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Requirement of cholesterol in the viral envelope for dengue virus infection

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ABSTRACT

The role of cholesterol in the virus envelope or in the cellular membranes for dengue virus (DENV) infection was examined by depletion with methyl-beta-cyclodextrin (MCD) or nystatin. Pretreatment of virions with MCD or nystatin significantly reduced virus infectivity in a dose-dependent manner. By contrast, pre-treatment of diverse human cell lines with MCD or nystatin did not affect DENV infection. The four DENV serotypes were similarly inactivated by cholesterol-extracting drugs and infectivity was partially rescued when virion suspensions were treated with MCD in the presence of bovine serum. The addition of serum or exogenous water-soluble cholesterol after MCD treatment did not produce a reversion of MCD inactivating effect. Furthermore, virion treatment with extra cholesterol exerted also a virucidal effect. Binding and uptake of cholesterol-deficient DENV into the host cell were not impaired, whereas the next step of fusion between virion envelope and endosome membrane leading to virion uncoating and release of nucleocapsids to the cytoplasm appeared to be prevented, as determined by the retention of capsid protein in cells infected with MCD inactivated-DENV virions. Thereafter, the infection was almost completely inhibited, given the failure of viral RNA synthesis and viral protein expression in cells infected with MCD-treated virions. These data suggest that envelope cholesterol is a critical factor in the fusion process for DENV entry.

1. Introduction

Dengue virus (DENV) belongs to the genus *Flavivirus* in the family *Flaviviridae* and is presently the most important causative agent of human viral disease transmitted by mosquitoes, with clinical presentations ranging from mild febrile illness and dengue fever to life-threatening dengue hemorrhagic fever and dengue shock syndrome (Guzmán et al., 2010; Kyle and Harris, 2008). The virion is an enveloped particle containing a positive-sense RNA genome and three structural proteins (envelope E, membrane M and capsid C proteins). There are four serotypes (DENV-1 to DENV-4) which co-circulate in over 100 countries from tropical and subtropical regions where the virus is endemic. Thus, vaccine strategies and antiviral therapies for DENV are urgently demanded and there is a great need to discover novel targets involved in DENV infection to establish successful approaches for treatment of the disease.

The initiation of infection by an enveloped virus relies on the binding to specific cellular receptors followed by the fusion of the viral envelope with either the endosomal or the plasma membrane of the cell. As such, alterations in the membrane lipid environment can interfere with this process and represent an interesting target to block infection. Especially, cholesterol was found to play an important role in different steps of virus life cycle, including virus entry to the host cell, but not all enveloped viruses are dependent on the presence of cholesterol in both the viral and cellular target membrane for proper infection. The infectivity of influenza virus (Sun and Whittaker, 2003), canine distemper virus (Imhoff et al., 2007) and hepatitis B virus (Bremer et al., 2009) is sensitive to cholesterol depletion only from the viral membrane whereas, in the reverse situation, murine leukemia virus (Lu et al., 2002), Ebola and Marburg virus (Bavari et al., 2002) are sensitive to cholesterol depletion only from the cellular membrane. Cholesterol in both membranes is required for infection with human immunodeficiency virus (Graham et al., 2003; Liao et al., 2001), transmissible gastroenteritis virus (Ren et al., 2008; Yin et al., 2010), borna disease virus (Clemente et al., 2009) and herpesviruses (Bender et al., 2003; Desplanques et al., 2008, 2010; Hambleton et al., 2007), whereas vesicular stomatitis virus infection was shown to be independent from cellular and viral cholesterol depletion (Imhoff et al., 2007; Moore et al., 1978; Ren et al., 2008; Thorp and Gallagher, 2004).

With respect to DENV, the results about cellular cholesterol function for infection are controversial. The entry of DENV-1 and DENV-2 was found independent of plasma membrane cholesterol depletion in mosquito C6/36 cells (Acosta et al., 2008, 2011; Mosso et al., 2008) as well as in monkey Vero cells (Acosta et al., 2009) and human endothelial-like ECV304 cells (Peng et al., 2009) by

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sequestering cholesterol with methyl-beta cyclodextrin (MCD), nystatin or filipin treatment. Similarly, the infection of C6/36 cells cholesterol-depleted by serial passages in medium containing delipidated serum demonstrated that DENV-1 and DENV-2 did not require cholesterol in the target cell membrane (Umashankar et al., 2008). By contrast, in other studies a requirement of cellular cholesterol was reported for DENV-2 infection by treatment of human monocytes (Reyes-del Valle et al., 2005) or mouse neuroblastoma cells (Lee et al., 2008) with MCD or filipin. It is not clear at present if differences may be due to the cell type or the treatment conditions to alter the membrane cholesterol content. Concerning to the importance of cholesterol as a constituent of the virion, preliminary data showed that pretreatment of virus suspensions with MCD before infection produced a drastic reduction in DENV-2 infectivity (Acosta et al., 2009). This prompted us to examine the effects of cholesterol content on the infectivity of the four DENV serotypes by using MCD or nystatin and to analyze the mechanism of inactivation of virion infectivity after treatment with these cholesterol-binding agents. Simultaneously, the effect of cholesterol extraction from different types of human cell lines on DENV serotype infectivity was also evaluated to understand whether cholesterol is important as a constituent of the virus, the host cell or both.

2. Materials and methods

2.1. Cells and viruses

Vero (African green monkey kidney) cells were grown in Eagle's minimum essential medium (MEM) (GIBCO, USA) supplemented with 5% fetal bovine serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%. The C6/36 mosquito cell line from *Aedes albopictus*, adapted to grow at 33 °C, was cultured in L-15 Medium (Leibovitz) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% MEM non-essential amino acids solution and 10% fetal bovine serum. The human myelomonocytic cell line U937, the human erythroleukaemic K562 cells, and the B cell line Raji/DC-SIGN$^+$, Raji cells transfected with DC-SIGN (Wu et al., 2004) kindly provided by Dr. J. Geffner (Facultad de Ciencias Médicas, Universidad de Buenos Aires, Argentina), were grown in RPMI medium 1640 supplemented with 10% fetal bovine serum and 50 μg/ml gentamycin. Medium was supplemented with $HCl/NaHCO_3$ when incubated at 37 °C under 5% CO_2.

The stocks of DENV-1 strain Hawaii, DENV-2 strain NGC, DENV-3 strain H87 and DENV-4 strain 8124 were prepared in C6/36 cells and titrated by plaque formation in Vero cells. Herpes simplex virus type 1 (HSV-1) strain KOS and vesicular stomatitis virus (VSV) strain Indiana were propagated and titrated in Vero cells.

