INFECTION OF DENGUE VIRUS TO A MEGAKARYOBLASTIC CELL LINE, MEG-01: A MODEL TO INVESTIGATE THE ROLES OF DENGUE VIRUS AND PLATELETS IN THE IMMUNOPATHOGENESIS OF DENGUE HEMORRHAGIC FEVER (DHF)

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ABSTRACT

Thrombocytopenia and bone marrow suppression occur in the early phase of dengue virus (DV) infection. Several studies have demonstrated that thrombocytopenia is a result of increased platelet consumption during DV infection. However, the effect of dengue virus on megakaryocytes (i. e. cells which produce platelets) remains unclear.

To investigate whether megakaryocytes are susceptible to dengue virus infection and replication, megakaryoblastic cell line (MEG-01) was infected with DV at multiplicity (MOI) of 1 and followed-up at days 1, 3, 5, and 7 after infection. The percentage of DV infection and secreted nonstructural protein 1 (NS1) and envelope (E) protein were determined by immuno-fluorescence staining and enzyme-linked immuno-sorbent assay respectively. Virus production was determined by focus forming unit assay.

All serotypes of DV infected MEG-01 and produced high titers of virus. The infection of DV induced apoptosis in a proportion of cells. 20% of cells were infected at day 1 and 3 which rose to 60% at day 5 and 7 after infection. 10^4 FFU/ml of virus were produced on day 1, rising to 10^7 FFU/ml on day 5 after infection. Compared with Vero cell, MEG-01 secreted less NS1 protein but an equal amount of E protein. On day 5 of infection, when 65% of cells were infected, 10^7 FFU/ml of virus were produced in MEG-01 compared to 10^6 FFU/ml from Vero cells.

In addition, small vesicles were detected in the culture supernatant by flow cytometry. These vesicles contained NS1 antigen as assayed by an NS1-specific ELISA. Lysate from the vesicles contained infectious virus.

In conclusion, megakaryocytes might be one of the major targets for virus replication and one of the sources producing infectious virus and viral proteins involved in the immunopathogenesis of dengue hemorrhagic fever. Infection of megakaryocytes and the subsequent events might be factors contributing to thrombocytopenia.

KEYWORDS : THROMBOCYTOPENIA / PLATELETS / DENGUE VIRUS (DV) / MEG-01 / MICROPARTICLES

94 pp.

การติดเชื้อไวรัสเด็งกี่และผลกระทบต่อ Megakaryoblastic cell line, MEG-01: ต้นแบบของการศึกษาผลกระทบของการติดเชื้อไวรัสเด็งกี่ต่อระบบเกล็ดเลือดในโรคไข้เลือดออก

(INFECTION OF DENGUE VIRUS TO A MEGAKARYOBLASTIC CELL LINE, MEG-01: A MODEL TO INVESTIGATE THE ROLES OF DENGUE VIRUS AND PLATELETS IN THE IMMUNO-PATHOGENESIS OF DENGUE HEMORRHAGIC FEVER (DHF))

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บทคัดย่อ

ภาวะเกล็ดเลือดต่ำและการลดจำนวนของเซลล์ไขกระดูกพบได้ในระยะแรกของผู้ป่วยติดเชื้อไวรัสเด็งกี่โดยเฉพาะในผู้ป่วยไข้เลือดออก การศึกษาที่ผ่านมาพบว่าในภาวะที่มีการติดเชื้อไวรัสเด็งกี่จะมีการใช้เกล็ดเลือดที่เพิ่มมากขึ้นและจำนวนเกล็ดเลือดลดลง ยังไม่มีงานวิจัยที่ศึกษาผลกระทบของการติดเชื้อไวรัสเด็งกี่ต่อเซลล์ Megakaryocytes ซึ่งเป็นเซลล์ต้นกำเนิดของเกล็ดเลือดและผลกระทบที่เกิดขึ้นจากการติดเชื้อ

ผู้วิจัยได้ใช้เซลล์เพาะเลี้ยง MEG-01 ซึ่งเป็นเซลล์ที่มีระยะอ่อนกว่า Megakaryocytes มาศึกษาผลกระทบของการติดเชื้อไวรัสเด็งกี่ต่อเซลล์ Megakaryocytes โดยผสมเซลล์ MEG-01 กับไวรัสเด็งกี่ในอัตราส่วนของจำนวนเซลล์ต่อไวรัสเป็น 1 ต่อ 1 แล้วนำเซลล์และน้ำเลี้ยงเซลล์ที่เก็บได้ในวันที่ 1, 3, 5, และ 7 หลังการผสมมาตรวจการติดเชื้อไวรัสเด็งกี่ในเซลล์ MEG-01 โดยการย้อมโปรตีนของไวรัสภายในเซลล์ พร้อมทั้งหาจำนวนของไวรัสโดยใช้วิธี focus forming unit assay (FFU) และใช้วิธี enzyme-linked immunosorbent assay (ELISA) เพื่อวัดปริมาณโปรตีน nonstructural protein 1 protein และ envelope protein ของไวรัสที่ถูกปล่อยออกมานอกเซลล์

ผลการศึกษาพบว่าเชื้อไวรัสเด็งกี่ได้ทั้ง 4 สายพันธุ์สามารถเพิ่มจำนวนและปล่อยไวรัสออกนอกเซลล์ MEG-01 ได้เป็นจำนวนมาก การติดเชื้อไม่ทำให้เซลล์ตาย (apoptosis) ในระยะแรกของการติดเชื้อ แต่เซลล์จะเริ่มตายมากขึ้นในวันที่ 5 เมื่อติดตามจำนวนเซลล์ที่ติดเชื้อไวรัสเด็งกี่พบว่าในวันที่ 1 และ 3 มีเซลล์ที่ติดเชื้อไวรัสเด็งกี่ประมาณ 20% และเพิ่มขึ้นเป็น 60% ในวันที่ 5 และ 7 ส่วนปริมาณไวรัสที่ถูกปล่อยออกนอกเซลล์นั้นมีประมาณ 10^4 FFU/ml ในวันที่ 1 และเพิ่มขึ้นเป็น 10^7 FFU/ml ในวันที่ 5 และเมื่อเปรียบเทียบการติดเชื้อไวรัสเด็งกี่ระหว่างเซลล์ MEG-01 และ Vero cell พบว่าเซลล์ทั้งสองชนิดปล่อย envelope protein ออกมาเท่ากัน แต่เซลล์ MEG-01 ปล่อย nonstructural protein1 1 ออกมาน้อยกว่า และในวันที่ 5 ซึ่งมีจำนวนเซลล์ที่ติดเชื้อไวรัสเด็งกี่เท่ากันที่ 65% แต่ MEG-01 กลับปล่อยไวรัสออกนอกเซลล์ ได้มากกว่า Vero cell (10^7 FFU/ml และ 10^6 FFU/ml ตามลำดับ) นอกจากนี้ยังพบ small vesicles ถูกปล่อยออกมาจากเซลล์ (Flow cytometry) และพบว่ามีโปรตีน nonstructural protein 1 (ELISA) และมีไวรัสเด็งกี่ภายใน (FFU)

การศึกษานี้สรุปได้ว่า Megakaryocytes อาจจะเป็นเซลล์เป้าหมายหนึ่งของเชื้อไวรัสเด็งกี่ และอาจเป็นหนึ่งในแหล่งที่สร้างไวรัส และโปรตีนของไวรัสซึ่งเกี่ยวข้องกับกลไกการก่อโรคของโรคไข้เลือดออก

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LIST OF ABBREVIATIONS

α	Alfa
ADE	Antibody dependent enhancement
ADP	Adenosine diphosphate
β	Beta
BFU	Burst-forming unit
BSA	Bovine serum albumin
C	Capsid protein of dengue
CMC	Carboxy methyl cellulose
CML	Chronic myelogenous leukemia
CMP	Common myeloid progenitor
cm^2	Square centimeters
COX-2	Cyclooxygenase-2
CO_2	Carbon dioxide
Cy3	Cyanine 3
DAB	3, 3’ diamino benzidine tetrahydrochloride
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
DV	Dengue virus
E	Envelope protein of dengue
ER	Endoplasmic reticulum
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
FcR	Fc receptor
FITC	Fluorescein isothiocyanate
FFU	Focus forming unit
γ	Gamma

LIST OF ABBREVIATIONS (Cont.)

G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte/macrophage progenitor
gp	Glycoprotein
GPI	Gycosylphosphatidylinositol
h	Hour
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HPP	High proliferative potential
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
L-15	Leiboritz's medium
mAb	Monoclonal antibody
MCMV	Murine cytomegalovirus
MCP	Monocyte chemotactic protein
MEP	Megakaryocyte/erythroid progenitor
MgCl	Magnesium chloride
mg	Milligram
min	Minute
MIP-1	Macrophage inducible protein-1
MK	Megakaryocytes
mM	Millimolar

LIST OF ABBREVIATIONS (Cont.)

mm	Millimeter
MOI	Multiplicity of inflection
MPs	Microparticles
NaCl	Sodium chloride
$NiCl_2$	Nickel chloride
nm	Nanometer
NS	Nonstructural protein of dengue
OPD	O-phenylene diamine
pAb	Polyclonal antibody
PBMC	Peripheral blood monolayer cell
PBS	Phosphate-buffered saline
Ph1	Philadelphia chromosome-positive
PI	Propidium iodide
PrM	Precocursor of the mature membrane protein
PS	Phosphatidylserine
RNA	Ribonucleic acid
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGF	Transforming growth fator
TNF	Tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TPO	Thrombopoietin
TPB	Tryptose phosphate broth
TUNEL	Terminal uridine deoxynucleotidyl transferase dUTP nick end labelling
µg	microgram
µM	micromolar
µm	micrometer

LIST OF ABBREVIATIONS (Cont.)

µl	microliter
U	Unit
WHO	World Health Organization

CHAPTER I
INTRODUCTION

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are important mosquito-borne viral diseases caused by dengue virus (DV) infection. Annually, there are 50 to 100 million cases of DF and 250,000 to 500,000 cases of DHF around the world. Over half of the world's population lives in areas at risk of DF (1). DV is transmitted from human to human by the mosquito *Aedes aegypti* and *Aedes albopictus* (2-4). Clinical manifestations of DV infection range from a nonspecific flu-like illness or classical DF to the most severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which is associated with a significant mortality risk (5). Some hypothesis has been proposed for the pathogenesis of DV infection. The antibody dependent enhancement (ADE) of infection involves preexisting antibodies to previous DV infection that cannot neutralize but instead enhance infection, resulting in higher viral load and the development of DHF/DSS (5). DHF is more common in secondary DV infection than in primary infection (6, 7).

Thrombocytopenia is one of the most common clinical findings in DF and DHF/DSS patients, indicating an abnormal hemostasis during infection (5, 8). However, this symptom does not occur in every DF and DHF/DSS patient. It occurs before the day of defervescence or shock and usually reaches the nadir at the day of defervescence or shock. The mechanisms involved in thrombocytopenia associated with DF and DHF/DSS are still not well understood. Several studies demonstrated that thrombocytopenia in DV could be due to platelet dysfunction (i.e., platelet activation and aggregation), infection increased destruction or consumption (9, 10). In addition, alteration in megakaryocytopoiesis (11, 12) and reduction of bone marrow cells (11, 13) have also been mentioned as causes of thrombocytopenia in DV infection.

Bone marrow suppression is also a well known phenomenon in DF and DHF/DSS. The degree of suppression is similar in both DF and DHF/DSS (14). The suppression of bone marrow occurs transiently during DV infection (15). During the febrile phase, bone marrow biopsy reveals a marked hypocellularity with arrest of the megakaryocyte maturation (13, 16). During the time of shock when the platelet count reaches a nadir, bone marrow is normal or hypercellular. During the convalescent phase, there is a rapid recovery of all marrow cells (16, 17). The mechanisms of the transient bone marrow suppression are still not completely understood. Several studies demonstrated that hematopoietic progenitor cells and stromal cells are susceptible to dengue virus infection leading to cell death and inhibition of the progenitor cell maturation (11, 15, 18, 19). Changes in hematopoietic progenitor regulator cytokines after dengue virus infection (i.e., hematopoiesis suppressive cytokines such as MIP-1α and β (20), IFN-α, and TGF-β) have also been suggested to cause bone marrow suppression in DV infection (10, 15).

Megakaryocytopoiesis is a term used to describe megakaryocyte processes including proliferation, maturation, and platelet production (21, 22). Mechanisms of megakaryopoiesis regulation are not well understood. Thrombopoietin (TPO), a ligand for the *c-mpl* proto-oncogene, plays an important role in megakaryocytopoiesis (23, 24). TPO promotes both the proliferation and maturation into platelet-producing megakaryocytes (25, 26). The level of circulating TPO, constitutively produced mainly in kidneys and liver, was controlled by binding of TPO to TPO receptors on the surface of platelets in circulation (27, 28).

Several studies demonstrated that hematopoietic progenitor cells could be infected with DV resulting in an abnormality in cell maturation (11, 15, 16, 19, 29). However, there was no report that DV could directly infect megakaryocytes which are the sources of circulating platelets.

Microparticles (MPs) are one of the hallmarks of cellular alterations. At present, there is no real consensus on the definition of MPs. It is accepted that MPs are submicron elements (from 0.1 to 1 μm) that express on their surfaces phosphatidylserine (PS) and membrane antigens that are characteristic of their cell of origin (30, 31). MPs are shed from the plasma membrane of the cells undergoing

activation or apoptosis. Since DV infection leads to activation and apoptosis of the cells (32), increased levels of MPs could potentially be observed.

This project aims to determine the susceptibility of megakaryocytes to DV infection. The infection of megakaryocytes by DV could affect the viability or proliferation of these cells. The particles and/or microparticles produced from DV infected-megakaryocytes could contain DV progeny or DV proteins that may play a role in DV distribution and immunopathogenesis in DV infection.

CHAPTER II
HYPOTHESIS

The hypothesis is based on a set of key questions aimed at understanding the mechanisms of thrombocytopenia that occurs in DHF/DSS. It is hypothesized that DV infects and replicates in megakaryocytes. Infection of megakaryocytes by the virus could lead to cell death or replication of virus. This would produce virus progeny and platelets that contain infectious virus capable of infecting other cells that phagocytose the platelets. Infected megakaryocytes could produce virus-infected particles and/or microparticle expressing viral antigens on their surface that are recognized by host anti-dengue antibodies. It is also hypothesized that DV-infected megakaryocytes generate microparticles which can contribute to dengue pathogenesis.

In order to answer the questions and the hypothesis, it is necessary to explore whether DV can infect and replicate in megakaryocytes and the consequences of the DV infection in that particular cell. The best source of megakaryocytes in clinical practice is from bone marrow; but it is virtually impossible to use this source for experimental purposes. In this thesis, it is planned that a megakaryoblast cell line will be used instead. The cell line is the human megakaryoblast MEG-01 (ATCC CRL-2021) as described by Ogura M, *et al*. in 1985. This cell has similar characteristics to the megakaryoblast cell in bone marrow.

OBJECTIVES

3.1 To investigate the infection of dengue virus in MEG-01 cells

3.1.1 To investigate the DV antigens expressed on DV-infected MEG-01 cells

3.1.2 To investigate the infectious virus progeny produced from DV-infected MEG-01 cells

3.2 To investigate the consequences of DV infection in MEG-01

3.2.1 To determine the cell proliferation and viability of DV-infected MEG-01 cells, compared with mock-infection

3.2.2 To investigate the mechanisms leading to cell death in DV-infected MEG-01

3.3 To investigate the susceptibility to DV infection of MEG-01

3.3.1 To determine the infectivity of DV in MEG-01, compared with Vero cells

3.3.2 To determine the viral proteins secreted from MEG-01, compared with Vero cells

3.3.3 To determine the DV virus progeny production from DV-infected MEG-01, compared with Vero cells

3.3.4 To determine susceptibility of MEG-01 to the infection of clinically isolated DV serotypes 1-4

3.4 To investigate whether DV infection leads to generation of particles and/or microparticles containing infective viruses

CHAPTER III
LITERATURE REVIEW

3.1 Dengue virus

Dengue virus (DV) is a positive sense, single stranded RNA virus belonging to the family *Flaviviridae* genus Flavivirus, which also includes yellow fever, West Nile, Japanese, and St. Louis encephalitis viruses. Its genome is approximately 11 kb in length and contains a type I methylated nucleotide cap structure at the 5' end but lacks a poly A tail at the 3' end. After translation, the single polyprotein is cleaved by either host or viral protease resulting in 3 structural proteins including Capsid (C) protein; Membrane (M) protein, and envelope (E) protein and 7 nonstructural proteins (NS) including NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 as shown in figure 1 (33-36).

In flavivirus infection (figure 2), it is hypothesized that virus enters the cell via a membrane fusion mechanism motivated by conformational changes of viral E protein (37) or the antibody-dependent enhancement (ADE) using Fc receptor on the host cell membrane and internalized by endocytosis (38, 39). When the virus is inside the cells, uncoating processes occur to release the viral RNA into the cytoplasm. The positive sense viral RNA is translated to a polyprotein which is later cut by viral and host cellular proteases into three structural proteins and seven nonstructural proteins. Following the replication and protein synthesis, the assembly and maturation of virus progenitors can take place at the endoplasmic reticulum (ER). Rapid packaging of viral RNA in the viral capsid protein and budding from the ER membrane (already embedded with the viral prM and E protein) generate intracellular virions (35). The intracellular virions are transported away from the infected cell through the secretory pathway and released by exocytosis (37, 40, 41). In addition, the direct budding of virions at the plasma membrane has been found in mosquito cell lines but it does not seem to be a major mechanism of the release of virions (42).

