NEUTRALIZATION AND ENHANCEMENT ACTIVITIES OF MONOCLONAL ANTIBODIES SPECIFIC TO THE ENVELOPE PROTEIN OF DENGUE VIRUSES

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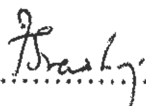

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Kriangkrai Chawansuntati

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ABSTRACT

Envelope (E) protein is one of the most important antigens responsible for dengue virus attachment and mediates virus-specific membrane fusion. Infection by dengue virus induces anti-E neutralizing antibody production, one of the defensive mechanisms to neutralize the virus. In the presence of non-neutralizing or sub-neutralizing concentrations, anti-E antibodies are, in turn, involved in the antibody-dependent enhancement (ADE) of virus infection via Fc receptor bearing cells. In this study, the *in vitro* neutralizing as well as enhancement activity of twelve anti-E monoclonal antibodies (Mabs) to 4 prototype strains of each dengue serotype and Japanese encephalitis virus Nakayama strain were determined. The dengue virus-susceptible cells, Ps clone D were used to determine the neutralizing activity of Mab, while Fc receptor-bearing cells, U937 were used for ADE assay. The results indicated that seven anti-E Mabs (4G2, 2B7, 1D10, 2C8, 8A1, 1H10, and J93) exhibited both neutralizing and enhancement activity to each virus serotype correlating to their binding properties. Two Mabs neutralized dengue virus infection, but enhanced none (for 3H5) or some serotypes (for 1D3) of dengue virus infection. The other two Mabs neutralized only dengue-2 virus infection, but enhanced both dengue-1 and 2 (for 1C2) or dengue-2 and 3 (for 5A1). One Mab (1F4), specific to dengue-2 virus, could not neutralize or enhance dengue virus infection. It was also found that infection enhancement by 3H5 anti-E Mab is dependent on the strain of virus, which is consistent with previous results. The identified functional activities of anti-E Mabs would be additional information to identify new epitopes contributing to dengue vaccine design.

KEY WORDS: NEUTRALIZING ANTIBODIES/ ENHANCEMENT ANTIBODIES/ MONOCLONAL ANTIBODIES/ DENGUE VIRUSES/ENVELOPE PROTEIN

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(NEUTRALIZATION AND ENHANCEMENT ACTIVITIES OF MONOCLONAL ANTIBODIES SPECIFIC TO THE ENVELOPE PROTEIN OF DENGUE VIRUSES)

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บทคัดย่อ

โปรตีนส่วนเปลือกหุ้มของเชื้อไวรัสเด็งกี่มีความสำคัญในกลไกการจับและเข้าสู่เซลล์เจ้าบ้าน เมื่อมีการติดเชื้อไวรัสเด็งกี่ กลไกหนึ่งที่สำคัญในการยับยั้งประสิทธิภาพของการเข้าสู่เซลล์เจ้าบ้านของเชื้อไวรัสคือการผลิตแอนติบอดี อย่างไรก็ตาม แอนติบอดีที่จำเพาะต่อโปรตีนส่วนเปลือกหุ้มที่ไม่มีคุณสมบัติของการยับยั้ง หรือ แอนติบอดีที่มีคุณสมบัติในการยับยั้งแต่ในปริมาณต่ำ กลับมีส่วนเกี่ยวข้องต่อการเพิ่มการติดเชื้อในเซลล์เจ้าบ้านที่มีตัวรับส่วนเอฟซีของแอนติบอดี ในการศึกษานี้ ได้ทำการตรวจสอบคุณสมบัติของโมโนโคลนอลแอนติบอดีที่จำเพาะต่อส่วนเปลือกหุ้มจำนวน 12 โคลน ในการยับยั้งไวรัสเข้าสู่เซลล์ที่มีความไวต่อการติดเชื้อไวรัส และ การเพิ่มความสามารถในการเพิ่มจำนวนของไวรัสในเซลล์ที่มีตัวรับส่วนเอฟซีของแอนติบอดี ต่อเชื้อไวรัสเด็งกี่สายพันธุ์ต้นแบบทั้ง 4 ซีโรทัยป์ และเชื้อไวรัสไข้สมองอักเสบ สายพันธุ์นากายามา ผลการศึกษาพบว่า มีโมโนโคลนอลแอนติบอดีจำนวน 7 โคลน (4G2, 2B7, 1D10, 2C8, 8A1, 1H10 และ J93) ที่มีคุณสมบัติทั้งยับยั้งและเพิ่มการติดเชื้อของไวรัสที่จำเพาะต่อแอนติบอดีในซีโรทัยป์ที่เหมือนกัน มีโมโนโคลนอลแอนติบอดีจำนวน 2 โคลน ที่มีความสามารถในการยับยั้งการติดเชื้อแต่ไม่มีคุณสมบัติในการเพิ่มการติดเชื้อต่อไวรัสที่จำเพาะ (3H5) หรือ ต่อไวรัสบางสายพันธุ์ (1D3) และมีโมโนโคลนอลแอนติบอดีจำนวน 2 โคลน (1C2 และ 5A1) ที่สามารถยับยั้งการติดเชื้อเฉพาะไวรัสเด็งกี่สายพันธุ์ที่ 2 แต่มีความสามารถในการเพิ่มการติดเชื้อทั้งไวรัสเด็งกี่สายพันธุ์ที่ 2 และสายพันธุ์อื่นๆที่แอนติบอดีสามารถจับได้ และยังพบว่ามีโมโนโคลนอลแอนติบอดีอีก 1 โคลน (1F4) ที่จับกับโปรตีนส่วนเปลือกหุ้มของไวรัสเด็งกี่สายพันธุ์ที่ 2 แต่ไม่มีคุณสมบัติทั้งยับยั้งและเพิ่มจำนวนของเชื้อไวรัสเด็งกี่ อย่างไรก็ตาม ยังพบว่าความสามารถในการเพิ่มจำนวนเชื้อไวรัสของโมโนโคลนอลแอนติบอดีโคลน 3H5 นั้นขึ้นอยู่กับสายพันธุ์ของไวรัส ซึ่งให้ผลสอดคล้องกับการศึกษาก่อนหน้านี้ ผลที่ได้จากการศึกษาความสามารถของโมโนโคลนอลแอนติบอดีนี้น่าจะเป็นข้อมูลสำคัญที่จะใช้ในการศึกษาหาตำแหน่งใหม่บนโปรตีนส่วนเปลือกหุ้มซึ่งอาจนำไปสู่การพัฒนาเป็นวัคซีนหรือยาต้านไวรัสได้ในอนาคต

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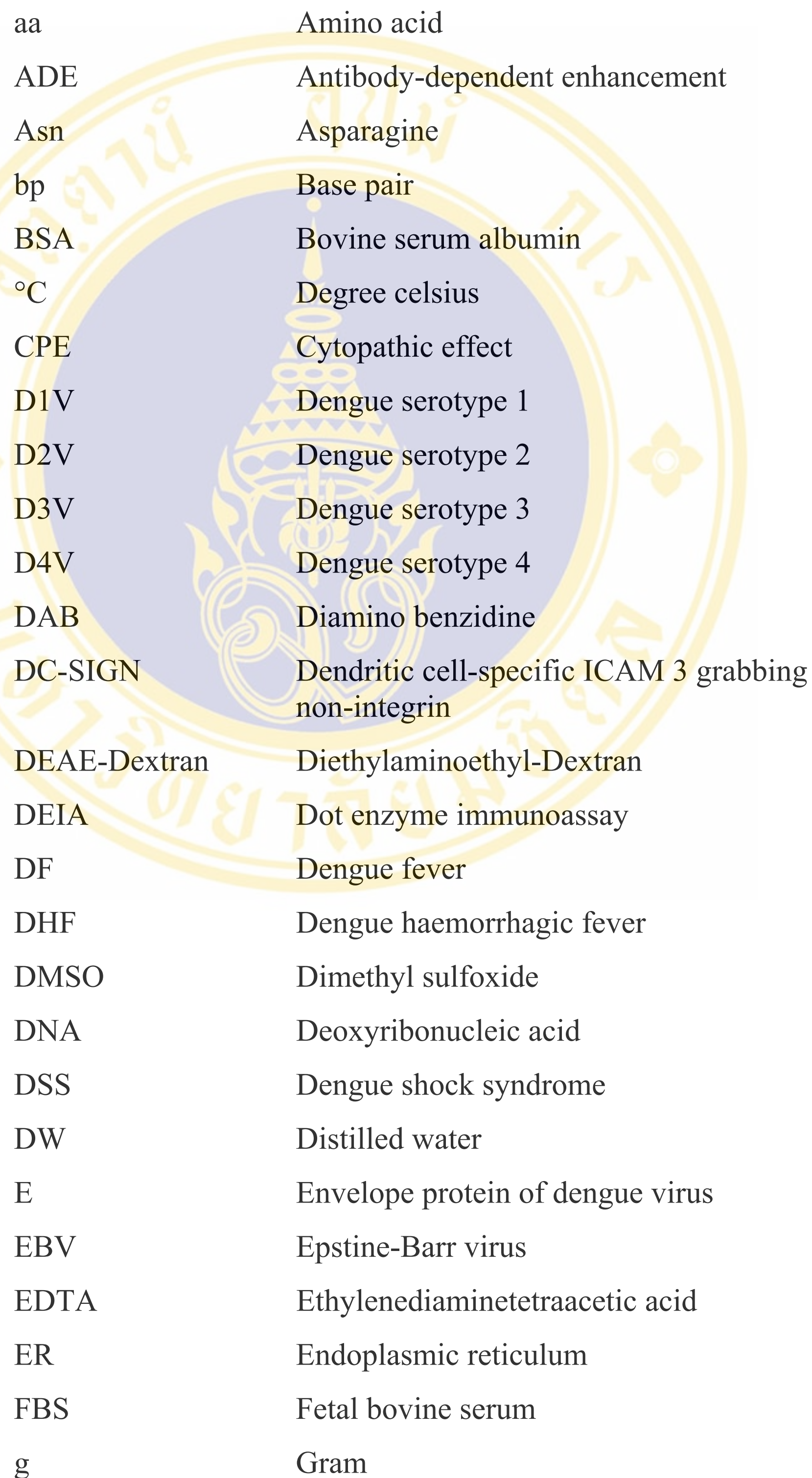

aa	Amino acid
ADE	Antibody-dependent enhancement
Asn	Asparagine
bp	Base pair
BSA	Bovine serum albumin
°C	Degree celsius
CPE	Cytopathic effect
D1V	Dengue serotype 1
D2V	Dengue serotype 2
D3V	Dengue serotype 3
D4V	Dengue serotype 4
DAB	Diamino benzidine
DC-SIGN	Dendritic cell-specific ICAM 3 grabbing non-integrin
DEAE-Dextran	Diethylaminoethyl-Dextran
DEIA	Dot enzyme immunoassay
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
DW	Distilled water
E	Envelope protein of dengue virus
EBV	Epstine-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
g	Gram

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LIST OF ABBREVIATIONS (cont.)

GRP 78	Glucose regulated protein 78
hr	Hour
hrs	Hours
HEK-293T	Human embryonic cells kidney 293T
HI	Haemagglutination inhibition
HRP	Horseradish peroxidase
HSP 70	Heat shock 70 kDa protein
IFA	Immunofluorescent assay
Ig	Immunoglobulin
JEV	Japanese encephalitis virus
kDa	Kilodalton
kb	Kilobase
L-15	Leibovitz' medium
l	Liter
M	Molar
MAb	Monoclonal antibody
MAbs	Monoclonal antibodies
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
NS	Non structural protein
nt	Nucleotide
NT	Neutralization
OD 280	Optical density at 280 nm
ORF	Open reading frame
PBS	Phosphate buffered saline
prM	Pre-membrane protein

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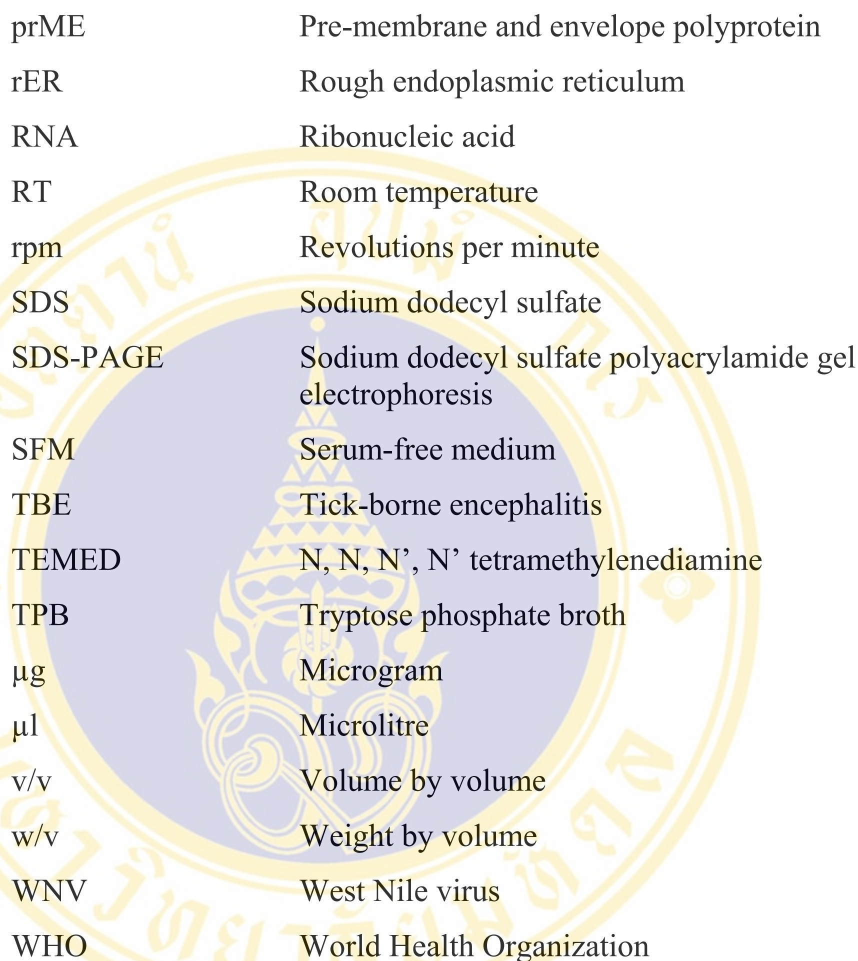

prME	Pre-membrane and envelope polyprotein
rER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RT	Room temperature
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFM	Serum-free medium
TBE	Tick-borne encephalitis
TEMED	N, N, N’, N’ tetramethylenediamine
TPB	Tryptose phosphate broth
µg	Microgram
µl	Microlitre
v/v	Volume by volume
w/v	Weight by volume
WNV	West Nile virus
WHO	World Health Organization

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CHAPTER I
INTRODUCTION

Dengue hemorrhagic fever is one of the most important arbovirus diseases of humans, and occurs in all major tropical areas of the world. Over 2.5 billion people are at risk of infection and it was the most important global public health problem in tropical countries at the beginning of the twenty-first century (1).

The four antigenically related serotypes of dengue virus, members of the Flavivirus genus (family *Flaviviridae*), are small (50 nm) enveloped particles with single-stranded, positive polarity RNA genome of approximately 11 kb. The viral genome encodes only one open-reading-frame translated into a polyprotein. After being processed with host and viral proteases, the polyprotein then produces three structural proteins and seven nonstructural proteins (2). The flavivirus envelope (E) glycoprotein, the major structural protein on the surface of the virion, encodes important phenotypic and immunogenic properties of the virion (3). This protein initiates infection through its binding to host cell surfaces, mediates virus-cell membrane fusion and elicits virus-neutralizing antibodies.

Production of human antibodies caused by virus infection is one of defensive mechanisms to neutralize virus. Several mechanisms have been proposed for the neutralization of viruses such as blockage of virus binding, fusion, and/or internalization into permissive cells. In dengue virus infection, human immune response induces the production of neutralizing antibodies to the desirable level that could eliminate viruses. In addition, administration of dengue virus antiserum protected mice from lethal dengue virus infection (4). However, the non-neutralizing antibodies have also been produced. These non-neutralizing antibodies, as well as subneutralizing concentration of neutralizing antibodies have been demonstrated to involve in enhancement of viral infection, defined to antibody-dependent enhancement (ADE) (5, 6). Mapping of neutralizing/enhancing epitopes by a panel of anti-dengue monoclonal antibodies (MAbs) are relevant to E glycoprotein (3, 7).

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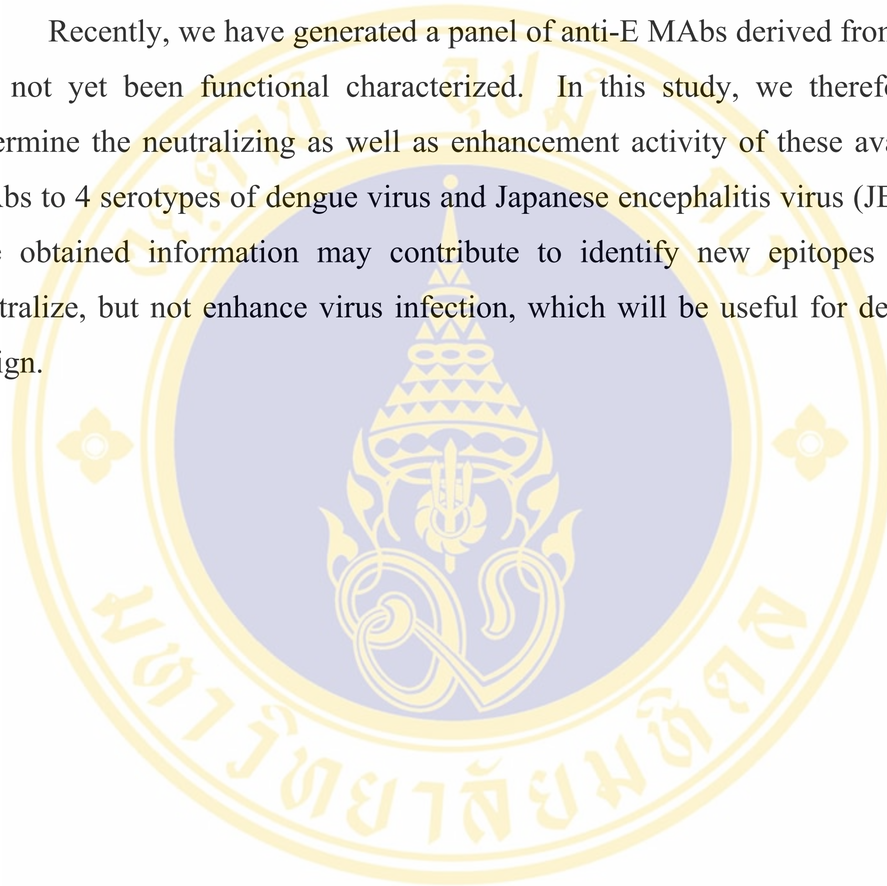
MAbs specific to dengue E protein domain III, which contains host cell receptor binding sites, have been shown to neutralize dengue-2 virus (D2V) by blocking of virus adsorption to Vero cells (8). Some of MAbs specific to E domain II, but none to E domain I, were also found to neutralize virus, probably by interference of fusion process (3, 8).

Recently, we have generated a panel of anti-E MAbs derived from D2V which has not yet been functional characterized. In this study, we therefore aimed to determine the neutralizing as well as enhancement activity of these available anti-E MAbs to 4 serotypes of dengue virus and Japanese encephalitis virus (JEV) infection. The obtained information may contribute to identify new epitopes which could neutralize, but not enhance virus infection, which will be useful for dengue vaccine design.



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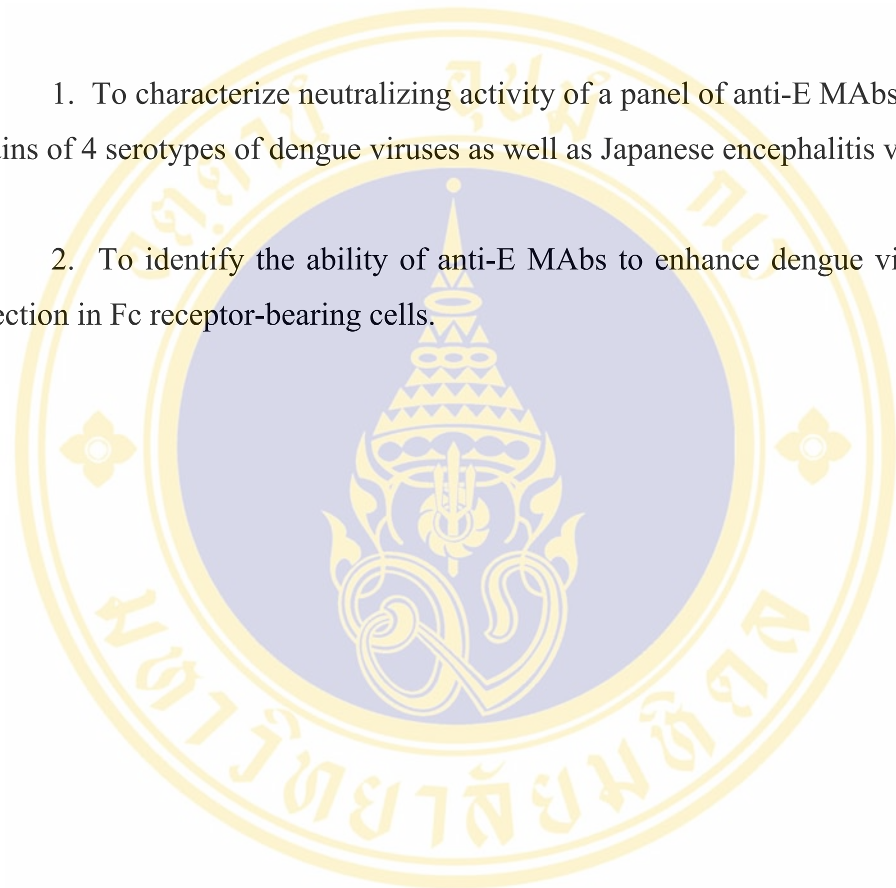
CHAPTER II
OBJECTIVE



1. To characterize neutralizing activity of a panel of anti-E MAbs in prototype strains of 4 serotypes of dengue viruses as well as Japanese encephalitis virus (JEV).

2. To identify the ability of anti-E MAbs to enhance dengue virus and JEV infection in Fc receptor-bearing cells.

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CHAPTER III
LITERATURE REVIEW

Dengue infection causes one of the most important mosquito-borne viral diseases of humans. The diseases cause a broad range of from asymptomatic infection, undifferentiated fever, mild febrile dengue fever (DF) to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (9-11). DF and DHF are increasingly important public health problems in tropical and subtropical regions in the world. Dengue virus has been recognized in over 100 countries and over 2.5 billion people are at risk of infection at the beginning of the 21st century (1). When people were infected with dengue virus, one of defensive mechanisms is to produce antibodies to neutralize virus. However, in dengue epidemiological study reported that the patients, who have been second infected with different serotype from the first infection, increased the risk of more disease severity (12).

3.1 Molecular biology of dengue virus

Dengue viruses belong to the genus Flavivirus in the family *Flaviviridae*, are arthropod-borne human pathogen transmitted by mosquitoes mainly *Aedes aegypti* and *Aedes albopictus*. There are four closely related antigenically serotypes of dengue viruses including dengue serotype 1 (D1V), dengue serotype 2 (D2V), dengue serotype 3 (D3V) and dengue serotype 4 (D4V).

Dengue virus appears as spherical particles, about 40 to 50 nm in diameter, containing the core protein encapsulating a positive single-stranded RNA. This viral RNA genome is approximately 11 kilobases in length, contains a 5’ cap at the 5’ end and lacks a polyadenylate (poly A) tail, consist of a single open reading frame (ORF). The genomic RNA is translated as a single polyprotein of over 3300 amino acids which is processed co- and post translationally by cellular proteases and virally serine protease into at least 10 discrete products, three structural proteins, the nucleocapsid or core (C) protein, premembrane (prM, the precursor of the mature

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membrane (M) protein) protein, and the envelope (E) protein and seven non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) proteins (13). The order of proteins encoded in dengue virus is 5' C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5. Based on the deduced hydrophobicity of the viral protein, their predicted membrane topology is illustrated in Figure 3.1.