2.2. Depletion and replenishment of cholesterol from virus

For extraction of viral envelope cholesterol, virus stocks were previously separated from cellular debris by centrifugation at 10,000 × g during 30 min. Then, the clarified virus suspensions containing 1×10^6 PFU/ml were incubated at 37 °C with phosphate-buffered saline (PBS) without serum containing or not MCD (Sigma–Aldrich, USA) in the range 0.078–1.25 mM during 1 h or nystatin (Sigma–Aldrich, USA), 3–100 μM during 2 h. Then, samples were chilled, diluted in MEM without serum at least 100-fold to avoid effects of drugs on cells, and remaining infectivity was determined by plaque formation in Vero cells. For cholesterol replenishment, the treatment with compounds and infection was performed in PBS supplemented with 2% fetal bovine serum. The addition of PBS with 2% serum after the previous MCD treatment of virus suspension without serum was also tested.

To analyze the effect of cholesterol treatment on virion infectivity, a DENV-2 suspension containing 1×10^6 PFU/ml was incubated with water-soluble cholesterol (Sigma–Aldrich), 0.19–1.5 μM, during 1 h at 37 °C. Then, samples were diluted and remaining infectivity was titrated by plaque formation in Vero cells.

2.3. Depletion of cholesterol from cells

For removal of cellular cholesterol, cells were washed with PBS and pretreated with MCD (0.625–5 mM) or nystatin (25–100 μM) in MEM without serum during 1 h at 37 °C. Thereafter, cells were washed three times with PBS to remove compound before virus infection, followed by incubation at 37 °C for 1 h. The virus inocula were then discarded; cultures were washed with PBS and further incubated at 37 °C in MM without compound. At 24 h p.i. for VSV and HSV-1 infected cultures, and 48 h p.i. for DENV infected cultures, supernatants were harvested to determine extracellular virus yields by plaque formation.

The time of treatment and range of concentrations adequate for each cell system were previously evaluated by a cell viability assay in order to avoid any toxic effects. Vero, U937, K562 and Raji/DC-SIGN$^+$ cells grown in 24-well microplates were incubated in MEM without serum containing or not different compound concentrations and the number of viable cells was then determined by Trypan blue exclusion. According to the viability data obtained (not shown) the treatment conditions for MCD and nystatin were chosen as above reported.

2.4. Determination of cholesterol

Cellular and viral cholesterol content was determined using the Amplex Red Cholesterol assay kit (Molecular Probes, USA). For cellular determinations, samples containing equal cell numbers were treated with MCD as described, then washed with PBS and centrifuged at 1000 × *g* for 2 min. The pellet was resuspended in 100 μl of reaction buffer and homogenized through a 25-gauge needle, followed by cholesterol determination according to the manufacturer's instructions, using a spectrofluorometer FLUOstar Optima (BMG Labtech) at 540-nm excitation and 590-nm emission wavelengths. For virus cholesterol content, clarified virus samples containing 1×10^7 PFU/ml of MCD-treated and untreated virion suspensions were filtered through cellulose membranes (Vivacon 500, 100,000 MWCO, Sartorius) to eliminate free drug and then the assay kit was used as above.

2.5. Virus-cell binding assay

A DENV-2 suspension containing 1.5×10^7 PFU/ml was treated or not with 1.25 mM MCD during 1 h at 37 °C. Then, control and inactivated virions were filtered through cellulose membranes to eliminate free drug and Vero cell monolayers were infected for 1 h at 4 °C. Thereafter, cells were extensively washed with cold PBS to remove unadsorbed virus and total RNA was extracted from cells by using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. For quantification of the amount of cell-bound viral RNA, a real time RT-PCR assay was conducted utilizing Taq Man technology as previously described (Talarico and Damonte, 2007).

2.6. Internalization assay

Vero cells were infected with control and MCD-treated DENV-2 as for binding assay. After 1 h adsorption at 4 °C, cells were washed with PBS and incubated at 37 °C for 1 h. Then, cells were washed with PBS and treated with proteinase K (Invitrogen, USA) for 45 min at 4 °C to remove adsorbed but non internalized virus. After proteinase K inactivation with 2 mM phenyl-methyl-sulfonide fluoride (PMSF) in PBS containing 0.5% bovine seroalbumin (PBS–BSA), cells

were washed with PBS–BSA by low speed centrifugation. Total RNA was extracted from cells by using TRIzol and the amount of internalized viral RNA was quantified by real-time RT-PCR as described above. The number of internalized DENV RNA molecules per culture was expressed after subtracting the amount of DENV RNA molecules present in cultures in which immediately after virus adsorption at 4 °C, proteinase K treatment was performed.

2.7. Uncoating assay by envelope-endosome membrane fusion

Vero cells grown in coverslips were infected with control and MCD-treated DENV-2 as for binding assay during 1 h at 4 °C. Thereafter, cells were washed with cold PBS, covered with MM and incubated al 37 °C. At different times (0, 10, 45 min) cells were fixed with methanol for 10 min at −20 °C. After methanol fixation, cells were washed with PBS and stained with a mouse monoclonal antibody (mAb) against DENV-2C capsid protein (clone 6F3.1) (Bulich and Aaskov, 1992), kindly provided by Dr. J. Aaskov (University of Queensland, Australia) followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma–Aldrich, USA). After a final washing with PBS, cells were mounted in a glycerol solution containing 1,4 diazabicyclo [2,2,2]octane (DABCO) and visualized under a fluorescence microscope Olympus BX51 with a 100× objective lens. For quantification, cells were visualized using a confocal microscope Olympus fv1000 with a 60× objective lens and images were analyzed with Fiji Software to express density of fluorescent particles per cell.

2.8. Synthesis of viral RNA

Vero cells were infected with control and MCD-treated DENV-2 as for binding assay. After adsorption MM was added and incubation was followed at 37 °C during 24 h. Thereafter, total RNA was extracted from cells by using TRIzol and the amount of viral RNA was quantified by real-time RT-PCR as described above.

2.9. Expression of viral proteins by immunofluorescence and Western blotting

Vero cells grown in coverslips were infected with a DENV-2 suspension containing 1×10^6 PFU/ml treated or not with 1.25 mM MCD and MM was added after adsorption. At 24 h p.i., cells were fixed with methanol for 10 min at −20 °C. Then, cells were stained with a mouse mAb against DENV-2 C capsid protein (clone 6F3.1) or a mouse mAb reactive against E glycoprotein (Abcam, United Kingdom). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) during 2 min at 37 °C and extensively washed before mounting in DABCO as described above.

