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SHORT COMMUNICATION

Ultrastructural aspects of sylvatic dengue virus infection in Vero cell

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Abstract

Introduction: Dengue virus (DENV), the causative agent of dengue disease exists in sylvatic and endemic ecotypes. The cell morphological changes and viral morphogenesis of two dengue ecotypes were examined at the ultrastructural level to identify potential similarities and differences in the surrogate model of enzootic host. *Materials and Methods:* Vero cells were inoculated with virus at a multiplicity of infection (MOI) of 0.1. Cell cultures were harvested over a time course and processed for transmission electron microscopic imaging. *Results:* The filopodia protrusions on cell periphery preceded virus entry. Additionally, sylvatic DENV infection was found spreading slower than the endemic DENV. Morphogenesis of both dengue ecotypes was alike but at different level of efficiency in the permissive cells. *Conclusions:* This is the first ultrastructural study on sylvatic DENV and this comparative study revealed the similarities and differences of cellular responses and morphogenesis of two dengue ecotypes *in vitro*. The study revealed the weaker infectivity of sylvatic DENV in the surrogate model of enzootic host, which supposed to support better replication of enzootic DENV than endemic DENV.

Keywords: Sylvatic dengue virus, endemic dengue virus, cell morphological changes, viral morphogenesis, transmission electron microscopy

INTRODUCTION

Dengue is a mosquito-borne disease caused by dengue virus (DENV), existing as serotype 1, 2, 3, 4 (DENV-1, DENV-2, DENV-3, DENV-4), and a newly characterised serotype 5 (DENV-5).[1] Two ecologically and evolutionarily distinct transmission cycles have been defined for DENV: a sylvatic/enzootic cycle and an endemic/human cycle.[2]

Thus far, studies related to sylvatic DENV are mostly focused on genotypic characterisation,[3] serological analyses,[4] phylogenetic and evolutionary inference,[5] and infection susceptibility or adaptation in cross-species vectors and hosts.[6, 7] To date, sylvatic DENV infection has never been studied at the ultrastructural level. On the other hand, ultrastructural studies of endemic DENV have been conducted, both *in vivo*[8] and *in vitro* at MOI ranging from as low as 0.08 up to 60,[9-11] which described several pathways for DENV internalisation and replication but lacking in visualisation on the release of dengue virion.

Considering the occurrence of cross-species infections of sylvatic DENV into human hosts have been reported in Africa[12, 13] and Malaysia[14, 15], an insight into the infection responses and morphogenesis of sylvatic DENV in susceptible host cells would complement the efforts in characterising the DENV that has been maintained in the sylvan cycle.

In this study, the cellular changes in response to the infections of two dengue ecotypes and viral morphogenesis in the Vero cell over a time course were examined and compared using transmission electron microscopy (TEM). The virus isolates used in this study, the DENV-1 strain D1.Malaysia.36046/05 (sylvatic) and 36000/05 (endemic) were isolated during the dengue outbreak in 2005 and identified by Teoh *et al*.[14] Soon after the report by Teoh *et*

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al. which inferred the relationship between sentinel monkey-derived P72-1244 and human-derived D1.Malaysia.36046/05, Vasilakis *et al*.[16] speculated that the origin of both strains was of human as the phylogenetic reconstruction of complete DENV genome does not place P72-1244 at the basal position within DENV-1.[17] Despite the uncertain origin, the sylvatic nature of P72-1244 and D1.Malaysia.36046/05 should be considered since the P72-1244 has been maintained in an enzootic cycle for decades. Vero, the monkey-derived cell lineage, was justified for the present study as serological and immunological data revealed the role of sentinel monkeys in the maintenance and transmission of sylvatic DENV in sylvan habitat.[2, 18] Therefore, the current study aimed to examine the cell morphological changes and viral morphogenesis of two dengue ecotypes at the ultrastructural level in the surrogate model of enzootic host.

