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Syncytial and Congregative Effects of Dengue and Zika Viruses on the *Aedes Albopictus* Cell Line Differ among the Viral Strains

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ABSTRACT

Objective: Dengue viruses (DENV) and Zika viruses (ZIKV) are transmitted from human to human or from non-human primates to humans by mosquito biting, so the viral interaction with mosquito cells is one key step within the viral life cycle. Therefore, our objective is to know how DENV or ZIKV interacts with mosquito cells.

Methods: Immunofluorescence assay and a direct visualization system are combined to monitor the syncytial or congregative effects of DENVs and ZIKVs on C6/36 cells. we studied the cytopathic effects of DENVs and ZIKVs on the mosquito cells, C6/36 which are widely used in the laboratory for the infections of DENV and ZIKV.

Results: Our results show that all strains of DENV-1 and DENV-2, most DENV-4 and some DENV-3 strains caused syncytial effects on C6/36 cells, while some DENV-3 and DENV-4 strains, and all the tested ZIKV strains caused cell congregation after infection but no cell fusion. In addition, we detected a range of pH environments from 6.0 to 8.0 that support the virus-caused cell fusion and figured out that the optimal pH condition is 7.5 at which the viral production is also the best. Furthermore, viral replication may be required for DENV's syncytial effects on C6/36 cells because the UV-inactivated virus failed to cause cell fusion.

Conclusion: Syncytial and congregative effects of DENV and ZIKV on the *Aedes albopictus* cells differ among the viral strains. Syncytial effects of DENV on C6/36 are important for viral replication.

INTRODUCTION

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Viruses of the *Flaviviridae* family comprise four genera: *Flavivirus, Hepacivirus, Pestivirus, and Pegivirus* (1). They are characteristically similar in genome structure, virion morphology, and life cycle. Their genome is an unsegmented, single-stranded, and positive-sense RNA with a length between 9.6 and 12.3 kb, which encodes a single polyprotein that is cleaved after translation, through the cooperation of host and viral proteases, into structural and nonstructural (NS) proteins: Envelop (E), PrM, Capsid, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (2, 3). While the structural proteins are the components of viral particles, NS proteins are required for viral RNA replication and viral maturation. Flaviviruses are enveloped and contain capsids with icosahedral and spherical geometries.

Dengue virus (DENV) and Zika virus (ZIKV) belong to the genera *Flavivirus* of the family *Flaviviridae*. DENV-infected human cases reach 100 million annually (4, 5) and ZIKV not only caused a wide-range pandemic in recent years but also are related to severe diseases such as microcephaly and other neurological symptoms of maldevelopment of the brain in neonates, and Guillain-Barré syndrome (GBS) in adults (2, 6-8). Most cases occur in tropical and subtropical areas where the mosquito of the genus *Aedes* becomes the major vector for the transmission of DENV and ZIKV (9-11). Although a wide range of hosts has been recognized for infection of DENV and ZIKV, the most important life cycle has been defined as mosquito transmission from human to human, or from non-human primate to human.

DENV enters the mosquito body through a viremic blood meal after biting a DENV-shedding patient (12). The virus replicates in the epithelial cells of the mid-gut. Newly formed virion particles then spread to various mosquito organs. DENV can replicate in different types of mosquito cells. For example, DENV can enter hemocytes but only replicate to a mild level. Its replication is limited by different innate mechanisms that are related to the different immune systems of mosquitoes. Due to the special immune system of the *Aedes aegypti* and *Aedes albopictus* mosquitos, they allow DENV production at a sub-pathogenic level. This interaction of DENV and mosquito probably keep both viral productions in different orangs including the salivary gland and the mosquito alive to consequently transmit DENV to the next host (human or monkey) in subsequent bites (13). Such experimental studies are still lacking for ZIKV that

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might use the same interaction to keep the mosquito to both produce viruses and infect the subsequent host for the transmission (14). We will start by investigating interactions of DENVs and ZIKVs with mosquito cells to reveal the mechanisms underlying how DENV or ZIKV infection causes a sub-pathogenic state in a mosquito.

