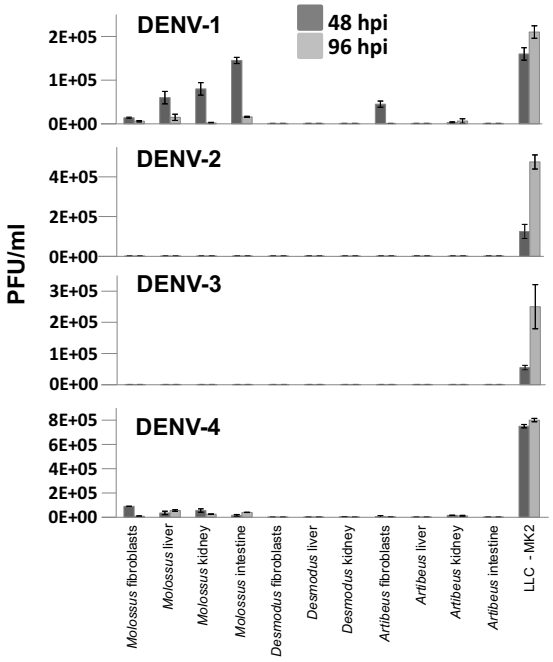

Fig. 2. Viral growth kinetics of DENV serotypes 1–4 in different bat primary embryonic cell lines derived from different bat species and LLC-MK2 cells. Cells were infected with DENV at an MOI of 2–3, absorbed at 37 °C during 1 h and incubated at 37 °C 5% CO_2. Supernatants were collected every 48 h. Titers of infectious virus were determined by a standard plaque assay in LLC-MK2 cells. One representative experiment of three is shown. Each value depicted represents the mean of triplicates (n = 3).

For viral growth kinetics, 2 different *Desmodus* derived bat primary embryonic cells and LLC-MK2 cells were infected with DENV-1 at a low MOI (0.1), adsorbed at 37 °C during 1 h and incubated at 37 °C 5% CO_2. Supernatants were collected every 24 h. Titers of infectious virus were determined by a standard plaque assay in LLC-MK2 cells.

For determination of permissivity for the 4 DENV serotypes, different bat primary embryonic cell cultures derived from different bat species and LLC-MK2 cells (as positive controls) were infected with DENV serotypes at a higher virus dose (MOI of 2–3), since lack of virus production was observed with a low virus dose, absorbed at 37 °C during 1 h and incubated at 37 °C 5% CO_2. Supernatants were collected and infectious virus titer was determined by a standard plaque assay in LLC-MK2 cells at 48 and 96 h post infection.

Primary embryonic bat cells were infected with DENV-1 and DENV-2 (MOI of 2) respectively and viral infection was monitored by immunofluorescence. After 24 h of infection, cells were fixed with methanol, incubated for 15 min with a permeabilization solution (PBS 1%, Saponin 0.1% and FBS 1%) and stained using an anti-NS3 protein antibody (GENETEX 124252), an anti- DENV 1–4 antibody (recognizing E and other structural proteins) (GENETEX 29202), and secondary antibody goat anti-rabbit IgG (H + L) Alexa Fluor 488 (Invitrogen) or goat anti-mouse IgG FITC (Sigma-Aldrich), respectively. Nuclei were counterstained with DAPI. Images were taken using a Cytation 3 Cell Imaging Multi-Mode reader (BioTek Instruments, Inc., USA). An image analysis pipeline was developed using Cell Profiler software 2.1.1 (http://www.cellprofiler.org/citations.shtml).

3. Results

3.1. Primary bat cell lines show restricted dengue virus replication

To analyze DENV virus infection of primary bat cells, first we assessed if 2 different cell lines sustained viral replication by