MATERIALS AND METHODS

Cell cultivation

The C6/36 (ATCC® CRL-1660) and Vero (ATCC® CCL-81) cells were cultured in minimum essential medium (MEM; biowest, France) supplemented with 10% fetal bovine serum (FBS; biowest, France) and 1X non-essential amino acids (NEAA; HyClone, US). The C6/36 cell cultures were incubated at 28°C whereas the Vero cells were incubated at 37°C, with 5% CO_2. All the cell lines were screened for mycoplasma contamination using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, USA) prior to and at the end of the experiments. The stained cell cultures were examined under the Olympus IX51® inverted fluorescent microscope (USA).

Virus stock preparation

The sylvatic DENV-1 (D1.Malaysia.36046/05) and endemic DENV-1 (D1.Malaysia.36000/05) isolates[14] were propagated in C6/36. Dengue viral titer was determined by focus forming assay (FFA)[19] in Vero cells. The number of distinct coloured foci was calculated to determine the virus titer and expressed as FFU/mL.

Virus infection

Vero cells were inoculated with virus suspension at a multiplicity of infection (MOI) of 0.1. An equal volume of complete culturing medium instead of virus was used as inoculum for mock-infection controls. Virus adsorption was performed at room temperature for 1 hour with gentle rocking. The inoculum was then removed and the cells were washed twice with 1X PBS. Next, MEM supplemented with 2% FBS and 1X NEAA was dispensed into the tissue culture flasks and the cell cultures were incubated at 37°C with 5% CO_2. Infected cell cultures were harvested at 24, 48, 72, and 96 hours post-infection (h.p.i), whereas, the mock controls were harvested at 0 h.p.i and 96 h.p.i.

Transmission electron microscopy

The harvested cells were fixed with 2.5% glutaraldehyde in phosphate buffer, pH 7.2 (EMS, USA) and post-fixed with 1% osmium tetroxide (EMS, USA). Then, the specimen was dehydrated with ethanol in a series of concentration (30%, 50%, 70%, 90%, and absolute ethanol). The specimens were embedded in resin prepared from Araldite 502 kit (EMS, USA). The ultrathin sections (60-70 nm) which were placed onto copper grids were stained with 2% uranyl acetate and lead citrate stain (EMS, USA) prior to TEM viewing. The samples were examined in the Hitachi HT7700 transmission electron microscope (USA) at an accelerating voltage of 100 kV.

RESULTS

After 24 hours of sylvatic DENV infection in Vero cells, over half of the cell population exhibited normal cell morphology, whereas, the other half was swollen with minimal disintegration of organelles. Extracellular virus particles as well as virus particles near to the electron-dense invagination of plasma membrane could be seen (Fig. 1A). DENV-induced filopodia protrusions from cell surface were prominent. Filopodia reached out to the virus particle and transported it towards the cell body (Figs. 1B and 1C). Smooth membrane-endocytic vesicles were seen frequently at the cell periphery. At 48 h.p.i, the ratio of swollen cells and cells with obvious organelles disintegration was in proportion. Progressing from 24 h.p.i to 72 h.p.i, increment in cytoplasmic volume accompanied by extensive vacuolization originating near the perinuclear position were the most pronounced features. Virus particles and degenerative cellular materials were observed in vacuoles that vary in size and shape. The nucleus was displaced to the cell periphery. At the site where viral replication had taken place, mitochondria, rough endoplasmic reticulum (rER), and Golgi complex were usually associated (Fig. 1D). An increment in the quantity and density of ribosomes