Dengue virus can infect in both vertebrates and arthropods. The virus has been isolated or detected in several organs such as lymphoid tissue, liver, spleen, kidney, and brain(14). The first step of virus infection is virus entry by receptor-mediated endocytosis, presumably mediated by the virion E protein (E) and plasma membrane receptors. Additionally, in the presence of either non-neutralizing or sub-neutralizing antibodies also mediated attachment uptake of virus-antibodies complex through Fc receptor(15). After virus binds to the cell, the acidic environment of the endosome triggers irreversible conformational change of E protein from dimer to trimer. This conformational change induces the fusion of viral and cell membrane(16, 17). Membrane fusion of flavivirus is occurred as class II viral fusion protein. The proposed mechanism for flavivirus fusion was demonstrated in Figure 3.2. Briefly, viral E protein binds to cell-surface receptor and this binding leads to endosomal uptake. Reduced pH in the endosome causes Domain II of E protein to turn outward, away from the virion surface, and to insert its fusion loop into the target-cell membrane and promotes trimer formation. Formation of trimer contacts spreads from the fusion loops at the trimer tip to domain I at the base. Domain III shifts and rotates, folding the C terminus of E back towards the fusion loop. Free energy released by this refolding can drive the two membranes to bend towards each other, forming apposing membrane. Creation of additional trimer contacts between the stem-anchor and domain II leads first to hemifusion and then to formation of a lipidic fusion pore. After fusion and uncoating process have occurred, the viral genome is released into the cytoplasm of host cells. The positive-sense RNA is initially translated into a single polyprotein by cellular protein synthesis machinery. The resulting polyprotein is processed co- and transitionally by cellular proteases and viral encoded serine protease into at least 10 discrete products. The assembly of virion begins with positive-stranded RNA and C protein forming the nucleocapsid in the lumen of the endoplasmic reticulum (ER). The nucleocapsid eventually becomes

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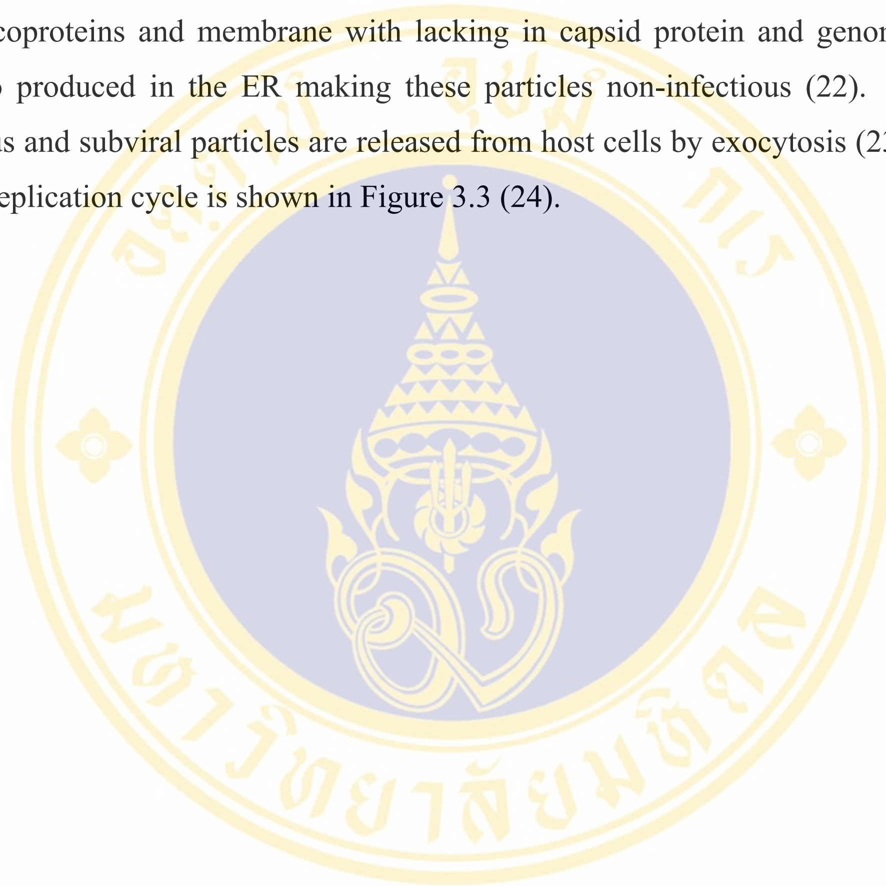
enveloped by budding through rough endoplasmic reticulum (rER). These immature particles, which contain E and prM proteins, lipid membrane and nucleocapsid, can not induce host cell fusion, making them non-infectious (18, 19). Once cleavage of pr-M occurs in the trans-Golgi network by furin-like protease, mature infectious virions are released (20, 21). However, subviral particles, which contain only the glycoproteins and membrane with lacking in capsid protein and genomic RNA are also produced in the ER making these particles non-infectious (22). Both mature virus and subviral particles are released from host cells by exocytosis (23). Schematic of replication cycle is shown in Figure 3.3 (24).



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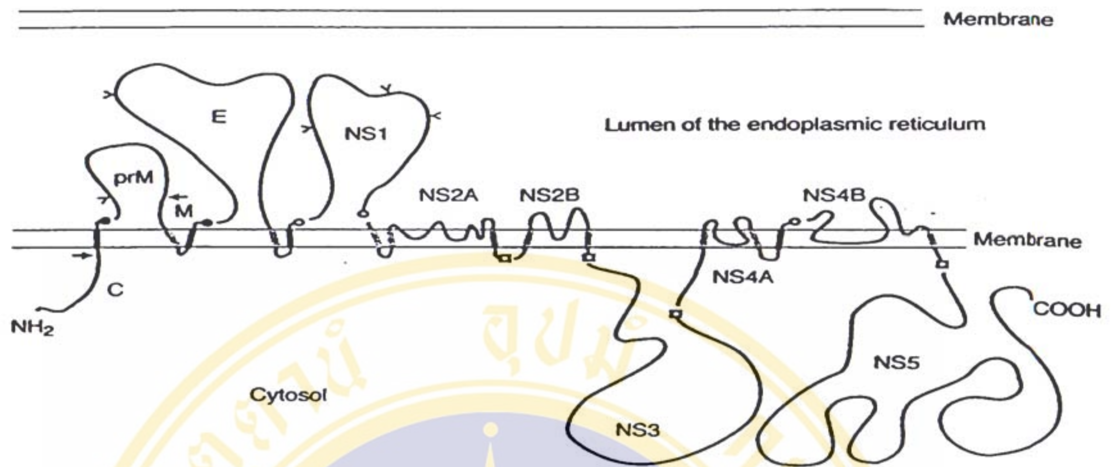

Figure 3.1 Membrane topology of flavivirus polyproteins (25).

The proposed orientation of the flavivirus polyprotein cleavage products with respect to ER membrane is shown. The proteins are draw to scale and arranged in order (left to right) of their appearance in the polyprotein.

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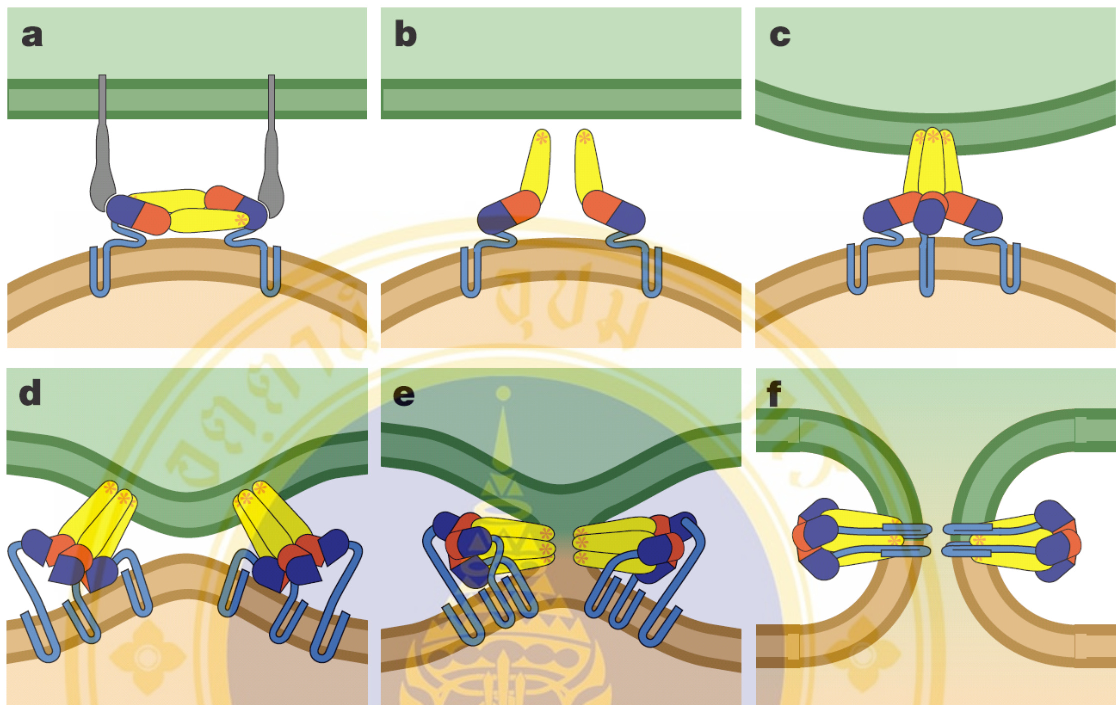

Figure 3.2 Proposed mechanism for fusion mediated by class II viral fusion proteins (17).

a) E binds to a receptor on the cell surface and the virion is internalized to an endosome.

b) Reduced pH in the endosome causes domain II to hinge outward from the virion surface, exposing the fusion loop, and allowing E monomers to rearrange laterally in the plane of the viral membrane.

c) The fusion loop inserts into the hydrocarbon layer of the host-cell membrane, promoting trimer formation.

d) Formation of trimer contacts spreads from the fusion loop at the tip of the trimer, to the base of the trimer. Domain III shifts and rotates to create trimer contacts, causing the C-terminal portion of E to fold back towards the fusion loop. Energy release by this refolding bends the apposed membranes.

e) Creation of additional trimer contacts between the stem-anchor and domain II leads first to hemifusion.

f) Formation of a lipidic fusion pore.

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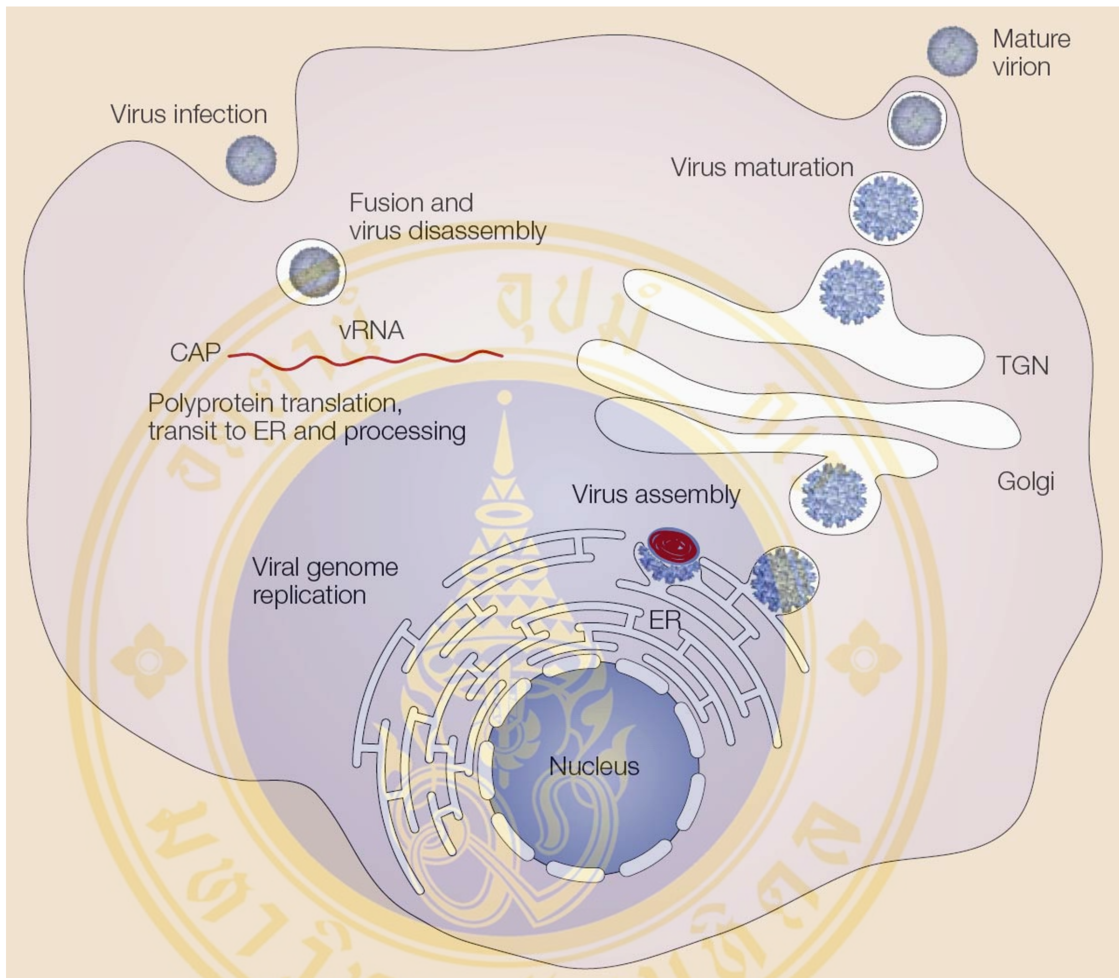

Figure 3.3 Schematic diagram of flavivirus life cycle (24).

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3.2 Clinical manifestation of dengue virus infection

Dengue virus can cause a spectrum of disease ranging from asymptomatic or mild undifferentiated fever, classical dengue fever (DF) to severe and fatal disease, dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (26). After a person is bitten by infective mosquito, the virus undergoes an incubation period which can vary from 3 to 14 days (average 4 to 7 days). The majority of infections, especially in children under age 15 years, are asymptomatic or undifferentiated fever which is clinically indistinguishable from other viral infection (27). Older children and adults may develop a mild febrile syndrome or classical dengue fever. There is a risk of progressive development into dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (28).

3.2.1 Dengue fever (DF)

Dengue fever (DF) is an acute biphasic febrile illness which is most common in older children and adults. The incubation period after the infective mosquito bite is 3 to 8 days. It is characterized by sudden onset of fever and a variety of nonspecific signs and symptoms such as headache, backache, body aches, nausea and vomiting, joint pains, weakness and rash. The patients may have anorexia, have taste aberration and have a mild sore throat. The body temperature may rise to between 39 to 40 °C and fever may be biphasic and tend to last for 2 to 7 days (26, 28-30). 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 gingival bleeding, gastrointestinal bleeding, epitaxis and menorrhagia. Hematuria occurs infrequently and jaundice is rare (29). In most cases, DF is generally self-limiting and is rarely fatal. The patient usually recovers from the symptoms without complications about 10 days after the onset of disease. The acute phase of illness lasts for 3 to 7 days, but the convalescent phase may prolong for weeks and probably associats with weakness and depression, especially in adults (27).

3.2.2 Dengue hemorrhagic fever (DHF)

Dengue hemorrhagic fever (DHF) is primarily a disease of children under the age of 15 years, although it may also occur in adults (31). DHF is an acute

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vascular permeability syndrome accompanied by abnormality in hemostasis, which is characterized by high fever, hemorrhagic phenomena and features of circulatory failure (32). Plasma leakage is the major patho-physiological feature observed in DHF and differentiates it from typical dengue fever (14). The World Health Organization (WHO) case definition of DHF is a patient with the following four criteria including acute sudden onset of high fever for 2 to 7 days, hemorrhagic manifestation with at least a positive tourniquet test, platelet count <100,000 / mm^3 and hemoconcentration (rising packed cell volume > 20%) or other evidence of plasma leakage such as ascites, pleural effusion or low level of serum protein/albumin. Furthermore, according to severity, DHF is categorized into four grades from less severe (grade I) to most severe (grade IV). Grade I and grade II are non-shock DHF. In grade I, only hemorrhagic manifestation is positive tourniquet test while grade II there are similar in grade I and has spontaneous bleeding. Grade III and grade IV are case of DHF with shock (Dengue shock syndrome, DSS). In grade II there are sign 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 and/or pulse (33).

The incubation period for DHF and DSS is similar to that of classical DF and also initially present with symptoms, including a high temperature (>39 °C), arthralgia, myalgia, headache and vomiting (34). The critical stage of DHF/DSS is reached after 3 to 7 days of fever. Approximately 24 h before and 24 h after fever defervescence, sign of circulatory failure of vary severity may appear. Several symptoms and signs occur before defervescence and may serve as warning signs that DHF and DSS are coming: generalized abdominal pain, persistent vomiting, change in the level of consciousness, a sudden drop in the platelet count and a rapid rise in the haematocrit. By this time, most patients also have evidence of pleural effusion on the chest radiograph (35). If plasma loss continues and become excessive, the patients can progress into profound shock. The outcome of DHF and DSS depends on early diagnosis and the immediate replacement of fluid.

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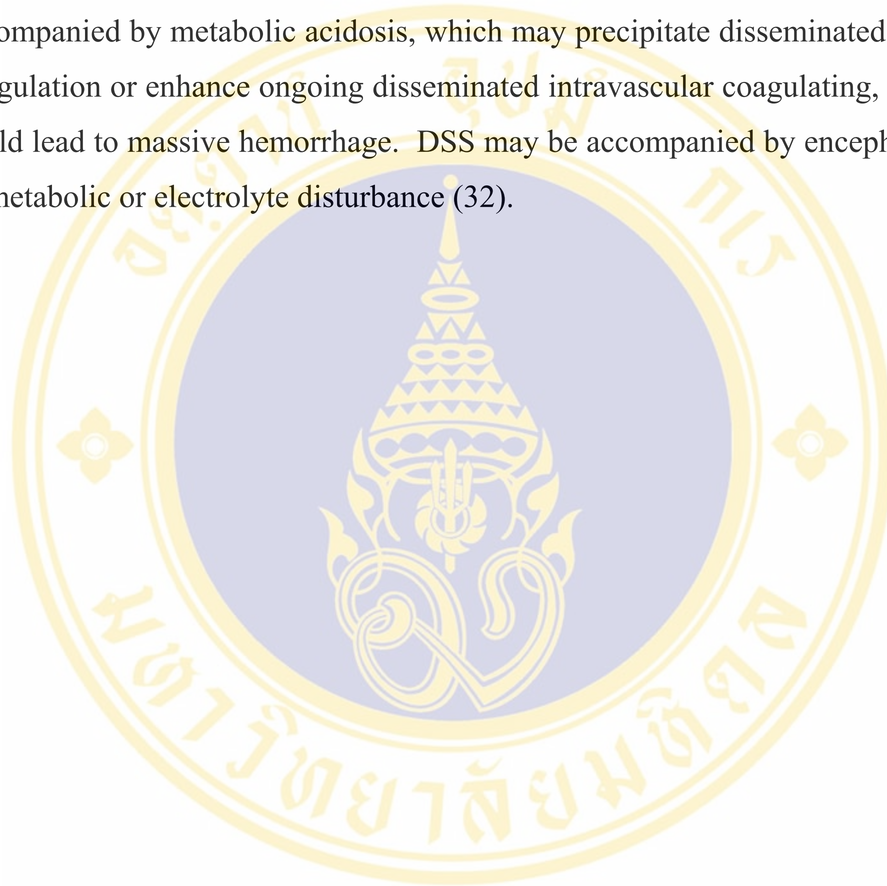
3.2.3 Dengue shock syndrome (DSS)

Dengue shock syndrome (DSS) is associated with very high mortality (around 9.3% increasing to 47% instance of profound shock). Acute abdominal pain and persisting 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 coagulating, which in turn could lead to massive hemorrhage. DSS may be accompanied by encephalopathy due to metabolic or electrolyte disturbance (32).



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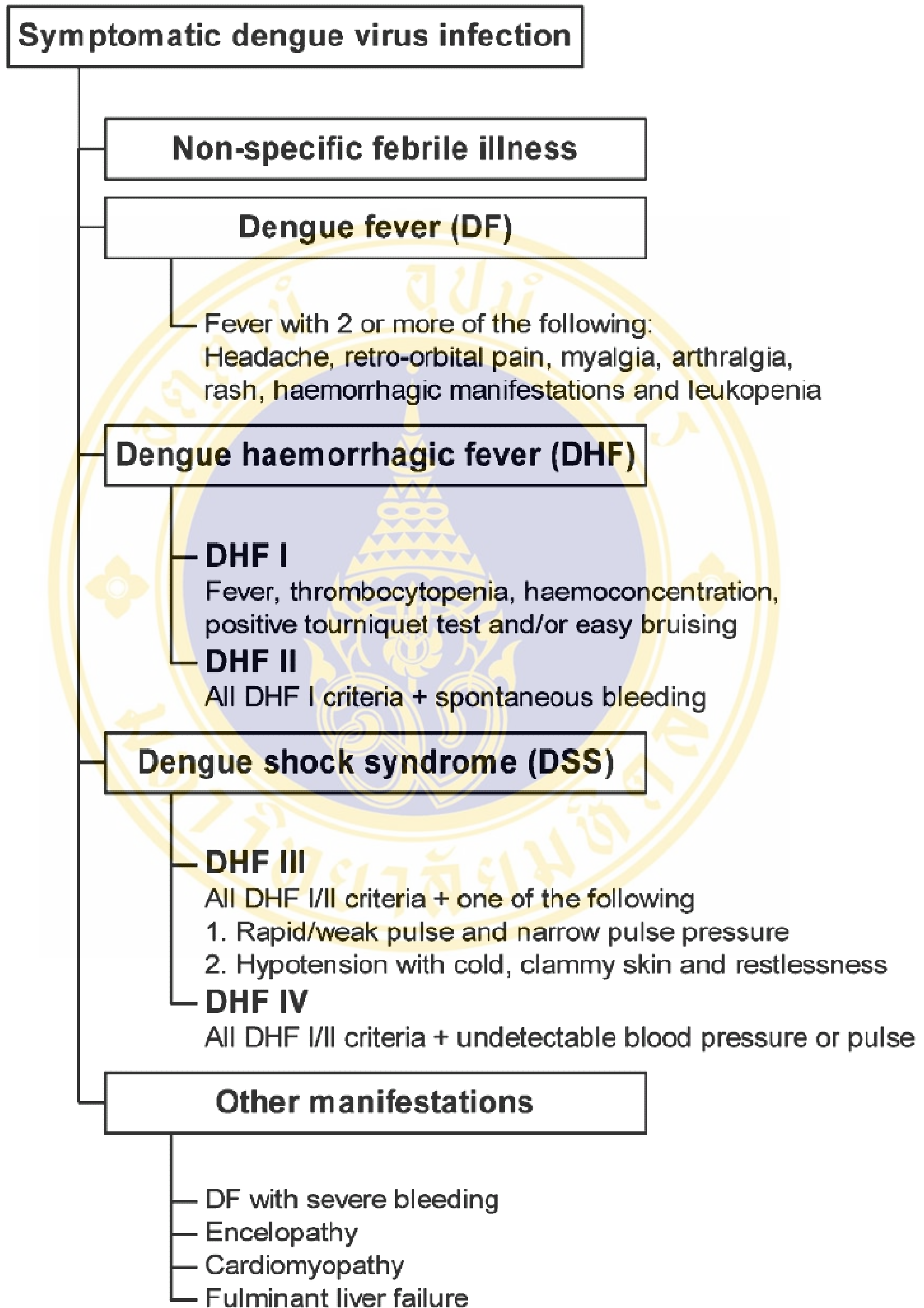

Figure 3.4 Classification of symptomatic dengue virus infection (32).

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3.3 Structure and function of dengue virus E protein

The E glycoprotein is the major structural protein on the surface of the mature dengue virions. The molecular weight of E protein is about 55 kilodalton (kDa), consists of approximately 495 amino acids. This protein is composed of 80% N-terminal ectodomain (which is exposed on the virion surface) and 20% C-terminal transmembrane hydrophobic domain (which anchors the molecule on the lipid bilyer surrounding the virion). The possible glycosylation sits of flavivirus E protein locate at Asparagine (Asn) – 67 (on domain II) and Asn – 153 (on domain I). However, the number of glycosylation sites are different between serotypes of dengue viruses and other flaviviruses (36).

The high resolution crystal structures of flavivirus E protein have been first elucidated in 1995, describing the ectodomain of the tick-borne encephalitis (TBE) virus E protein (37). It has been revealed that the E protein is an elongated head-to-tail homodimer lies parallel to the surface of the virion, anchored in the membrane at its distal ends. Each monomer consists of three structural distinct domains (I, II and III), which correspond to previously defined antigenic domains (C, A and B) (38). In 2003 and 2005, the crystal structures of DEN-2 and DEN-3 E proteins, respectively have also been reported (39, 40). They closely resemble the E protein from TBE in its dimeric structure, 3 distinct antigenic domains and in the details of protein fold (Fig 3.5).

Domain I, the central domain, contains 8-stranded β-barrel organized structure. It is predominately type-specific non-neutralizing epitopes and is theorized to be the molecular hinge region involved in low-pH-triggered conformational changes (39). This domain includes about 120 residues in three segments (residues 1-52, 133-193, and 281-296). The two loops between the segments form dimerization by domain II.

Domain II, the dimerization domain, makes important contacts with itself in the homodimer. It contains 12 β-strands and is involved in virus-mediated membrane fusion. This domain contains many cross-reactive epitopes eliciting neutralizing and non-neutralizing monoclonal antibodies (17, 37). Domain II consists of two separated segments of residues 53-132 and 194-280. The elongated part of the domain is a narrow sandwich of three stranded β-sheets and a β-hairpin. The loop at the tip of the

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domain, correspond to residues 98-110, is almost fully conserved in all flaviviruses. This residue portion is hydrophobic and glycine-rich and has been proposed to be important for the internal fusion activity (41, 42).

Domain III is characterized by an immunoglobulin-like structure, with 10 β-strands, containing the most distal projecting loops from the virion surface. It contains multiple type- and subtype-specific epitopes eliciting virus-neutralizing MAbs and involves in binding to the host cell receptors (3, 8, 43). The domain III consists of residues 297-394. The axis of the immunoglobulin β-barrel would be perpendicular to the surface of the virus, and its tip would project farther than any part of the E dimer. Differently from the TBE envelope structure, there are additional four residues (aa 382-385) in domain III of dengue virus which has been reported to be host cell receptor binding site (8). Mutations in the domain III region of flavivirus E protein (DIII) are associated with attenuated virulence or the ability of virus to escape immune neutralization, suggesting that domain III plays a role in receptor recognition (44-48).

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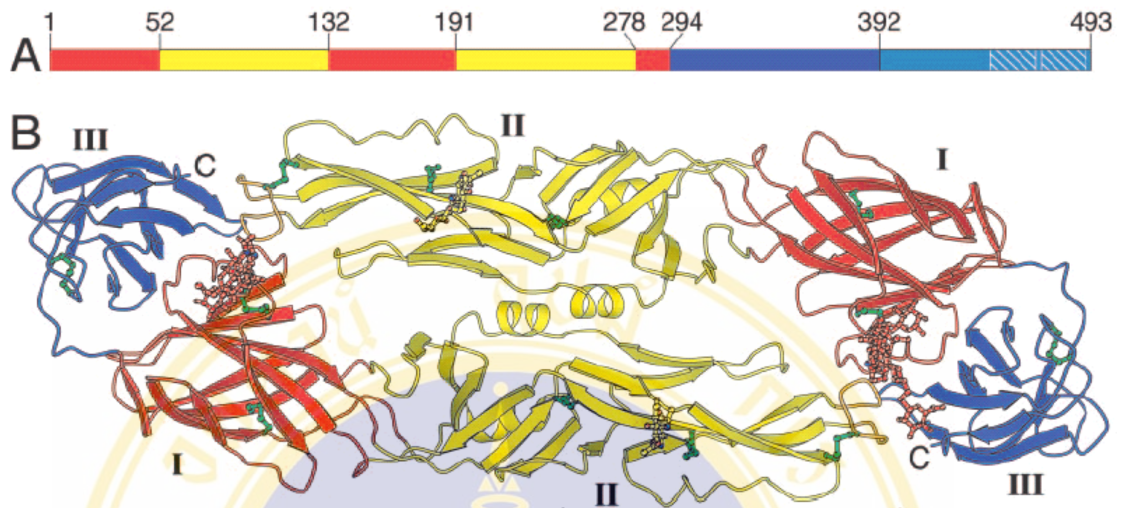

Figure 3.5 Structure of the homodimer of dengue virus envelope protein in the mature virus particle (40). (A) The three domains of dengue virus sE. Domain I is red, domain II is yellow, and domain III is blue. (B) The dengue E dimer viewed along its twofold symmetry axis.