Several cells were described as targets for DV infection including peripheral blood mononuclear cells (PBMC), hepatocytes, B and T lymphocytes, endothelial cells and fibroblasts (43-50). The studies in tissue and blood specimens from DHF/DSS patients using immunofluorescence and immunohistochemistry, show that viral antigens can be detected in Kupffer and endothelial cells in the liver; macrophages, multinucleated cells, and reactive lymphoid cells in the spleen; macrophages and vascular endothelium in the lung; and kidney tubules; monocytes, and lymphocytes in blood (51). In addition, *in vitro* studies on the infection of DV to hematopoietic progenitor cells show the susceptibility of these cells to DV infection (11, 19).

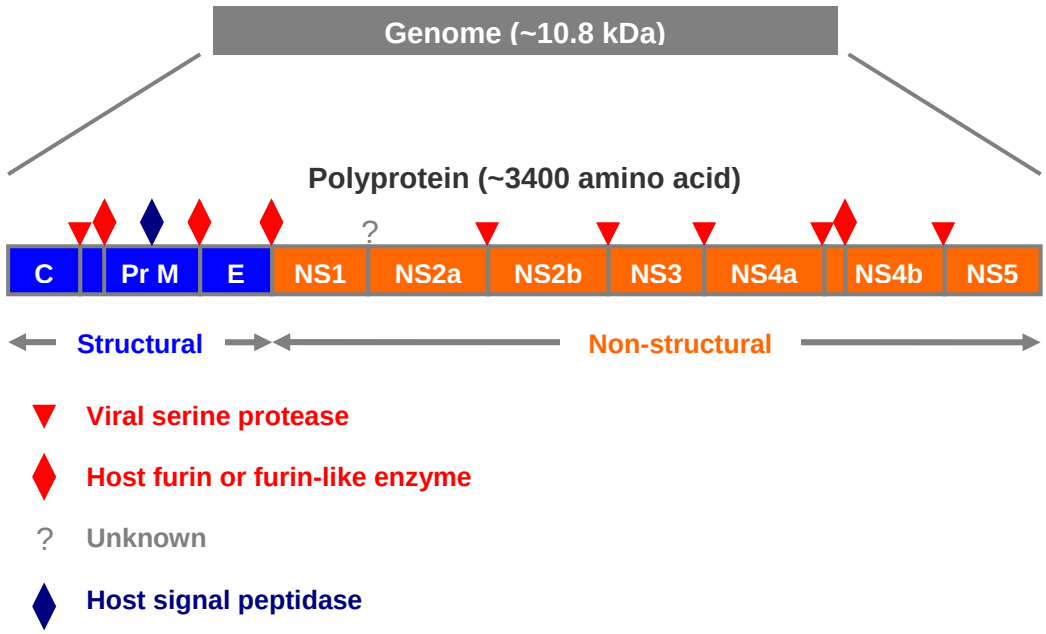


Figure 1 Flavivirus genome and polyprotein processing (36)

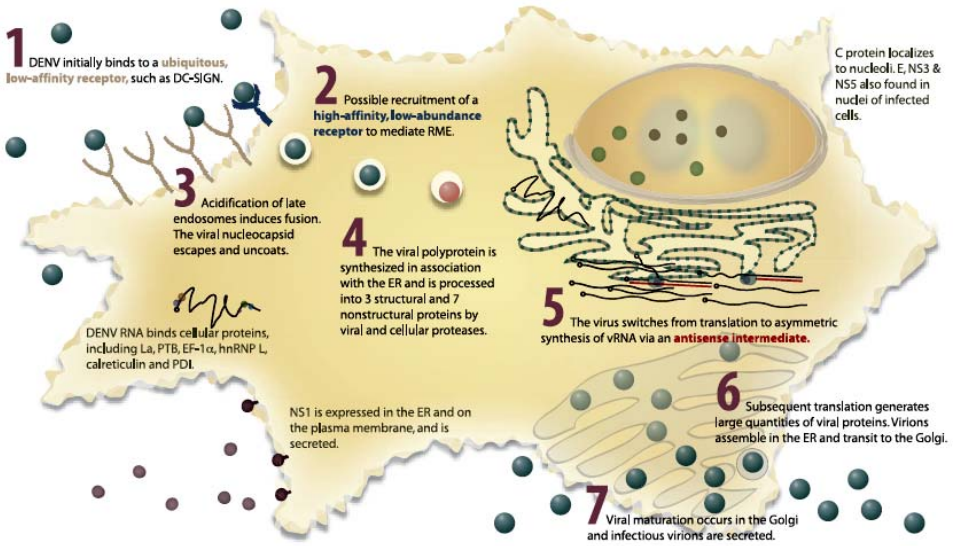


Figure 2 Intracellular life cycle of dengue virus (52)

3.2 Clinical manifestations of DV-infections

The prevalence of DV diseases is mostly in tropical and subtropical regions which are also endemic area of *Aedes aegypti*, a major transmitter for DV (2), as shown in figure 3 (53). Approximately 50–100 million individuals are infected every year, and in some years as many as 500,000 people have been admitted to hospitals (5). The incubation period of DV infection varies from 3-14 days. DV infection results in a wide spectrum of disease ranging from asymptomatic or mild undifferentiated fever, classical dengue fever (DF) to severe and fatal disease, dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS) (Figure 4). World Health Organization (WHO) divided DHF/DSS into 4 grades, DHF grade I to grade IV, using criteria showed in Figure 5 (54).

3.2.1 Dengue fever (DF)

DF is an acute biphasic febrile illness which is most common in older children and adults, infected with DV. The incubation period is 3-8 days after a mosquito bite. DF is characterized by sudden onset of fever and a variety of nonspecific signs and symptoms such as headache, backache, body ache, nausea and vomiting, joint pains, weakness, and rash. The body temperature is usually between 39 ^{o}C and 40 ^{o}C; the fever may be biphasic and tends to last for 2-7 days (55-57). Hemorrhagic manifestations in DF patients are uncommon and range from mild to severe. Skin hemorrhages, including petechiae and purpura are the most common, along with gastrointestinal bleeding, gingival bleeding, epitasis, and menorrhagia. Hematuria occurs infrequently, and jaundice is rare (56). DF is generally self-limiting and is rarely fatal. The acute phase of illness lasts for 3 to 7 days, but the convalescent phase may last for weeks and is probably associated with weakness and depression, especially in adults (58).

3.2.2 Dengue hemorrhagic fever (DHF)

DHF is characterized by high fever, hemorrhagic phenomena, and features of circulatory failure (59). The WHO case definition of DHF is a patient that meets the following four criteria: sudden onset of high fever for 2-7 days, hemorrhagic manifestations with at least a positive tourniquet test, platelet count < $100x10^9$/L and hemoconcentration (rising packed cell volume >20%) or other evidence of plasma leakage such as ascites, pleural effusion or low levels of serum protein/albumin. Furthermore, according to severity, DHF is divided into four grades. Grade I and grade II are non-shock DHF. In grade I, the only hemorrhagic manifestation is a positive tourniquet test Grade II is similar to grade I but in addition there is spontaneous bleeding. Grade III and grade IV are cases of DHF with shock (Dengue shock syndrome, DSS). In grade III there are signs of shock such as rapid and weak pulse, narrow pulse pressure (20 mmHg or less), hypotension with cold, clammy skin and restlessness. Grade IV cases are those with profound shock with undetectable blood pressure or pulse (59).

3.2.3 Dengue shock syndrome (DSS)

DSS is associated with very high mortality (around 9.3%, increasing to 47% in instances of profound shock). Acute abdominal pain and persistent vomiting are early warning signs of impending shock. Sudden hypotension may indicate the onset of profound shock. Prolonged shock is often accompanied by metabolic acidosis, which may precipitate disseminated intravascular coagulation or enhance ongoing disseminated intravascular coagulation, which in turn could lead to massive hemorrhage. DSS may be accompanied by encephalopathy due to metabolic or electrolyte disturbances (59).

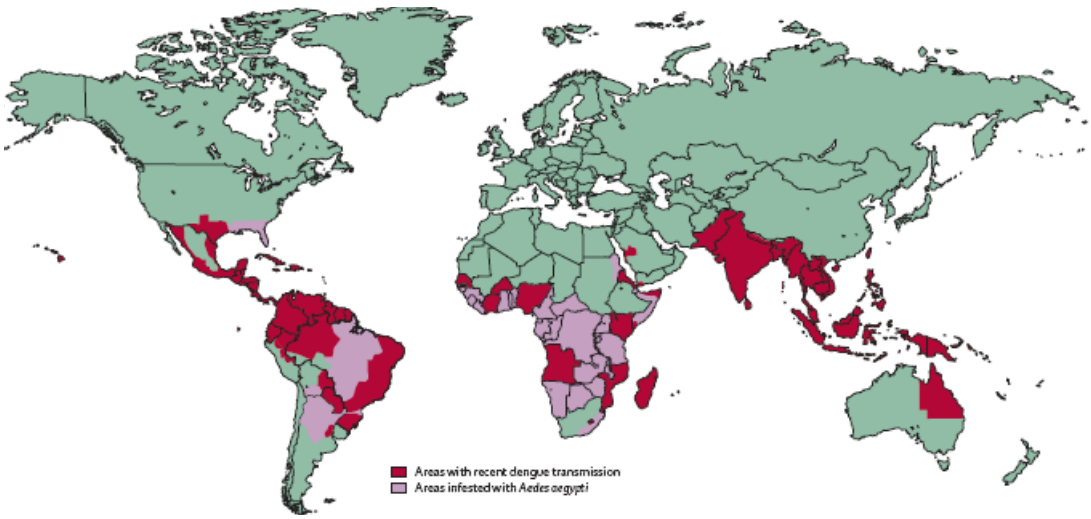


Figure 3 Approximate global distribution of dengue and *Aedes aegypti* in 2005 (53)

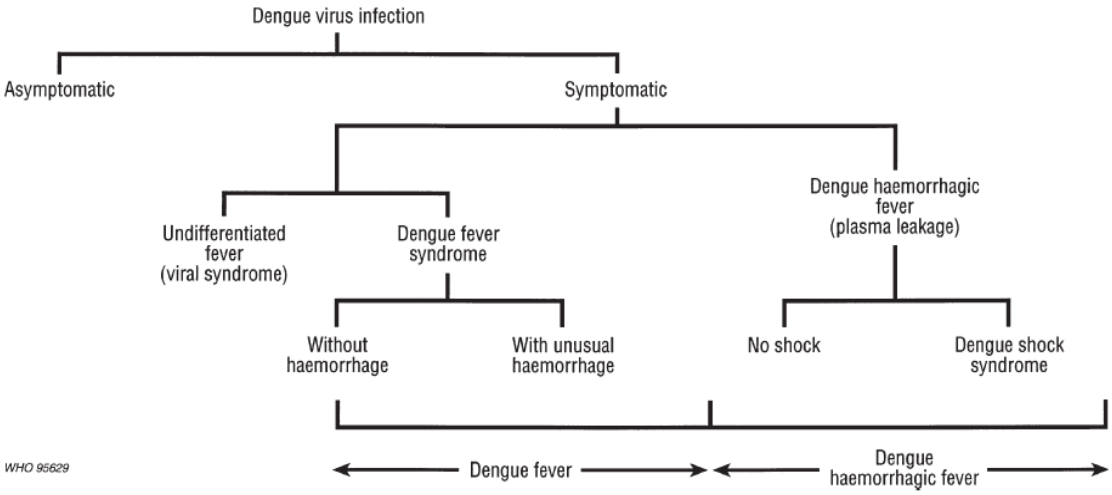


Figure 4 Manifestations of dengue virus infection (54)

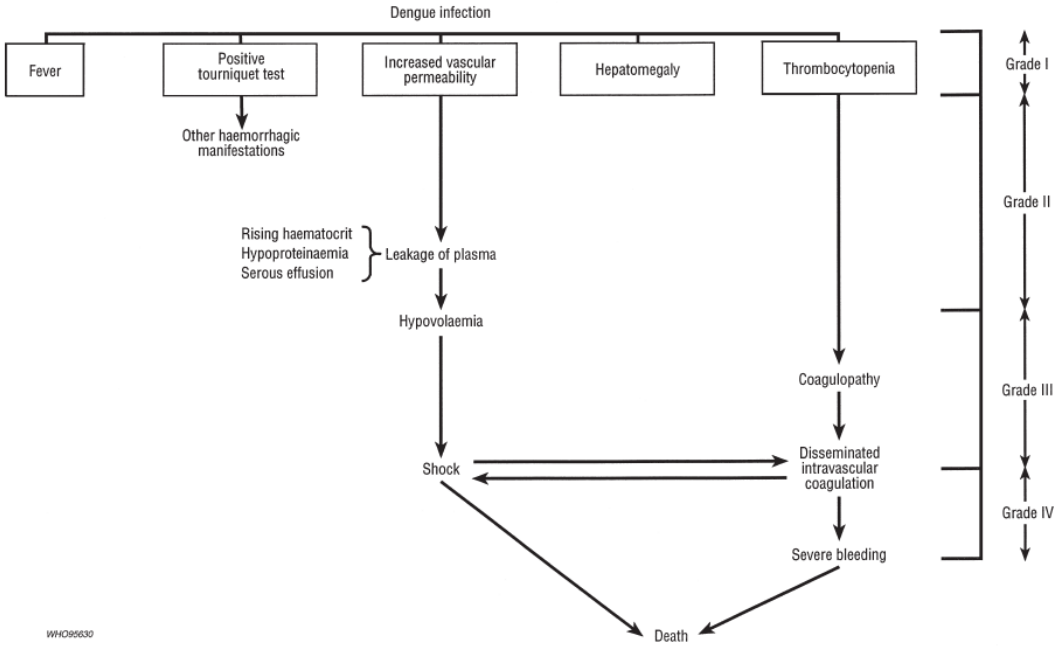


Figure 5 Spectrum of dengue hemorrhagic fever (54)

3.3 Thrombocytopenia in DF and DHF/DSS

Thrombocytopenia is common in DF and is always found in DHF/DSS (9, 13). This phenomenon occurs approximately 2 days before the onset of shock or defervescence and usually reaches a nadir on the day of shock or defervescence (60). Thrombocytopenia disappears in approximately 7 days after the onset of shock or defervescence. In DHF patients, the degree of thrombocytopenia shows a strong correlation with disease severity (61). However, the pathogenesis of thrombocytopenia is poorly understood. Several findings demonstrate that thrombocytopenia may be a result of platelet clearance via a direct binding of DV to human platelets in the presence or absence of DV-specific antibodies (10, 62, 63). Levels of anti-platelet autoantibody (IgM) in the circulation which lead to platelet clearance are higher in DHF/DSS than in DF patients (64). Moreover, generation of anti-dengue NS1 antibodies that can bind directly to normal platelets resulting in platelet destruction is a possible mechanism for thrombocytopenia in dengue virus infection (65-67). In 1987 the following mechanism of platelet consumption was proposed: injury to vascular endothelial cells by DV may allow the blood circulating in the vessel to interact with subendothelial collagen and lead to the promotion of platelet aggregation and lysis of platelets resulting in thrombocytopenia (10). In 1977 a mechanism of platelet destruction and dysfunction was also proposed. In DV-infected patients, platelets containing DV antigen-antibody complexes on their surfaces lead to the destruction and the dysfunction of platelets via a defect in the release of adenosine diphosphate (ADP) (9). Alternatively, alteration of platelet production which is the result of bone marrow suppression is a factor of thrombocytopenia in DV-infected patients (14, 15).

3.4 Bone marrow suppression in Dengue infection

Bone marrow suppression is a common phenomenon in DV infection. However, the degree of bone marrow suppression is similar in both DF and DHF. The suppression of bone marrow probably begins around 4-5 days after DV infection. This suppression occurs for approximately 7-10 days and ends in the acute febrile phase approximately 2-3 days before the onset of shock or defervescence. The bone marrow cells, including megakaryocytes, erythroblasts and myeloid precursors, show marked hypocellularity at the acute phase of DV infection (13, 16, 17, 68). During the period of bone marrow suppression, there is no change in the number of white blood cells and platelets in circulation. At the time of shock or defervescence when thrombocytopenia reaches the nadir, the bone marrow cells including megakaryocytes, erythroblasts, and myeloid precursors become normal or hypercellular (14, 15). Following the suppression, recovery of bone marrow occurs a few days before the onset of shock or defervescence (17). The numbers of leucocytes, neutrophils and platelets return to normal within 7-10 days after defervescence (14).

The pathogenesis of bone marrow suppression in DHF possibly involves three main factors (15). Firstly, direct infection of DV to the hematopoietic progenitor cells (11, 19, 29), Secondly, infection of stromal cells by DV (15, 18), and thirdly, alteration of the bone marrow cell regulatory cytokines including megakaryocytopoiesis regulator cytokines (i.e. thrombopoietin (TPO), and IL-6), or hematopoiesis depressive cytokines (i.e. MIP-1alfa/beta, TNF-alpha, and IFN-gamma) (15, 67, 69-75).