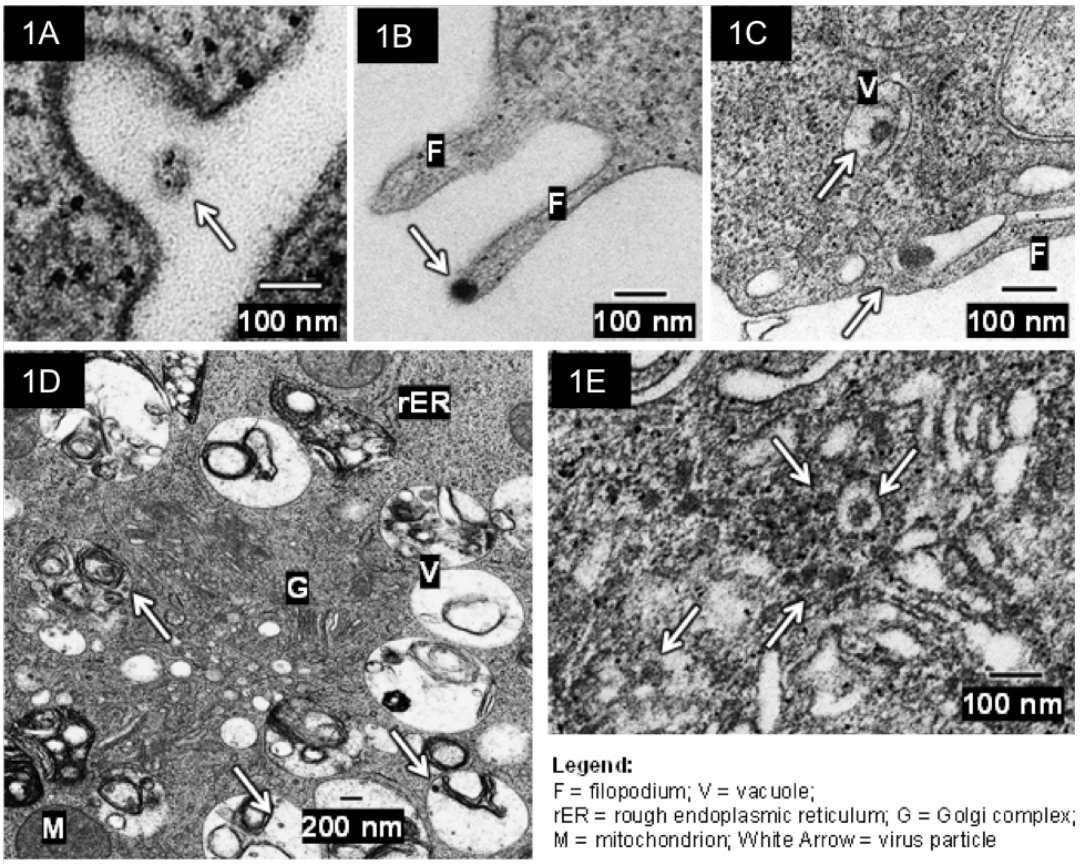

FIG. 1: Sylvatic dengue virus infection in Vero cells. Extracellular virus particles as well as virus particles near to the electron-dense invagination of plasma membrane can be seen at 24 h.p.i **(1A)**. DENV-induced filopodia protrusions from the cell surface were prominently reaching out to the virus particle **(1B)** and transporting it towards the cell body **(1C)**. The virus particles can be found within the cytoplasmic vacuoles and vesicles from 24 h.p.i onwards. At the viral replication site that was first noticed at 72 h.p.i, mitochondria, rough endoplasmic reticulum, and Golgi complex were being surrounded by virus-containing vacuoles **(1D)**. At this point, virus particles can also be found within the swollen cisternae of the Golgi complex **(1E)**.

especially those attached on the endoplasmic reticulum (ER) was seen. Golgi complex with cisternae closely stacked in a parallel array was observed at 72 h.p.i. The cell population was then dominated by those with extensive vacuolization and disintegrated organelles from 72 h.p.i to 96 h.p.i. At this time point, virus particles could be found within the swollen lumen of the Golgi complex (Fig. 1E) and vesicles.

