รายงานการวิจัย

เรื่อง

การวิเคราะห์และศึกษาการแสดงออกของโมเลกุลตัวรับบนผิวเซลล์ไมโครเกลียในเซลล์เพาะเลี้ยงและในสัตว์ทดลองเพื่อยับยั้งการติดเชื้อและการตายแบบทำลายตัวเองของเซลล์จากไวรัสไข้สมองอักเสบ Japanese encephalitis

โดย

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บทคัดย่อ

เชื้อไวรัสไข้สมองอักเสบเจอี (JEV) จัดอยู่ในกลุ่มแฟมิลี่ฟลาวิไวริเดอีซึ่งมียุงเป็นพาหะนำโรค ในทวีปเอเชียเชื้อไวรัสไข้สมองอักเสบเจอีเป็นสาเหตุหลักในการก่อโรคไข้สมองอักเสบที่เกิดจากการติดเชื้อไวรัส ถึงแม้ว่าเซลล์เป้าหมายหลักของเชื้อไวรัสไข้สมองอักเสบเจอีในระบบประสาทส่วนกลางคือเซลล์ประสาท แต่เซลล์ไมโครเกลียจะอยู่ในภาวะถูกกระตุ้นเมื่อมีการติดเชื้อไวรัสนี้เช่นกัน ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อที่จะศึกษาถึงความสัมพันธ์ระหว่างไวรัสไข้สมองอักเสบกับเซลล์ไมโครเกลีย โดยใช้เซลล์เพาะเลี้ยงไมโครเกลียสายพันธุ์ BV-2 ซึ่งได้จากหนู จากการตรวจสอบเซลล์ที่ติดเชื้อเมื่อเวลา 8,15 และ 24 ชั่วโมงภายหลังการได้รับเชื้อไวรัสไข้สมองอักเสบเจอีด้วยเทคนิค flow cytometry พบว่าเปอร์เซนต์เซลล์ที่ติดเชื้อมีค่าเท่ากับ 53.5, 71.3 และ 83.6 ตามลำดับ สำหรับการบ่งชี้โปรตีนบนผิวเซลล์เพาะเลี้ยงไมโครเกลียที่สามารถจับกับเชื้อไวรัสไข้สมองอักเสบเจอี คณะผู้วิจัยได้ทำการแยกโปรตีนที่ผิวเซลล์ด้วยเทคนิค One-dimensional และ Two-dimensional gel electrophoresis ตามด้วยเทคนิค virus overlay protein binding assay (VOPBA) และวิเคราะห์ชนิดโปรตีนด้วย liquid chromatography-mass spectrometry (LC/MS/MS) ผลการทดลองบ่งชี้ว่า laminin receptor precursor ที่มีขนาด 43 กิโลดาลตัน สามารถจับกับไวรัสไข้สมองอักเสบเจอีอย่างจำเพาะ เมื่อทดสอบการทำหน้าที่เป็นโปรตีนตัวต้อนรับด้วยเทคนิค infection inhibition assay จากผลการทดลองยืนยันการทำหน้าที่เป็นโปรตีนต้อนรับสำหรับเชื้อไวรัสไข้สมองอักเสบเจอีบนผิวเซลล์ไมโครเกลียเนื่องจาก anti-laminin receptor antibody ที่ปริมาณ 5,10 และ 20 µg และโปรตีน laminin ที่ปริมาณ 20 µg สามารถยับยั้งการติดเชื้อจากการตรวจสอบด้วยเทคนิค flow cytometry ภายหลังการได้รับเชื้อไวรัสเป็นเวลา 15 ชั่วโมง โดยเปอร์เซนต์การยับยั้งจะแปรผันตรงกับปริมาณ anti-laminin receptor antibody โดยสรุปจากผลการทดลอง คณะผู้วิจัยสามารถบ่งชี้และทดสอบการทำหน้าที่เป็นโปรตีนต้อนรับต่อไวรัสไข้สมองอักเสบเจอีของโปรตีน laminin receptor precursor ที่มีขนาด 43 กิโลดาลตันบนผิวเซลล์ไมโครเกลีย

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. This research aimed to investigate the relationship between JEV and microglial cells. The percentage of JEV infectivity in mouse microglial (BV-2) cell line at 8,15 and 24 hr post infection was determined by flow cytometry. It was found that the percentage of infected cells were approximately 53.5, 71.3 and 83.6 respectively. The JEV binding protein(s) expressed on the surface of BV-2 cells was also identified. Using One dimensional and Two-dimensional gel electrophoresis to separate the membrane proteins, we later identified the 43 kDa laminin receptor precursor protein as a JEV binding protein by virus overlay protein binding assay (VOPBA) followed with liquid chromatography-mass spectrometry (LC/MS/MS). This newly identified JEV binding protein was further characterized by infection inhibition assay. BV-2 cells were mock-infected or infected with JEV in the presence of either 0 (control), 5,10 and 20 µg anti-laminin receptor antibody or 20 µg soluble laminin. The percentage of inhibition of JEV infection was determined by flow cytometry. Results showed a dose dependent pattern of inhibition in the presence of anti-laminin receptor antibody, determined at 15 hr post infection, compared to non-relevant antibody and control. Taken together, 43 kDa laminin receptor precursor protein is verified as JEV putative receptor on mouse microglial cell surface.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF FIGURES