For Western blotting, Vero cells were infected as above with control and MCD-treated DENV-2. At 24 h p.i., cells were harvested in sample buffer for electrophoresis (5% sodium dodecyl sulfate (SDS), 2% 2-mercaptoetanol, 10% glycerol and 0.005% bromophenol blue in 0.0625 M Tris–HCl, pH 6.8) (Promega, USA). After boiling the lysates during 5 min, proteins were separated by 15% SDS-polyacrylamide electrophoresis (PAGE) and blotted onto a PDVF membrane (Millipore, USA). Membranes were incubated with the above mentioned anti-E mAb overnight at 4 °C. After washing with Tris-buffered saline (TBS, 20 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 0.1% Tween 20, a second incubation was performed with horseradish peroxidase-conjugated anti-mouse IgG during 1 h at room temperature. As control, the presence of β-actin was revealed by incubation with rabbit polyclonal anti-actin antibody (Cell Signalling Technology, USA) followed by incubation with peroxidase-conjugated anti-rabbit IgG (Amersham, USA). Secondary antibodies were visualized by enhanced chemiluminescence (ECL–Amersham, USA).

2.10. Effect of MCD treatment on virion antigenicity

A DENV-2 suspension containing 1.5×10^7 PFU/ml was treated or not with 1.25 mM MCD during 1 h at 37 °C. Then, control and inactivated virions were centrifugated at 46,000 rpm (Beckman ultracentrifuge equipped with a SW55Ti rotor) during 1.5 h and the pellet was lysed in 20 μl of sample buffer for electrophoresis. Proteins were fractionated by 15% SDS-PAGE and DENV-2 E glycoprotein was visualized by Western blotting with anti-E mAb as above described.

3. Results

3.1. Depletion of cholesterol from the viral envelope impairs DENV infection

The importance of cholesterol in viral envelope for DENV infectivity was first analyzed by depletion of cholesterol from virus suspension by MCD treatment prior to infection. This drug sequesters cholesterol by extraction of this lipid from the membranes without penetrating them (Ilangumaran and Hoessli, 1998; Zidovetzki and Levitan, 2007). To determine the virucidal activity of MCD, a suspension of DENV-2 was incubated with different drug concentrations during 1 h at 37 °C, then samples were diluted and remaining infectivity was titrated by plaque formation in Vero cells. The sample dilution effectively reduced the drug concentration to be incubated with the cells at least 100-fold to assess that any titer reduction was only due to virion inactivation. As depicted in Fig. 1A, increasing drug concentrations in the range 0.078–1.25 mM resulted in a dose-dependent decrease of DENV-2 infectivity with 50% reduction at a concentration of 0.0514 ± 0.0002 mM and more than 99% inhibition of virion infectivity at MCD concentrations higher than 0.312 mM. HSV-1 was assayed as positive control known to be dependent of virion-associated cholesterol for successful infection (Bender et al., 2003). As seen in Fig. 1A, HSV-1 virion infectivity was highly reduced by MCD treatment although the level of inhibition at very low concentrations of MCD was not so drastic as observed for DENV-2. VSV was included as negative control because it is known to bud from nonraft detergent sensitive membrane areas of the cell (Brown and Lyles, 2003; Scheiffele et al., 1999). Similarly as reported in other studies (Imhoff et al., 2007; Moore et al., 1978; Ren et al., 2008), VSV infectivity was almost unaffected in the presence of low doses of MCD and a weak reduction in virus titer, lower than 50% respect to control untreated virus, was observed at the highest MCD concentrations (Fig. 1A).

To further assess the requirement of virion-associated cholesterol for DENV infection, we also tried to inhibit infection by exposure of DENV-2 to nystatin, another cholesterol targeted drug that has been shown to bind cholesterol forming a complex at the membrane (Anderson et al., 1996). Nystatin also exerted an inactivating effect on DENV-2 virions, but the highest level of inhibition achieved was 87% at the maximum noncytotoxic concentration tested of nystatin (100 μM) (Fig. 1B) whereas MCD produced more than 99% inhibition in DENV-2 infectivity at noncytotoxic concentrations. This difference may be ascribed to the different mode of interaction with cholesterol exerted by both compounds (Anderson et al., 1996; Ilangumaran and Hoessli, 1998; Zidovetzki and Levitan, 2007). Again, HSV-1 and VSV were included as positive and negative controls, respectively: HSV-1 was similarly inhibited by nystatin as DENV-2 whereas VSV was not significantly affected (Fig. 1B).

It has been reported that the inhibitory effect against DENV of certain compounds may be variable according to virus serotype (Hidari et al., 2008; Kaptein et al., 2010; Talarico and Damonte, 2007; Talarico et al., 2005). However, in our case the virucidal activity of cholesterol-depleting drugs was not dependent on

