การศึกษาความสัมพันธ์ระหว่างไวรัสไข้สมองอักเสบกับเซลล์ไมโครเกลีย

นางสาวชุติมา รัตนารังสรรค์

INVESTIGATION INTO THE RELATIONSHIP BETWEEN

JAPANESE ENCEPHALITIS VIRUS AND MICROGLIAL CELLS

Miss Chutima   Rattanarungsan

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Biochemistry
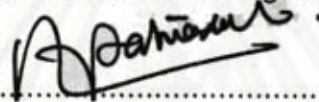
Department of Biochemistry

Faculty of Medicine

Chulalongkorn University

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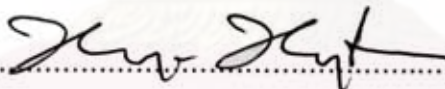
Thesis Title   Investigation into the relationship between Japanese
                    Encephalitis virus and microglial cells
By   Miss Chutima   Rattanarungsan
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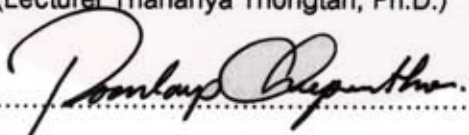Thesis Advisor   Thananya Thongtan, Ph.D.

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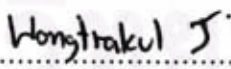
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เกลีย. (Investigation into the relationship between Japanese encephalitis virus
and microglial cells) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.ธนัญญา ทองตัน, 99 หน้า.

เชื้อไวรัสไข้สมองอักเสบเจอี (JEV) จัดอยู่ในกลุ่มแฟมิลี่ฟลาวิไวริเดอีซึ่งมียุงเป็นพาหะนำ
โรค ในทวีปเอเชียเชื้อไวรัสไข้สมองอักเสบเจอีเป็นสาเหตุหลักในการก่อโรคไข้สมองอักเสบที่เกิด
จากการติดเชื้อไวรัส ถึงแม้ว่าเซลล์เป้าหมายหลักของเชื้อไวรัสไข้สมองอักเสบเจอีในระบบ
ประสาทส่วนกลางคือเซลล์ประสาท แต่เซลล์ไมโครเกลียจะอยู่ในภาวะถูกกระตุ้นเมื่อมีการติดเชื้อ
ไวรัสนี้เช่นกัน ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อที่จะศึกษาถึงความสัมพันธ์ระหว่างไวรัสไข้
สมองอักเสบกับเซลล์ไมโครเกลีย โดยใช้เซลล์เพาะเลี้ยงไมโครเกลียสายพันธุ์ BV-2 ซึ่งได้จาก
สมองหนู จากการศึกษาความสามารถที่จะเป็นเซลล์เจ้าบ้านสำหรับเชื้อไวรัสไข้สมองอักเสบเจอี
ด้วยเทคนิค Immunocytochemistry ตรวจพบอนุภาคไวรัสในไซโตพลาสมของเซลล์ไมโครเกลีย
ในการศึกษาช่วงเวลาผลิตอนุภาคไวรัสใหม่ภายหลังการติดเชื้อซึ่งแสดงถึงภาวะการเจริญเติบโต
ของไวรัสครบหนึ่งรอบด้วยเทคนิค Plaque assay พบว่ามีอนุภาคไวรัสถูกผลิตออกมาในอาหาร
เพาะเลี้ยงในชั่วโมงที่ 10 ภายหลังการติดเชื้อไวรัส เซลล์เพาะเลี้ยงไมโครเกลียสายพันธุ์ BV-2 ที่
ติดเชื้อไวรัสไข้สมองอักเสบเจอีสามารถถูกเพาะเลี้ยงได้ต่อเป็นเวลา 16 สัปดาห์ และยังสามารถ
ผลิตไวรัสได้อย่างต่อเนื่องโดยมีปริมาณไวรัส 10^5 p.f.u./ml สำหรับการบ่งชี้โปรตีนบนผิวเซลล์
เซลล์เพาะเลี้ยงไมโครเกลียที่สามารถจับกับไวรัสไข้สมองอักเสบเจอีโดยใช้เทคนิค One
dimensional และ Two dimensional gel electrophoresis virus overlay protein binding
assay และวิเคราะห์ผลด้วย tandem mass spectrometry (LC/MS/MS) พบว่า โปรตีน laminin
receptor precursor ที่มีขนาด 43 กิโลดาลตัน สามารถจับกับไวรัสไข้สมองอักเสบเจอี การ
ค้นพบนี้แสดงให้เห็นถึงความเป็นไปได้ของโปรตีนดังกล่าวในการเป็นตัวรับบนผิวเซลล์ไมโครเกลีย
สำหรับเชื้อไวรัสไข้สมองอักเสบเจอี

ภาควิชา ชีวเคมี   ลายมือชื่อนิสิต ชุติมา รัตนารังสรรค์
สาขาวิชา ชีวเคมีทางการแพทย์   ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก
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KEYWORDS : JAPANESE ENCEPHALITIS VIRUS / MICROGLIA / PERSISTENCE / JAPANESE ENCEPHALITIS VIRAL BINDING PROTEIN(S) / TANDEM MASS SPECTROMETRY / VIRUS OVERLAY PROTEIN BINDING ASSAY / TWO DIMENSION GEL ELECTROPHORESIS

CHUTIMA RATTANARUNGSAN: INVESTIGATION INTO THE RELATIONSHIP BETWEEN JAPANESE ENCEPHALITIS VIRUS AND MICROGLIAL CELLS. THESIS ADVISOR: THANANYA THONGTAN, PhD, 99 pp.

Japanese encephalitis virus (JEV), a mosquito-borne virus that belongs to the family *Flaviviridae*, is a major cause of viral encephalitis in Asia. Even though the principle target cells for JEV in the central nervous system are neurons, the microglial cells are also activated in response to JEV infection. This study aimed to investigate the involvement of microglial cell upon JEV infection. Using mouse microglial (BV-2) cell line as a model, the JEV virions were detected in the cytoplasm of BV-2 cells by immunocytochemistry indicating cellular permissiveness to the virus. The extracellular virions were released into the culture medium, determined by standard plaque assay, implying one complete viral cycle at 10 hr post infection. It was proved possible to culture the persistent JEV-infected BV-2 cells for 16 weeks with a viral titer of 10^5 p.f.u./ml. For the identification of JEV binding protein(s) on the surface of BV-2 cells, one dimensional and two dimensional virus overlay protein binding assay, followed by tandem mass spectrometry (LC/MS/MS) were applied. A JEV binding protein band of 43 kDa laminin receptor precursor protein was identified. The newly identified JEV binding protein is a potential candidate JEV receptor protein on microglial cells.

Department : Biochemistry

Field of Study : Medical Biochemistry

Academic Year : 2009

Student's Signature _____

Advisor's Signature _____

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List of Abbreviations

%	Percent
μg	Microgram
μl	Microlitre
$^{\circ}C$	Degree Celsius
C6/36	Aedes Albopictus Cell Line
$CaCl_2$	Calcium Chloride
Da	Dalton
DAPI	4',6-Diamidino-2-Phenylindole
E glycoprotein	Envelope Protein
EBSS	Earle's Balanced Salt Solutions
FBS	Fetal Bovine Serum
g	Gram
H_2O	Water
hr(s)	Hour(s)
KCl	Potassium Chloride
kDa	Kilodalton
LLC-MK2	Macaca Mulatta (Rhesus Monkey) Cell Line
M	Molar
MEM	Minimum Essential Media
mg	Milligram (s)
$MgSO_4$	Magnesium Sulphate
min	Minute (s)
ml	Milliliter
MOI	Multiplicity of Infection
MW	Molecular Weight

NaCl	Sodium Chloride
NaHCO$_3$	Sodium Hydrogen Carbonate
NS	Nonstructural Protein
NS1	Nonstructural Protein 1
NS2A	Nonstructural Protein 2A
NS2B	Nonstructural Protein 2B
NS3	Nonstructural Protein 3
NS4A	Nonstructural Protein 4A
NS4B	Nonstructural Protein 4B
NS5	Nonstructural Protein 5
p.f.u.	Plaque-Forming Unit
PBS	Phosphate Buffered Saline
pH	The Negative Logarithm of the Concentration of Hydrogen Ions
rpm	Revolution per Minute
SDS	Sodium Dodecyl Sulfate
Seakem LE agarose	Seakem Low Electroendosmosis Agarose
Tris base	2-Amino-2-(Hydroxymethyl)-1,3-Propanediol
YE-LAH	Yeast Extract-Lactalbumin Hydrolysate

CHAPTER I

INTRODUCTION

Background and rational

Japanese encephalitis virus (JEV), a mosquito-borne virus, is the leading cause of epidemic encephalitis worldwide, especially in Asia. JEV is transmitted to human through the bite of the infected mosquitoes principally by *Culex tritaeniorhynchus*. JEV is a member of the genus *Flavivirus*, family *Flaviviridae* and is related to St. Louis encephalitis virus, Murray Valley encephalitis virus and West Nile virus. Like other flaviviruses, JEV contains a small glycoprotein-containing lipid envelope surrounding a nucleocapsid in which consists of one molecule of single-stranded positive-sense RNA. This 11-kb RNA molecule comprises 5'- and 3'-untranslated regions (UTRs), which lies a single open reading frame carrying genes for three structural proteins (capsid (C), premembrane (PrM), and envelope (E)) and seven nonstructural (NS) proteins in between. [1, 2] JEV have been classified into five genotypes based on limited nucleotide sequencing of C/PrM and E genes, representatives of three have been fully sequenced. Each differential genotype isolated form different place, such as, genotype I includes isolates from northern of Thailand, Cambodia, and Korea while genotype II includes isolates from southern of Thailand, Malaysia, Indonesia, and Northern Australia. For genotype III, this includes isolates from mostly temperate regions of Asia, including Japan, China, Taiwan, the Philippines and the Asian subcontinent. Genotype IV includes isolates from Indonesia and Malaysia. Some of the Indonesia, Malaysia and Singapore isolates are classified in the fifth genotype.[1]

The milestones of Japanese encephlalitis virus researches were summarized in Table 1.[3] Since Japanese encephalitis virus was emerged in Japan in 1871, there are attempt to isolated JEV from a clinical case and success in the first recorded epidemic in 1924.

Table1. History of Japanese encephalitis virus, 2002 [3]

Year	Incident
1871	First outbreak of JE attributable to JE.
1924	Isolation of JEV.
1934	Isolation of Nakayama strain.
1950	Elucidation of the route of transmission.
1970	Change in geographical location of viral transmission.

JEV infection leads to a wild spectrum of clinical manifestations, ranging from a febrile headache to acute and possibly fatal encephalitis. The incubation period in human is usually 5 to 15 days upon infection. Mortality rate is ranging from 15%-40% whereas the complete recovery is only one-third of the patients.[4] Most of the survivors have neurological sequelae such as mental retardation, epilepsy, paralysis, deafness and blindness. The degree of severity of the disease is rather dependent on the degree of neural destruction. However, the pathogenesis of Japanese encephalitis remains unclear.

Upon JEV infection, microglia, a type of glial cells that acts as primary immune effector cell in the central nervous system (CNS), was activated morphologically and the expression of the neuronal specific nuclear protein (NeuN) was down-regulated significantly indicating neuronal loss *in vivo*[5] and resulting in increased levels of cytokines in the serum and cerebrospinal fluid in Japanese encephalitis patients. Pathological studies of Japanese encephalitis have shown that the JEV specifically infect neurons in the brain. [6-8] Generally, when infectious agents are directly introduced to the brain or cross the blood-brain barrier, microglial cells which are primary immune effector cells of the CNS react quickly to undergo proliferate and generate numerous mediators involved in the inflammatory and immunomodulatory response in order to destroy the infectious agents, before they can damage the sensitive neural tissue. [9] Infection of JEV results in increased levels of cytokines such as macrophage-derived chemotactic factor, TNF-α and IL-8 in the serum and cerebrospinal fluid in Japanese encephalitis patients.[5,10] The virus is probably not directly involved in the destruction of brain tissue but may cause damage indirectly by triggering cell mediated immune response.[11-13]

Since there is no antiviral drug is commercially available for treatment of Japanese encephalitis, several studies have attempted to identify cell surface receptor(s) for JEV on different cell line with multiple approaches for inhibition of viral

entry, which ultimately lead to the production of effective antiviral agents. [14-16] The interaction between the viral attachment proteins and cellular receptor(s) is the first step for virus infection. Thus, the identification of the cell surface receptor(s) utilized by the JEV to gain entry into host cells may lead to the production of effective antiviral therapeutic agents.

As mentioned before, there is possibility in the relationship between microglia and Japanese encephalitis virus. This study aims to investigate the involvement of microglial cells upon JEV infection, regarding its permissiveness to JEV infection and JEV binding protein(s) identification, on microglial cell surface.

Research Questions

1. Are microglia permissive to Japanese encephalitis virus infection?

2. Does Japanese encephalitis virus persistently infect microglia?

3. Does microglia display specific binding protein for Japanese encephalitis virus?

Objectives

1. To investigate if Japanese encephalitis virus infect and replicate in microglia

2. To determine the early and long-term Japanese encephalitis viral production from mouse microglial cells

3. To identify Japanese encephalitis virus binding protein(s) in mouse microglial cells

Hypothesis

Microglia display specific surface binding proteins required for being permissive host to Japanese encephalitis virus

Conceptual framework
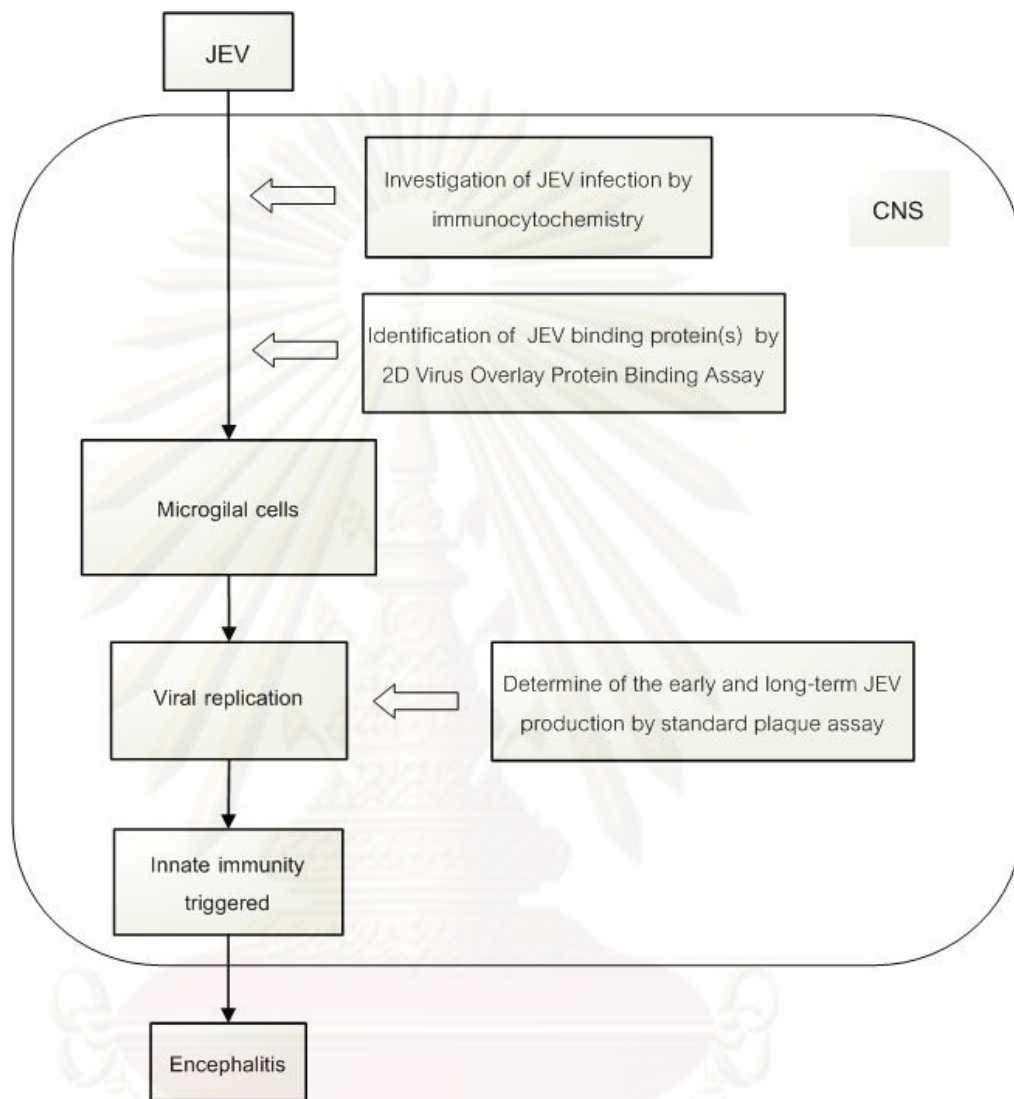
Assumption

All Instruments were tested for the precision and accuracy according to the corresponding standardizations.

Keywords

Japanese encephalitis virus

Microglia

Viral Persistence

Japanese encephalitis viral binding protein(s)

Tandem mass spectrometry

Virus overlay protein binding assay

Two dimensional gel electrophoresis

Operation Definition

Plaque assay is technique for viral titration. A plaque is formed as a result of infection of one cell by a single virus particle followed by the replication of that virus. The culture will then be stained with a permeant dye. Therefore, the dead cells will not be stained and will appear as a white plaque for counting and calculating the virus titer.

Viral overlay protein binding assay (VOPBA) is a technique for identifying matrix-immobilized proteins which interact with virus. Viruses are overlaid on the membrane and detected the viral protein complex by antibody specific to virus.

Research Design

Experimental Research

Benefits of Study

1. To elucidate the involvement of microglia on the pathogenesis of Japanese encephalitis

2. To identify the putative Japanese encephalitis virus receptor (s) on microglia for the inhibition of viral entry

3. To present a potential target for the development of Japanese encephalitis antiviral drug

4. To provide basic knowledge for the enhancement of Japanese encephalitis vaccine efficiency

Obstacles and Strategies to Solve the Problem

1. Cell culture contamination: All kind of procedures involving in the cell culture were performed using aseptic techniques

2. Cell line cross-contamination: Because this research used various cell types, cell lines should not be subcultured simultaneously .

3. Horizontal streaking or incompletely focused spots (anodic sample application spotted at the anodic end of the IPG strip): Possible cause from sample applied at too acidic pH. Concentration of IPG buffer in sample should be increased and Immobiline DryStrip in pH range of 3 to 5 should be applied.

