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Infectious dengue-1 virus entry into mosquito C6/36 cells

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ABSTRACT

The entry of dengue virus-1 (DENV-1) strain Hawaii into mosquito C6/36 cells was analyzed using a variety of biochemical inhibitors together with electron microscopy. The treatment with ammonium chloride, chlorpromazine, dansylcadaverine and dynasore inhibited virus yields, determined by infectivity titrations, whereas nystatin and methyl-β-cyclodextrin did not have any effect. The effect of the clathrin and dynamin inhibitors on DENV-1 entry was corroborated by detection of internalized virions using immunofluorescence staining. Furthermore, electron micrographs showed the incoming virions attached to electron-dense invaginations of the plasma membrane and within coated vesicles that resembled clathrin-coated pits and vesicles, respectively. The susceptibility to clathrin and dynamin inhibitors of clinical isolates from recent outbreaks was comparable to that shown by the cell culture-adapted reference strain. Similarly, DENV-3 strain H87 and DENV-4 strain 8124 were also inhibited in the presence of ammonium chloride, chlorpromazine and dynasore, allowing conclude that the infectious entry of DENV serotypes to mosquito cells occurs by low pH-dependent clathrin-mediated endocytosis.

1. Introduction

Dengue virus (DENV), a mosquito-borne member of the genus *Flavivirus* in the family *Flaviviridae*, is the causative agent of a relatively benign febrile illness in humans known as dengue fever or the more severe and potentially lethal forms of dengue hemorrhagic fever/dengue shock syndrome. Approximately 2.5 billion people live in more than 100 countries in tropical and subtropical areas at risk of DENV transmission and 50–100 million new infections resulting in about 25,000 deaths are estimated to occur each year (Gubler, 2002; Guzman and Kouri, 2002). There are four serotypes, DENV-1 to DENV-4, which all co-circulate in the endemic areas and are transmitted to humans through the bite of infected *Aedes* mosquitoes, mainly *Ae. aegypti* and *Ae. albopictus*, with continuous occurrence of mosquito–human–mosquito transmission cycles. No antiviral agents or vaccines for treatment or prevention are currently available.

Virions are small enveloped particles containing a nucleocapsid with a positive-sense RNA genome of approximately 11 kb. Virus entry to the host cell is the initial event of the infective cycle that involves virion attachment to the specific cell receptors followed by internalization into the cytoplasm. For DENV infection, this early interaction seems to be particularly complex in both vertebrate and mosquito cells (Acosta et al., 2008b; Kaufmann and Rossmann, 2011). The envelope E glycoprotein is involved in both attachment and internalization, representing a key determinant for infection, but the receptor in mosquito cells has not been fully identified. Several cellular proteins generally characterized only by molecular weight were reported as putative DENV receptors: two glycoproteins of 40 and 45 kDa were proposed as DENV-4 receptors in C6/36 cells, a cell line derived from *Ae. albopictus* larvae (Igarashi, 1978), as well as in diverse tissues from eggs, larvae and adult *Ae. aegypti* mosquitoes (Salas-Benito and del Angel, 1997; Salas-Benito et al., 2007; Yazi Mendoza et al., 2002); two proteins of 67 and 80 kDa were recognized by the four DENV serotypes in C6/36 cells and midgut cells of *Ae. aegypti* (Mercado-Curiel et al., 2006); diverse proteins in the range 37–77 kDa were able to bind the four DENV serotypes in *Ae. aegypti* and *Ae. polynesiensis* salivary glands (Cao-Lorneau, 2009). Only recent reports have characterized more precisely proteins expressed by mosquito cells as viral receptors: a laminin-binding protein was identified as candidate receptor for DENV-3 and DENV-4, but not DENV-1 and DENV-2, in C6/36 cells (Sakoonwatanyoo et al., 2006) whereas prohibitin was reported for DENV-2 binding in both *Ae. albopictus* and *Ae. aegypti* cell lines and mosquitoes (Kuadkitkan et al., 2010). Other proteins like heat shock cognate 70 (Hsc70), tubulin or tubulin-like were also shown to interact with DENV-2 E glycoprotein and proposed to participate in virus entry into mosquito cells (Chee and AbuBakar, 2004; Paingankar et al., 2010).