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3.4 Host Cell receptors of dengue virus

Numerous studies have attempted to identify the putative cell surface receptors (heparan sulfate, dendritic cell-specific ICAM 3 grabbing non-integrin (DC-SIGN), glucose regulated protein 78 (GRP 78 or (BiP)) and laminin receptor) utilized by the dengue viruses to gain entry into the different susceptible cells. The attachment of dengue and other flaviviruses *in vitro* to plasma membranes of mammalian and mosquito cell lines have been reported to be dependent on the presence of glycosaminoglycans, particularly heparan sulfate (49, 50) Pre-incubation of heparin or treatment with heparinase caused the inhibition of virus binding to target cells. But in some cell types such as myelomonocytic cell line (HL60) and the non-EBV transformed B-cell lines (BM), heparinase treatment increased the viral attachment (51). However, binding to glycosaminoglycans is non-specific but causes viruses come closer to the specific receptor, possibly multicomponent receptor or receptor complex (52). DC-SIGN is a cell-surface mannose-specific C-type (calcium-dependent) lectin. DC-SIGN was demonstrated to bind to dengue viruses and essential for dengue virus infection (53, 54). Anti DC-SIGN and soluble tetrameric ectodomain of DC-SIGN were demonstrated to inhibit dengue infection. In addition, GRP 78 (BiP) was demonstrated to interact with D2V. A 37/67 kDa high-affinity laminin receptor was proposed as a specific candidate receptor for D1V into liver cells (HepG2 cell) (55). However, Tio (56) demonstrated that the D1V, D2V and D3V interacted with 37/67 kDa high-affinity laminin receptor on PS clone D cell line and a 37/67 kDa high-affinity laminin receptor on C6/36 cell line was bound with D3V and D4V (57). Moreover, the putative receptors for dengue virus were identified by many investigators, however, there are different in result based on cell type or species origin as well as serotype of virus (58-65).

3.5 Neutralization of virus infection

It has been known that production of antibody caused by virus infection is one of defensive mechanisms to neutralize virus. One of the proposed mechanisms for the neutralization of virus infection is the blocking by antibodies to virus attachment and entry into host cells via primary receptor and/or coreceptors prior to endocytosis (12, 66). It has been known that dengue E protein which is responsible for the virus

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attachment, internalization and fusion into host cell, contains major immunodominants that elicit neutralizing antibodies (7, 67). Before the elucidation of high resolution structure was come out, the antigenic structure of the dengue E protein was investigated by using a set of MAbs (3, 44, 68-73). MAbs have not only been used to dissect the antigenic relatedness between dengue but also in studies aimed at defining epitopes on viral protein involved in a range of biological activities (such as neutralization, HI or fusion activity) (3, 7, 74-77). Most of mouse anti-E MAbs which contain neutralizing activity are specific to domain II and III of E protein (3, 8, 68, 71-73, 76-78) as summarized in table 3.1. Domain III is characterized by an immunoglobulin-like structure which is responsible for binding of virus to the host cells (79). It has been demonstrated that those of MAbs specific to domain III mediated neutralization by blocking dengue virus adsorption to Vero cells. Whereas, the domain II-specific neutralizing MAbs were the less blockers to adsorption but they have ability to interfere fusion process (8). One study in the West Nile virus (WNV) demonstrated that one MAb, recognized the distal lateral surface of domain III, have strongly neutralizing activity. These results also showed that such monoclonal antibody inhibits infection primarily at a step after viral attachment, potentially by blocking E glycoprotein conformational changes (80).

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Table 3.1 Summary of reported neutralizing epitopes that were determined by using anti-E MAbs.

MAbs	Isotype	Serotype specific	Neutralizing activity	Epitope specificity		References
				Domain	Amino acid	
4G2	IgG2a	Flavivirus	+	III (B) I (C) II (A)	298 - 397 169 275	(71, 77)
3H5	IgG1	D2V	+	III (B) III (B) III (B)	298 - 397 386 - 397 383-385	(44, 71, 72)
1B7	IgG2a	Flavivirus	+	I (C) & II (A) I (C) & II (A) III (B) II (A)	50 - 57 127 - 134 349 - 356	(3, 68)
G8D11		D2V	+	III (B)	307	(73)
9A3D-8 1A1D-2 10A4D-2 4E5 2H3 6B6C-1	IgG2A IgG2A IgG2A IgG2A IgG2A IgG2A	D2V D1V-D3V D1V-D4V D1V-D3V D2V Flavivirus	+ + + + + +	III (B) III (B) III (B) II (A) II (A) II (A)		(3)
D1-M10 D1-M17	IgM IgM	D1V, D3V D1V, D2V	+ +	I (C) I (C)	279 293	(76)
1H9	IgM	D3V	+	III (B)	386	(77)
1A5	Fab	Flavivirus	+	II (A) III (B)	106 317	(78)

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3.6 Antibody-dependent enhancement (ADE)

Although antibodies represent a major contribution of the host's immune response directed at eliminating virus infection, however, they may also enhance the susceptibility of cells to viruses. This phenomenon has generally been termed antibody-dependent enhancement (ADE) of virus infection. In dengue epidemiological studies, ADE has been suggested as one of important factors causes disease severity (DHF/DSS) especially in the patients who have been experienced in sequential heterologous dengue infection (i.e., infection with one of the four dengue serotypes followed by subsequent infection by a different serotype) (12). The good example demonstrating the severity of the diseases by sequential infection was the dengue outbreak in Cuba in 1981. D1V epidemic in 1977-1978 did not develop DHF, but introduction of D2V (Asian type) in 1981 resulted in a sharp DHF/DSS epidemic (81, 82). It was proposed that low-titer (subneutralizing or enhancing) antibody remaining from a prior dengue virus infection, which could not neutralize but enhance infection of subsequent heterologous dengue virus serotype, was responsible for the development of severe disease (83).

Many *in vitro* studies have shown that ADE of flavivirus replication in the presence of non-neutralizing antibody or sub-neutralizing antibody concentration can occur in peripheral blood cells of the monocyte-macrophage lineage (5, 6) as well as in murine macrophage cell-line (P388 D1) and human monocyte cell lines (U-937) (84, 85). The enhancement of virus production in these cells is due to infection of virus-antibody complexes via Fc receptor on the cell surface. Peiris and his colleagues demonstrated that MAbs against Fc receptors block infection of mouse macrophage cell lines with virus-antibody complexes (86). However, the other mechanism of ADE has been proposed. Huang and his team demonstrated that anti-prM have dual specificities, one binding to dengue virion with another to HSP60 protein. These antibodies could enhance dengue virus infection on non-Fc bearing cells (87). In addition, many studies proposed that the enhancement activity of several viral strains were differed, although the single MAb were used (88-90).

Several attempts have been performed to identify the viral epitopes that mediate enhancement by the use of MAbs in ADE assays. One study in West Nile virus demonstrated that the same antibody which at high concentration neutralizes the

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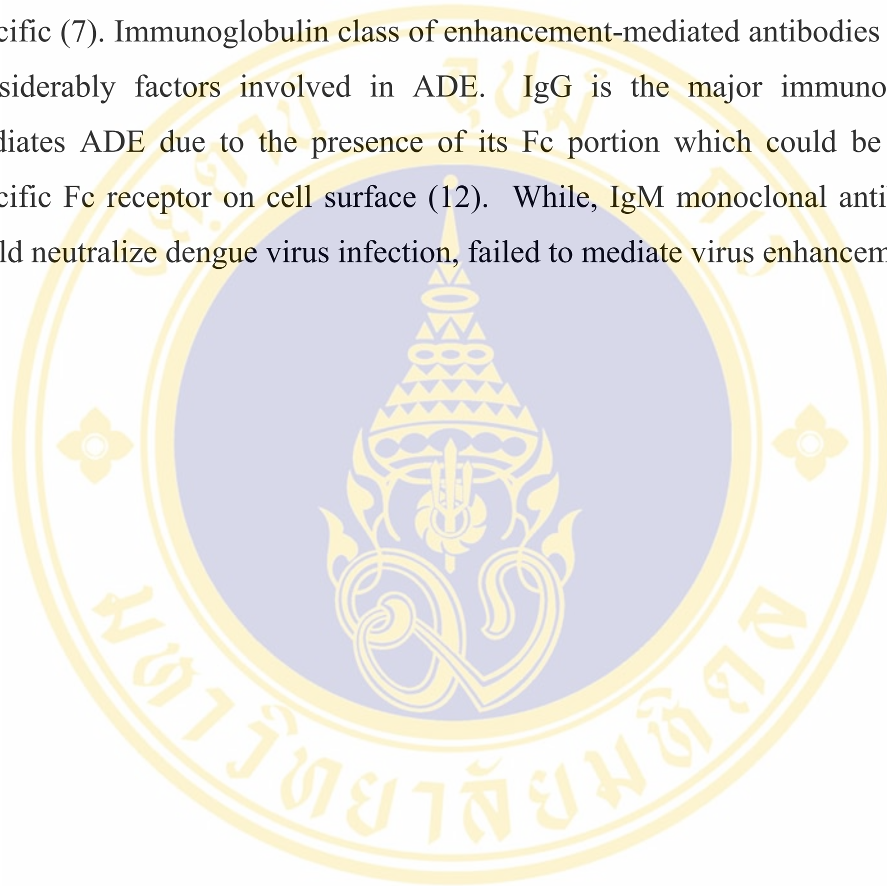
virus may cause ADE at sub-neutralizing concentration. It is implied that one epitope can be involved in both neutralization and enhancement of infectivity (91). In addition, broadly cross-reactive as well as type-specific MAbs could mediate ADE of Flavivirus infection (88, 92). Most of dengue specific MAbs that could enhance dengue virus infection in Fc-receptor bearing cells were identified as E glycoprotein specific (7). Immunoglobulin class of enhancement-mediated antibodies is also one of considerably factors involved in ADE. IgG is the major immunoglobulin that mediates ADE due to the presence of its Fc portion which could be interacted to specific Fc receptor on cell surface (12). While, IgM monoclonal antibodies which could neutralize dengue virus infection, failed to mediate virus enhancement (93).



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CHAPTER IV
MATERIALS AND METHODS

MATERIALS:

4.1 Antibodies

4.1.1 Anti-flavivirus MAbs

Twelve hybridoma clones producing anti–E MAbs used in this study are listed in Table 4.1. Five reference clones (4G2, 3H5, 8A1, 1H10 and J93) have been supplied from AFRIMS. Seven clones (2B7, 1F4, 1D10, 2C8, 1C2, 1D3, 5A1) were produced in our lab previously.

Table 4.1 List of anti–flavivirus MAbs.

No.	MAbs	Abbreviation name	Isotype	Serotype specificity	References
1	4G2	4G2	IgG2a	D1-4, JE	(7, 74, 75)
2	3H5	3H5	IgG1	D2	
3	8A1	8A1	IgG1	D3	(12)
4	1H10	1H10	IgG1	D4	(75)
5	J93	J93	IgG2a	JE	
6	1PF13F (2B7)	2B7	IgG1	D1-4, JE	(94)
7	1PF20F (1F4)	1F4	IgG1	D2	
8	1PF27F (1D10)	1D10	IgG1	D1-4, JE	
9	1Cap1B (2C8)	2C8	IgG2a	D2	
10	BF2/1C2-1	1C2	IgG1	D1-3	(95)
11	BF2/1D3-2	1D3	IgG2a	D1-4	
12	BF2/5A1-1	5A1	IgG2a	D2-3	

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4.1.2 Rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase (HRP) P260, Dakopatts, Denmark.

4.1.3 Goat anti-mouse immunoglobulin G conjugated with Cy3, Dakopatts, Denmark.

4.1.4 Goat anti-mouse immunoglobulin M conjugated with Cy3, Dakopatts, Denmark.

4.1.5 Mouse immunoglobulin isotype IgG2a, Sigma, USA

4.2 Viruses

Prototype strains of each serotype of dengue viruses (D1V Hawaii strain, D2V NGC strain, D3V H87 strain and D4V H241 strain) and Japanese encephalitis virus (JEV Nakayama strain) were used for the whole study.

4.3 Cell culture

4.3.1 C6/36 cell lines from *Aedes albopictus* (ATCC CRL-1660) were cultured at 28°C in L-15 medium (GIBCO BRL) containing 10% tryptose phosphate broth (TPB) (SIGMA), 10% fetal bovine serum (FBS)(GIBCO BRL), 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium).

4.3.2 Ps clone D cells, a swine fibroblast cell line, were cultured at 37°C in the same complete medium as C6/36 cells.

4.3.3 U937 cells, a human monocyte cell line, were cultured in RPMI 1640 (GIBCO BRL) supplement with 10% FBS (GIBCO BRL), 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium) at 37°C in humidified air containing 5% CO_2.

4.3.4 HEK 293T cells, human embryonal kidney cell were cultured in the same complete medium and condition as U937 cell line.

4.3.5 Hybridoma cells were cultured in the same complete medium and condition as U937 cell line.

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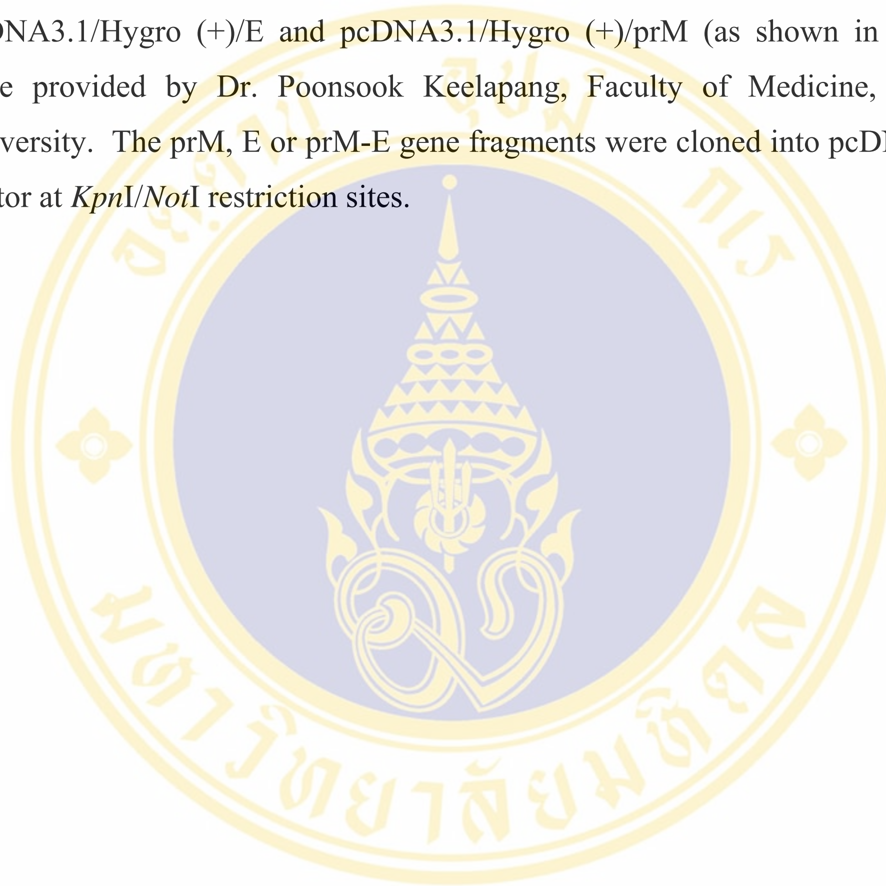
4.4 Vector

4.4.1 pcDNA3.1/Hygro™ (+) vector (Invitrogen, U.S.): the map of pcDNA3.1/Hygro vector is shown in Figure 4.1a.

4.4.2 A set of construct pcDNA3.1/Hygro plasmids containing dengue prM and E gene. The construct plasmids, including pcDNA3.1/Hygro (+)/prM-E, pcDNA3.1/Hygro (+)/E and pcDNA3.1/Hygro (+)/prM (as shown in Figure 4.1b) were provided by Dr. Poonsook Keelapang, Faculty of Medicine, Chiang Mai University. The prM, E or prM-E gene fragments were cloned into pcDNA3.1/Hygro vector at *Kpn*I/*Not*I restriction sites.



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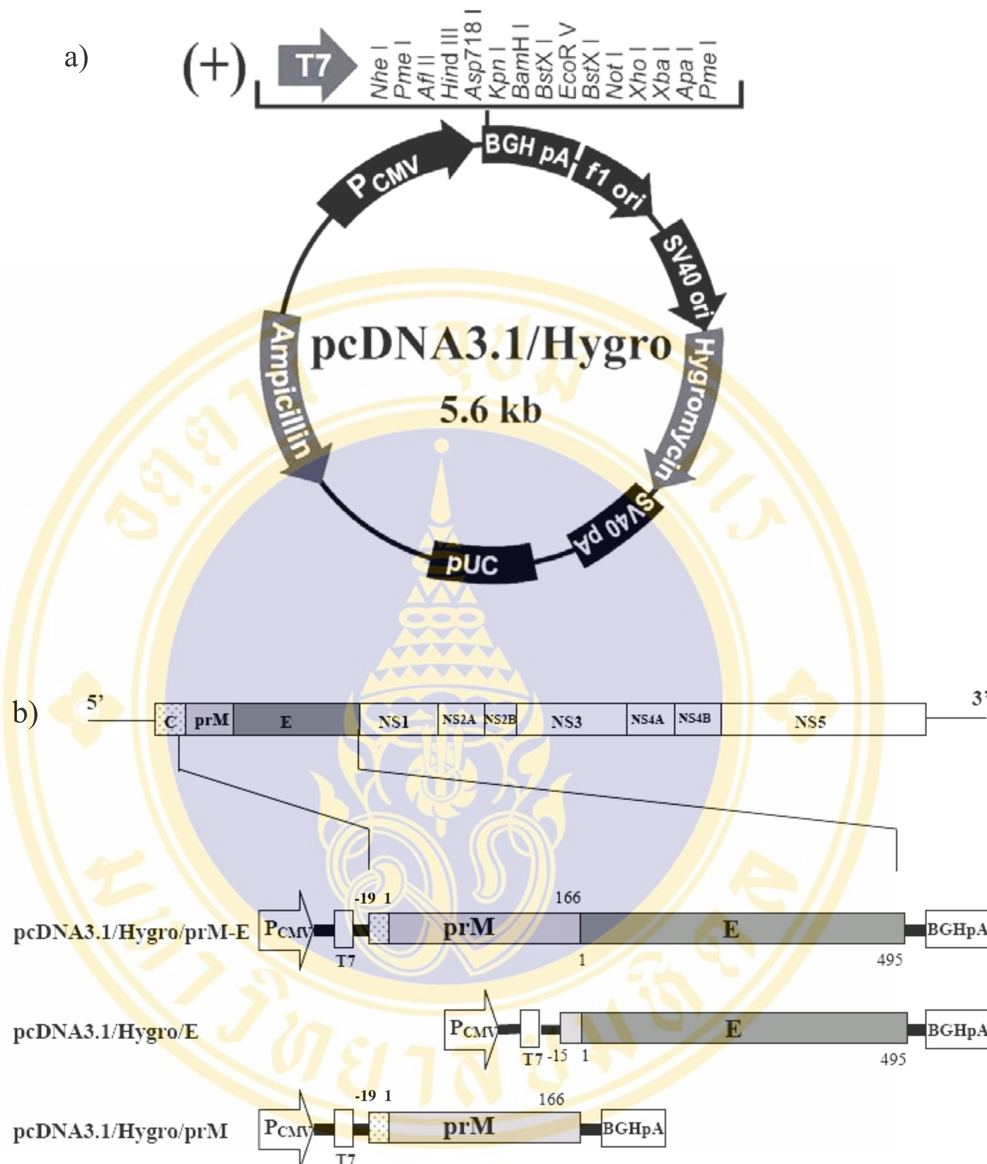

Figure 4.1 Schematic diagrams demonstrating the construction of plasmid cDNA encoding 3 patterns of dengue 2 viral prM and E (strain 16681).

a) The map of pcDNA3.1/Hygro (+) vector.

b) Maps of plasmid constructs. Amino acid positions represented for full-length of prM (1-166) and E proteins (1-495) are indicated. Upstream of prM coding sequence in pcDNA3.1/Hygro(+)/prM and pcDNA3.1/Hygro(+)/prM-E contain 19-aa residues of dengue C protein as native signal sequence (-19). The 15-aa prM residue upstream of E protein in pcDNA3.1/Hygro(+)/E is representing as an E signal sequence (-15). The full-length genome of dengue virus is shown at the top.

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METHODS:

4.5 Preparation of monoclonal antibodies

4.5.1 Large – scale production of MAbs

The production of anti-flavivirus MAbs were enlarged by 2 techniques, *in vivo* mouse ascitic fluid and *in vitro* hybridoma cell culture in serum-free media (SFM). All hybridoma cells producing anti- flavivirus MAbs were subjected to the first technique. The clones of hybridoma that fail to produce ascitic fluid were adapted to the other one.

For mouse ascitic fluid production, the hybridoma cells (5-10 x10^6 cells / dose) that produce anti-flavivirus MAbs were intraperitoneally injected into BALB/c mice which have been 7-10 days pre-treated with pristane. Ascitic fluids were harvested at 1-2 weeks after inoculation and clarified by centrifugation at 1,000 rpm for 10 min. The clarified ascites were aliquoted into 1 ml and stored at -20 °C.

For *in vitro* cell culture technique, hybridoma cells were adapted into serum-free media (Hybridoma SFM, Gibco BRL). Initially, hybridoma cells, cultured in RPMI 1640 supplemented with 10% FBS (10% FBS-RPMI), were subcultured into 10%FBS-RPMI containing 20% SFM and allowed the cells to grow well in this media composition. The adapted hybridoma cells were further subcultured in the same manner into a modified media in which the percentage of SFM is stepwise increased to 50%, 80% and 100%. Finally, the cells were adapted in solely SFM culture medium and grown in a large volume as desired, ready to be purified.

4.5.2 Purification of MAbs by Protein-G affinity chromatography

Mouse immunoglobulin G (IgG) in mouse ascetic fluids or in SFM culture supernatants were purified by Protein G affinity column chromatography. Briefly, HiTrap Protein-G HP column (Amersham Bioscience, Sweden) was initially equilibrated with a binding buffer (100mM sodium phosphate buffer, pH 7.2). Then, 10-times diluted ascitic fluid or SFM cultured supernatant were applied to the pre-equilibrated column, allowing the binding of IgG to the protein-G matrix. After washing with the binding buffer, the binding antibody were eluted from the column

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with the elution buffer (0.1M Glycine-HCl, pH 2.7) and immediately neutralized with 1M Tris-HCl pH 8.2. The purified MAbs were determined for the protein concentration at OD280 using spectrophotometer. The fractions containing purified antibody were pooled and dialyzed against PBS. The concentrations in mg/ml of IgG antibody were calculated by OD280/1.35. The purified MAb were stored at -20°C at 1 mg/ml concentration.

4.5.3 Verification of purified MAbs

To ensure purity, reactivity and specificity, each purified MAbs were confirmed those properties prior to be used in any experiments. The characterizations of those MAbs were performed as followed.

4.5.3.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Purity of the purified MAbs was determined by electro-separation of those MAbs in 12%SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Under reduced and heat conditions, the IgG antibody were separated into 2 protein bands of heavy chain (50 kDa) and light chain (25 kDa) as observed by Coomassie blue R250 staining.

4.5.3.2 Dot enzyme immunoassay (DEIA)

Specificity of purified MAbs to dengue serotypes (type-specific) or flavivirus group (group-specific) was determined by dot enzyme immunoassay. The C6/36 cell culture supernatants infected by each of dengue virus serotype 1 to 4 or Japanese encephalitis virus were separately dotted onto nitrocellulose membrane. Non-infected cell supernatant or (mock) were also added to the experiment as a negative control antigen. The membrane was blocked in 5% skim milk/PBS for 1 hour and 3-times washed by PBS. The dotted membranes were reacted to purified MAbs for 1 hour at 37 °C. After washing, the membrane was further incubated with the 1:1000 diluted rabbit anti-mouse Igs-HRP for 1 hour at room temperature. Antigen-antibody complex was detected by addition of chromogenic substrate solution (DAB-H_2O_2-$NiCl_2$) containing 0.06 mg/ml of diaminobenzidine (DAB) in PBS, 2 µl/ml of H2O2 and 5 µl of 8% NiCl2 for approximately 5 min in dark. Positive reactivity was visualized as a dark-brown dot on the membrane.

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4.5.3.3 Western blot analysis

Determination of immuno-reactivity of purified MAbs to dengue proteins was performed by western blot analysis. The dengue infected cell lysates were separated by SDS-PAGE in slab gel apparatus containing 3.5% stacking gel and 12% separating gel either reducing or non-reducing condition. Electrophoresis was performed in vertical direction in reservoir buffer with constant voltage of 150 volts from cathode to anode until the blue dye marker reached the bottom of the gel. The separated proteins were transferred to nitrocellulose membrane (PROTRAN®, Germany) in a Towbin buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, and 0.1% SDS) for 2 hours using a SemiPhor semi-dry transphor unit (Amersham Bioscience, Sweden). After transferring the protein, the blotted membrane was blocked with 5% skimmilk for 1 hour at room temperature, followed by 3-time washing with PBS for 5 min each. The blotted membranes were then reacted to purified MAbs at room temperature or 37 °C for 2-14 hours. After washing, the 1:1000 diluted rabbit anti-mouse Igs-HRP in 5% skimmilk/PBS, as a secondary antibody, was further incubated with the membrane at room temperature for 1 hour in dark. Finally the membrane was washed and the reaction was visualized by adding chromogenic substrate solution (DAB-H_2O_2-$NiCl_2$) for approximately 5 min in dark until the dark-brown target bands on the membrane were appeared. The size of reactive protein bands were estimated by comparing with standard protein molecular weight marker separated on the same polyacrylamide gel.