Flowchart of the experimental design

```
┌──────────────────────────┐
│ Propagation of Japanese  │
│ Encephalitis Virus (JEV) │
│      in C6/36 cells      │
└──────────────────────────┘
             │
             ▼
┌──────────────────────────┐                    ┌──────────────────┐
│      JEV titration by    │                    │    Microglial    │
│    standard plaque assay │                    │   (BV-2) cells   │
└──────────────────────────┘                    └──────────────────┘
             │                         ┌──────────────┴──────────────┐
             │                         ▼                             ▼
             │          ┌──────────────────────────┐   ┌──────────────────────────────┐
             └─────────▶│      Infected with JEV at │   │  Extraction of membrane protein │
                        │   Multiplicity Of Infection│   │  using sequential centrifugation │
                        │         (MOI) = 1         │   └──────────────────────────────┘
                        └──────────────────────────┘                  │
                                     │                                ▼
                        ┌──────────────────────────┐   ┌──────────────────────────┐
                        │ Using immunocytochemistry │   │     Identification of    │
                        │  for detection of JEV antigen│ │       JEV Binding        │
                        │        in BV-2 cells      │   │        Protein(s)        │
                        └──────────────────────────┘   └──────────────────────────┘
              ┌──────────────┴──────────────┐                        │
              ▼                             ▼                        ▼
   ┌──────────────────┐      ┌──────────────────┐   ┌──────────────────────────────┐
   │ Determination of │      │  Maintenance as  │   │ - Using 1D and 2D gel         │
   │early JEV production│     │   Persistently   │   │   electrophoresis for         │
   └──────────────────┘      │ infected BV-2 cells│  │   separating protein          │
              │              └──────────────────┘   │ - Blotted on to PVDF membranes│
              ▼                        │             │ - Using Virus overlay protein │
   ┌──────────────────┐      ┌──────────────────┐   │   binding assay (VOPBA) for   │
   │ Collect media hourly│   │Collect media weekly│  │   detection of JEV binding    │
   │ after infection for │   │after infection and │  │   protein(s)                  │
   │ 24 hours and        │   │determine virus titer│ │ - Reactive protein spots were │
   │ determine virus titer│  │by standard plaque  │  │   visualized by chemiluminescent│
   │ by standard plaque  │   │assay for 16 weeks  │  │ - Membranes were scanned and  │
   │ assay               │   └──────────────────┘   │   compared with the coomasie-blue│
   └──────────────────┘                             │   stained gel images          │
                                                    │ - Indentified proteins of the │
                                                    │   corresponding spots in the  │
                                                    │   coomassie-blue stained gel  │
                                                    │   with LC/MS/MS               │
                                                    └──────────────────────────────┘
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CHAPTER II

REVIEW LITERATURES

2.1 Japanese encephalitis

2.1.1 Epidemiology

Japanese encephalitis (JE) was found in Japan from the 1870s onwards. About 30,000 to 50,000 encephalitic cases were reported and 10,000 deaths annually[1]. JE leads to a recognized cause of childhood encephalitis in countries where the Japanese encephalitis virus (JEV) are endemic including China, Siberia, Korea, Japan, Taiwan, Guam, Saipan, Vietnam, Cambodia, Thailand, India, Nepal, and Sri Lanka.[1,17-19] (Figure1) After the first epidemic in Japan in 1924, JE has increasingly been found throughout most countries of east and south east Asia. Factors support disease in this area comprise the vector, the environmental conditions which are essential for the mosquito breeding cycle such as rainfall, humidity, tropical temperatures, and finally, the residence of the amplifying hosts which are pigs and birds.[20]

In Thailand, the first epidemic of JEV was reported in Chiang Mai valley and nearby areas in 1969. Later on, there was a study of JEV genotypic distribution in Thailand to detect epidemiologic patterns of Japanese encephalitis that occurred in 7 provinces[21]. These provinces represent 4 regions of Thailand consisting of the north (such as Chiang Mai province), the northeast (such as Khon Khen province), the midland (such as Nakhon Pathom, Ratchaburi, and Samut Songkram provinces), and the south (such as Phuket and Chumphon provinces) (Figure2).The dramatic increase of industrial pig farming and trading must have featured in the dispersion of JEV genotypes in Asia[21].

Figure1. Global epidemiology of Japanese encephalitis, 2006[20].
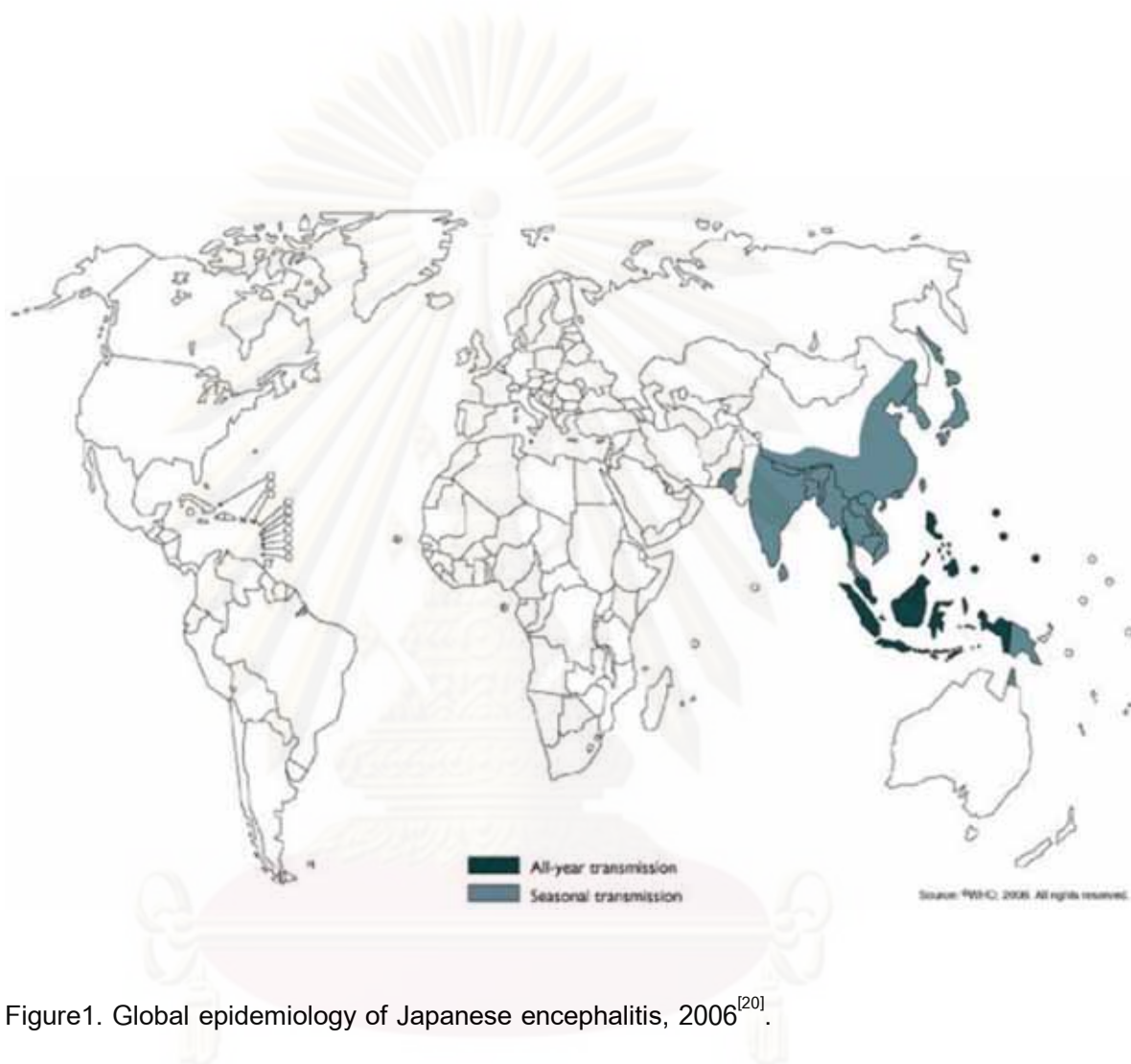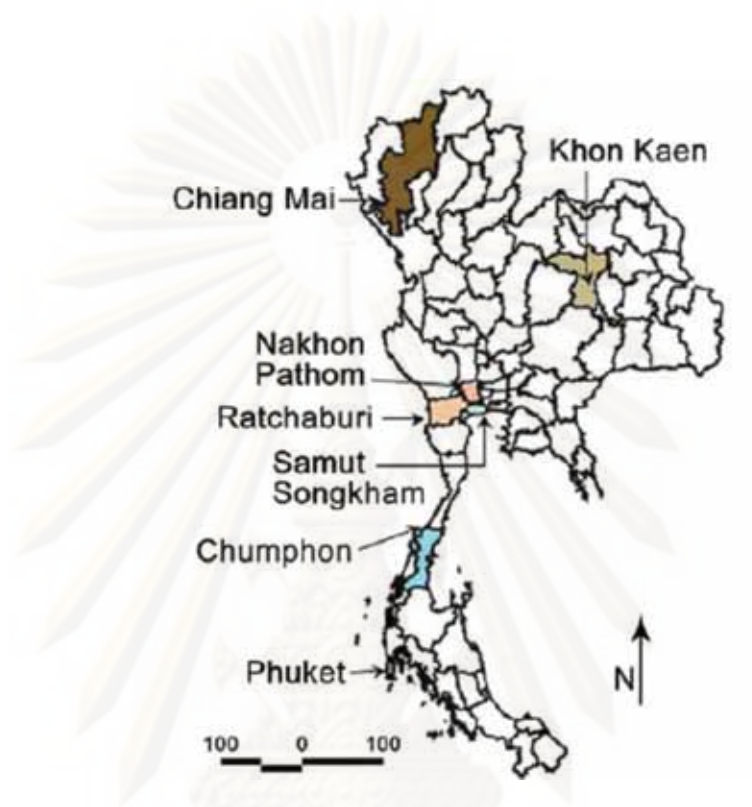
Figure2. JEV distribution in Thailand during 2003-2005[21].

       This figure shows the provinces of Thailand that had been studied sites of the JEV genotypic distribution. Genotype I of JEV was found in Chiang Mai province, a representative of the north, Nakhon Pathom, Ratchaburi, and Samut Songkram provinces, representatives of the midland including Phuket and Chumphon provinces, representative of the south. Genotype I and III of JEV were found in Khon Khen province, a representative of the northeast.[21]

2.1.2 Clinical features

After the virus has invaded the human body by a mosquito's bite, it primarily proliferates in the local lymph node, and subsequently enters into the blood causing a transient viraemia before invading the CNS. The genetic differences of JEV affects both their neuroinvasiveness and neurovirulency. The clinical manifestations onset after infection occurs at 6-16 days. Most patients show a non-specific febrile illness that may include headache, fever, cough, nausea, vomiting, diarrhea, and rigors. In some patients present with aseptic meningitis or acute flaccid paralysis with no encephalopathic features, especially in older children and adults [4,18,22]. There could be other abnormal movements, cranial nerve palsies, altered consciousness leading to high mortality and neurological sequel in some of those who survive[1,4,22]. The other extrapyramidal features include head nodding and pill rolling movements, opsoclonus myoclonus, choreoathetosis, and bizarre facial grimacing, and lip smacking (Figure3).

2.1.3 Treatment and prevention

To date, there is no specific antiviral therapy available for JE. The main treatment are supportive and symptomatic. The potential methods to prevent JEV transmission are summarized in Table2. Prevention of mosquito bite such as using nets and mosquito repellents, staying in screened houses, wearing long sleeved shirts and long trousers reduce the risk of exposure to vector mosquitoes. Measures against reservoirs include building the piggeries away from human dwellings and spraying residual insecticides around the piggeries.[3]

Vaccination for Japanese encephalitis, a main strategy of JE control, have been found for at least 70 years and were available worldwide. However, only the inactivated mouse brain–derived vaccine is currently in use commercially for travelers, even though the results from experimental research in phase III trial of an inactivated vaccine, derived from Vero cells, mark the arrival of a new vaccine for Japanese encephalitis.

Figure3. Facial grimacing in a Vietnamese boy with Japanese encephalitis.[4]

This vaccine is of concern because the viruses were grown in mouse brain. Acute disseminated encephalomyelitis is its possible adverse effect, although only one per million doses, similar to the risk with vaccines for other diseases, including measles[23,24].

Besides second-generation live inactivated, single-dose vaccine grown in Vero cells, chinese researchers had developed a live attenuated vaccine (SA 14-14-2) in 1988 and it was used widely in China (Chengdu Institute of Biological Products (CDIBP), China.). The SA 14-14-2 attenuated virus is proved for its safety and immunogenicity in a non-inferiority comparing with the JE-VAX formaldehyde inactivated mouse brain–derived vaccine[22]. The development of this new single dose vaccine is good news for natives of Japanese encephalitis endemic areas and for travelers.

2.1.4 Classification

Japanese encephalitis virus (JEV), a mosquito-borne virus, is a member of the genus *Flavivirus*, family *Flaviviridae*.[1,2] Other important members of the same JE serogroup which cause neurological disease are Murray Valley encephalitis virus in Australia, St Louis encephalitis virus in North America and West Nile virus in Africa, the Middle East, and parts of Europe.[1, 2, 14,18] The antigenic relationships of JEV isolated from different geographic regions and various time periods have been divided into at least five antigenic subgroups. The first genotype (I) comprises isolates from northern Thailand, Cambodia, and Korea and a second (II) comprises isolates from southern Thailand, Malaysia, Sarawak, Australia, and Indonesia. The largest genotype (III) consists of viruses from Japan, Okinawa, China, Taiwan, Vietnam, the Philippines, Sri Lanka, India, and Nepal. Some Indonesian isolates (two from Java, two from Bali, and one from Flores) are similar to each other and distinct from the other Indonesian isolates, identifying the fourth (IV) genotype. Finally, a single strain from Singapore, Indonesia and Malaysia comprises a possible the fifth genotype (V).[1,4,21] (figure4.)

Table2. Prevention for Japanese encephalitis virus transmission.[3]

1. Control of mosquito vectors.

2. Vaccination of humans.

3. Prevention of mosquitoes from biting humans.

4. Measures against reservoirs.
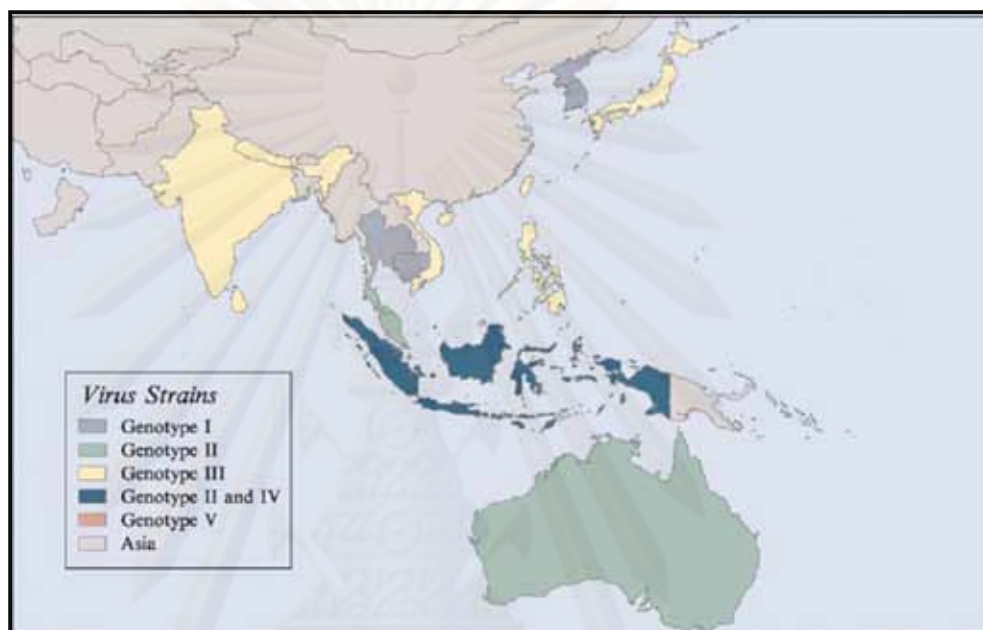
Figure4. Distribution of JE virus genotypes in Asia, 2001[21].

2.1.5 Genome structure

Japanese encephalitis viral genome is a 11 kilobases positive-sense single strand RNA, wrapped in a nucleocapsid and surrounded by a 50 nm glycoprotein-containing lipid envelope. The genomic RNA of JEV comprises 5'- and 3'-untranslated regions (UTRs), and a single open reading frame between them which carrying genes for a single polyprotein. This polyprotein is co- and post-translational cleaved by viral and host proteases into three structural proteins which consists of core (C) involved in packaging of the viral genome and forming the nucleocapsid, pre-membrane (PrM) as a chaperone for folding and assembly of the E protein and envelope (E) that contains a cellular receptor-binding site(s), For nonstructural (NS), the polyprotein is cleaved into seven nonstructural protein which are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. [1, 2,25, 26](figure5-6.)

2.1.6 Pathogenesis

Pathogenesis of JE remains unclear. After being infected, The virus comes to the lymphatic tissue of the whole body and the bone marrow. The viruses can proliferate and come to the tissue of the cranial nervous system. Pathway JEV uses to cross the blood-brain barrier is not well understood. The virion may transport across the cerebral blood vessel wall by attaching to the endothelial cells through an endocytic vesicle. Then, the virion is taken up in a perivascular cells and transported into the CNS. Patients with sequelae has revealed abnormal mainly in grey matter and the thalamus, substantia nigra, anterior horns of the spinal cord, cerebral cortex, and cerebellum. Neuronal inflammation is typically associated with mononuclear cell infiltration.[3] The pathogenesis of flavivirus encephalitis is consisting of direct, viral cytolytic and the indirect host inflammatory response. Cytolytic viruses are known to cause their host cells to disintegrate by increasing plasma membrane permeability, causing a loss of cellular
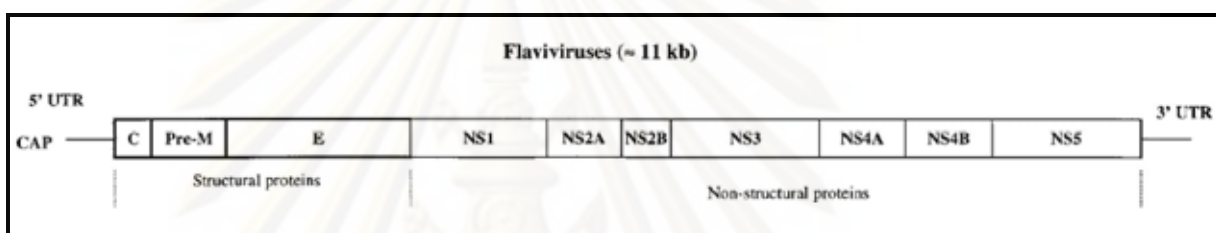
Figure5. Genomic organization of the *Flaviviridae*.[15]

This digram shows 11 kb single stranded RNA viral genome. A single-stranded RNA molecule is positive polarity and 5'capped. Untranslated regions (UTR) are present both at the 5' and 3' ends of the genome. Boxes indicate mature proteins generated by proteolytic processing.
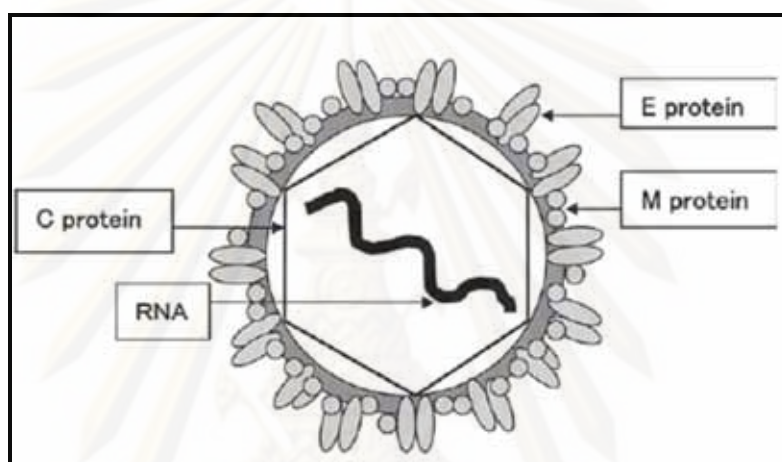
Figure6. Schematic presentation of Japanese encephalitis virion.[24]

JE virion has a spherical shape with 40 to 50 nm in diameter. The envelope is a lipid bilayer containing two envelope-associated proteins: the E and M proteins. The nucleocapsid, which is about 30 nm in diameter and covered by envelope, is composed of RNA genome and C protein.