Previous studies have shown that DENV and other flaviviruses infection induces the fusion of mosquito cells in a cell culture system (15-20). The induced cell fusion is important for viral replication because the mutated DENV that failed to cause cell-cell fusion presented a lower replication phenotype (15, 16). It remains unknown whether the different types of DENV and different strains of ZIKV cause different pathogenic effects on mosquito cells and it has been not experimentally defined how the cell fusion occurs during DENV or other flaviviruses' infection. In the present study, we report that different types, and strains of DENV and ZIKV behave differently in C6/36 cells in inducing mosquito cell-cell fusion.

MATERIALS AND METHODS

Cell lines, Tissue culture, and viruses. Vero cells (ATCC® CCL-81™) were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin (100 IU/ml)-streptomycin (100 ug/ml) and amphotericin B (2.5 ug/ml) (28). *Aedes albopictus* clone C6/36 cells (ATCC® CRL-1660™) was purchased from ATCC and maintained in RPMI1640 supplemented with 10% fetal calf serum (FCS) and penicillin (100 IU/ml)-streptomycin (100 ug/ml) and amphotericin B (2.5 ug/ml). L-15 Medium (Leibovitz) is purchased from Biological Industries USA (Cromwell, CT, USA) and buffered by the use of free basic amino acids, phosphate buffers, and higher levels of galactose and sodium pyruvate to help maintain physiological pH control. ZIKV strains MR766 (29), PRVABC59 (30) and DENV-2 (ATCC® VR-1584™) strain New Guinea C, DENV-1 strain Western Pacific 74 (WP 74) (31), DENV-3 strain H87 (32) and DENV-4 (33) were purchased from ATCC and propagated in C6/36 cells for less than 3 passages to generate stocks. Other strains of ZIKV and DENVs (Table 1) were from BEI Resources. The viral stocks were aliquoted and kept at -80°C for use.

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Antibodies. Mosquito antibodies are obtained from Bei Resources (MRA-249 Hybridoma 6D12 and MRA-253 Hybridoma 10C8 Anti-*Aedes aegypti* Salivary Glands). Other antibodies from BEI Resources include 1) anti-DENV-1 envelope protein antibodies (NR-4751 and NR-9549), 2) NR-2556 monoclonal anti-Dengue virus Type 2 Envelope Protein, 3) clone 3H5-1 (produced in vitro), 4) NR-15511 monoclonal anti-Dengue Virus Type 3 Envelope Protein, Clone E2 (produced in vitro), and NR-15540 monoclonal anti-Dengue virus type 4 envelope protein, clone E23 (produced in vitro).

ICC assay. Immunostaining was performed on cells grown on coverslips after fixation with 1% paraformaldehyde (10 min at room temperature) and permeabilization in 0.2% Triton (20 min on ice) by sequential incubation with primary and Texas red (TR)-labeled secondary antibodies (Vector Laboratories, Burlingame, Calif.) for 30 min each (all solutions in PBS). Finally, cells were equilibrated in PBS, stained for DNA with Hoechst 33258 (0.5 μg/ml), and mounted in Fluoromount G (Fisher Scientific, Newark, Del.).

Microscopy. Cells were examined with a Leica TCS SPII confocal laser scanning system. Two or three channels were recorded simultaneously and/or sequentially and controlled for any possible breakthrough between the fluorescein isothiocyanate and Texas Red signals and between the blue and red channels.

Cell membrane staining. To know whether the viral infection causes the fusion of C6/36 cells, we employed the method of the staining membrane with CellMask™ Deep Red Plasma membrane Stain kit (ThermoFisher, C10046) according to the manufacturer's protocol. Briefly, the cells were incubated with the staining agent (1:1000 dilution) for 5 min at 37°C. The cells were then fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. Then the cells were treated as ICC assay using an anti-ZIKV antibody (anti-Envelop).

