รายงานการวิจัย

เรื่อง

การวิเคราะห์และศึกษาการแสดงออกของโมเลกุลตัวรับบนผิวเซลล์ไมโครเกลียในเซลล์เพาะเลี้ยงและในสัตว์ทดลองเพื่อยับยั้งการติดเชื้อและการตายแบบทำลายตัวเองของเซลล์จากไวรัสไข้สมองอักเสบ Japanese encephalitis

โดย

อาจารย์ ดร. ธนัญญา ทองตัน
รองศาสตราจารย์ ดร.พูลลาภ ซีพสุนทร
ศาสตราจารย์ นายแพทย์เกียรติ รักษ์รุ่งธรรม

คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ทุนอุดหนุนการวิจัยจากเงินอุดหนุนทั่วไปจากรัฐบาล
ประจำปีงบประมาณ 2553

ACKNOWLEDGEMENTS

This research was financially supported by the National Research Council of Thailand year 2010. We wish to express our gratitute to Asst. Prof. Dr. Supang Maneesri le Grand, Department of Pathology, Faculty of Medicine, Chulalongkorn University for her expertise in animal experiment and immunohistochemistry. Also, the assistance from all of the staff of Division of Research Affairs, Faculty of Medicine, Chulalongkorn University concerning secretary work for this project is very much appreciated.

Thananya Thongtan. Ph.D.

Assoc. Prof. Poonlarp Cheepsunthorn, Ph.D.

Prof. Dr. Kiat Ruxrungtham MD.

บทคัดย่อ

เชื้อไวรัสไข้สมองอักเสบเจอี (JEV) จัดอยู่ในกลุ่มแฟมิลี่ฟลาวิไวริเดอีซึ่งมียุงเป็นพาหะนำโรค ในทวีปเอเชียเชื้อไวรัสไข้สมองอักเสบเจอีเป็นสาเหตุหลักในการก่อโรคไข้สมองอักเสบที่เกิดจากการติดเชื้อไวรัส ถึงแม้ว่าเซลล์เป้าหมายหลักของเชื้อไวรัสไข้สมองอักเสบเจอีในระบบประสาทส่วนกลางคือเซลล์ประสาท แต่เซลล์ไมโครเกลียจะอยู่ในภาวะถูกกระตุ้นเมื่อมีการติดเชื้อไวรัสนี้เช่นกัน การจับของไวรัสที่ผิวเซลล์เจ้าบ้านเป็นขั้นตอนสำคัญขั้นแรกของการติดเชื้อ ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์ที่จะบ่งชี้และทดสอบการทำหน้าที่เป็นโปรตีนต้อนรับไวรัสไข้สมองอักเสบเจอีบนผิวเซลล์ไมโครเกลีย คณะผู้วิจัยได้ทำการแยกโปรตีนที่ผิวเซลล์ด้วยเทคนิค One-dimensional และ Two-dimensional gel electrophoresis ตามด้วยเทคนิค virus overlay protein binding assay (VOPBA) และวิเคราะห์ชนิดโปรตีนด้วย liquid chromatography-mass spectrometry (LC/MS/MS) เพื่อบ่งชี้โปรตีนบนผิวเซลล์เพาะเลี้ยงไมโครเกลียที่สามารถจับกับเชื้อไวรัสไข้สมองอักเสบเจอี จากผลการทดลองพบว่าโปรตีน laminin receptor และโปรตีนนิวคลีโอลินสามารถจับกับไวรัสไข้สมองอักเสบเจอีอย่างจำเพาะ เมื่อทดสอบการทำหน้าที่เป็นโปรตีนต้อนรับสำหรับเชื้อไวรัสไข้สมองอักเสบเจอีบนผิวเซลล์ไมโครเกลียด้วยเทคนิค infection inhibition assay ด้วยการใช้แอนติบอดี้ต่อโปรตีนที่บ่งชี้ทั้งสองชนิดดังกล่าวรวมทั้งแอนติบอดี้ต่อ Hsp70 Hsp90 GRP78 CD14 และ CD4 จากผลการทดลองพบว่าแอนติบอดี้ต่อโปรตีน laminin receptor และ CD4 สามารถลดการติดเชื้อของไวรัสได้อย่างมีนัยสำคัญ (25-40%) นอกจากนี้การติดเชื้อไวรัสของเซลล์ไมโครเกลียในสภาวะที่มี lipopolysaccharide (LPS) ซึ่งนำไปสู่การยับยั้งการแสดงออกของโปรตีน CD4 สามารถลดการติดเชื้อของไวรัสได้ถึง 80% จากผลการทดลองชี้แนะว่าการเข้าสู่เซลล์ไมโครเกลียของไวรัสไข้สมองอักเสบเจอีอาศัยโปรตีนต้อนรับหลายชนิดบนผิวเซลล์โดยมีโปรตีนต้อนรับของ LPS ทำหน้าที่เป็นโปรตีนต้อนรับหลัก

ABSTRACT

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. Viral attachment to a host cell is the first step of the viral entry process and is a critical mediator of tissue tropism. This research aimed to identify molecules associated with JEV entry to microglial cells and examine their function as viral receptor. One dimensional and Two-dimensional gel electrophoresis were applied to separate the membrane proteins. Virus overlay protein binding assay (VOPBA) and liquid chromatography-mass spectrometry (LC/MS/MS) identified the laminin receptor protein and nucleolin as a potential JEV binding proteins. These newly identified JEV binding proteins were further characterized for their function as viral receptors by infection inhibition assay using anti-laminin receptor and anti-nucleolin. Other possible candidate receptor molecules including Hsp70, Hsp90, GRP78, CD14 and CD4 were also applied in the antibody mediated inhibition of infection experiments. Results showed that both anti-laminin receptor and anti-CD4 antibodies significantly reduced virus entry (25-40%) . Significant inhibition of virus entry (up to 80%) was observed in the presence of lipopolysaccharide (LPS) which resulted in a complete down regulation of CD4. These results suggest that multiple receptor proteins may mediate the entry of JEV to microglial cells, with LPS receptor molecules playing a major role.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

PAGE

LIST OF FIGURES

PAGE

FIGURES

LIST OF TABLES

PAGE

TABLES

LIST OF ABBREVIATIONS

%	Percent
1D	One dimensional
2D	Two dimensional
BSA	Bovine serum albumin
°C	Degree Celsius
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
EBSS	Earle's balanced salts solution
EDTA	Ethylenediaminetetraacetic acid
et al.	and others
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
hr	Hour(s)
Ig	Immnunoglobulin
kDa	Kilodalton(s)
µg	Microgram(s)
µl	Microliter(s)
µM	Micromolar
M	Molar
MEM	Minimum essential medium
mg	Milligram(s)
min	Minute(s)
ml	Milliliter(s)
mM	Millimolar
MW	Molecular weight
p.f.u	Plaque-forming units
SDS	Sodium dodecyl sulfate

LIST OF ABBREVIATIONS (continued)

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris- buffered saline
TEMED	N,N,N',N' tetramethylene-ethylenediamine
TNF	Tumor necrosis factor
Tris-HCl	Tris-(hydroxymethyl)-aminomethane hydrochloride
VOPBA	Virus overlay protein binding assay

CHAPTER I

INTRODUCTION

Japanese encephalitis virus (JEV), a positive sense single stranded RNA virus belongs to the *Flaviviridae* family and is transmitted to humans by *Culex* mosquitoes . The virus is prevalent in Southeast Asia and the Pacific Rim and in Southeast Asia JEV infections cause some 30,000 to 50,000 cases of encephalitis annually (Misra and Kalita, 2010; Tsai, 2000). Humans are not an amplifying host for JEV and the majority of infections with JEV are asymptomatic, but where infection is symptomatic, mortality rates are approximately 20-30% (Solomon, 2004; Solomon et al., 2000). Long term neurological sequelae such as cognitive impairment, seizures and movement disorders are found in 50% of those who survive encephalitis (Ghosh and Basu, 2009; Solomon et al., 2000). JEV mainly affects the thalamus, brainstem and spinal cord (Misra and Kalita, 2010), although the detailed pathogenesis of Japanese encephalitis (JE) remains unclear. JEV is proposed to reside and replicate in leukocytes, then reach the central nervous system (CNS) during the incubation period of 6-14 days (Ghosh and Basu, 2009). Productive JEV infection at sites peripheral to the CNS, where the primary target cells are neurons, results in the release of virus into bloodstream and, subsequently, increased levels of cytokines. In JE patients, levels of TNF-α in the serum and CSF are significantly increased (Babu et al., 2006) and in addition IFN-α, the proinflammatory cytokine IL-6 and the chemokine IL-8 are also increased in CSF (Singh et al., 2000; Winter et al., 2004) . Elevated levels of IFN-α , TNF-α and IL-8 are correlated with a fatal outcome (Singh et al., 2000). It is known that JEV not only directly causes neuronal cell death, but also causes damage in the brain indirectly by triggering cell mediated immune responses, which corresponds with the fact that the mortality rate is correlated with increased concentrations of cytokines in serum and cerebrospinal fluid of JE patients (Babu et al., 2006; Ravi et al., 1997).