3.5 Megakaryocytes

Megakaryocytes are the sources of circulating platelets (21, 22, 76). The hallmarks of megakaryopoiesis (megakaryocyte development) are: formation of a large cell mass (~50–100 μm diameter) and single, large, multilobulated, and polyploidy nuclei (77) which is a result of endomitosis. Nuclei do not go to anaphase or cytokinesis and lead to an increase of DNA content up to 256N per cell (78). Eventually, each megakaryocyte releases approximately 10^4 platelets (79). In addition, a normal adult human can produce approximately 1×10^{11} platelets a day and this can increase tenfold with demand (80).

3.6 Megakaryopoiesis

Megakaryopoiesis is the process by which mature megakaryocytes (MK) are derived from pluripotent hematopoietic stem cells (HSC) (Figure 7). HSC gives rise to two major lineages, the common lymphoid progenitor (CLP) (81) and the common myeloid progenitor (CMP) (82). CLP gives rise to lymphocytes (NK, T and B cells) and the CMP gives rise to both the granulocyte/macrophage progenitor (GMP) and the megakaryocyte/erythroid progenitor (MEP) (83). However, MEP could arise directly from the HSC to give rise to either the erythroid or megakaryocyte lineages without the CMP intermediate (84, 85). The most primitive MK progenitors are the high proliferative potential-colony-forming unit-megakaryocyte (HPP-CFU-MK) and burst-forming unit-megakaryocyte (BFU-MK). BFU-MK is thought to produce a more differentiated MK progenitor, termed colony-forming unit-megakaryocyte (CFU-MK). CFU-MK is the first in the MK lineage (86). CFU-MK then give rise to immature MK or megakaryoblasts, a heterogeneous population that undergoes endomitosis to increase in size and ploidy to a DNA content in excess of 16N (87). These cells are transitional cells to the mature MK, which is polyploid and no longer proliferates. Mature MK then begin the process of shedding their cytoplasm to produce platelets (88). Necessary components such as platelet granules, organelles and ribosomes are transported from the MK body to the ends of the proplatelet where platelet synthesis and release occurs (88). Platelet release does not occur within the bone marrow, but rather proplatelets either extrude into the marrow sinusoids (89) or

the entire MK enters into the circulation for release (90). Platelet formation and release is a terminal process for the mature MK that results in apoptosis and subsequent phagocytosis by macrophages (89, 91-93). The maturation of MK from the HSC is associated with the expression of characteristic CD antigens. The expression of CD34 and CD38 are typically used to analyze early megakaryopoiesis, while in mature MK, CD61, CD41 and CD42 are used (Figure 6) (94, 95).

Several cytokines have been associated with the process of megakaryopoiesis (96), thrombopoietin (TPO) is clearly the major physiological regulator of this process. TPO was identified and cloned as the factor that bound to c-mpl and stimulated megakaryopoiesis to lead to elevated platelet production (23, 97). TPO is expressed primarily in the liver and to a lesser extent in kidney, bone marrow stromal cells and other organs. TPO has been shown to affect all aspects of platelet formation (98) (Figure 6), beginning with the survival and entry into cell cycle of HSC (99, 100). During MK development, TPO has been demonstrated to be responsible for stimulating the expression of characteristic cell surface proteins including CD61/41 (gpIIb/IIIa) and CD42 (gpIb) (23, 101) as well as inducing endomitosis (23, 102). Several other cytokines, including interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), have been shown to be important for normal megakaryopoiesis. In contrast to TPO, which plays a role throughout megakaryopoiesis, other cytokines affect megakaryopoiesis during the early stages of MK lineage development (Figure 6). IL-3 was shown to act on bone marrow progenitor cells through the CFU-MK stage of MK development (103, 104). With respect to megakaryopoiesis, the effects of GM-CSF were also seen primarily in the BFU-MK and immature MK populations (104). GM-CSF was shown to increase proliferation and expansion of both primary bone marrow progenitors and MK cell lines (105). Although the primary affects of G-CSF have been demonstrated for the granulocytic lineage, G-CSF has been shown to have a stimulatory role in bone marrow progenitors of multiple hematopoietic lineages (99, 106).

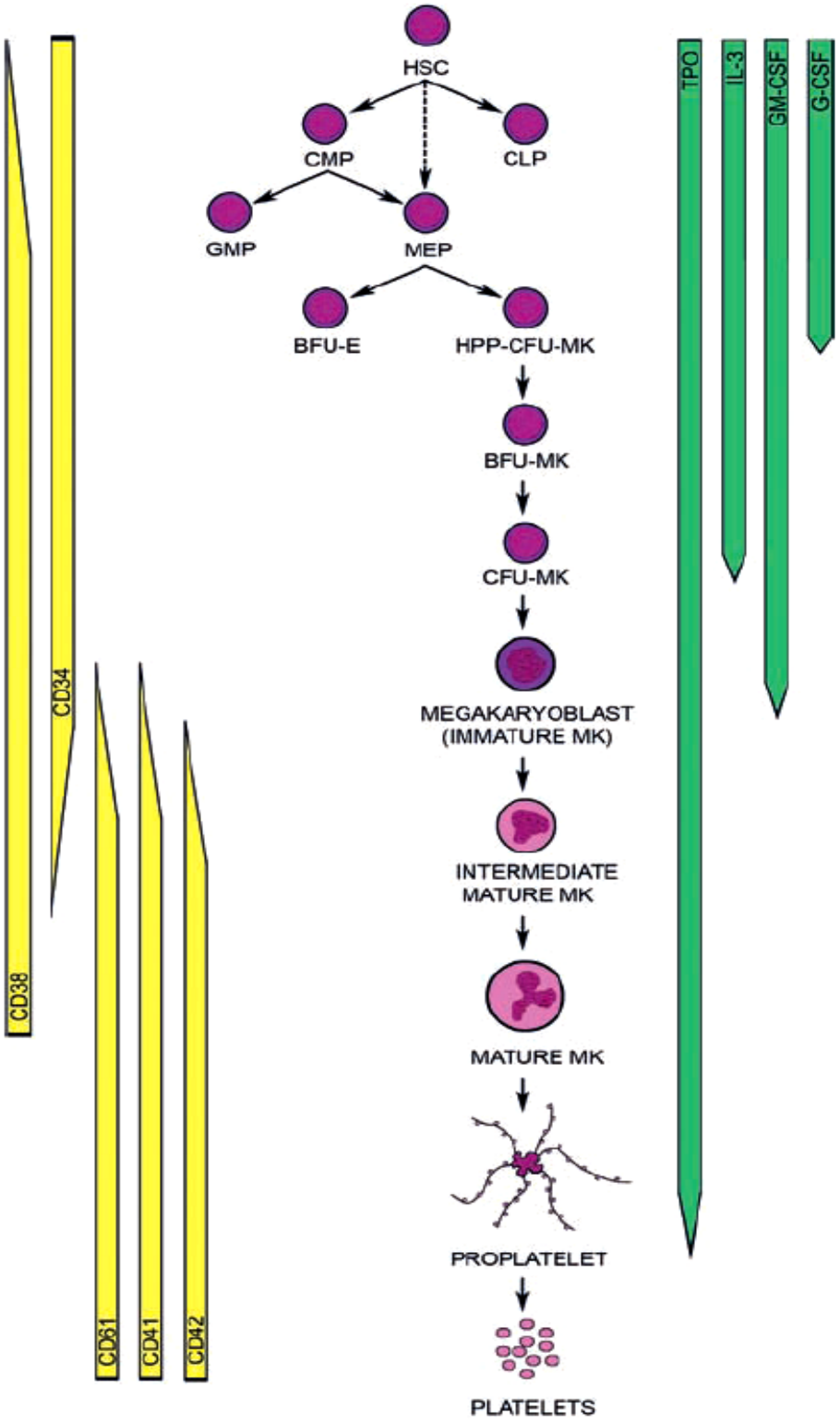


Figure 6 Overview of megakaryopoiesis (107)

3.7 Megakaryocytes and virus infection

Several studies show that a number of viruses have the ability to infect megakaryocytes leading to abnormalities in maturation and viability of virus-infected cells. The human immunodeficiency virus (HIV) could infect megakaryocytes through the HIV receptors expressed on the surface of megakaryocytes leading to inhibition of megakaryopoiesis and thrombocytopenia (108-114). From several *in vitro* studies, parvovirus-infected hematopoietic progenitor cells lead to significant suppression of CFU-MK (megakaryocyte colony formation) (115, 116). The significantly decrease of CFU-MK was also found in parvovirus-infected mice (116). Human cytomegalovirus (HCMV)-infection of the megakaryocyte cell line CHRF-288-11, results in apoptosis of the HCMV-infected cells (117). In murine cytomegalovirus (MCMV) infection, a reduction of CFU-MK, megakaryocyte progenitor cells, and spleen colony-forming cells have been observed in MCMV-infected mice (118). The Megakaryoblast cell line, MEG-01, could be infected with hepatitis C virus (HCV) from HCV patient sera (119). The human herpesvirus-7 (HHV-7) infection of $CD41^{+}/61^{+}$, megakaryocytes leads to apoptosis of this cell(120).

3.8 Microparticles

The presence of microparticles (MPs) is one of the hallmarks of cellular alteration. MPs are shed from the plasma membrane of the cells or platelets (121) undergoing activation (122-124) or apoptosis (125, 126) as shown in Figure 8. Although, the translocation of phosphatidylserine (PS) from the inner to the outer part of the cell membrane is a common phenomenon in MPs (127-129), there is some proportion of microparticles containing PS on their surfaces (130). There is no real definition for MPs, although it is accepted that MPs are submicron elements (from 0.1 to 1 μm) that express antigens characteristic of their cell of origin (30, 31) or phosphatidylserine (PS) (130) on their surfaces. The functional role of MPs is still largely unknown although several studies have tried to define their roles. Firstly, MPs control the coagulation process. MPs derived from activated monocytes and platelets provide a membrane surface that facilitates the induction of cell coagulation (31, 131, 132). In addition, platelet MPs provide for the aggregation of neutrophils via

formation of a bridge between GPIb and Mac-1 (133). Secondly, MPs serve as a transporter of cellular surface antigens. The cells that express receptors to HIV can transfer these receptors to the HIV receptor-lacking cells via their MPs leading to susceptibility to HIV infection (134-137). Thirdly, MPs induce cell and platelet activation. MPs from activated platelets can induce the activation of platelets and endothelial cells through the delivery of COX-2 and prostacyclin (PGI2) which are the activation markers expressed on MPs (138). Fourthly, MPs induce inflammatory responses. The monocyte/macrophage-derived MPs induced inflammatory responses through the up-regulation of proinflammatory mediators including IL-8, MCP-1, and ICAM-1 (139).

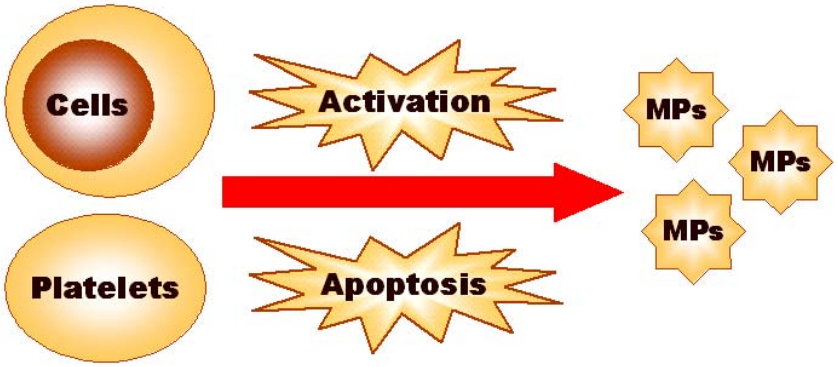


Figure 7 The formation of microparticles (MPs)

3.9 MEG-01, a megakaryoblastic cell line

MEG-01 (ATCC CRL-2021) is a human megakaryoblast cell line originally created from the bone marrow of a 55 year old patient in blast crisis caused by Philadelphia (Ph1) chromosome-positive chronic myelogenous leukemia (CML) as described by Ogura M, *et al*. (140). This cell line is similar to the human megakaryoblast with respect to megakaryoblast markers including CD36 (141, 142), CD9 (143), Fc gamma RIIA (CD32A) (144), and platelet GP IIb/IIIa (CD41) (145) all characteristic of bone marrow megakaryoblasts. The advantages of this cell line are 1) Cells readily proliferate in tissue culture. 2) Induction of differentiation by phorbol diesters, 12-O-tetradecanoylphorbol-13-acetate (TPA), results in the expression of cellular markers restricted to megakaryocytic lineage (146), and 3) They are able to form long beaded processes which split to platelet-like particles having size and function similar to normal platelets either spontaneously (147) or after stimulation with thrombopoiesis stimulating cytokines such as thrombopoietin (TPO) (145, 148).

CHAPTER IV
MATERIALS AND METHODS

4.1 Cell culture

C6/36, an *Aedes albopictus* cell line (ATCC CRL-1660) was cultured in L-15 medium (GIBCO BRL, Invitrogen, Grand Island,NY) containing 1% heat-inactivated fetal bovine serum (FBS, GIBCO BRL, Invitrogen, Grand Island, NY) 10% tryptose phosphate broth (TPB, SIGMA, St. Louis, MO) 100 U/ml penicillin and 100 µg/ml streptomycin (SIGMA, St. Louis, MO) at 28 ^{o}C.

Vero, a African green monkey kidney epithelial cell line (ATCC CCL-81) was cultured in MEM medium (GIBCO BRL, Invitrogen, Grand Island, NY) containing 3% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 ^{o}C in a 5% CO_2 incubator.

MEG-01, a human megakaryoblast cell line (ATCC CRL-2021) was cultured in RPMI 1640 (GIBCO BRL, Invitrogen, Grand Island, NY) containing 10% FBS, 2mM L-glutamine, without antibiotics at 37 ^{o}C in a 5% CO_2 incubator.

4.2 Antibodies

5.2.1 Rabbit anti-mouse IgS conjugated with HRP, P0260, DAKO

5.2.2 Rabbit anti-mouse IgS conjugated with FITC, P0261, DAKO

5.2.3 Goat anti-mouse IgG conjugated Cy3, Jackson Immuno Research Laboratories, Inc., West Grove, PA

5.2.4 Rabbit anti-mouse IgS, Z0259, DAKO, Denmark

5.2.5 Monoclonal antibodies (mAbs)

5.2.5.1 mAb 3H5 , specific for linear epitope of E protein of DV serotype 2, isotype IgG1 (149)

5.2.5.2 mAb 4G2 , specific for conformational epitope of E protein of DV serotype 1-4, isotype IgG2a (149)

5.2.5.3 mAb 2E11 (2NS 1F), specific for linear epitope of NS1 protein of DV serotype 1-4 and JE, isotype IgM (150)

5.2.5.4 mAb 2G6 (1PF 6S), specific for conformational epitope of NS1 protein of DV serotype 2, isotype IgG2a (unpublished data)

5.2.6 Polyclonal antibodies (pAb)

5.2.6.1 pAb specific for NS1 protein of DV serotype 1-4 (unpublished data)

4.3 Virus

4.3.1 Preparation of virus stock

In order to generate a large stock of DV-2 strain 16681 for this study, DV strain 16681 was propagated in C6/36 cells. Approximately $1x10^7$ C6/36 cells were grown in T75 cm^2 tissue culture flasks (Costar ,Cambridge, MA, USA) in growth medium L-15 containing 10% FBS, 10% TPB, 100 U/ml penicillin and 100 µg/ml streptomycin (SIGMA, St. Louis, MO) and incubated at 28°C for 1 day. The confluent cell monolayer was then incubated with the virus at a multiplicity of infection (MOI) of 0.01 in maintenance medium (1.5% FBS 10% TPB, 100 U/ml penicillin and 100 µg/ml streptomycin) in a total volume of 5 ml at 28°C for 3 h on a rocker. After that, supernatants were replaced with maintenance medium in a total volume of 15 ml/ T75 cm^2 flask and further incubated at 28°C until approximately 7 days after infection. The culture supernatants were then collected and clarified by centrifugation at 1000xg for 10 min at 4°C. Clarified supernatants were aliquot and stored at -70°C.

Clinical isolates of 1-4 strains of dengue viruses were isolated from clinical specimens collected in the project “Establishment of dengue clinical research centers supporting basic and clinical research T cell response in dengue virus infection” as stored at the Medical Molecular Biology Unit, Bangkok. Stocks of these strains were generated as described above.