RESULTS

1. Different types and strains of DENV and ZIKV cause various morphologic changes in C6/36 cells.

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Although DENV and ZIKV have shown different pathogenesis in humans, we wondered whether the DENVs and ZIKV cause different cytopathic effects (CPE) on mosquito cells. For that purpose, we selected C6/36 cells. C6/36 cells were generated from *Aedes albopictus* which transmits DENV and ZIKV. The C6/36 cells were infected with DENV or ZIKV at an MOI of 0.5, incubated at 28 ° C with a 5% CO_2, and were photographed every other day under a microscope in the DIC setting with the 10x len. The dynamic images are shown in Figure 1. Among the viruses (DENV-1, -2, -3, -4, ZIKV), all DENV-1, -2, most DENV-4 and two strains of DENV-3 infection in C36/6 cells resulted in syncytial morphology. At 0 hours post-infection (hpi), the cells are evenly distributed. The cells started fusion after 48 hpi, generating larger cells. At 4 dpi and later, the cell fusion expanded to form more fused cells and the number of unfused cells clearly decreased. For ZIKV or some DENV-3 and -4 infections, although the cells started congregation at 48 hpi, we have not observed any large cells caused by fusion. At 6 dpi and later, the congregation of cells became clearer, however, no cell fusions were seen.

To know whether it is a unique phenomenon for DENV-1 and -2, we examined multiple strains of DENVs covering all 4 serotypes and 7 strains of ZIKV. The strains of viruses were listed in Table 1. All the strains of DENV-1 and -2 caused cell fusion, most strains of DENV-4 and only two strains of DENV-3 caused cell fusion. We tested 7 strains of ZIKV, all the ZIKVs resulted in cell congregation but no cell fusion. Therefore, infection of C6/36 with different DENVs and ZIKV causes different morphological CPE at the same cell culture condition that was 28°C and 5% CO_2.

2. Network-like connections of the fused cells containing vesicle-like structures in C6/36 cells infected by DENV-1 and -2.

We next focused on the infection of DENV-1 and -2 in C6/36 cells. To show more details of the morphological change at the end of viral infection in C6/36 cells, we show the C6/36 cells that are infected with DENV or ZIKV for 12 days in a larger microscopy field with a 10x len. As can be seen in Figures 2A and 2B, DENV-1 or -2 infection caused cell fusions, and the large fused cells are still connected to other fused large cells, forming a network-like morphology, which

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was not observed in ZIKV-infected C6/36 cells (Figure 2C). Interestingly, most DENV-3 and DENV-4 and all ZIKV-caused cell congregations and the cells congregates were not connected (we only showed ZIKV infection-caused cell congregations in C6/36 cells here).

To demonstrate the cell fusion, we employed the method of the staining membrane with CellMask™ Deep Red Plasma membrane Stain kit (ThermoFisher, C10046). On day 12 post-infection of DENV-1 (TH-Sman), the cells were incubated with the staining reagent (1:1000 dilution) and DAPI for 5 min at 28°C. Then the cells were washed with PBS and observed under a fluorescent microscope at a 40x len. As shown in Fig. 2D, the fused cells were linked by an extended cell membrane that is stained in red. Therefore, we hypothesize that the network-like morphology is formed by DENV-1 or -2 infection that caused the fusion of cellular membranes.

To confirm the cell fusion by the DENV-1 or -2 infection, we need to demonstrate the viral infection in the fused cells. To that end, we performed an immunofluorescence assay (IFA) using anti-DENV antibodies. As shown in Figure 3A, two fused cell clusters are connected by cell membranes. The C6/36 cells mostly expressed viral proteins: Envelope (Env) in red and NS3 in green. We also observed the vesicle-like structures in the DENV-1 infected C6/36 cells as shown in Figure 2A. Our IFA results showed that vesicle-like structures are actually empty as shown in Figure 3B because there are no cells (by DAPI) inside of the structure.

To more directly show the cell fusion or congregation of C6/36 cells by DENV or ZIKV, DENV-2 (NGC strain), DENV-3 (NR-80) or ZIKV (MR766 strain) infection in C6/36 cells at 28°C with 5% CO_2 was recorded dynamically as shown in the movies (Supplemental data), which clearly recorded the cell fusion by DENV-2 infection (Movie 1) and cell congregation by DENV-3 and ZIKV (Movies 2 and 3). In Movie 1, we recorded the formation of the fibrilization of the cells to form a net-like structure but also the vesicle-like structures in the DENV-2-infected C6/36 cells. Therefore, we discovered not only that DENVs and ZIKV infection cause morphologically different CPE in C6/36 cells but also that DENV-1 or -2 infection-caused cell fusion of C6/36 has two types of structures: network-like and vesicle-like structures.