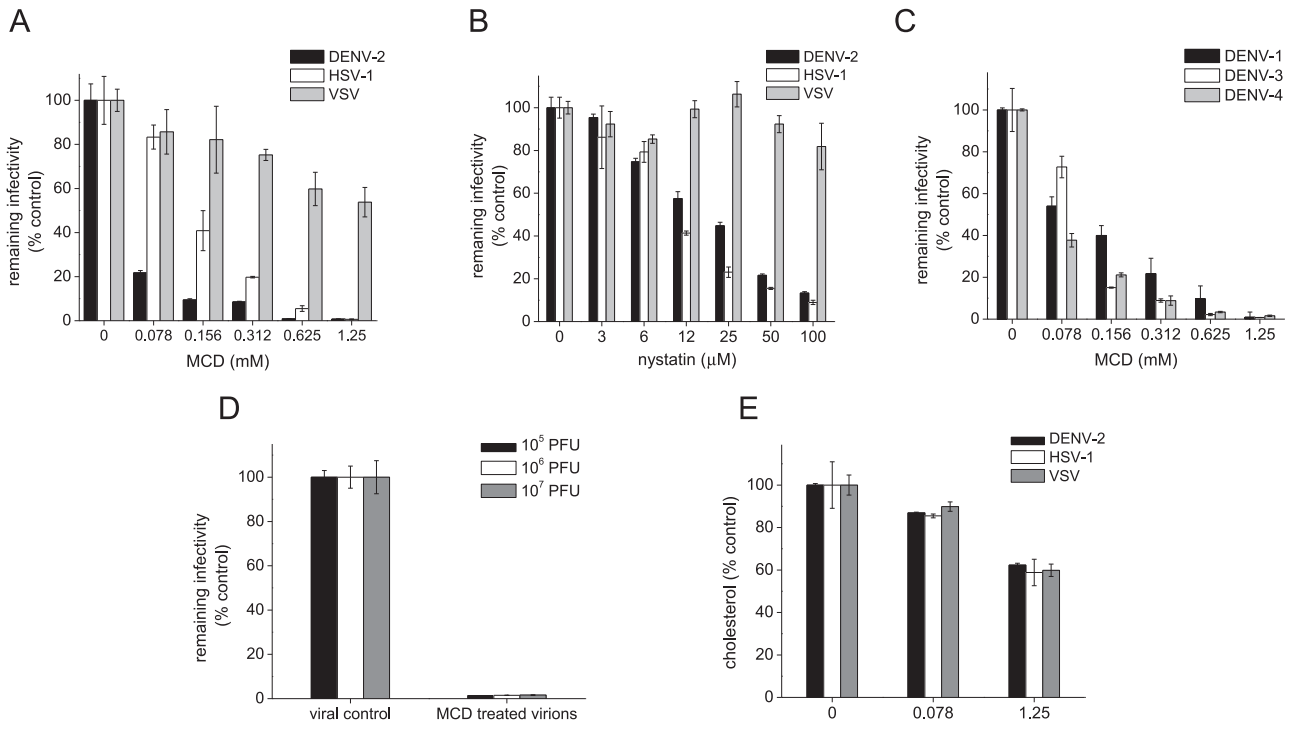

Fig. 1. Cholesterol depletion from the viral envelope. (A and B) Suspensions of DENV-2, VSV and HSV-1 containing 1×10^6 PFU/ml were treated with various concentrations of MCD (A) or nystatin (B) for 1 h. Then, samples were diluted and remaining infectivity was determined by PFU in Vero cells. (C) Suspensions of DENV-1, DENV-3 and DENV-4 containing 1×10^6 PFU/ml were treated with MCD and remaining infectivity was measured as in A. (D) Suspensions of DENV-2 containing 10^5, 10^6 or 10^7 PFU/ml were treated with 1.25 mM MCD and remaining infectivity was measured as in A. (E) After treatment of DENV-2, HSV-1 and VSV suspensions with MCD, the virion cholesterol content was determined using the Amplex Red Cholesterol assay kit. Values represent the mean ± SD of two independent experiments.

DENV serotype. A similar level of reduction in virus infectivity was observed after treatment of DENV-1, DENV-3 and DENV-4 suspensions with MCD (Fig. 1C). Furthermore, the effectiveness of MCD to reduce the infectivity of virions was not importantly affected by the initial virus inoculum. When the inactivation assay was performed by incubation of different amounts of DENV-2 with 1.25 mM MCD, the inhibition in remaining infectivity was similar in the range 10^5–10^7 PFU (Fig. 1D).

To corroborate that the strong decrease observed in infectivity was caused by cholesterol extraction from the virus envelope, the cholesterol content in MCD-treated and untreated DENV-2 suspensions was determined using a cholesterol assay kit. The cholesterol level of DENV-2 virions after incubation with 1.25 mM MCD was found to be reduced about 50% in comparison to untreated virions (Fig. 1E), a level of depletion similar to that described for other viruses after exposure to these doses of MCD (Ren et al., 2008; Sun and Whittaker, 2003). Similarly, the cholesterol levels were also reduced after treatment of HSV-1 and VSV suspensions with MCD (Fig. 1E).

To further analyze the critical role of cholesterol in virion envelope, MCD treatment of DENV-2 suspension was done in the presence of fetal bovine serum (FBS) to re-establish cholesterol levels. A partial and significant recovery of infectivity in virions treated with low concentrations of MCD in the presence of FBS was observed with respect to the samples incubated without serum, and also the level of cholesterol was increased in virion suspensions (Fig. 2A and C). By contrast, when serum was added after MCD treatment of virions in PBS without serum the infectivity was not recovered (data not shown), suggesting that the effect of MCD was not reversible and the simultaneous presence of serum cholesterol is required to compete for MCD and reduce the loss of infectivity. We also attempted to reverse the inactivating effect by restoring the cholesterol level through the addition of exogenous water-soluble cholesterol during the MCD treatment. But we were unable to test successfully this experimental approach due to the virucidal activity of free cholesterol against DENV-2. In fact, the treatment of a DENV-2 suspension with different concentrations of cholesterol showed a dose-dependent inhibition of virus infectivity with 50% inhibition at 1.32 ± 0.28 μM and about 90% inhibition at a very low concentration such as 1.5 μM (Fig. 2B). In spite of this reduction in infectivity, the level of cholesterol in virions after addition of 1.5 μM soluble cholesterol was similar to control values (Fig. 2D). Since the cholesterol concentrations usually employed for replenishment during MCD treatment are around 50–100 μM (Bremer et al., 2009; Imhoff et al., 2007; Ren et al., 2008; Sun and Whittaker, 2003; Yin et al., 2010), it was technically impossible to restore infection completely with this experimental protocol.

3.2. Depletion of cholesterol from cellular membranes of human cell lines does not prevent DENV infection

Otherwise, to investigate the functional importance of cholesterol in cell membranes for DENV infection experimental depletion of cholesterol by MCD was also intended from cell cultures previous to infection. We tested the monkey Vero cell line, a cell system routinely used for DENV titration, as reference system, and the lymphoid human cell lines U937, K562 and Raji/DC-SIGN+, more representative of the natural infection. Detrimental effects of MCD on cell viability, which might impair virus infection independently of cholesterol reduction, were first discarded by measuring cell viability after compound treatment. No effects on cell viability were detected for Vero and human cell lines by Trypan blue staining and cell count after 1 h MCD treatment up to a concentration of 2.5 mM for U937 cells and 5 mM for Vero, K562 and Raji/DC-SIGN+ cells whereas more prolonged times of cell treatment with the drug reduced the number of viable cells (data not shown).

To investigate the role of cellular cholesterol on DENV-2 infection, cells were pretreated with noncytotoxic concentrations of