4.3.2 Virus titration

Culture supernatants from DV-infected cells were titrated by focus forming assay (FFU assay) in Vero cell lines. Briefly, approximately $2x10^4$ Vero cells were plated on each well of 96 well plates in growth medium and incubated at 37°C in a humidified chamber for 24 h. The collected DV-supernatants were 10-fold serially diluted (10^{-1}-10^{-7}) in maintenance medium (MEM containing 3% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin) and subsequently 50 µl of diluted samples were added into each well in duplicate. Following 2 h incubation in a humidified chamber at 37°C, 100 µl of overlay medium (maintenance medium containing 2% Carboxy methyl cellulose (CMC) (SIGMA)) was added into each well, and incubated at 37°C in a humidified chamber for 72 h. After that, culture supernatants were removed and the cells were washed with phosphate buffered saline (PBS) pH 7.4 and 100 µl of fixative solution was added to the cells and incubated for 10 min. After discarding the fixative solution, 100 µl of permeablization solution were added and then incubated for 10 min. After washing with PBS, 50 µl of mouse mAb specific to flavivirus envelope protein (4G2) was added in each well and incubated at 37°C in a humidified chamber for 30 min. The cells were then washed, and incubated with 50 µl of HRP-conjugated rabbit anti-mouse Igs (DAKO) in the dark at 37°C in a humidified chamber for 30 min. After washing, substrate solution was used for color development. Calculation of virus titer was based on the following formula.

$$\textbf{Virus titer (ffu/ml)} = \frac{\textbf{(A+B)}}{\textbf{Total volume of virus x (A+B)}} \times 10^{C} \times 10^{3}$$

A = number of foci counted in the first dilution

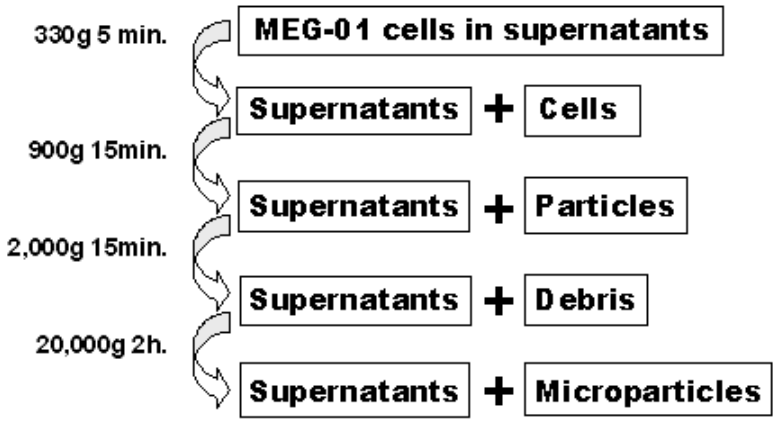
B = number of foci counted in the final dilution

C = the first dilution in which foci were counted

4.4 Virus infection and harvesting the infected MEG-01 cells

Approximately $5x10^6$ MEG-01 cells were grown in 25 cm^2 tissue culture flasks (Costar, Cambridge, MA, USA) in 5ml of growth medium (RPMI 1640 containing 10%FBS, 2mM L-glutamine) and incubated overnight at 37 ^oC in a 5% CO_2 incubator. Subsequently, the cells were pelleted by centrifugation and the growth medium replaced by mock or DV supernatant at a multiplicity of infection (MOI) of 0.1 or 1.0 in growth medium followed by incubation at 37 ^oC in a 5% CO_2 incubator for 3 hours. Later, the supernatants were replaced by fresh growth medium and then the cells were cultured at 37 ^oC in a 5% CO_2 incubator.

Mock or DV infected cells, platelet-like particles, and microparticles were collected at day 1, 3, 5 and 7 after dengue infection by serial centrifugation. Briefly, the DV infected cells (nucleated cells) were collected by centrifugation at 330g for 5 min. The platelet-like particles (un-nucleated cell) were collected by centrifugation at 900g for 15 min. The debris was removed from the supernatant by centrifugation at 2,000g two times for 15 min. The microparticles were pelleted by centrifugation at 20,000g for 120 minutes as diagram below.



4.5 Light microscopy

Mock or DV infected cells were diluted 1:100 before being mixed at the ratio of 1:1 with trypan blue dye. Subsequently, the numbers of total and dead cells (trypan blue positive cells) were counted using light microscopy. Cell proliferation of DV infected MEG-01 were calculated by comparing the total cells from mock and DV

infected MEG-01. The dynamics of cell expansion were assessed by collecting the cells every 2 days post infection.

4.6 Confocal microscopy

Finally, the cells were observed under a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany) with a 63x oil immersion lens. FITC was excited at 488 nm and emission collected at 505-530 nm and Cy3 was excited at 543 nm and emission collected at 560-615 nm to avoid an overlap in emission spectra. All background auto fluorescence was corrected on all slides and for all wavelengths. Photography was performed by using an image capture program (LSM 510 software version 3.2, Carl Zeiss).

4.7 Analysis of cell surface associated viral proteins

Approximately $1x10^6$ harvested cells were washed with washing medium (RPMI 1640 containing 5% FBS and 10 mM NaN_3) and incubated with 10% normal human AB serum for 30 min on ice to block FcR. After washing, the cells were incubated with 50µl of mouse mAbs clone 2G6 or 3H5 (specific to NS1 or E, respectively) on ice for 1 h and followed by 6 µg/ml FITC-conjugated rabbit anti mouse Igs (DAKO) or 1.5 ng/ml Cy3-conjugated rabbit anti mouse Igs (DAKO) for 30 min on ice in the dark. The cells were washed and analyzed by confocal microscopy.

4.8 Analysis of cytoplasmic viral proteins

Approximately $1x10^6$ harvested cells were washed with PBS and fixed with 2% formaldehyde (BDH, England) in PBS for 1 h at RT. After that, the fixed cells were washed once with a large volume of PBS and permeabilized with 0.1% Triton X-100 in PBS. Permeabilized cells were incubated with 50 µl of mouse mAbs clone 2G6 or 3H5 (specific to NS1 or E, respectively) for 1 h at RT. Subsequently, the cells were washed once with a large volume of 0.1% Triton X-100 in PBS and incubated with 6 µg/ml FITC labeled rabbit anti-mouse Igs (DAKO) or 1.5 ng/ml Cy3-conjugated rabbit anti mouse Igs (DAKO) for 30 min at RT in dark. After a final wash with a

large volume of 0.1% Triton X-100 in PBS, the cells were analyzed by confocal microscopy or flow cytometry.

4.9 Apoptosis detection

Apoptotic cells were analysed by annexinV-FITC detection kit I (BD biosciences, San Diego, CA) and TUNEL assay (APO-BRDU, CHEMICON, USA). Briefly, the detection of early apoptotic cells and total number of apoptotic cells were performed by AnnexinV/PI assay and TUNEL assay, respectively. Both assays were performed according to the manufacturer's instructions and analyzed by flow cytometry. The dynamics of cell death were measured by collecting the cells every 2 days post infection.

4.10 Western blotting

The expression of DV proteins, including NS1 and E protein were determined using western blotting. Briefly, mock or DV infected MEG-01 cells were washed once with PBS buffer. MEG-01 cells were lysed by lysis buffer (PBS containing 1% triton X-100, 0.5% BSA, and 10mM PMSF). Total proteins inside the cells were separated by 12% SDS-PAGE. The separated proteins were transferred to nitrocellulose membrane using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, USA). The membrane was blocked with 5% skimmed-milk/PBS for 2 hours at room temperature. After washing, 3 times the membrane was incubated with monoclonal antibodies against NS1 or E protein for 1 hour at room temperature. The membrane was washed and incubated in the dark with HRP conjugated rabbit anti mouse IgS (DAKO) for 30 minutes at room temperature. Finally, the membrane was washed and then reacted with substrate solution (PBS containing 3, 3' diaminobenzidine tetrahydrocholide (DAB), $NiCl_2$ and H_2O_2) for color development.

4.11 NS1-capture ELISA

Total amounts of secreted NS1 protein in DV infected MEG-01 supernatants were quantitated using an ELISA assay. Briefly, 0.5 μg/ml of monoclonal antibody against NS1 (2E11) was coated on the polystyrene micro ELISA plates (NUNC) at 4^o

C overnight. The plate was washed with 0.05% Tween-20/PBS and followed by 15%FBS/PBS to block the remaining free binding sites at room temperature for 2 hours. After washing, the serially diluted samples were added to the appropriate wells and incubated at room temperature for 1 hour. The plate was washed and followed by 50µg/ml of monoclonal antibody against dengue NS1 (2E3) and incubated at room temperature for 1 hour. The HRP-conjugated goat anti mouse IgG (DAKO) in a final dilution of 1:1,000 was added after washing and then incubated in the dark at room temperature for 1 hour. The enzyme reacting solution (0.06 mg of Ortho-phenylenediamine dihydrochloride (OPD, SIGMA) in 10 ml 0.1 M Citric acid phosphate buffer pH 5.5 containing H_2O_2 (Sahakarn)) was added and the reaction was stopped after incubation in the dark at room temperature for 10 minutes by adding 4N H_2SO_4. The optical density of each sample was read with an ELISA reader at the wave length of 492 nm.

4.12 E-capture ELISA assay

Total amounts of secreted E protein in DV infected MEG-01 supernatants was measured by using an ELISA assay. Briefly, the rabbit anti mouse immunoglobulin (DAKO), at a 1:5,000 dilution, was coated on the polystyrene micro ELISA plates (NUNC) at 4^{o} C overnight. The plate was washed with 0.05% Tween-20/PBS and followed by 0.5%BSA/PBS to block the remaining free binding sites at room temperature for 2 hours. After washing, the pooled convalescent serum (PCS, diluted 1:1,000) was added and then incubated at room temperature for 2 hours. The serially diluted samples were added to the appropriate wells and incubated at room temperature for 2 hours. The plate was washed followed by the addition of monoclonal antibody against E protein (3H5, diluted 1:500) and incubated at room temperature for 1 hour. After washing, the HRP-conjugated rabbit anti mouse IgS (DAKO) in a final dilution 1:1,000 was added and then incubated in the dark at room temperature for 1 hour. The color was developed by adding a solution of 0.06 mg of OPD (SIGMA) in 10 ml 0.1 M Citric acid phosphate buffer pH 5.5 containing H_2O_2 (Sahakarn) and the reaction was stopped after a 10 min incubation at room temperature in the dark by adding 4N H_2SO_4. The optical density from each sample was read with an ELISA reader at the wave length of 492 nm.

4.13 Detection of NS1 or E proteins

The expression of NS1 and E protein was determined using indirect fluorescence staining. Briefly, mock or DV infected MEG-01 cells or platelet-like particles were washed with PBS buffer and followed by fixing with 2% formaldehyde (BDH, England) in PBS for 1 hour at room temperature. Afterward, the fixed cells were washed with PBS and 1% Triton X-100 in PBS was added to the cells. After washing, the cells were incubated with monoclonal antibodies against NS1 or E protein for 1 hour at room temperature. The cells were washed and incubated in the dark with FITC conjugated rabbit anti mouse IgS (DAKO) for 30 minutes at room temperature before washing once with 0.1% TritonX-100 in PBS. Finally, the number of NS1 and E expressing cells were analyzed by flow cytometry.

Monoclonal antibodies against NS1 and E are 2G6 and 4G2, respectively. Both of the monoclonal antibodies were produced in our laboratory and some have been described by Puttikhunt *et al* (150).

4.14 DV distribution via DV infected MEG-01 derived-particles and/or microparticles

Particles and/or microparticles from DV infected MEG-01 were collected at day 5 post-infection by centrifugation at 100,000 g for 30 minutes followed by washing 3 times with PBS. The particles and/or microparticles were lysed by freeze-thawing 3 times to break the particles or microparticles. Subsequently, the lysates were clarified by centrifugation at 13,000 g for 10 minutes. The infectious virus particles in the lysate fractions were investigated by FFU assay.

4.15 Protein determination (Bradford assay)

The total protein concentration was determined by Bradford assay kit (Bio-rad laboratory, USA) according to the manufacturer's instructions. Briefly, the protein in lysate were mixed with 3 volume of Coomassie Brilliant Blue G-250 solution and incubated for 10 min. before analyzed by spectrophotometer at λ of 595 nm.

4.16 Statistical analysis

Data analysis was performed using software package Stat View for Windows version 5.0 (SAS Institute Inc., NC). Komolgorov samonov test was used to test for normality. Comparison between groups was done by t-test if the distribution of the variables was comparable to a normal distribution; otherwise the Mann Whitney test was used. Multiple comparisons were performed using ANOVA. $P \leq 0.05$ was considered to be statistical significant. All analyzed P values were 2-sided.

CHAPTER V
RESULTS

5.1 The study of DV infection in MEG-01

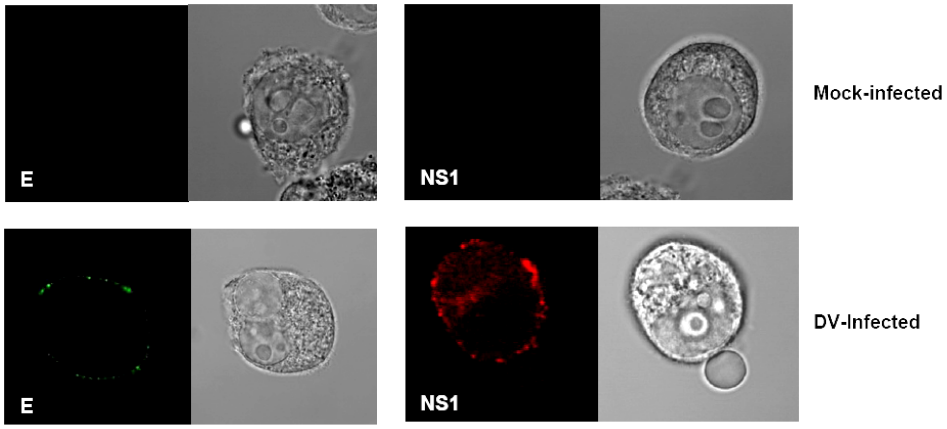
5.1.1 DV-infected MEG-01 cells express NS1 and E proteins on their surfaces and in the cytoplasm

NS1 and E protein expression on surface and cytoplasm of DV-infected MEG-01 cells was determined using 2 mAb clones (2G6 and 3H5, specific for NS1 and E proteins, respectively) and analyzed by confocal microscopy and flow cytometry. Dengue NS1 and E proteins were detected on the cell surfaces (Figure 8A) and in the cytoplasm (Figure 8B) of DV-infected but not mock-infected MEG-01 cells at 5 days after infection. Analysis of the kinetics of cytoplasmic E expression using 3H5 mAb was demonstrated in Figure 9. The result showed that approximately 20% of DV-infected MEG-01 cells at 1 and 3 days and 70% of DV-infected MEG-01 cells at 5 and 7 days after infection at MOI of 1.0 expressed E in their cytoplasm as detected by positive staining with 3H5 (Figure 9B).

5.1.2 DV-infected MEG-01 cells produce infectious virus progeny

Kinetics of infectious virus progeny production from DV-infected MEG-01 was determined by FFU assay. The infectious virus progenies were detected within 1 day after DV-infection (Figure 10). Infectious virus titer was approximately 1.5×10^4 FFU/ml at day 1 rising to 1.5×10^7 FFU/ml at day 5 after DV-infection, and remained unchanged until day 7 after DV-infection.

A



B

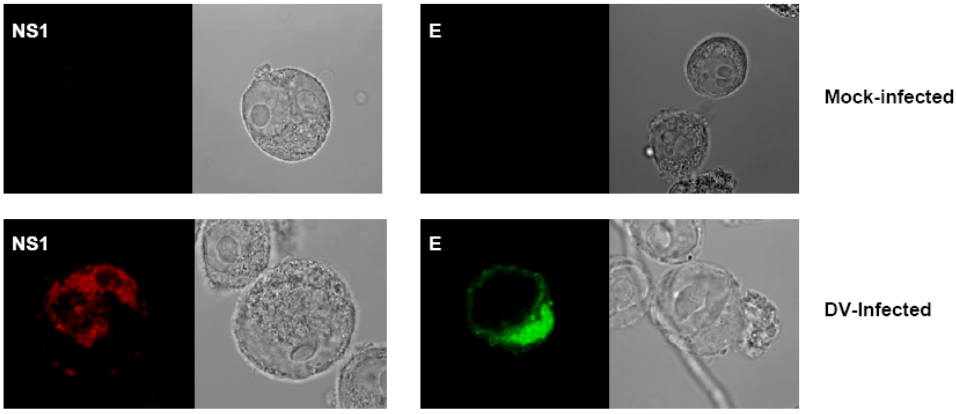
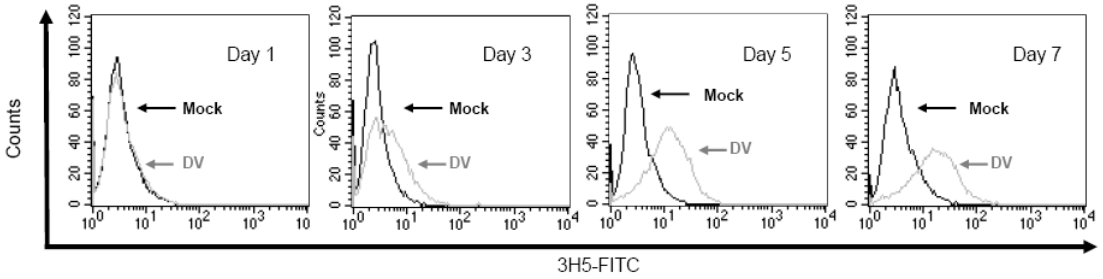


Figure 8 Localization of NS1 and E proteins on the surface and cytoplasm of DV-infected MEG-01 cells

Mock-infected or DV-infected MEG-01 cells were harvested at day 5 after infection. After washing the cells with PBS, NS1 and E on the cell surface (A) or in the cytoplasm (B) were stained with mAb specific to NS1, 2G6 (red, Cy3) and E, 3H5 (green, FITC) and followed by FITC-conjugated rabbit anti-mouse IgG or Cy3-conjugated goat anti-mouse IgG. The stained cells were observed under a Zeiss LSM 510 META confocal microscope. One representative experiment of three independent experiments is shown.