The mechanism for penetration in mosquito cells is also poorly understood. Earlier studies showed by electron microscopy observation that DENV-2 penetrated directly into the cytoplasm of C6/36 cells by fusion of the virion envelope with the plasma membrane at physiological pH (Hase et al., 1989). By the contrary, more recent

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studies have provided evidence that uptake of DENV-2 in C6/36 cells for productive infection occurs through low pH-dependent receptor-mediated endocytosis (Acosta et al., 2008a; Mosso et al., 2008). There is no knowledge about the mode of entry in mosquito cells for other DENV serotypes different from DENV-2. Interestingly, alternative endocytic routes were found for entry of different DENV serotypes in mammalian cells (Acosta et al., 2009; Van der Schaar et al., 2008).

In this report, the infective entry pathway of DENV-1 strain Hawaii into C6/36 cells was analyzed with biochemical inhibitors of endocytosis and electron microscopy observations. Studies were also partially extended to clinical isolates of DENV-1 and reference strains of DENV-3 and DENV-4. Taking in consideration the previous reports about DENV-2 (Acosta et al., 2008a; Mosso et al., 2008), our results assessed the functional requirements of pH-dependent clathrin-mediated endocytosis for the entry of the four DENV serotypes to mosquito cells.

2. Materials and methods

2.1. Cells and viruses

The C6/36 mosquito cell line from *Aedes albopictus* adapted to grow at 33 °C was cultured in L-15 medium (Leibovitz) (GIBCO, USA) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% minimum essential medium (MEM) non-essential amino acids solution and 5% fetal calf serum. Vero (African green monkey kidney) cells were grown at 37 °C in Eagle's MEM (GIBCO, USA) supplemented with 5% calf serum. For maintenance medium (MM) of L-15 and MEM serum concentration was reduced to 1.5%.

The stocks of the DENV-1 strain Hawaii (HW), DENV-3 strain H87, DENV-4 strain 8124 and the clinical isolates of DENV-1 ARG9920 and ARG0044 (provided by Dr. A. Mitschenko, Hospital R. Gutiérrez, Buenos Aires, Argentina) were prepared in C6/36 cells and titrated by plaque formation on Vero cells.

2.2. Cell viability assay

Monolayers of C6/36 cells were incubated in MM containing or not different concentrations of the compounds for 2 h at 33 °C. Cell viability was measured after each treatment by the [3-(4,5-dimethylthiazol-2-yl)]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich, USA) method as described previously (Talarico et al., 2005).

2.3. Inhibition of virus infection by inhibitor treatment

C6/36 cells grown in 24-well microplates were pretreated for 1 h at 33 °C with different concentrations of the inhibitors chosen according to the viability data: ammonium chloride (10–50 mM), chlorpromazine (10–50 μM), dansylcadaverine (25–200 μM), nystatin (12.5–200 μM), methyl-β-cyclodextrin (1–5 mM) and dynasore (30–150 μM). The drugs were purchased from Sigma–Aldrich (USA). Then, cells were infected at an m.o.i. of 0.1 in the presence of the drugs, except for nystatin and methyl-β-cyclodextrin where pretreated cultures were intensively washed with PBS before infection. Virus inocula were removed after 1 h of infection at 33 °C, and then cultures were washed with PBS and further incubated at 33 °C in MM without compound. At 48 h post-infection, supernatants were harvested to determine extracellular virus yields by plaque assay in Vero cells.

To assess the effect of ammonium chloride on the pH of acid intracellular vesicles, C6/36 cells, treated or not with the compound during 1 h at 33 °C were stained with 1 μg/ml acridine orange in MM without serum for 15 min at 33 °C. Cells were washed twice with PBS, mounted on PBS and visualized under an Olympus BX51 fluorescence microscope.

To corroborate the effectiveness of endocytic pathway inhibitor treatment, transferrin or cholera toxin uptake studies were performed. C6/36 cells treated for 1 h with chlorpromazine, dansylcadaverine or dynasore were then incubated with 15 μg/ml rhodamine (TRITC)-human transferrin (Molecular Probes, USA) for 1 h at 33 °C, washed with PBS, fixed with methanol for 10 min at −20 °C and visualized under the fluorescence microscope. Similarly, for nystatin or methyl-β-cyclodextrin treated cultures, cells were incubated with 0.3 μg/ml fluorescein isothiocyanate (FITC)-cholera toxin B subunit (Sigma–Aldrich, USA) for 1 h at 33 °C, washed and fixed as above.

