PRIMARY SEQUENCE OF THE ENVELOPE GLYCOPROTEIN OF A DENGUE TYPE 2 VIRUS ISOLATED FROM PATIENT WITH DENGUE HEMORRHAGIC FEVER AND ENCEPHALOPATHY

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Abstract. Dengue viruses exist in nature as a collection of highly similar but not identical members (quasispecies). In order to correlate the presence of viral quasispecies with rare occurrence of unusual clinical manifestations in dengue-infected individuals, a dengue type 2 virus was isolated from the peripheral blood of a 12-year-old boy who presented with fever, headache, drowsiness and tonic seizure of the left arm, and subsequently manifested symptoms and signs of dengue hemorrhagic fever. Analysis of the envelope glycoprotein sequence of the encephalopathy-associated virus and two other dengue type 2 viruses from the same epidemic season in Chiang Mai, Thailand revealed that all three viruses belonged to the subtype IIIa of the five-subtype phylogenetic nomenclature system for dengue type 2 virus. The encephalopathy-associated dengue virus was more divergent from the others and was characterized by an Ala → Val substitution at the position 173 of the envelope glycoprotein. This substitution mapped to the central domain I which was not known to be involved directly in envelope-receptor interaction.

INTRODUCTION

Dengue viruses are single-stranded RNA viruses in the family Flaviviridae. Infection of humans by any of the four serological types of dengue viruses may result in a wide range of illnesses including undifferentiated fever, classical dengue fever (DF), and dengue hemorrhagic fever (DHF) (Halstead, 1980, 1988; Monath, 1986). The majority of dengue infections are self-limited, but DHF, a potentially fatal disease, occurs in a small fraction of all infected individuals (Halstead, 1980). Recently, unusual clinical manifestations, such as hepatitis, encephalopathy, and renal impairment, have been observed in even rarer patients suffering from DF or DHF (George *et al*, 1988; Nimmannitya, 1993). While there is evidence indicating that the risk of developing DHF is increased in individuals secondarily infected with dengue viruses (Sangkavipha *et al*, 1980; Burke *et al*, 1989), the underlying cause and pathogenetic mechanism(s) involved in the generation of unusual manifestations remain unknown.

At any given point of time, RNA virus populations are usually composed of distinct members bearing similar but not identical sequences (Steinhauer and Holland, 1987; Domingo and Holland, 1994). For dengue viruses, the quasispecies have been clearly demonstrated by RNAse T1 oligonucleotide fingerprinting (Trent *et al*, 1983, 1989; Walker *et al*, 1987) and sequence analysis (Blok *et al*, 1989, 1991; Rico-Hesse, 1990; Lewis *et al*, 1993; Lee *et al*, 1993; Duangchanda *et al*, 1994). Rare occurrence of unusual manifestations following dengue virus infection in human may reflect this quasispecies nature. Conceivably, there may exist certain variants within dengue virus population which contain rare mutations and, as a result, exhibit distinct biological properties, such as reduced/enhanced receptor binding affinity, capability to interact with wider range of cell surface receptors, or ability to replicate in unusual cell type or target organ. Infection by these variants may possibly result in unusual clinical manifestations even though they are not readily propagatable in nature. In order to identify such virus for further study, we isolated a dengue type 2 virus from a DHF patient with encephalopathy and determined its envelope glycoprotein gene sequence. The primary

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sequences of this isolate and two other viruses derived from the same season in Chiang Mai revealed that all three viruses belonged to the subtype IIIa of the recent five-subtype phylogenetic classification (Lewis *et al*, 1993). However, the encephalopathy-associated isolate contained an amino acid substitution (Ala → Val) at the position 173.

MATERIALS AND METHODS

Dengue virus isolation and quantitation

Dengue serotype 2 viruses were isolated by inoculation onto C6/36 clone of *Aedes albopictus* mosquito cells (Igarashi, 1978). For serological classification, an indirect immunofluorescence assay (Henchal *et al*, 1982) was employed with the following monoclonal antibodies: 4G2, anti-flaviviruses; 3H5, anti-dengue virus type 2; 1F1, anti-dengue virus type 1; 10C10, anti-dengue virus type 3; and 1H10, anti-dengue virus type 4 (kindly provided by Drs B Innis and D Trent). Quantitation by a 4-step peroxidase-antiperoxidase method was performed in 96-well plates according to Okuno *et al* (1977) and Ishimine *et al* (1987) with some modifications (Viputtikul *et al*, 1993) and expressed as focus forming units (ffu). Neutralizing antibody level in the sera samples was determined as described previously (Ishimine *et al*, 1987). Clinical diagnosis and grading of disease severity were according to WHO criteria (1986).