A.



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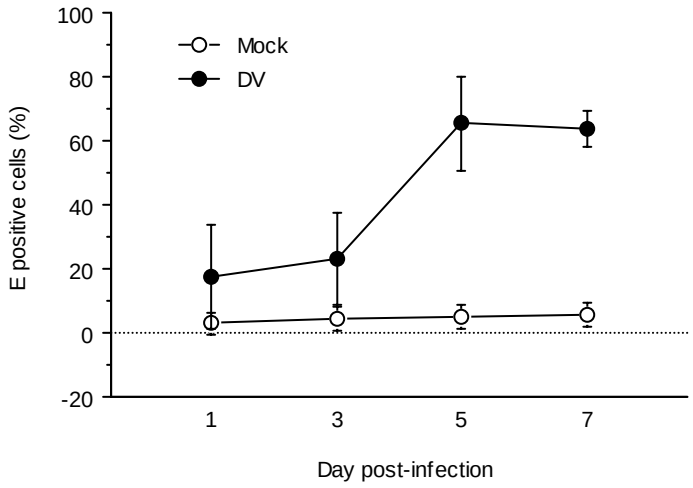


Figure 9 Kinetics of E protein expression in the cytoplasm of DV-infected MEG-01 cells

MEG-01 cells were infected with DV at MOI of 1. Mock-infected or DV-infected cells were harvested at 1, 3, 5, and 7 days after infection. The cells were stained with mAbs specific to dengue E protein and followed by FITC-conjugated rabbit anti mouse IgG. The intensity of E expression (A) was compared between mock-infected (dark line) and DV-infected (gray line) MEG-01 cells. Percentage of cells expressing E protein (B) was compared between mock-infected (open circle) and DV-infected (filled circle) MEG-01 cells. The error bars indicate SD corresponding to three independent experiments.

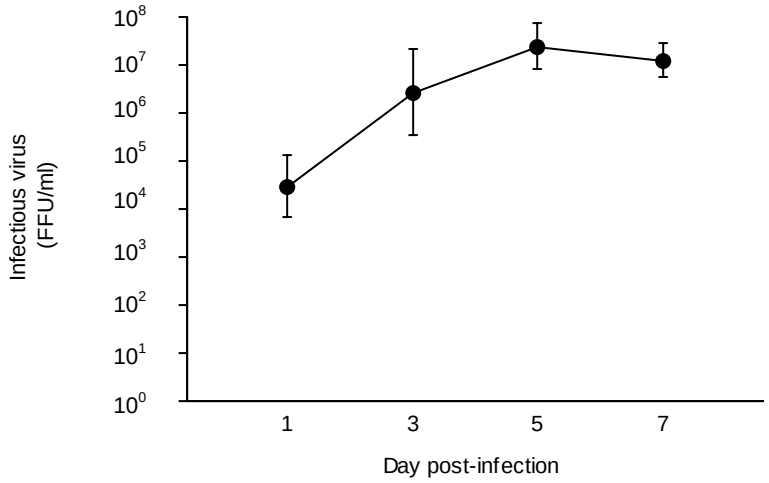


Figure 10 Kinetics of infectious virus progeny produced from DV-infected MEG-01 cells

MEG-01 cells were infected with DV at MOI of 1. The supernatants were collected at 1, 3, 5, and 7 days after infection. After removing the cells and debris by two centrifugations at 2,000g for 10 minutes, the infectious virus progeny in supernatants were measured by FFU assay. The error bars indicate SD corresponding to three independent experiments.

5.2 The study of consequences of DV infection in MEG-01

5.2.1 Proliferation of DV-infected MEG-01 cells

The cell proliferation rates between mock-infected and DV-infected MEG-01 cells were compared by total cell counts. The numbers of mock-infected and DV-infected MEG-01 cells at various time points after infection are shown in Figure 11. The numbers of cells in both groups were similar from day 1 to day 5 after DV infection. However, at day 7 after DV infection the number of DV-infected MEG-01 cell was slightly lower than mock-infected MEG-01 cell (1.86×10^7 cells and 2.29×10^7 cells, respectively).

5.2.2 Viability of DV-infected MEG-01 cells

The cell viability of mock-infected and DV-infected MEG-01 cells was determined by propidium iodide (PI) staining assay. The number of PI positive cells in mock-infected and DV-infected MEG-01 cells was analyzed by flow cytometry (Figure 12). The number of PI positive cells was not different between mock-infected and DV-infected MEG-01 from day1 to day 5 after DV-infection. However, the number of PI positive cells in DV-infected MEG-01 populations (60%) was significantly higher ($P < 0.05$) than mock-infected MEG-01 cell (79%) at day 7 after DV-infection.

5.2.3 Mechanisms leading to the death of DV-infected MEG-01 cells

To study mechanisms leading to the death of DV-infected MEG-01 cells, both early and late apoptotic markers were determined kinetically in mock-infected and DV-infected MEG-01 cells.

Early apoptotic marker was detected by an annexin V/PI staining assay. Apoptotic cells at early stages express phosphatidyl serine on their surface which specifically binds to annexin V but are negative for PI nuclear staining as shown in the lower-right quadrant of dot plots in figure 13A. The numbers of PI negative and annexin V positive cells were similar among mock-infected (19, 31, 32, and 34% at day 1, 3, 5, and 7) and DV-infected MEG-01 cells (21, 34, 31 and 34% at day 1, 3, 5, and 7 after DV-infection) (Figure 13B).

Late apoptotic cells have DNA fragmentation which can be identified by TUNEL assay. The TUNEL positive cells were detected by flow cytometry as demonstrated in the histograms of figure 14A. The number of TUNEL positive cells was significantly higher ($P < 0.05$) in DV-infected MEG-01 (25, and 48% at day 5, and 7 after DV-infection, respectively), than mock-infected MEG-01 (16, and 19% at day 5, and 7, respectively) (Figure 14B).

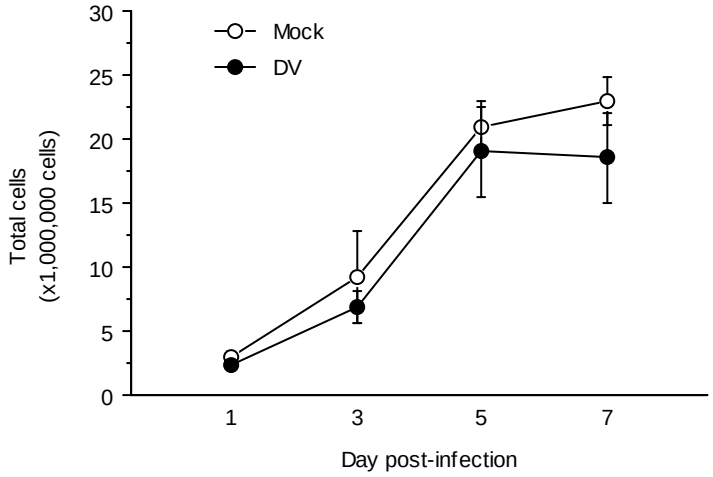


Figure 11 Total numbers of mock-infected and DV-infected MEG-01 cells

MEG-01 cells were infected with DV at MOI of 1. Mock-infected or DV-infected cells were harvested at 1, 3, 5, and 7 days after infection. The cells were diluted 100-fold with PBS and stained with trypan blue dye. The total cells were counted with a cell-counting hematocytometer. Total numbers of mock-infected (open circle) and DV-infected (filled circle) MEG-01 were compared. The error bars indicate SD corresponding to three independent experiments.

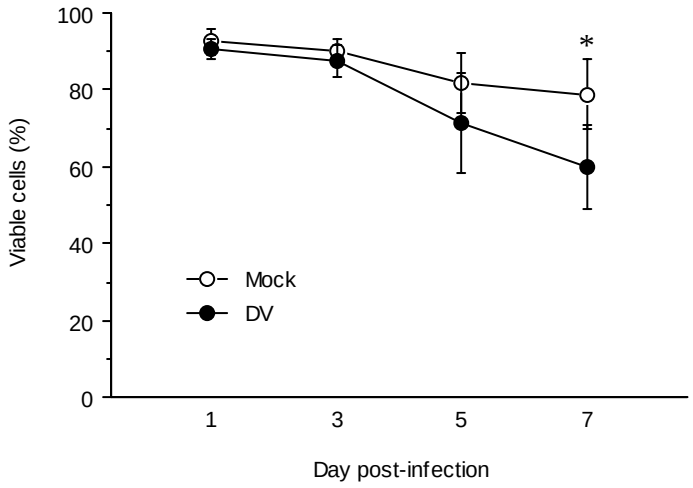
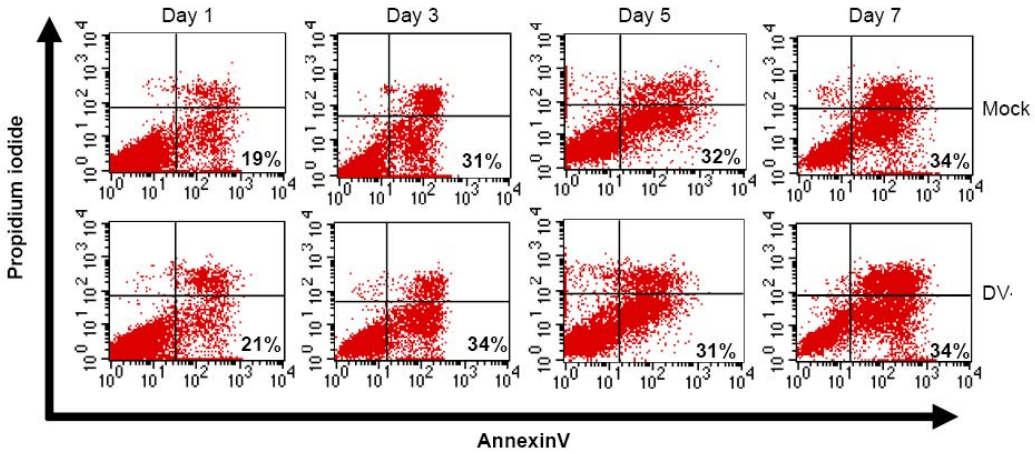


Figure 12 Viability of mock-infected and DV-infected MEG-01 cells

MEG-01 cells were infected with DV at MOI of 1. Mock-infected or DV-infected cells were harvested at 1, 3, 5, and 7 days after infection. After washing with 1% BSA/PBS, the cells were stained with propidium iodide (PI) and analyzed by flow cytometry. The percentage of PI negative cells in mock-infected (open circle) and DV-infected (filled circle) MEG-01 was compared. The error bars indicate SD corresponding to three independent experiments. Asterisks indicate differences that were statistically significant (mock-infected vs DV-infected, $P < 0.05$).

A.



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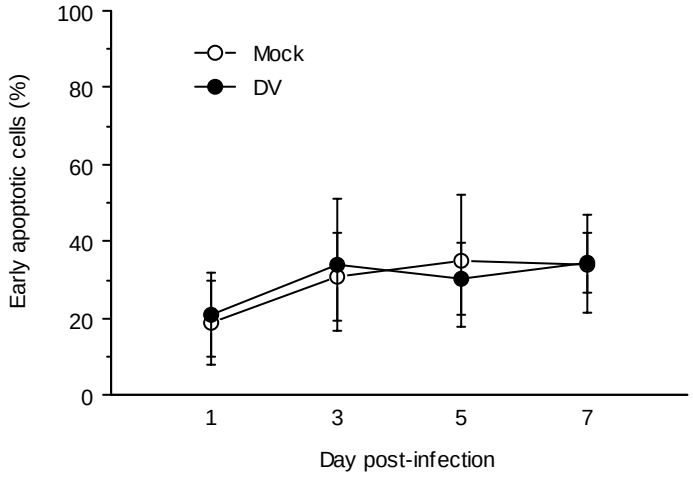
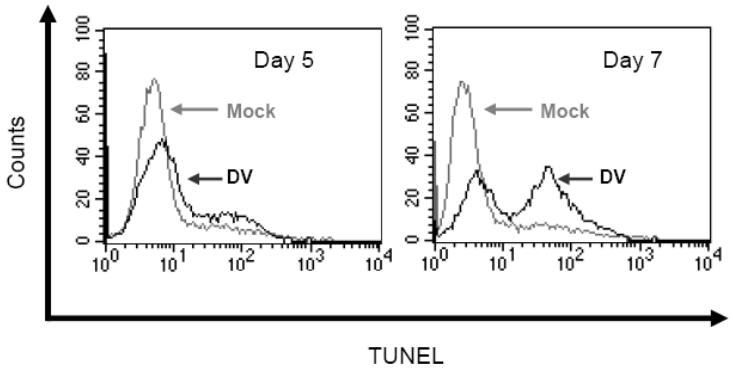


Figure 13 Kinetics of early apoptotic cells in DV-infected MEG-01 cells

MEG-01 cells were infected with DV at MOI of 1. Mock-infected or DV-infected cells were harvested at 1, 3, 5, and 7 day after infection. After washing with 1% BSA/PBS, the cells were stained with FITC-conjugated annexin V and PI and analyzed by flow cytometry. Early apoptotic cells were annexin V positive and PI

negative which are shown in the lower-right quadrant of dot plots (A). Percentages of early apoptotic cells between mock-infected (open bar) and DV-infected (filled bar) MEG-01 cells were compared (B). The error bars indicate SD corresponding to three independent experiments.

A.



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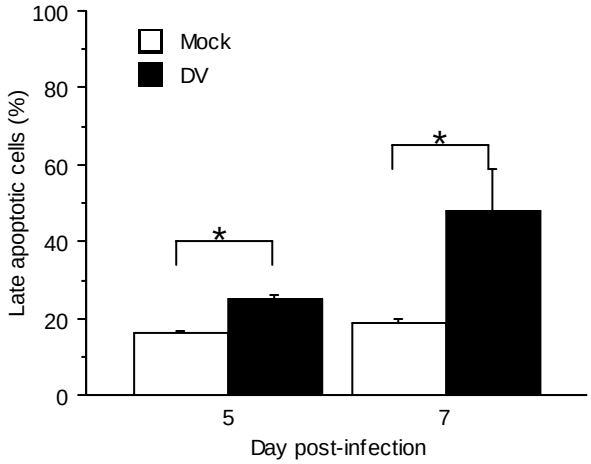


Figure 14 Kinetics of late apoptotic cells in DV-infected MEG-01 cells

MEG-01 cells were infected with DV at MOI of 1. Mock-infected or DV-infected cells were harvested at 5 and 7 days after infection and late apoptotic cells were detected with TUNEL assay. The TUNEL positive cells were analyzed by flow cytometry. The histograms of TUNEL positive cells of mock-infected (gray line) and DV-infected MEG-01 cell (dark line) were compared (A). Percentage of late apoptotic cells in mock-infected (open bar) and DV-infected (filled bar) populations are shown (B). Each bar represents the mean of late apoptotic cells from three independent experiments $\pm$ SD. * indicates P value < 0.05 (mock-infected vs DV-infected).

5.3 The study of the susceptibility of MEG-01 to DV infection

5.3.1 Infectivity of DV to MEG-01, compared with Vero cells

Kinetics of DV-infection in MEG-01 and Vero cells were determined by cytoplasmic staining of dengue envelope protein using mAb 3H5 (Figure 15). The percentage of MEG-01 cells infected with DV was significantly lower ($P < 0.05$) compared with Vero cells at day 1 and 3 and the number of infected cells increased to almost the same as Vero cell at day 7 after DV-infection. There were approximately 19%, 22%, and 64% of DV-infected MEG-01 cells at days 1, 3, and 5 after DV-infection, respectively while 50%, 78%, and 82% of Vero cells were infected by DV at day 1, 3, and 5 respectively.

5.3.2 Viral proteins secretion in MEG-01, compared with Vero cells

Kinetics of secreted NS1 and E proteins from DV-infected MEG-01 and Vero cell were determined by NS1-capture ELISA and E-ELISA, respectively. MEG-01 cells secreted considerably lower ($P < 0.05$) soluble NS1 protein compared with Vero cells at day 1 (3 ng/ml, MEG-01; 230 ng/ml, Vero), 3 (176 ng/ml, MEG-01; 3600 ng/ml, Vero), and 5 (300 ng/ml, MEG-01; 7500 ng/ml, Vero) after DV-infection (Figure 16). In contrast, MEG-01 and Vero cell secreted comparable levels of soluble E protein at all time points measured after DV-infection (Figure 17). The concentrations of secreted soluble E were 1, 7, 17 μg/ml for MEG-01, and 2, 7, 17 μg/ml for Vero cells at day 1, 3, and 5 after DV infection respectively.