PAGE

FIGURES

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

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) is a mosquito-borne, positive single-stranded RNA virus of the family *Flaviviridae*. This virus is the leading cause of viral encephalitis in many Asian countries including Japan, Korea, Thailand, Taiwan, China and India (1). Nearly 3 billion people, approximately 60% of the world population, are believed to be at risk for JEV infection. The infection results in high mortality approximately 30% and survivors are often left with permanent neuropsychiatric sequelae such as mental retardation, epilepsy, paralysis, deafness and blindness (2).

Up to present, the pathogenesis of Japanese encephalitis (JE) remains unclear. The incubation period in infected person is between 5-15 days. JEV is proposed to reside and replicate in leukocytes, then reach the central nervous system (CNS) during this incubating time (3). A productive JEV infection at a peripheral site to the CNS, where the primary target cells are neurons, results in the release of viruses into bloodstream and, subsequently, increased levels of cytokines such as macrophage-derived chemotactic factors, TNF-α and IL-8 in the serum and cerebrospinal fluid of JEV infected patients (4)(5) . In a neuroinvasive case, physical disruptions of the blood-brain barrier and a significant induction of brain inflammation occur. Pathologic changes comprising of focal neuronal degeneration, diffuse and focal glial activation/proliferation and perivascular cuffing are distributed principally in the basal ganglia, giving rise to the characteristic Parkinson's disease-like clinical features. At the microscopic level, infected neurons / glial cells contain JEV antigen in their cell bodies, axons and dendrites, suggesting that virus spreads from cell to cell within the brain (6)(7). It is known that the JEV not only directly causes neuronal cell death, but also cause damage in brain indirectly by triggering cell mediated immune response, corresponding with the fact that mortality rate correlated with increasing concentrations of cytokines in serum and cerebrospinal fluid of JE patients (3)(8).

Besides neuronal cells, astrocytes and microglia are also infected by JEV (9)(10)(11). As the primary brain immune effector cells and the target for many encephalitis viruses, microglial cells respond to viral infection by becoming activated, undergoing proliferation and releasing myriad of inflammatory molecules such as TNF-α, interleukin 6, RANTES, monocyte chemotactic protein 1(MCP1) (10)(12). Therefore, increased microglial activation has been viewed as a key factor in indirect neuronal cell death and eventually neuronal deficit (10).

The interaction between JEV virion and host cellular receptor is the first step for JEV infection . One of the strategies to identify surface proteins interacting with virus is to use 2D-gel electrophoresis/MS system. This proteomic techniques are successfully applied into virology research such as HIV virion proteome (13) and HIV receptor identification (14). The most important determinants of viral tropism in particular tissue are the cellular receptors. Since cell surface receptors are uniquely expressed on each cell type, they limit the possible cell types that can be infected by each virus. Most viral receptors are glycoproteins. To date, many cellular molecules have thus been identified as putative receptor(s) for JEV such as heat shock protein 70 on mouse neuroblastoma (Neuro2a) cells (15), 53 kD protein in C6/36 cells (16), 57 kDa protein in BHK-21(17) and 74 kDa heat shock cognate protein 70 on mosquito cells (18) Viral overlay protein binding assay (VOPBA) is an assay that has been widely used to characterize putative viral receptors (19)(16)(15). Besides determination of the percentage of JEV infected cells by flow cytometry, this research aimed to identify the JEV binding protein (s) on microglial cells using both one dimensional and two dimensional gel electrophoresis followed with VOPBA. The major virus binding protein bands revealed by VOPBA analysis would be excised from gels and subjected to in-gel proteolysis before further analyzed by LC/MS/MS. Also, infection inhibition assay would be performed to verify its function as JEV putative receptor, expressed on microglial cells.

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

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

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)	
FITC conjugated goat anti-mouse IgG antibody	KPL, Gaithersburg
Rabbit antimouse IgG-horseradish-peroxidase	SIGMA
Rabbit antigoat IgG-horseradish-peroxidase	SIGMA
soluble laminin (Engelbreth-Holm-Swarm murine sarcoma)	SIGMA
Anti-laminin receptor antibody	Santa Cruz
Anti-CD4 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 Detection of infectivity in JEV-infected BV-2 cells by flow cytometry

A total of 5×10^4 BV-2 cells were mock or JEV- infected at 1 pfu/cell and harvested at 8, 15, 24 hour post infection. The cells were blocked with 10% normal goat serum, washed twice with 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 BV-2 cells were incubated overnight with a 1:50 dilution with a pan specific anti-Flavivirus monoclonal antibody produced by hybridoma cell line HB-112 at 4 °C for 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.