3. The formation of the cell fusion needs viral replication.

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We next wondered whether the cell fusion by DENV-1 or -2 is viral replication independent. For that purpose, we inactivated DENV-1 (NGC strain) by UV irradiation. The inactivation was demonstrated by IFA at 24 hours after infection in C6/36 cells as shown in the lower panel of Figure 4A while the untreated DENV-2 infection was positive for Envelop protein shown in red in the upper panel of Figure 4A. Therefore, the UV treatment of the DENV-2 effectively inactivated the DENV-2.

We infected the C6/36 cells with either UV-treated DENV-2 or the untreated DENV-2 and took pictures at 24 or 144 hpi. As can be seen, the inactivated DENV-2 didn't cause any CPE but the untreated DENV-2 caused cell fusion. Our experiments also showed that a higher titer of viral infection causes cell fusion more rapidly (not shown). In addition, the cell fusion only occurred at later than 48 hpi. Therefore, viral replication is required for DENV-2 to cause the fusion of C6/36 cells.

4. Dengue viruses cause syncytial effects on C6/36 cells within a range of pH values of the medium.

Our aforementioned experiments were set up at 28°C with 5% CO_2 because it is the optimal growth condition for C6/36 cells. The pH of the medium in that condition is between 7.4 – 7.6 which is close to the physiological pH of cells. It was reported that another flavivirus, St Louis Encephalitis virus induced cell fusion at a low pH environment (21). Another study also supported that an acidic environment is important for DENV-caused cell fusion (18), which is different from our experimental condition. To know if DENV-2-induced cell fusion of C6/36 cells is pH dependent, we used a special medium, L-15, which doesn't need CO_2 to maintain the pH. Therefore, the L-15 medium can be used to culture C6/36 cells without CO_2.

We adjusted the pH of the L-15 from 5.0 to 9.0 as shown in Figure 5A. we infected the cells with the same amount of DENV-2 at the same time to observe the CPE daily under a microscope. As shown in Figure 5B, DENV-2 caused cell fusion of C6/36 cells at a range of 6.0 to 8.0. And the optimal fusion pH ranged from 6.5 to 7.5. Interestingly, viral production was detected with the highest yield at pH 7.5. Therefore, we found a correlation between the pH of the medium, syncytial CPE, and viral yield during the DENV-2 infection in C6/36 cell line.

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DISCUSSION

Cell fusion is often used by viruses to spread progeny viral particles from one cell to the other. Mosquito is the major vector for transmission of DENVs and ZIKV among humans and non-human primates, it is hence extremely important to understand the interaction of DENV or ZIKV with mosquito cells. Here, we employed both cell biological and virological approaches to discern the cytopathic effects (CPE) of the two members of flaviviruses on C6/36 cells, an *Aedes albopictus* cell line that was established in 1967 from freshly hatched *Aedes albopictus* larvae of unspecified ancestry (22). Our overarching findings are that DENVs and ZIKVs cause two types of CPE: syncytial or congregative effects and different strains cause different CPE. For example, DENV-1, -2, and most DENV-4 strains cause syncytial effects in c6/36 cells, while some DENV-4 strains, most DENV-3 strains, and all ZIKV strains engender congregative effects on C6/36 cells (Figure 1 and Video 1-3). To enforce our discoveries, we applied a number of strains of each type of DENV and ZIKV as shown in Table 1. Moreover, the strains of the viruses were selected from different countries and territories.

Whether DENV or ZIKV infection kills C6/36 cells remains arguable. In live mosquitos, DENV or ZIKV infection should not cause cell deaths even if the progeny viral particles have been produced within cells because the infected mosquitos are live, and able to both replicate and transmit viruses by biting. Indeed, several cell culture studies revealed that mosquito cells can be protected from DENV by the induced antioxidant defense as well as anti-apoptotic effects (23, 24). Another study also showed that DENV persistently infected C6/36 cells without affecting cell intactness, and the virus was retained in cells for 20 weeks without seeing CPE (25). Therefore, these seem to have explained from a cell culture perspective why mosquitos support both viral and host lives, placing the mosquito as a dangerous vector. However, other studies have shown that DENV caused cell fusion of C6/36 cells (17, 21, 26, 27). Our current studies also show the syncytial effects of DENVs on C6/36 cells. The syncytial effect of the virus is a type of CPE because the consequences of cell fusion are usually cell deaths. Therefore, some DENV infections may kill the infected cells in the mosquito.