Microglia have been implicated as a viral reservoir for CNS infection, owning to the high viral titer and persistency of JEV infection in these cells (Thongtan et al., 2010). The

parenchymal microglia comprises approximately 12% of the cells in the brain and these cells are important for monitoring the CNS environment and restoring homeostasis after the CNS injury. Their primary function is to phagocytose apoptotic neurons and microglial cells respond to viral infection by becoming activated, undergoing proliferation and releasing a myriad of inflammatory molecules such as TNF-α, interleukin 6, RANTES and monocyte chemotactic protein 1(MCP-1) (Dopp et al., 1997; Ghoshal et al., 2007). During JEV infection, activated microglial cells lead to inflammation in the central nervous system, and there is a contribution of peripheral macrophage that infiltrate into the CNS to neuronal death (Nazmi et al., 2011). Therefore, increased microglial activation has been viewed as a key factor in indirect neuronal cell death and eventual neuronal deficit (Ghoshal et al., 2007).

The interaction between the JEV virion and its cognate host cellular receptor is the first step in the JEV infection process and several cellular molecules have been identified as putative receptor(s) for JEV such as heat shock protein 70 on mouse neuroblastoma (Neuro2a) cells (Das et al., 2009), a 53 kD protein on C6/36 cells (Boonsanay and Smith, 2007), a 57 kDa protein in BHK-21 (Su et al., 2001) and the 74 kDa heat shock cognate protein 70 on mosquito cells (Ren et al., 2007). In addition, a number of proteins have been implicated in the internalization of other closely related flaviviruses including DC-SIGN (Tassaneetrithep et al., 2003), the 37/67kDa high affinity laminin receptor (Thepparit and Smith, 2004), GRP78 (Jindadamrongwech et al., 2004), heat shock proteins 70 and 90 (Reyes-del Valle et al., 2005), a CD-14 associated protein (Chen et al., 1999) and the mannose receptor (Miller et al., 2008) for the dengue virus as well as $\alpha_v\beta_3$ integrin for West Nile virus (Chu and Ng, 2004). Consistently therefore studies have shown that flavivirus receptor proteins are relatively diverse, and apparently show considerable cell type specificity. As yet the JEV receptor protein expressed by microglial cells remains to be identified. The current study sought to identify molecules involved in the internalization of JEV to microglial cells, and used a combined investigative and candidate approach.

CHAPTER II

MATERIALS& METHODS

2.1 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
Lipopolysaccharide	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 (MEM-α)	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 microglial cell

Miscellaneous

SuperSignal® West Pico Chemiluminescent kits	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	Amersham Pharmacia Biotech
Prestain Protein marker	Fermentas
Pan-specific anti-flavivirus monoclonal E protein antibody (A kind gift from Dr. Duncan R Smith, Mahidol University, Thailand)	
FITC conjugated goat anti-mouse IgG antibody	KPL, Gaithersburg
Rabbit antimouse IgG-horseradish-peroxidase	SIGMA
Rabbit antigoat IgG-horseradish-peroxidase	SIGMA
Goat antirabbitIgG-horseradish-peroxidase	PIERCE
soluble laminin (Engelbreth-Holm-Swarm murine sarcoma)	SIGMA
Anti tomato-Lactin antibody	Vector
Anti-laminin receptor antibody	Santa Cruz
Anti-CD4 antibody	Santa Cruz
Anti-nucleolin antibody	Santa Cruz
Anti-actin antibody	Santa Cruz
Anti-Hsp 70 antibody	Santa Cruz
Anti-Hsp 90 antibody	Santa Cruz
Anti-GRP78 antibody	Santa Cruz
Anti-CD14 antibody	Santa Cruz

DAPI (4',6-diamidino-2-phenylindole) Chemicon

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

C18 PepMap column (LC Packings, Amsterdam, The Netherlands)

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

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

BD FACSCalibur cytometer Becton Dickinson

2.2 Cell culture and virus stock

BV-2 immortalized mouse microglial cells (a kind gift of from Dr James R. Connor, Pennsylvania State University College of Medicine, USA) were cultured in Dulbecco's modified eagle's medium supplemented with 10% heat-inactivated fetal bovine serum,100 units of penicillin, 100 µg/ml streptomycin and 1% L- glutamine at 37°C in humidified incubator with 5% CO_2.

LLC-MK2 were cultured in DMEM supplemented with 10% v/v FBS, and the same antibiotics formula as mentioned above. Cells were incubated at 37°C in humidified incubator with 5% CO_2.

C6/36 cell line was grown in minimum essential medium supplemented with 10% FBS, 1% L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin. Cells were cultured at 28°C without CO_2

JEV 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

2.3 JEV propagation in C6/36 cells

C6/36 cell line was cultured in media until it reached 80% confluency. Cells were counted using hemocytometer. After cell concentration was determined, the culture medium was discarded and replaced with 3 ml of MEM-α without FBS containing JEV strain Beijing-1 at 1 pfu/ml. Viral absorption was allowed for 2 hr at 28°c with constant agitation. The cells were supplemented with fresh culture media and incubated

at 28°c for 3 days. Aliquots of the culture media were stored at -80°c until use. Virus titers were determined by standard plaque assay.

2.4 Virus titration by plaque assay

After propagating JEV in C6/36 cell, JEV was titrated by plaque assay. The 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% 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

2.5 Identification of viral binding protein(s) on microglial cells

2.5.1 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 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. 100μg of the membrane protein was loaded onto 10% SDS polyacrylamide gel for 1 dimensional (1D) gel electrophorsis.

2.5.2 2-dimensional (2D) gel electrophoresis

A total of 350μg of membrane proteins were prepared for electrophoresis by the TCA protein precipitation technique. Protein pellet was resolubilized in IPG strip rehydration solution at room temperature overnight. 125μl of the resulting supernatant were used for each IPG strip, pH 3-10. The IPG strip was put onto electrophoresis for 8 hr at 20°C using the IPGphor IEF system IPG strip was equilibrated by rocking for 10 min at room temperature in SDS equilibration buffer, followed by 10 min at room temperature in Iodoacetamide equilibration buffer. After this, the strip was 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 electrophoresis was achieved at a constant 12-15 mA/gel.

2.5.3 Virus overlay protein binding assay (VOPBA) and Western Blotting

1D and 2D protein gels were electrotransferred to PVDF membranes at 100 V for 150 min using Mini Trans-Blot® Electrophoresis Transfer Cell (BioRad, USA). Membranes were blocked in TBS containing 5% skimmed milk for 1 hr at room temperature. After blocking, membrane were overlaid with 10^7 p.f.u of JEV in TBS (50mM Tris HCl pH 7.6, 150mM NaCl) containing 1% skimmed milk for 2 hr at room temperature. The blots were then washed three times with TBS buffer and incubated with the pan-specific anti-flavivirus monoclonal antibody at a dilution of 1:50 in 5% skimmed milk in TBS for 2 hr at room temperature. After washing three times with 0.1% Tween20 in TBS, the blots were incubated with horseradish-peroxidase-conjugated rabbit anti-mouse 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 corresponding bands or spots in the coomassie blue-stained gels were picked out and identified by mass spectrometry. The blot was later stripped off and reprobed with a goat polyclonal anti-laminin receptor at dilution 1:1000 in 5% skimmed milk in TBS followed with horseradish-peroxidase-conjugated rabbit anti-goat IgG at dilution 1:3000 in 5% skimmed milk in TBS. 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.