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

2.6.1 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.

2.6.2 1D and 2D gel electrophoresis

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 overnight. 125 µl of the resulting supernatant were used for each IPG strip and rehydration was achieved for 8 hr at 20°C using the IPGphor IEF system. Subsequently IEF carried out for 30 min at 300 V, 30 min at 1000 V and 1.5 hr 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.

2.6.3 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 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 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.

2.6.4 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.

2.7 Infection inhibition assay in JEV-infected BV-2 cells

A total of 5×10^4 BV-2 cells were grown on 6-well plates for 2 days beforetime. The culture medium was discarded and the cells were pre-incubated with and either 20µg soluble laminin or 0, 5, 10, 20µg anti-laminin receptor antibody or anti-CD4 antibody which is non-relevant as a control for 1 hr with agitation every 10 min. The cells were mock or JEV- infected at a multiplicity of infection of 1 pfu/cell. The cells were then harvested at 8 and 15 hr post infection for the flow cytometry analysis.

2.8 Detection of infection inhibition in JEV-infected BV-2 cells by flow cytometry

The BV-2 cells, from the infection inhibition assays, harvested at 8 and 15 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 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 at 4 ^{o}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 FACSCalibur cytometer using CELLQuest™ software.

2.9 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 $P<0.05$

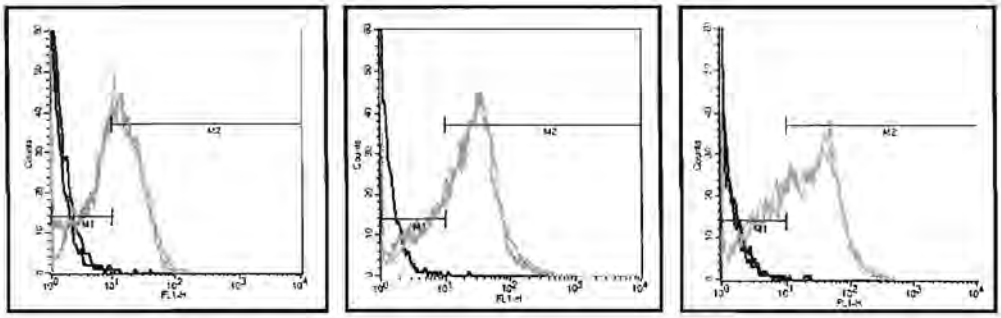
CHAPTER III

RESULTS

3.1 Detection of the % infectivity of JEV-infected microglial cells by flow cytometry

To determine the percentage of JEV-infected microglial cells, mouse microglial (BV-2) cells were infected with JEV at 1 pfu/cell at 37°C for 1 hr and 30 min and further incubated for 8,15 and 24 hr post infection. Analysis of the %infectivity by flow cytometry was performed with a pan specific anti-Flavivirus E protein monoclonal antibody, produced by hybridoma cell line HB-112, and a FITC conjugated goat anti-mouse IgG antibody. The percentage of infected cell were increased over time. Results (Figure 1) showed that approximately 53.5, 71.3 and 83.6% of cells were infected with JEV by 8, 15 and 24 hr post infection respectively.

A)



8h post-infection 15h post-infection 24h post-infection

B)

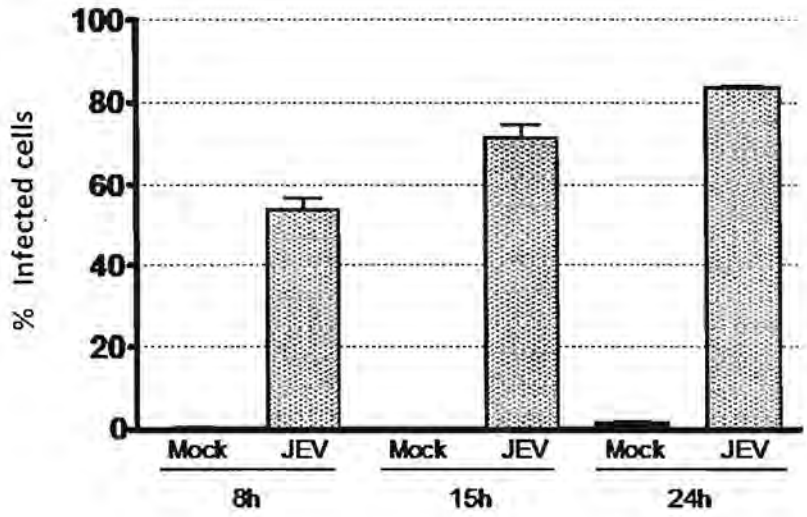


Figure 1. The percentage of JEV-infected BV-2 cells at 8,15 and 24 hr post infection, determined by flow cytometry

BV-2 cells were either mock-infected or infected with JEV at 1 pfu/cell. At 8, 15 and 24 h post infection, cells were incubated with a pan specific anti-*Flavivirus* monoclonal antibody (HB-112) raised against E protein, followed by a secondary antibody conjugated with FITC before analyzed by flow cytometry. Flow cytometry analysis of percent infectivity (A) M1 represent the population of uninfected cells . M2 represent the population of infected cells. (B) Histogram statistics of flow cytometry analysis shown as a bar graph. All experiments were undertaken independently in triplicate. Error bars represent SEM.