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We, for the first time, examined different strains of DENV or ZIKV for their effects on cell fusion of C6/36 cells. All tested DENV-1 and DENV-2 strains are able to cause syncytial effects on C6/36 cells, some DENV-4 strains, most DENV3 strains, and all ZIKV strains cause cell congregative effects but no cell fusion was seen. Our results suggest that DENV may cause two distinctive CPE: cell fusion and cell congregation. This may suggest that DENVs may have two modes of replication and spread within a live mosquito: cell fusion and "release and entry" infection.

An early experimental result suggested that cell fusion of C6/36 cells could be mediated by E protein because anti-E antibody blocked the virus-caused cell fusion (18). However, the anti-E antibody is able to prevent the entry, infection, and hence replication of the viruses. Our experimental results show that the UV-inactivated DENV failed to cause cell fusion. This suggests that the *de novo* produced viral proteins are important for DENV-caused cell fusion. We also found that the pH environment of the medium affects DENV-caused cell fusion. The viral replication assays show that cell fusion may benefit viral production.

Our studies are of limitations. For example, cell culture studies are different from *in vivo* experiments so many of the observations of DENV or ZIKV infection in mosquitoes cannot be completely explained by in cell culture study results. An *in vivo* study of DENV or ZIKV infection in the mosquito is needed to make clear whether different strains of DENV or ZIKV may have different replication mechanisms and spreading routes in mosquitoes. In addition, our results show that different strains DENV-3 and -4 cause different CPE in mosquito cells. However, the number used may be still not statistically enough to conclude which CPE is more predominant in DENV-infected cells. We will also plan to analyze the genomic sequences to figure out whether there are genomic census sequences that are related to the DENV-caused CPE in mosquito cells.

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The authors declare that they have no conflicts of interest with the contents of this article.

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Legends of the Figures

Figure 1. Cytopathic effects of DENVs and ZIKV on C6/36 cells. C6/36 cells were seeded on a 24-well plate and were infected with the viruses at an MOI of 0.5 as indicated on the left side when the cells are 60-70% confluent. Cells were photographed under a regular light in a phase mode with a 10x len every other day till 12 dpi (days post-infection).

Figure 2. Formation of a network-like syncytial morphology of C6/36 cells by DENV-1 and -2. Viral infection is performed as mentioned in Figure 1. The cells were photographed at 12 dpi (A-C). D. the cells on a coverslip were incubated with a membrane dye [CellMask™ Deep Red Plasma Membrane Stain kit (ThermoFisher, C10046)] according to the manufacturer's protocol and DAPI.

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Figure 3. Viral proteins in the fused C6/36 cells. C6/36 cells were seeded on coverslips and infected with DENV-2 or UV-inactivated DENV-2 at an MOI of 0.5. At 12 dpi, the cells were fixed and permineralized for IFA. Two types of cell fusions were detected: a larger number of C6/36 cells are fused together as shown in A (A1-DAPI, A2-envelop, and A3-NS3 protein). and a smaller number of cells together forming a network-like morphology as shown in B (B1-DAPI, B2-envelop, and B3-NS3 protein).

Figure 4. Viral entry and replication are needed for the viral syncytial effects on C6/36 cells. C6/36 cells were seeded on coverslips and infected with DENV-2 or UV-inactivated DENV-2 at an MOI of 0.5. A. viral protein production shows the UV-inactivated DENV-2 is not able to de novo produce viral proteins. B. photographed the cells at 12 dpi.

Figure 5. pH environment on the syncytial effects of DENV on C6/36 cells. The cells were seeded on a 24-well plate, when the cells are 60-70% confluent, the medium was changed to a laddering pH medium as shown in the top of A. cells were photographed at 12 dpi as shown in the bottom of A. Viral titers were detected by plaque forming unit (PFU) assay as shown in B.

Table 1. Cytopathic effects of DENV and ZIKV in C6/36 cells. Different strains of DENV and ZIKV were used to infect C6/36 cells and CPE modes, either the congregation or cell fusion, were summarized.