For Western analysis of lipopolysaccharide (LPS) treated cells, BV-2 cells from a confluent 10mm diameter dish were either not treated or treated with various amounts of lipopolysaccharide for 1 hr followed by washing twice with PBS. Cells were collected by scraping and used for total protein preparation as described previously (Panyasrivanit et al., 2009). A total of 80μg protein were separated by electrophoresis through a 10% polyacrylamide gel and transferred to a nitrocellulose membrane by electrotransfer. Membranes were blocked with 5% low fat skim milk and incubated over night at 4°C with a 1:500 dilution of a polyclonal goat anti-CD4 antibody followed by a 1:10,000 dilution of a HRP conjugated rabbit anti-goat IgG polyclonal antibody for 1 hr, or a 1:500 dilution of a rabbit polyclonal anti-nucleolin antibody over night at 4°C followed by a HRP conjugated goat anti-rabbit IgG for 1 hr, a 1:500 dilution of mouse monoclonal anti-actin antibody for 1 hr at room temperature followed by a 1:7,500 dilution of a HRP conjugated rabbit anti-mouse IgG for 1 hr. Signals were developed using the SuperSignal® West Pico Chemiluminescent Substrate Kits (PIERCE biotechnology, USA)

2.5.4 Liquid chromatography-mass spectrometry

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 coupled to a Q-TOF mass spectrometer 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. 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.

2.6 Immunohistochemistry

Male Wistar rats weighing 250-300 grams were used in this study. They were housed in a light/dark cycle with light on from 6 a.m. to 6 p.m. Food and water was provided ad libitum. All the protocols in this experiment were approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC). On the experimental day, rats were deeply anesthetized with an excessive dose of sodium pentobarbital and perfused transcardially with 250 ml of phosphate buffer, followed by 250 ml of 4% paraformaldehyde in 0.1 M PBS pH 7.4. The brain were removed and immediately immersed in 4% paraformaldehyde in 0.1 M phosphate buffer for overnight storage. After fixation, the brain at 7 mm anterior to the bregma were selected. All selected brain specimens were dehydrated, infiltrated and embedded in paraffin. 3μm thick transverse sections were cut and collected with gelatin coated slide. Sections were then washed 3 times with phosphate buffer saline and were further processed for Lectin and laminin immunohistochemistry.

For tomato-lectin immunohistochemistry, the rat brains were cut at 3 μm and collected on the super-frost plus slides. All sections were deparafinized in xylene and rehydrated in graded series of alcohol (100%, 70% and 50% respectively). Then, all sections were immersed in citrate buffer pH 6.0 (Dako) and heated in microwave with high power for 10 min, followed by medium power for 10 min. After the antigen retrieval step, the slides were cool down at room temperature for 20 min. Then all slides were incubated with 3% hydrogen peroxide for 5 min, rinsed with phosphate buffer saline (PBS) and incubated with Dako antibody diluents for 10 min, The sections were incubated overnight with rabbit anti tomato-Lactin at dilution 1: 2500. After overnight incubation, all sections were washed with PBS and then were incubated for 40 min. with the secondary antibody (labelled polymer-HRP anti rabbit, Dako Cytomation Envision+R System. After washing with PBS for three times, the bound peroxidase was revealed by incubating all sections in a solution containing 0.05% 3,3-diaminobenzidine, 0.005% hydrogen peroxide for 10

min. The reaction was stopped by repeated rinsing in PBS. The sections were mounted on gelatinized slides, dehydrated in graded series of ethanol, and coverslipped with mounting medium. The same procedures were employed for laminin immunohistochemical study, except goat anti-laminin receptor was used as a primary antibody. Rabbit anti goat immunoglobulin HRP at dilution 1:50 was used as a secondary antibody.

2.7 Infection inhibition assay

A total of 5×10^4 BV-2 cells were grown on 6-well plates. The culture medium was discarded and the cells were pre-incubated with 0, 5, 10, 20μg anti-laminin receptor antibody, or 10 or 20μg anti-Hsp70, anti-Hsp-90, anti-GRP78, anti-CD14, anti-nucleolin or anti-CD4 antibodies for 1 hr with constant agitation. Normal mouse serum and normal goat serum were used as controls. In addition cells were pretreated for 1 hr with lipopolysaccharide (LPS). The cells were mock or JEV- infected at a multiplicity of infection of 1 p.f.u/cell at 37 °C for 1 hr and 30 min with constant agitation. After this period, the cells were washed 3 times with PBS to remove unabsorbed viruses and then subsequently treated with acid glycine buffer (137mM NaCl, 5mM KCl, 0.49mM $MgCl_2.6H_2O$, 0.68mM $CaCl_2.2H_2O$, 99.84mM glycine, pH 3.0) for 1 min to inactivate any uninternalized extracellular viruses followed by washing with PBS. Fresh medium containing serum was later added to the cells and the cells were incubated under standard condition. The cells were then harvested at 8 hr post infection for the flow cytometry analysis. All analyses were undertaken independently in triplicate.

2.8 Detection of infection inhibition by flow cytometry

The BV-2 cells from the infection inhibition assays, harvested at 8 hr post infection were blocked with 10% normal goat serum, then washed twice with 1X PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. The BV-2 cells were washed twice with 1%BSA in PBS and permeabilized with 0.2% Triton-X 100 for 30 min at room temperature followed by washing twice with 1% BSA in PBS. The cells were incubated overnight with a 1:50 dilution with a pan specific anti-Flavivirus monoclonal antibody produced by hybridoma cell line HB-112 (Henchal et al., 1982) at 4 °C

overnight with constant agitation. After that, the cells were washed 6 times with 1% BSA in PBS and incubated with a 1:10 dilution of a FITC conjugated goat anti-mouse IgG antibody for 1 hr at room temperature. Following washing 6 times with 1% BSA in PBS, the cells were analyzed by flow cytometry on a BD FACSCalibur cytometer using CELLQuest™ software.

2.9 Detection of Inhibition infection by standard plaque assay

BV-2 cells were grown onto 6-well plates for 2 days beforetime. Cells were pre-incubated with and without either soluble laminin (basement membrane laminin, Engelbreth-Holm-Swarm murine sarcoma) 20µg of soluble laminin was used for 1 hr incubation. The cells were later infected with JEV at a multiplicity of infection of 10 p.f.u./cell. The cells were harvested at 8 hr post infection for the intracellular plaque assay and the supernatant were harvested at 0, 8 hour post infection for extracellular plaque assay. Inhibition infection was undertaken by standard plaque assay using LLC-MK_2 cells, which were cultured in 6-well plates. The cells were incubated with the supernatant for 1 hr and inoculated with 200 µl of 10-fold serially diluted virus solution in BA-1 medium. The plates were incubated at 37 ^{o}C, 5% CO_2 for 1.5 hr with agitation every 10 min. During this period, the overlay solution was prepared. Then, the mixure of autoclaved 1.6% SeaKem LE agarose and 2X nutrient overlay (Earle's Balanced Salts supplemented with 0.5% (w/v) yeast extract, 2.5% lactalbumin hydrolysate, 5%FBS) were added to each well and the plates were left at room temperature for 45-60 min until the overlay had fully set. The plates were then incubated upside down under standard LLC-MK_2 cell culture conditions for 7 day. Then ,The plaques were visible by fixing cells with the 3.7% formaldehyde for 1 hr before crystal violet staining. The plaque formations on the monolayer of LLC-MK2 cell line were counted

2.10 Statistical analysis

All data were analyzed using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA., USA). Error bars represent SEM and the statistically significant level in all experiments is $P<0.05$

CHAPTER III

RESULTS

3.1 Identification of JEV binding protein

To identify JEV binding protein expressed on microglial cells, both one- and two-dimensional polyacrylamide gel electrophoresis was used to separate microglial membrane proteins before transfer to PVDF membranes. The membranes were overlaid with JEV and later incubated with a pan-specific anti-flavivirus monoclonal antibody. Several JEV binding protein bands and spots were visualized by chemiluminescence using horseradish-peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Figure 1-2).