3.2 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 with 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).

3.2.1 One-dimensional gel electrophoresis VOPBA

100 µg of membrane proteins were separated on a 10% SDS-PAGE and transferred to PVDF (Polyvinylidene Fluoride) membrane. The membrane was 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 2-3. The major virus binding protein bands, as indicated by arrows in Figures 2, 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 2).

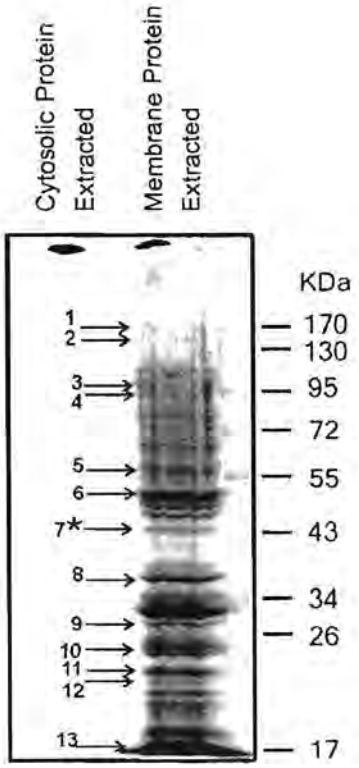


Figure 2. One-dimensional gel electrophoresis of extracted membrane proteins from BV-2 cells.

Using 10% gel SDS-PAGE to separated membrane protein followed by VOPBA technique for detection of virus binding protein. After comparing this Coomassie -stained 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 appendices.

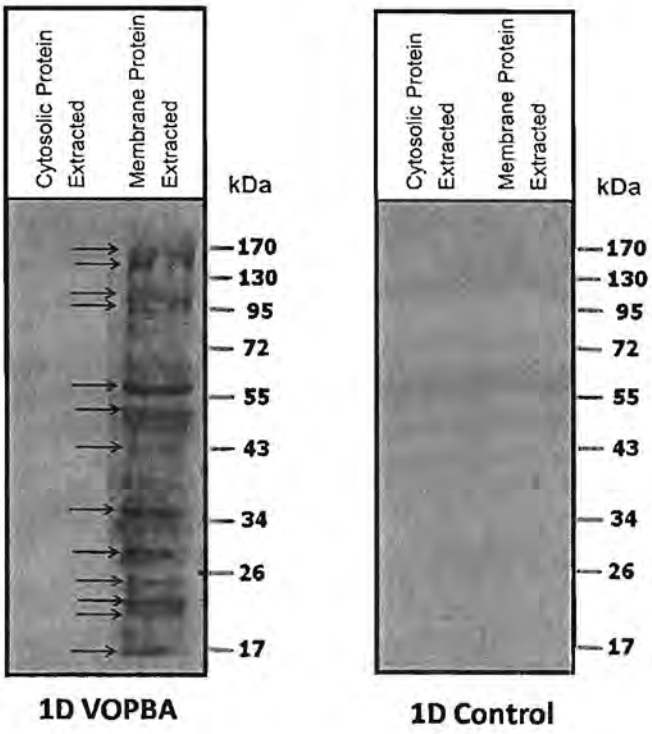


Figure 3. One-dimensional VOPBA analysis of JEV binding proteins on BV-2 cells

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.

3.2.2 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 4-5. 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 4).

3.2.3 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. 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 confirmed the result with Mascot search tool shown in Figure 6-7.

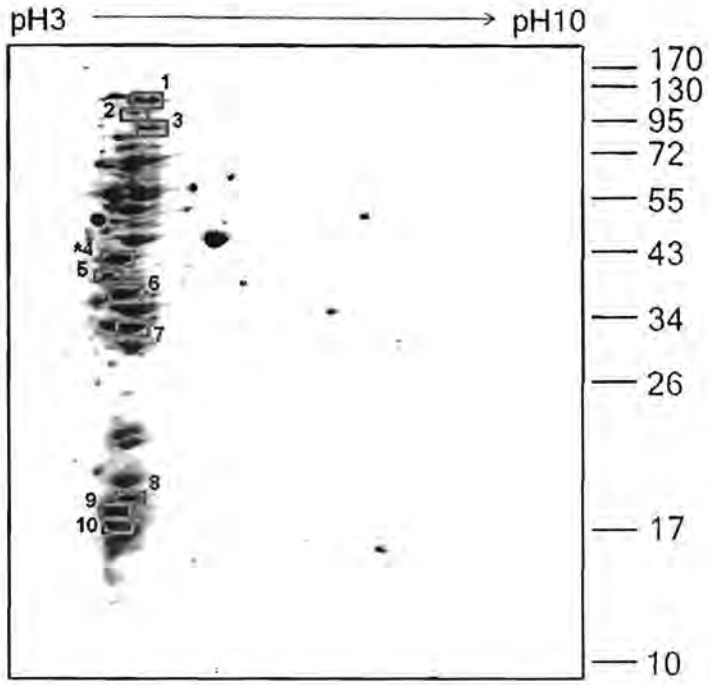


Figure 4. Two-dimensional gel electrophoresis of extracted membrane proteins from BV-2 cells

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 Coomassie-stained 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 appendices.

