GENETIC DIFFERENCES OF *E* GENE OF A THAI STRAIN OF JAPANESE ENCEPHALITIS VIRUS THAT DETERMINE SMALL PLAQUE SIZE PHENOTYPE BUT NOT NEUROVIRULENCE IN SUCKLING MICE

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Abstract. Two small plaque variants of Japanese encephalitis virus (JEV), S4P9 and S9P10, were recovered from the wild type of JEV strain KE-093 using plaque purification in combination with the temperature-shift induction technique. Growth patterns of the S4P9 and S9P10 in BHK-21 cells as well as neurovirulence in suckling mice were similar to that of KE-093. An amino acid substitution, lysine for glutamic acid, was present in envelope protein at residue E-83 in the small plaque variants. This study shows that small plaque phenotype is not always associated with attenuation *in vivo*.

INTRODUCTION

In Asia Japanese encephalitis virus (JEV) is one of the leading causes of acute encephalitis in children under 15 years of age. Approximately 25% of cases are fatal and 45% of surviving patients retain neurological deficit (Tsai, 2000). JEV is transmitted to humans by several species of *Culex* mosquitoes including *Culex tritaeniorhynchus, C. gelidus* and *C. fuscocephala*. The natural cycle occurs as a zoonotic cycle with either pig or bird serving as the vertebrate amplifying host, with man serving as an accidental host (Rosen, 1986). There is no specific drug available for treatment of JE. The promising strategies for the prevention of this disease are vaccination together with efficient mosquito control, protection of the amplifying hosts, and prevention of humans from being bitten by infected mosquitoes (Tiroumourougane *et al*, 2002).

Live attenuated vaccine derived from JEV strain SA14-14-2 has shown several advantages, viz. low dose of vaccination, low cost in vaccine production, and induction of a long-lasting immune response in immunized children as opposed to those of inactivated vaccines (Tsai, 2000; Bista *et al*, 2001; Tandan *et al*, 2007). This attenuated virus strain has a small plaque phenotype. The potential of small plaque size mutants to serve as materials to produce live-attenuated vaccine has been extensively studied. Temperature sensitive (ts) mutants of dengue virus type 2 and 4, which have small plaque phenotype, appear to be attenuated strains in mice and may be useful for the development of live attenuated vaccine (Blaney *et al*, 2002; Hanley *et al*, 2002; Huang *et al*, 2003). It has also been shown that the virulence of small plaque mutants of other flaviviruses, including West Nile virus (WNV), tick-borne encephalitis virus (TBE), Murray Valley encephalitis virus (MVE) and JEV, are attenuated in the mouse model (Mandl

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et al, 2001; Lee and Lobigs, 2002; Lee *et al*, 2004). In addition, almost all strains of JEV that are small plaque size variants in BHK-21 cells are nonvirulent in mice (Kimura *et al*, 1973). Thus, it is of interest to look for small plaque size variants of JEV for development of a live attenuated vaccine.

The objective of this work was to select variants with small plaque size from a Thai strain of JEV, strain KE-093, using the temperature-shift induction technique, and to test for their virulence in suckling mice.

MATERIALS AND METHODS

Viruses

JEV strain KE-093, isolated in 1983 from the brain tissue of a patient with encephalitis at Kamphaeng Phet Province, Thailand, was used throughout this study. The virus sample was originally obtained from the Department of Virology, Armed Forces Research Institute of Medical Science (AFRIMS), Bangkok, Thailand.

Cell line

BHK-21 cells (ATCC) were grown in Dulbecco's modified medium (DMEM) (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 0.01 M Tricine (Gibco BRL), 0.01 M Na_2HCO_3 (Scharlau Chemi, USA) and antibiotics at the final concentration of 100 units of penicillin and 100 µg of streptomycin per ml (Gibco BRL). Cell cultures were maintained at 37ºC under 5% CO_2 atmosphere.

Animals

Litters of 8-12 suckling Swiss ICR mice (4 days old) were purchased from National Laboratory Animal Center, Mahidol University, Salaya campus, Nakhon Pathom, Thailand. Animals were maintained at the laboratory animal facilities, Faculty of Science, Mahidol University according to the regulation of Laboratory Animal Committee, Mahidol University.

