Short Communication

Ultrastructural changes of cell morphology and viral morphogenesis of two ecotypes of dengue virus infection in human monocytic U-937 cell

Fish-Low, C.Y.[1], Abu Bakar, S.[2], Othman, F.[3] and Chee, H.Y.[1*]
[1]Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, University of Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Tropical Infectious Diseases Research and Education Centre (TIDREC), Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.
[3]Department of Human Anatomy, Faculty of Medicine and Health Sciences, University of Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
*Corresponding author e-mail: cheehy@upm.edu.my

Abstract. Dengue virus (DENV) is maintained and circulated in both sylvatic/enzootic and endemic/human cycles and spill over infection of sylvatic DENV into human populations has been reported. Extensive deforestation and increase human activities in forest may increase the risk of human exposure to sylvatic dengue infection and this may become a threat to human. Present study investigated the changes in cell morphology and viral morphogenesis upon infection with sylvatic and endemic ecotypes in human monocytic U-937 cells using transmission electron microscopy. Autophagy, a process that is either pro-viral or anti-viral, was observed in U-937 cells of both infections, however only the replication of endemic DENV was evidenced. An insight into the infection responses of sylvatic progenitors of DENV in susceptible host cells may provide better understanding on dengue emergence in human populations.

INTRODUCTION

Dengue is a mosquito-borne disease caused by the dengue virus (DENV). There are four DENV serotypes, termed dengue serotype 1, 2, 3 and 4 (DENV-1, DENV-2, DENV-3, DENV-4), and a controversial newly characterized DENV serotype 5 (DENV-5) (Normile, 2013). DENV is maintained and circulated in two ecologically distinct cycles, in a sylvatic cycle and in a human cycle. Spill over infection of sylvatic DENV into human populations has been reported in Malaysia (Cardosa *et al.*, 2009; Teoh *et al.*, 2010) and Africa (Vasilakis *et al.*, 2008; Franco *et al.*, 2011). Experimental studies revealed the possible threats of sylvatic DENV due to the absence of adaptive barrier for its emergence into human populations (Vasilakis *et al.*, 2007).

It is of great importance to study sylvatic DENV since it is the progenitor of endemic DENV. Thus far, the studies related to sylvatic DENV mostly focus on genotypic characterization (Rossi *et al.*, 2012), serological analyses (Wolfe *et al.*, 2001), phylogenetic and evolutionary (Wang *et al.*, 2000), as well as susceptibility to infection or adaptation in cross-species vectors (Moncayo *et al.*, 2004, Diallo *et al.*, 2005) and hosts (Vasilakis *et al.*, 2007). This report serves as the first ultrastructural study of sylvatic DENV in human monocytic cell. The present study aimed to compare the infections

of two dengue ecotypes in U-937 cell at ultrastructural level.

MATERIALS AND METHODS

The sylvatic DENV-1 (D1.Malaysia.36046/05) and endemic DENV-1 (D1.Malaysia. 36000/05) isolates (Teoh *et al.*, 2010) were propagated in C6/36 (ATCC® CRL-1660), where the cells were cultured in minimum essential medium (MEM; biowest, France) supplemented with 10% foetal bovine serum (FBS; biowest, France) and 1X non-essential amino acids (NEAA; HyClone, US). The C6/36 cell cultures were incubated at 28ºC with 5% CO_2. Dengue viral titre was determined by focus forming assay (FFA) (Okuno *et al.*, 1979) in Vero cells (ATCC® CCL-81). The number of distinct coloured foci was calculated to determine the virus titre and expressed as FFU/mL. The U-937 (ATCC® CRL-1593.2) cells were cultured using the same culturing medium as of C6/36, and incubated at 37ºC, with 5% CO_2. Then, the U-937 cells were inoculated with virus to make an input multiplicity of infection (MOI) of 0.1. Virus adsorption was performed at room temperature for 1 hour with gentle rocking. The cell cultures were incubated at 37ºC with 5% CO_2. Cell cultures were harvested at 24, 48, 72, and 96 hours post-infection (h.p.i) and 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). Ultrathin sections were stained with 2% uranyl acetate and lead citrate stain (EMS, USA). The samples were examined in the Hitachi HT7700 transmission electron microscope (USA) at an accelerating voltage of 100 kV.