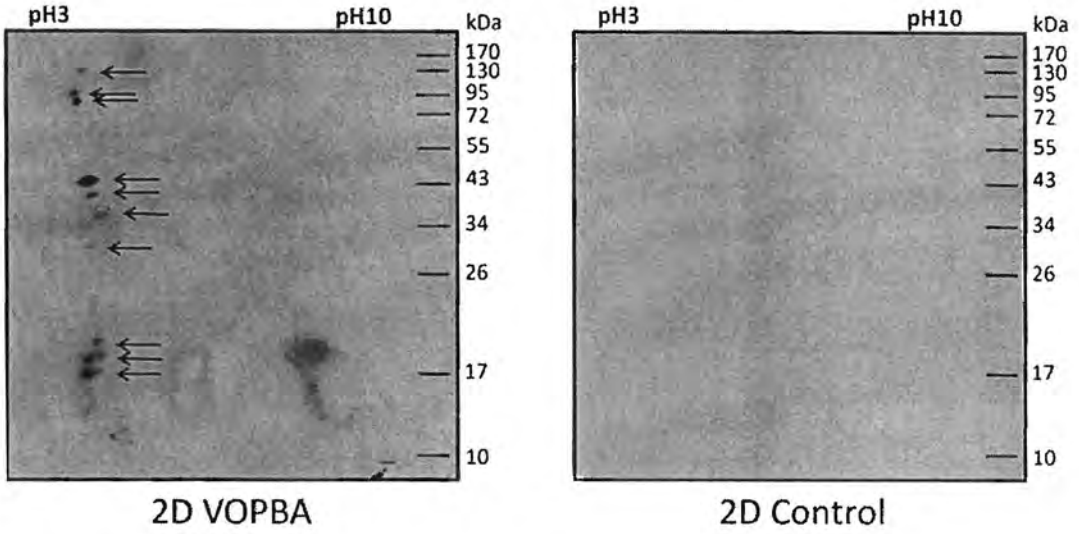
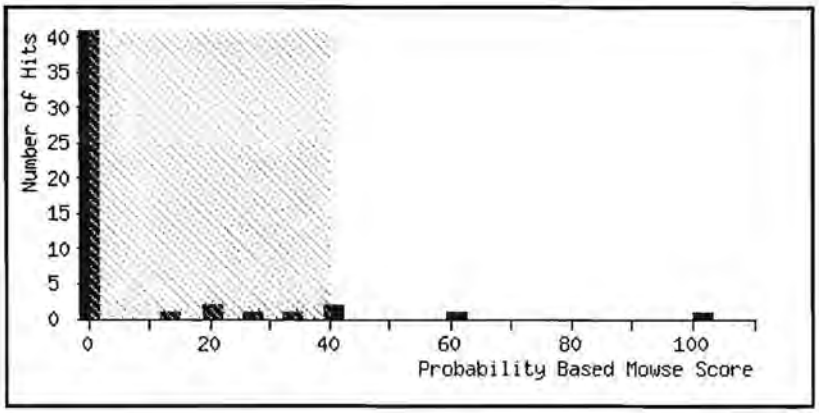


Figure 5. Two-dimensional VOPBA analysis of JEV binding proteins on BV-2 cells

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.