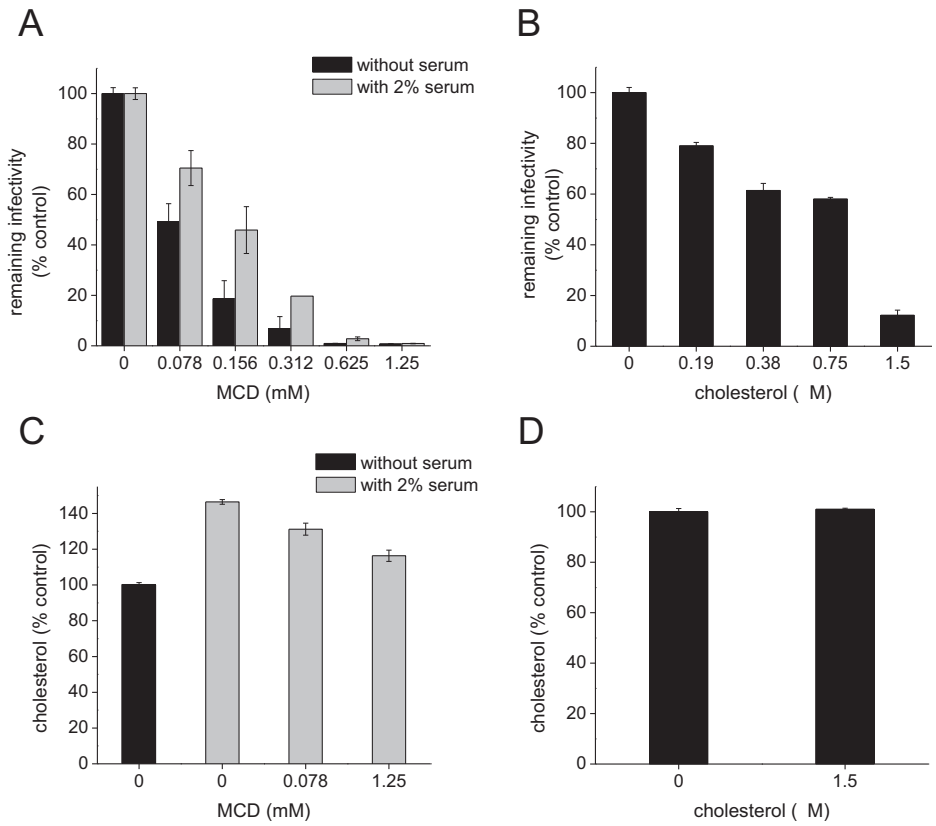

Fig. 2. Effect of cholesterol replenishment on virion infectivity. (A) A suspension of DENV-2 was treated with various concentrations of MCD for 1 h in PBS containing or not 2% fetal bovine serum. Then, remaining infectivity was determined by PFU in Vero cells. (B) A suspension of DENV-2 was treated with various concentrations of water-soluble cholesterol for 1 h, and then remaining infectivity was determined by PFU in Vero cells. Values represent the mean ± SD of two independent experiments. (C and D) Virion cholesterol was determined in DENV-2 suspensions treated with MCD (C) or cholesterol (D) as described in A or B. Values represent the mean ± SD of two independent experiments.

MCD for 1 h at 37 °C. Then, cultures were extensively washed and infected with DENV-2 in the absence of compound. It is important to note that our experimental protocol was designed to avoid the effects of MCD on virion envelope. These treatment conditions allowed to test the effects of MCD on DENV-2 infection by affecting the lipid content of cell membrane but without a direct contact between drug and virions that could lead to virus inactivation. As seen in Fig. 3A, the efficacy of DENV-2 infection in Vero cells, determined by virus yield at 48 h p.i., was not reduced at any MCD concentration. The profile of the dose-response curve for DENV-2 was similar to that obtained for VSV (Fig. 3A), tested as virus control reported to successfully infect cells irrespective of cholesterol depletion from cell membranes (Imhoff et al., 2007; Ren et al., 2008; Thorp and Gallagher, 2004). By contrast, infection of Vero cells by HSV-1, a virus dependent on viral and cellular cholesterol (Bender et al., 2003), was highly reduced even at a very low concentration of 1.25 mM MCD, indicating the effectiveness of the compound treatment.

Next, the human cell lines U937, K562 and Raji/DC-SIGN$^+$ were tested for DENV-2 infection in the same treatment conditions as Vero cells. DENV-2 infection was supported in the three human cell systems after MCD treatment with the same effectiveness as in non-treated cells (Fig. 3B). A similar lack of effect on DENV-2 infection of human cells was detected by cell treatment with nystatin (Fig. 3C), confirming that cholesterol is not required for infection. As described for MCD, the cytotoxicity of nystatin was evaluated in the different cell lines previous to assay the effect of this drug on DENV-2 infectivity, resulting in a maximum noncytotoxic concentration of 100 μM.

The efficiency of cholesterol reduction in the MCD-treated monkey and human cell lines was assessed by determining cellular cholesterol content after treatment. A dose-dependent cholesterol reduction was observed in the four cell systems although no alterations on DENV-2 multiplication were detected. Similar results were previously obtained in mosquito C6/36 cells (Acosta et al., 2008, 2011) indicating that the independence of DENV-2 infection respect to cellular cholesterol depletion is a property common to diverse vertebrate and invertebrate cell types.

3.3. Mechanism of blockade of infection by cholesterol-depleted virions

We next analyzed the infection of Vero cells with cholesterol depleted virus to determine which step is arrested in the inactivated virus life cycle and is consequently critically affected by the cholesterol failure in DENV-2 envelope. First, virus cell binding was investigated. To this end, DENV-2 was treated with MCD, then free compound was depleted by filtration and virions were attached to Vero cells during 1 h at 4 °C. Virus control was similarly processed but without MCD treatment. The amount of cell-bound virus was measured by quantitative real-time RT-PCR. MCD-inactivated DENV-2 particles were attached to Vero cells with the same efficacy as control infectious virions (Fig. 4A).

Next, the effect of MCD on DENV-2 internalization into host cell was analyzed. Vero cells were allowed to adsorb virions treated or not with MCD during 1 h at 4 °C, and then infected cultures were incubated at 37 °C for 1 h. Thereafter, cultures were processed to determine the amount of viral RNA inside the cells. As seen in