3.2 Liquid Chromatography-Mass spectrometry analysis

The major protein bands and spots were picked from identical Coomassie-stained parallel gels and subjected to in-gel proteolysis with trypsin before peptide mass fingerprinting analysis with LC/MS/MS. The generated data was searched against the ProteinLynx database (Table 1). Two proteins, the 37/67kda high affinity laminin receptor and nucleolin were identified by mass spectroscopy consistently in both 1- and 2-D VOPBA analysis (Table 1). The 2D VOPBA blot was subsequently stripped and reprobed with a goat polyclonal anti-laminin receptor antibody, followed with a rabbit anti-goat IgG secondary antibody conjugated with horseradish-peroxidase which confirmed that the JEV binding protein detected was the laminin receptor protein (Figure 3).

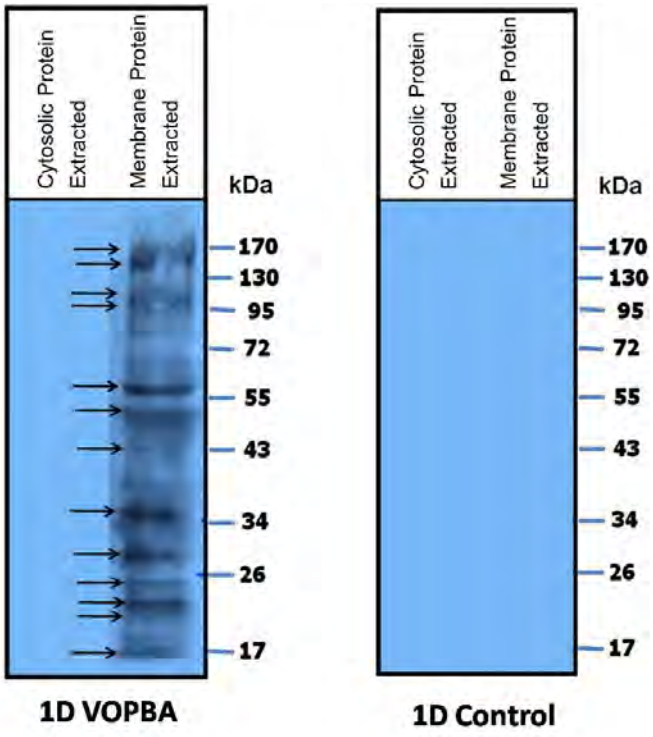

Figure 1. 1D VOPBA analysis of Japanese encephalitis virus on membrane proteins from mouse microglial (BV-2) cells.

100μg extracted membrane proteins was separated on 10% SDS-PAGE before VOPBA for detection of virus binding proteins. The virus binding protein bands were visualized by a pan-specific anti-flavivirus monoclonal antibody and horseradish-peroxidase-conjugated secondary antibody. Many bands were picked from the Coomassie-stained gel that run in parellel for further identification by LC/MS/MS (arrow). Negative control with no virus overlaid in VOPBA analysis was included.

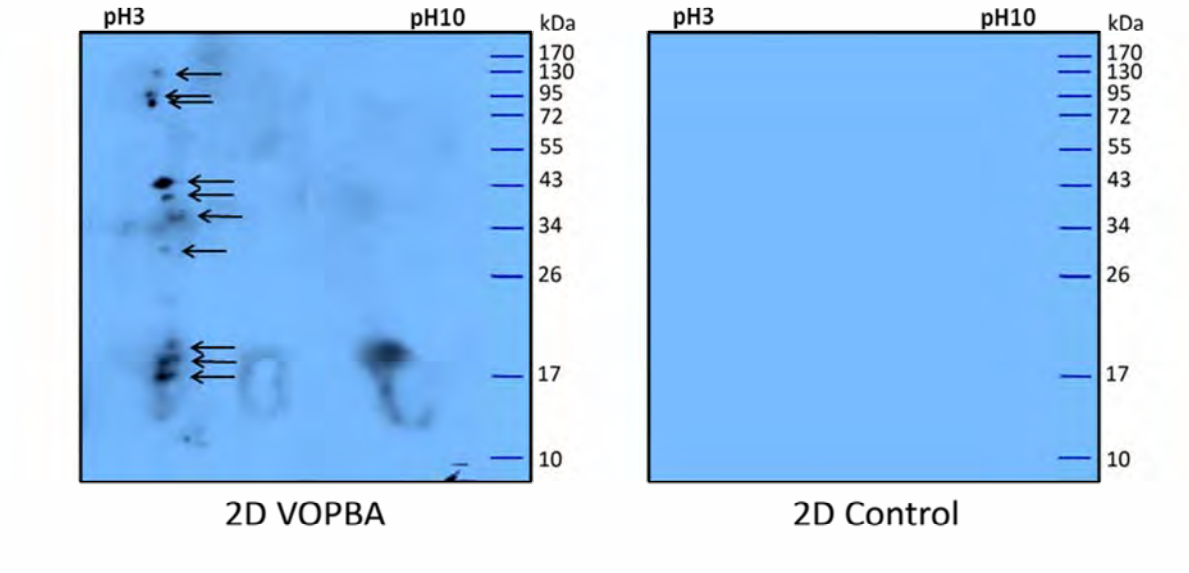

Fig 2. 2D VOPBA analysis of Japanese encephalitis virus on membrane proteins from mouse microglial (BV-2) cells.

350μg extracted membrane proteins was separated by two dimensional gel electrophoresis before VOPBA for detection of virus binding proteins. The first dimension was run on non linear IPG strip, pH3-10. The second dimension was 12.5% SDS-PAGE. The virus binding protein bands were visualized by using a pan-specific anti-flavivirus monoclonal antibody and horseradish-peroxidase-conjugated secondary antibody. Many spots were picked from the Coomassie-stained gel that run in parellel for further identification by LC/MS/MS (arrow). Negative control with no virus overlaid in VOPBA analysis was included.

Table 1. 1D gel electrophoresis-VOPBA analysis followed with 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

2Dgel electrophoresis-VOPBA analysis followed with 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

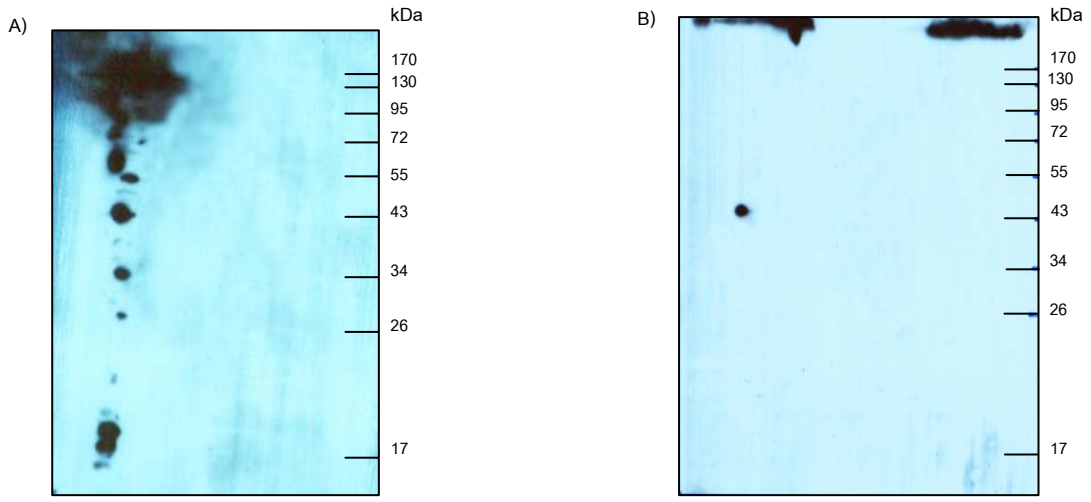

Figure 3. Western Blotting of laminin receptor protein

350 µg of membrane proteins extracted from BV-2 cells were firstly separated on 2D gel electrophoresis, followed by western blotting and VOPBA. The virus binding protein bands were detected by using a pan-specific anti-flavivirus monoclonal antibody and secondary conjugated with HRP antibody (a). The blot was later stripped off and reprobed with a goat polyclonal anti-laminin receptor antibody, followed with rabbit - antigoat secondary antibody conjugated with HRP. The signal of laminin receptor protein was clearly detected by chemiluminescence (b)

3.3 In vivo expression of laminin receptor protein

In vivo expression of laminin receptor protein on microglial cell surface was examined to confirm the biological significance. The examination of brain slide obtain from the normal rats demonstrated that the cells with tomato lectin immunoreaction(TL-IR) were widely distributed throughout the brain section. Lectin staining represented the clear morphology of resting uninfected microglial cells. The high density of TL-IR cells were observed in hippocampus (Figure 4A). At the higher magnification (X400), the TL-IR cell demonstrate the small cell body with thin to medium ramified process which is the characteristic of resting microglia (Figure 4B).