Three dengue type 2 isolates were included in this study: TH91-CM JE 6A was isolated from a patient with dengue hemorrhagic fever and encephalopathy; TH91-CMC 15 and TH91-CMS 41 were derived from patients with dengue hemorrhagic fever without unusual manifestation. They were all isolated during the 1991 epidemic season in Chiang Mai area.

Preparation of genomic RNA and cDNA synthesis

Genomic RNA was prepared from culture fluid of infected C6/36 cells by phenol-chloroform extraction as described previously (Sittisombut *et al*, 1996). Briefly, virions in the culture fluid were lysed in 1% SDS and 100 μg/ml proteinase K at 56°C for 1.5 hours. Following extraction with phenol and chloroform, the RNA was recovered by precipitating with ethanol and collected by centrifugation. For synthesis of cDNA, genomic RNA derived from 4×10^4 ffu was reverse transcribed in 10 mM Tris-HCl, pH 8.3 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 10 mM dithiothrietol, 0.5 mM each of dNTPs, 20 pmol of antisense primer, and 150 U of recombinant MMLV RNaseH$^-$ reverse transcriptase (GIBCO/BRL, Gaithersburg, MD) at 45°C for 1 hour. The cDNA products were used directly in the amplification reactions without any purification.

Amplification and nucleotide sequence analysis of envelope gene

The cDNA was amplified in 100 μl of the mixture containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 0.1 mM each of dNTPs, 10 pmol of sense primer, and 2.5 U of Taq DNA polymerase. The amplification was allowed to proceed for 35 cycles according to the following parameters: denaturation, 94°C for 1 minute; annealing and extension, 72°C for 5 minutes. The last extension step was at 72°C for 7 minutes. Products were analyzed by electrophoresis through 1% agarose gel in Tris/acetate/EDTA buffer and the 1,700 bp DNA band was purified by using the GlassMax DNA isolation system (GIBCO/BRL).

Nucleotide sequence analysis was performed by using the dsDNA cycle sequencing system (GIBCO/BRL). One picomol of sequencing primer was end-labeled with [γ^{-32}P] ATP (> 5,000 Ci/mmol, Amersham) with T4 polynucleotide kinase and were added, without removing of unincorporated [γ^{-32}P] ATP, with approximately 50 fmol of the PCR product and 2.5 U Taq DNA polymerase in 37.5 mM Tris-HCl, pH 9.0, 6.2 mM $MgCl_2$, 37.5 mM KCl and one of the four dideoxynucleotide mixes. Depending on the melting temperature of each sequencing primers, either or both of the quick cycle or normal cycle of the cycling temperature parameters was employed according to the manufacturer's protocols. The products of cycle sequencing reaction were analyzed on a 6% or 8% denaturing polyacrylamide gel within 24 hours. Oligonucleotide primers for cDNA synthesis, amplification and sequence determination were as described previously (Sittisombut *et al*, 1996).

RESULTS

Clinical features of a patient with DHF and encephalopathy

A 12-year-old boy (HN. 1765524) was presented at Maharaj Nakhon Chiang Mai Hospital, Chiang Mai, with high fever for two days with mild running nose, headache and vomiting. Focal tonic seizures of the left arm with eyes rolling upward and marked drowsiness occurred on the second day of illness. The patient was previously healthy. He never received Japanese B encephalitis vaccine. Pigs were raised near the house which situated near rice paddy.

The physical examination revealed marked drowsiness in otherwise normal child. The cerebrospinal fluid (CSF) study revealed 17 cells/mm^3. (15 lymphocytes, 2 polymorphonuclear cells). The CSF sugar was 91 mg% in the presence of 124 mg% of blood sugar. The CSF protein was 21 mg%. No organism was found on CSF Gram and acid fast stains. CSF culture was negative for bacterial organisms.