ion gradients and leakage of essential compounds from the cell, which leads to necrosis. When a person has been exposed to one flavivirus, cross-reacting antibodies may affect the outcome of infection with a second flavivirus. reducing in secondary flavivirus infections.[3]

2.1.7 Transmission

This virus is transmitted naturally in an enzootic cycle among birds, pigs and other vertebrate hosts through the bite of the mosquitoes and specially by *Culex tritaeniorhynchus*.[1, 2] Human is its dead end host. A complex life cycle of JEV involves pigs, birds as amplifying hosts and mosquitoes as vectors which transporting JEV from the reservoir or amplifying host via bloodsucking. Other domesticated animals, such as dogs, sheep, cows, and chickens, and rodents may become infected, however fail to develop a sufficient viremia to support further viral amplification (Figure7)

2.2 Strategies of viral replication

Viral relication starts with virions-host cell surface receptor(s) binding and then subsequently enters the cell by receptor-mediated endocytosis. Upon internalization, acidification of the environment of endosome triggers conformational changes in the virion to an irreversible trimerization of the E protein. After the fusion of viral and vesicular membranes, the nucleocapsid is retried into the cytoplasm and genome uncoating. The capsid protein and RNA dissociate replication of the RNA genome and particle assembly is initiated. Then, the virus switches from translation to synthesis of a negative-strand intermediate, which serves as a template for the production of multiple copies of positive-strand viral RNA. The positive-sense RNA is translated into a single polyprotein that is processed co- and post-translationally by viral and host proteases to produce high levels of viral proteins; the structural protein capsid or core (C), premembrane (prM), and envelope (E) proteins and seven nonstructural proteins.[15,26,27]
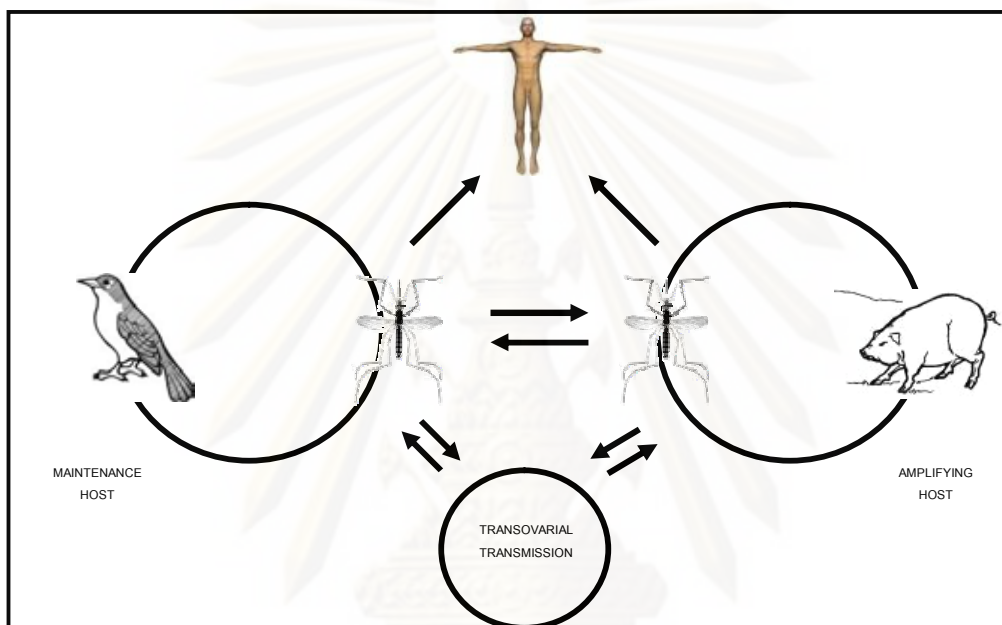
Figure7. Transmission cycle of Japanese encephalitis virus

Human is dead end host for the JEV. It transmitted in an enzootic cycle among its principle vector mosquitoes, vertebrate-amplifying hosts, mainly domestic pigs and its maintenance host.

The replication of viral genome occurs on the surface of the endoplasmic reticulum (ER). Then, JEV are assembled into progeny virions, when the structural proteins and newly synthesized RNA buds into the lumen of the ER. Subsequently, cleavage of prM occurs in the trans-Golgi network and the mature virions including subviral particles are transported through the Golgi compartment and secreted finally by exocytosis.[15, 16, 26, 27] (Figure8)

2.3 Persistent infection

Persistent virus infection are associated with chronic progressive, even lethal, diseases. Neuropsychiatric sequelae, after JEV acute infection phase, occur in 45%-70% of the survivors and are particularly severe in children raises the possibility that persisting virus may lead to progressive deterioration of neurologic function. Normally, JEV resulting in acute illness but sometimes it may run a protracted course with acute exacerbations. The main feature of JEV infection is evasion of the host immune response which cause a failure to eliminate the virus. Evidence of persistent infection with JEV has been described in many experimental such as mammalian and mosquito cell lines and from studies in bats[28,29]. Defective interfering particles have been found to be responsible for establishment and maintenance of JEV persistent infection *in vitro* [28,29]. In 1978, an attempt to study the establishment of persistence, latency and reactivation of JEV in pregnant and non-pregnant mice using JEV-mouse model for transplacental transmission of the virus. This phenomenon observed in human cases. The results shown that JEV can persisted for 16 weeks in pregnant mice[28]. Although a number of viruses can establish persistent infection in the host but the mechanism of virus persistence is complex[28]. However, the precise mechanism for JEV persistency *in vitro* and *in vivo* has not been fully understood. There have been successfully JEV persistency established in several immortalized cell lines including murine neuroblastoma cells (N18), murine astrocytoma cells (DBT), and Vero cells. These
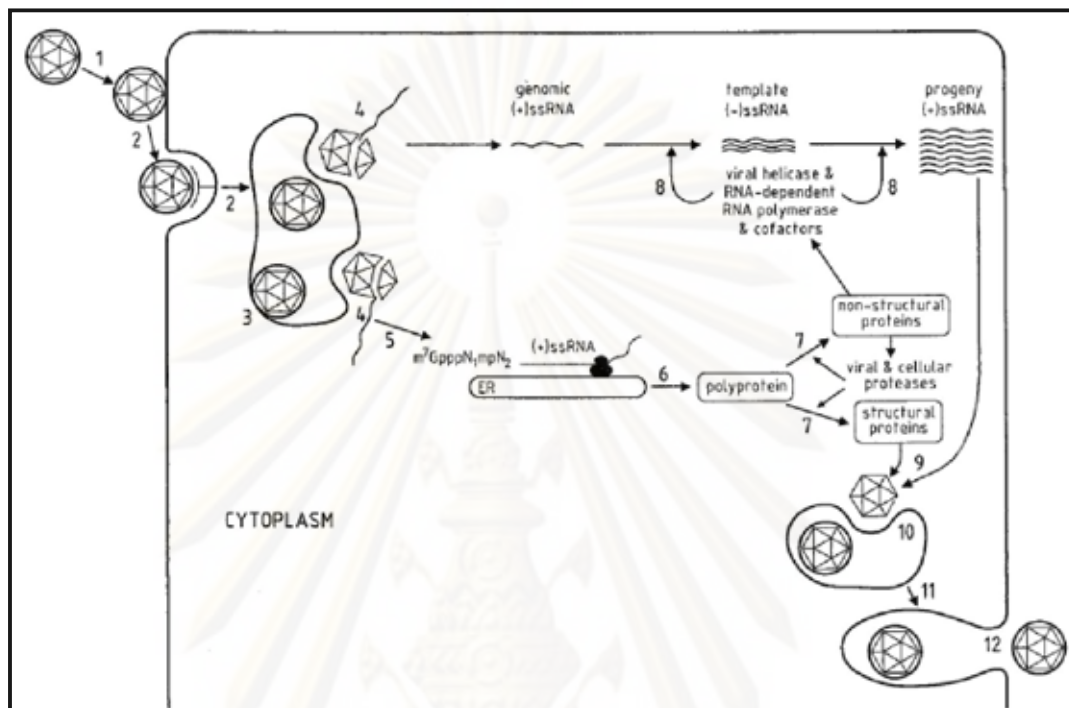
Figure8. Replicative cycle of members of the *Flaviviridae*.[15]

The presumed replication cycles of the flaviviruses are shown. 1, adsorption; 2, receptor-mediated endocytosis; 3, low-pH fusion in lysosomes; 4, uncoating; 5, cap-mediated initiation of translation; 6, translation of the viral RNA into viral precursor polyprotein; 7, co- and posttranslational proteolytic processing of the viral polyprotein by cellular and viral proteases; 8, membrane-associated synthesis of template minus-strand RNA and progeny plus-strand RNA; 9, assembly of the nucleocapside; 10, budding of virions in the endoplasmatic reticulum; 11, transport and maturation of virions in the endoplasmatic reticulum and the Golgi complex; 12, vesicle fusion and release of mature virions. ss, single stranded.

results suggest a probable involvement of dysfunctional NS1 in the establishment and/or maintenance of JEV persistency in culture systems.[30]

2.4 Cellular targets of virus

Among all the neural cells in fetal rat brain culture, developing neurons showed the highest rate of infection by Japanese encephalitis virus (JEV).[29] JEV specifically bound to these cells as measured by immuno-staining. This indicated that the developing neurons are the main target of JEV. After infection in neuron, JEV can replicates and matures in the neuronal secretory system, mainly the rough endoplasmic reticulum and Golgi apparatus, eventually destroying them. In mammalian host cells, JEV tropism to neurons in the CNS, indicating the presence of specific receptors with strong affinity for the virus[3]. However, clinically, the infection of JEV results in increased levels of inflammatory mediators in the serum and cerebrospinal fluid (CSF) of nonsurvivors compared to survivors. The involvement of microglia for these immunological response during JEV viral infection is of interest.

Microglia are the resident macrophage population in the brain which is about 12% of all populations in the central nervous system (CNS). It is recognized as the primary components of an intrinsic brain immune system, and plays a central role in cellular neuroimmunology, neuroinflammation, acute and chronic neurodegenerative diseases of the CNS. (Figure9-10) The mechanism by which JEV causes neurological disease remains unclear . Infection of JEV results in increased levels of cytokines such as macrophage-derived chemotactic factor, TNF-α and IL-8 in the serum and cerebrospinal fluid in Japanese encephalitis patients.[5]

Mortality rate is increased with increasing concentrations of cytokines in serum and CSF of JE patients.[22] The virus is probably not directly involved in the destruction of brain tissue but may cause damage indirectly by triggering cell mediated immune

Figure9. Microglia functions in brain.

  Microglia typically exist in a resting state, characterized as ramified cells Microglia continually extend (green) and retract (yellow) processes to monitor their immediate environment within the brain. The processes move rapidly toward a site of injury, such as a damaged blood vessel in the brain, in response to the localized release of a chemoattractant (gradient of orange) from the injured sited. Once at the target site, the processes form a barrier to protect healthy tissue. This figure can be accessed at http://pmokeefe.blogspot.com/2005/07/brain-under-surveillance-microglia.html
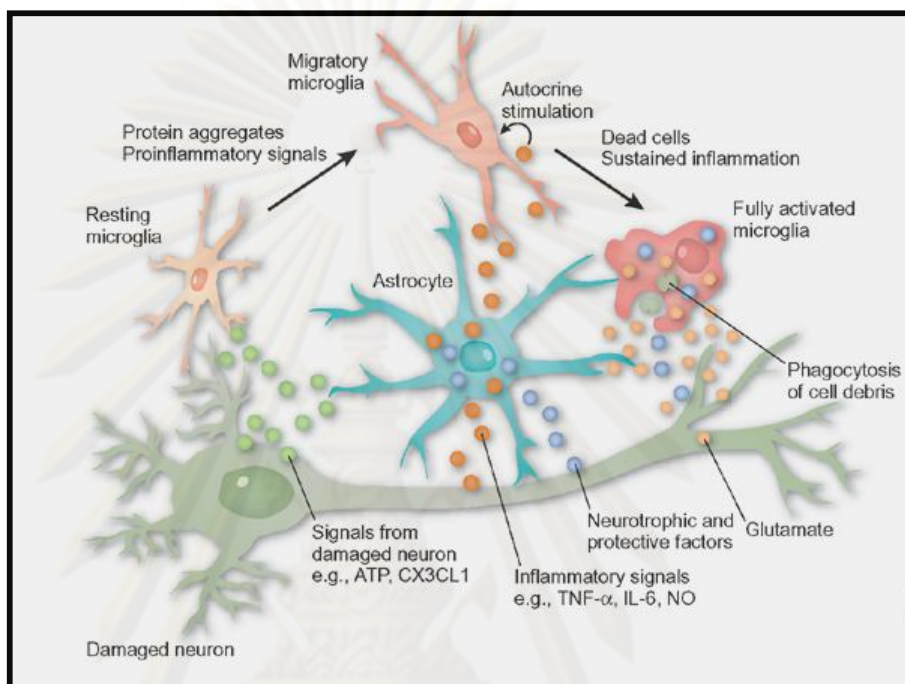
Figure10. Microglia activation

In response to central nervous system insults, activated microglia become amoeboid-like cells that show such macrophage properties as phagocytosis, release of cytokines at sites of neural damage or inflammation.

response. Microglia is directly involved in responsibility of brain immune system to JEV infection.[11-13]

After infection, microglia, a primary immune cell responds to the presence of pathogens by migrating to the site of injury, where they may proliferate. Activated microglia at the site of inflammation express increased levels of MHC antigens and become phagocytic. Like other tissue macrophages, microglia release inflammatory cytokines and potential neurotoxins that amplify the inflammatory response by recruiting cells to the site of injury.[11]

Upon JEV infection, microglia was activated morphologically and functionally, as determined by elevation of the proinflammatory mediators, in vivo.[5] The microglial activation suggests the role of microglia in the pathogenesis of JE.

2.5 Receptor(s) for JEV

The major step of understanding enveloped viral entry at the molecular level is to obtain structural information on the viral surface glycoproteins.[31,32] A glycoprotein JEV envelope (E) is a major component of the virion surface that play a crucial role in attachment to host cells. The envelope protein of JEV has been found to be associated with all the biological properties of the virus such as attachment to cell receptor, penetration, fusion with the endosomal membrane, host cell range and cell tropism, neutralization and virulence. However, the receptor interacting with envelope protein has not been conclusively identified.[9,31,33]

In the first step of JEV infection requires interaction between the E protein and the cellular receptor present on the surface of the host cell. However, the accurate molecular events which involved in JEV entry into permissive cells are not well explained. Because JEV is both lead to neurovirulent, neuroinvasive and causes life-threatening CNS infections, elucidating the functional role of JEV-E protein in virus binding and interactions with cellular proteins of host cells is important. The E protein

was proved to mediate the primary attachment of the virus to its target cell indicating the host-cell tropism[33]. (Figure11)

Based on previous studies of flaviviruses receptor for dengue virus and West Nile virus such as DC-SIGN, GRP-78, the 37/67kDa high-affinity laminin receptor and heat shock proteins 70 and 90 [15,31,32-34], the dissimilarity of viruses require different receptor(s). Although the interaction of viral binding protein and its cellular receptors is known to contribute to its host range, numerous studies have attempted to identify the cell surface receptor(s) utilized by the JEV to gain entry into cells for leading to the production of effective antiviral therapeutic agents.[9, 14, 27] At present, there have been experimental studies to identify the nature of the molecules involved in the initial JEV entry. A recent report is confirmed that a 74 kDa heat shock cognate protein 70 (Hsc 70) act as a penetration receptor for JEV on C6/36 mosquito cells and mouse neuroblastoma (Neuro2a) cells.[9,14,33] The domain of JEV E protein revealed in the conformational change together with cellular receptors could be a target of neutralizing antibodies or antiviral drugs in the future.[15]

2.6 Application of Mass Spectrometry in Proteomics

Mass spectrometry (MS) has become the method of choice for analysis of complex protein samples. The application of mass spectrometry based techniques for the qualitative and quantitative analysis of global proteome samples derived from complex mixtures for understanding of cellular function. Generally, mass spectrometers consist of three basic components; an ion source, a mass analyser that measures the mass-to-charge ratio (m/z) of the ionized analytes, and an ion detector that registers the number of ions at each m/z value. Both electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two techniques most commonly used to volatize and ionize the proteins or peptides for mass spectrometric analysis.[35,36]
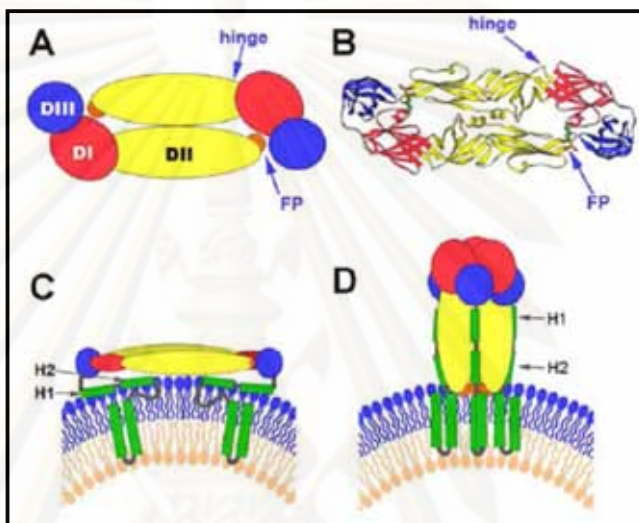
Figure11. Summary of the structural organization and conformations of the Flavivirus Envelope Protein.[14]

Panel (A) Top view of the organization of the E protein dimer as present at the surface of mature virions. Panel (B) Ribbon diagram of the E protein dimer. Panel (C) Side view of the E protein dimer Panel. This is the conformation of E protein in the mature virus particle. Panel (D) The proposed organization of full-length E in its postfusion conformation.

ESI is based on spraying an electrically generated fine mist of ions into the inlet of a mass spectrometer at atmospheric pressure before analyzing out of a solution. The instrument is therefore readily coupled to liquid-based such as chromatographic or electrophoresis separation tools (Figure 12). This technique ionises molecules directly from solution, so it can easily be interfaced with liquid separation methods. In proteomics researches, liquid-chromatography ESI-MS systems (LC-MS) was preferred for the analysis of complex samples.[35, 36]