Examination of brain slide demonstrated the cells with the immunorection of laminin (laminin-IR cell) distributed throughout the brain section including hippocampus (Figure 5A). At the high magnification, It was clearly demonstrated that the laminin immunoreactions are limited on the cell membrane which confirmed the characteristic of laminin membrane receptor (Figure 5B-C)

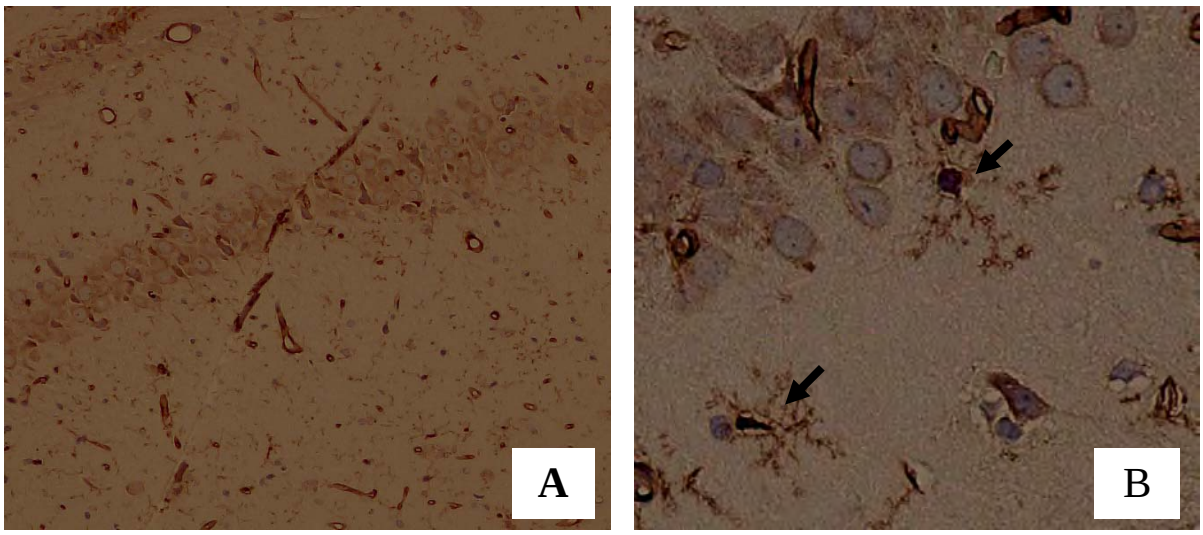

Figure 4. Immunohistochemistry of microglial cells in rat brain

Immunohistochemistry showing the distribution of tomato lectin immunoreactive cells (TL-IR cells) in hippocampus (A). At the higher magnification (X400), the TL-IR cells (arrow) demonstrate the small cell body with thin to medium ramified process (B).

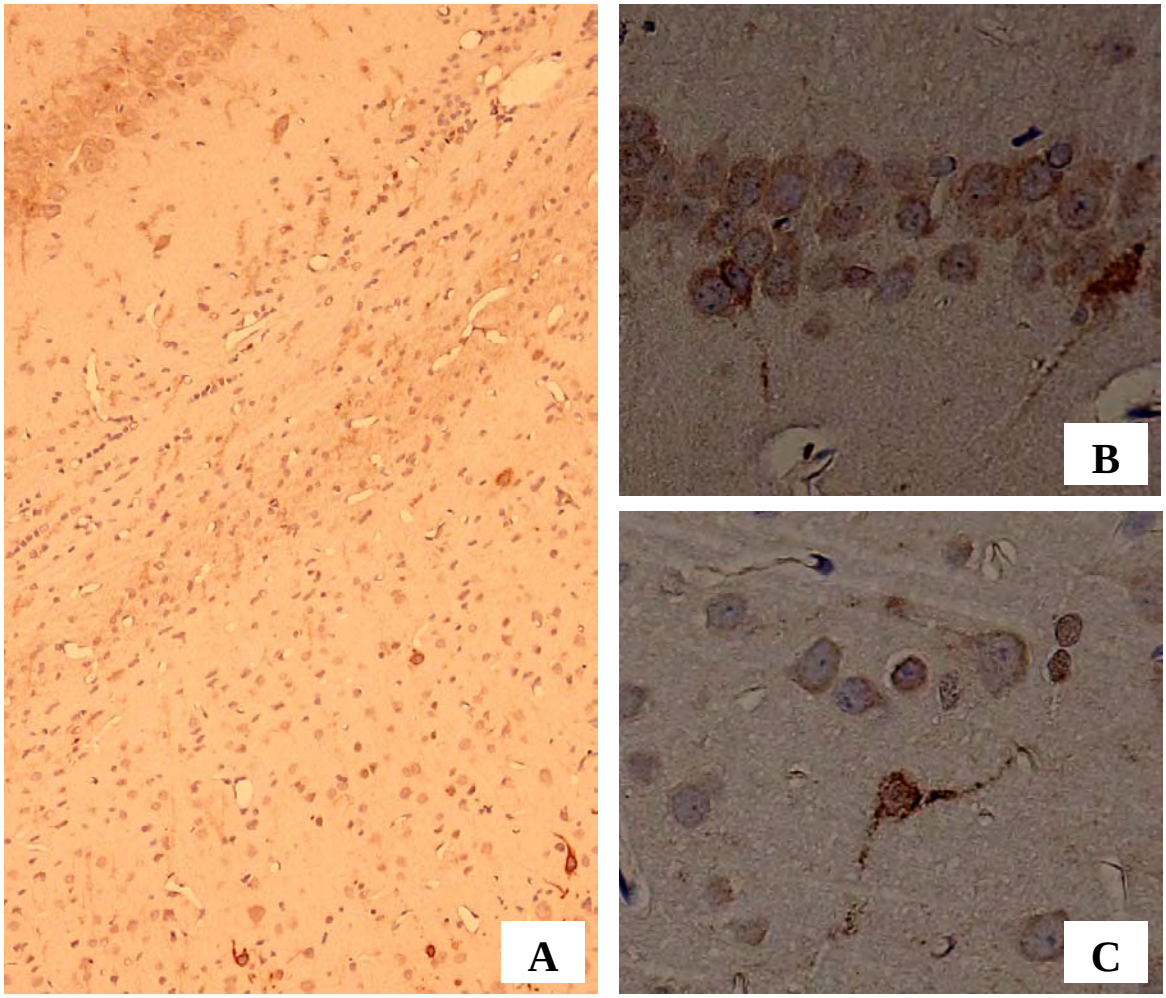

Figure 5. Immunohistochemistry of laminin receptor protein in rat brain

The immunohistochemistry showing the distribution of Laminin immunoreactive cells (laminin-IR cells) in hippocampus at low magnification (A) and at the higher magnification ,X400, (B,C).

3.4 Inhibition of infection with soluble laminin

To verify that laminin receptor protein can function as a JEV receptor BV-2 cells were preincubated with either 0 µg (control) or 20 µg of soluble laminin which is the highest level of antibody inhibition assays, that were reported in the previous study (Thepparit and Smith, 2004) before infection with JEV at 1 pfu/cell at 37^{o}C for 1 hr and 30 min. Analysis of infectivity was undertaken by flow cytometry using a pan specific anti-Flavivirus E protein monoclonal antibody. By 8 hr post infection, soluble laminin inhibited JEV infection approximately 25.3% when compared to control infections (Figure. 6). The time of 8 hr post infection was selected since one replication cycle of JEV, detected by extracellular virion is 10 hr post infection (Thongtan et al.,2010). and as positive cells originate through the initial infection, and not through a secondary infection from newly produced virions. The viral production was determined at 8 hr post infection both intracellular and extracellular after pre-incubation with 20µg soluble laminin. (Figure 7) At 8 hr post infection, the level of extracellular virus show no significant different compare to that of 0 hr post infection. Pre-incubatation of cell with 20 µg soluble laminin have no effect on virion production, both intracellular and extracellular. This implies that the laminin receptor protein plays a minor role in the viral entry step of JEV into the microglial cells.