Morphogenesis of the endemic DENV in Vero cells followed a similar but more active pattern as that of the sylvatic DENV infection. At 24 h.p.i, the cytoplasm of the endemic DENV-infected cells were filled with vacuoles containing virus particles and the nucleus was dislocated to the cell edge. These were not spotted in the sylvatic DENV-infected cells at 24 h.p.i. Virus particles were found internalised and present freely in the cytoplasm and within the vesicles (Fig. 2A) in addition to those enclosed in the cytoplasmic vacuoles. Over a period of 48 hours of infection, rER, mitochondria, and Golgi apparatus were in position at the site where virus particles-containing vacuoles accumulated (Fig. 2B). At 72 h.p.i, dengue virus particles that were arranged in crystalloid pattern, as well as isolated virus particles were present within the distended cisternae of rER where smooth membrane structures co-occurred (Fig. 2C).

DISCUSSION

Ultrastructural studies on endemic DENV have been well characterised in Vero cells[20,21]; however, none have been conducted for sylvatic DENV. In the present study, there were noticeable

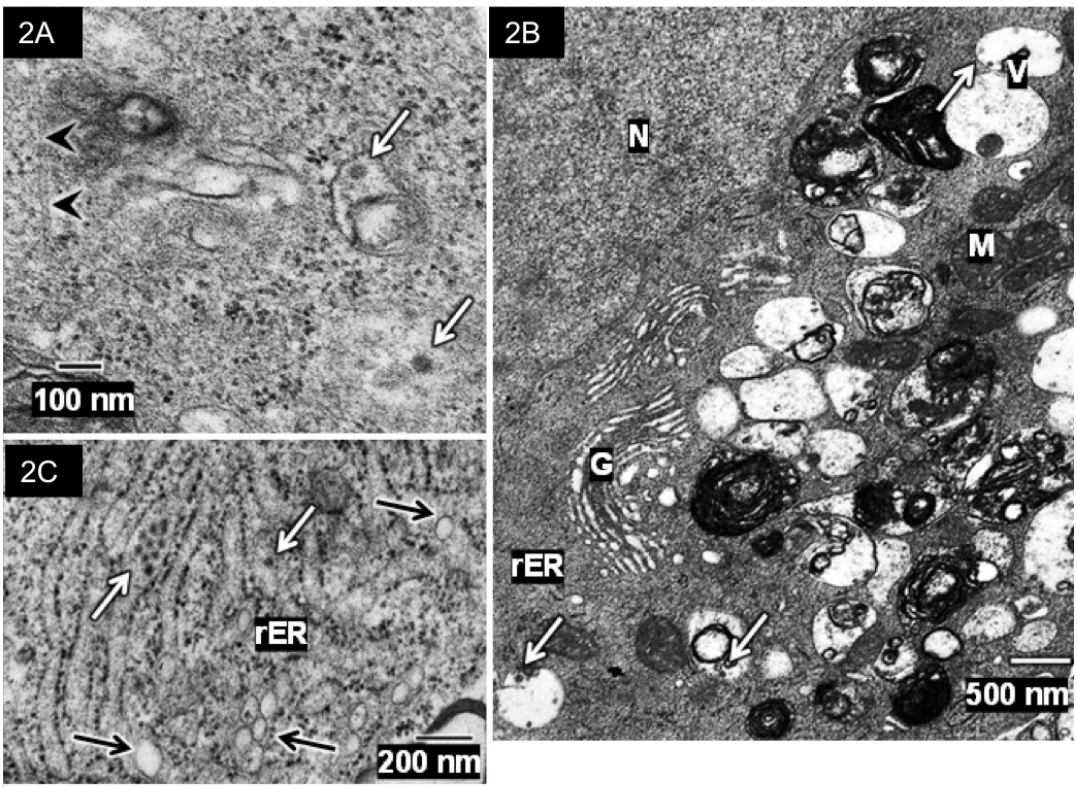

FIG. 2: Endemic dengue virus infection in Vero cells. At 24 h.p.i, internalised virus particles were present freely in the cytoplasm or within the vesicle and microtubule (black arrowhead) was seen together **(2A)**. Over 48 hours of infection, mitochondria, rough endoplasmic reticulum, and Golgi complex associated intimately at the viral replication site where virus particles-containing vacuoles accumulated **(2B)**. At 72 h.p.i, isolated dengue virus particles or those arranged in crystalloid pattern, and smooth membrane structures (black arrow) co-occurred within the distended cisternae of rough endoplasmic reticulum **(2C)**.