1. gi|226005 Mass: 32732 Score: 102 Queries matched: 9 emPAI: 0.72
protein 40kD

☐ Check to include this hit in error tolerant search

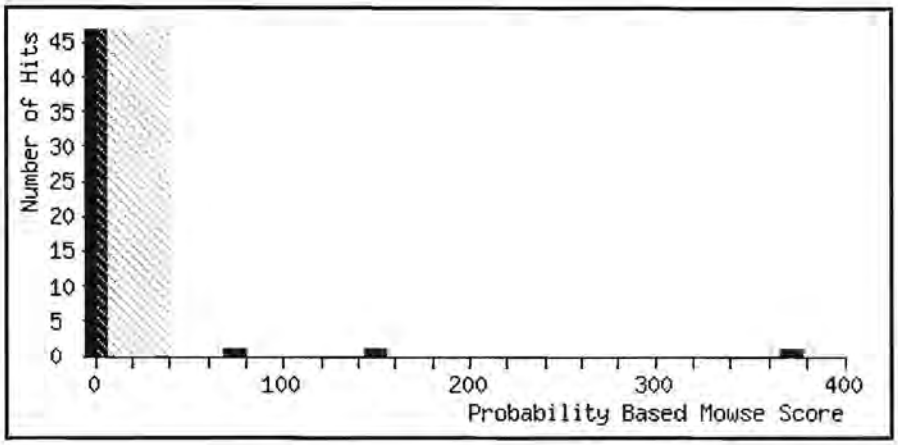
	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	147	568.2867	1134.5588	1134.6284	-0.0696	0	43	0.03	1	K.SDGIYIINLK.R
☑	155	602.3110	1202.6074	1202.6408	-0.0333	0	66	0.00017	1	K.FAAATGATPIAGR.F
☑	156	602.8134	1203.6122	1202.6408	0.9715	0	(34)	0.26	1	K.FAAATGATPIAGR.F
☑	157	602.8254	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	0.0886	1	37	0.085	1	R.DPEEIEKEEQAAAEK.A

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]

Figure 6. 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: 1.96
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.FLAAGTHLGGTNLDFQMEQYIYK.R

Proteins matching the same set of peptides:

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

Figure 7. 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.

The 2D gel electrophoresis-VOPBA was again reconfirmed. The blot was then stripped off and reprobed with a goat polyclonal anti-laminin receptor antibody, followed with rabbit - antigoat secondary antibody conjugated with HRP. The signal was detected by chemiluminescence. Result (Figure 8) reconfirmed the expression of 43 kDa laminin receptor precursor protein on microglial cell surface and this protein is one of the JEV binding proteins.

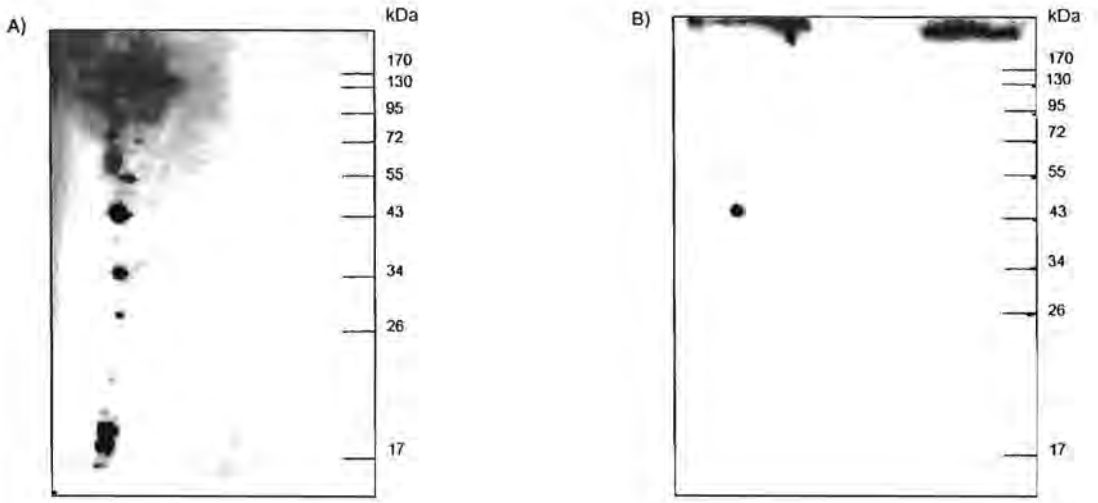


Figure 8. Western Blotting of 43 kDa laminin receptor precursor protein

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 43 kDa laminin receptor precursor protein was clearly detected by chemiluminescence (b)

3.3 Detection of JEV infection inhibition in microglial cells by flow cytometry at 8 hr post infection

To determine inhibition of the JEV infection in microglia, 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 (19) before infection with JEV at 1 pfu/cell at 37°C for 1 hr and 30 min. Analysis of infectivity 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 compare to control (Figure 9.) The time of 8 hr post infection was selected since one replication cycle of JEV, detected by extracellular virion is 10 hr (11).