2.4. Internalization of virion particles by indirect immunofluorescence

C6/36 cells grown in 24-well microplates were pretreated for 1 h at 33 °C with chlorpromazine 50 μM, dansylcadaverine 200 μM, nystatin 200 μM, methyl-β-cyclodextrin 5 mM or dynasore 150 μM. Then, cells were infected with DENV-1 at an m.o.i. of 10 PFU/cell in the presence of the drugs. At 30 min p.i., cells were washed with PBS, fixed with methanol for 10 min at −20 °C and stained with monoclonal antibody against the E glycoprotein (Abcam, UK) followed by FITC-labelled goat anti-mouse IgG (Sigma–Aldrich, USA).

Phase contrast observation of cells treated with the clathrin and dynamin inhibitors under the same conditions were also performed.

2.5. Electron microscopy

C6/36 cells grown in 6 well microplates were infected with DENV-1 (m.o.i. = 60), incubated for 60 min at 4 °C and then shifted to 33 °C for 25 min. At this time, cells were washed with cold PBS and fixed with 1.5% glutaraldehyde in phosphate buffer 0.1 M during 4 h at 4 °C. Cells were scraped and incubated overnight at 4 °C with 0.32 M sucrose. Then, cells were pelleted and further incubated with 1.5% OsO_4 containing 0.32 M sucrose for 2 h at 4 °C. Thereafter, cells were pelleted and washed with distilled water. Cells were incubated overnight with 2% uranyl acetate and dehydrated with a series of ethanol gradients followed by propylene oxide, embedded in Epon 812 Resin mixture (TAAB), and polymerized at 70 °C for 2 days. Ultrathin sections were re-stained with 2% uranyl acetate and observed in an electronic microscope C10 Zeiss.

3. Results

3.1. Low pH-dependence

In order to assess if DENV-1 entry into mosquito cells is pH-dependent, we first determined if infection of C6/36 cells with DENV-1 strain HW requires low endosomal pH. The importance of endosome acidification was studied with ammonium chloride, a lysosomotropic weak base that immediately raises the pH of intracellular acidic vesicles. Treatment of C6/36 cells with ammonium chloride before infection and during the first hour of infection at 33 °C reduced the amount of virus release from infected cells in a dose-dependent manner, with maximum effect at a concentration of 50 mM and without affecting cell viability (Fig. 1A). To ensure that the drug treatment used effectively increased the pH of intracellular vesicles in C6/36 cells, we performed acridine orange staining. Untreated cells showed the typical cytoplasmic orange fluorescence of the acid compartments, but cells treated with ammonium chloride did not exhibit this fluorescence pattern (Fig. 1B).

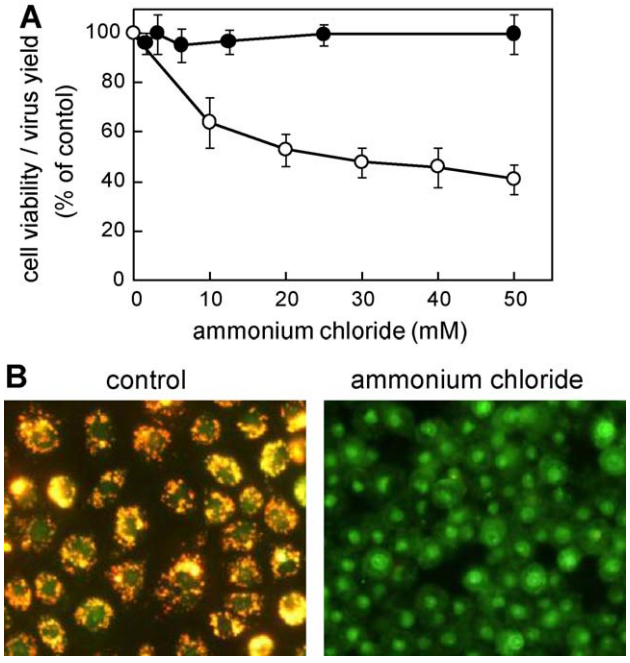

Fig. 1. Low pH-dependence of DENV-1 entry into mosquito cells. (A) Cells were treated with various concentrations of ammonium chloride, and then infected with DENV-1. Virus yields were quantified by PFU at 48 h p.i. and results are expressed as % of virus multiplication with respect to a control of infected cells without drug treatment (○). Each bar is the mean value of two independent experiments ± standard deviation. Other set of cultures were treated with compound as above and then cell viability was determined by MTT method (●). (B) Cells were treated or not with 50 mM ammonium chloride and then stained with acridine orange.