Selection of small plaque variants by temperature-shift induction technique

BHK-21 cells were grown in T25 cm^2 flasks (Costar, USA) until 90-100% confluent cell monolayers were obtained. Monolayers were then inoculated with 0.1 ml of 10^3 PFU/ml of KE-093 seed virus, washed twice with phosphate-buffered saline and subsequently supplied with fresh medium containing 2% FBS. One of two flasks was incubated at 37ºC while the other flask was incubated at 40ºC. Supernatants were harvested on the day that 90-100% cytopathic effect (CPE) was observed. Supernatants were clarified by centrifugation at 1,500*g* (4ºC) for 15 minutes. An aliquot of the harvested viruses was kept at -70ºC for subsequent inoculation. The same procedure was used to produce further passages of the virus replicating in BHK-21 cells.

Virus samples taken from each passage were examined for plaque morphology by plaque assay at 37ºC. Virus samples that produce small plaque sizes were directly picked from infected cells grown in 6-well plates (Costar, USA) by capillary pipette with an aid of a microscope. Selected samples were kept at -70ºC in aliquots of 0.5 ml of growth medium containing 10% FBS. Selected samples that had small plaque phenotype were prepared as virus stocks for further study.

Plaque assay

Samples (100 µl) of serial ten fold dilutions of virus were inoculated onto BHK-21 monolayer. Inoculated cultures were incubated at 37ºC for 30 minutes and subsequently overlaid with a medium consisting of equal volume of a mixture of 2X DMEM containing of 4% FBS and 2% gum tragacanth (Vidthayasom, Thailand). One set of culture plates was incubated at 37ºC and another at 40ºC in 5% CO_2 for 5 days. Infected cells were fixed and stained with 1% crystal violet in 10% formaldehyde, and subsequently rinsed under running tap water, air-dried, and the virus titer determined.

Kinetics of viral multiplication

The selected samples and wild type strain of JEV were examined for their replication activity in BHK-21 cell monolayer grown in 6-well plates using virus inocula at a concentration of approximately 1 x 10^3 PFU/ml. Each of two sets was separately incubated at 37ºC and 40ºC, after viral adsorption at 37ºC for 30 minutes. Virus samples grown at both temperatures were harvested at intervals of 6 hours, 12 hours, 24 hours, 2, 3, 4, and 5 days post-infection. Viral samples were assayed for their infectivity by plaque assay technique.

Isolation of viral RNA and reverse transcriptase-polymerase chain reaction (RT-PCR) of envelope *E* gene

Extraction of viral genomic RNA of selected samples and wild type JEV was made from virus stocks using QIAamp Viral RNA Mini kit (Qiagen, Germany). Viral RNA was subjected to first strand cDNA synthesis using 1 µM 2501r primer, 5′ GAT GTC AAT GGC ACA TCC AGT 3′ (Bioservice Unit, NSTDA, Thailand), 10 units of AMV reverse transcriptase (Promega, USA), 10 unit of RNase inhibitor (Promega) and 1 mM dNTP mixture in 20 µl volume. Samples were incubated at 42ºC for 90 minutes. The cDNA sample was then subjected to PCR amplification using JEV *E* gene specific primers, sense 5′ CTC GGA TCC GCA CCA TGG TCC GTC CGG CTT ACA GTTT 3′ and antisense 5′ GCA GAA TTC TTA GAT GTC AAT GGC ACA TCC AGT GTC 3′. PCR was performed as previously described (Ali and Igarashi, 1997).

Cloning and sequencing of JEV *E* gene

PCR products of the selected samples and wild type JEV were cloned into pDrive vector for *E* gene sequencing using the Qiagen's protocol. Sequences of both strands were determined by primer walking method at Cybergene AB, Huddinge, Sweden. The nucleic acid sequence was converted to amino acid sequence by EMBOSS transeq program (EMBL, EBI, UK) and was analyzed by multiple sequence alignment with program ClustalW (EMBL, EBI, UK). The GenBank/EMBL/DDBJ accession number for envelope *E* gene sequence of the small plaque mutant S4P9 and S9P10 obtained in this study is EF363694 and EF363695 respectively.

