CHAPTER V
DISCUSSION AND CONCLUSIONS

Japanese encephalitis (JE) remains a public health problem in Asia (26). The clinical presentations of JE are ranging from nonspecific flu-like illness to acute fatal encephalitis (2). Upon Japanese encephalitis virus (JEV) infection, neuronal cell is a primary target of this virus in central nervous system. However, microglia, residential brain macrophages, are activated and secreted many pro-inflammatory cytokines leading to a greatly increased level of neuronal damage (10, 31, 34). Therefore, this study aimed to determine the involvement of autophagy in human microglia upon JEV infection.

Tryptan blue exclusion assay were used to determine the number of life and death cells. Infection with MOI 10 and 100 showed a few number of percent cell death (less than 10%). In addition, the proliferation rate in JEV infected CHME-5 cultures with an MOI of 100 was slower when compared to either mock-infected or JEV-infected CHME-5 cultures with an MOI of 10. This data suggested that, with a higher multiplicity of infection, a process for maintaining cellular homeostasis and survivability is triggered. The cell cycle analysis determined by flow cytometry after propidium iodine staining will provide the evidence of cell cycle arrest, resulting in a slower cell proliferation rate in response of JEV infection at last. Also, another flavivirus, Dengue virus serotype 4 (DEN-4) was reported to slow down human hematopoietic (K562) cell proliferation rate without cytopathic effects (59).

Concerning the susceptibility of human microglial cell in response to JEV infection, immunocytochemistry technique was performed. The results were quantitated as percentage of infectivity. At 48 hr post infection, approximately 60% and 78% of JEV-infected human microglial cell (CHME-5) were detected when at MOI 10 and 100 were used, respectively. Viral E protein was clearly observed within cytosol. These results showed that human microglial cells are susceptible to JEV infection, correlating to the previous study in mouse microglial (BV-2) cells (12). Interestingly, the amount of extracellular virions produced in culture supernatant of mouse microglial cells ($5x10^9$ p.f.u./ml) was approximately 2 log higher than that of human microglial cells ($4x10^7$

p.f.u./ml) at 48 hr post infection, even though mouse microglial cells were challenged with 100 times less multiplicity of infection. These imply host cell tropism and human is a dead-end host of this virus.

Autophagic cells death in human microglial cells in response to JEV infection was revealed at ultrastructural level by transmission electron microscope. In addition, immunocytochemistry and western blotting for detection of autophagic specific marker, LC3, were also performed for confirmation. These results are consistent to JEV infection in a pluripotent human testicular embyonal carcinoma cell line NT-2 cells with two different strains of viral virulence, Rp-9 and Rp-ms (attenuated) (56).

From this study, colocalization between viral E protein and LC3 was detected by using immunocytochemistry. This implies that the autophagic vacuoles may be the replication sites for JEV infection in microglial cells. To confirm that autophagosome is a replicative site for JEV , colocalization of LC3 and viral dsRNA should be performed (42).

Many Flaviviruses have been shown to subvert cellular autophagy for their benefit of viral replication. Recently research in Chikungunya virus, Dengue virus and Japanese encephalitis virus strain RP-9 and RP-ms show the induction of autophagy and suggest that autophagy may play a crucial role during flavivirus infection (16, 55-57).

Effect of autophagy on JEV viral production was studied using 3-Methylanine (3-MA) as a pharmacological autophagy inhibitor. It was found that JEV replication is dramatically decreased at 48 hr post infection. Interestingly, at 24 hr post infection, no virion was detected in supernatants of JEV-infected CHME-5 culture with 3-MA pretreatment (data not show). To further support this finding, pretreatment with autophagy inducer, Rapamycin, and genetic knockdown of autophagy essential gene Beclin-1 and Atg5 should be performed to confirm autophagy mediated JEV replication.

Recently, the reduction of viral yields was reported using genetic knockdown of Atg5 in JEV infected NT-2 cells. The level of JEV NS3 protein expression also decreased (56).

Furthermore, in 2013, Jin et al., found that the suppression of autophagy in JEV-infected neuronal cell correlated with an enhanced interferon type-1 activation. Additionally, autophagy can prolong cell survivor. This study suggests that the

autophagy negatively regulates the innate immune response to facilitate JEV infection. (60).

Understanding of details about relationship between autophagy and viral replication will improve the knowledge of JEV pathogenesis. Our finding might be applied for antiviral drug design which target to inhibit autophagosome formation resulting in preventive of viral production in host cells.