After ionisation, the sample reaches the mass analyser, which separates ions by their mass-to-charge (m/z) ratios. Ion motion in the mass analyser can be manipulated by electric or magnetic fields to direct ions to a detector, which registers the numbers of ions at each individual m/z value. In proteomics research, four basic kinds of mass analysers are time-of-flight (TOF), ion trap, quadrupole, and Fourier transform ion cyclotron resonance (FTICR) analysers. But, ESI is most frequently coupled to ion traps (three-dimensional and linear ion traps) and hybrid tandem mass spectrometers like quadrupole time-of-flight (Q/TOF) instruments. The ESI with Q/TOF analyzer was used in this research.[35, 36]

In this research, JEV strain Beijing-1 and microglial cells were used as a model for the study of viral permissiveness and virus-host interaction. Even though this viral strain is not endemic in Thailand, it induces high level of neutralizing antibodies. The induced antibodies are protective against heterologous JEV strains such that the Beijing-1 was used for the production of the first-generation, mouse brain-derived JE vaccine. This vaccine effectively decreases the incidence of JE in Thailand. Productive infection of microglial cells was demonstrated by immunocytochemistry and standard plaque assay. One dimensional and two dimensional virus overlay protein binding assay, followed by liquid chromatography-mass spectrometry were also applied for the identification of JEV binding protein(s) on microglial cells.
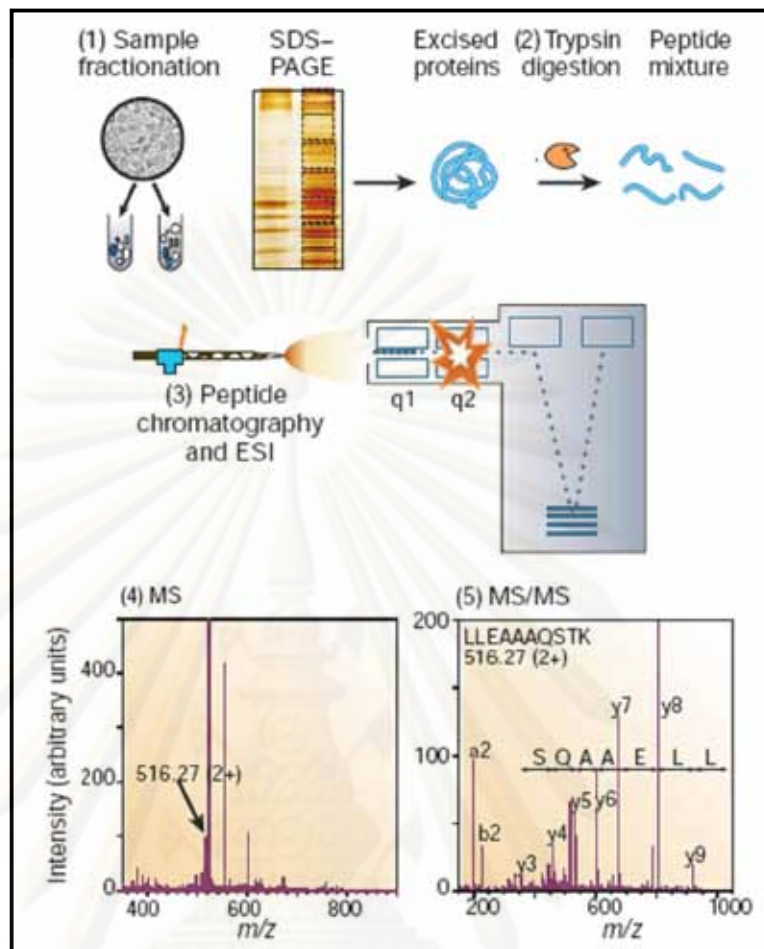
Figure12. Generic mass spectrometry (MS)-based proteomics experiment.[35]

The typical proteomic experiment consists of five stages. In stage 1, the proteins to be analyzed were isolated from cell lysate or tissues by biochemical methods before separation by gel electrophoresis. In stage 2, proteins were degraded enzymatically into peptides usually by trypsin. In stage 3, the peptides were separated by one or more steps of high-pressure liquid chromatography in very fine capillaries and eluted into an electrospray ion source. After evaporation, multiply protonated peptides entered the mass spectrometer. In stage 4, a mass spectrum of the peptides eluted at particular time point was taken. The computer generated a prioritized list of these peptides for fragmentation and a series of tandem mass spectrometric or 'MS/MS' experiments ensued (in stage 5). The MS and MS/MS spectra were typically acquired and stored for matching against protein sequence databases. The outcome of the experiment is the identity of the peptides and therefore the proteins.

CHAPTER III

MATERIALS AND METHODS

1. Source of Materials

Chemicals

Acrylamide	BIORAD
SeaKem LE Agarose	CamBrex
COOMASIE BRILLIANT BLUE R 250	BIO BASIC Inc.
Urea Ultrapure	ICN Biomedicals,Inc
Thiourea	SIGMA
CHAPS	USB Corporation
Iodoacetamide (IAA)	Amersham Bioscience
SERVALYT 40% w/v (ampholine)	SERVA
Dithiothreitol (DTT)	USB Corporation
Trichloroacetic acid (TCA)	MERCK
Sodium Dodecyl Sulfate-Lauryl (SDS)	SIGMA
Phosphate Buffered Saline (PBS)	SIGMA
Trypan Blue dye	SIGMA

Other general chemicals and solvents used but not listed here were purchased from a variety of suppliers. All chemicals used were analytical grade.

Cell culture reagents

Dulbecco's Modified Eagle's medium (DMEM)	GIBCO
DMSO (Dimethyl sulfoxide)	SIGMA
Minimum essential medium (MEMα)	GIBCO
Fetal Bovine Serum (FBS)	GIBCO

Penicillin-Streptomycin solution	HyClone
HEPES, Free acid	HyClone
Sodium Pyruvate powder	SIGMA

Cell lines

LLC-MK2 : kidney cell line from *Macaca mulatta* (rhesus monkey)

C6/36 : whole hatch larva of mosquito cell line from *Aedes albopictus*

BV-2 : mouse microgial cell

Japanese encephalitis virus

JEV strain Beijing-1 (BJ-1)   (accession No. L48961)

Source: Human from China

Miscellaneous

ECL + Western blotting detection reagents	Pierce
PVDF (Polyvinylidene Fluoride) membrane	Pall Corporation
IPG Buffer pH 3-10 NL	GE Healthcare
Immobiline DryStrip pH 3-10 NL, 7 cm	GE Healthcare
Ettan™ IPGphor™ II IEF Unit	GE Healthcare
Prestain Protein marker	Fermentas

Pan-specific anti-flavivirus monoclonal E protein antibody

(A kind gift from Dr. Duncan R Smith, Mahidol University, Thailand)

Goat anti mouse IgG-*Alexaflor 594*	Invitrogen
Rabbit antimouse IgG-horseradish-peroxidase	SIGMA
DAPI	Chemicon

Mass spectrometry (Q-ToF micro; Micromass, UK)

UV-visible spectrophotometer (UV-1700 PharmaSpec; SHIMADZU)

Automatic pH meter (Mettler-Toledo AG, Process Analytics CH-8902 Urdof)

Culture media

LLC-MK2 : Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), 1% L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin

BV-2 : Dulbecco's Modified Eagle's Medium (DMEM), 10% FBS, 1% L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin

C6/36 : Minimum essential medium (MEMα), 10% FBS, 1% L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin

2. Cell culture

The *Aedes albopictus/Stegomyia albopicta*-derived C6/36 cells line was grown in minimum essential medium (MEMα) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin. Cells were cultured at 28°C.

Monkey kidney LLC-MK2 cell line and mouse microglial BV-2 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplement with 10% FBS, 1% L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin. Cells were cultured in humidified atmosphere (5% CO_2/95% air) at 37°C.

3. Cell counting

The number of the cells was determined by using a hemocytometer (Figure13). To prepare the counting chamber, the mirror-like polished surface chamber and the coverslip were carefully cleaned with lens paper. The coverslip was placed over the counting surface before putting on the cell suspension. Cells were pelleted by centrifugation at 1,200 rpm for 5 min at room temperature and resuspended in appropriate volume of media. Subsequently, 20 µl of the cell suspension was mixed with 50 µl of 0.4% Trypan Blue dye and 30 µl of PBS to make a ratio of 1:5 dilution of cell suspension before counting. The reaction was briefly vortex and incubated for 5 min
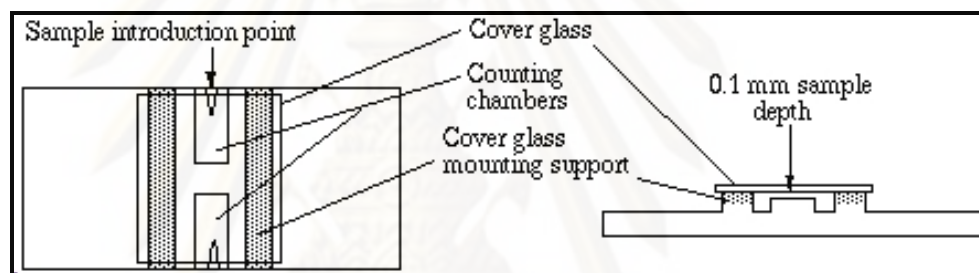
Figure13. Hemocytometer and components

at room temperature. The suspension was introduced into one of the V-shaped wells for counting with hemocytometer under a light microscope. Dead cells were identified by blue color staining. (Figure14) By counting the number of cells in several of the 1x1 mm squares, the cell number / ml was then calculated.

4. JEV propagation in C6/36 cells

The *Aedes albopictus/Stegomyia albopicta*-derived C6/36 cell line were grown in 75-cm^2 tissue culture flasks containing minimum essential medium (MEMα) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin. Cell culture were incubated at 28°c. The Japanese encephalitis virus strain Beijing-1 (genotype III) was propagated in C6/36 cells and stored at -80°c until use.

C6/36 cell line was cultured in media until 80% confluency. Cells were counted using hemocytometer. After cell concentration was determined, the culture medium was discarded and replaced with 3 ml of MEMα without FBS containing JEV strain Beijing-1 at an MOI of 1. Proceeding of viral absorption was allowed for 2 hrs at 28°c with constant agitation. The cells were supplemented with fresh culture medium and incubated at 28°c for 3 days. Aliquots of the culture medium were stored at -80°c until use. Virus titers were determined by standard plaque assay.

5. Virus Titration by plaque assay

After propagating JEV in C6/36 cell, JEV was titrated by plaque assay. The monkey kidney LLC-MK2 cells were plated in 6-well plates for 2 days before time. Cell were washed with PBS and inoculated with 12-fold dilution of JEV in DMEM without FBS. Viral absorption was allowed to proceed for 90 min at 37°c with constant agitation. The JEV-infected monolayer cell were overlaid with 2x nutrient mixed 2% seakem agarose and incubated at 37°c for 7 days. The plaques were visible by fixing cells with the 3.7%
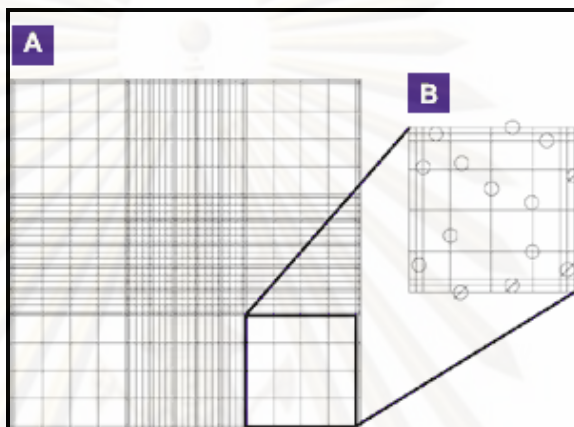
Figure14. Cell counting by hemocytometer.

Panel A is a one-sided of the hemocytometer chamber. Panel B is an enlarged view of the ninth square, with cells. In this sample, only the cells marked as circles should be counted. The cells with diagonal lines through them should not be counted. For trypan blue dye exclusion assay, the viable cells appear white and the non-viable cells appear blue.

formaldehyde for 1 hr before crystal violet staining. The plaque formations on the monolayer of LLC-MK2 cell line were counted and calculated titers of JEV. (Figure15)

6. Immunocytochemistry

Mouse microglial BV-2 cell line were plated in 24-wells plates for 24 hrs. When the cells reached density of 5×10^4 cell/well, the culture medium was discarded and washed once with PBS. Later on, cells were incubated in 100 µl of MEMα containing JEV strain Beijing-1 at an MOI of 1 for 2 hrs at 37°c with constant agitation. Cells were washed once with PBS and followed by acid glycine treatment for 1 min. After washing three times with PBS, normal growth media added and then cells were fixed with cold-methanol for 10 min. Cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked by incubation with 1% goat serum for 45 min.

After washing with PBS, cells incubated with 1° antibody (mouse monoclonal anti flavivirus E-protein) for 1 hour at 37°C and subsequently incubated with 2° antibody (goat anti mouse IgG - Alexaflor 594) for 45 min at 37°C. Finally, nuclei were counterstained with DAPI for 15 min. After washing with PBS, cells were viewed under a fluorescent microscope.

7. Early and Long-term Japanese encephalitis viral production from mouse microglial (BV-2) cells

Mouse microglial BV-2 cell line were grown in 75-cm^2 tissue culture flasks. When the cells reached density of 1.6×10^7 cells/flask, the culture medium was discarded and replaced with 3 ml of DMEM containing JEV at MOI of 1. After viruses were absorbed into the cells for 2 hours at 37°c, the infected cells were washed once with PBS and then treated with acid glycine buffer (pH 3.0) for 1 min to wash any un-internalized viruses. Following acid glycine treatment, cells were again washed with PBS. After washing normal growth media was added and cells incubated under standard conditions.
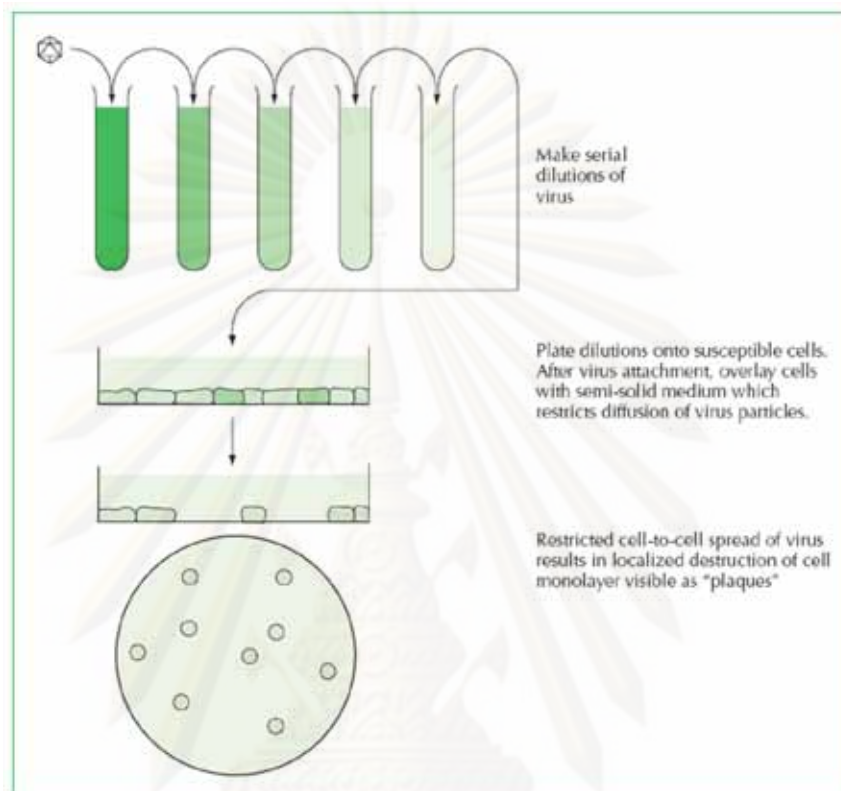
Figure15. Virus titration by standard plaque assays

After allowing time for the serial of viral dilutions to infect the LLC-MK2 cells, liquid medium was replaced by a semisolid culture medium containing agarose, which restricts diffusion of virus particles from infected cells. At day 7, the medium was removed and the cells stained to make the holes in the monolayer (plaques) more easily visible. Each plaque therefore results from infection by a single plaque-forming unit (p.f.u.).

Aliquots of the growth medium were collected hourly for the first 24 hrs after infection and assayed for virus titer in duplicate by plaque assay. For long-term viral production, the JEV infected BV-2 culture was subcultured 2-3 times per week .The growth media was collected every week for 16 weeks for viral titration.

8. Identification of viral binding protein in microglial cell

8.1) Cell membrane preparation

The confluent 75-cm^2 tissue culture flasks of the BV-2 cells were used in the preparation of membrane extracts for separation by SDS-PAGE. The extracted protein was differentially centrifuged only for membrane protein. The membrane proteins were separated and electrotransferd to PVDF (Polyvinylidene Fluoride) membranes.

Cells were scraped in TBS buffer and pelleted by centrifugation at 1,200 x g for 4 min. The pellet of cells was lysed by vigorous vortexing in ice-cold modified buffer M (100 mM NaCl, 20 mM Tris-HCl (pH 8), 2 mM MgCl$_2$, 1 mM EDTA, 0.2% Triton X-100, 1 X Cocktail Protease Inhibitor). The nuclei and debris were removed by centrifugation at 600 x g for 3 min, and the membranous organelles were pelleted from the supernatant by further centrifugation at 6,000 x g for 5 min. Finally, the membrane proteins were pelleted by ultracentrifugation at 35,000 rpm for 30 min and resuspended in modified buffer M. The concentration of protein was quantified by the Bradford dye protein assay. (Figure16)

8.2) 1D and 2D SDS-PAGE

The extracted protein was differentially centrifuged and collected only for membrane protein, before loading onto 10% SDS polyacrylamide gel of 1D gel electrophoresis. For 2D gel electrophoresis, the membrane proteins were prepared for electrophoresis by TCA protein precipitation technique. Protein pellets were resolubilized in lysis buffer and IPG strip rehydration solution at room temperature for
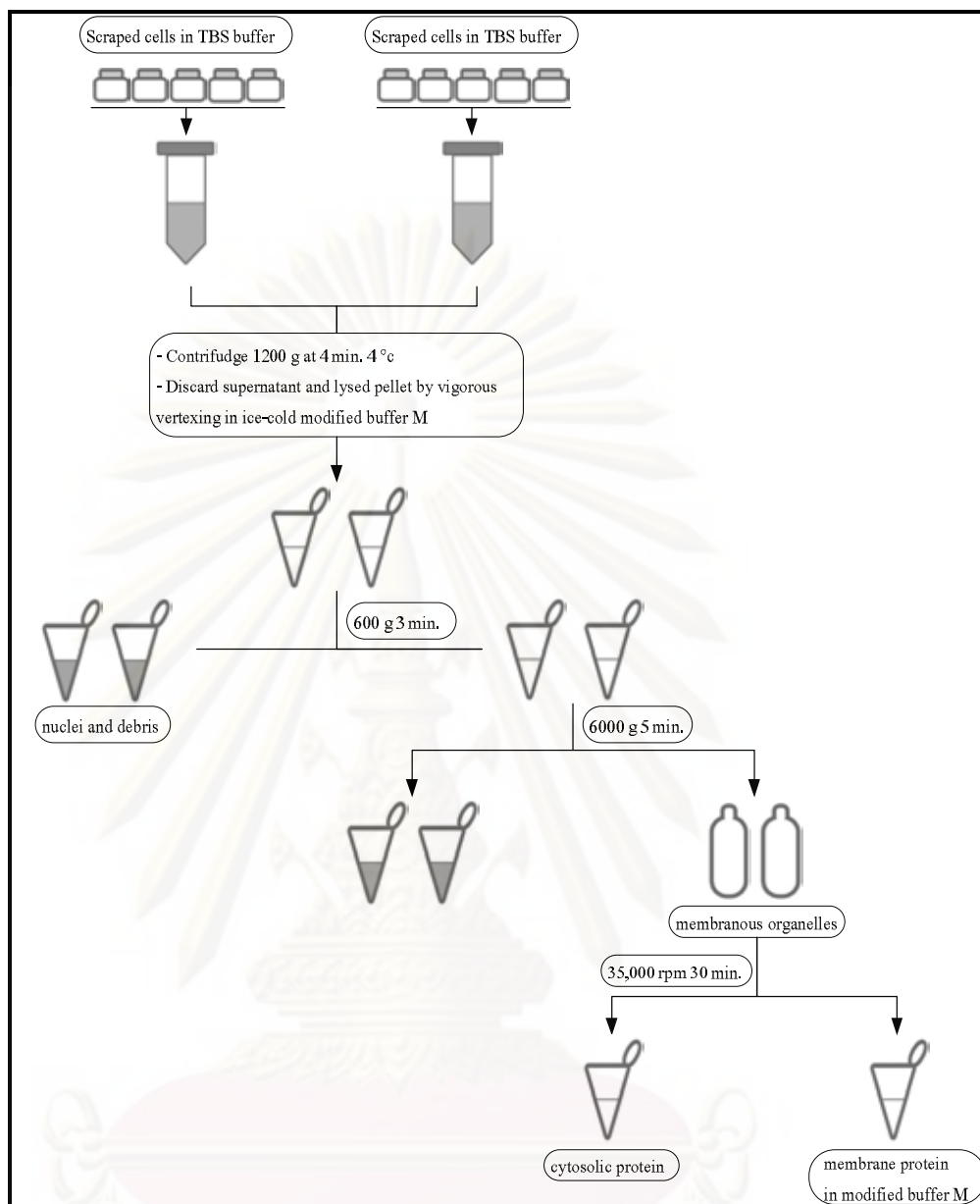
Figure16. Steps of membrane extraction by sequential centrifugation technique