5.3.3 Infectious virus progeny production from MEG-01, compared with Vero cells

Kinetics of infectious virus progeny production from DV-infected MEG-01 and Vero cells were determined by FFU assay. The infectious virus progeny production from DV-infected MEG-01 and Vero cells were similar at day 1 (1.5×10^4 FFU/ml, MEG-01 and Vero), and 3 (1.5×10^6 FFU/ml, MEG-01; 1.5×10^5 FFU/ml, Vero) after DV infection. However, infectious virus progeny production from DV-infected MEG-01 was significantly higher than Vero cells at day 5 after DV-infection (1.5×10^7 FFU/ml, MEG-01; 1.0×10^6 FFU/ml, Vero) (Figure 18A). Moreover, the

efficiency to produce infectious virus and less of non-infectious virus is a noticeable phenomenon of DV infected cells. The infectious virus produced from DV-infected MEG-01 and Vero cells were calculated from the ratio of infectious virus titers per amount of secreted soluble E. The efficiency of infectious virus production from MEG-01 and Vero cells were similar at day 1 (2.2×10^5 FFU/μg of secreted soluble E protein, MEG-01 and 2.3×10^5 FFU/μg of secreted soluble E protein, Vero) and day 3 (2.0×10^4 FFU/μg of secreted soluble E protein, MEG-01 and 1.1×10^4 FFU/μg of secreted soluble E protein, Vero) after DV infection. While, at day 5 after DV infection, MEG-01 produced higher amount of infectious virus than Vero cell (1.6×10^6 FFU/μg of secreted soluble E protein, MEG-01 and 7.9×10^4 FFU/μg of secreted soluble E protein, Vero) (Figure 18B).

5.3.4 Susceptibility to the infection of MEG-01 by clinical isolates of DV serotypes 1-4

To determine the susceptibility of MEG-01 to DV 1-4 infection, the percentage of DV-infected cells and the production of infectious virus progeny were investigated.

Infection of DV 1-4 in MEG-01 cell was determined by a cytoplasmic staining assay using 4G2, a flavivirus cross-reactive mAb against E protein. Histogram profiles of cytoplasmic dengue E protein expression are shown in Figure 19A. Approximately, 20-30% of MEG-01 cells were susceptible to infection by clinical dengue isolates, serotypes 1-4. Moreover, a low expression of E protein in cytoplasm of DV 1-4 infected MEG-01 cells as indicated by mean fluorescence intensity (Figure 19A).

The production of infectious virus progeny from DV 1-4-infected MEG-01 cells was determined by FFU assay. The infectious virus progenies were detected from all DV 1-4-infected MEG-01 (Figure 20). Infectious virus titers at day 5 after DV infection were 1.0×10^7, 1.5×10^7, 1.0×10^5, and 1.5×10^5 FFU/ml for DV-1, 2, 3, and 4, respectively.

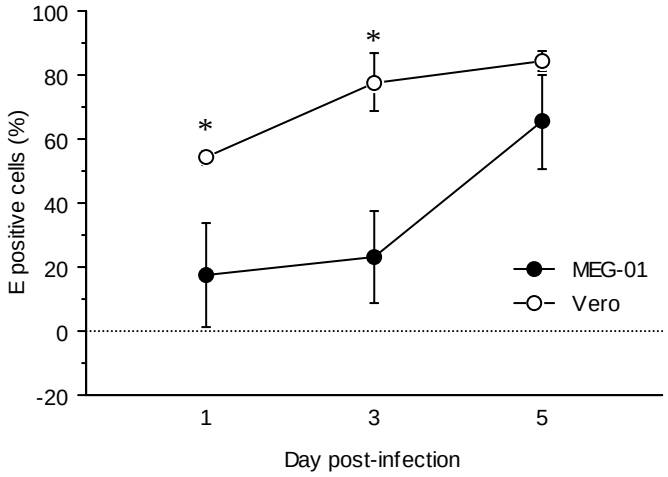


Figure 15 Percentages of DV-infected MEG-01 cells, compared with Vero

MEG-01 or Vero cells were infected with DV at MOI of 1. DV-infected cells were harvested at 1, 3, and 5 days after infection. The cells were stained with mouse monoclonal antibody specific to dengue E protein followed by FITC-conjugated rabbit anti mouse IgG. Percentages of E expressing cells between DV-infected Vero cells (open circle) and DV-infected MEG-01 cells (filled circle) were compared. Each point represents the average of percentage of E expressing cells from three independent experiments $\pm$ SD. * indicated P value < 0.05 (Vero vs MEG-01).

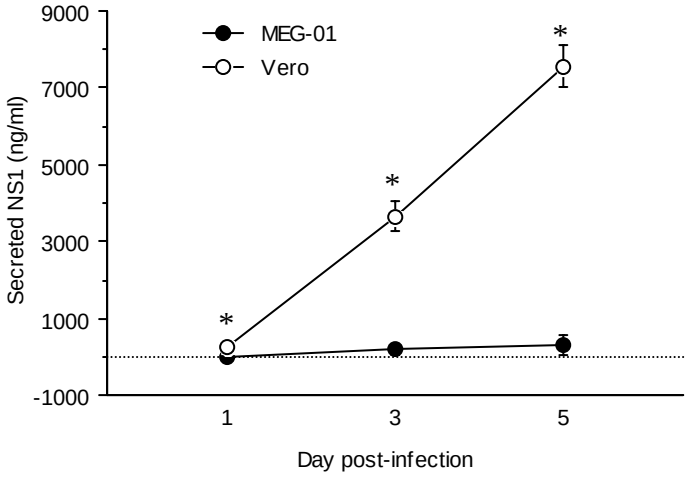


Figure 16 Levels of soluble NS1 secretion in DV-infected MEG-01, compared with Vero

MEG-01 or Vero cells were infected with DV at MOI of 1. The supernatants from DV-infected cells were collected at 1, 3, and 5 days after infection. The secreted NS1 levels in supernatants were determined by NS1-capture ELISA. The levels of secreted NS1 in supernatants from DV-infected Vero cells (open circle) and DV-infected MEG-01 cells (filled circle) were compared. Each bar represents the average of NS1 secretion levels from three independent experiments $\pm$ SD. * indicated *P* value < 0.005 (Vero vs MEG-01).

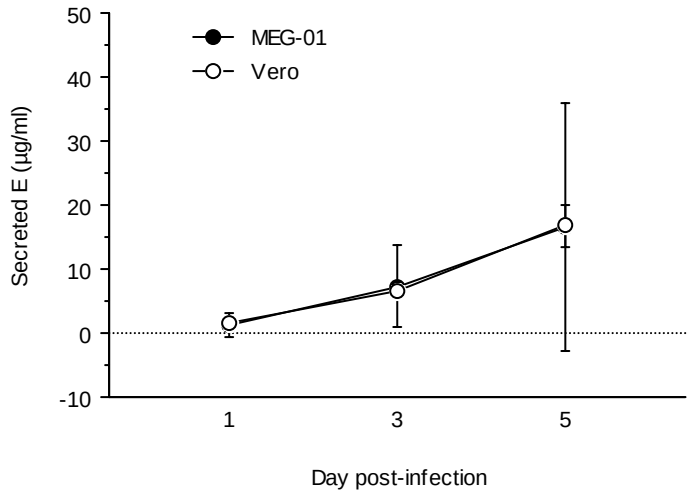
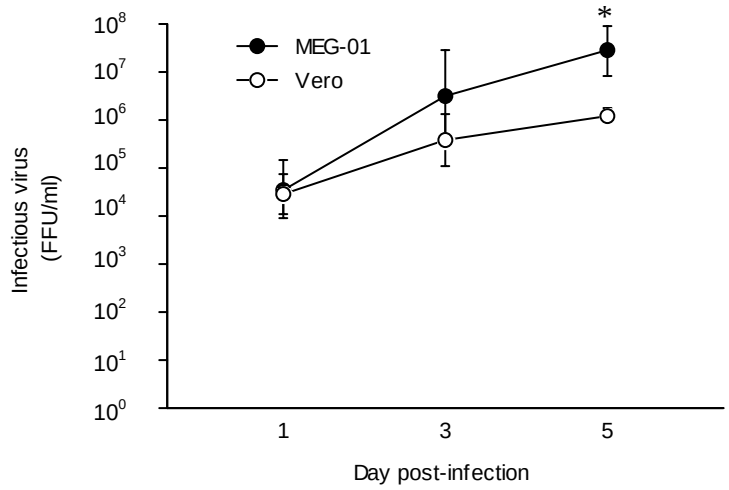


Figure 17 Levels of soluble E secretion in DV-infected MEG-01, compared with Vero

MEG-01 or Vero cells were infected with DV at MOI of 1. The supernatants from DV-infected cells were collected at 1, 3, and 5 days after infection. The secreted E levels in supernatants were determined by E-ELISA. The levels of secreted E in supernatants from DV-infected Vero cells (open circle) and DV-infected MEG-01 cells (filled circle) were compared. Each bar represents the average of E secretion levels from three independent experiments $\pm$ SD.

A.



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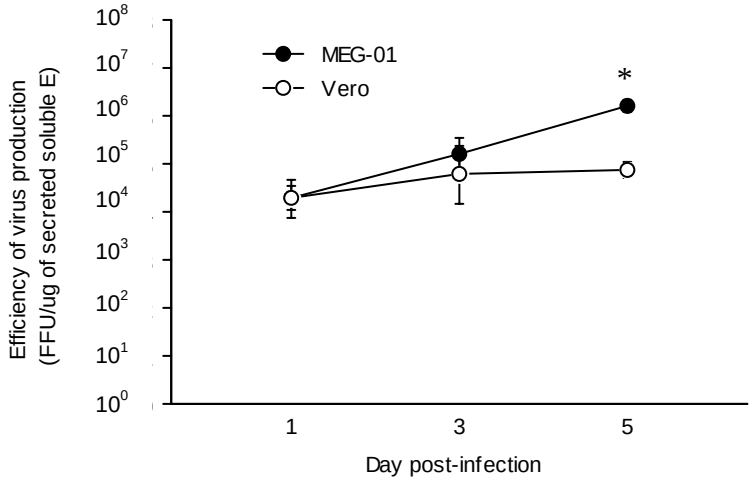
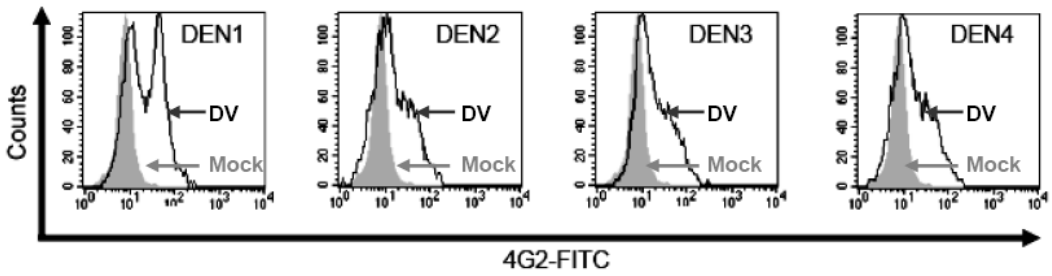


Figure 18 Efficiency of infectious virus production from DV-infected MEG-01, compared with Vero

MEG-01 and Vero cells were infected with DV at MOI of 1. The supernatants from DV-infected cells were collected at 1, 3, and 5 days after infection. The production of infectious virus was determined with FFU assay. The infectious virus progeny titer from Vero (open circle) and MEG-01 (filled circle) were compared (A). Each point represents the average of infectious virus titers from three independent experiments $\pm$ SD. * indicated *P* value < 0.005 (Vero vs MEG-01). (B), The efficiency of infectious virus production, calculated from the ratio of infectious

virus titers per amount of secreted soluble E, indicates the ability of cells to produce infectious virions over defective non-infectious virus particles of Vero (open circle) and MEG-01 (filled circle). Each point represents the average of infectious virion production per µg of secreted soluble E from three independent experiments ± SD. * indicated *P* value < 0.05 (Vero vs MEG-01).

A.



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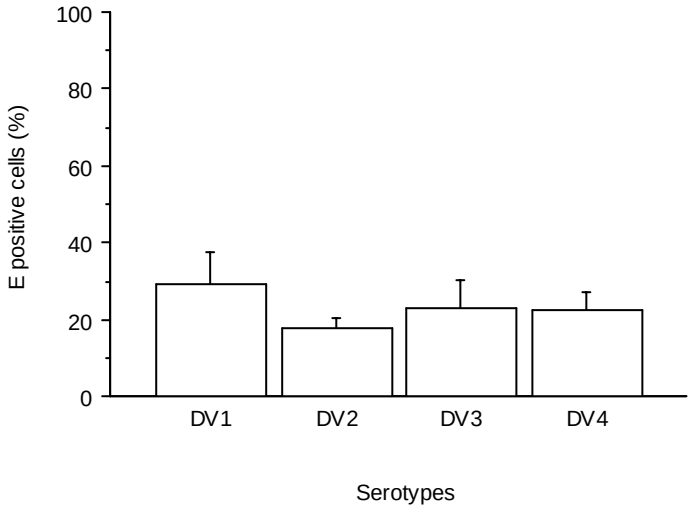

Figure 19 Infectivity of MEG-01 cells to DV clinical isolates, serotypes 1-4

MEG-01 cells were infected with DV 1-4 at MOI of 1. Mock-infected or DV-infected cells were harvested 5 days after infection. The cells were stained with mouse monoclonal antibody specific to dengue E protein followed by FITC-conjugated rabbit anti mouse IgG. (A), The intensity of E expression was compared between mock-infected (gray histogram) and DV-infected (dark line) MEG-01 cells. (B), Percentage of E expression indicates the infectivity of DV serotypes 1-4 to MEG-01 cells. Each bar represents the average of DV 1-4 E expressing cells from three independent experiments $\pm$ SD.

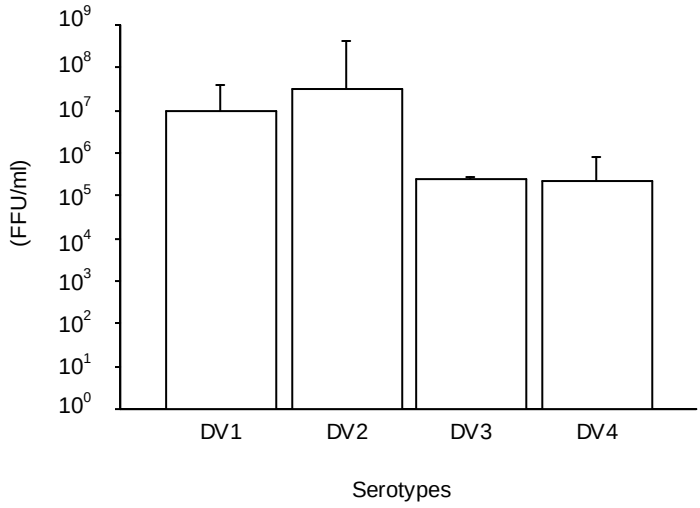

Figure 20 Infectious virus progeny production in DV 1-4 –infected MEG-01 cells

MEG-01 cells were infected with DV serotypes 1-4 at MOI of 1. The supernatants were collected 5 days after infection. After removing the cells and debris by centrifuging twice at 2,000g for 10 minutes, the infectious virus progeny in supernatants was determined by FFU assay. Each bar represents the average of infectious virus titers from three independent experiments $\pm$ SD.

5.4 Generation of particles and microparticles carrying infectious virus particles by DV-infected MEG-01

A microparticle is a small membrane vesicle, approximately 0.1-1 μm in size, budding from either activated cells or apoptotic cells that are involved in several pathways of pathogenesis as described above. DV infection leading to both activation and death of infected cells via apoptosis may be the source of MPs. Morphological identification is a possible way to differentiate MPs from small debris because microparticles have more granularity than debris.

5.4.1 Identification of particles and microparticles derived from DV-infected MEG-01

Since the sizes of particles and microparticles derived from MEG-01 differ; ~1-10 μm and ~0.1-1μm in size for particles and microparticles respectively. To classify these two types of vesicles, size identification was performed using FACS analysis. Ps and MPs were fractionated by the serial centrifugation at day 5 after infection which is the peak of DV infection and beginning of the death of DV-infected cells by apoptosis. Subsequently each fraction was identified as particles or microparticles based on forward scatter (FSC) and sideward scatter (SSC) of particles and microparticles (Figure 21A and B). The particles fraction show more particle population, compared with microparticles fraction (10±0.6 vs. 2±1.3, respectively). Conversely, microparticles fraction show markedly increased in microparticles population, compared with particles fraction (45±8.2 vs. 2±1.2, respectively).

5.4.2 Total protein concentration in particles and microparticles derived from DV-infected MEG-01

To normalized the protein levels from particles and microparticles fraction the total protein concentrations from corresponding fraction were determined by Bradford assay. The total protein in microparticles fraction was lower than particles fraction (300±13 μg/ml vs. 430±76 μg/ml) (Figure22)

5.4.3 Viral protein expression in the particles and microparticles derived from DV-infected MEG-01

DV-infected cells express NS1 and E proteins on their surface membranes. The particles and microparticles derived from DV-infected cells should contain these proteins on their surface. At day 5 after infection, DV-infected MEG-01 cells were harvested and fractionated by serial centrifugation. The NS1 and E protein on DV-infected MEG-01-derived Ps and MPs were determined by NS1-captured ELISA or E-ELISA. The levels of NS1 and E protein were normalized by division with the total protein concentration of each fraction. Both NS1 and E protein were detected in MPs fraction (116±29 pg/µg of total protein and 8±3 ng/µg of total protein respectively), although under detectable on Ps fraction (Figure23). The total NS1 and E proteins in each fraction are determined by Western blotting, NS1-capture ELISA and E-ELISA using freezed-thawing lysates. The total E protein concentrations detected in DV-infected MEG-01 Ps, and MPs, were 12 ng/µg of total protein and 18 ng/µg of total protein respectively (Figure 25). Interestingly, high levels of total NS1 antigens were detected in DV-infected MEG-01 derived MPs (380 pg/µg of total protein) compared with Ps (70 pg/µg of total protein) (Figure 24).

