West Nile Virus Entry Requires Cholesterol-Rich Membrane Microdomains and Is Independent of αvβ3 Integrin


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West Nile virus (WNV) has been the leading cause of viral encephalitis in the United States since 1999. The endocytic processes involved in the internalization of infectious WNV by various cell types are not well characterized, and the involvement of cholesterol-rich membrane microdomains, or lipid rafts, in the life cycle of WNV has not been investigated previously. In this study, we found that the depletion of cellular cholesterol levels by brief treatment with methyl-β-cyclodextrin resulted in a 100-fold reduction of the titers of infectious WNV released into the culture supernatant, as well as a reduction in the number of WNV genome copies in the cholesterol-depleted cells. The addition of exogenous cholesterol to cholesterol-depleted cells reversed this effect. Cholesterol depletion postinfection did not affect WNV growth, suggesting that the effect occurs at the level of WNV entry. We also showed that while WNV entry did not require αvβ3 integrin and focal adhesion kinase, WNV particles failed to be internalized by cholesterol-depleted cells. Finally, we showed the colocalization of the WNV envelope protein and cholera toxin B, which is internalized in a lipid raft-dependent pathway, in microdomain clusters at the plasma membrane. These data suggest that WNV utilizes lipid rafts during initial stages of internalization and that the lipid rafts may contain a factor(s) that may enhance WNV endocytosis.

West Nile virus (WNV) belongs to the family Flaviviridae and the genus Flavivirus, which includes pathogens of global concern such as dengue virus (DENV), Japanese encephalitis virus, and yellow fever virus. Severe forms of WNV disease manifest as neurological symptoms such as meningitis, encephalitis, and poliomyelitis. WNV is a single-stranded positive-sense RNA virus. The 11-kb WNV RNA genome encodes a single polyprotein that is cleaved by the host and viral proteases into three structural (capsid [C], membrane [M], and envelope [E]) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (3). WNV entry into the host cells is mediated by clathrin-coated vesicles (6, 19). Virus delivery into acidic endosomes leads to the uncoating of the virion, releasing viral RNA into the cytoplasm, followed by RNA replication via minus-strand RNA intermediates (3). The mechanism of WNV entry and the relative roles of different endocytic pathways in WNV internalization have not been well characterized.

Lipid rafts are membrane microdomains enriched with cholesterol and glycosphingolipids (10). They perform crucial roles in the establishment of cell polarity and act as platforms mediating signal transduction and protein trafficking. Proteins with posttranslational modifications, such as a glycosylphosphatidylinositol anchor or palmitoylation, are associated with lipid rafts and are responsible for the functions ascribed to lipid rafts. Some of the raft-associated proteins include Src family kinases, receptor tyrosine kinases, G protein-coupled receptors, integrins, microtubules, and cytoskeletal and intermediate filament proteins (12). Many viruses utilize raft-mediated trafficking pathways for entering or exiting cells. While some animal viruses like human immunodeficiency virus type 1, coxsackievirus, simian virus 40, and severe acute respiratory syndrome coronavirus depend on lipid rafts for binding to and entry into the host, others like rotavirus, Newcastle disease virus, influenza virus, and Ebola and Marburg viruses utilize raft-mediated pathways for assembly and egress (4, 22, 26, 27). The involvement of lipid rafts in the life cycle of WNV has not been investigated. A previous study with DENV has indicated a role for lipid rafts in the flaviviral life cycle (29). However, the exact step at which lipid rafts play a role and the mechanism involved remain uncharacterized. In the present study, we provide evidence to support the role of lipid rafts in WNV entry. Our results provide insights into the endocytic pathway involved in WNV internalization, which will further our understanding of the cellular factors involved in WNV endocytosis.