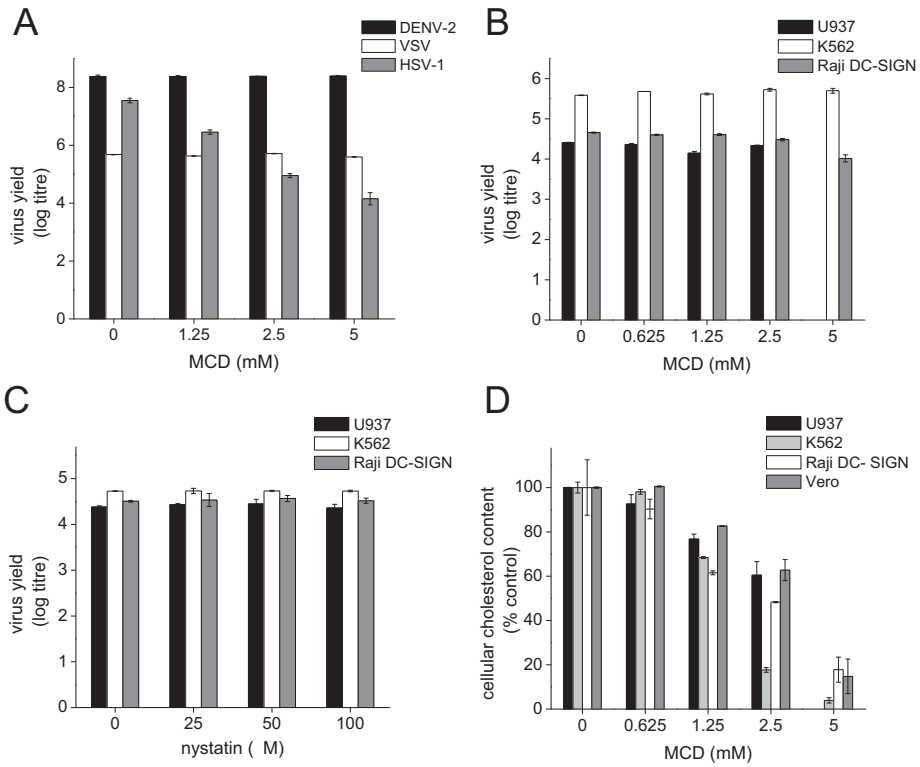

Fig. 3. Cholesterol depletion from cellular membranes. (A) Vero cells were pretreated with various concentrations of MCD during 1 h. Then, monolayers were washed and subsequently infected with DENV-2, HSV-1 or VSV. Virus yields were determined by plaque formation at 24 h p.i. (HSV-1 and VSV) or 48 h p.i. (DENV-2). (B, C) U937, K562 and Raji/DC-SIGN$^+$ cells were pretreated with various concentrations of MCD (B) or nystatin (C) during 1 h. Thereafter, cells were washed and infected with DENV-2, and virus yields were determined at 48 h p.i. by plaque formation in Vero cells. (D) After treatment of Vero, U937, K562 and Raji/DC-SIGN$^+$ cells with various concentrations of MCD during 1 h, the cellular cholesterol content was measured with Amplex Red cholesterol assay kit. Results represent the mean ± SD of two independent experiments.

Fig. 4B, the number of internalized DENV-2 RNA molecules was similar in cells infected with infectious or inactivated virions.

The following step in flavivirus multiplication cycle is virion uncoating triggered by low pH-induced fusion of the viral envelope with the endosomal membrane leading to nucleocapsid release into the cytoplasm. To evaluate whether cell-associated cholesterol-depleted DENV-2 was capable of undergoing fusion and uncoating in the endosomes, we monitored the distribution of capsid protein in cells infected with control or inactivated-DENV-2 within the initial period of incubation at 37 °C after virion binding at 4 °C. It has been previously reported that when DENV-2 envelope-endosomal membrane fusion takes place the genome is released to the cytoplasm together with the capsid protein which consequently first disappears from endocytic vesicles, a process that is almost completed after 30 min of infection at 37 °C, and then from cytoplasm (Acosta et al., 2012). As expected, in Vero cells infected with infectious DENV-2 a bright dotted fluorescence pattern for C protein was observed in the cytoplasm at 10 min post-adsorption (Fig. 4C). In agreement with the kinetics of fusion reported for DENV-2 infection in Vero cells (Acosta et al., 2012), the amount of fluorescence corresponding to protein C was highly reduced resulting almost undetectable at 45 min post-attachment (Fig. 4C). By contrast, in cells infected with MCD inactivated-DENV-2 the dotted fluorescence of C protein remained constant between 10 and 45 min after the beginning of internalization, indicating the blockade of uncoating during infection with cholesterol-depleted DENV-2.

The maintenance of the fluorescence levels corresponding to C protein staining was verified by quantification of the fluorescent signal. The number of fluorescent particles/cell/100 μm^2 at 10 min p.i were 11.4 ± 3.6 and 10.2 ± 1.9 for control and MCD-treated virions, respectively; at 45 min p.i., the value in cells infected with control virions was reduced to 2.2 ± 1.5 (81% reduction respect to 10 min p.i.) whereas in cells infected with treated virions fluorescence remained at 9.5 ± 2.6 (only 6.6% reduction respect to 10 min p.i.), confirming the retention of C protein in the cells infected with cholesterol-depleted DENV-2.

To further examine whether the viral RNA uncoating was affected in inactivated virions, intracellular viral RNA was harvested from cells infected with control and MCD-treated virion suspensions and analyzed by real-time RT-PCR. Quantification of DENV-2 RNA synthesized after infection with MCD-treated virions showed a strong level of inhibition, with almost 3 log reduction in comparison to DENV-2 control infected cells (Fig. 4D), confirming that viral RNA cannot be released to the cytoplasm and subsequently replicated.

We also analyzed the synthesis of DENV-2 proteins in infected cells to verify that viral protein expression was blocked due to the prevention of uncoating of MCD-inactivated virions. As shown in Fig. 4E, the expression of both the capsid C protein and the envelope E glycoprotein, as determined by indirect immunofluorescence staining at 24 h p.i., were prevented in Vero cells infected with inactivated DENV-2 in comparison to control virus infection. The unability of inactivated virions to be translated was further assessed through other experimental approach such as Western blotting. Again, DENV-2 E glycoprotein was not detected in cells infected with MCD-inactivated DENV-2 in contrast to the strong expression observed after infection of Vero cells with control untreated DENV-2 (Fig. 4F), assessing that inactivated virions were unable to perform the process of protein synthesis.

3.4. MCD does not affect virion surface antigenicity

To further examine the effects of MCD treatment on DENV-2 virus particles, we analyzed if the extraction of viral cholesterol