3.2. Clathrin and caveolae-dependence

The pH-dependent entry of DENV-1 suggests that the virus is internalized from the cell surface by receptor-mediated endocytosis and reaches an endosomal compartment where the fusion occurs. To determine if DENV-1 enters mosquito cells through the well-defined clathrin-mediated pathway, as recently reported for the serotype DENV-2 in *Ae. albopictus*-derived cells (Acosta et al., 2008a; Mosso et al., 2008), the effect on virus infection of two pharmacological inhibitors of this endocytic via, chlorpromazine and dansylcadaverine was tested. Chlorpromazine is a cationic, amphiphilic molecule that acts by disturbing the assembly of clathrin lattices at cell surfaces and endosomes (Wang et al., 1993), whereas dansylcadaverine is an extensively used monoamine that inhibits receptor internalization by preventing its clustering in clathrin-coated pits (Haigler et al., 1979; Levitzki et al., 1980; Zheng et al., 2008). As seen in Fig. 2A, a dose-dependent inhibition of DENV-1 infection was obtained in C6/36 cells treated with any of both compounds at noncytotoxic concentrations during the first hour of infection. The internalization of TRITC-labelled transferrin, a ligand known to enter into the cell by clathrin-mediated endocytosis, was used as a functional control to assess that the action of the inhibitors was effectively exerted on receptor-mediated endocytosis from clathrin-coated pits. In control untreated cells a dotted fluorescence pattern inside the cell cytoplasm was observed, whereas cultures treated with chlorpromazine or dansylcadaverine exhibited a widespread fluorescence only at the cell surface, indicating that transferrin uptake was efficiently blocked (Fig. 2B).

The clathrin-dependent endocytic pathway for DENV-1 entry into mosquito cells was further characterized by probing the participation of dynamin, a GTPase essential for pinching off endocytic vesicles. The effect of dynasore, a recently developed dynamin inhibitor (Macia et al., 2006), on DENV-1 infection of C6/36 cells was evaluated. The treatment with this drug during the first hour of infection did not affect cell viability but reduced virus yield more than 1–2 log at the highest concentrations tested (Fig. 2A) and as a control of drug effect, the internalization of TRITC-labelled transferrin was proved (Fig. 2B).

We next corroborated that the effect of clathrin- and dynamin-dependent inhibitors was exerted at the internalization of DENV-1 into mosquito cells. C6/36 cells were infected with DENV-1 (m.o.i. 10 PFU/cell) in the presence of chlorpromazine, dansylcadaverine or dynasore, and after 30 min of infection cells were fixed and the internalized virions were detected by fluorescence staining with an antibody reactive against E glycoprotein. In control infected cultures, DENV-1 particles showed a dotted fluorescence profile in cytoplasm indicative of the virus localization in endocytic vesicles, whereas in drug-treated cells the incorporation of viral particles was drastically reduced exhibiting a diffuse fluorescence similar to the pattern observed for transferrin staining in treated cultures (Fig. 2C). No background fluorescence staining was detected in uninfected cells (Fig. 2C). Furthermore, the treatment with clathrin and dynamin inhibitors did not affect cell morphology as corroborated by phase microscopy in cultures treated under the same conditions (Fig. 2C).

If a clathrin-mediated endocytosis is used by DENV-1 to enter into mosquito cells, it can be predicted that agents perturbing caveolae will not affect virus infection. To verify this supposition, we examined the effect on DENV-1 entry of two sterol-binding drugs, methyl-β-cyclodextrin and nystatin. Methyl-β-cyclodextrin sequesters cholesterol by extraction of this lipid from membranes and nystatin forms a complex with cholesterol at the membrane (Anderson et al., 1996; Ilangumaran and Hoessli, 1988; Pelkmans et al., 2001). As cholesterol is a prominent component of lipid rafts involved in caveolae formation, the cholesterol-depleting activity of these drugs lead to inhibition of caveolae-mediated endocytosis. The specific action of both compounds was tested using FITC-labelled cholera toxin as a marker of this endocytic pathway (Fig. 2B). Neither DENV-1 virus yield determined by infectivity determination nor the cellular uptake of virions detected by immunofluorescence staining were reduced by nystatin or methyl-β-cyclodextrin (Fig. 2A and C). By the contrary, an increase in virus production from infected C6/36 cells was detected in nystatin-treated cultures (values higher than 100% of control in Fig. 2A).