MATERIALS AND METHODS

Cell culture and virus strains. WHO-certified Vero cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% Fetalplex serum (Gemini Bio-Products), penicillin, streptomycin, and glutamine. SK-N-MC neuroblastoma and HeLa cells were grown in DMEM containing 10% fetal bovine serum with penicillin, streptomycin, and glutamine. CS-1 hamster melanoma cells were grown in suspension in RPMI 1640 medium containing 10% fetal bovine serum with penicillin, streptomycin, and glutamine (11, 37). Wild-type and β3 integrin-deficient mouse embryonic fibroblasts (MEFs) were grown as described previously (16, 30). Focal adhesion kinase-deficient (FAK−/−) and FAK+/+ cells were grown as described previously (33, 35). The WNV strain (NY385-99) used in this study has been described before (25). The infection of...
cells with WNV and the determination of viral titers by plaque assays were performed as described previously (15, 25). Vesicular stomatitis virus (VSV) Indiana (strain ATCC VR-158) was purchased from the ATCC. VSV infections and plaque assays were performed in the same manner as described for WNV except that VSV plaque assay preparations were fixed and stained 48 h post-infection (hpi).

**Cholesterol depletion and repletion.** Cells grown in 12-well plates were washed three times with phosphate-buffered saline (PBS), and DMEM containing 2% delipidated serum (Biomeda) with or without the following concentrations of methyl-β-cyclodextrin (MBCD; Sigma) was added to the cells: 7.5 mM for 1 h for Vero cells, 10 mM for 30 min for HeLa cells, and 5 mM for 30 min for SK-N-MC cells. Plates were incubated at 37°C on a rocker for the indicated periods. After treatment, the cells were washed three times with PBS and infected with WNV in the above-mentioned medium without MBCD. Cholesterol replenishment was done as described in earlier studies (7, 9, 28). Briefly, cholesterol was depleted as described above, and cells were washed three times with PBS and incubated with 400 μg of water-soluble cholesterol (Sigma)/ml in the above-mentioned medium with 2 mM MBCD for 1 h. Cells were washed again three times with PBS and infected with WNV as described above. The level of viability of untreated or MBCD-treated cells was found to be >90%, as measured by trypan blue exclusion. MBCD treatment did not affect cell proliferation, as evaluated by cell counting before and after a 48-h treatment (data not shown).

**Quantitative real-time RT-PCR.** Cells were collected in 0.5 to 1 ml of TRIzol reagent (Invitrogen), and RNA was prepared according to the instructions of the reagent manufacturer. One hundred nanograms of RNA was used for determining WNV genome copy numbers by analysis with a one-step reverse transcription-PCR (RT-PCR) system (Applied Biosystems) as described previously (25). Genomic DNA copy numbers were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or β-actin gene copy numbers by using the comparative cycle threshold values determined in parallel (25). An Applied Biosystems inventoried assay (assay identification no. Hs00168352_m1) was used to estimate the copy numbers of 3-hydroxy-methylglutaryl coenzyme A reductase (HMGR) mRNA at the time points indicated in Fig. 3 by one-step RT-PCR using RNA isolated from SK-N-MC cells infected with 10 PFU of WNV/cell.

**Virus attachment.** Vero cells (3×10⁵) were grown in 12-well plates and treated with MBCD as described above. After a 1-h treatment with MBCD, cells were shifted onto ice and washed three times with cold PBS. Cells on ice were infected with WNV diluted in cold DMEM containing 2% delipidated serum at a multiplicity of infection (MOI) of 10 PFU/cell (200 μl of virus medium/well). Cells were incubated at 4°C for 1 h with intermittent mixing. After incubation, virus medium was removed and cells were washed three times with cold PBS. Cells were collected in 500 μl of TRIzol reagent per well for RNA isolation and WNV genome copy number determination as described above.

**Immunofluorescence microscopy.** Cells were grown on 12-mm coverslips in 24-well plates. MBCD treatment and WNV infection were performed as described above. At 24 hpi, cells were washed twice with PBS, fixed in 4% paraformaldehyde (PFA) for 20 min, permeabilized in PBS containing 0.1% Triton X-100, rinsed in PBS, and blocked with normal goat serum in the same buffer for 10 min. Samples were incubated with anti-E antibodies (purified from D1-4G2 hybridomas from the ATCC) for 1 h, followed by rhodamine or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody (Invitrogen).