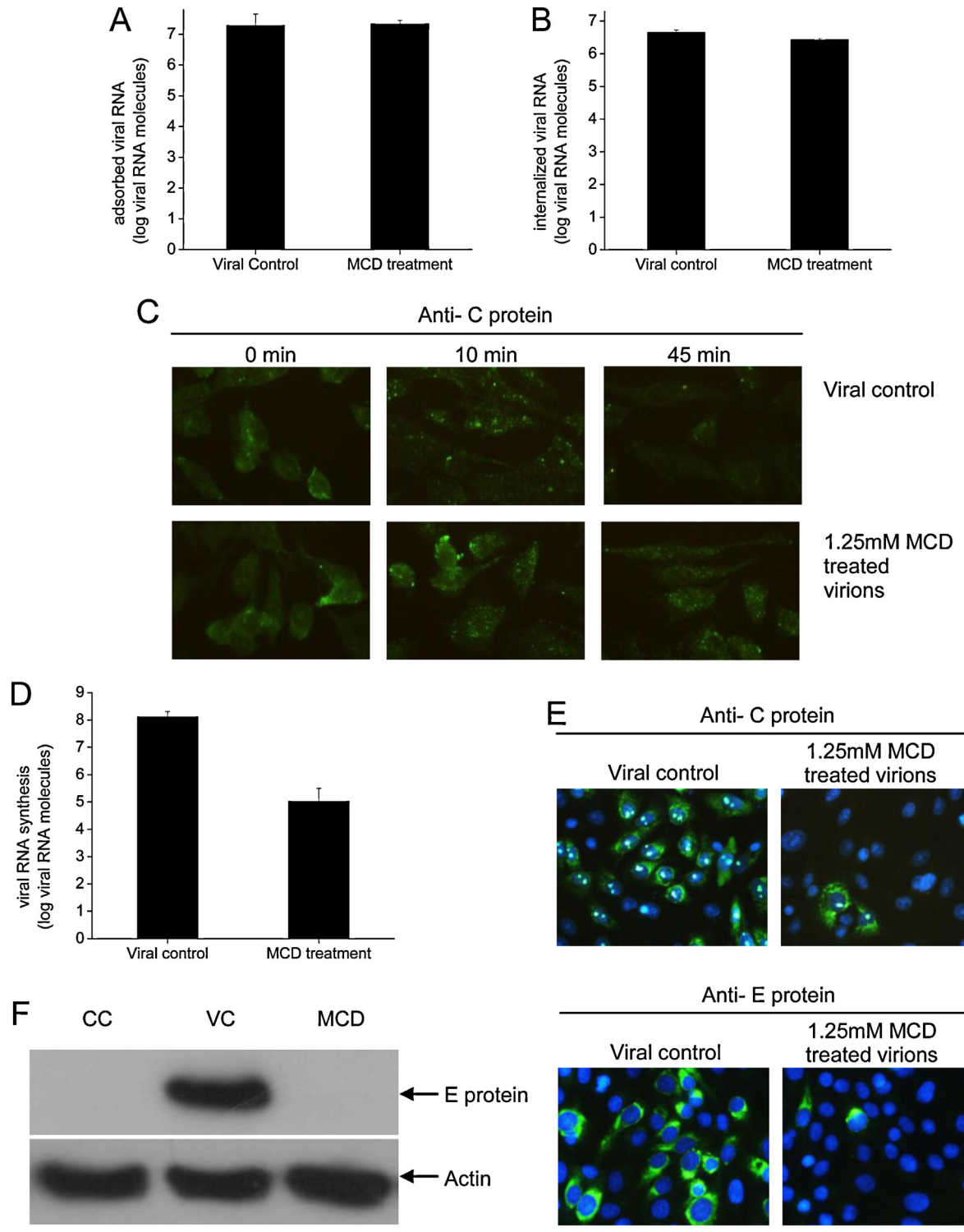

Fig. 4. Infection with MCD-inativated virions. (A) Virus binding. DENV-2 suspensions treated or not with 1.25 mM MCD during 1 h at 37 °C were filtered through cellulose membranes to eliminate free drug and Vero cell monolayers were infected for 1 h at 4 °C. Thereafter, total RNA was extracted and the amount of bound DENV-2 RNA molecules was determined by quantitative real-time RT-PCR. (B) Virus internalization. Control and MCD-treated DENV-2 was adsorbed to Vero cells at 4 °C for 1 h. Then, cells were incubated at 37 °C for 1 h and treated with proteinase K. Total RNA was extracted from the cell pellet and real-time RT-PCR was performed to determine the amount of internalized viral RNA molecules. (C) Virus uncoating. Control or MCD-treated DENV-2 were adsorbed during 1 h at 4 °C and then cultures were shifted to 37 °C. At different times, cells were fixed and processed to reveal C protein by immunofluorescence using a mouse anti-C mAb followed of FITC-labeled goat anti-mouse IgG. (D) Virus RNA synthesis. Vero cells were infected as for binding assay and total RNA was extracted after 24 h of infection. Real time RT-PCR was performed to determine the amount of viral RNA in the cytoplasm. (E, F)Virus protein expression. Vero cells were infected with control and MCD-treated DENV-2. At 24 h p.i., a set of cell cultures were fixed and stained with a mouse mAb against DENV-2 C capsid protein or a mouse mAb reactive against E glycoprotein followed of FITC-labeled goat anti- mouse IgG (E). Other set of cell cultures were lysed in sample buffer, electrophoresed on 15% SDS-PAGE and E glycoprotein was detected in cell extracts by Western blot with anti-E mAb (F). (CC: cellular control, VC: cells infected with viral control, MCD: cells infected with MCD treated virions).

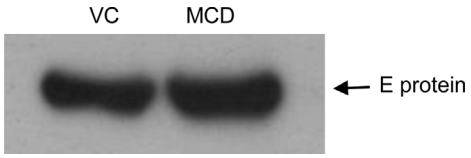

Fig. 5. Effect of MCD on virion antigenicity. Clarified DENV-2 suspensions were treated or not with 1.25 mM MCD during 1 h at 37 °C, then samples were lysed in sample buffer, resolved by electrophoresis in 15% SDS-PAGE and E glycoprotein was detected by Western blot with anti-E mAb. (VC: control untreated virions, MCD: MCD treated virions).

with this compound alters the antigenicity of the virion surface protein. MCD-treated or untreated DENV-2 suspensions were assayed against a mouse anti-E glycoprotein mAb by Western blotting. As shown in Fig. 5 the reactivity of E glycoprotein with mAb was not affected by MCD treatment and the electrophoretic pattern of the envelope DENV-2 protein was similar in treated and untreated virions. Then, MCD treatment with the observed cholesterol depletion inactivated the infectivity of DENV-2 virions without significantly affecting the antigenicity of surface glycoprotein.

4. Discussion

In conclusion, the results presented in this study show a differential requirement of cholesterol in cellular and viral membranes for the initiation of a productive DENV infection. Pre-treatment of different human cell lines with cholesterol-reactive compounds like MCD or nystatin did not affect the subsequent infection with DENV-2, whereas the incubation of virions with these compounds previous to cell infection lead to a strong inhibition of infectivity. Furthermore, the virion cholesterol requirement for infectivity seems to be a conserved feature of the four DENV serotypes.