3.3. Ultrastructural analysis

Finally, electron microscopy studies were performed to assess the entry route of DENV-1 into C6/36 cells by other experimental approach and visualize the cell structures involved. Cells infected with DENV-1 were first incubated at 4 °C for virus adsorption, and then shifted to 33 °C for 30 min to allow virus internalization. The virus particles were in the size-range of 44–52 nm, with an electron-dense core enveloped in a membrane bilayer (Fig. 3). Virions at different stages of the entry pathway could be observed: particles in the extracellular space close to thickened regions of the plasma membrane (Fig. 3A), then incoming virions linked to electron-dense invaginations that resembled those of clathrin-coated pits (Fig. 3 B), and finally single virions within coated vesicles with a diameter about 100–150 nm, according to the size range described for clathrin-coated vesicles (Fig. 3C) (Bishop, 1997; Ehrlich et al., 2004).

3.4. Clinical isolates

Both pharmacological inhibitors-mediated inhibition and electron microscopy studies indicated that DENV-1 HW enters mosquito cells through clathrin-coated vesicles. Since DENV-1 HW is a reference virus strain adapted to in vitro propagation after

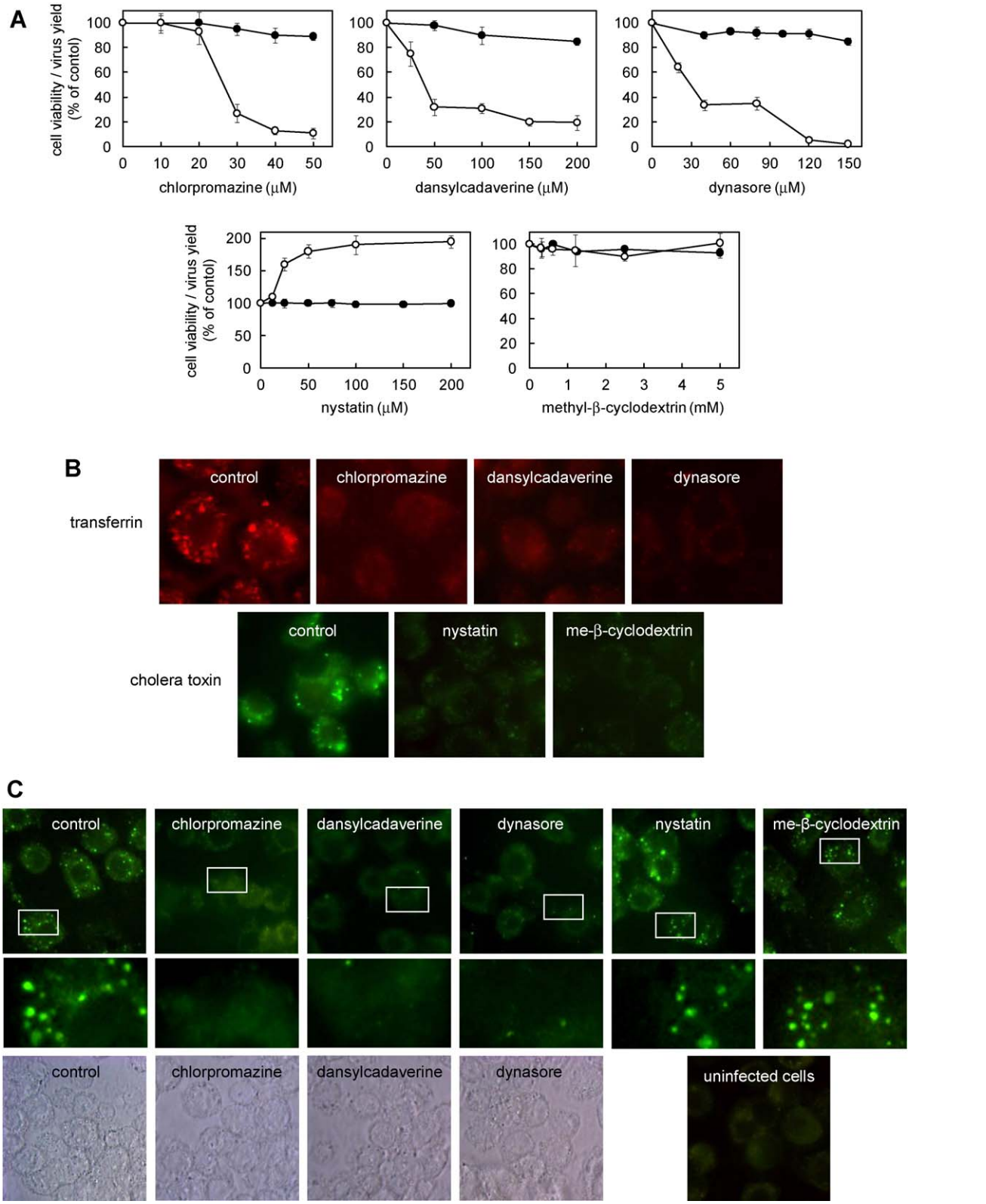

Fig. 2. Effect of endocytosis inhibitors on infection of C6/36 cells with DENV-1. (A) Cells were treated with increasing concentrations of chlorpromazine, dansylcadaverine, dynasore, methyl-β-cyclodextrin and nystatin, and then infected with DENV-1. Virus yields were quantified by PFU at 48 h p.i. and results are expressed as % of virus multiplication with respect to a control of infected cells without drug treatment (○).Titer of virus control: 5.0×10^5 PFU/ml. Each bar is the mean value of two independent experiments ± standard deviation. Other set of cultures was treated with compound as above and cell viability was determined by MTT method (●). (B) Cells were treated with 50 