**CTB and WNV endocytosis.** For cholera toxin B (CTB) endocytosis, cells were washed twice with PBS and incubated for 30 min at 4°C with DMEM containing 0.5% bovine serum albumin with or without 10 μg of FITC-conjugated CTB (Invitrogen) and with or without purified WNV at an MOI of 10 PFU/cell. After attachment at 4°C, cells were transferred to 37°C for 10 min to allow the endocytosis of CTB and WNV. Cells were washed twice with PBS, fixed with PFA, and processed for immunofluorescence analysis as described above.

In WNV endocytosis experiments, virus attachment was permitted as described above and virus internalization was allowed for 30 min. Cells were fixed and stained as described above with monoclonal anti-E and anti-early endosome antigen 1 (anti-EAA1) antibodies (BD Biosciences), followed by isotype-specific Alexa dye-conjugated goat anti-mouse secondary antibodies (Alexa 488-IgG2a [E] and Alexa 594-IgG1 [EAA1], respectively; Invitrogen). After final washes, coverslips were mounted onto glass slides in Prolong antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) to stain the nuclei (Invitrogen). Images were acquired with a DeltaVision real-time image station (Applied Precision Instruments), a wide-field deconvolution microscope system using SoftWoRx as the software application. This system has normal band-pass filters and a Nikon CoolSnap HQ camera. Images were acquired with the 60×/1.45-numerical-aperture lens objective at 0.2 μm for Z-stacks in red (Alexa 594; excitation wavelength, 594 nm; emission wavelength, 617 nm), green (Alexa 488; excitation wavelength, 488 nm; emission wavelength, 538 nm), and blue (DAPI; excitation wavelength, 360 nm; emission wavelength, 460 nm). These stacks were then deconvolved using nine iterations. All images were captured under identical settings. Color levels were adjusted using Adobe Photoshop with the same template file for all images. For a proportion of six stacks in the middle, see Fig. 2. For a single Z-stack image, see Fig. 5 and 6.

**Western blots.** Total protein was isolated from the TRizol organic phase after RNA isolation per the instructions of the TRizol manufacturer (Invitrogen). Equal amounts of protein were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel, transferred onto polyvinylidene difluoride (Immobilon-Milipore), and probed with rabbit polyclonal antibody against NS5 (a kind gift from Ian Lipkin) and monoclonal anti-β-actin antibodies (Sigma), followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (GE Health-care). Blots were visualized by using a SuperSignal West Pico chemiluminescent substrate according to the protocol of the manufacturer (Pierce).

**Statistical analyses.** GraphPad Prism software was used for graphical representations and statistical analyses. P values were obtained from a two-tailed, unpaired Student t test.

**RESULTS**

The depletion of cholesterol leads to a decrease in WNV titers in the supernatant. In order to characterize the role of cholesterol-rich membrane microdomains, or lipid rafts, in the life cycle of WNV, we treated Vero, SK-N-MC, and HeLa cells with MBCD, which is widely used to sequester cholesterol from the plasma membrane, prior to infection with WNV and measured viral titers in the culture supernatant 22 hpi. As shown in Fig. 1A, we observed a 100-fold reduction in the viral titers in various cell types treated with MBCD, suggesting that the lipid rafts play an essential role in the life cycle of WNV and that this effect is not cell type specific. The concentrations of MBCD and the periods of MBCD treatment used in our study have been shown to be nontoxic for many cell lines (39), and we have verified the same by trypan blue exclusion and found that ~90% of the cells were viable under these conditions (data not shown).

The acute depletion of cholesterol by MBCD has been shown previously to transiently inhibit clathrin-mediated endocytic pathways (31). To rule out the possibility that the reduction in viral titers was due to a gross disruption in the membrane architecture and the inhibition of all cellular uptake pathways upon cholesterol depletion, we treated SK-N-MC and HeLa cells with MBCD as described above and infected the cells with VSV, which enters cells via clathrin-dependent endocytosis (36). We measured viral titers in the supernatants 16 hpi. Under our conditions of cholesterol depletion of these cells, MBCD treatment did not affect VSV titers (Fig. 1B). These data suggest a specific requirement for lipid rafts for normal WNV replication.