Assay of virulence in suckling mice

Eight to 12 suckling Swiss ICR mice (4 days old) were inoculated intracerebrally with a series of 10-fold serial dilutions of the selected virus samples and of wild type JEV strain KE-093 in 0.01 ml of virus suspension per animal. Mice were observed daily for 21 days after inoculation for signs of encephalitis and death. The virus dose used was checked by back titration. Fifty-percent lethal dose (LD_{50}) was estimated by the method of Reed and Muench (1938).

RESULTS

Isolation of small plaque variants by temperature-shift induction technique

Two small plaque variants, S4P9 and S9P10, were obtained from the 9^{th} and 10^{th} passage of sequential passaging of JEV strain KE-093 in BHK-21 cells using temperature-shift induction technique. The plaques size produced by S4P9 and S9P10 was 1.85 ± 0.24 and 2.05 ± 0.28 mm in diameter respectively, compared to plaques produced by the wild type virus of about 4.1 ± 0.32 mm.

Comparison of growth kinetics of small plaque variants with wild type virus

Replication kinetics of the small plaque variants, S4P9 and S9P10, and wild type virus were studied at 37ºC and 40ºC. As shown in Fig 1, the titers of virus gradually increased and reached a peak at 48 hours post-infection for both temperatures. However, both small plaque variants replicated to a log higher value than that of the wild type at 37ºC, but not at 40ºC.

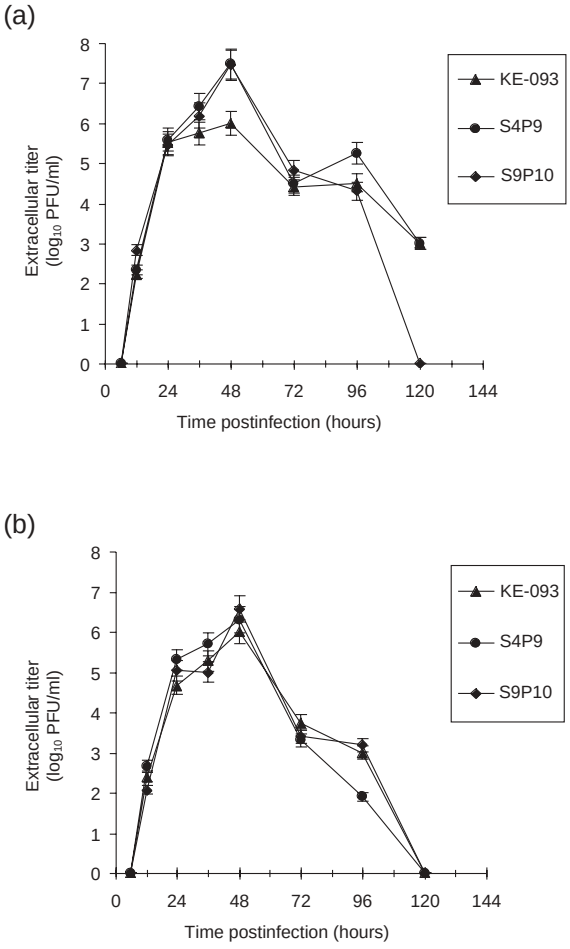

Fig 1–Viral kinetics of wild type and small plaque variant JEV in BHK-21 cell monolayers at 37ºC (a) and 40ºC (b). Monolayers of BHK-21 cell were infected with 10^3 PFU/ml of virus inocula. The titer of virus released during each time interval was determined by plaque assay.

Neurovirulence assay in suckling mice

Selected variants were tested for virulence in suckling mice. LD_{50} value in suckling mice of wild type virus and small plaque variant S4P9 and variant S9P10 was equivalent to 1.84 ± 1.55, 3.30 ± 0.77, and 1.32 ± 0.65 PFU, respectively. Although it appeared that the S4P9 and S9P10 JEV variants were less virulence than wild type JEV, differences were not significant ($p > 0.05$).