RESULTS AND DISCUSSION

The mock-infected controls, both at the beginning (0 h.p.i) as well as at the last time-point (96 h.p.i) of infection were examined in order to confirm that the cell morphological alterations observed were in response to the infection. Morphologically, both the mock- and DENV-infected U-937 cell showed smooth outline.

Sylvatic DENV did not undergo active morphogenesis in U-937 cells throughout the course of infection. The shape and size of the cells as well as the cytoplasmic organelles were mostly well-preserved. At 24 h.p.i and 48 h.p.i, only a few virus particles were found enclosed in the vacuoles and vesicles (Figure 1a and 1b). At 72 h.p.i to 96 h.p.i, virus particle-containing autophagosomes were observed (Figure 1c). Autophagosome is characterized by its double-membrane structure and confinement of cellular materials and organelles. Isolated virus particles could be detected at the Golgi complex, where the cisternae were closely stacked in a parallel array (Figure 1d).

On the other hand, morphogenesis of endemic DENV in U-937 was found more evidenced than in the sylvatic strain. At 24 h.p.i, virus containing-vacuoles and vesicles were at close distance to the replication sites that were associated with rough endoplasmic reticulum (rER), Golgi complex and mitochondria (Figure 2a). From 48 h.p.i onwards, virus particles were mostly located within the cisternae of rER that were associated with electron-dense ribosomes (Figure 2b). Vesicles (Figure 2c) and autophagosomes (Figure 2d) that contained virus particles, which were near to the perinuclear position and at the viral replication site were observed from 72 h.p.i onwards.