Complete blood count revealed hemoglobin of 12.2 gm%, hematocrit 37 vol%, total WBC count 12,600 cells/mm^3 with 91% lymphocytes and adequate platelets on the peripheral blood smear. Serum sodium was 135 mEq/l while serum potassium, chloride and CO_2CP were 4.2, 98, and 17 mEq/l, respectively. Urinalysis was normal.

Serum antibodies to dengue type 2, type 3 and JE antigens as measured by hemagglutination inhibition method showed more than 4-fold rise in titer, *ie* from the level of 1 : 20 or below 1 : 20 to 1 : 2,560, within seven days. Changes in neutralizing antibody titers against dengue and Japanese encephalitis viruses are shown in Table 1. No IgM antibody to Japanese encephalitis virus was detected in cerebrospinal fluid by IgM capture ELISA. Dengue serotype 2 viruses were recovered from frozen plasma samples taken on the second day (isolate TH91-CM JE 6A) and fourth day (isolate TH91-CM JE 6C1) of illness.

Following admission, symptomatic and supportive treatments were given. No corticosteroids were prescribed. The patient's high fever declined rapidly on the fourth day of illness concurrent with the manifestation of definite signs of hemoconcentration. The hemoglobin level was increased to 16.1 gm%, hematocrit to 43 vol% and platelets were markedly reduced to 25,000 per mm^3. Enlargement of liver was also detected with blood pressure dropped down to 90/60 mmHg. Intravenous fluid replacement together with other supportive treatments were given to stabilize the hemodynamic status. The patient fully recovered on the eighth day of illness. No neurological deficit was encountered when the patient was discharged home after 7 days of hospitalization. He could do normally in school as he could previously.

Table 1

Antibody titers to dengue viruses and Japanese encephalitis virus at three different days of illness.

	Serum neutralization titer against				
	Den-1	Den-2	Den-3	Den-4	JEV
Day 2	< 10	< 10	< 10	< 10	10
Day 4	< 10	27	< 10	< 10	< 10
Day 10	1,700	980	65	1,600	10

In the view of typical clinical course, hematological changes, significant rises of HI and neutralizing antibody titers and virus isolation, the patient was considered to suffer from DHF with encephalopathy.

Deduced amino acid sequence of envelope glycoprotein gene

Envelope glycoprotein gene of the isolate TH91-CMJE 6A and two other dengue serotype 2 viruses (TH91-CMC 15 and TH91-CMS 41) derived from the Chiang Mai area in the year 1991 contained 1,485 bases encoding a single stretch of 495 amino acids typical of dengue envelope glycoprotein. When compared with published sequences of dengue type 2 prototypic strains and other Thai strains (Deubel *et al*, 1986; Hahn *et al*, 1988; Blok *et al*, 1989), all nucleotide differences were detected as single base substitutions. Deduced amino acid sequences revealed conserved cysteine residues and potential N-glycosylation sites (Fig 1).

Since 1980, dengue serotype 2 viruses circulating in Bangkok have been classified into two subtypes, IIIa and IIIb, based on phylogenetic rela-

Figure 1.

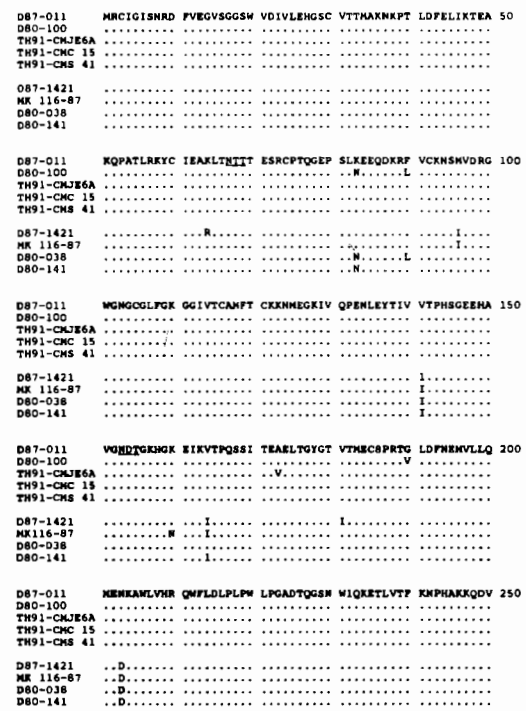

Figure 1 (continued).

