Neurovirulence of four encephalitogenic dengue 3 virus strains isolated in Malaysia (1992–1994) is not attributed to their envelope protein

Mun-Yik Fonga,*, Roziyah Yusupb, Rohana Yusofc, Sai-Kit Lamb

a Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
b Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
c Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

Received 24 July 2003; received in revised form 7 November 2003; accepted 20 November 2003

KEYWORDS
Dengue virus; Encephalitis; Neurovirulence; Envelope protein; Malaysia

Summary The amino acid sequences of the envelope (E) protein of four encephalitogenic and five non-encephalitogenic dengue 3 virus strains isolated in Malaysia were determined and compared. Multiple sequence alignment revealed a high degree of similarity in the E protein of the strains suggesting that neurovirulence of these four encephalitogenic strains is not attributed to this protein.

© 2004 Royal Society of Tropical Medicine and Hygiene. Published by Elsevier Ltd. All rights reserved.

Dengue viruses are members of the genus Flavivirus, and they occur as four distinct serotypes (DENV1, DENV2, DENV3, and DENV4). These mosquito-borne viruses are of global public health concern as they are the causative agents of dengue. Millions of dengue virus infections occur annually, particularly in the tropical and subtropical regions of the world. Dengue virus infection may be asymptomatic or may lead to dengue fever, a mild self-limiting febrile illness. In some cases, however, vascular and haemostatic abnormalities occur, progressing to the severe form known as dengue hemorrhagic fever. Dengue shock syndrome (DSS) occurs when the patient’s condition deteriorates abruptly with signs of circulatory failure (Gubler, 1998).

Over the past two decades, an increasing number of dengue infections with atypical clinical manifestations have been reported, including those involving the central nervous system (CNS). It is believed that these CNS manifestations are due to leakage of plasma into serous space and abnormal homeostasis, resulting in hypovolaemic shock, haemorrhage and metabolic disturbances in the brain (Lam, 1996). In most of these cases, no virus infection of the CNS was detected. Therefore, these cases have been referred to as dengue encephalopathy. However, recent reports have provided convincing evidence of encephalitis following direct invasion of the CNS by dengue virus. In Malaysia, six serologically confirmed dengue cases presenting with encephalitis were reported, and DENV3 was successfully isolated from the cerebrospinal fluid (CSF) of four of the cases (Lum et al., 1996). Detection of dengue virus in the CNS of dengue patients has been reported in French Guiana, Mexico, Brazil and Southeast Asia (Cam et al., 2001; Hommel et al., 1998; Nogueira et al., 2002; Ramos et al., 1998), emphasizing the global emergence of neurovirulence in dengue viruses.
Like other flaviruses, the mature virions of dengue virus contain three structural proteins: a core (C) nucleocapsid protein, a small transmembrane (M) protein, and a major surface envelope (E) protein (Chambers et al., 1990). The E protein has been shown to mediate receptor binding and membrane fusion, and it induces a protective immune response. Mutations in the E protein gene are believed to be the molecular basis of neurovirulence in encephalitogenic dengue virus strains (McMinn, 1997).

In a previous study, we analysed the nucleotide and encoded amino acid sequences of the E protein of the four Malaysian encephalitogenic DENV3 strains described above (Fong and Lam, 2001). Comparison of the sequences of these strains (isolated in 1992–1994) with those of non-encephalitogenic strains isolated 10–15 years earlier (1974–1981) revealed amino acids at nine positions that were unique for the encephalitogenic strains. We postulated then that these amino acids might contribute to the neurovirulence of the strains.

In the present study, we determined the nucleotide sequences of the E gene of three additional Malaysian non-encephalitogenic DENV3 strains, which were isolated in 1994, and compared the encoded amino acid sequences with those obtained in our previous study. The availability of sequences of non-encephalitogenic strains isolated around the same time as the encephalitogenic ones should provide a better basis for comparison and inference on the significance of the unique amino acids identified in our previous study.

All the DENV3 strains used in the study were passaged in C6/36 mosquito cell line, grown in 25 ml tissue culture flasks. None of the strains were

![Figure 1](Alignment of the amino acid sequences of the envelope protein of Malaysian dengue 3 virus strains. Strains 1300, 29586, LN7029, LN7933, and LN8180 were non-encephalitogenic, whereas strains LN5547, LN1746, LN2632, and LN6083 were encephalitogenic. Conserved cysteine residues are underlined, N-linked glycosylation sites are arrowed, membrane anchor domains are shaded, and the flavivirus fusion domain is boxed. Amino acids unique for the strains isolated in the 1990s are in italics. Dots indicate amino acids identical to the reference strain, 1300.)
Envelope protein of encephalitogenic dengue 3 viruses

passed more than five times. The E gene of each strain was amplified by reverse-transcriptase PCR, and the PCR products were directly sequenced. Figure 1 shows the alignment of the amino acid sequences of the E protein of the Malaysian DENV3 strains. The sequences of strains 1300 and 29586 are from Lanciotti et al. (1994). The E protein of both the encephalitogenic and non-encephalitogenic strains possessed sequences of amino acids postulated to play key roles in the processing and folding of the E protein. Conserved cysteine residues for the formation of disulfide bridges in the protein were retained. The two potential asparagine N-linked glycosylation sites, the valine—glutamine—alanine (VQA) proteolytic cleavage site at the C-terminus and the membrane anchor domain were all conserved. The flavivirus fusion domain (amino acid residues 98 to 111) was conserved as well. Furthermore, pair-wise comparison among the sequences, especially that of strains isolated in the 1990s, revealed very high similarity values (>98.8%).

Eight of the unique amino acids identified in our previous study were found in the E protein of both the encephalitogenic and non-encephalitogenic strains isolated in the 1990s, thus suggesting that these amino acids do not contribute to neurovirulence. The valine (V) at position 489 was found only in the E protein of the encephalitogenic strains. However, it is unlikely that this amino acid has any role in conferring the neurovirulence phenotype, as it is located in the anchor domain. This anchor domain is embedded deep in the membrane matrix of the virus particle, and therefore cannot be involved in the interaction with receptors on the neuron cell of the host.

Our findings in this study thus show that neurovirulence determinants of the four encephalitogenic DENV3 strains are not located on their E protein. As pointed out by McMinn (1997), neurovirulence can be attributed also to mutations in other genes and non-coding or untranslated regions of the flavivirus RNA genome. Hence, a thorough analysis of the genomes of the encephalitogenic DENV3 strains is necessary in order to unravel the basis of their neurovirulence.

Note
Nucleotide sequences of the E protein gene reported in this study have been deposited into the GenBank database (http://www.ncbi.nlm.nih.gov). These sequences can be obtained using the following accession numbers: AF147457, AF147458, AF147459, AF147460, AY338492, AY338493, and AY338494.

Conflicts of interest statement
The authors have no conflicts of interest concerning the work reported in this paper.

Acknowledgements
This research was supported by the University of Malaya, and the Ministry of Science, Technology and Environment, Malaysia (IRPA Grant No. 06-02-03-0306).

References