The remarkable difference noticed in the morphogenesis of both dengue ecotypes in the U-937 cells was the infection by either dengue ecotype activated autophagy. In addition to the anti-viral role of autophagy in the host immunity, its pro-viral functions in elevating viral replication are established for coronavirus (Prentice *et al.*, 2004), chikungunya virus (Krejbich-Trotot *et al.*, 2011), and hepatitis B virus (Tian *et al.*, 2011). The participation of autophagy in enhancing DENV replication has also been demonstrated in Huh7 (Lee *et al.*, 2008),
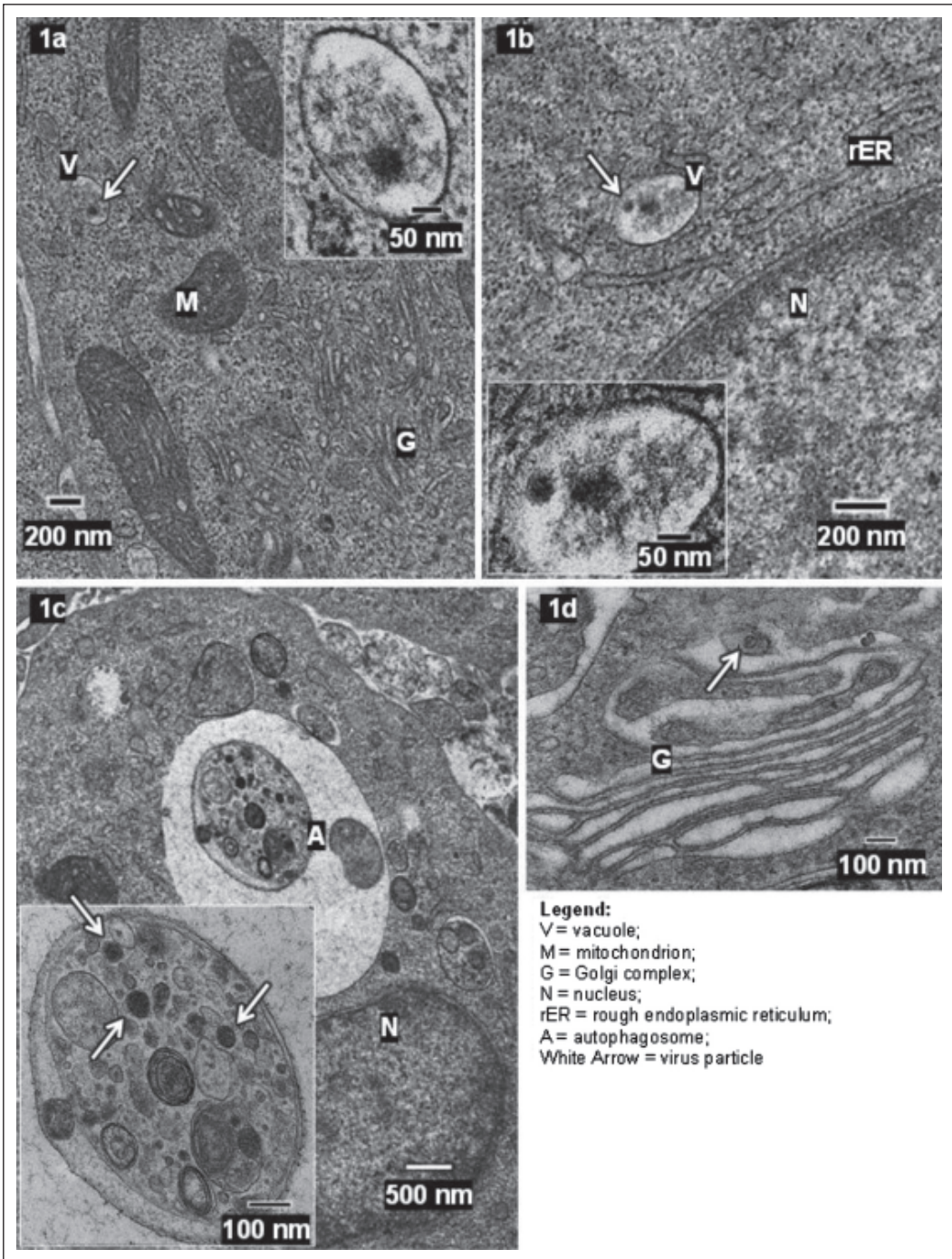
Figure 1. Sylvatic dengue virus infection in U-937 cells.