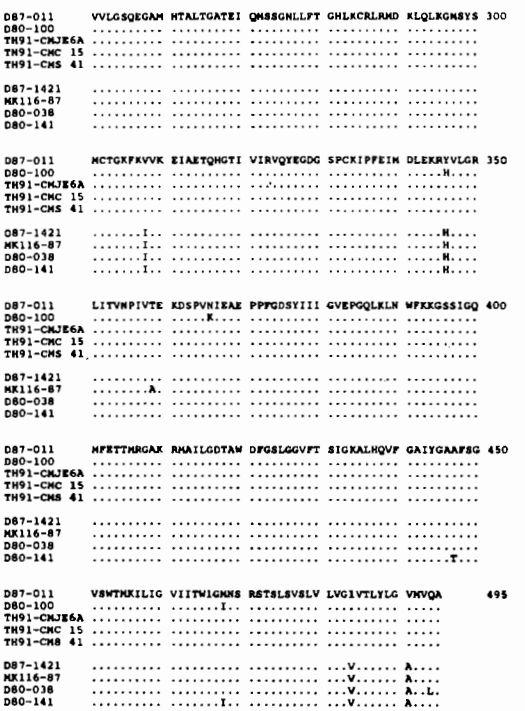

Fig 1–Deduced amino acid sequences of the envelope protein of strains TH91-CMJE 6A, TH91-CMC 15, TH91-CMS 41 and selected dengue type 2 viruses from the 1980 and 1987 epidemic seasons in Thailand. Only the amino acids that differ from D87-011 sequence are indicated for other strains. Two potential glycosylation sites are underlined. According to the nomenclature system by Lewis *et al* (1993), strains D87-011 and D80-100 belong to subtype IIIA while strains D87-1421, MK 116-87, D80-038 and D80-141 belong to subtype IIIb. Sequences of the 1980 and 1987 strains were from Blok *et al* (1989), Duangchanda *et al* (1994), and Sittisombut *et al* (1996).

tionship (Lewis *et al*, 1993). Direct comparison of the necleotide sequences of Chiang Mai isolates with other dengue type 2 viruses derived from patients in Bangkok (Blok *et al*, 1989; Sittisombut *et al*, 1996) and Maha Sarakham Province (Duangchanda *et al*, 1994) indicated closer genetic relationship among all three Chiang Mai isolates with the subtype IIIa viruses found in Bangkok (Fig 1). They all shared five amino acid residues (Val141, Asn203, Val308, Ile484 and Val491) characteristic to the subtype IIIa viruses of the 1980 and 1987 epidemic seasons in Bangkok (Sittisombut *et al*, 1996). In addition, all Chiang Mai isolates contained Tyr residue at the position 346 similar to the subtype IIIa viruses of the 1987 epidemic season in Bangkok.

Comparison among the three Chiang Mai isolates revealed that the strain TH91-CMJE 6A was more divergent from the other two strains (Table 2). While strains TH91-CMC 15 and TH91-CMS 41 differed from each other by 13 bases, they varied from TH91-CMJE 6A by up to 20 and 23 bases, respectively. With one exception, all of these substitutions were conservative changes. A C → T transition at the nucleotide position 518 resulted in an amino acid substitution, Ala → Val, at the position 173 in TH91-CMJE 6A. As observed previ-

Table 2

Summary of nucleotide (above diagonal axis) and amino acid (below diagonal axis) differences among three dengue type 2 viruses isolated from Chiang Mai in 1991.

	TH91-CMJE6A	TH91-CMC 15	TH91-CMS 41
TH91-CMJE6A	-	20	23
TH91-CMC 15	1	-	13
TH91-CMS 41	1	0	-

ously in the comparison of Bangkok isolates (Blok *et al*, 1989; Sittisombut *et al*, 1996), there was no obvious hypervariability region in the entire envelope protein gene in this group of dengue type 2 viruses.