4.5.3.4 Indirect immunofluorescent assay (IFA)

To confirm antigen-specificity of purified MAbs. The transfected cells that expressed prM, E and PrM-E antigen were used to determine by indirect immunofluorescent assay.

4.5.3.4.1 Transfection of the plasmid DNA by calcium phosphate in 293T cell

293T cells were plated in a 6-wells tissue culture plate (Costar, Cambridge, MA, USA.) at 5x10^5 cells/well in 2 ml of RPMI-1640 media supplemented with 10% FBS and incubated overnight in 5% CO_2 incubator at 37°C. Three hours before transfection, the cell culture media was replaced with 2 ml of plain DMEM. To prepare transfection solution, 5 µg of plasmid DNA in 87.5 µl of

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deionized water was mixed with 87.5 µl of 0.2 M $CaCl_2$ to obtain the DNA-Ca_2PO_4 complex. The complex was drop-by-drop added with 175 µl of 2x HBS and mixed vigorously. After incubating for 15 min, 350 µl of transfection solution was added into each well of 6-well plate containing 50-70% confluent of 293T cells. The transfected cells were incubated in 5% CO_2 incubator at 37°C for 8-15 hrs and the culture media was replaced by 2 ml of complete medium (RPMI 1640 supplemented with 10% FBS). The transfected cells were harvested at 72 hrs post transfection and detected for protein expression by indirect immunofluorescence.

4.5.3.4.2 Cytoplasmic indirect immunofluorescent staining

The cover slip containing monolayer of transfected cells was transfered in 24-well plate and gently washed once with 1 ml of PBS. Cells were fixed with 1 ml of 4% paraformaldehyde (freshly prepared) for 20 min and washed twice with 1 ml of PBS. The fixed cells were then permeabilized with 1 ml of 0.2% Triton X-100 in PBS for 10 min and washed 3 times with 0.1% Triton X-100 in PBS. The treated cells were stained by 250 µl of specific monoclonal antibody for 1 hr at room temperature. The antibody was discarded and the cells were washed 3 times with 1 ml of 0.1 % Triton X-100 in PBS for 5 min each with rocking. The 250 µl of 1:10,000 diluted goat anti-mouse IgG (or IgM) conjugated with Cy3 (Dakopatts, Denmark) were next added and incubated in dark for 30 min with rocking. The cells were washed in the same early manner and the cytoplamic stained protein was observed under confocal laser scanning microscope.

4.6 Preparation of viruses

4.6.1 Preparation of stock viruses

In order to generate large stock of viruses for the whole study, prototype strain of each virus was propagated in C6/36 cells. Approximately $3x10^7$cells were grown into a T-175 tissue culture flask (Costar, Cambridge, MA, USA) in L-15 growth medium (L-15 containing 10% fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB), 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (SIGMA, St.Louis, MO)) and incubated at 28°C for 2 days or until 80% confluent. The monolayer cells were then incubated with the virus at a multiplicity of

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infection (MOI) of 0.1 in maintenance medium (L-15 containing 1.5% FBS, 10% TPB, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) in total volume of 15 ml at 28°C for 3 hrs with rocking. After that, maintenance medium was added to total volume of 40 ml and incubated at 28°C until 7 days after infection. The culture supernatants were collected and clarified by centrifugation at 1000xg for 10 min at 4°C. Clarified supernatants were aliquots and stored at -70°C. Maintenance medium was added into cultured flask again and incubated for 3 days (10 days after infection). The culture supernatants were collected again and processed as the same early manner.

4.6.2 Virus titration

Culture supernatants from virus-infected cells were titrated by focus forming assay in PS clone D cell lines. Briefly, approximately $1x10^4$ and $7.5x10^3$ of PS clone D cells were plated on each well of 96-well tissue culture plate and incubated at 37°C in humidified chamber for 2 and 3 days, respectively. The virus supernatants were 10-fold serially diluted in diluting medium (L-15 containing 3% FBS, 10% TPB, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). The 50 μl of each diluted sample was added into each well in duplicates (at least). After 2 hrs incubation at 37°C in humidified chamber, 125 μl of overlayer medium (diluting medium containing 1.5% carboxylmethyl cellulose (CMC)) was added into each well and incubated at 37°C in humidified chamber for 1, 2 and 3 days for JEV D4V and D1V, D2V, D3V, respectively.

After incubation, the infected foci in each well were stained by focus immunoassay. Briefly, the culture supernatant was discarded and cells were washed with phosphate buffer saline (PBS) pH 7.4. Then, infected cells were fixed and permeabilized by 100 μl of 3.7% formaldehyde in PBS for 10 min and 100 μl of 2% Triton X-100 in PBS for 10 min, respectively. After washing with PBS, 50 μl of 1:500 mouse MAb specific to flavivirus envelope protein (4G2) was added in each well and incubated at 37°C in humidified chamber for 1 hr. After that, the cells were washed and incubated with 50 μl of working rabbit anti-mouse Igs conjugated with HRP (1:1000 diluted rabbit anti-mIgs-HRP in PBS containing 2% FBS and 0.05%

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Tween-20) in dark at 37°C in humidified chamber for 1 hr. After washing, the antigen-antibody complex was detected by using substrate solution (3, 3' diaminobenzidine tetrahydrochloride (DAB) in PBS containing hydrogen peroxide and nickel chloride) for color development. The dark-brown foci of infected cells was visualized and counted. The concentration of virus titer was calculated as focus forming unit per milliliter (ffu/ml), which based on the following formula.

$$\text{Virus titer (ffu/ml)} = \frac{(Na + Nb + Nc)}{[Va+(Vb \times 0.1) + (Vc \times 0.01)]} \times Ra \times 10^3$$

Na = number of foci counted at the end point dilution

Nb = number of foci counted at the dilution next above the end point (2nd dilution)

Nc = number of foci counted at the second dilution above the end point dilution (3rd dilution)

Va = total volume of virus in first dilution

Vb = total volume of virus in 2nd dilution

Vc = total volume of virus in 3rd dilution

Ra = the reciprocal of the first dilution that can count

Mean of virus titer in ffu/ml from separate triplicated experiments was used as the final titer of the stock virus.

4.7 Determination of neutralizing antibodies by Focus Reduction Neutralization Test (FRNT)

To determine neutralizing activity of anti-E MAbs, those MAbs should have the ability to reduce the number of infected foci compared to that of virus control (without antibody). Briefly, stock purified MAbs (1 mg/ml) were 4-fold serially diluted in diluting medium (L-15 medium containing 3%FBS, 10%TPB, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin) (i.e., 1:4, 1:16, 1:64 and 1:256). The virus seeds containing approximate 50 ffu in 25 µl were mixed with 25 µl of each diluted antibodies or diluting medium (as a negative control) on ice and incubated at 37 °C for 1 hour. Then the mixture were added onto the monolayer of

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PS clone D cells in 96-well plates and incubated at 37 °C in humidified chamber for 2 hours. The 125 µl of overlayer medium were added into each well and the plates were incubated at 37 °C in humidified chamber for 1, 2 or 3 days for JEV, D4V or D1V, D2V, D3V, respectively. The infected foci in each well were stained by focus immunoassay as described above. The number of foci in each antibody dilution was counted and the percentage of foci reduction (%FRNT) compared to that of virus control was calculated by the following formula.

$$\%FRNT = \frac{(C - A)}{C} \times 100$$

C = average number of foci counted in virus control

A = average number of foci counted in each antibody dilution

The 50%FRNT titer, the reciprocal dilution of antibody that resulted in 50% reduction of infected foci, was determined by PROBIT regression analysis using SPSS software.

4.8 *In vitro* antibody-dependent enhancement assay

To assess whether these anti-E antibodies can enhance dengue virus infection via Fc receptor, *in vitro* antibody-dependent enhancement (ADE) assay were performed with Fc receptor-bearing cells, the human monocyte cell line (U937). Briefly, stock purified MAbs (1 mg/ml) were serially diluted in ten-fold steps with complete medium (RPMI 1640 containing 10%FBS, 100 U/ml penicillin and 100 µg/ml streptomycin) and mixed with the virus at an MOI of 0.2 for D1V, D2V D4V, JEV and MOI of 0.06 for D3V. The mixtures were incubated at 37°C for 1 hour. The virus-antibody complex or virus control (without antibody) was added to suspension of $2x10^5$ U937 cells in 24 well-tissue culture plates. After 4 days post-infection at 37°C in humidified air containing 5% CO_2, the extracellular virus in U937-culture supernatant were harvested with containing 20% FBS and store at -70 °C. The culture supernatants were titrated in PS clone D cells as described above. The titer of viral progeny which enhanced by various dilutions of antibody was compared to that of the virus control.

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CHAPTER V
RESULTS

5.1 Preparation of anti-E monoclonal antibodies

5.1.1 Large-scale production and purification of anti-E MAbs

The production of anti-E MAbs were enlarged by both ascitic fluid and cell culture technique. Eleven hybridoma clones producing anti-E MAb (4G2, 3H5, 8A1, 1H10, J93, 1F4, 1D10, 1C2, 1D3, 5A1 and 2C8) were succeeded to induce ascitic fluid in Balb/c mice. Whereas, only one hybridoma clone, 2B7, could not induce mouse ascitic fluid with unknown reason. Therefore this clone was large-scale produced by cell culture technique in serum-free media (SFM) to minimize the contaminated calf immunoglobulins which are normally appeared in normal hybridoma cell culture media. The volume of mouse ascitic fluid obtained in each clones were varied from less than 1 ml to several ml. The anti-E immunoglobulins were then purified from mouse ascetic fluid and SFM culture media by Protein G affinity chromatography. Total amount of purified anti-E MAbs in mg were varied in each clone. The discrepancy among purified anti-E MAbs per ml of original mouse ascitic fluid or SFM culture supernatant (for 2B7) was shown in Table 5.1. The results indicated the variation in amount of anti-E MAbs production in ascitic fluid from each hybridoma clone. Proportion of purified 2B7 per ml of SFM media was very low (less than 0.1 mg/ml), indicating the diluted MAb produced in normal culture flask. Purification of 2B7 from several hundred ml of SFM could achieve the adequate amount of antibody required for overall study. The purified anti-E MAbs of twelve clones were adjusted to a concentration of 1 mg /ml for further studies.

The purity of MAbs was investigated by SDS-PAGE analysis as shown in Fig. 5.1. Under reduced and heat condition, the purify MAbs were separated to only heavy chain (~50 kDa) and light chain (~25 kDa) bands (lane P), comparing to several bands of the non-purified ascitic fluids (lane A).

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Table 5.1 Discrepancy of scale-up and purification of anti-E MAbs in this study.

No.	MAb	Isotype	Source of Ig		Total purified MAb (mg)	[Ig] [a] (mg/ml)
			Type	Volume (ml)		
1	4G2	IgG2a	Ascitic Fluid	5.5	10.35	1.9
2	3H5	IgG1	Ascitic Fluid	5.6	10.64	1.9
3	8A1	IgG1	Ascitic Fluid	1.0	5.90	5.9
4	1H10	IgG1	Ascitic Fluid	1.0	7.70	7.7
5	J93	IgG2a	Ascitic Fluid	1.0	5.15	5.4
6	2B7	IgG1	SFM [b]	425.0	12.80	0.03
7	1F4	IgG1	Ascitic Fluid	2.0	2.47	1.2
8	1D10	IgG1	Ascitic Fluid	0.5	3.62	7.2
9	1C2	IgG2a	Ascitic Fluid	1.0	6.85	6.9
10	1D3	IgG1	Ascitic Fluid	1.5	2.14	1.4
11	5A1	IgG2a	Ascitic Fluid	1.0	8.78	8.8
12	2C8	IgG2a	Ascitic Fluid	2.3	11.99	5.3

[a] The concentration of purified immunoglobulin (Ig) in 1 ml of ascitic fluid (or SFM).

[b] Serum-free medium cultured supernatant

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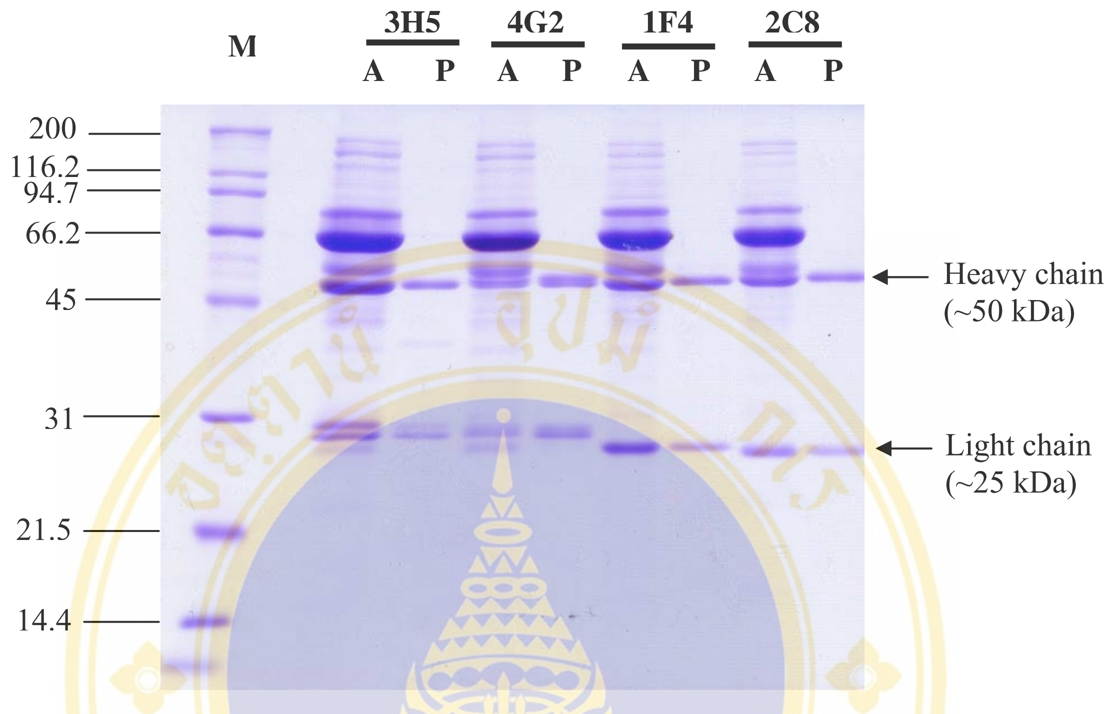

Figure 5.1 SDS-PAGE analysis of purified anti-E MAbs.

Anti-E MAbs (as indicated on the top) in purified form (lane P) or non-purified ascitic fluid (lane A) were separated by SDS-PAGE under β-ME reduced condition followed with Coomassie blue R-250 staining. Lane M was standard protein molecular weight markers (in kDa). Two forms of immunoglobulins were indicated by arrows.

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5.1.2 Verification of purified anti-E MAbs

Although these anti-E MAbs were previously characterized and reported by others (7, 12, 74, 75, 94, 95), however we also would like to verify some properties of these purified MAbs before applying to determine their functional activity in this study.

5.1.2.1 Verification of virus serotype specific/cross reactivity by dot enzyme immunoassay (DEIA)

To verify the serotype specificity as well as cross reactivity to other flavivirus (JEV) of the purified MAbs, the DEIA technique was used. Each MAb was reacted to 5 separated viral cultured supernatant dots of D1V, D2V, D3V, D4V and Japanese encephalitis virus (JEV), including mock antigen dot as a negative control antigen. The results were shown in Fig. 5.2. Three anti-E MAbs (4G2, 2B7 and 1D10), designated as a Flavi-cross reactive group, reacted to all dengue and JE viral antigen dots. Other three anti-E MAbs, designated as dengue-cross reactive group, containing 1D3 (reacted to D1V-D4V), 1C2 (reacted to D1V-D3V) and 5A1 (reacted to D2V and D3V). The last group was designated as dengue-serotype/JEV specific, including six anti-E MAbs; 3H5, 1F4 and 2C8 (D2V-specific), 8A1 (D3V-specific), 1H10 (D4V-specific) and J93 (JEV-specific). Most of these results were similar to the serotype specific/cross-reactive properties of these anti-E MAbs which have been previously reported. But only 5A1 MAb differed from previous report, as shown in Table 5.2. In previous study, 5A1 was characterized as D2V – specific MAb. But we characterized as D2V and D3V cross-reactive MAb.

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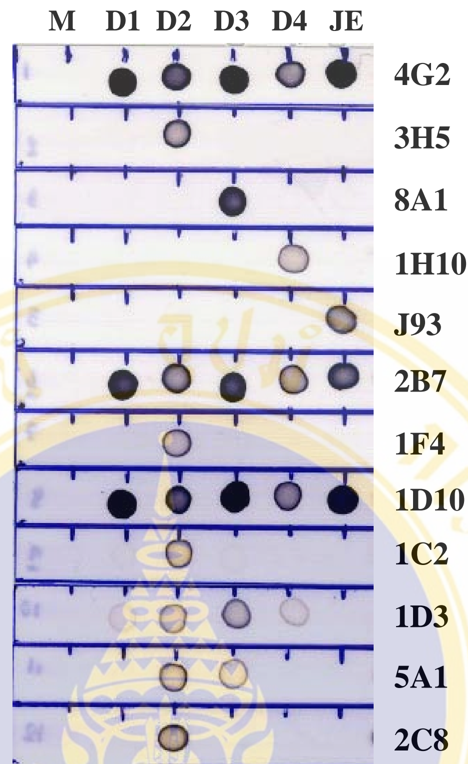

Figure 5.2 Serotype specificity of purified anti–E MAbs by DEIA.

The nitrocellulose membranes containing 5 dots of virus infected C6/36 cells cultured supernatants were reacted to purified anti-E MAbs as indicated on the right. D1 to D4 were dengue virus serotype 1 to serotype 4, JE was Japanese encephalitis virus. Mock infected culture supernatant (M) was included as a negative control.

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5.1.2.2 Specificity of purified MAbs to viral envelope protein

To verify the specificity of purified MAbs to viral envelope protein, western blot analysis to dengue infected C6/36 cells and indirect immunofluorescent staining to plasmid DNA transfected 293T cells were performed.

5.1.2.2.1 Western blot analysis

D2V infected C6/36 cells lysates were electrophoretically separated by SDS-PAGE under two treated conditions; reduced and heat or non-reduced and non-heat. Mock infected cell lysate was also included as a negative control. After blotting onto nitrocellulose membrane, all purified MAbs, except 8A1, 1H10 and J93, were then reacted by western blot analysis. The 8A1, 1H10 and J93 MAbs were instead reacted to C6/36 cell lysates infected by D3V, D4V and JEV, respectively. The results were shown in Fig. 5.3. It was found that nine MAbs, including 4G2, 3H5, 8A1, 1H10, J93, 2B7, 1F4, 1D10 and 1D3 were reacted to dengue E protein of approximately 55 kDa, though weak reactivity were observed with 2B7 and 1D3. Most of reactive bands were found in non-reduced condition (lane 3), except for 1F4 and 3H5 which both reduced and non-reduced conditions were observed. 3H5 reacted very weak to reduced E compared to the non-reduced condition, whereas 1F4 reacted comparably strong to E treated by both conditions. These results indicated linear epitope specificity of 1F4 and 3H5, while others were of conformational epitope specific. The other three MAbs; 1C2, 5A1 and 2C8 were found no reactivity to E in either condition by western blot analysis. None of tested MAbs reacted to mock proteins (lane 1).

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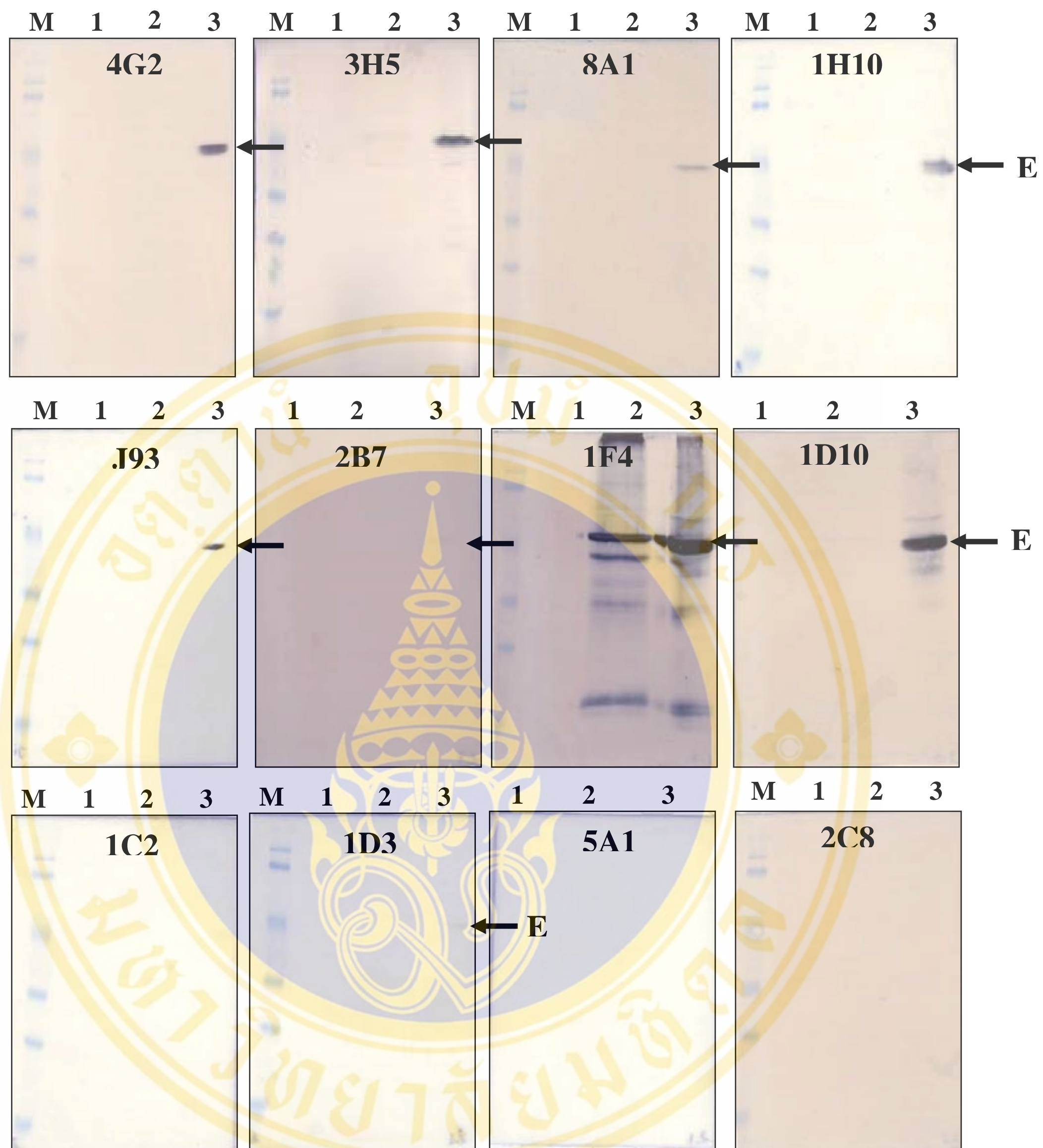

Figure 5.3 Reactivity to E protein of purified anti-E MAbs by western blot analysis.

Virus infected C6/36 cells lysate was separated by 12% SDS-PAGE under reduced/heat (lane 2) and non-reduced/non-heat (lane 3) conditions. Mock infected cell lysate (lane 1) was included. The proteins were blotted onto nitrocellulose membranes and reacted to indicated purified anti-E MAbs by western blot analysis. Most of MAbs were reacted to D2V infected cell lysate, except for 8A1, 1H10 or J93 were reacted to D3V, D4V or JEV infected cell lysates, respectively. The E protein reacted to anti-E MAbs were indicated by arrows.

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5.1.2.2.2 Indirect immunofluorescent assay (IFA)

To further validate the specificity of MAb to dengue E protein, especially to the clones which have no reactivity to western blot analysis, we therefore demonstrated by IFA to plasmid DNA transfected cells. Four different pcDNA3.1/Hygro (+)-based plasmids containing gene encoding for D2V prM or E protein or prME polyprotein or none (as a negative control), were transfected into HEK 293T cells. The transfected cells were cytoplasmic immunofluorescent stained by a panel of MAbs as shown in Fig. 5.4. It is included 4G2 and 1D10 which were shown specificity to conformational E protein, 3H5 and 1F4 which were shown specificity to linear E protein, 2B7 and 1D3 which were weak reactive, and 1C2, 5A1 and 2C8 which were found no reactivity to E by western blot analysis. We found that all our tested MAbs were reacted to 293T cells transfected by E-DNA and prME-DNA, but not by prM-DNA or vector only (mock). This result confirmed that those MAbs were specific to dengue E protein, especially for the ones that were no reactivity by western blot analysis. As a control of the experiment, anti-prM MAb (1H10) reacted to 293T cells transfected by prM-DNA and prME-DNA, but not E-DNA or vector only. Whereas 8A1 (anti-D3V specific), as a negative control antibody, stained none of transfected cells since the plasmids were constructed by D2V background.

From both western blot analysis and transfected cells IF staining results, it is demonstrated that all 12 MAbs used in this study were E protein specific MAbs. The properties of all anti-E MAbs demonstrating in this study compared to the previous reports were summarized in Table 5.2.