This diagram shows steps of membrane protein extraction. Cells were pelleted by centrifugation at 1,200 x g for 4 min and subsequently resuspended in ice-cold modified buffer M and lysed by vigorous vortexing. Nuclei and debris were removed by centrifugation at 600 x g for 3 min, and membranous organelles were separated from the supernatant by centrifugation at 6,000 x g for 5 min. Membrane proteins were pelleted by centrifugation at 35,000 x g for 30 min and resuspended in modified buffer M.

overnight. 125 µl of the resulting supernatant were used for each IPG strip and rehydration was achieved for 8 hrs at 20°C using the IPGphor IEF system (Amersham Pharmacia Biotech, Uppsala, Sweden). Subsequently IEF carried out for 30 min at 300 V, 30 min at 1000 V and 1.5 hrs at 5000 V with a step-and-hold gradient until an approximately total of 7000 volt-hr had been achieved.

IPG strips were equilibrated by rocking for 10 min at room temperature in SDS equilibration buffer containing 10 mg/ml DTT followed by 10 min at room temperature in IAA equilibration buffer containing 25 mg/ml, allowing for at least 1.5 ml of buffer per strip. After this, the strips were placed on the top surface of the second dimension gel which was a 12.5% SDS polyacrylamide gel. Molecular weight markers were applied next to each strip on the top of each gel. The second dimension separation of proteins by molecular mass was achieved at a constant 12-15 mA/gel.

8.3) Western Blotting and VOPBA

The 1D and 2D gels were electrotransferred proteins to PVDF (Polyvinylidene Fluoride) membranes at 100 V for 150 min using Mini Trans-Blot® Electrophoresis Transfer Cell. Membranes were blocked by skimmed milk to prepare for VOPBA.

After blocking for overnight in TBS containing 5% skimmed milk, the proteins on membrane were overlaid with JEV for identification of JEV viral binding protein. The membranes were incubated with rocking at room temperature for 1 hr followed by overnight at 4°c with 10^7 pfu/cm^2 of JEV in TBS containing 1% skimmed milk. The blots were then washed with TBS and incubated with the pan-specific anti-flavivirus monoclonal antibody at a dilution of 1:50 in 5% skimmed milk in TBS for 2 hrs at room temperature. After washing 3 times with 0.1% TBS-tween and TBS, the blots were incubated with horseradish-peroxidase-conjugated rabbit antimouse IgG at a dilution of 1:3000 in 5% skimmed milk in TBS for 1 hr at room temperature. The reactive protein spots were visualized by developing with SuperSignal® West Pico Chemiluminescent

Substrate Kits. Then, the reactive protein images were compared with the Coomasie staining gel scanned previously.

The viral binding protein(s) have been seen as reactive bands and spots on the blots. The corresponding bands and spots in the coomassie blue-stained gel were picked out and identified by mass spectrometry.

8.4) Liquid chromatography-mass spectrometry (LC/MS/MS)

All of this part in this method was facilitated by Chulabhorn Research Institute. The selected bands were subjected into in-gel proteolysis with trypsin before analyzed by LC-MS/MS. LC/MS/MS analyses were carried out using a capillary LC system (Waters) coupled to a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray ion-source working in the nanoelectrospray mode. Glu-fibrinopeptide was used to calibrate the instrument in MS/MS mode. The tryptic peptides were concentrated and desalted on a 75 μm ID x 150 mm C18 PepMap column (LC Packings, Amsterdam, The Netherlands). Eluents A and B were 0.1% formic acid in 97% water, 3% acetonitrile and 0.1% formic acid in 97% acetonitrile respectively. 6 μl of sample was injected into the nanoLC system, and separation was performed using the following gradient: 0 min 7% B, 35 min 50% B, 45 min 80% B, 49 min 80% B, 50 min 7% B, 60 min 7% B. The database search was performed with ProteinLynx screening SWISS-PROT and NCBI. For some proteins that were difficult to find, the Mascot search tool available on the Matrix Science site screening NCBInr was used.

CHAPTER IV
RESULTS

4.1 Propagation of JEV in C6/36 cells

C6/36 cells were cultured in media until they reached 80% confluences. The cell number was determined using hemocytometer. Cells were later infected with JEV strain Beijing-1 at an MOI of 1. After proceeding of viral absorption was allowed for 120 min at 28°c with constant agitation, cells were supplemented with fresh culture medium and incubated at 28°c for 3 days. Aliquots of the culture medium were stored at -80°c and virus titer was subsequently determined by standard plaque assay.

4.2 Virus Titration by plaque assay

The LLC-MK2 cell monolayer in 6-well plate was infected with a serial dilution of the virus suspension to be titrated for 90 min at 37°c with constant shaking. Subsequently, 2% (w/v) Seakem LE agarose mixed with nutrient overlay was added to each well. The plates were further incubated at 37°c in 5% CO_2 for 7 days. Agarose plugs were removed after cells were fixed with 3.7% formaldehyde for 1 hr at room temperature and then stained with 1% crystal violet in ethanol. Plaques were easily counted at seven days after infection at 10^{-6} dilution of the original virus stock (Figure17). The virus titer was calculated as 1.8×10^6 p.f.u./ml

4.3 Infection of BV-2 cells with JEV

To determine whether microglial BV-2 cells can permissive to JEV or not. BV-2 cells were plated in 24-wells plates at 37°C for 24 hrs. After incubation, the cells were washed once with PBS and then cells were incubated with JEV strain Beijing-1 at an MOI of 1 for 2 hours at 37°c. The excess unbound viruses were removed by being washed once with PBS and followed by acid glycine treatment for 1 min.
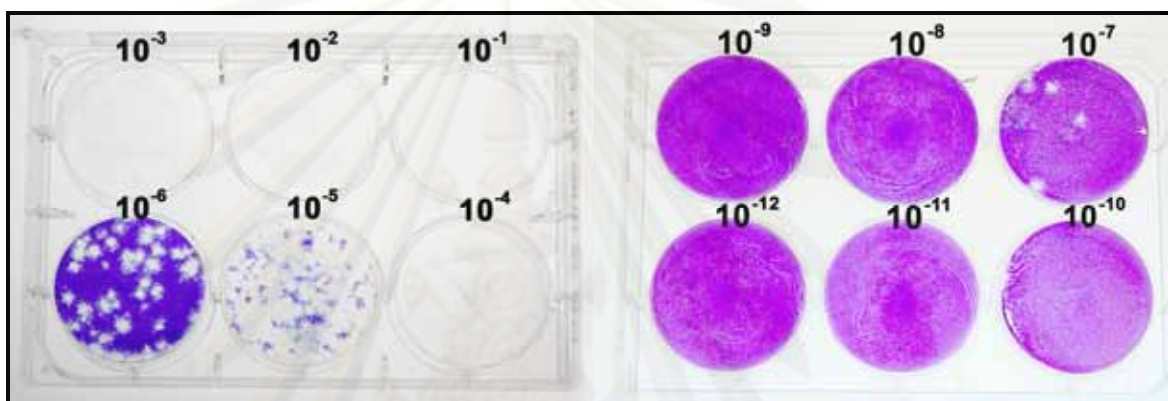
Figure17. Standard plaque assay on LLC-MK2 cells after incubation for 7 days

This figure shows the result of virus titration. JEV infected LLC-MK2 cell monolayer was infected with a serial dilution of the virus suspension to be titrated, and covered with agar. The plates were incubated and plaques were counted at 7 days after infection. Each well of 6-well plate represented a dilution of original virus stock as indicated.

After being fixed with ice cold-methanol for 10 min, the cells were permeabilized with 0.1% Triton X-100 and blocked by incubation with 1% goat serum. JEV were detected using primary antibody (mouse monoclonal anti JEV E-protein) and secondary antibody (goat anti mouse IgG - Alexaflor *594*). Nuclei were counterstained with DAPI and cells were viewed under a fluorescent microscope shown in Figure18. The result reveals that JEV can infect microglial cells. The JEV virions were detected in cytosol as shown in red color.

4.4 Japanese encephalitis viral production in BV-2 cells

4.4.1 Early JEV production

To determine the time course of JEV virions produced from JEV infected BV-2 cells in early-term, viral absorption was allowed to proceed for 2 hrs at 37°c with constant agitation. After this period cells were washed three times with PBS to remove unabsorbed viruses. After that cells were later treated with acid glycine (pH 3.0) for 1 min to wash any un-internalized extra cellular viruses followed by washing in PBS. Fresh culture media was added to the cells and the cells incubated under standard conditions. Aliquots of the growth medium were taken hourly for the first 24 hrs post infection. Result in Figure19 revealed that JEV infected BV-2 cells released infectious progeny virus into the culture media at 10 hrs post infection.
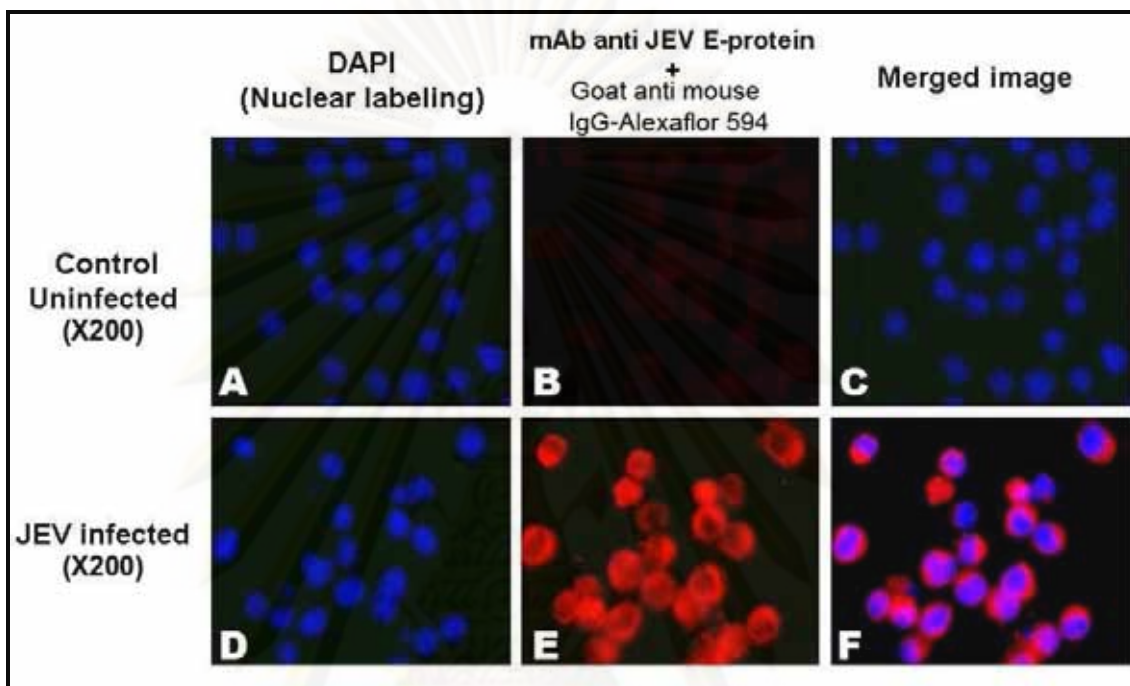
Figure18. Immunocytochemistry of JEV infected BV-2 cells

Panel (A-C) Uninfected BV-2 cells as negative control. (A) Cells were nuclear stained with DAPI (shown in blue). (B) No red color of JEV viral E protein was detected in cytosol of BV-2 cells after staining with 1°Ab (pan-specific anti-flavivirus monoclonal antibody) and 2°Ab antibody (goat anti mouse IgG-*Alexaflor 594*). (C) Merged images of A and B. Panel (D-F) JEV infected BV-2 cells at an MOI of 1. At 24 hrs post infection, (D) cells were nuclear stained with DAPI (shown in blue), (E) JEV viral E protein was detected in infected BV-2 cells as shown in red after staining with 1°Ab (pan-specific anti-flavivirus monoclonal antibody) and 2°Ab antibody (goat anti mouse IgG-*Alexaflor 594*). (F) Merging images of D and E. JEV infected BV-2 cells were identified in red color staining of viral E protein in their cytoplasm.
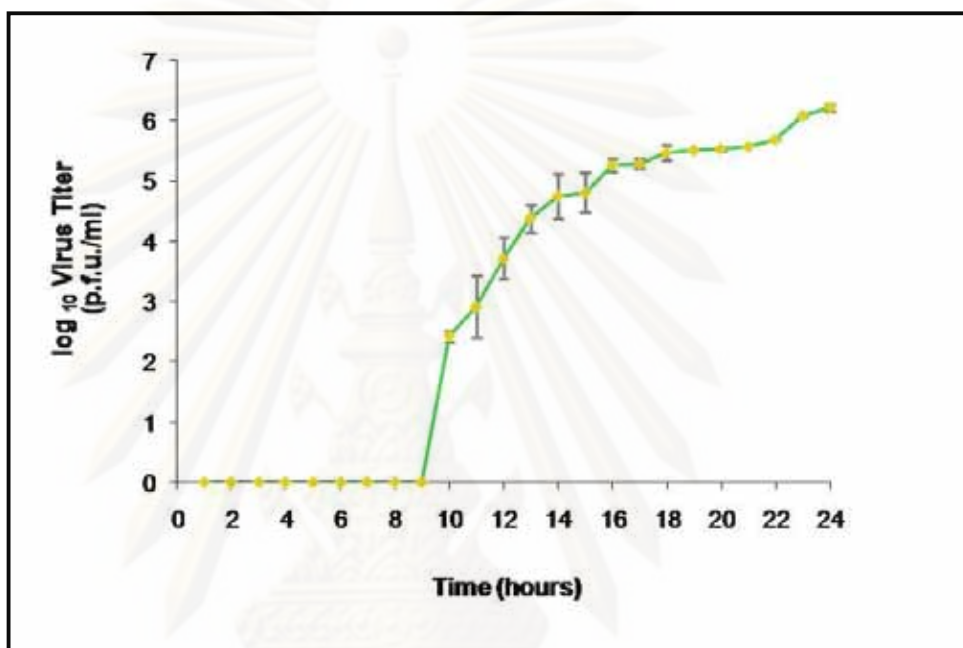
Figure19. Early Japanese encephalitis viral production from BV-2 cell lines

The BV-2 cells were infected with JEV at an MOI of 1. After 2 hrs of viral absorption, the supernatants were aliquoted hourly for 24 hrs post infection. The virus titers were determined by standard plaque assay on LLC-MK2 cells at the indicated time points. The values represent the mean $\pm$ SEM of three independent experiments.

4.4.2 Long-term JEV production

For determination of viral production in long-term, the persistently infected BV-2 cells were maintained in DMEM with 5% FBS. The culture medium was collected weekly for 16 weeks and viral titers were determined. Results (Figure 20) revealed that JEV infected BV-2 cells were still producing virus for 16 weeks. In addition, morphology of persistently infected BV-2 cells were compared to that of normal cells (uninfected) under light microsocopy (Figure21). It was shown that the morphology of the persistently infected BV-2 cells were different from the normal cells slightly. Persistently infected BV-2 cells were more spiked-shape and have many multinucleated cells.

4.5 JEV binding protein(s) on BV-2 cell surface

To identify JEV binding protein (s) on the surface of mouse microglial cells, one dimensional gel electrophoresis / two dimensional gel electrophoresis followed by virus overlay protein binding assay (VOPBA) were investigated. The excised bands were subjected to LC/MS/MS for peptide mass fingerprinting analysis to further identify JEV binding protein(s) detected by VOPBA.

4.5.1 One dimensional gel electrophoresis - VOPBA

100 µg of membrane protein was separated on a 10% SDS-PAGE and transferred to PVDF (Polyvinylidene Fluoride) membranes. The membranes were incubated with JEV and subsequently with a pan-specific anti-flavivirus monoclonal antibody, followed by a secondary anti-mouse IgG conjugated with horseradish peroxidase. After western blotting, several virus binding protein bands were detected compared with coomasie staining gel as shown in Figure 22-23. The major virus binding protein bands, as indicated by arrows in those figures, were excised and subjected to in-gel proteolysis with trypsin followed by LC/MS/MS. After PLGS Databank searching analysis, the result identified laminin receptor precursor (shown as band 7* in figure 22) protein (43 kDa) as candidates for JEV receptors on microglial cells.
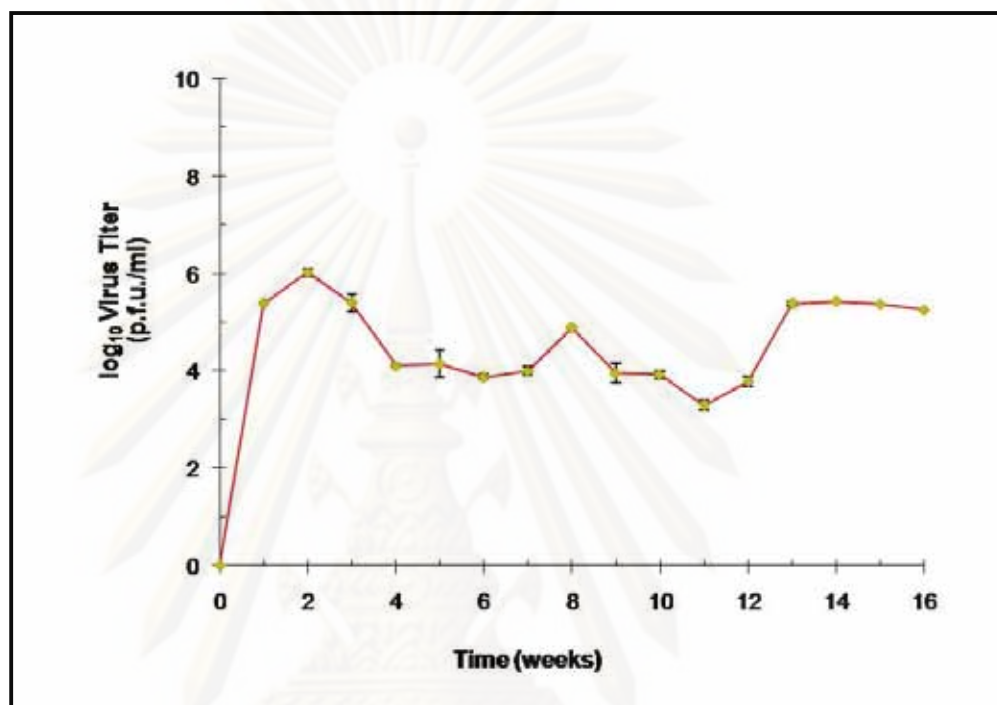
Figure20. Long-term Japanese encephalitis viral production from BV-2 cell line

After cells were infected with JEV at an MOI of 1, cells were subcultured 2-3 times per week for 16 weeks. The growth media was collected weekly to determine virus titration by standard plaque assay on LLC-MK2 cells. Experiments were undertaken independently in duplicated with triplicate titer of samples. Each point represents the triplicate count. Error bars represent SEM.

(A)

(B)
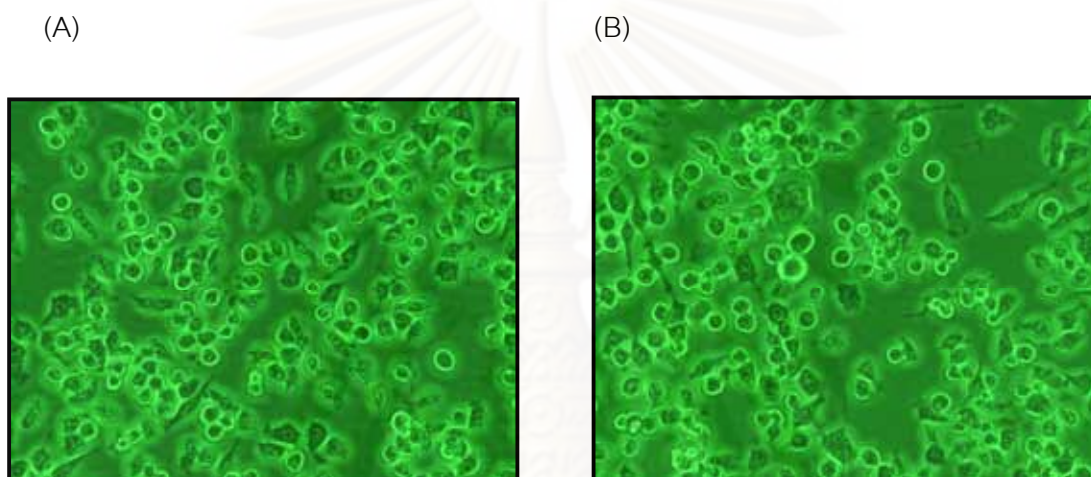
Figure21. Morphology of normal BV-2 and persistently JEV-infected BV-2 cells

       Morphological comparison between (A) normal BV-2 cells and (B) persistently infected BV-2 cells at 16 weeks culture. Cells were observed under light microscope (X200).
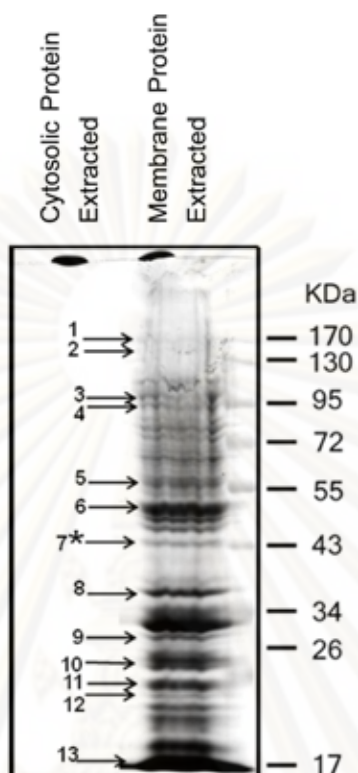
Figure22. One-dimensional gel electrophoresis of extracted membrane proteins from BV-2 cells.

Using 10% gel SDS-PAGE to separated membrane protein followed by VOPBA technique for detection of virus binding protein. After comparing this gel pattern with western blotting results, many bands were selected and cut for identification by LC/MS/MS (arrow). The band 7* of gel was later identified as laminin receptor precursor protein (43 kDa). Description of other JEV binding protein bands, after PLGS databank searching analysis, was also included in appendix.

Figure23. One-dimensional VOPBA analysis of JEV binding proteins on BV-2 cells

The virus binding protein bands were detected by using a pan-specific anti-flavivirus monoclonal antibody and secondary conjugated with HRP antibody. Many bands were selected for identification by LC/MS/MS (arrow). Negative control of no virus was also applied in parallel.

4.5.2 Two-dimensional gel electrophoresis -VOPBA

350 μg of membrane protein was separated using 2D technique and transferred to PVDF (Polyvinylidene Fluoride) membranes for VOPBA analysis. Several virus binding protein spots were also detected as shown in Figure 24-25. The major virus binding protein spots were excised and subjected to in-gel proteolysis with trypsin followed by LC/MS/MS. After PLGS Databank searching analysis, laminin receptor precursor protein (43 kDa) was again confirmed as JEV binding protein on microglial cells. (shown as spot no.4 in figure 24).