5.4.4 DV-infected MEG-01-derived particles and microparticles harbor infectious virus particles

The infectious virus progeny in DV-infected MEG-01 cells, Ps, and MPs were determined by FFU assay. The levels of infectious virus progeny were normalized by division with the total protein concentration of each fraction. The infectious virus progeny were detected in DV-infected MEG-01 Ps and MPs at day 5 after DV-infection (Figure 26). The infectious virus titers were 480 FFU/µg of total protein, and 1,500 FFU/µg of total protein in DV-infected MEG-01 Ps and MPs respectively.

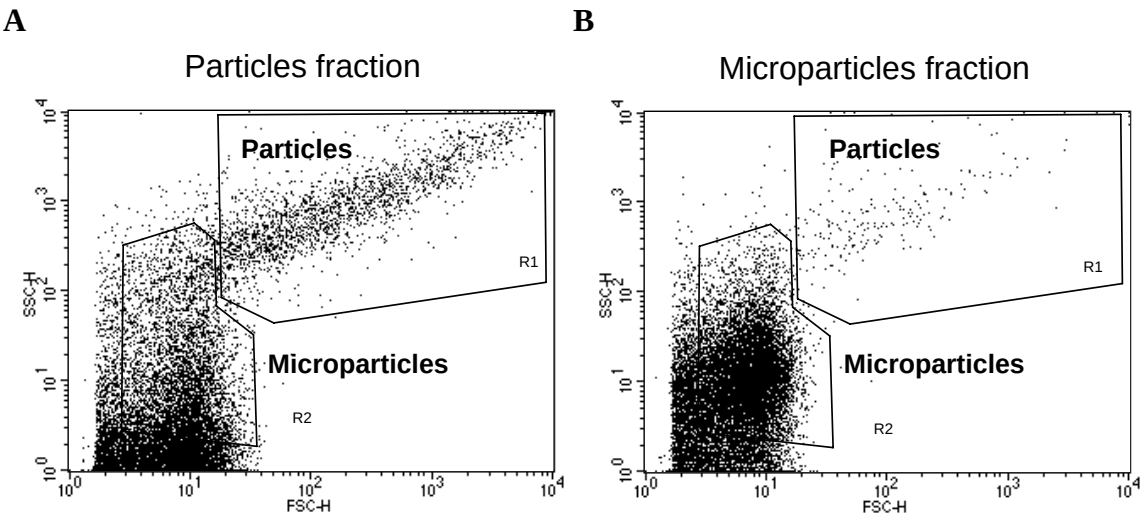

Figure 21 Identification of DV-infected MEG-01-derived Ps and MPs

MEG-01 cells were infected with DV at MOI of 1. The supernatants from DV-infected cells were collected at 5 day after infection. DV-infected MEG-01-derived particles, and microparticles were fractionated by serially centrifugation and each fraction was identified by FACS. The particles fraction (A) and microparticles fraction (B) were identified using FSC and SSC analysis. The population of platelet-like particles and microparticles were show in R1 and R2 region, respectively. The protein concentration was determined by Bradford assay. Each fraction was lysed and the membrane debris was removed by centrifugation before assays. Each bar represents the average of total protein concentration from three independent experiments $\pm$ SD. * indicated P value < 0.05.

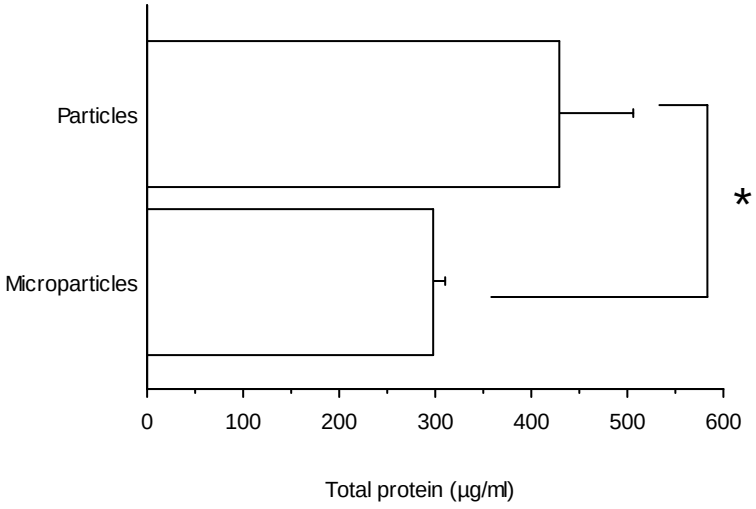

Figure 22 Total proteins in DV-infected MEG-01-derived Ps and MPs

MEG-01 cells were infected with DV at MOI of 1. The supernatants from DV-infected cells were collected at 5 day after infection. DV-infected MEG-01-derived particles and microparticles were fractionated by serially centrifugation. The total protein concentration was determined by Bradford assay. Each fraction was lysed and the membrane debris was removed by centrifugation before assays. Each bar represents the average of total protein concentration from three independent experiments $\pm$ SD. * indicated P value < 0.05.

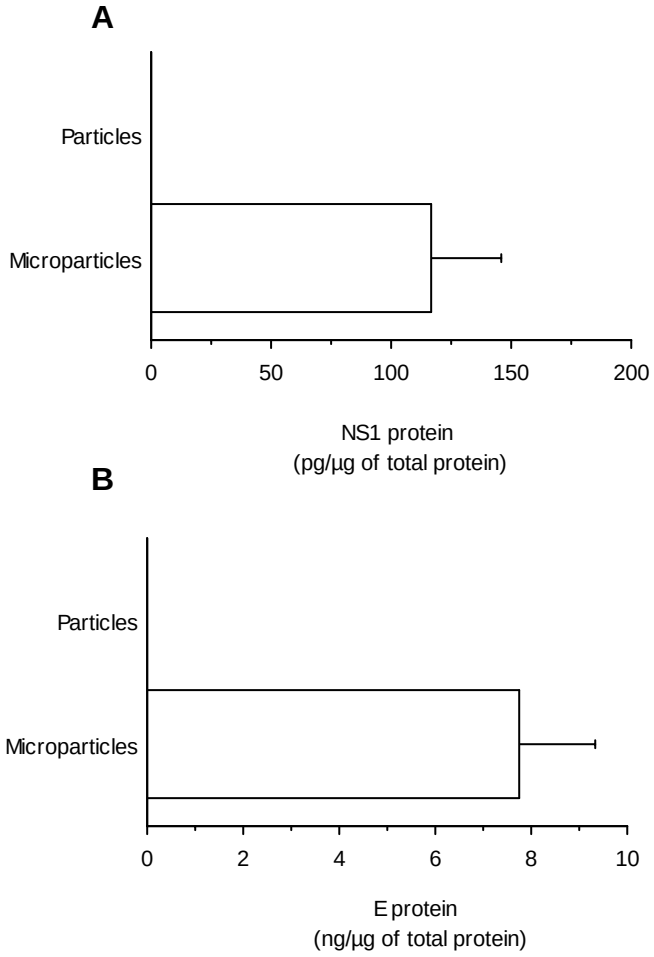

Figure 23 NS1 and E proteins on DV-infected-derived MEG-01-derived Ps and MPs

MEG-01 cells were infected with DV at MOI of 1. The supernatants from DV-infected cells were collected at 5 day after infection. DV-infected MEG-01-derived particles and microparticles were fractionated by serially centrifugation. After two times washing, NS1 and E proteins in each fraction were determined by NS1-captured ELISA and E-ELISA, respectively. The NS1 and E protein levels in each fraction were normalized by total protein concentration of a corresponding fraction. Each bar represents the average of levels of NS1 and E protein expressed on the surface of particles and microparticles from three independent experiments $\pm$ SD.

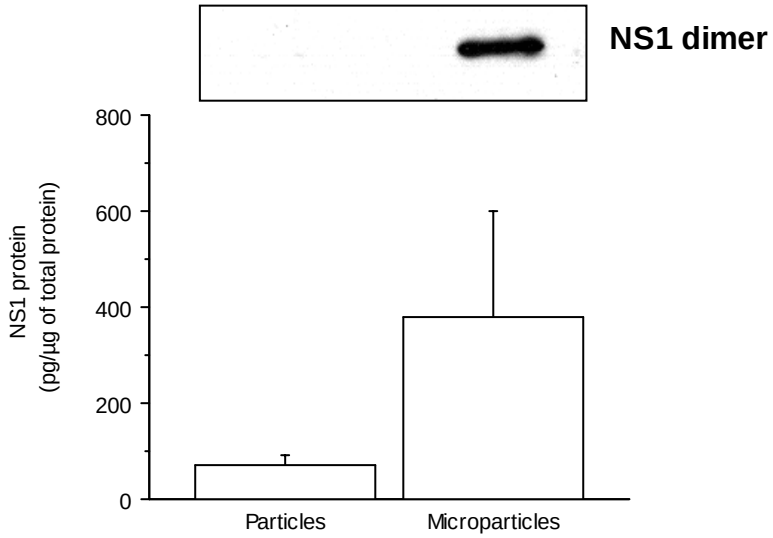

Figure 24 Total NS1 proteins in DV-infected MEG-01 Ps and MPs

MEG-01 cells were infected with DV at MOI of 1. The supernatants from DV-infected cells were collected at 5 day after infection. DV-infected MEG-01 cells, particles, and microparticles were fractionated by serial centrifugation and each fraction was subsequently lysed by repeatedly freezing and thawing. NS1 levels in each fraction were determined by NS1-capture ELISA. The NS1 protein level in each fraction was normalized by total protein concentration of a corresponding fraction. Each bar represents the average of NS1 levels from three independent experiments $\pm$ SD. Expression of NS1 protein in each fraction was confirmed by western blot using mouse monoclonal antibody specific to DV NS1 protein.

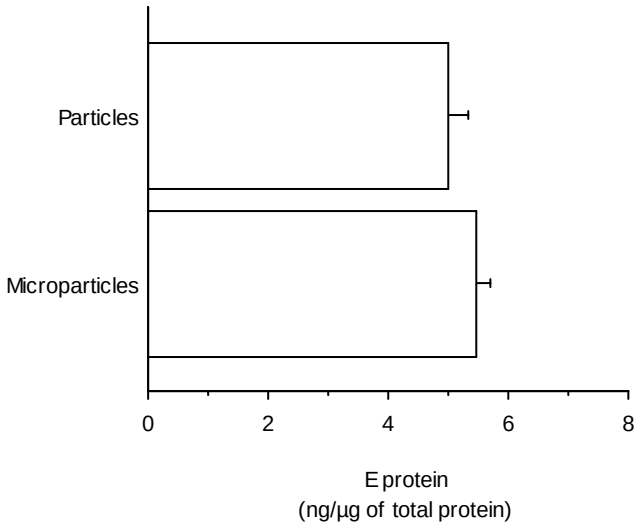

Figure 25 Total E proteins in DV-infected MEG-01-derived Ps and MPs

MEG- cells were infected with DV at MOI of 1. The supernatants from DV-infected cells were collected at 5 days after infection. DV-infected MEG-01-derived particles, and microparticles were fractionated by serial centrifugation and lysates from each fraction were prepared by repeatedly freezing and thawing. E protein levels in each fraction were determined by E-ELISA. The E protein level in each fraction was normalized by total protein concentration of a corresponding fraction. Each bar represents the average of levels of E protein expression from three independent experiments $\pm$ SD.

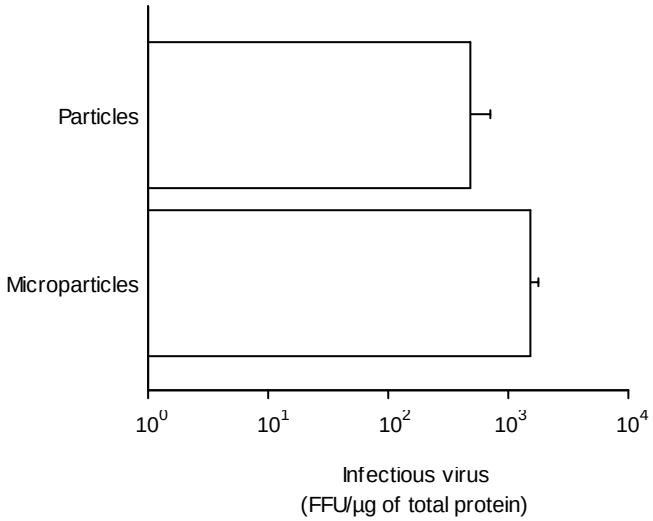

Figure 26 Infectious virus particles in lysates of DV-infected MEG-01-derived Ps and MPs

MEG-01 or Vero cells were infected with DV at MOI of 1.0. The supernatants from DV-infected cells were collected 5 days after infection. DV-infected MEG-01-derived particles and microparticles were fractionated by serial centrifugation and lysates from each fraction were prepared by repeatly freezing and thawing. Then infectious virus titers in each fraction were determined by FFU assay. The infectious virus titer in each fraction was normalized by total protein concentration of a corresponding fraction. Each bar represents the average of infectious virus titers from three independent experiments $\pm$ SD.

CHAPTER VI

DISCUSSION

The key finding of the present study is the susceptibility of the megakaryoblast cell line, MEG-01, to the infection and replication of dengue virus (DV). In the past, several studies tried to demonstrate that hematopoietic progenitor cells in bone marrow can be infected with DV leading to a hypocellular condition in the acute phase of DV infection (11, 15, 151). Thrombocytopenia is also mostly observed in the acute phase of DF and DHF/DSS patients as well as bone marrow suppression (9, 13, 16). However, the nadir of platelet counts occurs at the time of defervescence or shock, which later than the bone marrow suppression period (60). Thrombocytopenia can be caused by either 1) consumption or destruction of platelets in circulation (9, 152, 153) or 2) alteration of platelet production which is the result of bone marrow suppression (14). At present, no study has demonstrated that DV can directly infect megakaryoblasts or megakaryocytes which are the origin of platelets in circulation. This is the first study showing that the megakaryoblast cell line, MEG-01, which has virtually characteristics to megakaryoblast cells in bone marrow (140, 146, 147) are susceptible to DV infection.

The low percentages of DV infection on MEG-01 at the early period of infection suggest that the delay of infection arise on DV-infected MEG-01 cells. Inhibition of cytokine or chemokine, production by DV infection is one possible mechanism. Interferon (IFN) inhibits the infection of DV at the translational stage of the infectious viral RNA. This occurs via viral RNA attached ribosomes when they are independent, but not during virus binding and entry stages (154). *In vitro* and *in vivo* studies demonstrated that upregulation of chemokines, including CXCR3 and CXCL10/IFN-gamma-inducible protein 10 (IP-10), occur in DV infection (155, 156).

CXCL10/IP-10 inhibits the DV-infection via competition of virus binding to heparin sulfate on the surface of target cells (155). However, measurements of these cytokine and chemokine produced from DV-infected MEG-01 cells should be performed in order to show that this is the mechanism of the delay of DV infection on MEG-01.

Reduction in total cell count (Figure 11) and the increase in the number of dead cells (Figure 12) in DV-infected MEG-01 are not observed until day 7 after DV-infection. The death of DV-infected cells, which are the sources of platelets, might result to thrombocytopenia. A few amounts of DV-infected cells have no effect on DV replication, viral protein production, and virus progeny production as shown in figure 3. Additionally, the delay of DV infection in MEG-01 is the cause of these results.

Several studies have demonstrated that DV infection leads to the death of DV-infected cells by apoptosis. *In vitro* studies demonstrate that DV infection could lead to the death of DV-infected cell by the apoptotic pathway (32, 157-162). Moreover, studies in DV-infected patient tissues show that apoptosis can be observed (161, 163, 164). As expected, the infection of DV in MEG-01 also leads to the death of DV-infected cells via apoptosis (Figures 13 and 14). Although there are no differences when detected by annexin V/PI staining, the apoptotic cell population in DV-infected MEG-01 can be observed by TUNEL assay. Phosphatidylserine (PS), locates on the inner membrane of normal cells and is exposed on the surface membrane of early apoptotic cells and recognized by annexin V. Then early apoptotic cells can be determined by annexin V positive signals and PI which binds directly to the DNA in nucleus negative cells. Conversely, late apoptotic cells contain fragmented DNA in the nucleus which can be determined by TUNEL assay. The similar amount of early apoptotic cells in mock and DV-infected MEG-01 may be the result of the rapid death of DV-infected cells at the late period of infection.

The levels of virus in circulation correlate well with the severity of dengue disease (165, 166). However, the source of virus production in DV-infected patients

is still unknown. Although several studies demonstrated that DV-targeted cells, including monocytes, B lymphocytes, and endothelial cells, can produce infectious viruses at low levels (45, 49, 167), no virus production has been detected in DV-infected human Kupffer cells (168). However, *in vitro* studies have shown a high titer after infection of DV in primary isolated hepatocytes and HepG2 cell, a hepatocyte cell line (169). Although the percentages of infection are not over 25% of total cells at days 1 and 3 after infection the infectious virus productions are high (approximately 10^4-10^5 FFU/ml). This is a very interesting phenomenon that a few of DV-infected MEG-01 cells produce high virus titer, compared with Vero cell. Megakaryoblast cells may be the source of virus propagation in DHF/DSS patients although the megakaryoblast and megakaryocyte populations in normal bone marrow are very low (approximately 0.1-0.5% of total cells) (170).