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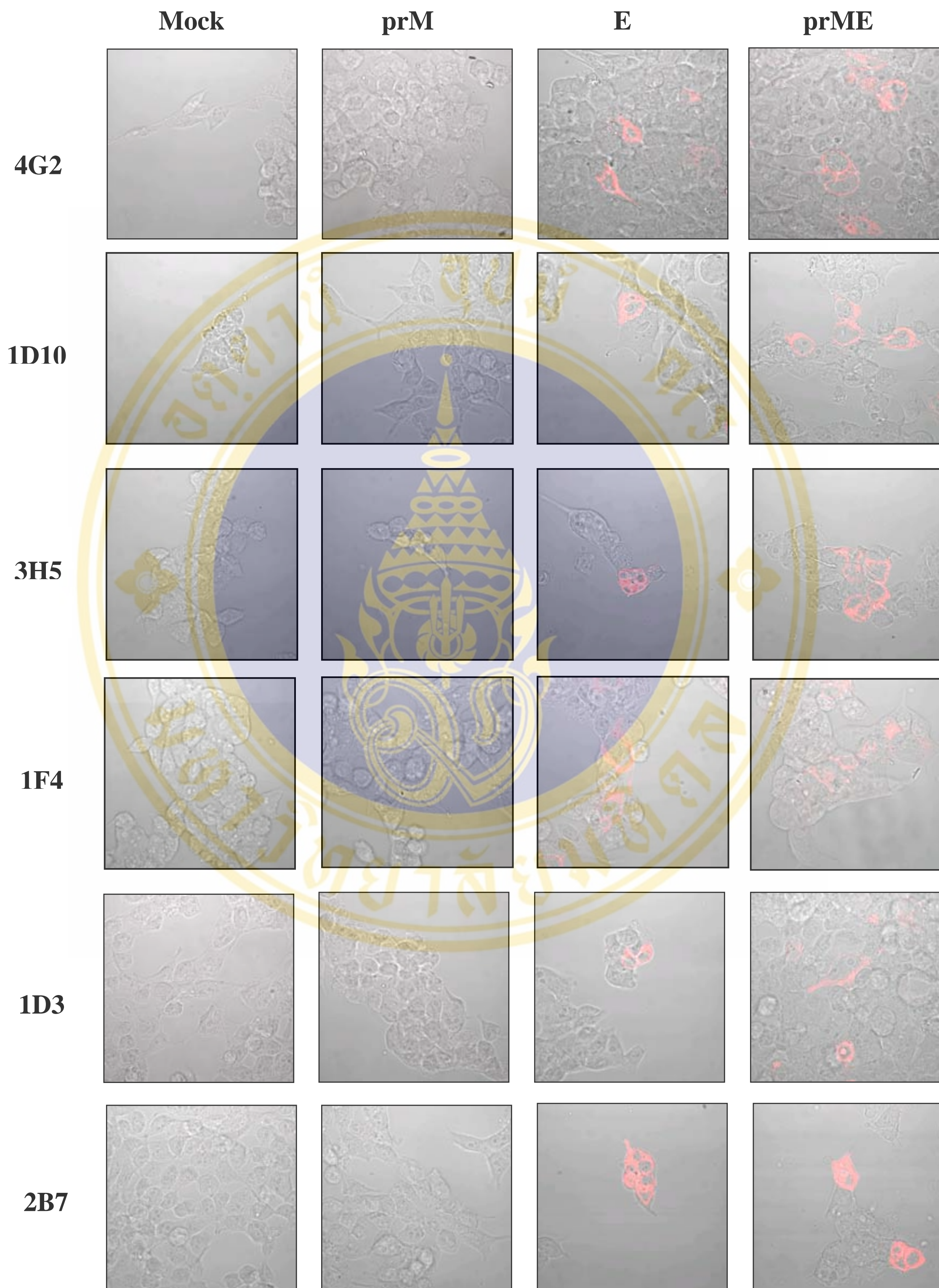

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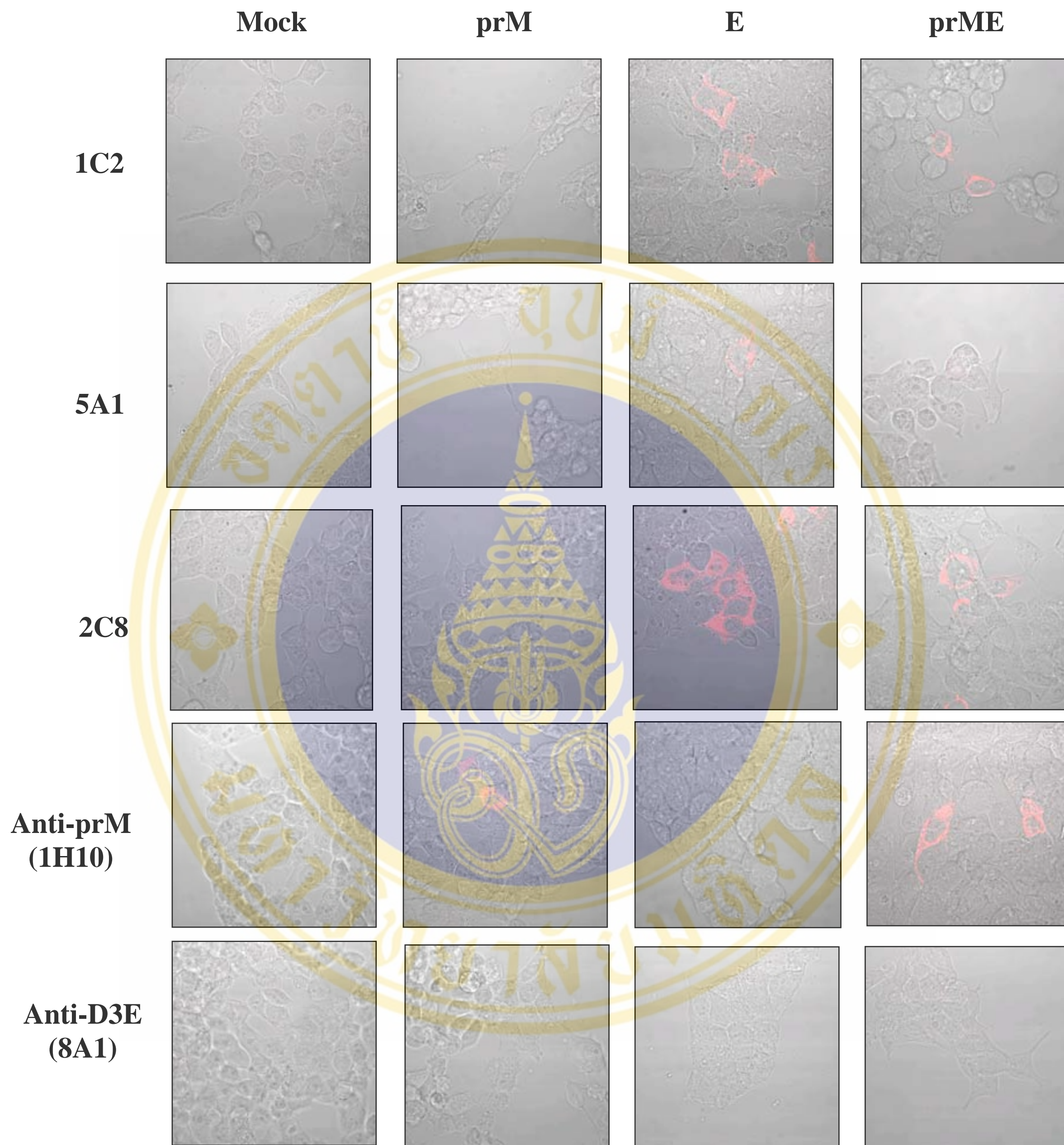

Figure 5.4 The reactivity of anti-E MAbs to plasmid DNA transfected cells by indirect immunofluorescent assay.

293T cells were transfected with pcDNA3.1/Hygro(+) (Mock), pcDNA3.1/Hygro(+)/prM (prM), pcDNA3.1/Hygro(+)/E (E), or pcDNA3.1/Hygro (+) /prME (panel prME). Transfected cells were cytoplasmic stained with anti-E MAbs as indicated on the left, followed with Cy3 –conjugated anti-mouse IgG. The stained cells were examined under confocal laser scanning microscopy.

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Table 5.2 Verification of anti-E MAbs properties compared to previous reports.

No.	MAbs	References	Previous properties / method of characterization			This study properties / methods of characterization		
			Serotype specificity	protein specificity	Linear/conformation specific	Serotype specificity	protein specificity	Linear/conformation specific
1	4G2	(7, 74, 75)	Flavivirus	E	Conformational	Flavivirus	E	Conformational
2	3H5	(7, 74, 75)	D2V	E	Linear	D2V	E	Linear
3	8A1	(12)	D3V	E		D3V	E	Conformational
4	1H10	(75)	D4V	E	Conformational	D4V	E	Conformational
5	J93	-	JEV	-	-	JEV	E	Conformational
6	2B7	(94)	Flavivirus	E	Conformational	Flavivirus	E	Conformational
7	1F4	(94)	D2V	E	Linear	D2V	E	Linear
8	1D10	(94)	Flavivirus	E	Conformational	Flavivirus	E	Conformational
9	1C2	(95)	D1-3	-	-	D1-3	E	Conformational
10	1D3	(95)	D1-4	-	-	D1-4	E	Conformational
11	5A1	(95)	D2V	-	-	D2-3	E	Conformational
12	2C8	(94)	D2V	E	Conformational	D2V	E	Conformational

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5.2 Determination of neutralizing antibodies by Focus Reduction Neutralization Test (FRNT)

All 12 clones of MAbs were tested for their ability to neutralize viral infection by FRNT. Fix amount of viruses (50 foci) were incubated with 4-fold serial dilutions of purified anti-E MAbs (starting from 1 mg/ml) before infecting to PS clone D cells. The infectious foci at each dilution of MAbs was stained and counted. % Reduction of foci at each MAb dilution compared to the control infection without antibody was plotted against corresponded MAb dilution. NT activity of each anti-E MAb to 4 serotypes of dengue viruses as well as JEV were compared by 50% FRNT titer which indicated the dilution or amount of MAbs in ng that sufficient to reduce the infectious foci to 50%. The 50% FRNT titer were determined by PROBIT regression analysis by SPSS software. We reported here the NT activities of tested MAbs as 3 categorized groups according to their serotype specific-property.

5.2.1 Flavivirus-cross reactive group

This group contains three anti-E MAbs; 4G2, 2B7 and 1D10. Their NT results are shown in Fig. 5.5. Percentage of foci reduction became lower when more dilution of MAbs were used, indicating the neutralizing activity of these MAbs. 4G2 and 1D10 neutralized all 4 dengue serotypes as well as JEV with different efficacy. NT activity to D2V of both MAbs was highest among others (50%FRNT of 4G2 and 1D10 were 8.5 and 16.5 ng, respectively), while those to JEV was the least (50%FRNT of 4G2 and 1D10 were 168 and 397.8 ng, respectively). 2B7 neutralized 4 dengue serotypes with the highest efficacy to D2V (50%FRNT was 2.4 ng) and the lowest to D3V (50%FRNT = 1024 ng). 2B7 also gave a pattern of neutralization to JEV, but as it was less than 50% reduction to all MAb dilutions, so 50%FRNT titer could not be determined. It was therefore identified as weak NT activity to JEV. It is noticed that neutralization to tested viruses by 2B7 gave a wider range of efficacy (50% FRNT were ranged from 2.4 ng to 1,024 ng) than those by 4G2 (8.5 – 168 ng range) and 1D10 (16.5 – 397.8 ng range).

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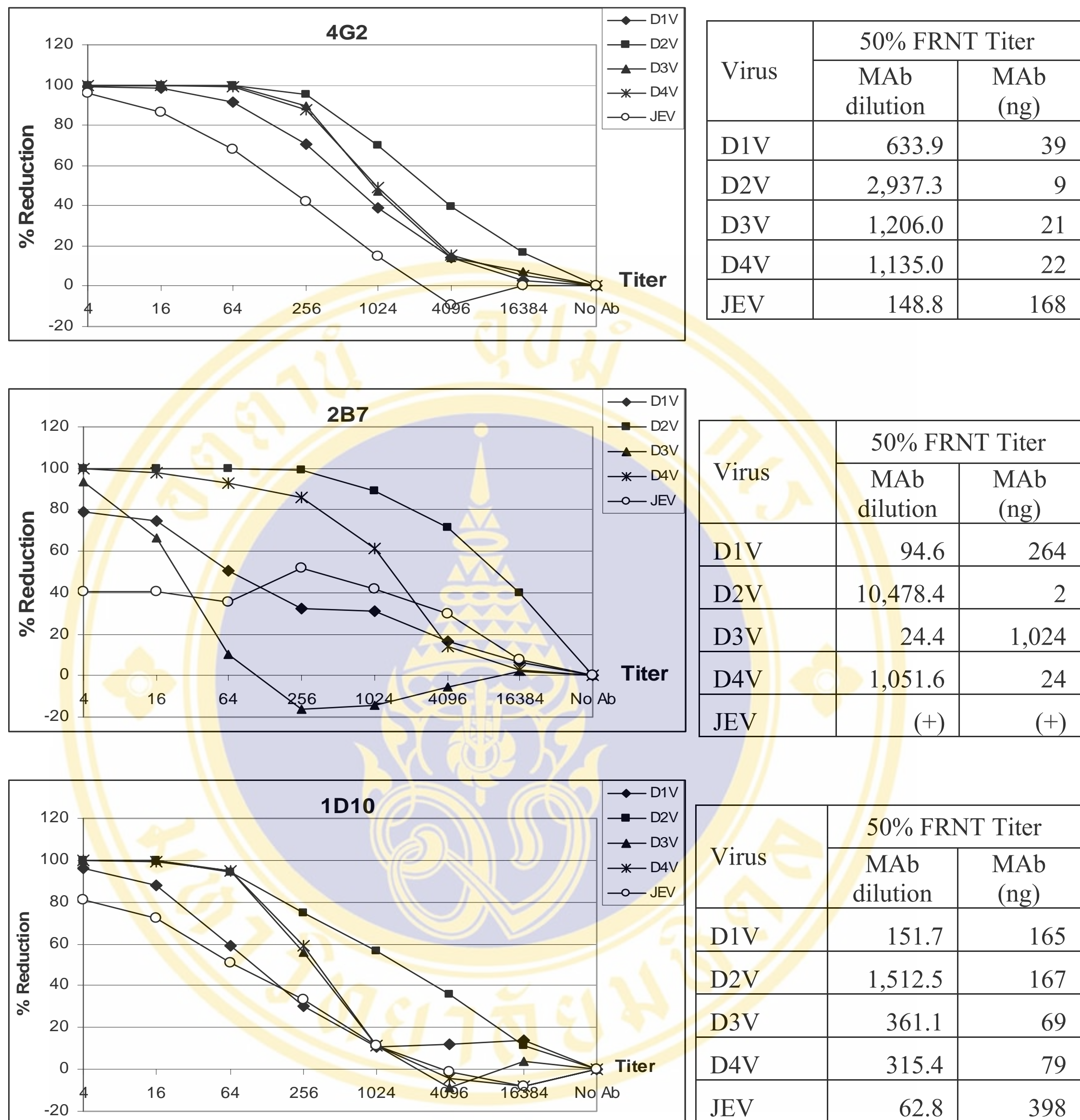

Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	633.9	39
D2V	2,937.3	9
D3V	1,206.0	21
D4V	1,135.0	22
JEV	148.8	168

Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	94.6	264
D2V	10,478.4	2
D3V	24.4	1,024
D4V	1,051.6	24
JEV	(+)	(+)

Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	151.7	165
D2V	1,512.5	167
D3V	361.1	69
D4V	315.4	79
JEV	62.8	398

Figure 5.5 Focus reduction neutralization by Flavivirus-cross reactive anti-E MAbs.

% Reduction of foci (Y-axis) in the absence of MAb (no Ab) or in the presence of 4-fold serially diluted MAbs were plotted as shown in the left panel. X-axis represents the reciprocal dilution of MAb. D1V to D4V and JEV were indicated. The antibodies included: 4G2, 2B7 and 1D10. The 50%FRNT titer of each MAb to five different viruses (D1V-D4V and JEV) were calculated as reciprocal dilution of MAb (1/dilution) or total amount of MAb (ng) as shown in the corresponded table on the right panel. (+) represents % reduction of foci less than 50%

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5.2.2 Dengue-cross reactive group

The three clones of MAbs were in this group; i.e., 1D3, 1C2 and 5A1. Neutralizing patterns and 50% FRNT titers of 3 MAbs are shown in Fig. 5.6. 1D3 neutralized 4 dengue serotypes with equivalent highest efficacy to both D2V and D4V (50%FRNT titers were 8.5 and 9.1 ng, respectively), while the least to D1V (50%FRNT titer was 192.2 ng). No NT activity to JEV was identified for 1D3. 1C2 neutralized only D2V with comparatively high efficacy (50% FRNT titer = 13.5 ng). Though 1C2 was identified as D1, D2 and D3-specific by DEIA (Fig. 5.2), but its reactivity to D1V and D3V were very weak compared to D2V. In FRNT, 1C2 at various dilutions partially reduced D1V and D3V foci at 10 to 40% reduction, but it could not be determined for 50% FRNT titer. Different to D4V and JEV, 1C2 was completely non-neutralized to both viruses. 5A1 has no NT activity to all tested viruses, except D2V. However, the pattern of neutralization of 5A1 to D2V was rather strange from others which have been described above. % Reduction of foci was maintained in the range of approximately 50 to 70%, even though the lowest (1:4) or the highest dilution of 5A1 (1:16,384) was used. We therefore identified that 5A1 has NT activity to D2V but 50% FRNT titer could not be determined.

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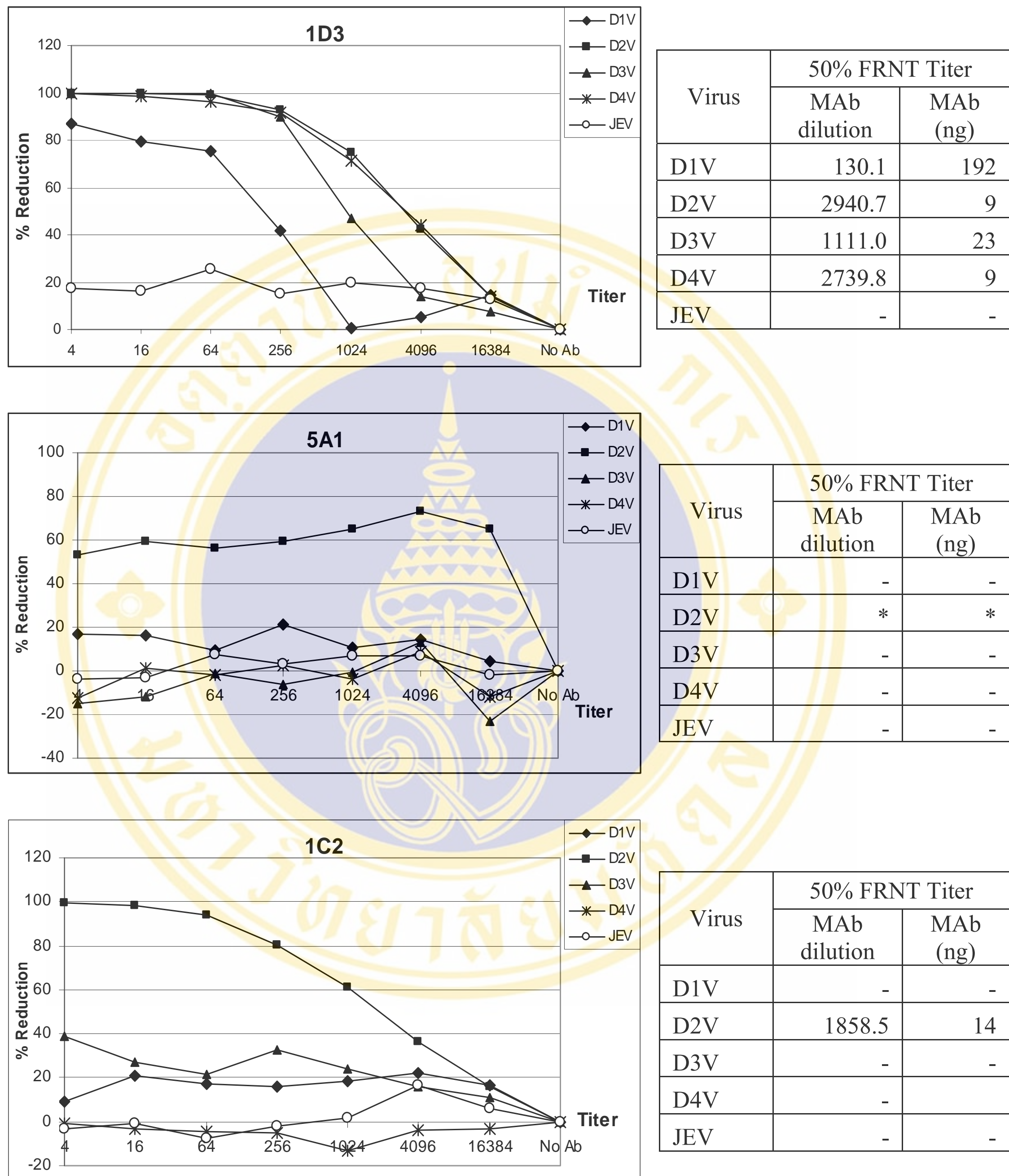

Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	130.1	192
D2V	2940.7	9
D3V	1111.0	23
D4V	2739.8	9
JEV	-	-

Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	-	-
D2V	*	*
D3V	-	-
D4V	-	-
JEV	-	-

Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	-	-
D2V	1858.5	14
D3V	-	-
D4V	-	
JEV	-	-

Figure 5.6 Focus reduction neutralization by Dengue-cross reactive anti-E MAbs.

The figure caption was similar to Figure 5.5. The antibodies included: 1D3, 5A1 and 1C2. * indicated the MAb which retains NT activity but 50% FRNT titer could not be determined.

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5.2.3 Dengue-serotype/ JEV specific group

This group contains 6 MAbs; 3H5, 2C8 and 1F4 are specific to D2V, 8A1 and 1H10 are specific to D3V and D4V, respectively, and J93 is specific to JEV. Neutralization patterns as well as 50%FRNT titer are shown in Fig. 5.7. In D2V-specific subgroup (Fig. 5.7), 3H5 and 2C8 neutralized specifically to D2V with very high efficacy, as 50%FRNT titers of both were less than 1 ng (50%FRNT of 3H5 = 0.9 ng and 2C8 = 0.5 ng). No NT activity was found to other viruses. However, 1F4 showed no NT activity to all tested viruses, even D2V which is reacted to 1F4 by DEIA.

As expected, 8A1 and 1H10 serotype-specifically neutralized D3V and D4V, respectively, with moderately high efficacy (50%FRNT of 8A1 = 50 ng, that of 1H10 = 62.6 ng). J93 also neutralized only JEV, but not to others dengue viruses. 50%FRNT titer of J93 was also quite high (9.6 ng) (Fig. 5.7).

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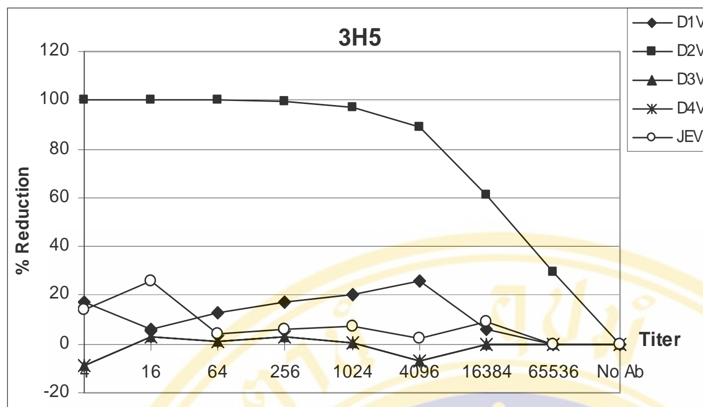
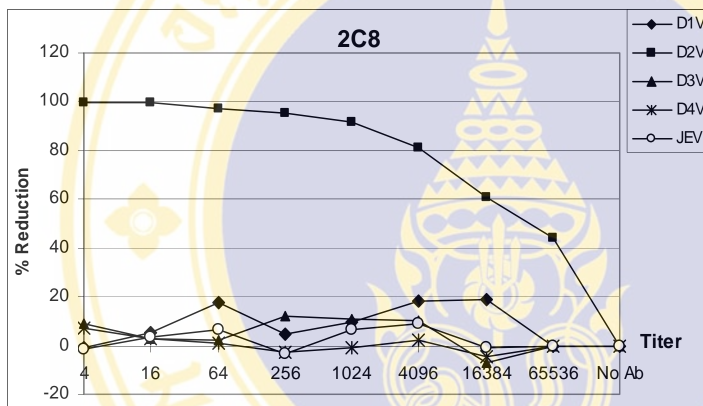
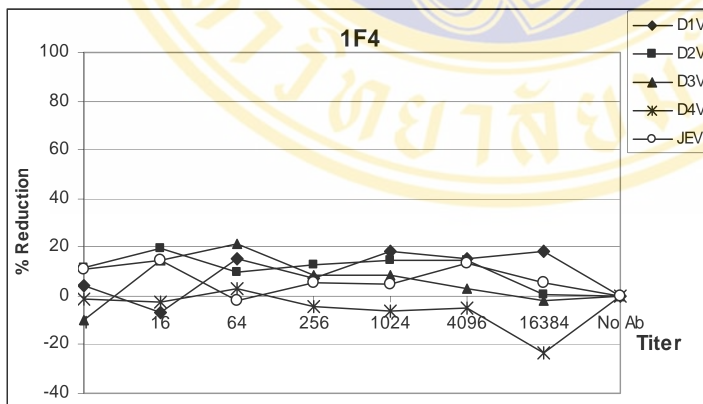


Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	-	-
D2V	26908.2	0.9
D3V	-	-
D4V	-	-
JEV	-	-



Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	-	-
D2V	53989.6	0.5
D3V	-	-
D4V	-	-
JEV	-	-



Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	-	-
D2V	-	-
D3V	-	-
D4V	-	-
JEV	-	-

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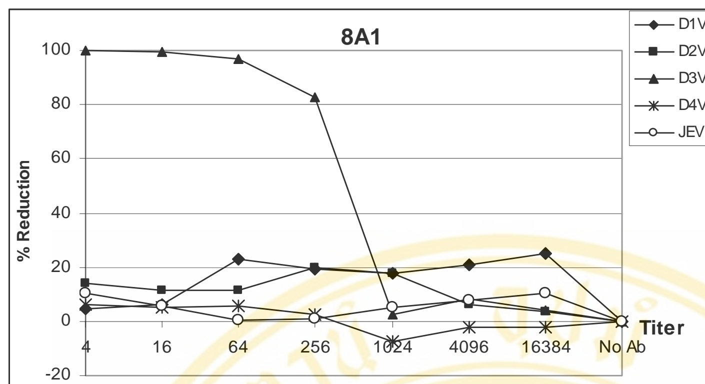
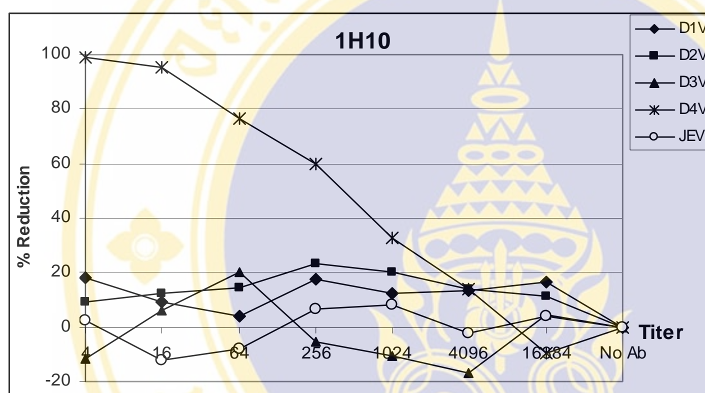
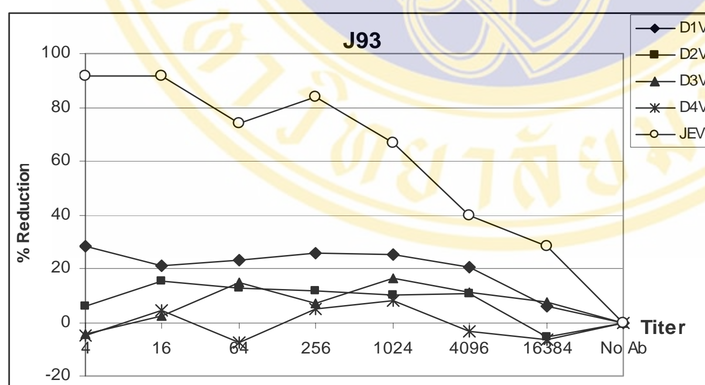


Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	-	-
D2V	-	-
D3V	499.4	50
D4V	-	-
JEV	-	-



Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	-	-
D2V	-	-
D3V	-	-
D4V	399.2	63
JEV	-	-



Virus	50% FRNT Titer	
	MAb dilution	MAb (ng)
D1V	-	-
D2V	-	-
D3V	-	-
D4V	-	-
JEV	2592.9	10

Figure 5.7 Focus reduction neutralization by Dengue-serotype / JEV specific reactive anti-E MAbs.