4.5.3 Liquid Chromatography-Mass spectrometry analysis

The major virus binding protein bands were subjected into in-gel proteolysis with trypsin and then LC/MS/MS analyses. The database search was performed with ProteinLynx screening SWISS-PROT and NCBI. For some proteins that were difficult to find, the Mascot search tool available on the Matrix Science site screening NCBInr was used and summarized in Table3. After PLGS Databank searching analysis, the results identified laminin receptor precursor protein (43 kDa) both in 1D - VOPBA and 2D - VOPBA as candidates for JEV receptors on microglial cells. We confirm the result with Mascot search tool shown in Figure 26-27.
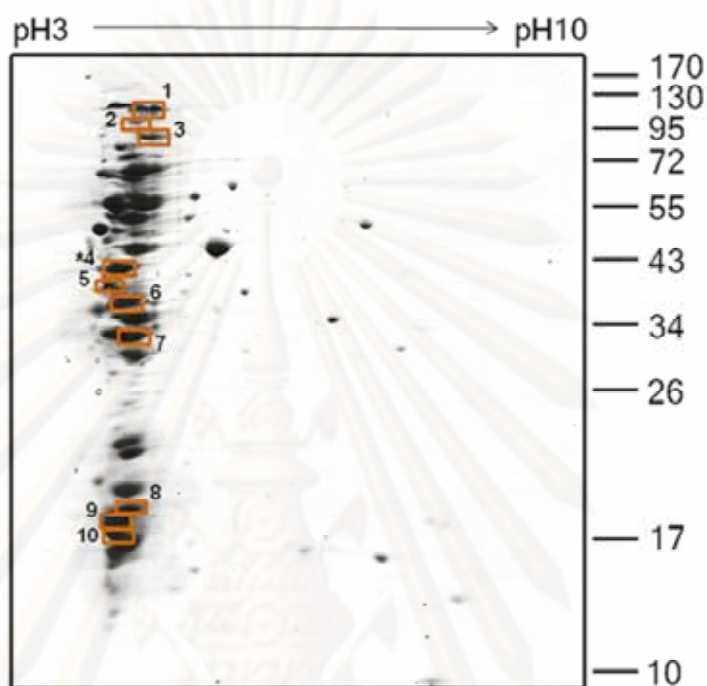
Figure24. Two-dimensional gel electrophoresis of extracted membrane proteins from BV-2 cells

After the first dimension gel electrophoresis, using 12.5% gel SDS-PAGE in second dimension to separated membrane protein followed by VOPBA technique for detection of virus binding protein. After compared this gel pattern with western blotting results, many bands were selected and cut for identification by LC/MS/MS. The spot no.4 of the gel was identified as laminin receptor precursor protein (43 kDa). Description of other JEV binding protein spots, after PLGS databank searching analysis, was also included in appendix.
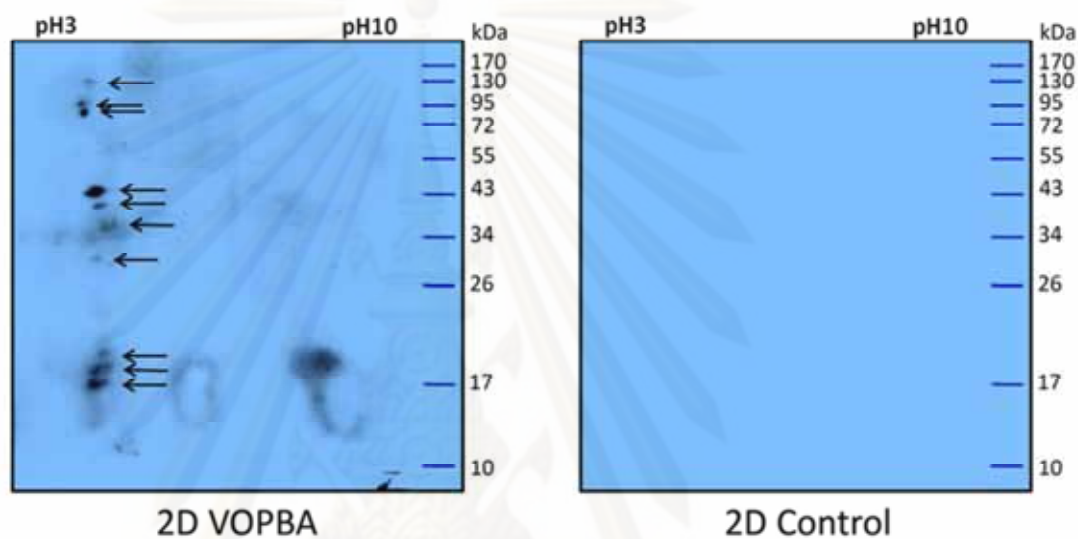
Figure25. Two-dimensional VOPBA analysis of JEV binding proteins on BV-2 cells

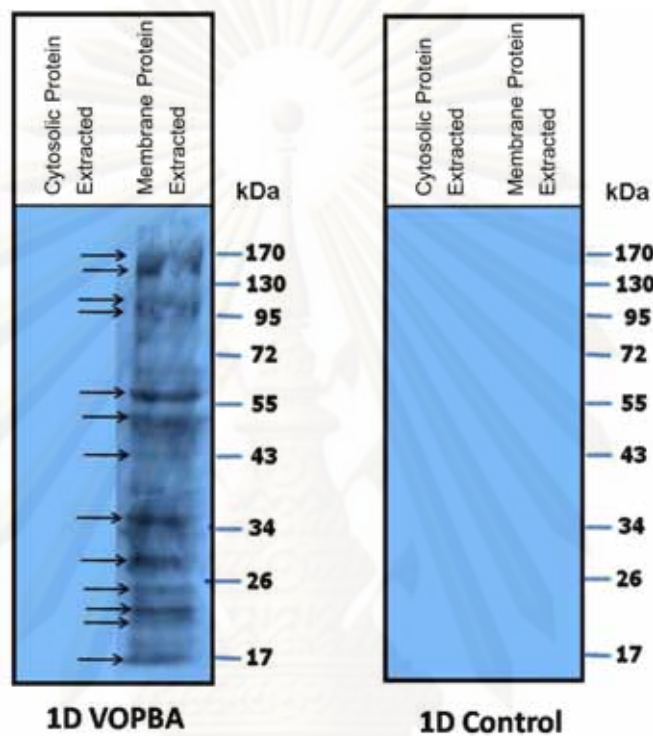The virus binding protein bands were detected by using a pan-specific anti-flavivirus monoclonal antibody and secondary conjugated with HRP antibody. Many bands were selected for identification by LC/MS/MS (arrow). Negative control of no virus was also applied in parallel.

Table3. Results of LC/MS/MS spectra matches from the NCBInr protein database

Match*	Accession No.	Protein Description	Mol mass (Da)	Score
1	1405340A	protein 40 KD	32,732	102
2	AAA39413	laminin receptor	32,698	102
3	1405340A	protein 40 KD	32,732	372
4	ACB59248	laminin receptor	32,829	372
5	XP_620036	similar to 67 kda laminin receptor	27,991	155

*Matches 1 to 2 are from the spectra obtained from the 1D VOPBA samples, while matches 3 to 5 are from the spectra obtained from the 2D VOPBA samples.

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
127	456.7717	911.5288	911.5440	-0.0152	0	16	15	1	R.LLVVTDPR.A
147	568.2867	1134.5588	1134.6284	-0.0696	0	43	0.03	1	K.SDGIYIINLK.R
155	602.3110	1202.6074	1202.6408	-0.0333	0	66	0.00017	1	K.FAAATGATPIAGR.F
156	602.8134	1203.6122	1202.6408	0.9715	0	(34)	0.26	1	K.FAAATGATPIAGR.F
157	602.8254	1203.6362	1202.6408	0.9955	0	(6)	1.5e+02	1	K.FAAATGATPIAGR.F
200	849.9354	1697.8562	1697.8526	0.0037	0	20	6.2	1	R.FTPGTFTNQIQAAFR.E
203	572.2731	1713.7975	1714.7897	-0.9922	1	(12)	30	1	R.DPEEIEKEEQAAAEK.A
204	572.6211	1714.8415	1714.7897	0.0518	1	(19)	5.5	1	R.DPEEIEKEEQAAAEK.A
205	858.4464	1714.8782	1714.7897	0.0896	1	37	0.085	1	R.DPEEIEKEEQAAAEK.A

1. gi|226005 Mass: 32732 Score: 102 Queries matched: 9 emPAI: 0.72
   protein 40kD
   ☐ Check to include this hit in error tolerant search

Proteins matching the same set of peptides:
gi|293694 Mass: 32698 Score: 102 Queries matched: 9
laminin receptor

gi|62024907 Mass: 32821 Score: 102 Queries matched: 9
Ribosomal protein SA [Mus musculus]

Figure26. MASCOT analysis of 1D VOPBA

This diagram represents the homology search using MASCOT. X-axis represents probability based mouse score and y-axis represents the number of hits. Result shown that the MASCOT analysis exhibited a score of 102 matching with laminin receptor.
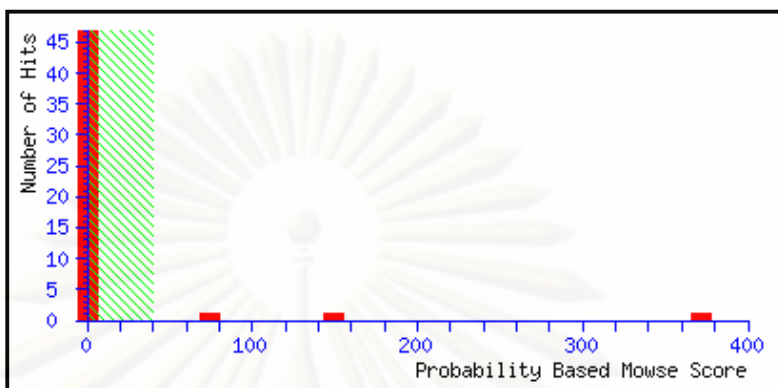
1. gi|226005   Mass: 32732   Score: 372   Queries matched: 16   emPAI: 1.98
   protein 40kD
   ☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	93	430.2285	858.4424	858.3970	0.0454	0	(15)	36	1	R.DPEEIEK.E
☑	94	859.4576	858.4503	858.3970	0.0533	0	39	0.12	1	R.DPEEIEK.E
☑	102	456.8075	911.6004	911.5440	0.0564	0	42	0.042	1	R.LLVVTDPR.A
☑	104	457.3302	912.6458	911.5440	1.1018	0	(3)	3.3e+02	1	R.LLVVTDPR.A
☑	128	568.3519	1134.6892	1134.6284	0.0608	0	47	0.013	1	K.SDGIYIINLK.R
☑	136	1203.7168	1202.7095	1202.6408	0.0688	0	(4)	2.4e+02	1	K.FAAATGATPIAGR.F
☑	137	602.3893	1202.7640	1202.6408	0.1233	0	68	0.00011	1	K.FAAATGATPIAGR.F
☑	143	632.3978	1262.7810	1262.7234	0.0576	1	79	7.8e-06	1	R.KSDGIYIINLK.R
☑	162	538.9922	1613.9548	1613.7959	0.1589	0	23	2.1	1	K.GAHSVGLMWWMLAR.E
☑	163	807.9879	1613.9612	1613.7959	0.1653	0	(23)	2.3	1	K.GAHSVGLMWWMLAR.E
☑	167	850.0104	1698.0062	1697.8526	0.1537	0	118	7.6e-10	1	R.FTPGTFTNQIQAAFR.E
☑	169	858.4916	1714.9686	1714.7897	0.1790	1	94	1.6e-07	1	R.DPEEIEKEEQAAAEK.A
	170	572.6965	1715.0677	1714.7897	0.2780	1	(1)	2.8e+02	3	R.DPEEIEKEEQAAAEK.A
☑	175	871.0712	1740.1278	1739.9417	0.1861	0	80	3e-06	1	R.AIVAIENPADVSVISSR.N
☑	181	941.5286	1881.0426	1880.8556	0.1871	0	46	0.0092	1	R.EHPWEVMPDLYFYR.D
☑	210	873.2198	2616.6376	2616.2683	0.3693	0	15	4.5	1	K.FLAAGTHLGGTNLDFQMEQYIVK.R

Proteins matching the same set of peptides:

gi|171948782   Mass: 32829   Score: 372   Queries matched: 16
laminin receptor [Mus musculus]

Figure27. MASCOT analysis of 2D VOPBA

This diagram represents the homology search using MASCOT. X-axis represents probability based mouse score and y-axis represents the number of hits. Result shown that the MASCOT analysis exhibited a score of 372 matching with laminin receptor.

CHAPTER V
DISCUSSION AND CONCLUSIONS

Japanese encephalitis virus (JEV) leads to a wild spectrum of clinical manifestations ranging from febrile illness to fatal encephalitis. Even though the target of JEV infection is neuronal cell, some case reports revealed that microglia, a type of glial cells that acts as primary immune cell in the CNS, was activated morphologically, resulting in increased levels of cytokines in the serum and cerebrospinal fluid in patients. [5, 6-8, 10] Therefore, this research aimed to study virus – host interaction in the aspect of microglia cell permissiveness and JEV binding protein(s) identification upon JEV infection

Immunocytochemistry technique was applied to study the infection of JEV in microglial cells. The result of immunocytochemistry revealed that mouse microglial BV-2 cells are susceptible to JEV infection. E protein was detected in cytosol of JEV infected microglia BV-2 cells at 24 hr post infection with the multiplicity of infection at 1. To quantitate the % infectivity, flow cytometry for detection of the stained cell should be performed.

JEV could replicate in microglia cells as demonstrated by virus plaque-forming units. The extracellular virions were released into the supernatant at 10 hr post infection, comparing to the released infectious progeny virus in the culture fluid after 24 hr in JEV infected astrocyte.[37] JEV could replicate even more efficiently in microglial (BV-2) cells than in its target neuroblastoma (HTB-11) cells [38] as demonstrated by higher plaque titer. Although Nakayama-NIH strain of JEV, instead of Beijing -1, was used for their study, both viral strains were classified in genotype III and were used for the production of mouse brain inactivated JEV vaccine because for their high virulence.[23,24]

In addition to the typical vegetative replication cycle, many viruses can establish long term persistent infection in host organisms and cultured cells. The underlying mechanisms of persistency are the suppression of viral cytopathic effects and evasion of cellular defenses, resulting in a failure to eliminate the virus. [28]. Viral persistence in the human nervous system immunologically and virologically was reported in 5% of JEV

infected patients [38]. Persistent infection with JEV has been described in rabbit kidney (MA-111)[29], monkey kidney (Vero)[29] and murine neuroblastoma N18 cell lines .[30]

Our results demonstrate that JEV could replicate very efficiently in microglial cells. Moreover, we cultured persistently infected microglial cells by routinely subcultured 2-3 times per week for 16 weeks. The virion released into the supernatant proved to be infectious to neuronal cells. (Chaiworakul,V. personal communicate) This result implies that microglia BV-2 cells may perform as a viral reservoir in nervous system. However, the in vivo experiments need to be performed for the confirmation.

Many cellular molecules have thus been identified as putative receptor(s) for JEV such as heat shock protein 70 on mouse neuroblastoma (Neuro2a) cells[33] and 53 kD protein on C3/36 cells[9]. Viral overlay protein binding assay (VOPBA) is an assay that has been widely used to characterize putative viral receptors[9,27,32,33]. For the identification of JEV binding protein(s) on microglial cells, the major virus binding protein bands revealed by VOPBA analysis were excised from identical parallel gels and subjected to in-gel proteolysis followed by LC/MS/MS. Result from two dimensional gel electrophoresis virus overlay protein binding assay (2D-VOPBA), followed by LC/MS/MS, identified many protein spots as potential JEV binding protein(s) on microglial cells. After PLGS Databank searching analysis and confirmed with MASCOT analysis, nucleolin, tumor rejection antigen gp96, peptidylprolyl isomerase A (cyclophilin A), nucleophosmin 1, laminin receptor precursor protein and many unknown proteins are on the list. Nucleolin is a nonhistone nucleolar phosphoprotein which ubiquitously expressed as acidic phosphoprotein of exponentially growing cells. It is involved mainly in the synthesis and maturation of ribosomes.[40] Tumor rejection antigen gp96 protein, a glycoprotein of 96 kDa localized to the endoplasmic reticulum but can also be present at the cell surface, is known as heat shock protein gp96 in which related to one of the heat shock proteins, hsp90. This protein is complexed with the transcription factor heat shock factor 1 (HSF1), a key regulatory protein responsible in Hsp synthesis. [41, 42] Peptidylprolyl isomerase A (cyclophilin A), is ubiquitous enzyme expressed in both prokaryotic and eukaryotic cell. Its function is to facilitate the cis-trans isomerization of peptide bond and accelerate protein folding.[43] Nucleophosmin is a nucleolar phosphoprotein implicated in ribosome biogenesis, stabilizes and regulates the

transcriptional activity of *p53*. This protein acts as a molecular chaperone and shuttle between the nucleus and cytoplasm[44]. The 43 kDa mouse laminin receptor precursor protein is thought to be a precursor protein generating a mature 67-70 kDa mouse laminin receptor (MLR)[45] by dimerization and acylation[34]. The MLR is a nonintegrin cell surface laminin receptor that is an imperative molecule both in cell adhesion to the basement membrane and in signaling transduction following the laminin binding. However, being a receptor is not the only function of this precursor protein, the immunological detection of P40, localized to the small subunit of the ribosome, implying its function as an initiator for translation[46-48].

Laminin receptor protein has been well characterized as a receptor protein for many RNA viruses, such as Sindbis virus and dengue virus on different cell line with multiple approaches. [4, 20-22, 31, 48] Our data from 1D-VOPBA followed with LC/MS/MS firstly identified laminin receptor precursor protein (43 kDa as estimated by SDS-PAGE) as JEV binding protein on mouse microglial cells. This finding was further confirmed with the result from 2D-VOPBA, as mentioned above. Therefore, a 43 kDa mouse laminin receptor precursor protein is a potential candidate JEV receptor on microglial cells.

Due to the fact that the structure of the virus attachment proteins of mosquito-borne flaviviruses are very similar, it was proposed that the same receptor molecules for mosquito-borne flaviviruses may present on the surface of C6/36 mosquito cells[14]. Identification of the 37/67-kDa high-affinity laminin receptor protein for dengue virus serotype 1 on the liver cells[32] correlated with our finding of 43 kDa laminin receptor precursor protein as a JEV binding protein on microglial cells. However, coimmunoprecipitation to confirm the interaction between laminin receptor and JEV virion and antibody inhibition study to block JEV entry into microglia need to be performed to verify laminin receptor as a putative receptor for Japanese encephalitis virus later on.

REFERENCES

[1]   Solomon T, Ni H, Beasley DW, Ekkelenkamp M, Cardosa MJ, Barrett AD. Origin
        and   evolution of Japanese encephalitis virus in southeast Asia. J Virol.
        2003 Mar;77(5):3091-8.

[2]   Solomon T. Recent advances in Japanese encephalitis. J Neurovirol. 2003
        Apr;9(2):274-83.

[3]   Tiroumourougane SV, Raghava P, Srinivasan S. Japanese viral encephalitis.