NS1 was first characterized as a soluble (non-virion-associated) complement fixing (SCF) antigen in infected suckling mice brain and cell culture (171). NS1 has been found to co-localize with marker of RNA replication in association with membrane structures that are presumed sites of replication (172, 173). A potential role of NS1 in immunopathogenesis of DV-infection has also been proposed based on the finding that high level of NS1 protein was detected in acute phase sera from patients experiencing primary or secondary DV-infection (174, 175). Its level correlates with disease severity as the amount of circulating NS1 is higher in DHF than DF patients (176). Moreover, secreted NS1 has shown a potential to activate complement leading to inflammation and vascular leakage (177). However, the sources of NS1 production are still unknown. In MEG-01, secreted NS1 levels are very low, compared with Vero cell. From this result, we could highlight that megakaryoblast cell line, MEG-01 are not the NS1 producing cells.

Microparticles (MPs) are generated from activated (122-124) or apoptotic (125, 126) cells or platelets (121) in the circulation. MPs are involved in several processes of cell response and pathogenesis including 1) control of the coagulation process (31, 131-133), 2) transporter of cellular surface antigens (134-137), 3) induction of cell and platelet activation (138) and, 4) induction of inflammatory responses (139). From our result, MPs derived from DV-infected MEG-01 containing high levels of NS1

which may locate on their surface (Figure 24) although the total protein concentration was lower than Ps. The membrane associated NS1 recognized by antibody is capable of activating complement (178). Then DV-infected MEG-01-derived MPs may activate complement in circulation and/or bind to endothelial cells resulting in vascular endothelial damage. Additionally, the infectious virus was found inside MPs and Ps derived from DV-infected MEG-01 (Figure 26). As with complement activation, the infectious virus might distributed via MPs resulting in enhanced DV infection or increased risk of DV infection by DV receptors on the surface of MPs. While the MPs can bind to platelets as endothelial cells, destruction of MPs binding platelets might arise as on endothelial cells. From these phenomena we could suggest that MPs may play an important role in the immunopathogenesis of DV infection and thrombocytopenia.

CHAPTER VII
CONCLUSION

Thrombocytopenia and bone marrow suppression mostly occur in DF and DHF/DSS patients. However, the mechanism of thrombocytopenia associated with bone marrow cells especially megakaryocytes, the platelet-producing cells, in DF and DHF/DSS are still not well understood. The present study provides a clear hypothesis on thrombocytopenia and immunopathogenesis in DHF/DSS via direct infection of DV in megakaryocytes. The susceptibility of the megakaryoblast cell line, MEG-01, to the infection and replication of all 4 serotypes of dengue virus (DV) was observed in this study. Infection with DV leads to the death of DV-infected MEG-01 cells via apoptosis. Although megakaryoblast cells are not the source of soluble NS1 in circulation, a high titer of dengue virus progeny is produced from this cell. Additionally, NS1 protein and infectious virus particles are observed on the particles and microparticles derived from DV-infected MEG-01. From these results, the direct infection of DV in megakaryocytes in bone marrow could be a potential mechanism of thrombocytopenia and immunopathogenesis occurring in DHF/DSS.

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APPENDIX

APPENDIX

1 Chemical/substances

Chemicals	Molecular weight (g/mol)	Source
Absolute ethanol (C_2H_5OH)	46.07	BDH, England, UK
Absolute methanol (CH_3OH)	32.24	Lab-Scan, Thailand
Acetic acid glacial (CH_3COOH)	60.05	Carlo Erba, Milan, Italy
Acrylamide (C_3H_5NO)	71.08	Sigma, USA
N, N'-Methylene bis-acrylamide	154.20	Sigma, USA
Ammonium persulfate (NH_4HCO_3)	228.20	USB, USA
Bovine Serum Albumin		Sigma, USA
Carboxy methyl cellulose (CMC)		Sigma, USA
3, 3-Diaminobenzidine tetrahydrochloride,anhydrous (DAB)	360.10	Sigma, USA
Fetal bovine serum		GibcoBRL, England, UK
Formaldehyde 40% m/v (HCOH)	30.026	Carlo Erba, Milan, Italy
L-Glutamine		Sigma, USA
Glycine ($H_2NCH_2CO_2H$)	75.27	USB, USA
Gum tragacanth		Sigma, USA
Hydrogen peroxide (H_2O_2)	74.015	Sahakarn-Osos (1996), Bangkok
Hydrochloric acid (HCl)	36.50	E. Merck, Germany
Isopropanol ($CH_3CHOHCH_3$)	60.10	BDH, England, UK
ß-Mercaptoethanol ($HSCH_2CH_2OH$)	78.13	Fluka, Italy
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	203.30	E. Merck, Germany

Chemicals	**Molecular weight (g/mol)**	**Source**
Nickel chloride hexahydrate ($NiCl_2.6H_2O$)	237.70	Sigma, USA
Paraformaldehyde		Sigma, USA
Penicillin ($C_{16}H_{17}N_2O_4SNa$) 6130 U/mg	356.40	Sigma, USA
Potassium chloride (KCl)	74.56	E. Merck, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	136.09	E. Merck, Germany
Skim milk (Instant non fat milk powder)		Mission, Thailand
Sodium azide (NaN_3)	65.01	E. Merck, Germany
Sodium bicarbonate ($NaHCO_3$)	84.01	E. Merck, Germany
Sodium carbonate (Na_2CO_3)	105.99	E. Merck, Germany
Sodium chloride (NaCl)	58.44	E. Merck, Germany
Sodium Hydroxide (NaOH)	40.00	E. Merck, Germany
Sodium dodecyle sulfate or SDS ($C_{12}H_25O_4SNa$)	288.38	Sigma, USA
Streptomycin sesquisulfate (750U/mg)		Sigma, USA
N,N,N',N'-Tetramethyl Methylene-Diamine or TEMED ($C_4H_{11}NO_3$)	166.21	Bio-Rad, USA
Tris (Hydroxymethyl aminomethane)	121.10	Sigma, USA
Triton X-100		Fluka, Italy
Tryptose phosphate broth		Sigma, USA
Ultra DOMA-PF		Cambrex Bio Science Walkersville, USA

2 Instruments

2.1 Autoclave, Mode HA-240M, Tokyo, Japan.

2.2 Automatic Pipettes, Gilson, Villiers-le-B4el, France.

2.3 Beckman Microfuge E, Califonia,USA.

2.4 Beckman Optima LE-80K Ultracentrifuge, USA.

2.5 Biofreezer (-70˚C), Forma Scientific, Marietta, Ohio, USA.

2.6 Bio-Rad, Econo Systems, Japan.

2.7 CO_2 incubator, Forma Scientific, Marietta, Ohio, USA.

2.8 Confocal Microscopy, Zeiss LSM 510 META, Germany.

2.9 Vacuum pump, Sartorius, Gottingen, Germany.

2.10 Digital camera, Nikon, coolpix 950, Japan.

2.11 Digital, refrigerated centrifuge IEC Centra-8R, Internation Equipment Company

2.12 Dotted Apparatus, Bio-Rad, USA.

2.13 Electronic analytical and Precision Balance, Sartorius, Gottingen, Germany.

2.14 ELISA-reader, Titer Multiskan, Flow laboratories, North Ryde, Australia.

2.15 Filter Sterilization Unit, Sartorius, Gottingen, Germany.

2.16 Flow cytometer, FACScan, Becton Dickinson Immunocyometry System, SanJose, CA, USA.

2.17 Fluorescent microscope, Zeiss, Oberkochn, Germany.

2.18 Freezer (-20˚C), Sanyo Medical freezer model MDF 0535, Sanyo ElectricCo.Ltd., Japan.

2.19 Fume Hood, TOXICAP 1000, CARTATR LABX, USA.

2.20 High speed refrigerated centrifuge MTX-150, Tomy Seiko, Tokyo, Japan

2.21 Incubator, Ehret, Germany.

2.22 Inverted microscope, Olympus CK2, Tokyo, Japan.

2.23 Laboratory centrifuge, Biofuge pico Heraeus, Kendro Laboratory Product,Germany.

2.24 Laminar airflow equipment, NuAir Biological Safty Cabinrts, USA.

2.25 Magnetic stirrers Hotplate, Stuart Scientific, Bibby Sterilin Ltd., UK.

2.26 Milli-Q Plus, Millipore Corporation Massachusetts, USA.

2.27 Model J2-MC centrifuge, Beckman, California,USA.

2.28 Multichannel pipette, Biohit praline, Biohit Oyj, Helsinki, Finland.

2.29 pH meter, Orion 520A, Boston, USA.

2.30 Pipetboy acu, integra Bioscience

2.31 Power supply E-C Apparatus Corporation, St.Peterberg, Florida, USA.

2.32 Refrigerator, Sanyo New touch, Sanyo Electric Co. Ltd., Japan.

2.33 Refrigerator, Traflo Fraame model Expo 310 PT/E, san Giorgio Monf.(AL), Italy

2.34 Rocker, Hoefer model PR 55, Hoefer Scientific Instruments, San Giorgio Monf (AL), Italy.

2.35 Semi-dry blotting apparatus, Hoefer^{TM} TE77 semiphor transphor Unit, Amersham Bioscience, Uppsala, Sweden.

2.36 Shaking water bath Julabo SW-20C, Julabo Labortecjnik.Germany.

2.37 UV-160A UV-visible recording spectrophotometer, Shimadzu, Japan.

2.38 Vertical gel electrophoretic apparatus model AE-6410E, ATTO Corporation, Japan.

2.39 Vortex mixer, Vortex Gene 2, Scientific Industries, Bohemia, N.Y, USA.

3 Protein markers

3.1.1 SDS-PAGE Molecular weight standards (Broad Range), Bio-Rad Laboratories, Hercules, CA, USA.

3.1.2 SDS-PAGE Molecular weight standards (Low Range), Bio-Rad Laboratories, Hercules, CA, USA.

4 Reagents

4.1 Cell culture reagent

4.1.1 Phosphate buffered-saline (PBS), pH 7.4

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.20 g

These chemicals were mixed; well dissolved in deionized water, and adjusted the final volume to 1 liter prior to sterilize by autoclave.

4.1.2 Tryptose phosphate broth

Dissolve 29.5 g of tryptose phosphate broth powder in 1 liter of deionized water, and sterilized by autoclave at 121 ˚C for 15 min.

4.1.3 Penicillin G-Streptomycin solution

Penicillin G	301.81 mg
Streptomycin sulfate	500.00 mg

These antibiotics were mixed in 0.9% normal saline solution (Siiraj Hospital) and stirred until completely dissolved prior to adjust 100 ml in volumetric flask. The solution was sterilized by filtrate though 0.2 μm of cellulose acetate filter membrane under sterile condition, aliquot and stored at –20 ˚C until use.

4.1.4 200 mM L-Glutamine

L-Glutamine 1.46 g was dissolved in 50 ml deionized water and sterilized by filtrate through 0.2 μM cellulose acetate filter membrane.

4.1.5 Cell dissociation solution

4.1.5.1 2.5 mM EDTA in PBS

EDTA.Na2.2H_2O 0.4653 g was dissolved in PBS, pH 7.4, stirred, and adjusted at a final volume to 500 ml in volumetric flask. The reagent was sterilized by autoclave at 121 ˚C for 15 min.

4.1.5.2 Trypsin solution (10% Trypsin in 2.5 mM EDTA/PBS)

2 g of trypsin was dissolved in 20 ml of 2.5 mM EDTA/PBS, stirred until completely dissolved and sterilized by filtrated through 0.2 μM cellulose acetate filter membrane. The regent was diluted to the desire concentration with sterile 2.5 mM EDTA/PBS before use.

4.2 Focus Forming Unit (FFU) reagents

4.2.1 Overlay medium

4.0 g of Carboxy methyl cellulose (CMC) was dissolved in 100 ml distilled water and sterilized by autoclaved. Equal volume of 4% (w/v) CMC was mixed together with MEM medium then FBS and PenG-Streptomycin solution were added up to 3% and 1.2%, respectively.

4.2.2 Fixative solution (3.7% formaldehyde in PBS)

37% (v/v) formaldehyde was 10-fold diluted with PBS pH 7.4 and stored in-dark at room temperature.

4.2.3 Permeable solution (1% Triton X-100 in PBS)

1 ml of Triton X-100 was added to 99 ml of PBS pH 7.4 and stored in-dark at room temperature.

4.2.4 Substrate solution

3, 3-Diaminobenzidine tetrahydrochloride anhydrous (DAB)	0.6 mg
Hidrogen peroxide (H_2O_2, 6% v/v)	20.0 µl
Nickel chloride ($NiCl_2$, 8% w/v)	90.0 µl

DAB was dissolved in 10 ml PBS buffer and mixed with H_2O_2 and $NiCl_2$ before used.

4.3 SDS-PAGE, Nodenaturing PAGE and Western Blotting Analysis.

4.3.1 30.8% (w/v) Acrylamide-Bisacrylmide

Acrylamide	30.0 g
Bis-acrylamide	0.8 g

These chemicals were dissolved in deionized water, and adjust the final volume to 100 ml. The reagent was filtrated through 125 mm diameter-filter paper (Whatman No.1).

4.3.2 Resolving gel buffer pH8.8.3 M Tris–HCl

Tris	36.3 g
1 M HCL	48.0 ml

Tris was added to 1 M HCl and adjusted the final volume to 100 ml with dionized water. The solution was adjusted the pH to 8.8 with 1 M CL, and stored at 4 ˚C

4.3.3 10% (w/v) Sodium dodecyl sulphate

Sodium dodecyl sulphate (SDS) 10 g was dissolved in 100 ml deionized water and stored at room temperature.

4.3.4 10% (w/v) Ammonium persulfate

Ammmonium persulfate 1.0 g was dissolved in deioized water and adjusted the final volume to 10 ml

4.3.5 Stacking gel buffer pH 6.8:0.5 M Tris-HCL

Tris	6	g
1 M HCL	48	ml

Tris was dissolved in 1 M HCL and adjusted the final volume to 100 ml with deionized water. The solution was adjusted the pH to 6.8 with 1 M hcl and tore at 4 ˚C.

4.3.6 10% Resolving gel of SDS-PAGE (for 1 PAGE)

30.8% (w/v) Acrylamide-bisacrylamide	1.670	ml
3.0 M Tris-HCL (pH 8.8)	0.630	ml
10%SDS	0.050	ml
10%Ammonium persulfate	0.038	ml
Distilled water	2.630	ml
TEMED	0.0025	ml

4.3.7 3.85%Stacking gel of SDS-PAGE (for 1 PAGE)

30.8% (w/v) Acrylamide-bisacrylamide	0.250	ml
0.5 M Tris-HCl (pH 6.8)	0.500	ml
10%SDS	0.020	ml
10%Ammonium persulfate	0.015	ml
Distilled water	1.220	ml
TEMED	0.0015	ml

4.3.8 10X Running buffer pH 8.3

(0.25 M Tris-HCl, 1.92 M Glycine, 1% (w/v) SDS)

Tris	30.3 g
Glycine	144 g
SDS	10 g

These chemicals were dissolved and adjusted the final volume to 1 liter with deionized water. The solution was diluted to 1X with deionized water before use.

4.3.9 1X Towbin buffer pH 8.3

Tris	3.0 g
Glycine	14.4 g
SDS	1.0 g

These chemicals were dissolved in 600 ml deionized water; 200 ml of methanol was added a brought to 1 liter with deionized water. The reagent was stored at 4 ˚C.

4.4 Enzyme linked-immunosorbent assay (ELISA)

4.4.1 Coating buffer pH 9.6

Na_2CO_3	0.1696 g
$NaHCO_3$	0.2856 g

Dissolved in 80 ml of DW, adjusted to pH 9.6 and made up to volume to 100 ml with DW

4.4.2 Blocking buffer

BSA	0.5 g

Dissolved in 100 ml of PBS

4.4.3 Diluent buffer

BSA	0.1 g

Dissolved in 100 ml of PBS

4.4.4 Washing buffer

Tween 20	500 µl
Dissolved in 1 litter of PBS	

4.4.5 Chromogenic substrate

O-Phenylenediamiine	6.0 mg
5-7% H_2O_2	10.0 µl
Citrate phosphate buffer pH 5.0	10.0 ml

The solution was mixed well and used immediately.

5 Consumable supplies

5.1 Microcentrifuge tubes (1.5 ml), Treff, Switzerland.

5.2 15 ml centrifuge tube, Costar, Coring Incorporated, Coring, NY, USA.

5.3 50 ml centrifuge tube, Costar, Coring Incorporated, Coring, NY, USA.

5.4 96-well cell culture clusters flat bottom with lid 3599, Costar, Coring Incorporated, Coring, NY, USA.

5.5 25-cm^2 cell culture flask phenolic style cap 3055, Coring Incorporated, Coring, NY, USA.

5.6 75 cm^2 cell culture flask phenolic style cap 3055, Coring Incorporated, Coring, NY, USA.

5.7 162 cm^2 cell culture flask phenolic style cap 3055, Coring Incorporated, Coring, NY, USA.

BIOGRAPHY

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