μM chlorpromazine, 200 μM dansylcadaverine, 150 μM dynasore, 200 μM nystatin, or 5 mM methyl-β-cyclodextrin, and then incubated with TRITC-labelled transferrin or FITC-cholera toxin. Controls without drugs were performed and samples were visualized by fluorescence microscope. (C) Cells were treated with compounds as in B and then infected with DENV-1 (m.o.i. 10 PFU/cell). After 30 min of infection with or without drugs, cells were fixed with methanol and internalized virions were revealed by immunofluorescence staining with anti-E monoclonal antibody. Infected and uninfected cells (lower panel, right image) without drug treatment were performed and visualized. Enlarged details of the boxed areas at a magnification of 4× are shown in the intermediate panel. Phase contrast images of control and treated cells is shown in the lower panel.

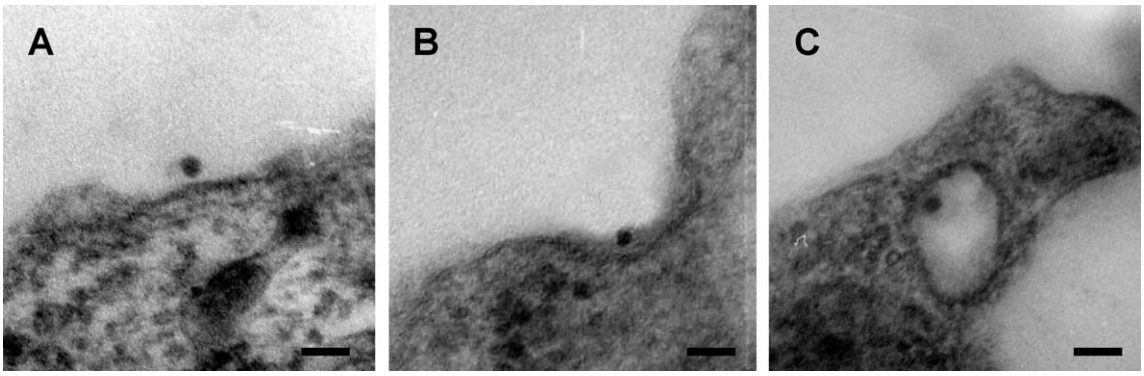

Fig. 3. Ultrastructural study of DENV-1 entry into C6/36 cells. DENV-1 was adsorbed to C6/36 cells during 1 h at 4 °C and then cultures were shifted to 33 °C to allow internalization. After 30 min, cells were fixed and processed for electron microscopy. (A) Virus particle at the extracellular medium close to the cell surface. (B) Virus particle attached within electron-dense invaginations of the plasma membrane. (C) Virus particle within endocytic coated vesicle. Bar: 100 nm.

several passages in mosquito-derived cells, we decided to evaluate if recent clinical isolates with few tissue culture passages employ the same entry pathway. As seen in Fig. 4A, the susceptibility to the clathrin and dynamin inhibitors chlorpromazine, dansylcadaverine and dynasore of two clinical isolates from recent outbreaks in Argentina, named ARG9920 and ARG0044 (Barrero and Mistchenko, 2004), was comparable to that shown by HW strain. Thus, the clathrin-mediated endocytic pathway appears to be the route for DENV-1 internalization into mosquito cells independently of the virus source.