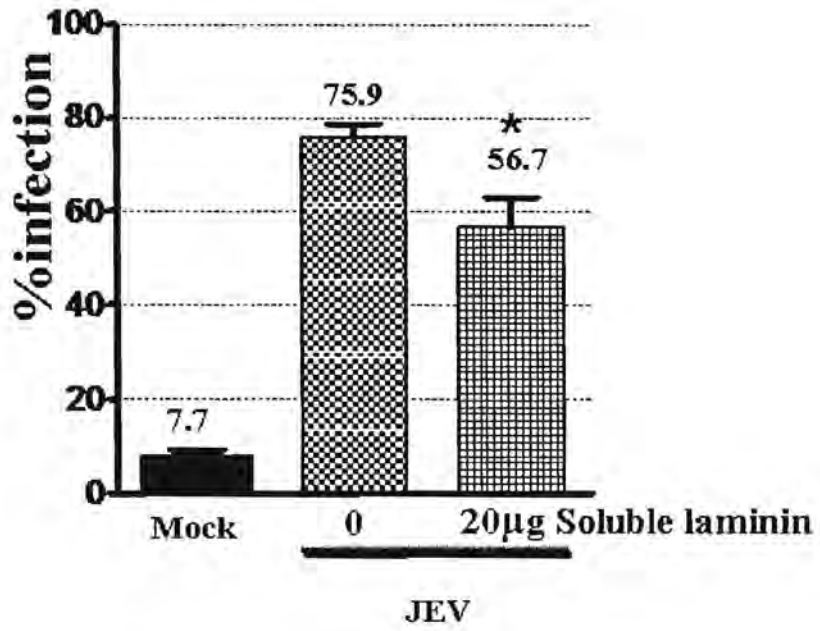


Figure 9. Infection inhibition of BV-2 with soluble laminin at 8 hr post infection

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) ($P<0.05$).

3.4 Detection of JEV infection inhibition in microglial cells by flow cytometry at 15 hr post infection

To determine the inhibition of the JEV infection in microglia, BV-2 cells were preincubated with the various amounts of antibody directed against the 37/67 kDa laminin receptor, that were reported in the previous study (16)(19). The cells were preincubated with either 0 or 20μg of soluble laminin and 0, 5, 10, 20μg of anti-laminin receptor antibody as well as 20μg of anti-CD4 antibody. The cells were later infected with JEV at 1 pfu/cell at 37°C for 1 hr and 30 min. Analysis of the percentage of inhibition by flow cytometry using a pan specific anti-Flavivirus E protein monoclonal antibody produced by hybridoma cell line HB-112 . By 15 hr post infection 20μg soluble laminin inhibited JEV infection approximately 16.41% compared to that of 0μg soluble laminin (control) (Figure 10.) .

For the preincubation with 5, 10 and 20μg anti-laminin receptor antibody, the percentage of JEV infection was inhibited approximately 13.4, 22.6 and 27.9% respectively, when compared to that of 0 μg anti-laminin receptor antibody (control) (Figure 11.) Anti-laminin receptor can inhibit JEV infection of BV-2 cells in a dose dependent pattern.

These results showed a significant inhibition of infection of BV-2 cells with JEV in the presence of a either 20 μg soluble laminin or the 5, 10 and 20μg anti-laminin receptor antibody by 15 hr post infection. The time of 15 hr post infection was selected since it is one cycle and a half of JEV replication cycle (11). It is insufficient time for viral progeny produced from the initial infection to reinfect the cells. However, preincubation with 20μg of anti-CD4 antibody was no different than that obtained with 0 μg anti-laminin receptor antibody suggesting the inhibition of JEV infection is antibody specific.

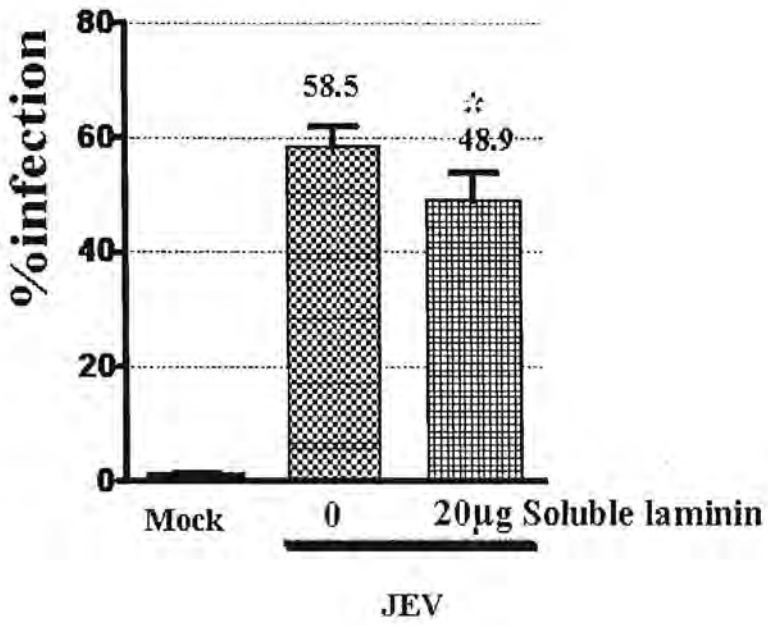


Figure 10. Infection inhibition of BV-2 with soluble laminin at 15 hr post infection

BV-2 cells were preincubated with either 0 (control) or 20 µg of soluble laminin before infection with JEV at 1 pfu/cell. At 15 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) ($P<0.05$).