Cholesterol-depleted cells have reduced levels of WNV RNA. Cholesterol-rich membrane microdomains are present not only within the plasma membrane but also in the membranes of intracellular organelles and are involved in protein trafficking from the plasma membrane and in the secretory pathway. The depletion of plasma membrane cholesterol by MBCD may cause the depletion of cholesterol stores at the endoplasmic reticulum as the cell attempts to restore a cholesterol balance. Therefore, the disruption of lipid rafts by MBCD may have an effect on WNV entry, RNA replication, assembly, or egress. To identify the stage(s) at which lipid rafts play a role in the WNV life cycle, we pretreated Vero cells with MBCD and infected them with WNV as described above. Twenty-four hours
postinfection, cells were fixed and analyzed by immunofluorescence with anti-E antibodies. Simultaneously, lysates from mock- or WNV-infected cells were prepared for Western blot analysis. As shown in Fig. 2A, MBCD-treated cell samples had fewer infected cells than mock-treated samples, as seen by staining with WNV E antibody. Western blot analysis of the lysates also showed smaller amounts of WNV NS5 protein in MBCD-treated cells than in mock-treated cells (Fig. 2B). Similar results were obtained for SK-N-MC cells (data not shown).

We next determined the WNV RNA copy numbers in mock- or MBCD-treated, WNV-infected cells 24 hpi by quantitative RT-PCR (qRT-PCR). As shown in Fig. 2C, we found 100-fold-lower levels of WNV RNA copies in cells treated with MBCD than in the mock-treated cells. These results indicated that lipid rafts either play a direct role in RNA replication or function at an earlier stage in the virus life cycle, possibly during virus entry.

**Cholesterol depletion postinfection does not affect WNV titers.** A recent study has indicated a role for cholesterol and the cholesterol biosynthetic pathway in the replication of the RNA of Kunjin virus (a nonpathogenic Australian variant of WNV) (21). We determined if cholesterol depletion affects postentry events in the WNV life cycle by infecting cells with WNV and treating them with MBCD postinfection. Cells were treated with MBCD 6 hpi as described above, and the viral titers in the culture supernatants were determined by plaque assays at 6 and 16 h post-MBCD treatment (12 and 22 hpi, respectively). As shown in Fig. 3A, cholesterol depletion by MBCD postinfection had no effect on viral titers. Similar results were obtained when cells were treated with MBCD 3 hpi (data not shown), suggesting that the effect of MBCD is at a step prior to WNV RNA replication. Since it is impossible to completely deplete cellular cholesterol without affecting viability, it is possible that the residual cholesterol present in the cells was sufficient to participate in WNV replication. Alternatively, it is possible that cellular cholesterol levels were replenished by de novo synthesis upon the removal of the drug, allowing for cholesterol participation in viral RNA replication, assembly, or egress.

To determine if cholesterol biosynthesis is upregulated during WNV infection, SK-N-MC cells were infected with WNV
and cells were collected 8, 24, and 48 hpi for RNA isolation. The levels of mRNA for HMGCR, an enzyme involved in cholesterol biosynthesis, were measured by qRT-PCR. We saw a modest induction of HMGCR in WNV-infected cells at early time points, and by 48 h, HMGCR levels were similar to those in uninfected controls (Fig. 3B). In agreement with our results, Mackenzie et al. also observed much less induction of HMGCR in cells infected with WNV strain NY99 than in those infected with Kunjin virus (21). This finding suggests that, unlike the Kunjin virus, WNV does not significantly induce cholesterol biosynthesis and that de novo synthesis of cholesterol may not be required for WNV RNA replication.