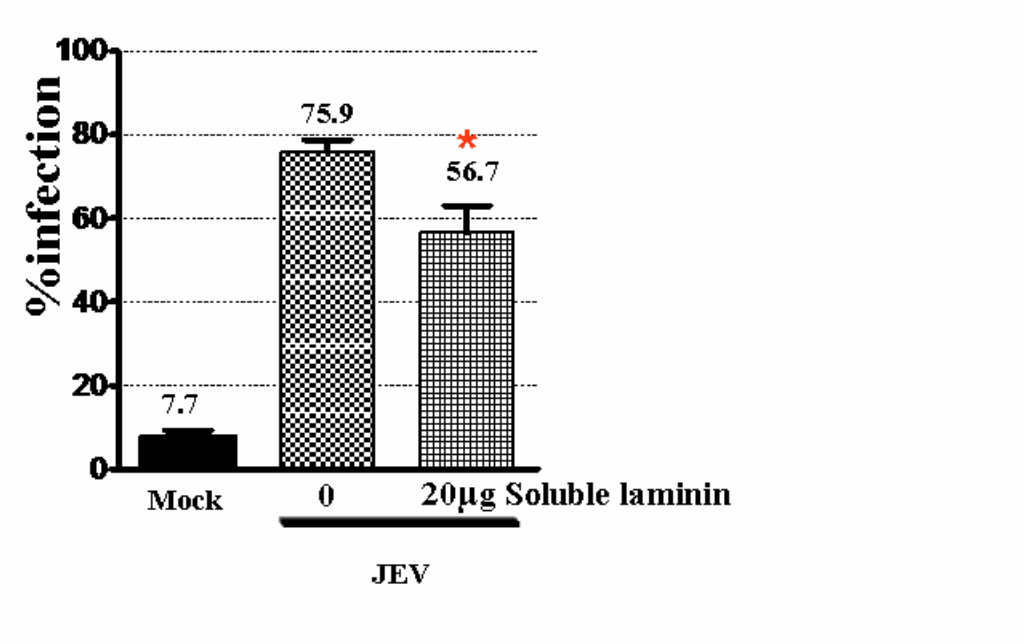

Figure 6. Infection inhibition of BV-2 with soluble laminin at 8 hr post infection

BV-2 cells were preincubated with either 0 (control) or 20 µg of soluble laminin before infection with JEV at 1 pfu/cell . At 8 hr post infection cells were incubated with a pan specific anti-*Flavivirus* monoclonal antibody (HB-112) raised against E protein, followed by FITC-conjugated goat anti-mouse IgG polyclonal antibody and analyzed by flow cytometry. All experiments were undertaken independently in triplicate. Error bars represent SEM. Asterisk indicates a statistically significant difference from the control (one-sample *t* test) ($P<0.05$).

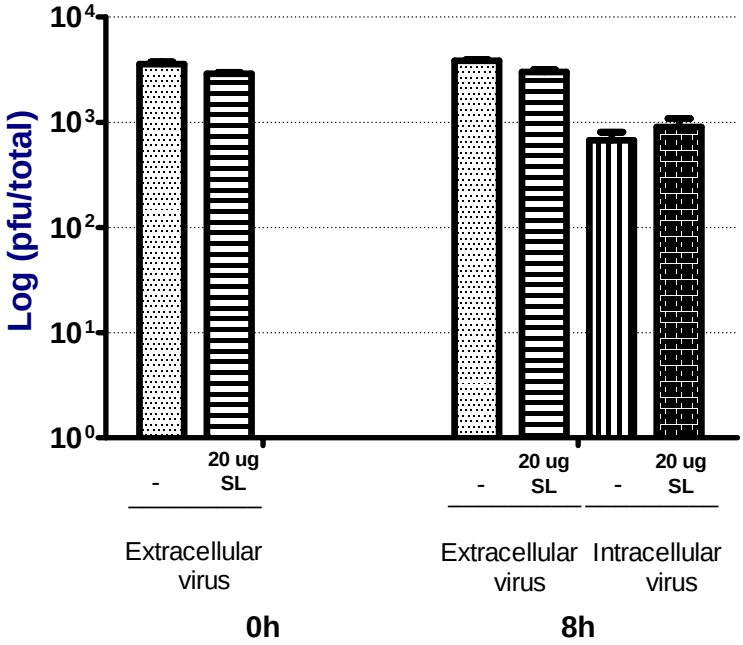

Figure 7. Viral production in infection inhibition assay by soluble laminin

At 0 hr post infection indicated the levels of background leftover after washing step . At 8 hr post infection, the level of extracellular virus show no significant different compare at 0 hour post infection. Pre-incubation of cell with soluble laminin have no effect on virion production. All experiments were undertaken three times independently in duplicate. Error bars represent SEM

3.5 Analysis of receptor candidates

BV-2 cells were preincubated with increasing concentrations of antibodies directed against the laminin receptor and nucleolin for 1 hr, prior to infection with JEV at a MOI of 1. At 8 hr post infection cells were analyzed by flow cytometry after incubation with a pan specific anti-flavivirus E protein monoclonal antibody (HB112, (Henchal et al., 1982) and an appropriate secondary antibody. Results showed a dose dependent inhibition of infection in response to pre-incubation with anti-laminin receptor antibodies to a maximum of approximately 75% of wild type levels (25% inhibition) while no significant inhibition was seen when the infection was undertaken after pre-incubation with anti-nucleolin antibodies (Figure 8). Control infections undertaken after pre-incubation with normal mouse serum (NMS) and normal goat serum (NGS) similarly showed no inhibition of infection (Figure 8)

We further assessed the role of a number of molecules implicated as receptor molecules for other viruses.The inhibition of infection experiment was repeated with antibodies directed against Hsp (Heat shock proteins) 70 and 90 , GRP78 (Glucose regulated protein 78 or Bip) CD14 and CD4. Results showed a modest but statistically significant reduction in infection in response to pre-incubation with Hsp70 and 90, and CD 14, while no reduction was seen in response to pre-incubation with anti-GRP78 antibodies (Figure 8). Interestingly, the most significant level of inhibition was seen in response to preincubation with antibodies directed against CD4, where an approximately 40% reduction in infection levels was seen (Figure 8).

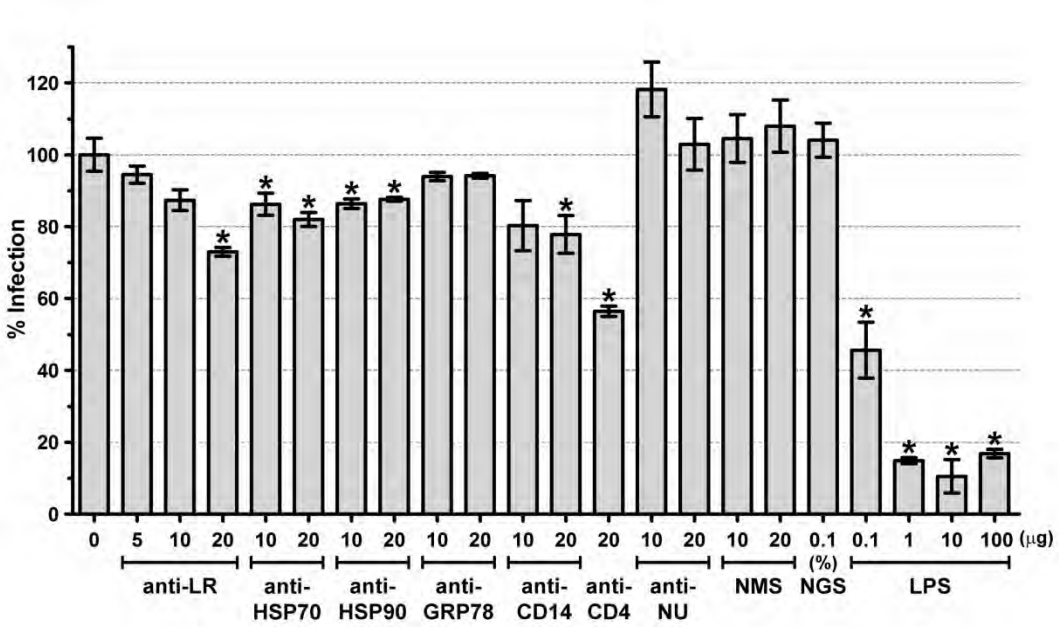

Figure 8. Inhibition analysis of receptor candidates

BV-2 cells were preincubated with varying amounts (as indicated) of antibodies directed against the 37/67 laminin high affinity receptor protein, GRP78, Hsp 90, Hsp70, CD14, nucleolin and CD4 or with increasing amounts (as indicated) of LPS prior to infection with JEV at 1 pfu/hr pocell. At 8 st infection, the cells were collected and incubated with a pan specific anti-*flavivirus* E protein monoclonal antibody (HB-112), followed by FITC-conjugated goat anti-mouse IgG antibody before analysis by flow cytometry. All experiments were undertaken independently in triplicate. Error bars represent SEM. Asterisks indicate a statistically significant difference from the control (one-sample *t* test) ($P<0.05$).