At 24 h.p.i (a) and 48 h.p.i (b), virus particles are found enclosed in the vacuoles (magnified view as shown in the inset). The cell morphology and organelles are mostly well-preserved. The virus particle-containing autophagosome [(c) magnified view as shown in the inset] is noticed at 72 h.p.i. At 96 h.p.i, isolated virus particles can infrequently be detected at the Golgi complex (d), where the cisternae are stacked in a parallel array.
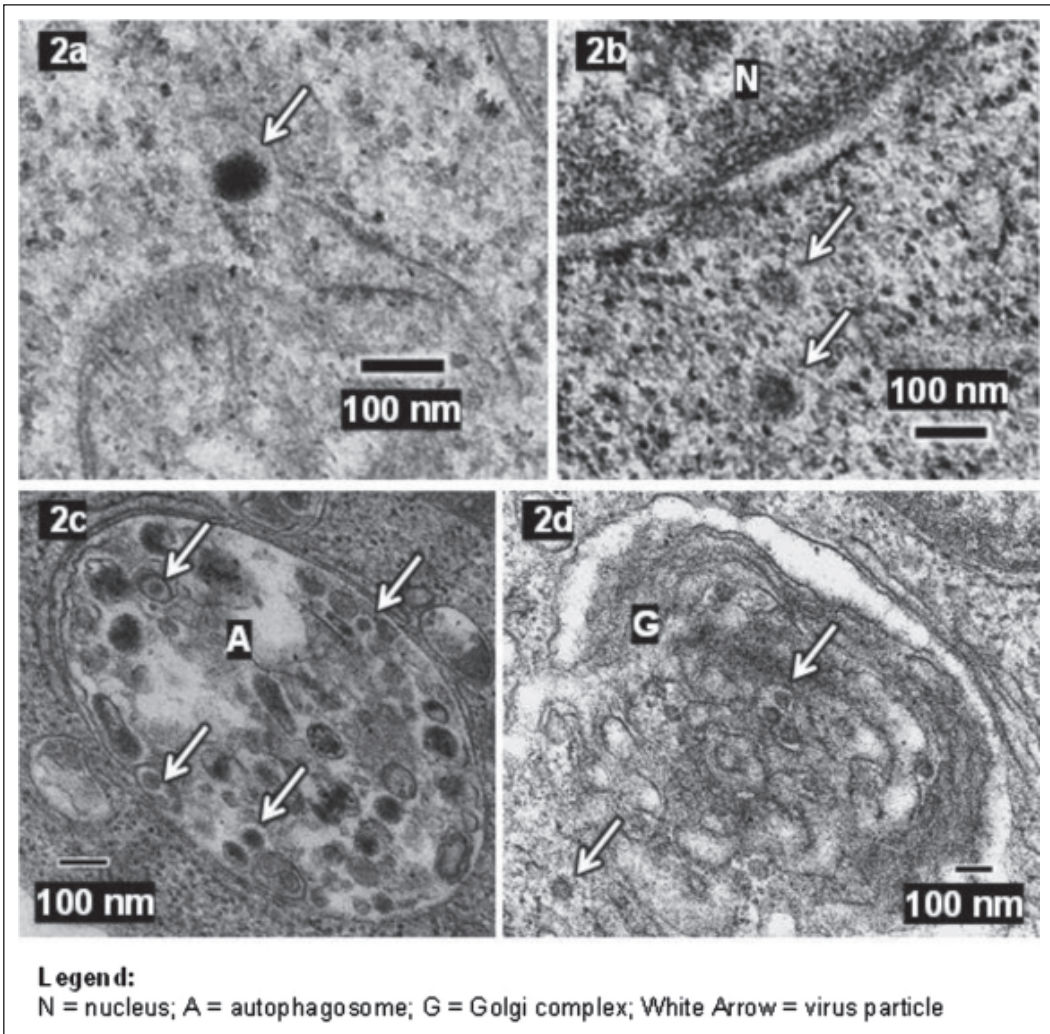
Figure 2. Endemic dengue virus infection in U-937 cells.
At 24 h.p.i, virus particles enclosed in vacuoles are found at close distance to the viral replication site, where rough endoplasmic reticulum, Golgi complex, and mitochondrion are present (a). From 48 h.p.i onwards, virus particles are mostly located within the distended cisternae of rER that are associated with electron-dense ribosomes (b). Vesicles (c) and autophagosomes (d) that contained virus particles were observed from 72 h.p.i onwards.

HepG2 (Khakpoor *et al.*, 2009) cells, and in suckling mice (Lee *et al.*, 2013). In the present study, autophagosomes were noticed at 72 h.p.i in the infections of both dengue ecotypes. However, progressing through 96 hours of infection, active viral morphogenesis was only seen in the endemic DENV but not in the sylvatic DENV infections. Hence, the active role of autophagy in the infection of sylvatic DENV remains to be elucidated. Future investigation can be expanded onto the study of sequence difference between these two

dengue ecotypes on NS4A, which has been shown to prolong dengue viral replication (McLean *et al.*, 2011), genes involved in RNA replication and other replication determinants.