**WNV entry is dependent on lipid rafts and independent of αvβ3 integrin.** We next tested directly if cholesterol depletion by MBCD affects WNV entry. Vero cells were pretreated with MBCD and infected with WNV as before. After 1 h of incubation at 37°C, cells were treated with trypsin to release any attached viral particles (see the supplemental material) that failed to be internalized and washed extensively with PBS. RNA was isolated from the cells, and the relative number of internalized WNV genome copies was determined by qRT-PCR. We observed an 85% reduction in the amount of internalized WNV RNA in MBCD-treated cells compared to that in mock-treated samples (Fig. 4A). These results demonstrate the requirement for cholesterol-rich membrane microdomains in the internalization of WNV. To further determine that the observed effect was due to cholesterol depletion and not due to a pleiotropic effect of MBCD on other membrane components, we supplemented cholesterol-depleted cells with exogenous cholesterol in the same experiment. As shown in Fig. 4A, the addition of cholesterol to the cholesterol-depleted cells completely eliminated the defects in WNV entry and restored normal viral titers in the culture supernatants 24 hpi were determined by plaque assays. Error bars represent SD (n = 10). (B) Vero cells were treated as described in the legend to panel A, and viral titers in the culture supernatants 24 hpi were determined by plaque assays. Error bars represent SD (n = 3). Results representative of at least two independent experiments performed with triplicates are shown.

WNV has been proposed to enter cells by endocytosis in clathrin-coated vesicles. αvβ3 integrin was identified previously as a receptor for WNV, and it was shown that the internalization of WNV leads to the activation of FAK (5, 6). We tested the requirement for αvβ3 integrin and FAK in WNV infection by infecting wild-type MEFs and MEFs lacking β3 integrin or FAK and found no difference in the viral titers in the culture supernatants of infected cells between the control and knockout MEFs (Fig. 5A and B), suggesting that WNV is capable of infecting and replicating in cells lacking αvβ3 inte-
We further infected hamster melanoma CS-1 cells that have been shown previously to lack αβ3 or αβ5 integrin complexes at the plasma membrane (11, 37) with WNV and measured viral titers in the supernatant 24 hpi. WNV efficiently infected CS-1 cells, and the titers in the supernatant were comparable to those in the supernatants of the other cell lines used in this study (Fig. 5C). These results suggest that WNV can utilize an alternate receptor in the absence of αβ3 or αβ5 integrin and that these integrins are not essential for WNV infection.

**FIG. 5.** αβ3 integrins are not required for WNV entry. (A) Wild-type (WT) and β3 integrin−/− MEFs were infected with WNV (MOI of 3 PFU/cell) as described in Materials and Methods. WNV titers in the culture supernatants at 20 hpi were determined by plaque assays on Vero cells. Error bars represent SD (n = 6). (B) FAK−/− and FAK−/− cells were infected with WNV (MOI of 3 PFU/cell), and 18 to 24 hpi, WNV titers in the culture supernatants were determined by plaque assays on Vero cells. Error bars represent SD (n = 6). (C) CS-1 cells were infected with WNV (MOI of 3 PFU/cell), and 24 hpi, WNV titers in the culture supernatants were determined by plaque assays on Vero cells. Error bars represent SD (n = 6). Results representative of at least two independent experiments performed with two or more replicates are shown. ND, not detected.

**FIG. 6.** Effect of cholesterol depletion on WNV attachment and internalization. (A) Vero cells were treated with MBCD for 1 h, infected on ice at an MOI of 10 PFU/cell, and incubated at 4°C for 1 h as described in Materials and Methods. Cells were collected in TRIzol for RNA isolation and WNV RNA copy number determination. Results from two independent experiments performed in duplicate are shown. Error bars represent SD (n = 4). (B) Vero cells grown on coverslips were infected at an MOI of 10 PFU of WNV/cell and incubated for 30 min on ice. Cells were shifted to 37°C for 30 min, after which they were fixed in 4% PFA and stained with WNV E and EEA1 antibodies, followed by Alexa 488- and Alexa 594-conjugated secondary antibodies. The nuclei were stained by DAPI. RGB overlay images are shown. Arrows indicate WNV particles in EEA1-positive endosomes in mock-treated cells and the sparse peripheral distribution of WNV particles in MBCD-treated cells. UI, uninfected cells; scale bars, 10 μm.