The figure caption was similar to Figure 5.5. The antibodies included: 3H5, 2C8, 1F4 (for D2V specific), 8A1 (for D3V-specific), 1H10 (for D4V specific) and J93 (for JEV specific).

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5.3 *In vitro* antibody-dependent enhancement assay

To determine functional activity of anti-E MAbs to enhance dengue virus infection *in vitro*, ADE experiment was performed with prototype strains of dengue virus using Fc receptor bearing cells, U937. The titers of viral progeny obtained by preincubation of virus with various dilutions of each MAb or none (no MAb) were determined as shown in Appendix A. We demonstrated ADE activity of MAbs at each dilution as infection-enhancement fold, which was defined as the ratio of virus titer in the presence of antibody and the virus titer in the absence of antibody. The infection-enhancement folds by each antibody were plotted against various antibody dilutions as shown in Fig 5.8 – 5.10. ADE of anti-E MAbs were analyzed as three groups of MAbs.

5.3.1 Flavivirus-cross reactive group

This group contains three anti-E MAbs; 4G2, 2B7 and 1D10. Their ADE results were shown in Fig. 5.8. All of them could enhance dengue/JE virus infection in U937 cells with different activity. 4G2 enhanced D2V infection up to 348-fold, highest among other viruses, at the Ab concentration of 0.1 µg/ml compared to no Ab. It is noticed that the concentration of Ab at higher or lower than the peak concentration reduced ADE activity as the reduction of enhancement fold was found. Almost no ADE activity was observed at 0.1 ng/ml for all dengue and JEV. 4G2 at 10 µg/ml demonstrated the reduction of D2V virus titer lower than that with no Ab. This phenomenon was also observed in D2V infection with 2B7 at the same concentration. It might be indicated that Ab at too high concentration may cause some neutralizing effect to D2V infection in U937 cells.

2B7 also enhanced DV and JEV infection in U937. The peak enhancement of D1V, D3V and JEV were comparably high upto 94-103 folds at the Ab concentration of 10 µg/ml. Whereas, 1D10 enhanced virus infection to U937 with approximately 45-60 fold for D1V-D3V and JEV. It is noticed that the infection-enhancement fold of three MAbs to D4V were very low compared to others (less than 10-fold enhancement).

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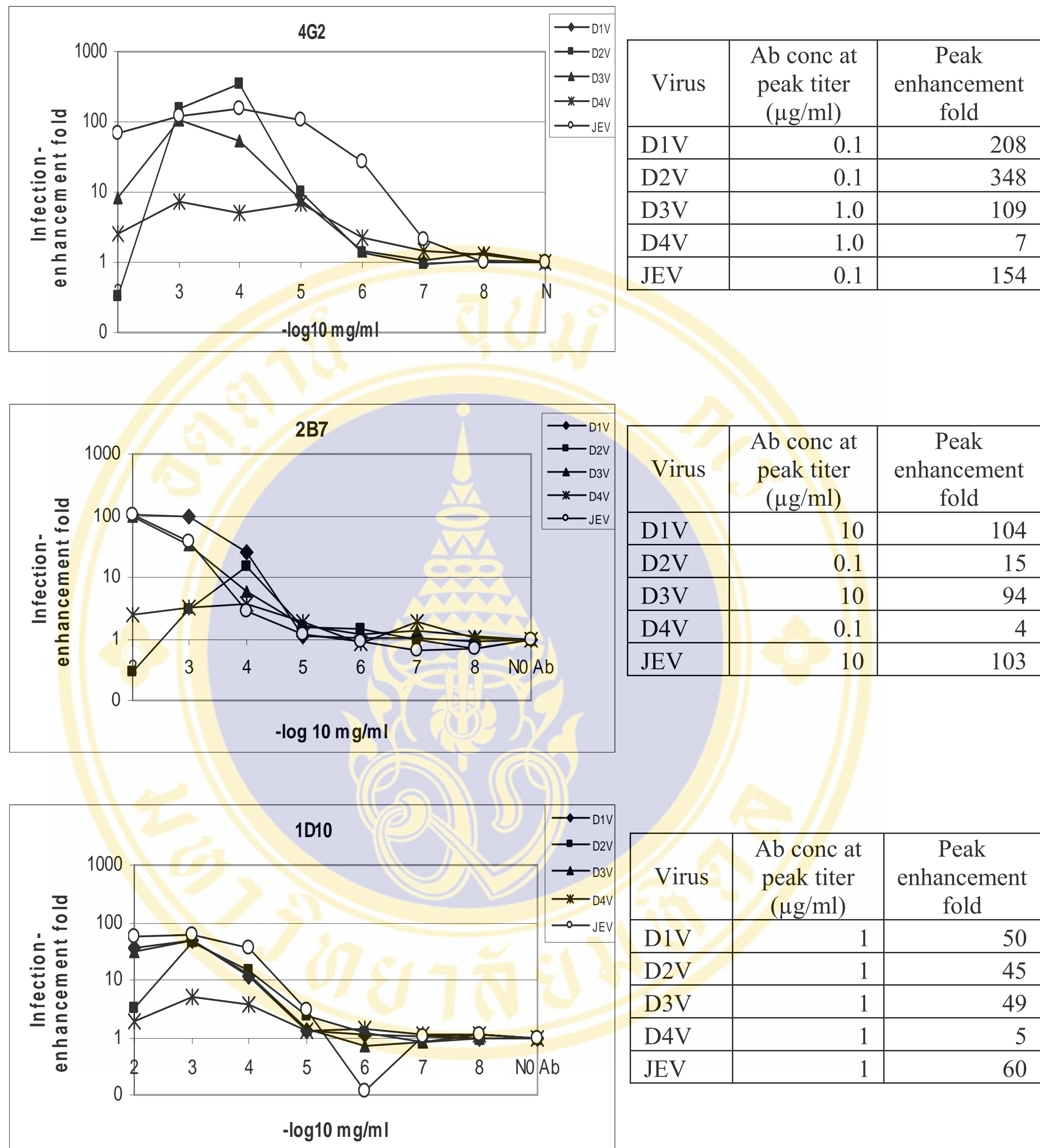

Virus	Ab conc at peak titer (µg/ml)	Peak enhancement fold
D1V	0.1	208
D2V	0.1	348
D3V	1.0	109
D4V	1.0	7
JEV	0.1	154

Virus	Ab conc at peak titer (µg/ml)	Peak enhancement fold
D1V	10	104
D2V	0.1	15
D3V	10	94
D4V	0.1	4
JEV	10	103

Virus	Ab conc at peak titer (µg/ml)	Peak enhancement fold
D1V	1	50
D2V	1	45
D3V	1	49
D4V	1	5
JEV	1	60

Figure 5.8 Antibody-dependent enhancement of dengue infection to U937 cells by Flavivirus-cross reactive anti-E MAbs.

Infection-enhancement fold (Y-axis) was plotted against corresponded MAb concentration (-log10 mg/ml) on the X-axis as shown on the left panel. Dengue 1 to 4 (D1V to D4V) and Japanese encephalitis virus (JEV) were indicated. The antibodies included; 4G2, 2B7 and 1D10. The right panel was corresponded tables determined antibody concentrations which give a peak enhancement of infection (in µg/ml) and peak enhancement fold for each virus.

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5.3.2 Dengue-cross reactive group

This group contains 1D3 (D1V to D4V cross reactive), 5A1 (D2V and D3V specific) and 1C2 (D1V to D3V specific). Different from the previous group, 1D3 enhanced only D1V and D3V infection to U937 cells, though it is cross reactive to all 4 serotypes (Fig. 5.9). The peak enhancement fold was moderate (ranged from 22 to 80 fold) at the Ab concentration of 10 µg/ml. Whereas, 5A1 enhanced both D2V and D3V infection to U937 cells. The ADE activity of 5A1 to enhance D2V infection was very high as the peak enhancement was found upto 845 fold (Fig. 5.9), while only 32 fold was enhanced for D3V at the same concentration of Ab (0.1 µg/ml). 1C2 enhance only D1V and D2V infection to U-937 (Fig. 5.9). Peak enhancements were found moderately at 130 to 213 fold at different concentration of Ab for D1V (1 µg/ml) or D2V (0.1 µg/ml). Infection of D3V could not be enhanced in U937 by 1C2, though it is reactive.

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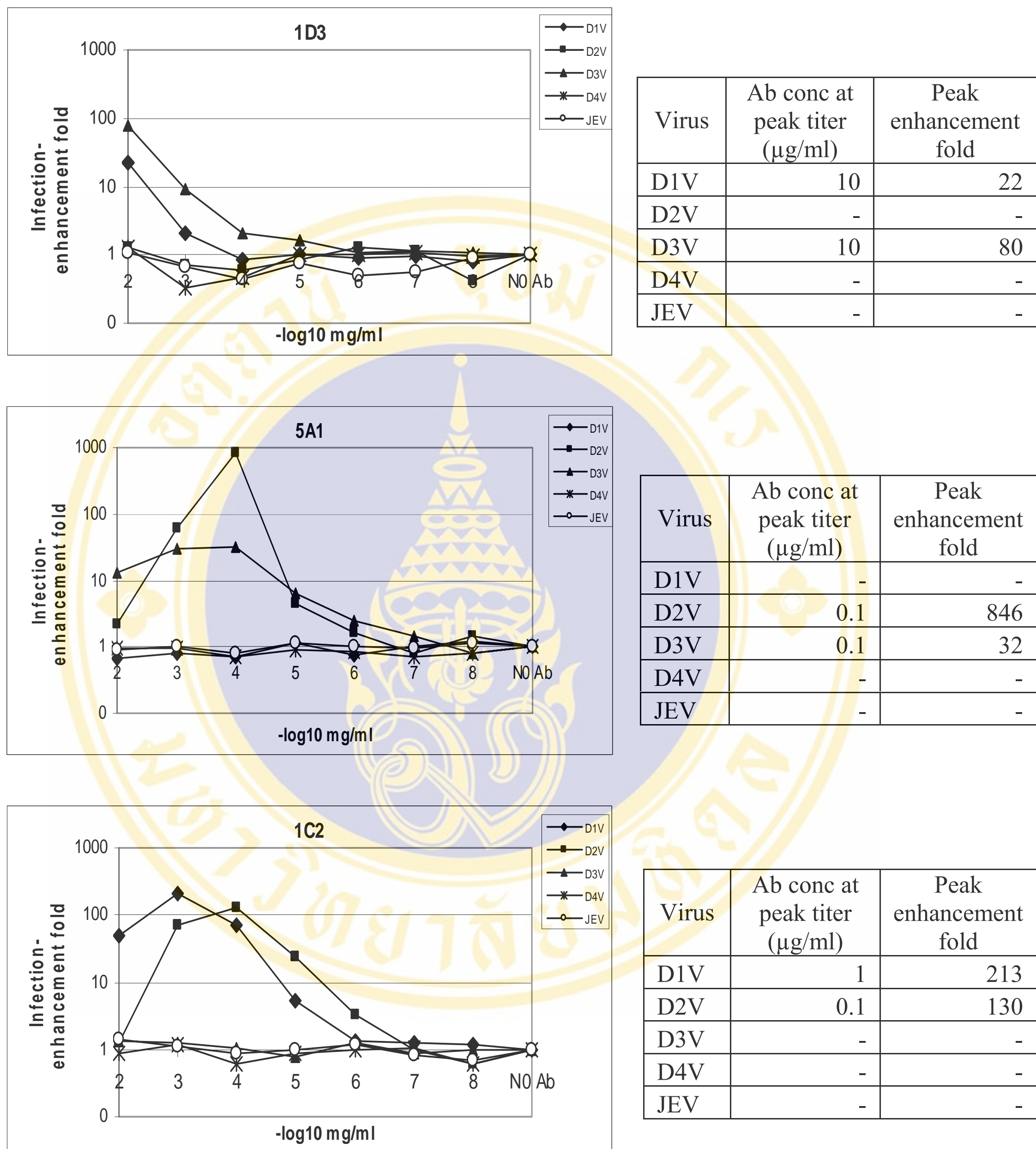

Virus	Ab conc at peak titer (μg/ml)	Peak enhancement fold
D1V	10	22
D2V	-	-
D3V	10	80
D4V	-	-
JEV	-	-

Virus	Ab conc at peak titer (μg/ml)	Peak enhancement fold
D1V	-	-
D2V	0.1	846
D3V	0.1	32
D4V	-	-
JEV	-	-

Virus	Ab conc at peak titer (μg/ml)	Peak enhancement fold
D1V	1	213
D2V	0.1	130
D3V	-	-
D4V	-	-
JEV	-	-

Figure 5.9 Antibody-dependent enhancement of dengue infection to U937 cells by Dengue-cross reactive anti-E MAbs.

The figure caption was similar to Figure 5.8. The antibodies included: 1D3, 5A1 and 1C2.

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5.3.3 Dengue-serotype/ JEV specific group

This group includs three MAbs specific to D2V (3H5, 1F4 and 2C8) and D3V specific (8A1), D4V specific (1H10) and JEV specific (J93). The last six clones are serotype specific antibodies. For D2V-specific MAbs, only 2C8 enhanced D2V infection to U937, whereas no other virus infection was enhanced. (Fig 5.10). The peak enhancement was upto 1195 fold, highest among other MAbs in this study, at the Ab concentration of 0.1 µg/ml. It is unexpected that 3H5 which is the most established clone has no ADE activity to enhance D2V infection in this study. This will be discussed in the next chapter.

For the other three clones, as expected, 8A1 enhanced only D3V infection in U937 at moderate activity (peak enhancement fold = 56) at Ab concentration of 1 µg/ml. 1H10 gave very low ADE activity (peak enhancement fold = 7) to enhance D4V infection in U937 at 0.1 µg/ml of MAb. J93 also specifically enhanced only JEV with the peak enhancement was 10^3-fold at the concentration of 1 ng/ml. However, it is noticed that JEV is normally infected into U937 cells at approximately 3-7 x 10^4 ffu/ml even in the absence of Ab (Table B. in appendix A), however, in the presence of some MAbs (i.e., 4G2, 2B7, 1D10 and J93), JEV infection could be further enhanced.

Three clones (3H5, 1F4 and 2C8) are dengue serotype 2 specific MAbs. Only 2C8 MAb could enhance viral propagation. The highest increasing ratio of viral progeny is 1194.99-fold at 100 ng of MAb. None of all dilution of both 3H5 and 1F4 MAbs could enhance the viral propagation (Figure 5.10 and Table B. in appendix A). For 8A1 MAb, 1H10 MAb and J93 are dengue serotype 3, 4 and JEV specific MAbs, respectively. They could enhance only the virus that they are specific. The increasing ratio of these MAbs are 56.11-fold at 1000 ng of 8A1 MAb, 6.74-fold at 100 ng of 1H10 MAb and 103.01-fold at 1 ng of J93 MAb (Figure 5.10 and Table B. in appendix A).

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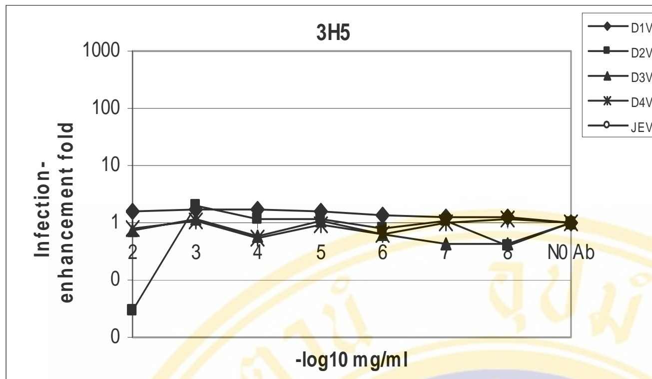
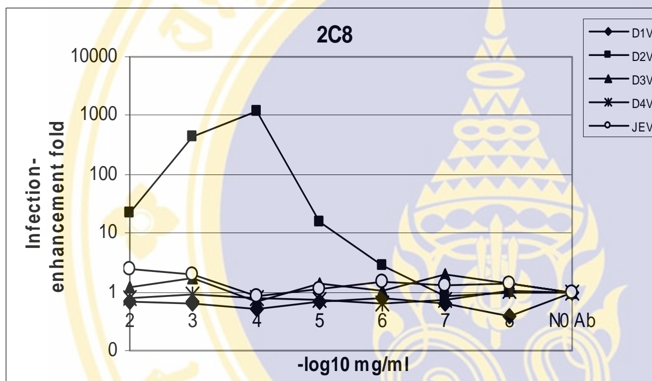
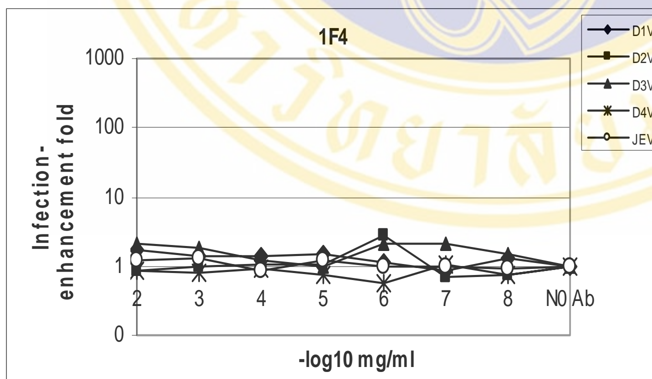


Virus	Ab conc at peak titer (µg/ml)	Peak enhancement fold
D1V	-	-
D2V	-	-
D3V	-	-
D4V	-	-
JEV	-	-



Virus	Ab conc at peak titer (µg/ml)	Peak enhancement fold
D1V	-	-
D2V	0.1	1195
D3V	-	-
D4V	-	-
JEV	-	-



Virus	Ab conc at peak titer (µg/ml)	Peak enhancement fold
D1V	-	-
D2V	-	-
D3V	-	-
D4V	-	-
JEV	-	-

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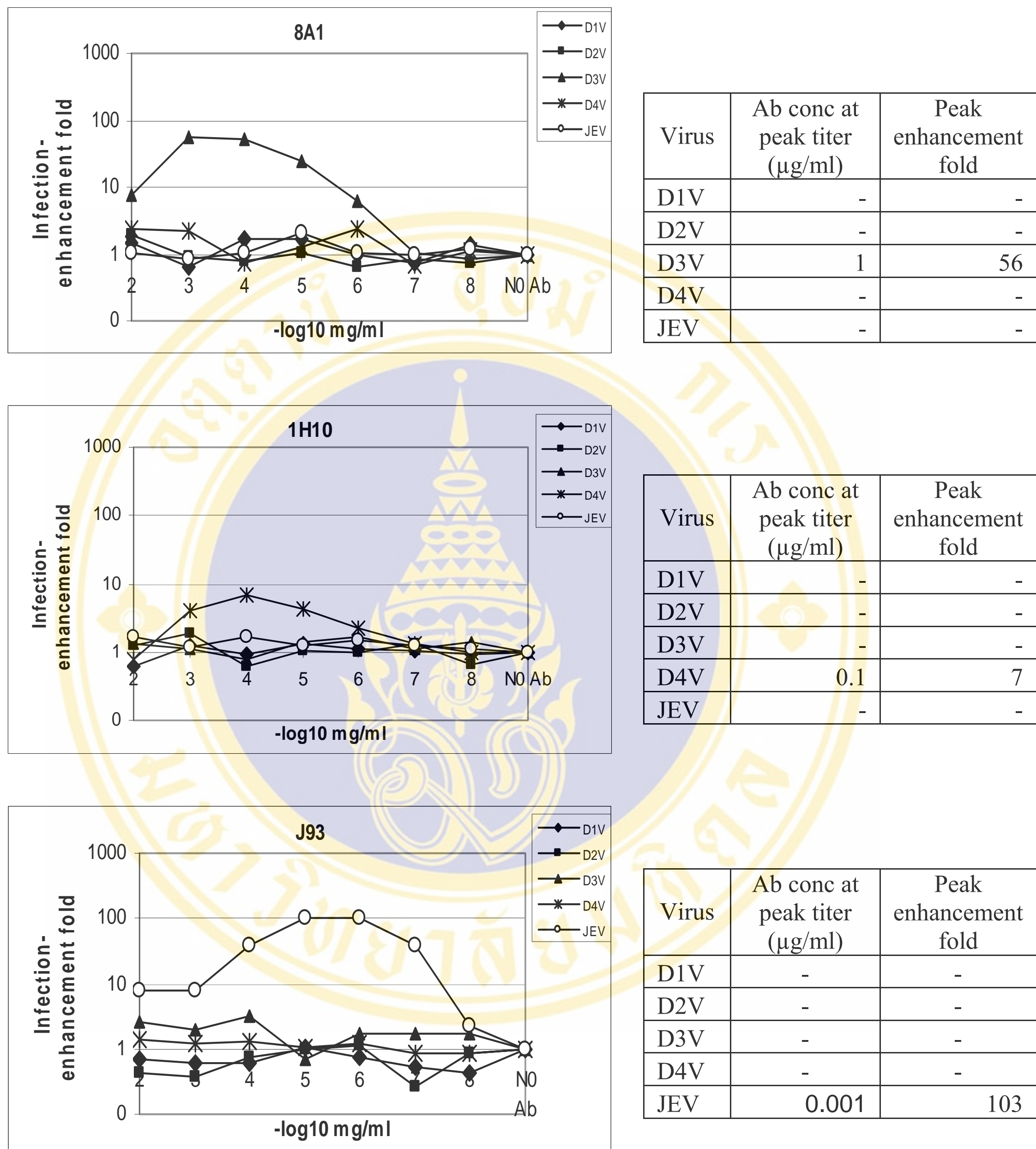

Virus	Ab conc at peak titer (µg/ml)	Peak enhancement fold
D1V	-	-
D2V	-	-
D3V	1	56
D4V	-	-
JEV	-	-

Virus	Ab conc at peak titer (µg/ml)	Peak enhancement fold
D1V	-	-
D2V	-	-
D3V	-	-
D4V	0.1	7
JEV	-	-

Virus	Ab conc at peak titer (µg/ml)	Peak enhancement fold
D1V	-	-
D2V	-	-
D3V	-	-
D4V	-	-
JEV	0.001	103

Figure 5.10 Antibody-dependent enhancement of dengue infection to U937 cells by Dengue-serotype / JEV specific anti-E MAbs.

The figure caption was similar to Figure 5.8. The antibodies included: 3H5, 2C8, 1F4 (for D2V specific), 8A1 (for D3V-specific), 1H10 (for D4V specific) and J93 (for JEV specific).

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CHAPTER VI
DISCUSSION

In this study, an available panel of anti-E MAbs has been determined for their functional activity to *in vitro* neutralization and/or enhancement of virus infection. The assays were performed directly to all four serotypes of dengue virus as well as Japanese encephalitis virus as a representative of other flavivirus members. Twelve anti-E MAbs used in this study were originally produced by either our group or other investigators. Among these, 4G2 and 3H5 have been previously determined for both functional activities (7, 74, 85, 88, 96-98), thus both were used as NT and ADE reference MAbs in this study.