        Postgrad Med J. 2002 Apr;78(918):205-15.

[4]   Solomon T, Dung NM, Kneen R, Gainsborough M, Vaughn DW, Khanh VT.
        Japanese   encephalitis.   J   Neurol   Neurosurg   Psychiatry.   2000
        Apr;68(4):405-15.

[5]   Ghoshal A, Das S, Ghosh S, Mishra MK, Sharma V, Koli P, et al. Proinflammatory
        mediators released by activated microglia induces neuronal death in
        Japanese encephalitis. Glia. 2007 Apr 1;55(5):483-96.

[6]   Kimura-Kuroda J, Ichikawa M, Ogata A, Nagashima K, Yasui K. Specific tropism
        of Japanese encephalitis virus for developing neurons in primary rat
        brain culture. Arch Virol. 1993;130(3-4):477-84.

[7]   Das S, Basu A. Japanese encephalitis virus infects neural progenitor cells and
        decreases their proliferation. J Neurochem. 2008 Aug;106(4):1624-36.

[8]   Kumar S, Kalita J, Saxena V, Khan MY, Khanna VK, Sharma S, et al. Some
        observations on the tropism of Japanese encephalitis virus in rat brain.
        Brain Res. 2009 May 1;1268:135-41.

[9]   Boonsanay V, Smith DR. Entry into and production of the Japanese encephalitis
        virus from C6/36 cells. Intervirology. 2007;50(2):85-92.

[10]   Khanna N, Agnihotri M, Mathur A, Chaturvedi UC. Neutrophil chemotactic factor
        produced by Japanese encephalitis virus stimulated macrophages. Clin
        Exp Immunol. 1991 Nov;86(2):299-303.

[11]   Gehrmann J, Matsumoto Y, Kreutzberg GW. Microglia: intrinsic immuneffector cell of the brain. Brain Res Brain Res Rev. 1995 Mar;20(3):269-87.

[12]   Chen CJ, Chen JH, Chen SY, Liao SL, Raung SL. Upregulation of RANTES gene expression in neuroglia by Japanese encephalitis virus infection. J Virol. 2004 Nov;78(22):12107-19.

[13]   Ravi V, Parida S, Desai A, Chandramuki A, Gourie-Devi M, Grau GE. Correlation of tumor necrosis factor levels in the serum and cerebrospinal fluid with clinical outcome in Japanese encephalitis patients. J Med Virol. 1997 Feb;51(2):132-6.

[14]   Ren J, Ding T, Zhang W, Song J, Ma W. Does Japanese encephalitis virus share the same cellular receptor with other mosquito-borne flaviviruses on the C6/36 mosquito cells? Virol J. 2007;4:83.

[15]   Leyssen P, De Clercq E, Neyts J. Perspectives for the treatment of infections with Flaviviridae. Clin Microbiol Rev. 2000 Jan;13(1):67-82.

[16]   Mori Y, Okabayashi T, Yamashita T, Zhao Z, Wakita T, Yasui K, et al. Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. J Virol. 2005 Mar;79(6):3448-58.

[17]   Yadav J.S. A Special Issue on Japanese encephalitis. ENVIS News Letter. 2006 Jan;1(3).

[18]   Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol. 2005 Jan;3(1):13-22.

[19]   Su CM, Liao CL, Lee YL, Lin YL. Highly sulfated forms of heparin sulfate are involved in japanese encephalitis virus infection. Virology. 2001 Jul 20;286(1):206-15.

[20]   National Travel Health Network and Centre. http://www.nathnac.org/pro/factsheets/japanese_enc.htm. United Kingdom: National Travel Health Network and Centre, 2009 May.

[21]   Nitatpattana N, Dubot-Peres A, Gouilh MA, Souris M, Barbazan P, Yoksan S, et

al. Change in Japanese encephalitis virus distribution, Thailand. Emerg

Infect Dis. 2008 Nov;14(11):1762-5.

[22]   Gould EA, Solomon T. Pathogenic flaviviruses. Lancet. 2008 Feb

9;371(9611):500-9.

[23]   Solomon T. New vaccines for Japanese encephalitis. Lancet Neurol. 2008

Feb;7(2):116-8.

[24]   Shlim DR, Solomon T. Japanese encephalitis vaccine for travelers: exploring the

limits of risk. Clin Infect Dis. 2002 Jul 15;35(2):183-8.

[25]   Lin RJ, Liao CL, Lin YL. Replication-incompetent virions of Japanese

encephalitis virus trigger neuronal cell death by oxidative stress in a

culture system. J Gen Virol. 2004 Feb;85(Pt 2):521-33.

[26]   Clyde K, Kyle JL, Harris E. Recent advances in deciphering viral and host

determinants of dengue virus replication and pathogenesis. J Virol. 2006

Dec;80(23):11418-31.

[27]   Tio PH, Jong WW, Cardosa MJ. Two dimensional VOPBA reveals laminin

receptor (LAMR1) interaction with dengue virus serotypes 1, 2 and 3.

Virol J. 2005;2:25.

[28]   Mathur A, Arora KL, Rawat S, Chaturvedi UC. Persistence, latency and

reactivation of Japanese encephalitis virus infection in mice. J Gen Virol.

1986 Feb;67 (Pt 2):381-5.

[29]   Schmaljohn CS, Blair CD. Clonal analysis of mammalian cell cultures persistently

infected with Japanese encephalitis virus. J Virol. 1979 Sep;31(3):816-

22.

[30]   Chen LK, Liao CL, Lin CG, Lai SC, Liu CI, Ma SH, et al. Persistence of Japanese

encephalitis virus is associated with abnormal expression of the

nonstructural protein NS1 in host cells. Virology. 1996 Mar 1;217(1):220-

9.

[31]   Rey FA. Dengue virus envelope glycoprotein structure: new insight into its
         interactions during viral entry. Proc Natl Acad Sci U S A. 2003 Jun
         10;100(12):6899-901.

[32]   Thepparit C, Smith DR. Serotype-specific entry of dengue virus into liver cells:
         identification of the 37-kilodalton/67-kilodalton high-affinity laminin
         receptor as a dengue virus serotype 1 receptor. J Virol. 2004
         Nov;78(22):12647-56.

[33]   Das S, Laxminarayana SV, Chandra N, Ravi V, Desai A. Heat shock protein 70
         on Neuro2a cells is a putative receptor for Japanese encephalitis virus.
         Virology. 2009 Mar 1;385(1):47-57.

[34]   Buto S., Tagliabue E., Ardini E., Magnifico A., Ghirelli C., van den Brule F.,
         Castronovo V., Colnaghi M.I., Sobel M.E., Menard S. Formation of the 67-
         kDa laminin receptor by acylation of the precursor. J. Cell. Biochem.
         69:244-251(1998).

[35]   Guerrera IC, Kleiner O. Application of mass spectrometry in proteomics. Biosci
         Rep. 2005 Feb-Apr;25(1-2):71-93.

[36]   Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003 Mar
         13;422(6928):198-207.

[37]   Suri NK, Banerjee K. Growth and cytopathic effect of Japanese encephalitis
         virus in astrocyte-enriched cell cultures from neonatal mouse brains.
         Acta Virol. 1995 Jun;39(3):143-8.

[38]   Yang KD, Yeh WT, Chen RF, Chuon HL, Tsai HP, Yao CW and Shaio MF. A
         model to study neurotropism and persistency of Japanese encephalitis
         virus infection in human neuroblastoma cells and leukocytes. J Gen Virol
         (2004); 85:635-642.

[39]   Ravi V, Desai AS, Shenoy PK, Satishchandra P, Chandramukhi A. and Gourie-
         Devi M. Persistence of Japanese encephalitis virus in the human nervous
         system. J Med Virol. (1993); 40: 326-329

[40]   Farin K, Di Segni A, Mor A, Pinkas-Kramarski R. Structure-function analysis of nucleolin and ErbB receptors interactions. PLoS One. 2009;4(7):e6128.

[41]   Mkaddem SB, Werts C, Goujon JM, Bens M, Pedruzzi E, Ogier-Denis E, et al. Heat shock protein gp96 interacts with protein phosphatase 5 and controls toll-like receptor 2 (TLR2)-mediated activation of extracellular signal-regulated kinase (ERK) 1/2 in post-hypoxic kidney cells. J Biol Chem. 2009 May 1;284(18):12541-9.

[42]   Prof Dr H Ibelgaufts. http://www.copewithcytokines.de/cope.cgi?key=heat%20shock%20protein%20GP96. German: Cytokines & Cells Online Pathfinde Encyclopaedia, 2005 December.

[43]   Shaw PE. Peptidyl-prolyl isomerases: a new twist to transcription. EMBO Rep. 2002 Jun;3(6):521-6.

[44]   Dhar SK, St Clair DK. Nucleophosmin blocks mitochondrial localization of p53 and apoptosis. J Biol Chem. 2009 Jun 12;284(24):16409-18.

[45]   Rao C, Castronovo V, Schnitt MC, Wewer U, Claysmith A, Liotta LA, Sobol M, Evidence for a precursor of the high-affinity metastasis associated murine laminin receptor. Biochemistry 1989; 28: 7476-7486

[46]   Fulcher KD, Welch JE, Davis CM, O'Brien DA, Eddy EM. Characterization of laminin receptor messenger ribonucleic acid and protein expression in mouse spermatogenic cells. Biol Reprod. 1993 Mar;48(3):674-82.

[47]   Rabacchi SA, Neve RL, Drager UC. A positional marker for the dorsal embryonic retina is homologous to the high-affinity laminin receptor. Development 1990;109:521-531.

[48]   Makrides S, Chitpatima ST, Bandyopadhyav R, Brawerman G. Nucleotide sequence for a major messenger RNA for a 40 kilodalton polypeptide that is under translational control in mouse tumor cells. Nucleic Acids Res. 1988; 16:2349.

APPENDICES

APPENDIX A

Buffers and Reagents

1. Dulbecco's Modified Eagle's medium (DMEM) stock medium   1 liter

    Sodium Pyruvate powder   110   mg

    Penicillin-Streptomycin solution   11   ml

    HEPES, Free acid   11   ml

    sterilized by filtering through a 0.45 µm membrane filter

    store at 4°c

2. Minimum essential medium (MEMα) stock medium   1   liter

    Penicillin-Streptomycin solution   11   ml

    HEPES, Free acid   11   ml

    sterilized by filtering through a 0.45 µm membrane filter

    store at 4°c

3. 1X Phosphate Buffered Saline (PBS)   1 liter

    Phosphate Buffered Saline dry powder in foil pouch   1 packet

    Add ddH_2O up to 1 liter and sterilize by autoclaving

4. BA-1 buffer   100   ml

    20X m199   5   ml

    1M Tris-Cl, pH 7.6   5   ml

    2% BSA fraction V   50   ml

    7.5% $NaHCO_3$ 1   ml

    Penicillin-Streptomycin solution   1   ml

    Add ddH_2O up to 100 ml

    ** Prepare freshly before use

5. 2X Nutrient solution   100   ml

     20X EBSS   9.8   ml

     Ye-lah   6.6   ml

     Fetal Bovine Serum (FBS)   6   ml

     Gentramycin   0.5   ml

     7.5% $NaHCO_3$ 6   ml

     Add ddH_2O up to 100 ml

     ** Prepare freshly before use

6. 2% Seakem LE Agarose   100   ml

     Seakem LE Agarose   2   g

     Add ddH_2O up to 100 ml

     Melted gel by microwave

     ** Prepare freshly before use

7. Ye-lah solution   100   ml

     Yeast extract   1   g

     Lactralbumin Hydrolysate   5   g

     Add ddH_2O up to 100 ml

     sterilized by filtering through a 0.45 µm membrane filter

     store at 4°c

8. 2% BSA fraction V   500   ml

     BSA fraction V powder   10   g

     Add ddH_2O up to 500 ml

     sterilized by filtering through a 0.45 µm membrane filter

     store at 4°c

9. 10x TBS buffer   1   liter

     Tris-Cl   60.57   g

     NaCl   87.6   g

     ddH_2O 800   ml

     Adjust pH to 7.6

     Add ddH_2O up to 1 liter

10. 1x TBS buffer   1   liter

     10x TBS buffer   100   ml

     Add ddH_2O up to 1 liter

11. 10x Transfer buffer   1   liter

     Tris-Cl   15.15   g

     Glycine   72.1   g

     Add ddH_2O up to 1 liter

12. 1x Transfer buffer   1   liter

     10x Transfer buffer   80   ml

     Methanol   200   ml

     Add ddH_2O up to 1 liter

     store at 4°c

13. 5% skim milk in TBS buffer   50   ml

     skim milk powder   2.5   g

     TBS buffer   50   ml

     ** Prepare freshly before use

14. 1x buffer M

	100	ml
Tris-Cl	0.242	g
NaCl	0.584	g
$MgCl_2 \cdot 6H_2O$	40.7	mg
EDTA	37.2	mg
Triton X-100	2	µl
ddH_2O	80	ml

Adjust pH to 8

Add dH_2O up to 100 ml

Add 100X Protease inhibitor before use

** Prepare freshly before use

15. 12.5% Separating solution

	11	ml
1.5M Tris-Cl, pH 8.8	2.75	ml
30% Acrylamide	4.47	ml
10% SDS	0.11	ml
10% Ammonium persulfate	55	µl
TEMED	5.5	µl
ddH_2O	3.62	ml

16. 4% Stacking solution

	2.5	ml
0.5M Tris-Cl, pH 6.8	0.625	ml
30% Acrylamide	0.325	ml
10% SDS	25	µl
10% Ammonium persulfate	12.5	µl
TEMED	2.5	µl
ddH_2O	1.525	ml

17. 10X SDS-PAGE running buffer   1   liter

       Tris-Cl

       Glycine

       SDS

       ddH_2O

18. 1X SDS-PAGE running buffer   1   liter

       10X SDS-PAGE running buffer   100   ml

       Add ddH_2O to 1 liter

19. Coomasie blue staining   400   ml

       Coomasie Brilliant Blue R250   0.4   g

       Methanol   160   ml

       Glacial acetic acid   40   ml

       Add ddH_2O to 400 ml

       filter through a filter paper

20. Destaining solution   1   liter

       Methanol   400   ml

       Glacial acetic acid   100   ml

       Add ddH_2O to 1 liter

21. 20X Earle's Balanced Salt Solutions (EBSS) 500 ml

$CaCl_2 \cdot 2H_2O$	2.65	g
KCl	4	g
$MgSO_4 \cdot 7H_2O$	2	g
NaCl	68	g
$NaH_2SO_4 \cdot H_2O$	1.25	g
Glucose	10	g

Add ddH_2O to 500 ml

sterilized by filtering through a 0.45 µm membrane filter
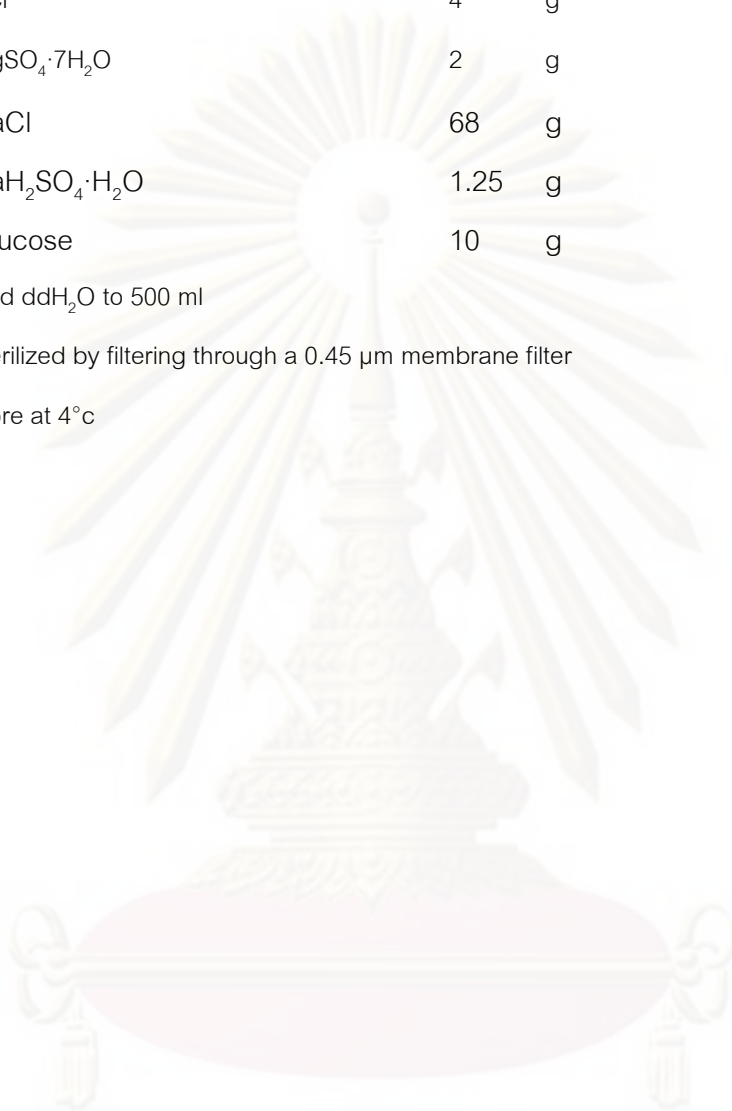
store at 4°c

APPENDIX B

Results of 1D VOPBA followed LC/MS/MS spectra matches from the NCBInr protein database

No. of band	Accession No.	Protein Description	Mol mass (Da)	Score
1	AAH66038	Eif4g1 protein	174,472	35
2	AAA90910	p162 protein	161,852	68
3	AAH05460	Nucleolin	76,733	94
4	AAH05460	Nucleolin	76,733	205
5	NP_079555	heterogeneous nuclear ribonucleoprotein K	50944	95
6	BAC40253	unnamed protein product	42,194	150
7	1405340A	protein 40kD	32,732	102
	AAA39413	laminin receptor	32,698	102
8	BAE40130	unnamed protein product	23,370	179
9	BAC40485	unnamed protein product	24,160	125
10	BAB27107	unnamed protein product	24,032	66
11	NP_035422	ribosomal protein L9	21,868	101
12	BAC40369	unnamed protein product	17,796	103
13	NP_001004	ribosomal protein S9	22,578	96