WNV particles fail to be internalized in cholesterol-depleted cells. We next examined if the effect of MBCD on WNV entry is due to the inefficient attachment of the virus at the cell surface. MBCD-treated cells were incubated with the virus at 4°C for 1 h to allow binding, after which the unbound virus was removed and the amount of bound virus was measured by determining the WNV genome copy numbers by qRT-PCR. As shown in Fig. 6A, cholesterol depletion did not affect the number of attached virions at the cell surface, suggesting that virus attachment does not require lipid rafts. We further examined the endocytosis of WNV by infecting Vero cells at an MOI of 10 PFU/cell. Cells were incubated with the virus at 4°C to allow virus attachment and then shifted to 37°C for 30 min to allow internalization, after which the cells were subjected to fixation and immunofluorescence staining with antibodies specific for WNV E and EEA1, a marker for early endosomes. As shown in Fig. 6B, most of the internalized WNV particles were in EEA1-positive early endosomes in control cells, whereas no
intracellular WNV particles could be detected in MBCD-treated cells. A few of the WNV particles that were present in the cholesterol-depleted cells were at the cell peripheries. These data further confirm our qRT-PCR and viral titer data and demonstrate that cholesterol depletion affects WNV internalization.

**WNV E and CTB colocalize during entry.** To investigate if WNV entry is mediated by cholesterol-rich membrane microdomains, we examined the internalization of WNV and FITC-conjugated CTB by Vero cells. The CTB entry pathway has been well-characterized, and CTB has been shown previously to enter cells in a lipid raft-dependent manner by binding to its receptor GM1 ganglioside (13). Vero cells were infected with WNV at an MOI of 10 PFU/cell and incubated with 10 μg of FITC-conjugated CTB/ml at 4°C for 30 min to allow for attachment. Cells were then shifted to 37°C for 10 min, washed extensively, fixed, and analyzed by immunofluorescence microscopy using anti-E antibodies. We observed discrete areas of colocalization of WNV E and CTB at the plasma membrane (Fig. 7). These data suggest that both WNV E and CTB associate with lipid rafts at the plasma membrane and that lipid rafts are required for the initial stages of WNV internalization.

**DISCUSSION**

In this study, we showed that cholesterol-rich membrane microdomains at the plasma membrane were important for normal WNV growth. The depletion of plasma membrane cholesterol from cultures of various cell lines by MBCD affected WNV titers in the culture supernatants. We observed a reduction in (i) the number of WNV-positive cells, as detected by immunofluorescence analysis, (ii) the amounts of viral proteins, and (iii) the levels of WNV RNA in cholesterol-depleted cells. The observed effects were due to a block in WNV entry into the cells. WNV particles in cholesterol-depleted cells were undetectable in our internalization assays, further suggesting that WNV failed to bind to its receptor or an attachment factor in these cell samples. Additionally, WNV particles colocalized with CTB, which utilizes lipid rafts for internalization, in microdomain clusters at the plasma membrane. Together, our data suggest that WNV utilizes lipid rafts for entry by binding either to a receptor present in the lipid rafts or to an accessory protein (an attachment factor or a signaling molecule) in the lipid rafts that promotes WNV binding to the receptor and WNV internalization.