6.1 Large scale production and purification of anti-E MAbs

Large scale production of most MAbs in mouse ascitic fluid indicated the variation of MAb obtained from each clone in term of ascite volume and immunoglobulin concentration. This phenomenon is sometime unpredictable because of various uncontrollable factors such as BALB/c mice lot, mouse environment, etc. However, the most important factor is the ability of hybridoma which produces different level of antibodies. 2B7 is one of the hybridoma clones which could not induce mouse ascitic fluid. We therefore had to scale up this clone in normal cell culture system. Serum-free culture media (SFM) were used instead of general 10%FBS-RPMI media as to minimize the contaminant calf immunoglobulins in the downstream Protein-G purified MAbs. However, since the lower concentration of MAb is released in normal cell culture (about 1-100 µg/ml for generally, but we got 30 µg/ml for 2B7, see table 5.1), comparing to 1-10 mg/ml in ascites, therefore, to produce large scale of MAb at higher concentration (more than 1 mg/ml), a membrane-based cell culture bioreactor is recommended.

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6.2 Verification of purified MAbs properties

Once the purified MAbs were obtained, some necessary properties of MAbs were verified before use in further steps. It is including serotype specific/cross reactivity, E protein specificity and conformation-dependent epitope specificity. Most properties of all MAbs, except 5A1, are concordant between previous reports and this study with some notifications (Table 5.2). In the previous results, 1C2, 1D3 and 5A1 reacted to no dengue protein on western blot, but they neutralized D2V infection by PRNT assay, so they were identified as E-specific MAbs. In this study, we reconfirmed no reactivity with 1C2 and 5A1, but weakly reactive with 1D3 by western blot (Fig. 5.3). 2C8 were previously reported as weakly reactive to E protein by western blot (94), but we could not detect any reactive band in this study, which might be due to the variation of infected cell lysate preparation. However, for all MAbs which weakly or negatively reactive to E by western blot, in this study, we could clearly demonstrate their E-specific property by IFA staining to E or prME transfected cells. It is indicated that those MAbs recognize highly conformational epitope of E which may be lost or disappeared under SDS-treatment. For 5A1, it was reported as D2V specific, but we found cross reactive to D2V and D3V (Table 5.2). The different strains of D3V were used in both studies; CH5 3489 strain for the previous, but H-87 strain was used in this study. It is suggested that variation of virus strains should also be concerned to define virus specificity.

Different to others, 1F4 which was demonstrated as D2V specific MAb by DEIA (Fig. 5.2), reacted to E protein as well as other smaller proteins by western blot (Fig 5.3). These smaller bands were appeared only in dengue infected cell lysate but not in mock infection. This might be speculated that 1F4 may cross react to unknown viral proteins, or degraded E protein, or unknown host proteins which are induced by viral infection. However, 1F4 showed specificity to E by IFA (Fig 5.4). It is not known whether this cross-reactivity may affect to the absence of both functional activities of 1F4 in neutralization and/or enhancement of dengue virus infection in this study or not.

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6.3 Neutralization of virus infection by anti-E MAbs

Neutralization of flavivirus infectivity by antibody specific to viral protein has been documented mainly with anti-E MAbs (7). 3H5 is well-characterized among anti-dengue E MAbs. It was mapped to amino acid residue 386-397 (72) and 383-385 (44) located on domain III of dengue-2 E protein. 3H5 showed *in vitro* neutralizing activity to inhibit D2V infection in various cell types (8, 66, 99). The exact mechanism involved in the protective role of dengue antibody is not fully understood. He *et al* (1995) demonstrated that 3H5 mediated neutralization of dengue virus by blocking virus attachment to monkey kidney cells (Vero), so that no virus entry and membrane fusion to release viral genome for replication in host cells (66). Whereas, Se-Thoe, *et al* (2000) proposed that 3H5 neutralized D2V infection to LLC-MK2 cells by alteration mode as demonstrated by ultrastructure study using transmission electron microscopy. They showed that 3H5 neutralized D2V by coating surrounded virion and these coated virions were allowed to enter the cells via endocytosis, but not via fusion at the plasma membrane as observed in non-neutralized virions. Neutralized virions were trapped within the cellular vesicles, but no uncoating process occurred, and later degraded by lysosome activity (99). Most of neutralizing MAbs which are specific to domain III of dengue E protein can block virus adsorption more efficiently than those to other domains (8). Domain II specific MAbs neutralized virus infection partly by inhibition of virus binding, but blocking of the fusion step was observed (8). In this study, most of anti-E MAbs has not been identified for specific domain yet. Besides 3H5 (D2V-specific), only 8A1 (D3V-specific) and 2C8 (D2V-specific) were mapped to domain III of E protein (Mongkolsapaya J, unpublished data), whereas 4G2 (Flavi-cross reactive) was published as multi-epitope specific on domain III (E349-359) and hinge region of domain I and II (E274-283) (100). It is noticed that most MAbs, except 1C2, 5A1 and 1F4, exhibited neutralizing activity to each virus serotype correlating to their binding properties, though very low efficient NT activity of 2B7 was obtained in JEV infection (Table 6.1). For cross-reactive MAbs, NT activities to D2V were more efficient than those to other serotypes. This might be due to the generation of these MAbs were by D2V-based immunization. It is not much surprised that 1C2 neutralized only D2V since D1V and D3V were weakly reacted by this MAb. 1F4 exhibited non-neutralizing activity,

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though it is D2V-specific. It is suggested that 1F4 may bind to the epitope not responsible for virus entry or fusion process. Domain-I specific anti-E MAbs are of the examples (8). For 5A1, the pattern of neutralization is unusual, dose-independent (Fig 5.5). Since 5A1 neutralized only D2V, so the possibility of toxicity to the cells would be excluded. Further study for the mechanism of 5A1-mediated neutralization would also be of interest.

6.4 *In vitro* enhancement of virus infection by anti-E MAbs

In this study, ADE was observed in U937 cell lines with both cross reactive and serotype-specific MAbs. Three Flavivirus-cross reactive MAbs (4G2, 2B7 and 1D10) enhanced all five tested viruses, whereas dengue-cross reactive MAbs (1D3, 5A1, and 1C2) enhanced only some specific serotypes. Four serotype-specific (2C8, 8A1 and 1H10) and JE-specific (J93) MAbs enhanced infection of correlated virus serotype. However, 3H5 and 1F4 (D2V-specific) MAbs showed no ADE to D2V infection. Previous ADE studies have been reported the enhancement of virus production in the presence of anti-E MAbs into either of two Fc receptor bearing cell lines, human monocyte (U937) or mouse macrophage (P388D1). Henchal found that infection enhancement of D2V (strain NGC) in U937 cells was MOI dependent, and at low MOI (0.005-0.01) it was restricted to flavivirus group-reactive MAbs and was not seen with serotype specific MAbs, including 3H5. (7). However, Halstead *et al* (1984) has identified infection enhancement of seven D2V strains by a panel of MAbs in P388D1 cell lines. He found ADE to be a property of antibody interactions with both DEN-complex and serotype-specific determinant. Five tested MAbs exhibited ADE to most of D2V strains, except 3H5 MAb (to PR 159 and NGC strains) and 4G2 MAb (to PR159, NGC, AHF110-80 and AHF191-80 strains) (88). D2V -16681 strains could be infection-enhanced into P388D1 in the presence of 3H5 and 4G2 (88). It is notified that ADE determinant on different strains of the same serotype may be varied. This notification was further confirmed by Moren *et al* (1987). They demonstrated that two of 19 D2V-isolates, PUO-280 and D80-038, could not be infected enhancement to in P388D1 cells by 3H5 (89). In addition to virus strain, cell type that was used in ADE study may be one of the factors to be concerned. Halstead

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(1984), which studied in P388D1 cells, found no ADE to D2V-NGC by 4G2 (88). Whereas, 4G2 was found to enhance D2V-NGC infection significantly in U937 by Brandt (1982) (85) and Henchal (1985) (7), as well as this study.

In this study, we found similar ADE negative result of 3H5 to D2V-NGC strain as previously reports (7, 85, 88). However, we have further performed ADE of D2V with different strain-16681 using 3H5 and other two MAbs, 2C8 (ADE positive to D2V-NGC) and 1D3 (ADE negative to D2V-NGC). We found that three of them could enhance D2V infection in U937 (Fig 6.1). The result consistency of ADE by 3H5 suggested the different ADE-mediated mechanisms by 3H5 to both strains (NGC and 16681). One of the possible mechanisms may be relevant to the different avidity of 3H5 to both D2V strains. Once the virus-Ab complex was endocytosed through Fc receptor, internal acidic environment may cause dissociation of the complex, and allows the virus fused to the membrane. Virus strain that binds with high avidity to 3H5 may not be released in the acidic endosome, so that the membrane fusion could not be occurred, thus no replication as a consequence. If this hypothesis is correct, 3H5 may bind to NGC with higher avidity than that to 16681 strain. However, more experiments should be performed to address this hypothesis.

1F4 was also found to have no ADE to D2V-NGC strain in U937 cells. As variation of strains may affect to ADE by some MAbs, so it could not exclude the possibility that 1F4 may retain ADE functional activity to other strains. Therefore, ADE of 1F4 with D2V-16681 should be further performed to address this suspicion. In addition, as mentioned above that 1F4 reacted to unknown proteins smaller than E as detected by western blot, we do not know whether or not these unknown proteins may cause somehow interrupt both ADE and/or NT activity of 1F4.

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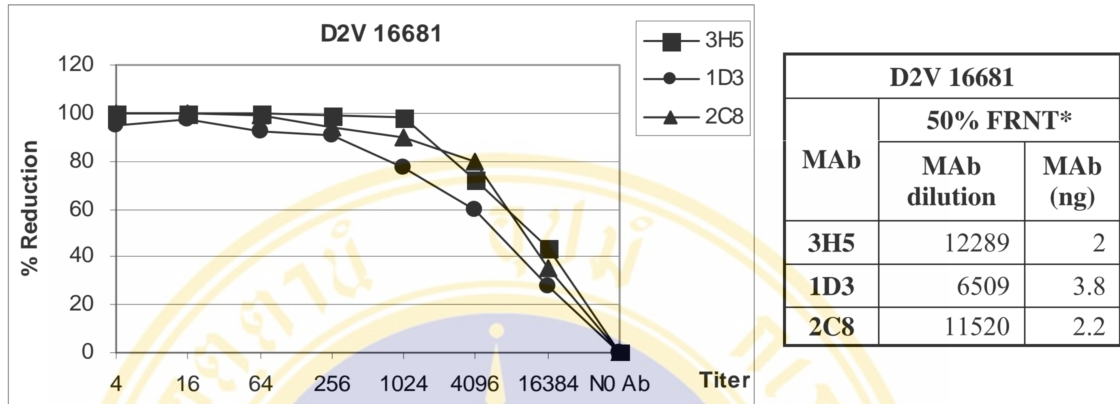
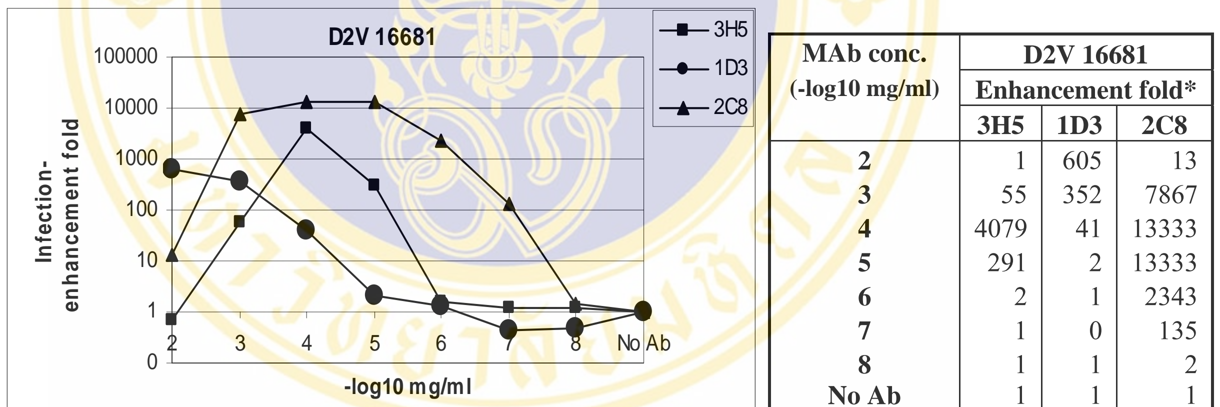
A.



MAb	D2V 16681 50% FRNT*	
	MAb dilution	MAb (ng)
3H5	12289	2
1D3	6509	3.8
2C8	11520	2.2

B.



MAb conc. (-log10 mg/ml)	D2V 16681 Enhancement fold*		
	3H5	1D3	2C8
2	1	605	13
3	55	352	7867
4	4079	41	13333
5	291	2	13333
6	2	1	2343
7	1	0	135
8	1	1	2
No Ab	1	1	1

Figure 6.1 Neutralization and enhancement activities of anti-E MAbs to D2V 16681 strain. A) FRNT assay, B) ADE assay. 3H5 (D2V-specific), 1D3 (DV-cross reactive) and 2C8 (D2V-specific) MAbs were used.

*The results were obtained from only one experiment.

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6.4 Concordance / discordance of neutralizing and infection-enhancement activity of anti-E MAbs

In this study, both functional activities, NT and ADE, of each MAbs to each dengue serotypes and/or JEV were determined. Concordance or discordance of both activities could be classified into four groups as shown in Table 6.1.

6.4.1 Positive to neutralizing and enhancement activity (NT +, ADE +)

This group represents MAbs which retain both NT and ADE activity to all particular viruses accordingly. It contains both Flavivirus-cross reactive MAbs (4G2, 2B7 and 1D10) and serotype-specific MAbs (2C8, 8A1, 1H10 and J93). At lower dilution of MAb, virus could be neutralized, whereas at higher dilution (sub-neutralizing titer) of MAb, infection enhancement of virus could be appeared. 4G2, as a reference MAb, has been identified for both NT and ADE activities only to D2V, while other dengue serotypes, only NT activity were identified (85, 88, 98). So, this study is a first report demonstrated both NT and ADE activities of 4G2 against four serotypes of dengue viruses as well as JEV.

6.4.2 Positive to neutralization but no enhancement activity (NT +, ADE -)

This group represents MAbs which retain NT activity but no specific correlated serotype ADE activity. It contains D2V serotype specific MAb, 3H5, and dengue-cross reactive MAb, 1D3. 3H5, the other reference MAb in this study, has been identified for NT and ADE by several research groups (7, 74, 85, 88, 96-98). As it is D2V-specific, most of functional studies therefore directed to dengue serotype 2. In this study, we have confirmed its NT activity specific to D2V, not to others. The observation that 3H5 has no ADE activity to NGC strain, but do retain in 16681 strain, was consistent to previous reports as discussed above. 1D3 showed ADE to D1V and D3V, but not completely related to NT activity in D1V to D4V.

The studies of MAbs against West Nile virus shown that one strong neutralizing MAb which had no ADE activity was specific to domain III (101). This MAb inhibits West Nile virus infection primarily at a step after viruses attach which potentially by blocking envelope glycoprotein conformational changes (80). According to WNV study, 3H5 may bind to D2V-NGC E epitope involved in the conformational changes for fusion step, but this may has no effect to D2V-16681 strain. It is still interesting to further investigate for the different ADE mechanisms of

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3H5 between the two strains. Besides the strain variation effect, this MAb group is of interest since the recognition determinants or epitopes may be contributed to vaccine development by the capability of inducing Ab which always neutralize but not enhance virus infection.

6.4.3 No neutralization but positive to enhancement activity (NT -, ADE +)

This group represents MAbs which retain ADE activity but no specific correlated serotype NT activity. It contains dengue-cross reactive MAbs, 1C2 and 5A1. Both MAbs showed NT and ADE activity to D2V, whereas 1C2 enhanced, but not neutralized D1V infection, and 5A1 enhanced, but not neutralized D3V infection. This might be explained that the binding epitope to other serotypes (except D2V), may not responsible for virus attachment, so that the Ab could not neutralize virus infection, but at sub-neutralizing titer of Ab, virus-Ab complexes could be internalized into U937 cells via Fc receptor.

6.4.4 Negative to neutralization and enhancement activity (NT -, ADE -)

This group represents a MAb which have neither NT activity nor ADE activity. It contains only dengue-serotype 2 specific MAb, 1F4. It is not surprised for non-neutralizing activity of 1F4 since it may recognize epitope that is not important for virus attachment or fusion process. However, at low antibody titer, it could bind to E protein of virion and mediated internalization to host cells via Fc receptor. The absence of ADE by 1F4 might be strain dependent, and need to be further investigated. In addition, viral proteins cross reactivity should be concerned as discussed above that may be involved to NT and ADE activities.

In this study, we have produced a set of functional data of anti-E MAbs which including both reference MAbs (4G2, 3H5, 8A1, 1H10 and J93) and our generated MAbs (2B7, 1D10, 2C8, 1D3, 1C2, 5A1 and 1F4). Some of reference MAbs (4G2 and 3H5) have been identified for NT or ADE activities to dengue virus, but not to all serotypes nor JEV. Whereas other reference MAbs (8A1, 1H10 and J93) have no data of both functional activities yet. Comprehensive data obtained in this study for the reference and our generated MAbs were therefore beneficial to confirm and add up new information of their NT and ADE activities to all four serotypes of dengue and JEV which are important to the field. The epitopes or

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molecular mechanisms relevant to neutralization but not enhancement would be one of the targets for dengue vaccine or drug development.

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Table 6.1 Summary of reactivity, neutralizing activity and enhancement activity of anti-E MAbs

Group	Anti-E MAbs [a]	Reactivity [b]					NT [c]					ADE [d]				
		D1	D2	D3	D4	JE	D1	D2	D3	D4	JE	D1	D2	D3	D4	JE
I (NT+,ADE+)	**4G2**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2B7	+	+	+	+	+	+	+	+	+	(+)	+	+	+	+	+
	1D10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2C8	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-
	8A1	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-
	1H10	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-
	J93	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+
II (NT +,ADE -)	**3H5**	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
	1D3	+	+	+	+	-	+	+	+	+	-	+	-	+	-	-
III (NT -,ADE +)	1C2	(+)	+	(+)	-	-	-	+	-	-	-	+	+	-	-	-
	5A1	-	+	+	-	-	-	*	-	-	-	-	+	+	-	-
IV (NT -, ADE -)	1F4	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

a: MAbs in bold represent reference antibodies.

b: Determined by DEIA, +; Positive, -; Negative, (+); Weak positive

c: +; 50% FRNT titer can be determined, (+); % Reduction of foci less than 50%,

* ; % Reduction of foci more than 50% over all tested Ab dilution

d: +; peak of infection-enhancement fold is more than 2

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CHAPTER VII
CONCLUSION

Envelope is one of the major dengue virion surface proteins responsible for virus attachment and fusion to susceptible cells as well as induction of host protective immunity. Production of antibody caused by virus infection is one of defensive mechanisms to neutralize virus. On the other hand, antibody at subneutralizing level could, in turn, enhance virus infection. MAbs to E protein of dengue virus have been shown to possess both neutralization and enhancement of virus infection at different level of antibody concentration.

We have previously generated a panel of anti-dengue E MAbs in our lab. Though the antigenic and some properties were identified but the functional properties have not yet known. In this study, we therefore aimed to determine the neutralizing as well as enhancement activity of 12 anti-E MAbs to 4 serotypes of dengue virus and Japanese encephalitis virus infection. They are included 7 generated anti-E MAbs (2B7, 1D10, 2C8, 1D3, 1C2, 5A1 and 1F4) and 5 referenced anti-E MAbs (4G2, 3H5, 8A1, 1H10 and J93). All anti-E MAbs were large-scale preparation in mouse ascitic fluid, except 2B7 which were produced in normal cell-culture in serum-free media. The MAbs were purified by Protein-G affinity chromatography. The purified MAbs were verified for their specificity/cross reactive to dengue serotypes by DEIA. Three groups were identified, i.e. flavivirus-cross reactive group (4G2, 2B7 and 1D10), dengue-cross reactive group (1C2, 1D3, 5A1) and dengue serotype/JE specific group (3H5, 8A1, 1H10, 2C8, 1F4, J93). Specificity to E protein of all purified MAbs was also verified by either western blot analysis and/or IFA.

Anti-E MAbs were analyzed for neutralizing activity in Ps clone D cells. Dengue virus of 4 serotypes as well as JEV at 50 ffu was mixed with 4-fold serial dilution of purified anti-E MAbs before infection to Ps clone D cells. MAbs which caused reduction of infectious foci as dose-dependent manner were identified as neutralizing antibody. The 50%FRNT titer were determined by a dilution of MAbs

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that reduced infectious virus foci to 50%. Most of anti-E MAbs have neutralizing activity to dengue serotypes or JE which they are antigenically specific, except 5A1 (which neutralized dengue 2 in dose-independent manner) and 1F4 (which showed no neutralizing activity). 3H5 and 2C8 gave the most efficient NT activity which 50%FRNT titers were less than 1 nanogram in this study.

For antibody-dependent enhancement (ADE) activity, anti-E MAbs were analyzed in the Fc receptor-bearing cells, U937. All dengue serotypes as well as JEV were mixed with 10-fold serial dilution of anti-E MAbs before infection to U937 cells. Virus progeny were harvested and their titers were determined in Ps cells. ADE activity of MAbs at each dilution were demonstrated as infection-enhancement fold, which was defined as the ratio of virus titer in the presence of antibody and the virus titer in the absence of antibody. Dengue serotypes/JEV infection in U937 cells were enhanced by flavi-cross reactive anti-E MAbs (4G2, 2B7 and 1D10) and most of dengue serotype/JE specific anti-E MAbs (2C8, 8A1, 1H10 and J93). Some dengue cross reactive anti-E MAbs (1D3 and 1C2) enhanced only some dengue serotypes, but not all serotypes which they are antigenically specific. 2C8 gave upto 1195-fold of dengue 2 infection enhancement in U937 cells, highest among other MAbs. Two anti-E MAbs were found no enhancement to dengue 2 infection, i.e. 3H5 and 1F4. However, our further investigation demonstrated that 3H5 can enhance dengue 2 strain 16681, but not NGC, in U937 cells, indicating that ADE activity of 3H5 is strain-dependent which confirmed with the previous reports by Halstead *et al* (1984) and Moren *et al* (1987).

In conclusion, we produced a set of *in vitro* functional data, NT and ADE activities, of anti-E MAbs in all serotypes of dengue virus as well as JEV in this study. According to concordance/discordance of both functional activities, twelve anti-E MAbs were classified into 4 groups. They are including;

Group I: Positive to neutralizing and enhancement activity (NT +, ADE +). It contains both Flavivirus-cross reactive MAbs (4G2, 2B7 and 1D10) and serotype-specific MAbs (2C8, 8A1, 1H10 and J93).

Group II: Positive to neutralization but no enhancement activity (NT +, ADE -). It contains D2V serotype specific MAb, 3H5, and dengue-cross reactive MAb, 1D3.

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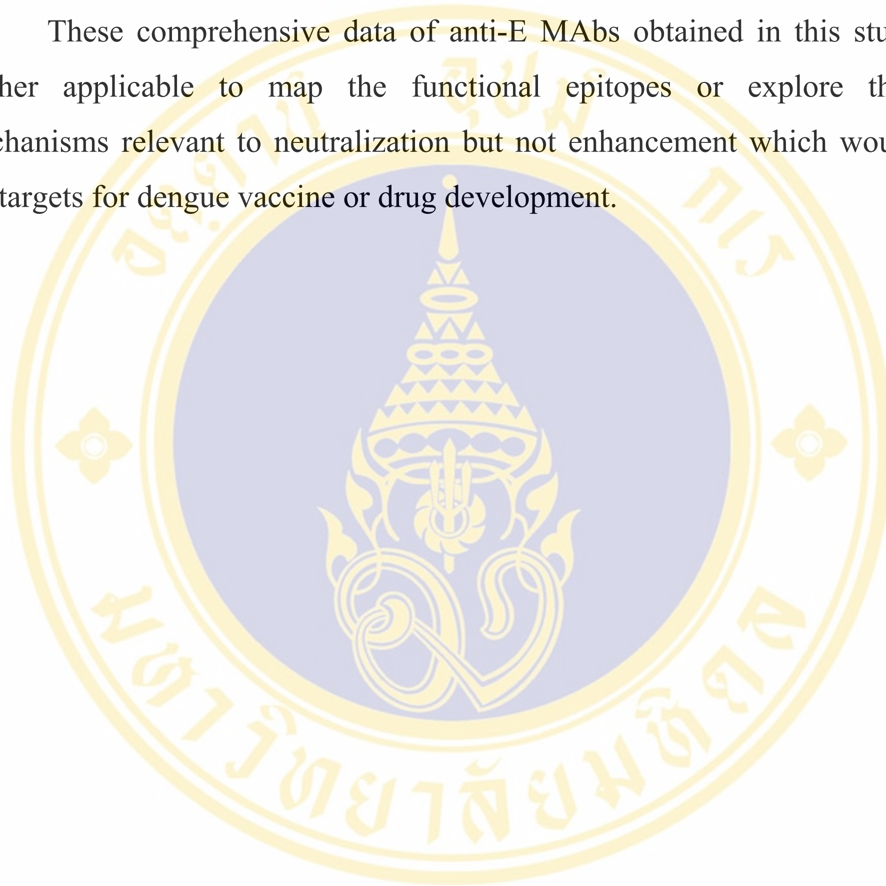
Group III: No neutralization but positive to enhancement activity (NT -, ADE +). It contains dengue-cross reactive MAb, 1C2 and 5A1.

Group IV: Negative to neutralization and enhancement activity (NT -, ADE -). It contains only dengue-serotype 2 specific MAb, 1F4.

These comprehensive data of anti-E MAbs obtained in this study would be further applicable to map the functional epitopes or explore the molecular mechanisms relevant to neutralization but not enhancement which would be one of the targets for dengue vaccine or drug development.