DENV is transmitted to a human host by the bite of an infectious mosquito, where dendritic cells in the skin are believed to be the primary target (Wu *et al.*, 2000). Soon after the infection, human monocytes and macrophages disregard of the state of cell differentiation are responsible in

disseminating DENV to other target tissues (Chen & Wang, 2002), including liver, lung, kidney, and spleen (Jessie *et al.*, 2004). A wide spectrum of human-origin cell types is available for dengue-related research, depends on the suitability of its functional characteristics. Human monocytic U-937 cell lineage has been described susceptible to DENV only if differentiated becomes macrophage prior to the infection (O'Sullivan & Killen, 1994) or in the presence of infection-enhancing antibodies (Brandt *et al.*, 1982). Meanwhile, this study has demonstrated that U-937 does serve as a permissive host for DENV replication even if it is in an undifferentiated state and without the enhancement of antibodies, and autophagy might have an anti-viral role in sylvatic dengue infected U-937 cells.

Comparative to the endemic DENV infection in terms of cell morphological responses and morphogenesis, this study has presented weaker infectivity of sylvatic DENV in human monocytic U-937 cell. Ultrastructural study should be included as one of the important fields of study especially to elucidate the structural responses upon virus infection. The results from this transmission electron microscopic study provide future directions for further research on this sylvatic dengue virus, especially the role of autophagy.

REFERENCES

Brandt, W.E., McCown, J.M., Gentry, M.K. & Russell, P.K. (1982). Infection enhancement of dengue type 2 virus in the U-937 human monocyte cell line by antibodies to flavivirus cross-reactive determinants. *Infection and Immunity* **36**: 1036-1041.

Cardosa, J., Ooi, M.H., Tio, P.H., Perera, D., Holmes, E.C., Bibi, K. & Abdul Manap, Z. (2009). Dengue virus serotype 2 from a sylvatic lineage isolated from a patient with dengue hemorrhagic fever. *PLoS Neglected Tropical Diseases* **3**: e423.

Chen, Y.C. & Wang, S.Y. (2002). Activation of terminally differentiated human monocytes/macrophages by dengue virus: productive infection, hierarchical production of innate cytokines and chemokines, and the synergistic effect of lipopolysaccharide. *Journal of Virology* **76**: 9877-9887.

Diallo, M., Sall, A.A., Moncayo, A.C., Ba, Y., Fernandez, Z., Ortiz, D., Coffey, L.L., Mathiot, C., Tesh, R.B. & Weaver, S.C. (2005). Potential role of sylvatic and domestic African mosquito species in dengue emergence. *The American Journal of Tropical Medicine and Hygiene* **73**: 445-449.

Franco, L., Palacios, G., Martinez, J.A., Vázquez, A., Savji, N., De Ory, F., Sanchez-Seco, M.P., Martín, D., Lipkin, W.I. & Tenorio, A. (2011). First report of sylvatic DENV-2-associated dengue hemorrhagic fever in West Africa. *PLoS Neglected Tropical Diseases* **5**: e1251.

Jessie, K., Fong, M.Y., Devi, S., Lam, S.K. & Wong, K.T. (2004). Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and *in situ* hybridization. *The Journal of Infectious Diseases* **189**: 1411-1418.

Khakpoor, A., Panyasrivanit, M., Wikan, N. & Smith, D.R. (2009). A role for auto-phagolysosomes in dengue virus 3 production in HepG2 cells. *Journal of General Virology* **90**: 1093-1103.

Krejbich-Trotot, P., Gay, B., Li-Pat-Yuen, G., Hoarau, J.J., Jaffar-Bandjee, M.C., Briant, L., Gasque, P. & Denizot, M. (2011). Chikungunya triggers an autophagic process which promotes viral replication. *Virology Journal* **8**: 432.

Lee, Y.R., Hu, H.Y., Kuo, S.H., Lei, H.Y., Lin, Y.S., Yeh, T.M., Liu, C.C. & Liu, H.S. (2013). Dengue virus infection induces autophagy: an *in vivo* study. *Journal of Biomedical Science* **20**: 65.

Lee, Y.R., Lei, H.Y., Liu, M.T., Wang, J.R., Chen, S.H., Jiang-Shieh, Y.F., Lin, Y.S., Yeh, T.M., Liu, C.C. & Liu, H.S. (2008). Autophagic machinery activated by dengue virus enhances virus replication. *Virology* **374**: 240-248.