We also showed that WNV entry was αvβ3 integrin independent and that FAK was nonessential for WNV growth. Previous studies have used a lab strain of WNV (Sarafend) to study WNV endocytosis and have identified αvβ3 integrin as a receptor for WNV. WNV Sarafend is a nonpathogenic strain whose source, year of isolation, and passage history are not known. Phylogenetic analyses based on sequences of E and NS5 proteins and 5' ends of the 3' untranslated regions of multiple WNV isolates have been used to classify WNV Sarafend as a lineage II WNV strain. WNV Sarafend also falls into a separate antigenic group, as anti-E monoclonal antibodies recognizing all other WNV isolates fail to recognize WNV Sarafend (32). It is possible that this virus differs from the infectious WNV strain NY99 that we used in our study in its receptor usage. This theory is further supported by the fact that while WNV Sarafend is reported unable to infect CS-1 cells that lack αvβ3 integrin (5), the infection of these cells with WNV NY99 resulted in high viral titers comparable to those in Vero cells. While our studies were in progress, Mackenzie et al. reported that infection with Kunjin virus (a nonpathogenic
Australian variant of WNV) induces cholesterol biosynthesis and that the viral replication is inhibited by drugs that inhibit cholesterol biosynthesis (lovastatin) but not by drugs that deplete cellular cholesterol (MBCD) (21). We have shown that, unlike Kunjin virus, WNV does not significantly induce cholesterol biosynthesis and that cholesterol depletion affects WNV at the level of entry. In all the experiments described by Mackenzie et al., MBCD or other drugs were added 90 min postaddition of virus and were left on the cells for the duration of the experiment. Therefore, any effect of cholesterol on WNV entry would not be observed under their experimental conditions. They found that cholesterol depletion by MBCD postentry has no effect on Kunjin virus RNA replication or protein expression, but a roughly 10-fold reduction in viral titers was observed upon prolonged MBCD treatment, suggesting a possible role for cholesterol in later stages of the viral life cycle, such as assembly and egress. In our experiments, we added MBCD for 30 min at 3 or 6 hpi and then removed the drug (we found prolonged exposure to result in significant toxicity), and we have not observed an effect on viral genome copies or titers. We cannot rule out the possibility of the presence of residual intracellular cholesterol in the cells sufficient to participate in WNV replication. Alternatively, it is possible that cellular cholesterol levels were replenished by de novo synthesis upon the removal of the drug, allowing for cholesterol participation in viral replication, assembly, or egress.

Our results do not dispute the role of clathrin in WNV endocytosis (6, 19) but suggest that the lipid rafts are also required for WNV internalization. Several viruses from the family Flaviviridae have been shown previously to exploit lipid rafts for entry (DENV, hepatitis C virus [HCV], and bovine viral diarrhea virus) or replication (Kunjin virus and HCV) (1, 17, 18, 21, 29). The HCV receptors tetraspanin CD81 and scavenger receptor B type 1 localize at specialized plasma membrane domains enriched with cholesterol. CD81-mediated HCV entry was shown previously to require plasma membrane cholesterol (17). Similarly, Hsp90 and Hsp70 have been proposed to form a receptor complex for DENV and these proteins were shown previously to be associated with the lipid rafts (29). In all these studies, the depletion of plasma membrane cholesterol by MBCD led to a reduction in the viral titers in culture supernatants. Furthermore, membrane fusion studies involving fusion between the liposomes and flavivirus or alphavirus virions have shown that cholesterol plays a crucial role in promoting fusion, suggesting that the involvement of cholesterol-rich membrane microdomains in viral entry may be a general feature of the entry pathways of flaviviruses and the closely related alphaviruses (20, 34).

In addition to entry, viruses utilize lipid rafts at various other stages of the life cycle. HCV and human immunodeficiency virus have been shown previously to modify the compositions of the lipid rafts so as to facilitate viral replication (2, 23). Lipid rafts act as signaling platforms due to the association of signaling proteins, such as various growth factor receptors, Src family kinases, and GTPases, in these membrane microdomains (12). Therefore, hijacking lipid raft machinery by using one of the lipid raft-associated proteins as a receptor or an attachment factor would enable the virus to gain access to various components of signaling and protein-trafficking machinery in the lipid rafts for later stages of viral replication (for an example with poliovirus, see reference 8). Further studies are required to examine how lipid rafts facilitate WNV growth and if WNV, like HCV, modifies host cell lipid raft compositions at late stages of virus replication. There is increasing evidence that a number of viruses utilize caveolin-independent, cholesterol- and clathrin-dependent endocytic pathways for internalization (14, 24, 38). Understanding the relative roles of clathrin-dependent and clathrin-independent endocytic processes in WNV internalization warrants further investigation. Identifying other proteins of the endocytic pathway that are involved in WNV internalization will provide further insights into the mechanism of WNV entry and the pathway involved. Our results demonstrate the importance of plasma membrane cholesterol for WNV entry. This observation provides new opportunities to further understand the mechanisms of WNV entry and replication and also to develop reagents that can block WNV entry and prevent WNV disease in the susceptible host.

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