Results of 2D VOPBA followed LC/MS/MS spectra matches from the NCBInr protein database

No. of band	Accession No.	Protein Description	Mol mass (Da)	Score
1	AAH05460	Nucleolin	76,733	159
2	NP_035761	tumor rejection antigen gp96	92,418	178
3	NP_032328	heat shock protein 1, beta	83,229	261
4	1405340A	protein 40kD	32,732	372
	ACB59248	laminin receptor	32,829	372
	XP_620036	similar to 67 kda laminin receptor	27,991	155
5	NP_032748	nucleophosmin 1	32,540	141
6	AAH03833	Ribosomal protein, large, P0	34,165	130
7	BAC36106	unnamed protein product	29,170	142
8	NP_001009	ribosomal protein S15	17,029	108
9	XP_991849	similar to hCG1640785	13,730	172
10	NP_032933	peptidylprolyl isomerase A	17,960	131

APPENDIX C

Poster 094

IDENTIFICATION OF JAPANESE ENCEPHALITIS VIRUS BINDING PROTEIN ON MICROGLIAL CELLS USING VIRUS OVERLAY PROTEIN BINDING ASSAY AND PROTEOMIC ANALYSIS

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Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is a major cause of viral encephalitis in Asia. Even though the principle target cells for JEV in the central nervous system are neurons, the microglia is activated in response to JEV infection. Also, the JEV antigens have been detected in microglial cells. Viral attachment to the host cells is the first step of the viral entry in which is required for the viral infection. This study sought to identify the JEV binding protein(s) on the surface of mouse microglial cells (BV-2). Using virus overlay protein binding assay (VOPBA) followed by liquid chromatography-mass spectrometry (LC/MS/MS), we identified JEV binding protein bands of 43 kDa laminin receptor precursor protein and 60 kDa chaperonin. Two-dimensional gel electrophoresis to separate the membrane proteins before VOPBA confirmed the 43 kDa laminin receptor precursor protein as a JEV binding protein. This newly identified JEV binding protein is a potential candidate JEV receptor protein on microglial cells.

Keywords: Japanese encephalitis virus, Microglia, Virus overlay protein binding assay (VOPBA), two-dimensional gel electrophoresis

Introduction

Japanese encephalitis virus (JEV) is a neurotropic virus that cause 35,000 - 50,000 encephalitis cases annually (Tsai, 2000). This virus is transmitted to human through the bite of the infected mosquitoes, principally by *Culex tritaeniorhynchus*. JEV is a member of the genus *Flavivirus*, family *Flaviviridae* that includes St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus and Dengue virus. Like other flaviviruses, JEV contains a small glycoprotein-containing lipid envelope surrounding a nucleocapsid which consists of one molecule of single-stranded positive-sense RNA. JEV infection leads to a wild spectrum of clinical manifestations, ranging from a febrile illness to acute and possibly fatal encephalitis. Most of the survivors have neurological sequelae including motor deficits, cognitive and language impairments, learning difficulties (Desai et al., 1995) However, the pathogenesis of Japanese encephalitis remains unclear. Upon JEV infection, microglia, a type of glial cells that acts as primary immune effector cell in the central nervous system (CNS), was activated morphologically and the expression of the neuronal specific nuclear protein (NeuN) was down-regulated significantly indicating neuronal loss *in vivo* (Ghoshal et al., 2007). Since no antiviral drug is commercially

available for the treatment of Japanese encephalitis, several studies have attempted to identify cell surface receptor (s) on different cell lines for inhibition of viral entry (Su et al., 2001; Boonsaney and Smith, 2007; Das et al., 2009) The interaction between the viral attachment proteins and cellular receptor(s) is the first step for virus infection. Thus, the identification of the cell surface receptor(s) utilized by the JEV to gain entry into host cells may lead to the production of effective antiviral therapeutic agents. In short, this study aimed to identify the JEV binding protein(s) on microglial cell surface using virus overlay protein binding assay and proteomic analysis.

Materials & Methods

Cell culture and virus stocks. C6/36 was cultured at 28°C in α-MEM (Thermo Fisher Scientific Inc., USA) supplemented with 10% FBS (Gibco, USA), 1% L-Gln (Thermo Fisher Scientific Inc., USA), 100U/ml of penicillin and 100 μg/ml of streptomycin (Thermo Fisher Scientific Inc., USA). Japanese encephalitis virus strain Beijing-1 (accession No.L48961) was propagated in C6/36 cells. The virus was partially purified and quantitated by standard plaque assay using LLC-MK2 cells before storage at -80°C. BV-2 mouse microglial cells were cultured in DMEM supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% L-Gln at 37°C in a 5% CO_2 incubator.

Cell membrane preparation. Cells were scraped in TBS buffer and pelleted by centrifugation at 1,200 x g for 3 min. The cell pellet was lysed by vigorous mixing in ice-cold modified buffer M (100 mM NaCl, 20mM Tris-HCl pH 8.0, 2mM $MgCl_2$ 1mM EDTA, 0.2% Triton X-100, 1X Protease Inhibitor Cocktail). The nuclei and debris were removed by centrifugation at 600 x g for 3 min, and the membranous organelles were pelleted from the supernatant by further centrifugation at 6,000 x g for 5 min. Finally, the membrane proteins were pelleted by ultracentrifugation at 35,000 rpm for 30 min and resuspended in modified buffer M. The concentration of protein was quantified by the BCA protein assay kit (Pierce Biotechnology, Inc., USA).

One and two-dimensional gel electrophoresis. The confluent 75-cm^2 tissue culture flasks of the BV-2 cells were used in the preparation of membrane extracts for separation by one dimensional SDS-PAGE (1D) and two-dimensional gel electrophoresis (2D). The extracted protein was differentially centrifuged and collected only for membrane protein, before loading onto 1D gel electrophoresis. For 2D gel electrophoresis, membrane protein was resolubilized in IPG strip rehydrating solution at room temperature overnight, then IPG strip was put onto electrophoresis for 8 hr at 20°C using the IPGphor IEF system (Amersham Pharmacia Biotech, Sweden). IPG strip was equilibrated by rocking at room temperature in SDS equilibration buffer. After this, the strip was placed on the top surface of the second dimension gel which is a 10% SDS polyacrylamide gel.

Virus Overlay Protein Binding Assay. The 1D and 2D gels were electrotransferred onto nitrocellulose membranes at 100 V for 120 min (Mini Trans-Blot® Electrophoretic Transfer Cell) and blocked in TBS containing 5% skimmed milk for 1 hr at room temperature. After blocking, the membranes were overlaid with 10^7 PFU of JEV in 1% skim milk in TBS for 2 hr at room temperature and washed three times with TBS buffer. Subsequently, the membranes were incubated with the pan-specific anti-flavivirus monoclonal antibody (a kind gift from Dr.Duncan R. Smith, Mahidol University) at a dilution of 1:50 in 5% skim milk in TBS for 2 hr at room temperature. After washing with TBS, the blots were incubated with horseradish-peroxidase-conjugated rabbit anti-mouse IgG at a dilution of 1:3,000 (Sigma-Aldrich, Inc., USA)

in 5% skim milk in TBS for 1 hr at room temperature. The signals were visualized by developing with SuperSignal® West Pico Chemiluminescent Substrate Kits (Pierce Biotechnology, Inc., USA). The corresponding spots in the coomassie blue-stained gel were picked and indentified by quadrupole-time of flight tandem mass spectrometry (Q-TOF micro; Micromass, UK)

Liquid chromatography-mass spectrometry (LC/MS/MS). The selected bands were subjected into in-gel proteolysis with trypsin and then LC/MS/MS analyses were carried out using a capillary LC system (Waters) coupled to a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray ion-source working in the nanoelectrospray mode. Glu-fibrinopeptide was used to calibrate the instrument in MS/MS mode. The tryptic peptides were concentrated and desalted on a 75 μm ID x 150 mm C18 PepMap column (LC Packings, Amsterdam, The Netherlands). Eluents A and B were 0.1% formic acid in 97% water, 3% acetonitrile and 0.1% formic acid in 97% acetonitrile respectively. Six μl of sample was injected into the nanoLC system, and separation was performed using the following gradient: 0 min 7% B, 35 min 50% B, 45 min 80% B, 49 min 80% B, 50 min 7% B, 60 min 7% B. The database search was performed with ProteinLynx screening SWISS-PROT and NCBI. For some proteins that were difficult to find, the Mascot search tool available on the Matrix Science site screening NCBInr was used.

Results & Discussions

To identify JEV binding proteins on microglial cells, 100 μg of membrane protein was separated on a 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with JEV and subsequently with a pan-specific anti-flavivirus monoclonal antibody, followed by a secondary anti-mouse IgG conjugated with horseradish peroxidase. Several virus binding protein bands were detected as shown in Figure 1. The major virus binding protein bands were excised from identical parallel gels and subjected to in-gel proteolysis with trypsin followed by LC/MS/MS. After PLGS Databank searching analysis, the results identified laminin receptor precursor protein (43 kDa) and chaperonin (60 kDa) as candidates for JEV receptors on microglial cells. Due to the fact that the structure of the virus attachment proteins of mosquito-borne flaviviruses are very similar, it was proposed that the same receptor molecules for mosquito-borne flaviviruses may present on the surface of C6/36 mosquito cells (Ren et al., 2007). Identification of the 37/67-kDa high-affinity laminin receptor protein for dengue virus serotype 1 on the liver cells (Thepparit and Smith, 2004) correlated with our finding of 43 kDa laminin receptor precursor protein as a JEV binding protein on microglial cells. Two dimensional gel electrophoresis was also investigated for the confirmation. 350 μg of membrane protein was separated using 2D technique and transferred to nitrocellulose membranes for VOPBA analysis. Several virus binding protein bands were also detected as shown in Figure 2. The major virus binding protein bands were excised from identical parallel gels and subjected to in-gel proteolysis with trypsin followed by LC/MS/MS. After PLGS Databank searching analysis, laminin receptor precursor protein (43 kDa) was again confirmed as JEV binding protein on microglial cells.
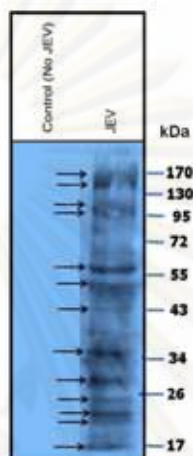
Figure 1 One-dimensional VOPBA analysis of JEV binding proteins on microglial cells. The virus binding protein bands were detected by using a pan-specific anti-flavivirus monoclonal antibody and secondary conjugated with HRP antibody. Many bands were selected for identification by LC/MS/MS (arrow). Negative control of no virus was also applied in parallel.
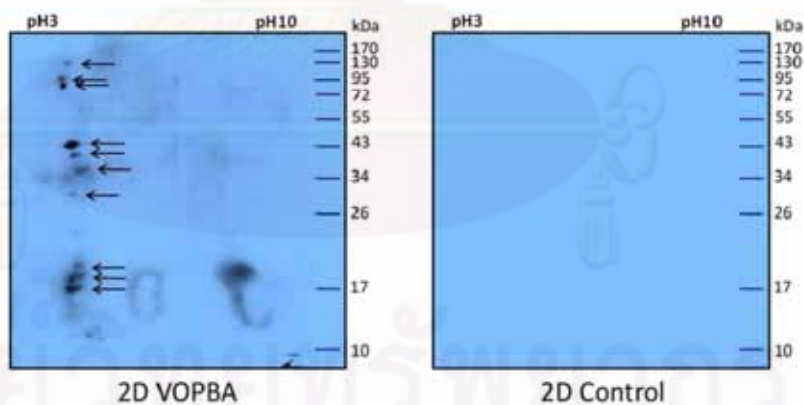

Figure 2 Two-dimensional VOPBA analysis of JEV binding proteins on microglial cells. The virus binding protein bands were detected by using a pan-specific anti-flavivirus monoclonal antibody and secondary conjugated with HRP antibody. Many bands were selected for identification by LC/MS/MS (arrow). Negative control of no virus was also applied in parallel.

Conclusions

In this study, using a combination of VOPBA and mass spectrometry analysis, we have identified a 43 kDa laminin receptor precursor protein as a JEV binding protein on microglial cells.

Acknowledgements

References

1. Boonsanay V, Smith DR. Entry into and production of the Japanese encephalitis virus from C6/36 cells. Intervirology. 2007;50(2), 85-92.

2. Das S, Laxminarayana SV, Chandra N, Ravi V, Desai A. Heat shock protein 70 on Neuro2a cells is a putative receptor for Japanese encephalitis virus. Virology. 2009; 385(1), 47-57.

3. Desai A, Shankar SK, Ravi V, Chandramuki A, Gourie-Devi M. Japanese encephalitis virus antigen in the human brain and its topographic distribution . Acta Neuropathology. 1995;89, 368-373.

4. Ghoshal A, Das S, Ghosh S, Mishra M, Sharma V, Koli P, Sen E and Basu A. Proinflammatory mediators released by activated microglia induces neuronal death in Japanese encephalitis. Glia . 2007;55, 483-496.

5. Ren J, Ding T, Zhang W, Song J, and Ma W. Does Japanese encephalitis virus share the same cellular receptor with other mosquito-borne flaviviruses on the C6/36 mosquito cells? Virology Journal. 2007; 4, 83.

6. Su CM, Liao CL, Lee YL, and Lin YL. Highly sulfated forms of heparin sulfate are involved in Japanese encephalitis virus infection. Virology. 2001; 286(1), 206-215.

7. Thepparit C and Smith DR. Serotype-specific entry of dengue virus into liver cells:Identification of the 37- kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor. Journal of Virology. 2004;78(22), 12647-12656.

8. Tsai TF. New initiatives for the control of Japanese encephalitis by vaccination. Vaccine. 2000;18, 1-25.

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