3.6 Inhibition of infection in response to LPS

Nucleolin, CD14, Hsp 70 and Hsp90 have all been implicated in the cell surface recognition of LPS (Dobrovolskaia and Vogel, 2002; Fujihara et al., 2003; Wang et al., 2011) while CD4 has been shown to be down regulated in human macrophages by exposure to LPS (Herbein et al., 1995; Neate et al., 1992). Given the varying degrees of inhibition of infection seen in response to incubation with antibodies against CD14, Hsp70 and 90 and particularly CD4, we sought to determine the effect of pre-treatment of microglial cells with LPS. Cells were therefore pre-treated with LPS for 1 hr, before washing and infection with JEV at a moi of 1. Results (Figure 8) showed an extremely strong and somewhat dose dependent inhibition of infection, with less than 20% of the cells being infected in the presence of greater than 1μg LPS suggesting a role for the previously reported CD-14 associated protein implicated in dengue virus entry to monocytes and macrophages (Chen etal.,1999).

The two highest levels of inhibition seen occurred in response to pre-incubation with either anti-CD4 antibody or pre-treatment with LPS, and studies have shown that LPS is able to down regulate CD4 expression in human macrophages and monocytes (Herbein et al., 1995; Neate et al., 1992). We therefore sought to investigate whether LPS pre-treatment affected CD4 expression in microglial cells. Treatment of BV-2 cells with LPS showed a clear dose dependent reduction in expression of CD4 as assessed by Western blotting, while no alteration in expression was seen for either nucleolin or actin (Figure 9).

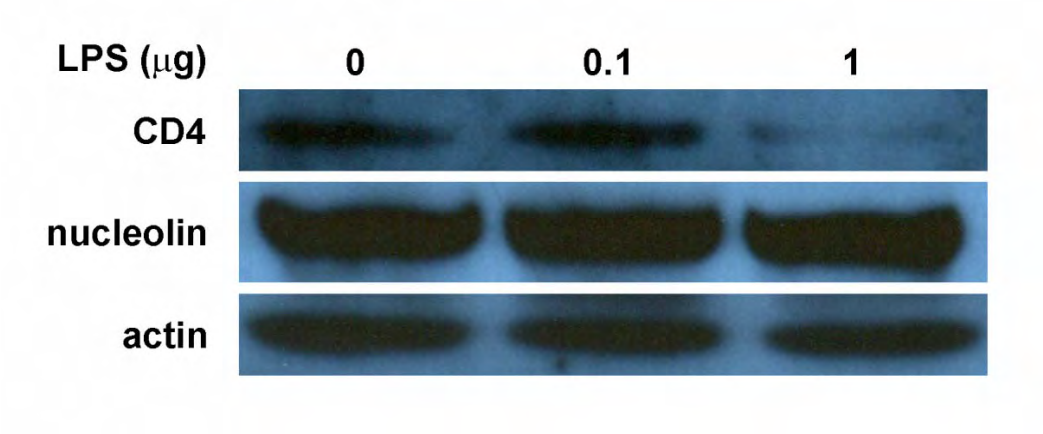

Figure 9. Expression of CD4 in response to LPS treatment.

BV-2 cells were incubated with 0, 0.1 or 1 μg of LPS for 1 hr after which total proteins were extracted, separated by electrophoresis and transferred to solid matrix support before being probed with antibodies directed against CD4, nucleolin and actin.

CHAPTER IV

DISCUSSION AND CONCLUSION

Japanese encephalitis virus (JEV) is a mosquito-borne *flavivirus*. This neurotopic virus is a major public health problem especially in Asia. JEV infection mainly affects the central nervous system (CNS) and the clinical manifestations of Japanese encephalitis depend on which part of the nervous system is affected. Many survivors of the disease acquire permanent neuropsychiatric sequelae (Ghosh and Basu,2009). Even though the target of JEV infection is neuronal cell, microglial cells are also permissive to its infection and involved in Japanese encephalitis pathogenesis (Thongtan et al.,2010; Ghoshal et al., 2007; Swarup et al., 2008).

Enveloped viruses use a number of different mechanisms to enter into cells although among the most common is receptor mediated endocytosis where the virus binds to a receptor or receptor complex prior to entering the cell through endocytosis (Cosset and Lavillette, 2011). The virus-receptor interaction may be preceded by an initial low affinity interaction which serves to concentrate the virus at the cell surface before transfer to a high affinity receptor (Bartosch and Cosset, 2006). Identification of proteins that serve to mediate either the initial binding or the high affinity binding leading to internalization of the virus is problematic, particularly for flaviviruses, where the evidence suggest that receptor usage is cell type specific (Cabrera-Hernandez and Smith, 2005) By implication, cell type specificity means that a single virus may be able to utilize a number of different proteins to enter into cells and susceptibility therefore being determined at least in part by the differential expression of these proteins on the cell surface.

For the identification of JEV binding protein(s) on microglial cells, we employed VOPBA analysis coupled with LC/MS/MS analysis. After PLGS Databank searching analysis and confirmation with the MASCOT software, laminin receptor protein and nucleolin were identified as JEV binding proteins on microglial cells by both one-dimensional and two-dimensional gel electrophoresis VOPBA (Table 1).

Using an affinity interaction screen, we identified that a number of different proteins were able to bind to JEV. The VOPBA methodology while useful has several important limitations the most important of which is that the assay relies upon binding of the virus to largely denatured proteins as a consequence of the methodology. While a degree of renaturation is believed to occur in the hybridization step proteins that would bind the virus under physiological conditions may not bind under experimental conditions. Conversely, denaturation may reveal binding epitopes that would not normally be exposed to the virus, resulting in binding that would not occur under physiological conditions. Furthermore, VOPBA is unable to discriminate between a virus binding protein, and a virus receptor protein, and a number of proteins may be able to bind the virus, or at least the major virion surface protein, the E protein, without the protein having a role in internalization of the virus. This is of particular significance as studies have shown that there is the potential for multiple contact points between the flavivirus E protein and the host cell machinery (Jindadamrongwech et al., 2004; Limjindaporn et al., 2009).

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). Of the two identified proteins characterized, blocking of the high affinity laminin receptor protein showed and approximately 25% inhibition of virus entry, while no inhibition of entry was seen when anti-nucleolin antibodies were used. This suggests that the laminin receptor protein plays some role in the internalization of JEV to microglial cells. This protein has previously been characterized as a dengue virus receptor

protein, mediating the internalization of dengue serotype 1 to liver cells (Thepparit and Smith, 2004) and given the significant similarities between the two viruses, it is perhaps unsurprising that laminin receptor protein, which has also been shown to have a receptor function for Sindbis virus (Wang et al., 1992) is also able to mediate entry of JEV.

BV-2 cells were mock- infected or infected with JEV in the presence of either 0,5,10 and 20μg anti-laminin receptor antibody or 20μg soluble laminin. The percentage of inhibition of JEV infection was determined by flow cytometry. It was found that the inhibitory effect of 20μg soluble laminin at 8 hr post infection was about as effective as that of 20μg anti-laminin receptor antibody. However, both soluble laminin and anti-laminin receptor antibody were not able to completely inhibit the JEV infection. This indicates other mechanisms/receptors/coreceptors are required for JEV to enter microglial cells.