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APPENDIX

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Appendix A

Table A. Data of neutralizing activity of anti – E MAbs

4G2 (D1-4, JEV)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	99.22	100.00	100.00	100.00	96.17
16	98.59	100.00	100.00	99.67	86.71
64	91.37	100.00	100.00	98.83	67.79
256	70.49	95.40	89.77	87.38	42.35
1024	38.78	70.11	46.97	48.85	15.10
4096	14.29	39.56	14.15	15.42	-9.45
16384	2.67	16.90	7.50	5.56	0.01
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**633.87**	**2937.25**	**1205.98**	**1135.01**	**148.79**
Ab (ng)	**39.44**	**8.51**	**20.73**	**22.03**	**168.02**

3H5 (D2V)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	16.91	100.00	-9.07	-2.74	13.92
16	6.13	100.00	2.68	6.51	26.12
64	12.61	100.00	0.76	11.30	4.11
256	17.17	99.67	2.98	4.17	6.31
1024	20.21	97.37	0.45	-3.86	7.31
4096	26.01	89.16	-7.17	1.27	2.11
16384	6.08	61.24	-0.20	-4.64	9.31
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**0.00**	**26908.16**	**0.00**	**0.00**	**0.00**
Ab (ng)		**0.93**			

8A1 (D3V)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	4.67	13.88	99.86	6.13	10.14
16	6.09	11.44	99.57	4.92	5.61
64	22.78	11.57	96.83	5.61	0.44
256	19.10	19.64	82.73	2.51	1.09
1024	17.97	17.72	2.59	-7.50	4.97
4096	21.08	6.06	8.06	-1.98	7.55
16384	25.04	3.62	4.17	-1.98	10.14
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**0.00**	**0.00**	**499.38**	**0.00**	**0.00**
Ab (ng)			**50.06**		

1H10 (D4V)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	18.29	9.14	-11.61	99.14	2.45
16	9.08	12.22	6.11	95.45	-12.18
64	4.11	14.59	20.28	76.41	-8.00
256	17.57	23.14	-5.42	59.95	6.63
1024	12.38	20.05	-10.74	32.47	8.03
4096	13.33	13.89	-16.93	13.86	-2.42
16384	16.63	11.27	4.33	-9.31	3.85
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**0.00**	**0.00**	**0.00**	**399.23**	**0.00**
Ab (ng)				**62.62**	

J93 (JEV)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	28.52	5.84	-4.60	-4.70	91.93
16	21.15	15.57	2.12	4.31	91.93
64	23.33	12.71	14.68	-7.70	74.00
256	25.52	11.92	6.94	5.11	83.86
1024	24.97	9.98	16.15	8.31	66.82
4096	20.60	10.71	11.18	-3.30	39.92
16384	5.87	-5.35	7.67	-6.50	28.26
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**0.00**	**0.00**	**0.00**	**0.00**	**2592.85**
Ab (ng)					**9.64**

2B7 (D1-4, JEV)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	78.99	100.00	93.57	99.60	40.82
16	74.90	100.00	66.25	98.20	40.84
64	50.62	99.76	10.01	93.19	35.44
256	32.58	99.03	-16.43	85.99	51.58
1024	31.25	88.81	-14.09	61.36	41.71
4096	16.78	71.53	-5.62	13.92	30.06
16384	6.42	39.90	1.83	2.51	7.64
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**94.60**	**10478.44**	**24.41**	**1051.57**	**0.00**
Ab (ng)	**264.27**	**2.39**	**1024.17**	**23.77**	

* The percentage of reduction was calculated from three separately experiments.

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Appendix A

Table A. Data of neutralizing activity of anti – E MAbs (continue)

1F4 (D2V)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	4.20	11.66	-9.74	-1.24	11.17
16	-7.11	19.53	14.44	-2.84	14.60
64	15.51	9.95	21.15	2.91	-1.80
256	7.22	13.03	8.49	-4.22	5.57
1024	18.16	14.90	8.29	-6.52	4.87
4096	15.51	14.39	2.73	-5.14	13.33
16384	18.53	0.72	-1.68	-23.55	5.46
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Ab (ng)					

1D10 (D1-4, JEV)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	96.02	100.00	100.00	100.00	80.93
16	87.76	100.00	100.00	99.58	72.37
64	58.98	94.31	95.01	94.82	50.75
256	30.25	75.04	55.77	58.88	33.19
1024	10.58	56.64	11.30	11.49	11.57
4096	12.25	36.05	-8.44	-4.30	-1.35
16384	13.77	11.52	4.17	-8.23	-8.23
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**151.65**	**1512.53**	**361.08**	**315.41**	**62.84**
Ab (ng)	**164.85**	**16.53**	**69.24**	**79.26**	**397.84**

1C2 (D1-3)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	8.96	99.82	38.88	-0.65	-3.12
16	20.65	98.40	26.92	-3.41	-1.03
64	16.93	93.95	21.61	-4.22	-7.30
256	16.04	80.43	32.90	-5.36	-1.73
1024	18.52	61.39	24.26	-13.31	1.76
4096	22.24	36.48	15.63	-3.90	16.39
16384	16.40	15.84	10.98	-3.25	5.94
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**0.00**	**1858.53**	**0.00**	**0.00**	**0.00**
Ab (ng)		**13.45**			

1D3 (D1-4)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	86.85	100.00	98.83	99.80	17.50
16	79.35	100.00	97.08	98.80	16.32
64	75.58	99.36	92.60	96.20	25.36
256	41.77	92.55	78.38	91.39	15.10
1024	0.48	75.03	50.13	71.57	19.91
4096	5.15	42.25	22.87	44.15	17.50
16384	14.56	14.03	12.54	14.12	12.73
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**130.08**	**2940.70**	**1110.96**	**2739.82**	**0.00**
Ab (ng)	**192.19**	**8.50**	**22.50**	**9.12**	

5A1 (D2-3)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	16.57	53.38	-14.93	-12.66	-3.82
16	16.22	59.08	-11.61	1.30	-3.12
64	9.13	56.41	-0.98	-1.95	7.33
256	21.53	59.43	-6.30	2.27	3.15
1024	10.73	65.30	-0.32	-3.90	6.63
4096	14.62	73.31	12.97	8.60	6.63
16384	4.35	65.30	-22.97	-11.85	-1.73
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Ab (ng)					

2C8 (D2V)	%Reduction *				
	D1V	D2V	D3V	D4V	JEV
4	-0.70	99.62	8.78	6.99	-1.50
16	5.24	99.36	2.59	2.85	3.67
64	17.68	97.18	2.45	1.13	6.26
256	4.96	95.26	12.23	-2.84	-3.44
1024	9.76	91.80	10.79	-0.77	6.26
4096	18.25	81.42	10.50	2.51	8.84
16384	19.10	60.53	-7.05	-4.40	-0.85
No Ab	0.00	0.00	0.00	0.00	0.00
50% FRNT	**0.00**	**53989.61**	**0.00**	**0.00**	**0.00**
Ab (ng)		**0.46**			

* The percentage of reduction was calculated from three separately experiments.

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Appendix A

Table B. Viral titer of enhancement activity of anti – E MAbs

MAb conc. (-log$_{10}$ mg/ml)	4G2 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	0.50	0.07	0.72	0.66	2444.44
3	31.77	33.36	9.34	1.94	4200.00
4	41.66	72.98	4.55	1.32	5522.22
5	4.04	2.11	0.69	1.79	3744.44
6	0.40	0.29	0.13	0.57	971.86
7	0.26	0.19	0.09	0.37	73.42
8	0.21	0.22	0.11	0.33	35.89
No Ab	0.20	0.21	0.09	0.26	35.89

MAb conc. (-log$_{10}$ mg/ml)	1H10 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	0.26	0.42	0.16	0.46	37.80
3	0.53	0.63	0.13	2.39	26.10
4	0.39	0.21	0.09	4.09	38.20
5	0.55	0.35	0.17	2.64	28.20
6	0.46	0.33	0.20	1.34	34.50
7	0.45	0.45	0.14	0.80	28.20
8	0.38	0.22	0.17	0.60	25.20
No Ab	0.42	0.34	0.12	0.61	22.70

MAb conc. (-log$_{10}$ mg/ml)	3H5 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	0.45	0.01	0.10	0.89	9.73
3	0.48	0.34	0.16	1.18	9.36
4	0.48	0.20	0.08	0.62	5.91
5	0.48	0.20	0.15	1.02	5.82
6	0.39	0.14	0.09	0.71	7.64
7	0.38	0.19	0.06	1.07	11.70
8	0.36	0.07	0.06	1.25	6.91
No Ab	0.29	0.17	0.14	1.11	5.36

MAb conc. (-log$_{10}$ mg/ml)	J93 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	0.25	0.11	0.16	0.99	518.56
3	0.21	0.10	0.13	0.91	535.33
4	0.22	0.19	0.20	0.93	2592.22
5	0.37	0.25	0.05	0.77	6622.22
6	0.27	0.30	0.11	0.90	6922.22
7	0.19	0.07	0.11	0.65	2544.56
8	0.15	0.22	0.11	0.64	149.07
No Ab	0.36	0.26	0.06	0.74	67.20

MAb conc. (-log$_{10}$ mg/ml)	8A1 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	0.21	0.57	0.73	0.39	36.90
3	0.09	0.27	5.48	0.35	29.70
4	0.24	0.23	4.99	0.12	37.30
5	0.23	0.31	2.40	0.21	71.80
6	0.14	0.19	0.59	0.39	37.80
7	0.10	0.25	0.10	0.11	33.30
8	0.19	0.21	0.08	0.18	40.90
No Ab	0.14	0.29	0.10	0.16	35.50

MAb conc. (-log$_{10}$ mg/ml)	2B7 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	33.84	0.05	7.36	0.37	4422.22
3	31.73	0.48	2.58	0.49	1684.22
4	8.23	2.36	0.45	0.55	117.82
5	0.36	0.24	0.13	0.28	49.03
6	0.33	0.23	0.09	0.13	37.73
7	0.33	0.15	0.10	0.29	28.52
8	0.29	0.11	0.09	0.16	29.79
No Ab	0.33	0.16	0.08	0.15	42.88

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Appendix A

Table B. Viral titer of enhancement activity of anti – E MAbs (continue)

MAb conc. (-log$_{10}$ mg/ml)	1D10 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	10.11	0.35	2.71	0.96	4144.44
3	13.69	4.83	4.25	2.53	4377.78
4	3.25	1.54	1.11	1.92	2597.00
5	0.36	0.25	0.12	0.66	212.72
6	0.31	0.13	0.06	0.69	8.24
7	0.30	0.09	0.07	0.55	78.99
8	0.27	0.12	0.08	0.58	79.30
No Ab	0.28	0.11	0.09	0.50	73.52

MAb conc. (-log$_{10}$ mg/ml)	1D3 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	6.45	0.26	6.66	0.31	41.80
3	0.59	0.15	0.76	0.08	25.50
4	0.24	0.12	0.17	0.11	17.30
5	0.30	0.17	0.13	0.24	29.10
6	0.27	0.25	0.09	0.24	19.10
7	0.29	0.24	0.10	0.25	20.90
8	0.24	0.09	0.09	0.23	33.60
No Ab	0.29	0.20	0.08	0.23	38.20

MAb conc. (-log$_{10}$ mg/ml)	1F4 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	0.46	0.18	0.17	0.57	68.50
3	0.37	0.22	0.15	0.55	76.00
4	0.36	0.23	0.10	0.61	50.00
5	0.38	0.23	0.08	0.50	68.50
6	0.29	0.59	0.17	0.36	57.30
7	0.23	0.15	0.17	0.72	58.20
8	0.35	0.16	0.12	0.48	53.60
No Ab	0.26	0.22	0.08	0.67	57.30

MAb conc. (-log$_{10}$ mg/ml)	5A1 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	0.12	0.33	2.05	0.41	35.00
3	0.14	9.18	4.91	0.41	38.70
4	0.12	128.99	5.05	0.31	29.70
5	0.20	0.69	1.00	0.40	43.20
6	0.13	0.25	0.40	0.37	38.70
7	0.18	0.12	0.23	0.31	36.90
8	0.21	0.23	0.13	0.35	43.20
No Ab	0.17	0.15	0.16	0.44	37.80

MAb conc. (-log$_{10}$ mg/ml)	1C2 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	12.23	0.13	0.15	0.69	55.90
3	53.86	7.45	0.14	0.91	43.60
4	18.10	13.71	0.12	0.48	34.20
5	1.30	2.45	0.09	0.71	38.70
6	0.33	0.34	0.14	0.80	45.90
7	0.32	0.11	0.09	0.83	32.70
8	0.30	0.06	0.11	0.48	26.40
No Ab	0.25	0.11	0.11	0.79	38.70

MAb conc. (-log$_{10}$ mg/ml)	2C8 Virus titer (x10^3 ffu/ml)				
	D1V	D2V	D3V	D4V	JEV
2	0.21	4.26	0.07	0.61	57.30
3	0.19	83.41	0.10	0.71	45.50
4	0.16	222.40	0.04	0.61	20.00
5	0.21	2.97	0.08	0.54	27.30
6	0.24	0.53	0.06	0.47	35.50
7	0.19	0.16	0.12	0.55	30.00
8	0.12	0.18	0.08	0.77	32.70
No Ab	0.31	0.19	0.06	0.76	23.60

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APPENDIX B

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
Calcium chloride dehydrate ($CaCl_2.2H_2O$)	147.02	E. Merck, Germany
Carboxymethyl cellulose (CMC)		Sigma, USA
3, 3-Diaminobenzidine tetrahydrochloride, anhydrous (DAB)	360.1	Sigma, USA
Disodium hydrogen phosphate anhydrous (Na_2HPO_4)	141.96	E. Merck, Germany
Dulbecco's modified eagle medium	-	GibcoBRL
Ethylenediaminetetraacetic acid ($EDTA.Na_2.2H_2O$)	372.24	USB, USA
Fetal bovine serum		GibcoBRL,
Formaldehyde 40% m/v (HCOH)	30.026	Carlo Erba, Milan, Italy
L-Glutamine		Sigma, USA
Glycine ($H_2NCH_2CO_2H$)	75.27	USB, USA
HEPES (no sodium salt)	238.3	Sigma, USA
Hybridoma-SFM (Serum-free Hybridoma medium)	-	Gibco, Invitrogen Corporate, USA

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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
Leibovitz-15 medium (L-15)	-	GibcoBRL
ß-Mercaptoethanol ($HSCH_2CH_2OH$)	78.13	Fluka, Italy
Nickel chloride hexahydrate($NiCl_2.6H_2O$)	237.7	Sigma, USA
Paraformaldehyde		Sigma, USA
Penicillin ($C_{16}H_{17}N_2O_4SNa$) 6130 U/mg	356.4	Sigma, USA
Polyoxyethylenesorbitan monolaurate (Tween 20)	-	Sigma
Potassium chloride (KCl)	74.56	E. Merck, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	136.09	E. Merck, Germany
RPMI medium1640	-	GibcoBRL
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 ($C_{12}H_{25}O_4SNa$)	288.38	Sigma, USA
Streptomycin sesquisulfate 750U/mg	-	Sigma, USA
N,N,N’,N’-Tetramethyl ethylene-Diamine or TEMED ($C_4H_{11}NO_3$)	166.21	Bio-Rad Laboratories, USA
Tris (Hydroxymethyl aminomethane)	121.10	Sigma, USA
Triton X-100	-	Fluka, Italy
Trypan blue stain	-	GibcoBRL
Trypsin (1:250)	-	GibcoBRL
Tryptose phosphate broth	-	Sigma, USA

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2. Consumable Supplies

2.1 1.5 ml Microcentrifuge tubes, Treff, Switzerland.

2.2 0.6 ml Microcentrifuge tubes, Treff, Switzerland

2.3 15 ml centrifuge tube, Costar, Coring Incorporated, Coring, NY, USA.

2.4 50 ml centrifuge tube, Costar, Coring Incorporated, Coring, NY, USA.

2.5 2 ml Biofreeze vial, Costar, Cambridge, MA, USA.

2.6 6-well cell culture clusters flat bottom with lid 3524, Costar, Coring Incorporated, Coring, NY, USA.

2.7 24-well cell culture clusters flat bottom with lid 3524, Costar, Coring Incorporated, Coring, NY, USA.

2.8 96-well cell culture clusters flat bottom with lid 3599, Costar, Coring Incorporated, Coring, NY, USA.

2.9 25-cm2 cell culture flask phenolic style cap 3055, Coring Incorporated, Coring, NY, USA.

2.10 75 cm2 cell culture flask phenolic style cap 3055, Coring Incorporated, Coring, NY, USA.

2.11 162 cm2 cell culture flask phenolic style cap 3055, Coring Incorporated, Coring, NY, USA.

2.12 Cellulose acetate filter membrane pore size 0.2 µm, Sartorius, Goettingen,Germany.

2.13 Cellulose acetate filter membrane pore size 0.45 µm, Sartorius, Goettingen,Germany

2.13 PROTRAN ® Nitrocellulose Transfer Membrane, Schleicher & Schuell BioScience, Germany

2.14 HiTrap™ Protein G HP 1ml column, Amersham Biosciences AB, Sweden

3 Instruments

3.1 ACTAprime, Amersham Biosciences AB, Sweden

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

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

3.4 Beckman Microfuge E, Califonia,USA.

3.5 Biofreezer (-70°C), Forma Scientific, Marietta, Ohio, USA.

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3.6 Bio-Rad, Econo Systems, Japan.

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

3.8 Confocal Microscopy, Zeiss LSM 510 META, Germany.

3.9 Vacuum pump, Sartorius, Gottingen, Germany.

3.10 Digital camera, Nikon, coolpix 950, Japan.

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

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

3.13 Filter Sterilization Unit, Sartorius, Gottingen, Germany.

3.14 Fluorescent microscope, Zeiss, Oberkochn, Germany.

3.15 Freezer (-20°C), Sanyo Medical freezer model MDF 0535, Sanyo Electric Co.Ltd., Japan.

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

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

3.18 Incubator, Ehret, Germany.

3.19 Inverted microscope, Olympus CK2, Tokyo, Japan.

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

3.21 Laminar airflow equipment, NuAir Biological Safty Cabinets, USA.

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

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

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

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

3.26 Pipetboy acu, integra Bioscience

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

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

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

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

3.31 Semi-dry blotting apparatus, HoeferTM TE77 semiphor transphor unit, Amersham Bioscience, Uppsala, Sweden.

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

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

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3.34 Vertical gel electrophoretic apparatus model AE-6410E, ATTO corporation, Japan.

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

3.36 Water Jacket Incubator, Forma Scientific Inc., 3548, Ohio, USA.

4 Protein marker

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

5 Reagents

5.1 Cell culture: reagent

5.1.1 1x Phosphate buffered-saline (PBS), pH 7.4

NaCl	8	g
KCl	0.2	g
Na_2 HPO_4	1.15	g
KH_2 PO_4	0.2	g

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

5.1.2 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.

5.1.3 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 cellulose acetate filter membrane under sterile condition, aliquots and stored at –20 °C until use.

5.1.4 200 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.

5.1.5 Cell dissociation solution

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5.1.5.1 2.5 mM EDTA in PBS

EDTA.Na_2.2H2O 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.

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

Trypsin 2 g 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.

5.1.6 10 mM Paramethyl sulfonyl fluoride (PMSF)

PMSF 17.4 mg was dissolved in 10 ml of isopropanol, and sterilized by filtrate through 0.2 µM cellulose acetate filter membrane. The reagent was adjusted to final concentration of 1 µM in 1% TritonX-100/PBS.

5.1.7 Substrate solution for focus forming unit assay

5.1.7.1 Tris-HCl Buffer, pH 7.4

Stock A: 2.42 g Tris base in 100 ml deionized water (DW)

Stock B: 1.7 ml concentrated HCl in 100 ml DW

To make Tris–HCl buffer, pH 7.4 2.5 ml of Stock A and 2.07 ml of Stock B were mixed and adjusted the final volume to 10 ml with DW.

5.1.7.2 Working substrate solution (freshly prepared)

DAB (0.06 g/ml in DW)	100	µl
Tris-HCL buffer	10	ml
5-7 %H2O2 (W/V)	20	µl
8% NiCl2 (W/V in DW)	50	µl

5.2 Cell Culture: Media

5.2.1 Stock Leibovitz-15 (L-15) media

Leibovitz-15 medium (GibcoBRL)	1	pack
200 mM L-glutamine	10	ml

Dissolved in DW and adjusted the volume to 1,000 ml. The media was filter-sterilized by 0.2 µm Millipore membrane and stored at 4°C.

5.2.2 Maintenance medium for C6/36 and PS Clone D cells

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Stock Leibovitz-15 (L-15) media	80	ml
Fetal bovine serum	10	ml
Tryptose phosphate buffer	10	ml
Penicillin (5000u/ml)& Streptomycin (10,000 µg/ml)	1.2	ml

5.2.3 Stock RPMI-1640 media

RPMI medium1640 (GibcoBRL)	1	pack
$NaHCO_3$	2	g

Dissolved in DW and adjusted the volume to 1,000 ml. The media was filter sterilized by 0.2 µm Millipore membrane and stored at 4°C.

5.2.4 Maintenance medium for 293T cells and U937 cells

Stock RPMI-1640 media	90	ml
Fetal bovine serum	10	ml
Penicillin (5000u/ml)& Streptomycin (10,000 µg/ml)	1.2	ml

5.2.7 Stock Dulbecco's modified eagle media (DMEM)

Dulbecco's modified eagle medium (GibcoBRL)	1	pack
$NaHCO_3$	3.7	g

Dissolved in DW and adjusted the volume to 1,000 ml. The media was filter sterilized by 0.2 µm Millipore membrane and stored at 4°C.

5.2.8 Maintenance medium for 293T cells

Stock Dulbecco's modified eagle media (DMEM)	90	ml
Fetal bovine serum	10	ml
200 mM L-glutamine	1	ml
Penicillin (5000 u/ml)& Streptomycin (10,000 µg/ml)	1.2	ml

5.3 Transfection reagent for Calcium-phosphate method

5.3.1 0.25M $CaCl_2$

Dissolve $CaCl_2 2H_2O$ (Merck) 3.68 g in Milli-Q water 100 ml and sterile by autoclave at 121°C, 15 min, aliquot 10 ml each store at 4°C.

5.3.2 10X HBS (stock)

NaCl	40	g
HEPES (no sodium salt,MW 238.3)	29.7	g
Na_2HPO_4	1	g

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Adjust pH to 7.0 with NaOH (add NaOH= 0.9 g or 1 M NaOH 23 ml) and make up volume to 500 ml with DW

5.4 Dot enzyme immunoassay (DEIA)

5.4.1 1x Phosphate buffer saline (PBS) pH7.4

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.2	g

These chemicals were mixed; well dissolved in deionized water, and adjusted the final volume to 1 liter

5.4.2 Blocking buffer: 5% skimmilk (w/v) in PBS pH 7.4

Skimmilk (Carnation)	5	g

Dissolved in 100 ml of PBS pH 7.4

5.4.3 Chromogenic substrate

DAB (0.06 g/100ml in PBS pH 7.4)	10	ml
6% H2O2 (w/v)	20	µl
8% NiCl2 (w/v in DW)	50	µl

5.5 SDS-PAGE and Western Blotting Analysis.

5.5.1 30.8% (w/v) Acrylamide-Bisacrylmide

Acrylamide	30.0	g
Bis-acrylamide	0.8	g

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

5.5.2 Resolving gel buffer pH8.8.3 M Tris–HCl

Tris	36.3	g
1 M HCL	48	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 HCl, and stored at 4°C

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5.5.3 10% (w/v) Sodium dodecyl sulfate

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

5.5.4 10% (w/v) Ammonium persulfate

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

5.5.5 Stacking gel buffer pH 6.8:0.5 M Tris-HCl

Tris	6.0	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 store at 4 °C.

5.5.6 TEMED (N,N,N',N'-tetramethylethylenediamine)

Used directly from the supplied bottle that should be stored in the dark.

5.5.7 Loading buffer (4X reducing buffer)

Mercaptoethanol	4	ml.
0.5M Tris-HCl pH 6.8	8	ml.
SDS	1.6	g.
Glycerol	8	ml.
Bromophenol blue	4	mg.

Stored at -20°C

5.5.8 Standard protein markers (broad range marker)

Protein marker	4	µl
4X reducing buffer	10	µl
Deionized water	26	µl

5.5.9 Coomassie blue staining solution

Coomassie brilliant blue R-250	0.25	g
Methanol : deionized water (1:1 v/v)	90	ml
Glacial acetic acid	10	ml

The solution was kept at RT.

5.5.10 Destaining solution

Glacial acetic acid	100	ml

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Methanol	300	ml
Deionized water	600	ml

The solution was kept at RT.

5.5.11 12% Resolving gel of SDS-PAGE (for 1 PAGE)

30.8% (w/v) Acrylamide-bisacrylamide	3.99	ml
3M Tris HCl, pH 8.8	2.5	ml
10% SDS	0.1	ml
10% Ammonium persulfate	0.075	ml
TEMED	0.005	ml
Distilled water	5.905	ml

This reagent were mixed together in the bottle and used immediately

5.5.12 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	1.22	ml
TEMED	0.0015	ml

5.5.13 10X Running buffer pH 8.3 (0.25 M Tris-HCl, 1.92 M Glycine, 1%(w/v) SDS)

Tris	30.3	g
Glycine	144.0	g
SDS	10.0	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 just before use.

5.5.14 Towbin buffer (25 mM Tris, 192 mM Glycine, 20% (v/v) Methanol, 0.1%SDS, pH 8.3)

Tris	3	g
Glycine	14.4	g
SDS	1	g

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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.

5.6 Purification of monoclonal antibody

5.6.1 1M Na_2HPO_4

$Na_2HPO_4.12H_2O$	35.81	g

Adjust volume to 100 ml by deionized water.

5.6.2 1M NaH_2PO_4

$NaH_2PO_4.H_2O$	13.799	g

Adjust volume to 100 ml by deionized water.

5.6.3 Binding buffer (100 mM sodium phosphate, pH 7.0)

1M Na_2HPO_4	5.8	ml
1M NaH_2PO_4	8.4	ml

Dissolved in 400 ml of DW, adjusted pH to 7.0 with HCL/ NaOH and made up volume to 500 ml with deionized water. The reagent was filter by 0.45 µm membrane before used.

5.6.4 Elution buffer (0.1 M Glycine-HCl, pH2.7)

Glycine	0.75	g

Dissolve in deionized water and adjust pH to 2.7 with 0.2 M HCL and adjust volume to 100 ml with deionized water. The reagent was filtered by 0.45 µm membrane before used.

5.6.5 Neutralizing buffer (1M Tris, pH8.0)

Tris	6.057	g

Dissolve in deionized water and adjust pH to 8.0 and adjust volume to 50 ml with deionized water.

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BIOGRAPHY

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