Regarding to cellular cholesterol, our results are coincident with previous studies discarding the requirement of cholesterol in the vertebrate or invertebrate target cell for effective DENV infection (Acosta et al., 2008, 2009; Mosso et al., 2008; Peng et al., 2009, Umashankar et al., 2008) but are contradictory with other studies performed with this virus in MCD-treated cells (Lee et al., 2008; Reyes-del Valle et al., 2005). These contrasting results could reflect variations in the characteristics of endocytosis process or the requirements for cholesterol during virus internalization among different cells or virus strains. For example, Reyes-del Valle et al. (2005) reported MCD inhibition for infection of human peripheral blood monocytes with the 16,681 strain of DENV-2 in a range of concentrations up to 15 mM MCD, whereas in the cell systems we assayed here, shown in Fig. 3A and B, MCD was cytotoxic at concentrations higher than 2.5–5 mM. The variations reported about the role of cholesterol in cell membranes can also be ascribed to differences in the treatment conditions to achieve cholesterol depletion and the time of infection. It must be considered that the virucidal activity of cholesterol-depleting drugs here reported may be a factor complicating the inference about the role of cellular cholesterol-rich microdomains for fusion and infection. In fact, a direct effect of MCD or similar agents against DENV was not analyzed in other studies reporting a requirement of cellular cholesterol. Concerning to this point, Lee et al. (2008) concluded that cholesterol depletion by MCD reduced DENV-2 infection of the mouse neuroblastoma cells N18 after a continuous treatment of cells with MCD during infection, a treatment condition that did not exclude the virucidal action of MCD against DENV-2. Due to this virus-inactivating ability of MCD and nystatin, we only performed cell-pretreatment and did not include the compounds during virus inoculation and all the period of infection. Consequently, under the experimental conditions used here we can conclude that the presence of cholesterol in cellular membranes is not essential for the initial events of virus entry, but it cannot be discarded a role for cellular cholesterol in later steps of the virus cycle.

Concerning to virion cholesterol, for several enveloped viruses belonging to quite diverse families it has been reported that cholesterol depletion from the viral membrane results in reduction of infectivity. In this group are included human immunodeficiency virus 1 (Graham et al., 2003), human herpesvirus 6 (Huang et al., 2006), canine distemper virus (Imhoff et al., 2007), transmissible gastroenteritis virus (Ren et al., 2008), varicella-zoster virus (Hambleton et al., 2007), pseudorabies virus (Desplanques et al., 2010), duck and human hepatitis B virus (Funk et al., 2008; Bremer et al., 2009), influenza virus (Sun and Whittaker, 2003), and Lassa virus (Schlie et al., 2010). In the family *Flaviviridae*, a critical role of virion-associated cholesterol was also demonstrated for the human pathogen hepatitis C virus and, as here shown for DENV, the presence of cholesterol in hepatitis C virions appeared more crucial for infection than the content in plasma membrane (Aizaki et al., 2008). Our results here have demonstrated, for the first time, a very high susceptibility of the four DENV serotypes to the inactivating effect of MCD. This loss of infectivity was associated to a reduction in cholesterol content of virions, however the provision of exogenous cholesterol by fetal bovine serum or as water-soluble cholesterol did not allow a full recovery of virion infectivity. Only the simultaneous incubation of virions with MCD and serum cholesterol produced a partial retrieval of infectious particles, but the inactivating action was not reversible when cholesterol or serum was added after treatment with MCD. This irreversible action of MCD on virus infection has been also reported by other authors, linked to failure in cholesterol replenishment (Desplanques et al., 2010; Lee et al., 2008; Sun and Whittaker, 2003). Then, it appeared that MCD mainly affect DENV infectivity by cholesterol extraction but a secondary cholesterol-independent effect of the compound may be also occurring. It cannot be discarded that MCD also extracted other lipids from the viral envelope, but several studies reported only minimal release of phospholipids from membranes in comparison with cholesterol after exposure to cyclodextrins (Zidovetzki and Levitan, 2007).

Noticeably, the incubation of virions only with exogenous cholesterol exerted also an inactivating effect on infectivity. This inhibitory action of cholesterol was not related to an increase in the cholesterol amount detected in virions in comparison to control untreated DENV particles. Interestingly, a blockade in DENV-2 infection by cholesterol treatment of virions was also observed by Lee et al. (2008), and the authors suggested that it could be due to an increase in envelope rigidity resulting in a barrier for membrane fusion and leading to a freezing in the step of virus entry.

It is also noticeable the high susceptibility of DENV to MCD treatment, since the infectivity of DENV was reduced by compound concentrations significantly lower than those required to affect infectivity of other viruses: a 50% reduction in DENV infectivity was detected in the presence of 0.05 mM MCD whereas the values reported for other viruses are higher than 1 mM. The cholesterol content of viral envelope may be related to this behavior. For arboviruses belonging to *Togaviridae* such as Sindbis virus and Mayaro virus, the lipid composition of the viral envelopes as well as their cholesterol content resemble that of the host cell membrane from which viruses bud (Hafer et al., 2009; Sousa et al., 2011). Flaviviruses acquire their envelope at the endoplasmic reticulum and are then presumably secreted through the constitutive secretion pathway (Welsch et al., 2009). The endoplasmic reticulum typically contains only a very small fraction, less than 1%, of the cell cholesterol (Lange et al., 1999). Furthermore, in our experiments DENV stocks were grown in C6/36 cells, a line derived from *Aedes albopictus*. Mosquito cells are cholesterol auxotrophs and contain low levels of this sterol, only provided by the bovine serum present in culture medium, in comparison to mammalian cells (Clayton, 1964;

Krebs and Lan, 2003). Then, the content of cholesterol in mosquito cells-grown DENV-2 is very low in comparison to Vero cells-grown virus, and concomitantly the MCD concentration required to produce a similar level of reduction in infectivity is 10-fold higher for DENV-2 produced in Vero cells respect to DENV-2 propagated in C6/36 cells (data not shown).

In the present study, we were especially interested in mechanistic studies to elucidate how the interaction of the lipid viral envelope with MCD affects virion infectivity. As shown, neither virus attachment nor virus uptake were blocked in infection with MCD-inactivated DENV virions in comparison to infection with control virus particles, indicating that the initial interaction of E glycoprotein with the host cell receptor was not affected. Similarly, the functional structure of the viral envelope protein required for antigenic reactivity did not appear to be altered as determined by monoclonal antibody recognition in Western blotting assay. By contrast, the next step of fusion between virion envelope and endosome membrane appeared to be prevented, stopping the loss of capsid protein observed in control infected cells as consequence of virion uncoating. Thereafter, the infection is almost completely inhibited, given the failure of viral RNA synthesis and viral protein expression in cells infected with MCD-treated virions. Envelope cholesterol depletion by MCD also markedly affected the fusion process of influenza virus (Sun and Whittaker, 2003). Still it remains to precisely establish how the compound interacts within the envelope of DENV particle to produce the rearrangements in the lipid bilayer that affect fusion of viral and cellular membranes and block the endosomal escape of the incoming virions.

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