Modest inhibitory effects on JEV entry were seen in response to pre-incubation with antibodies against CD14, Hsp70 and Hsp90, while significant inhibitory effects were seen by pre-incubation with anti-CD4 antibody or by pre-treatment with LPS. Both CD14 and Hsp70 and Hsp90 can act as receptor proteins for LPS (Dobrovolskaia and Vogel, 2002; Fujihara et al., 2003) as can nucleolin (Wang et al., 2011). Treatment with LPS has been shown to significantly inhibit dengue virus entry to human monocytes and macrophages (Chen et al., 1999). The most significant effect seen upon antibody inhibition was observed with antibodies directed against CD4. CD4 is well characterized as a receptor mediating the entry of the human immunodeficiency virus to CD4 bearing cells (Dalgleish et al., 1984). Western analysis here showed that LPS treatment completely down regulated CD4 expression on microglial cells as has been shown by other authors working with monocytes and macrophages (Herbein et al., 1995; Neate et al., 1992), and resulted in less than 20% virus entry. The results seen with antibody inhibition and LPS treatment are therefore consistent, and point to CD4 as being the major receptor molecule mediating JEV entry into microglial cells.

All together our results suggest that a number of receptor molecules or complexes serve to mediate JEV entry to microglial cells. These molecules are CD4, the laminin receptor protein, CD14 and Hsp70 and Hsp90. The identification of a number of proteins with a role in internalization of JEV is not particularly surprising as having a range of usable entry molecules serves to increase the possible cell tropism of the virus. The identification of these JEV putative receptors may lead to the development of specific receptor - based prophylaxis and therapy at last.

REFERENCES

Babu, G.N., Kalita, J., Misra, U.K., 2006. Inflammatory markers in the patients of Japanese encephalitis. Neurol Res 28, 190-192.

Bartosch, B., Cosset, F.L., 2006. Cell entry of hepatitis C virus. Virology 348, 1-12.

Boonsanay, V., Smith, D.R., 2007. Entry into and production of the Japanese encephalitis virus from C6/36 cells. Intervirology 50, 85-92.

Cabrera-Hernandez, A., Smith, D.R., 2005. Mammalian dengue virus receptors. Dengue Bulletin 29, 119-135.

Chen, Y.C., Wang, S.Y., King, C.C., 1999. Bacterial lipopolysaccharide inhibits dengue virus infection of primary human monocytes/macrophages by blockade of virus entry via a CD14-dependent mechanism. J Virol 73, 2650-2657.

Chu, J.J., Ng, M.L., 2004. Interaction of West Nile virus with alpha v beta 3 integrin mediates virus entry into cells. J Biol Chem 279, 54533-54541.

Cosset, F.L., Lavillette, D., 2011. Cell entry of enveloped viruses. Adv Genet 73, 121-183.

Dalgleish, A.G., Beverley, P.C., Clapham, P.R., Crawford, D.H., Greaves, M.F., Weiss, R.A., 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 312, 763-767.

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

Dobrovolskaia, M.A., Vogel, S.N., 2002. Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes Infect 4, 903-914.

Dopp, J.M., Mackenzie-Graham, A., Otero, G.C., Merrill, J.E., 1997. Differential expression, cytokine modulation, and specific functions of type-1 and type-2 tumor necrosis factor receptors in rat glia. J Neuroimmunol 75, 104-112.

Fujihara, M., Muroi, M., Tanamoto, K., Suzuki, T., Azuma, H., Ikeda, H., 2003. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacol Ther 100, 171-194.

Ghosh, D., Basu, A., 2009. Japanese encephalitis-a pathological and clinical perspective. PLoS Negl Trop Dis 3, e437.

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

Henchal, E.A., Gentry, M.K., McCown, J.M., Brandt, W.E., 1982. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am J Trop Med Hyg 31, 830-836.

Herbein, G., Doyle, A.G., Montaner, L.J., Gordon, S., 1995. Lipopolysaccharide (LPS) down-regulates CD4 expression in primary human macrophages through induction of endogenous tumour necrosis factor (TNF) and IL-1 beta. Clin Exp Immunol 102, 430-437.

Jindadamrongwech, S., Thepparit, C., Smith, D.R., 2004. Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. Arch Virol 149, 915-927.

Limjindaporn, T., Wongwiwat, W., Noisakran, S., Srisawat, C., Netsawang, J., Puttikhunt, C., Kasinrerk, W., Avirutnan, P., Thiemmeca, S., Sriburi, R., Sittisombut, N., Malasit, P., Yenchitsomanus, P.T., 2009. Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitates dengue virus production. Biochem Biophys Res Commun 379, 196-200.

Miller, J.L., de Wet, B.J., Martinez-Pomares, L., Radcliffe, C.M., Dwek, R.A., Rudd, P.M., Gordon, S., 2008. The mannose receptor mediates dengue virus infection of macrophages. PLoS Pathog 4, e17.

Misra, U.K., Kalita, J., 2010. Overview: Japanese encephalitis. Prog Neurobiol 91, 108-120.

Nazmi, A., Dutta, K., Das, S., Basu, A., 2011. Japanese Encephalitis Virus-Infected Macrophages Induce Neuronal Death. J Neuroimmune Pharmacol.

Neate, E.V., Greenhalgh, A.M., McPhee, D.A., Crowe, S.M., 1992. Bacterial lipopolysaccharide mediates the loss of CD4 from the surface of purified peripheral blood monocytes. Clin Exp Immunol 90, 539-544.

Panyasrivanit, M., Khakpoor, A., Wikan, N., Smith, D.R., 2009. Co-localization of constituents of the dengue virus translation and replication machinery with amphisomes. J Gen Virol 90, 448-456.

Ravi, V., Parida, S., Desai, A., Chandramuki, A., Gourie-Devi, M., Grau, G.E., 1997. Correlation of tumor necrosis factor levels in the serum and cerebrospinal fluid with clinical outcome in Japanese encephalitis patients. J Med Virol 51, 132-136.

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

Reyes-del Valle, J., Chavez-Salinas, S., Medina, F., Del Angel, R.M., 2005. Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol 79, 4557-4567.

Singh, A., Kulshreshtha, R., Mathur, A., 2000. Secretion of the chemokine interleukin-8 during Japanese encephalitis virus infection. J Med Microbiol 49, 607-612.

Solomon, T., 2004. Flavivirus encephalitis. N Engl J Med 351, 370-378.

Solomon, T., Dung, N.M., Kneen, R., Gainsborough, M., Vaughn, D.W., Khanh, V.T., 2000. Japanese encephalitis. J Neurol Neurosurg Psychiatry 68, 405-415.

Su, C.M., Liao, C.L., Lee, Y.L., Lin, Y.L., 2001. Highly sulfated forms of heparin sulfate are involved in japanese encephalitis virus infection. Virology 286, 206-215.

Swarup, V., Ghosh, J., Das, S., Basu, A., 2008. Tumor necrosis factor receptor-associated death domain mediated neuronal death contributes to the glial activation and subsequent neuroinflammation in Japanese encephalitis. Neurochem Int 52, 1310-1321.

Tassaneetrithep, B., Burgess, T.H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M.A., Pattanapanyasat, K., Sarasombath, S., Birx, D.L., Steinman, R.M., Schlesinger, S., Marovich, M.A., 2003. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J Exp Med 197, 823-829.

Thepparit, C., Smith, D.R., 2004. 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 78, 12647-12656.

Thongtan, T., Cheepsunthorn, P., Chaiworakul, V., Rattanarungsan, C., Wikan, N., Smith, D.R., 2010. Highly permissive infection of microglial cells by Japanese encephalitis virus: a possible role as a viral reservoir. Microbes Infect 12, 37-45.

Tsai, T.F., 2000. New initiatives for the control of Japanese encephalitis by vaccination: minutes of a WHO/CVI meeting, Bangkok, Thailand, 13-15 October 1998. Vaccine 18 Suppl 2, 1-25.

Wang, K.S., Kuhn, R.J., Strauss, E.G., Ou, S., Strauss, J.H., 1992. High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. J Virol 66, 4992-5001.

Wang, Y., Mao, M., Xu, J.C., 2011. Cell-surface nucleolin is involved in lipopolysaccharide internalization and signalling in alveolar macrophages. Cell Biol Int 35, 677-685.

Winter, P.M., Dung, N.M., Loan, H.T., Kneen, R., Wills, B., Thu le, T., House, D., White, N.J., Farrar, J.J., Hart, C.A., Solomon, T., 2004. Proinflammatory cytokines and chemokines in humans with Japanese encephalitis. J Infect Dis 190, 1618-1626.