sequential changes on the cell morphology of the host cell in accommodating the viral replication. The TEM microscopic observations showed that the cell morphological responses were correlated to the DENV morphogenesis. Despite the different dengue serotype/ecotype and the substantially lower MOI used in this study, the morphological aspects of dengue infection by either ecotype in the Vero cell showed a similar profile as reported by Matsumura *et al*[20] and Zargar *et al* [21] Cytoplasmic vacuolization appeared as a cellular response that was induced following DENV internalisation. The virus particles could be found confined in the membrane-bound vesicles and vacuoles, where microtubules and microfilaments that are usually responsible for the movement of the vesicles and organelles were seen together. Extensive cytoplasmic vacuolization has also been reported in DENV-infected C6/36,[22] LLC-MK2,[23] and BHK-21[24] cell cultures.

Internalisation of extracellular virion was found to be associated with plasma membrane invagination and filopodia. The known functional entry of DENV into a host cell is via the interaction of DENV envelope (E) protein with the receptor on plasma membrane of the target cell, in which the E protein-receptor interaction triggers plasma membrane invagination to form endocytic vesicle, either without[25] or with clathrin-coated[26]. In addition, filopodia on the exterior of the Vero cell periphery preceded DENV entry, consistent with the findings that DENV-2 requires filopodia to internalise into HMEC-1 cell.[27]

Although uncoating of nucleocapsid in releasing the viral RNA genome into the cytoplasm was not directly resolved in this study,

an increment in the size and electron density of the ribosomes in the cytoplasm as well as those attached on ER indicated active synthesis of RNA and glycoproteins, which has been described in other studies.[11,28] Virus particles could be seen individually or arranged in crystalloid pattern within the distended cisternae of rER, with the co-existence of smooth membrane structures. The smooth membrane structure is important for concentrating the viral RNA and possibly for its subsequent encapsidation into the virus particles.[29]

During the active phase of DENV multiplication, rER, Golgi apparatus together with ER- and Golgi-derived vesicles associated intimately and assembled to form the viral replication factory. Expansion and distention of the Golgi apparatus cisternae that had initially stacked in parallel array could be interpreted as intensive virus maturation and getting ready for exocytosis. Earlier electron microcopy studies revealed that budding of DENV during exocytosis is rare.[20,24,30] An exception is seen in the study of DENV-1-infected human leukaemic leukocyte-derived J-111 cell.[31] Taking together the observations of the infections of both dengue ecotypes in this study, DENV progeny might be able to leave the cell via interaction of the virus-contained vesicle and plasma membrane or until the cell lysed. Both pathways matched the observations of Matsumura *et al*[20] in DENV-2-infected Vero cell.

CONCLUSION

Ultrastructural study should be included as one of the important fields of study especially in elucidating the structural responses upon virus infection. This is the first ultrastructure report on sylvatic DENV and it revealed the similarities and differences of cellular responses and morphogenesis of two dengue ecotypes in Vero cells. The study revealed the weaker infectivity of sylvatic DENV in the surrogate model of enzootic host *in vitro*. The results from this transmission electron microscopic study provide future directions to identify the factors of enzootic or human hosts in transmitting two dengue ecotypes.

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Ethics approval: The dengue virus isolate was obtained from patient recruited under the ethic reference number: NMRR-11-1125-9109

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