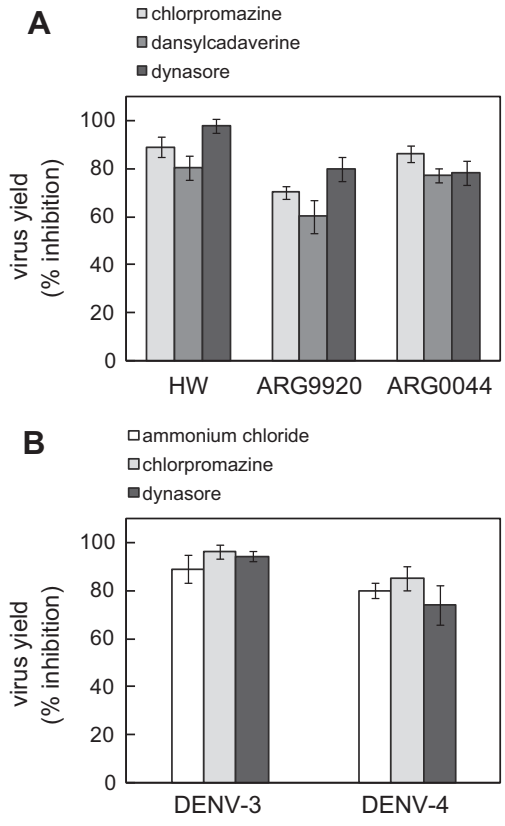

Fig. 4. (A) Effect of inhibitors on DENV-1 strains infection. C6/36 cells were treated with 50 μM chlorpromazine, 200 μM dansylcadaverine, or 150 μM dynasore and then infected with DENV-1 HW, ARG9920 or ARG0044. Virus production was quantified at 48 h p.i. Results are expressed as percentage of inhibition of virus multiplication with respect to a control without drug treatment. Titers of virus control were 5.0×10^5 PFU/ml for DENV-1 HW, 5.8×10^3 PFU/ml for ARG9920 and 1.7×10^3 PFU/ml for ARG0044. (B) Effect of inhibitors on DENV-3 and DENV-4 infection. C6/36 cells were treated with 50 mM ammonium chloride, 50 μM chlorpromazine or 150 μM dynasore and then infected with DENV-3 H87 or DENV-4 8124. Virus production was determined at 48 h p.i. and results are expressed as in A. Titers of virus controls were 3.0×10^5 and 2.0×10^5 PFU/ml for DENV-3 and DENV-4, respectively. Each bar shows the mean ± standard deviation of two independent experiments.

3.5. Endocytic pathway of DENV-3 and DENV-4 serotypes

According to the above described results and the previous reports about DENV-2 (Acosta et al., 2008a; Mosso et al., 2008) both serotypes use a similar endocytic route for entry into mosquito cells. Then, it was considered interesting to test if the two other serotypes, DENV-3 and DENV-4, share the same internalization pathway by testing their susceptibility to the biochemical inhibitors. A significant inhibition of infection with both viruses was observed after treatment of C6/36 cells with ammonium chloride, chlorpromazine and dynasore, under similar conditions to those employed for DENV-1 infection (Fig. 4B), suggesting that a low pH- and clathrin-mediated entry pathway is employed by the four DENV serotypes in mosquito cells.

4. Discussion

In conclusion, the sum of all our results, obtained by using diverse biochemical inhibitors, infectivity determinations, histochemical immunofluorescence staining and electron microscopy, demonstrate that the entry of DENV-1 to mosquito cells occurs by receptor-mediated endocytosis dependent on clathrin-coated vesicles and independent of caveolae and plasma membrane cholesterol. This lack of dependence on cholesterol for DENV-1 infection of mosquito cells is in agreement with a previous report in which efficient infection was achieved by DENV-1 strain Western Pacific and clinical isolates in C6/36-cholesterol depleted cells obtained by continuous passage in medium containing delipidated serum (Umashankar et al., 2008). Noticeably, a consistent and significative increase in DENV-1 yield was observed when infection was performed in the presence of nystatin (Fig. 2A). It must be noted that a similar result with nystatin was detected for DENV-1 entry into Vero cells (Acosta et al., 2009) and HepG2 cells (Suksanpaisan et al., 2009), by independent investigators. Perhaps, the caveola-mediated pathway may be a non-infective route of entry for DENV-1 and, consequently, its blockade by nystatin results in an enhancement in the utilization of the infective clathrin pathway with a concomitant higher production of infectious virus. Further research is required to assess this possibility but the usage of alternative routes for infective or non infective virus entry has become a more frequently described phenomenon in recent years