McLean, J.E., Wudzinska, A., Datan, E., Quaglino, D. & Zakeri, Z. (2011). Flavivirus NS4A-induced autophagy protects cells against death and enhances virus replication. *The Journal of Biological Chemistry* **286**: 22147-22159.

Moncayo, A.C., Fernandez, Z., Ortiz, D., Diallo, M., Sall, A., Hartman, S., Davis, C.T., Coffey, L., Mathiot, C.C., Tesh, R.B. & Weaver, S.C. (2004). Dengue emergence and adaptation to peridomestic mosquitoes. *Emerging Infectious Diseases* **10**: 1790-1796.

Normile, D. (2013). Surprising new dengue virus throws a spanner in disease control efforts. *Science* **342**: 415.

Okuno, Y., Fukunaga, T., Srisupaluck, S. & Fukai, K. (1979). A modified PAP (peroxidase-anti-peroxidase) staining technique using sera from patients with dengue hemorrhagic fever (DHF): 4 step PAP staining technique. *Biken Journal* **22**: 131-135.

O'Sullivan, M.A. & Killen, H.M. (1994). The differentiation state of monocytic cells affects their susceptibility to infection and the effects of infection by dengue virus. *The Journal of General Virology* **75**: 2387-2392.

Prentice, E., Jerome, W.G., Yoshimori, T., Mizushima, N. & Denison, M.R. (2004). Coronavirus replication complex formation utilizes components of cellular autophagy. *The Journal of Biological Chemistry* **279**: 10136-10141.

Rossi, S.L., Nasar, F., Cardosa, J., Mayer, S.V., Tesh, R.B., Hanley, K.A., Weaver, S.C. & Vasilakis, N. (2012). Genetic and phenotypic characterization of sylvatic dengue virus type 4 strains. *Virology* **423**: 58-67.

Teoh, B.T., Sam, S.S., Abd-Jamil, J. & AbuBakar, S. (2010). Isolation of ancestral sylvatic dengue virus type 1, Malaysia. *Emerging Infectious Diseases* **16**: 1783-1785.

Tian, Y., Sir, D., Kuo, C.F., Ann, D.K. & Ou, J.H. (2011). Autophagy required for hepatitis B virus replication in transgenic mice. *Journal of Virology* **85**: 13453-13456.

Vasilakis, N., Shell, E.J., Fokam, E.B., Mason, P.W., Hanley, K.A., Estes, D.M. & Weaver, S.C. (2007). Potential of ancestral sylvatic dengue-2 viruses to re-emerge. *Virology* **358**: 402-412.

Vasilakis, N., Tesh, R.B. & Weaver, S.C. (2008). Sylvatic dengue virus type 2 activity in humans, Nigeria, 1966. *Emerging Infectious Diseases* **14**: 502-504.

Wang, E., Ni, H., Xu, R., Barrett, A.D., Watowich, S.J., Gubler, D.J. & Weaver, S.C. (2000). Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. *Journal of Virology* **74**: 3227-3234.

Wolfe, N.D., Kilbourn, A.M., Karesh, W.B., Rahman, H.A., Bosi, E.J., Cropp, B.C., Andau, M., Spielman, A. & Gubler, D.J. (2001). Sylvatic transmission of arboviruses among Bornean orangutans. *The American Journal of Tropical Medicine and Hygiene* **64**: 310-316.

Wu, S.J., Grouard-Vogel, G., Sun, W., Mascola, J.R., Brachtel, E., Putvatana, R., Louder, M.K., Filgueira, L., Marovich, M.A., Wong, H.K., Blauvelt, A., Murphy, G.S., Robb, M.L., Innes, B.L., Birx, D.L., Hayes, C.G. & Frankel, S.S. (2000). Human skin Langerhans cells are targets of dengue virus infection. *Nature Medicine* **6**: 816-820.