Nucleic acid and amino acid analysis

In a preliminary search of genotypic basis for the small plaque phenotype, the DNA and amino acid sequences of S4P9 and S9P10 were compared to those of wild type virus (data not shown). Sequences of the envelope *E* gene of the small plaque variants demonstrated that one amino acid substitution occurred at residue E-83, glutamic acid for lysine (Table 1). This amino acid is located in domain II region of E protein. Two other amino acids changes in E protein (Thr40Ala and Ala362Val) were found in S9P10. Amino acid changes in E protein of S4P9 and S9P10 would result in an increase in the net positive charge of the E protein.

DISCUSSION

There is a good correlation between small plaque size and attenuation of viruses, including several members of flavivirus (Mandl *et al*, 2001; Lee and Lobigs, 2002; Huang *et al*,

Table 1
Comparison of nucleotide and amino acid sequences of *E* gene of wild type and small plaque variants of JE virus.

Nucleotide position	KE-093	S4P9	S9P10	Amino acid position	KE-093	S4P9	S9P10
118	A	A	G	E-40	Thr	Thr	Ala
247	G	A	A	E-83	Glu	Lys	Lys
507	A	G	A	E-169	Val	Val	Val
1085	C	C	T	E-362	Ala	Ala	Val

2003; Lee *et al*, 2004). Thus attempts were made to isolate small plaque variants from JEV, resulting in two small plaque variants, S4P9 and S9P10.

Growth patterns of these two selected small plaque variants in BHK-21 cells were similar to that of the wild type virus at both 37ºC and 40ºC. Both viruses replicated and reached the peak by 48 hours. The numbers of infectious particles in supernatant gradually declined after 48 hours. This decline could be attributed to extracellular viral lability at 37ºC or viral cytopathic effect which damages host cells (Makino and Jenkin, 1975; Masud *et al*, 1988). However, these small plaque variants replicated better than the wild type at 37ºC suggesting that the small plaque variants have adapted to grow better in cell cultures after several passages in BHK-21 cells. It has been shown that sequential passages of virus in cell culture lead to cell-adapted mutants and attenuated virulence in mice. For example, JEV strain SA 14-14-2 replicates in C6/36 cells better than the parent strain (Bhatt *et al*, 2000). TBE mutant virus (Oshima C1-1) replicates better in the same host than the parent virus (Oshima 5-10), and is also attenuated for neuroinvasiveness in a mouse model (Goto *et al*, 2003). These data suggest the usefulness of cell culture techniques to select attenuated virus strains.

E protein plays roles in virion assembly, receptor binding and membrane fusion, and is a major target for neutralizing antibodies (Chambers *et al*, 1990). Mutations in E protein are, therefore, often responsible for significant alterations in the biology of the virus and affect virulence of flaviviruses (Chambers *et al*, 1990; Burke and Monath, 2001; Tiroumourougane *et al*, 2002; Heinz, 2003). In our study, amino acid substitution in JEV E protein of the small plaque variants, S4P9 and S9P10, occurred at different positions from those reported by others. Attenuated vaccine clones, SA 14-14-2/PHK and SA 14-14-2/PDK, contain seven mutations, 4 of which are located in E protein, *viz*. Glu138Lys, Ileu176Val, Ala315Val and Lys439Arg (Ni *et al*, 1995). Two other attenuated viruses, Nakayama-O and an Indian 826309, contain different mutations, including alanine to valine change at E-366 (Cao *et al*, 1995). Thus JEV attenuated phenotype obtained from serial passage in cell culture system is not determined by a common mutation.

The mutation Glu83Lys present in the two small plaque variants may affect envelope protein fusion of the virus. An amino acid residue E-83 is located in the segment between the B and C sheets of dimerization domain II (Rey *et al*, 1995) and the fusion domain, residue 98-113, is located at the tip of domain II (Stiasny and Heinz, 2006). Mutation at E-83 may interfere with the activity of fusion domain resulting in reduction of viral dissemination. However, the E-83 mutation is not involved in neurovirulence as S9P10 and S4P9 have the same level of virulence as the wild type virus. Therefore, mutations in S9P10 and S4P9 determine plaque size phenotype.

In summary, two small plaque variants of JEV strain KE-093 were generated by using plaque purification in combination with temperature-shift induction technique.

Mutation at E-83, glutamic acid to lysine, determines small plaque phenotype but not neurovirulent characteristics, indicating that small plaque size does not always represent an attenuation *in vivo*.

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