(Acosta et al., 2009; Rust et al., 2004; Quirin et al., 2008; Spoden et al., 2008).

The DENV-1 traffic through a low pH compartment for functional entry was here demonstrated by inhibition with ammonium chloride, in accordance with the recent conformational transition reported for DENV-1 E glycoprotein under acidic conditions to trigger membrane fusion and genome uncoating (Nayak et al., 2009). However, a lower susceptibility of DENV-1 infectivity to ammonium chloride was observed in comparison to the other DENV serotypes. The reduction in virus production in DENV-1 infected mosquito cells in the presence of ammonium chloride never exceeded 60–65% inhibition at the highest drug concentrations in repeated experiments (Fig. 1A) whereas 80–85% inhibition was detected in DENV-2, DENV-3 and DENV-4 infected C6/36 cells under the same treatment conditions (Acosta et al., 2008a) (Fig. 4B). At present we cannot explain this small difference. Interestingly, structural studies reported a more stable postfusion structure for the envelope E glycoprotein from DENV-1 in comparison to DENV-2 and the authors suggested that this subtle difference could result in altered fusion properties for DENV-1 such as a higher pH threshold (Nayak et al., 2009). The minor susceptibility of DENV-1 to ammonium chloride inhibition appears to provide another evidence for this hypothesis, but additional confirmation will require a detailed analysis of the ability of virus serotypes to fuse with target membranes at different pHs.

The clathrin-dependence here shown for DENV-1 internalization in mosquito cells was a property shared not only with DENV-2 (Acosta et al., 2008a; Mosso et al., 2008) but also with other human pathogens of the genus *Flavivirus* such as West Nile virus (Chu et al., 2006; Mizutani et al., 2003) and Japanese encephalitis virus (Nawa, 1998). This conclusion is valid for the cell culture-adapted reference strain HW as well as for recent human isolates of DENV-1. Furthermore, clathrin-mediated endocytosis is also the primary route of DENV-1 entry into the mammalian cell line of monkey origin Vero (Acosta et al., 2009). This conservation of the entry route in mosquito and mammalian cells for different strains of DENV-1 is contrasting with data reported for DENV-2. This serotype uses also clathrin-dependent endocytosis to penetrate into mosquito cells, as above mentioned, and in certain mammalian cells such as A549, HeLa and BSC-1 cells (Acosta et al., 2009; Krishnan et al., 2007; Van der Schaar et al., 2008), whereas a non-classical endocytic pathway independent of clathrin, caveolae and lipid rafts but dependent of dynamin was demonstrated for DENV-2 entry into Vero cells.

Significant advances towards understanding the diverse strategies exploited by a virus to enter into its host cell and initiate the process of infection have been made in recent years. In particular, the mode of entry of a flavivirus in an insect cell culture is representative of the route of entry in its insect vector in nature which is responsible of virus transmission to human. Consequently, the knowledge of entry mechanism will provide relevant tools to the design of means for control against the infection of DENV in mosquitoes prompting an alternative to combat virus spreading. Interestingly, it was also shown here that the two remaining serotypes DENV-3 and DENV-4 appeared to enter into mosquito cells by a low pH- and clathrin-dependent endocytic pathway. The simultaneous cocirculation of the four serotypes in diverse regions from Asia and America with increasing reemergence of DENV makes mandatory the requirement of effectiveness against all serotypes for an adequate control intervention. Recently, the complete genome of *Aedes aegypti*, in addition to other mosquitoes, was sequenced allowing the possibility of genetic manipulation of cellular genes (Megy et al., 2009; Waterhouse et al., 2008). Thus, the conservation of the entry mechanism into mosquito cells among the four DENV serotypes may lead to the development of bioengineered mosquito vectors resistant to infection as an alternative strategy to prevent virus transmission.

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