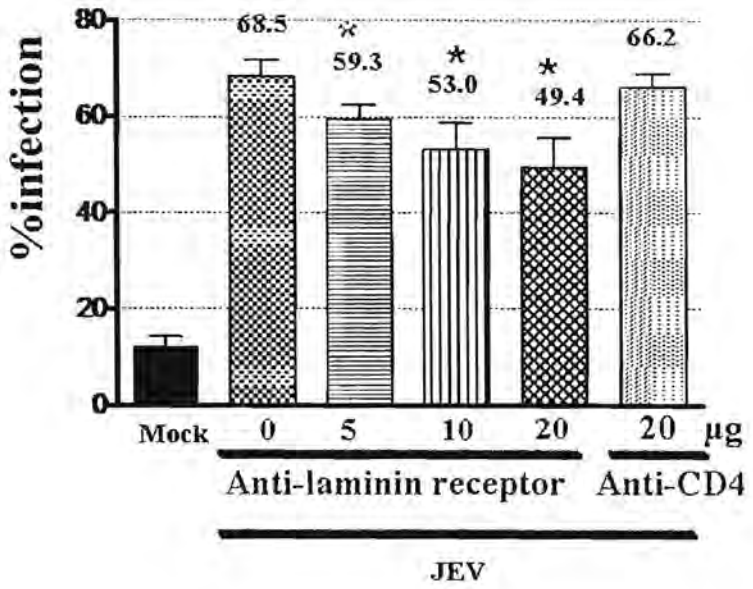


Figure 11. Infection inhibition of BV-2 with anti-laminin receptor antibody at 15 hr post infection

BV-2 cells were preincubated with various concentration of anti-laminin receptor antibody. 20µg of anti-CD4 was included as a non-relevant antibody. Cells were later infected with JEV at 1 pfu/cell. By 15 hr post infection, the 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 antibody before analyzed 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) ($P<0.05$).

CHAPTER IV

DISCUSSION AND CONCLUSION

Japanese encephalitis virus (JEV), a mosquito-borne *flavivirus*, that causes Japanese encephalitis disease which is a major public health problem especially in Asia. JEV infection mainly effects the central nervous system (CNS) causing fever, headache, neurological sequelae including motor deficits, cognitive and language impairment and learning difficulties. Even though the target of JEV infection is neuronal cell, microglial cells are also permissive to its infection and involved in Japanese encephalitis pathogenesis. (10)(11)(12)(20)(21) Results from the present study showed the percentage of infection in mouse microglial (BV-2) cells, a class of mononuclear phagocytes in the CNS, determined by flow cytometry at 8, 15 and 24 hr post infection. The percentage of infection was approximately 53.5, 71.3 and 83.6% respectively when microglial cell was infected with JEV at 1 pfu/ml

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 Coomassie-stained 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 (22).

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 (23). 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 (24). 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 (25).

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) (26) by dimerization and acylation (27). 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 (28) (29). 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 lines with multiple approaches (30)(31)(32)(33). 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 (18). Identification of the 37/67-kDa high-affinity laminin receptor protein for dengue virus serotype 1 on the liver cells (19) correlated with our finding of 43 kDa laminin receptor precursor protein as a JEV binding protein on microglial cells.

The in vitro virus binding ability of 43kDa laminin receptor precursor protein was verified by infection inhibition assay. Interestingly, at 15 hr post infection, the inhibitory effect of 20 μg of soluble laminin appeared to be less effective than that of 20μg anti-laminin receptor antibody, although the larger size of soluble laminin (900kDa) should be able to block the 43kDa laminin receptor precursor protein expressed on microglial cells more effectively than its antibody. Moreover, soluble laminin is a glycoprotein with multiple protein binding domains. It can bind to heparin, besides 43kDa laminin receptor precursor protein, that is known to be involved in many flavivirus entry. In addition, highly sulfated forms of heparin sulfate are involved in JEV infection in BHK-21 and CHO cell lines (17) (34). Even though the inhibitory effect of 20μg of soluble laminin at 8 hr post infection was about as effective as that of 20μg anti-laminin receptor antibody at 15 hr post infection, inhibitory effect of anti-laminin receptor antibody appeared to be more effective, in the aspect of dose dependent pattern of inhibition, than soluble laminin. However, both soluble laminin and anti-laminin receptor antibody were not able to completely inhibit the JEV infection indicating that there are other JEV binding proteins for entering into microglial cells besides using laminin receptor.

In conclusion, the 43 kDa laminin receptor precursor protein was identified as JEV binding protein on microglial cells by both one-dimensional and two-dimensional gel electrophoresis VOPBA . Its function as a JEV putative receptor was further verified by infection inhibition using soluble laminin and anti-laminin receptor antibody. Even though the inhibition only reaches the maximum of about 30%, determined by the presence of viral antigen in infected BV-2 cells, the effects of functional inhibition on JEV replication, determined by standard plaque assay, will be investigated. Also, in vivo expression of 43 kDa laminin receptor precursor protein assessed by western blotting, for the question of the biological significance paradigm, will be performed. The identification of this putative receptor may lead to the development of specific receptor - based prophylaxis and therapy at last.

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APPENDICES

Result of One dimensional 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

Result of Two dimensional 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	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