DISCUSSION

Limited sequence variation had been detected in a small number of flavivirus variants which manifested phenotypic characteristics distinct from known precursors. For example, an isolate derived from a case of yellow fever vaccine-associated viral encephalitis displayed only two non-conservative changes in the entire envelope protein gene (Jennings *et al*, 1994). A dengue type 2 vaccine candidate, 16681-PDK 53, differed from the virulent counterpart by three amino acids in the envelope protein (Blok *et al*, 1992). When the whole genome was taken into consideration, a total of 27 amino acid changes in all coding regions together with seven additional nucleotide variations in the 5' and 3' untranslated region were sufficient to reduce the infection, dissemination and transmission of strain 16681-PDK 53 in mosquito (Khin *et al*, 1994). Similarly, other attenuated strains of yellow fever virus (Hahn *et al*, 1987), dengue type 2 virus (Hahn *et al*, 1988) and Japanese encephalitis virus (Nitayaphan *et al*, 1990; Cecilia and Gould, 1991) all displayed less than 1% of nucleotide and amino acid variations from respective virulent precursors. These findings revealed clearly the significance of limited sequence variation on the biologic properties of RNA viruses. Although it has been difficult to correlate the position and type of sequence variations with specific phenotypic changes due to the occurrence of multiple nucleotide/amino acid substitutions in each variant and the rarity of such viruses, the recent crystallographically determined structure of flaviviral envelope glycoprotein, however, allows the placement of several mutations affecting virulence and tissue tropism of flaviviruses into three distinct clusters (Rey *et al*, 1995).

Low levels of nucleotide and amino acid variations were also present in field isolates of dengue viruses. Although it was likely that sequence changes were distributed throughout the entire genome, the envelope glycoprotein coding region had been studied most intensively because of its role in receptor binding and interaction with neutralizing antibody or infection-enhancing antibody (Blok *et al*, 1989; Lewis *et al*, 1993; Lee *et al*, 1993; Chungue *et al*, 1993; Duangchanda *et al*, 1994; Sittisombut *et al*, 1996). It appeared that there was no correlation between sequence variation in this region and the clinical severity in patients from whom viruses were isolated (Blok *et al*, 1989; Lee *et al*, 1993; Chungue *et al*, 1993; Duangchanda *et al*, 1994; Sittisombut *et al*, 1996). In one example, dengue type 2 isolates which were obtained from patients suffered from DF, DHF and DSS were found to be identical in the entire envelope glycoprotein sequence (Duangchanda *et al*, 1994). The lack of correlation between envelope protein variation and clinical disease severity (*ie* DF *vs* DHF/DSS), however, did not exclude the possibility that certain structural variations of the envelope glycoprotein were involved in the generation of unusual manifestations in some dengue-infected individuals. As observed in the case of yellow fever virus variant (Jennings *et al*, 1994), it is possible that the abilities to pass through blood brain barrier and to cause disease in the central nervous system are acquired by appropriate change in the envelope glycoprotein of encephalopathy-associated dengue virus.

In this study, the Ala → Val substitution at the position 173 of the envelope protein of strain TH91-CMJE 6A mapped to the Go β-pleated segment on the membrane proximal surface of the disulphide-linked central domain I of flaviviral envelope protein (Rey *et al*, 1995). This domain was not known to be involved directly in envelope-receptor interaction or determination of tissue tropism because single mutations which affected virulence, attentuation and tissue tropism of flaviviruses generally did not cluster in it (Rey *et al*, 1995). Nevertheless, the amino acid positions 150-174 which formed part of this Go segment were quite variable in tick-borne encephalitis viruses (TBEV), a member of the family Flaviviridae (Chambers *et al*, 1990). One of the three known mutations which influenced the threshold pH for fusion activating conformational change of flaviviral envelope protein was located in this Go segment in a TBEV variant (Mandl *et al*, 1989; Rey *et al*, 1995). Moreover, a mutation in the neurovirulent yellow fever virus variant also mapped to the juxtaposed Fo segment in the same domain (Jennings *et al*, 1994; Rey *et al*, 1995). In a similar manner, the substitution at the position 173 in strain TH91-CMJE 6A may possibly cause subtle change of the envelope protein conformation; the exact nature of

this change and how it affects the pathophysiologic potential of the virus remain to be investigated.

Changes in other coding and non-coding regions of the genome of strain TH91-CMJE 6A may also be present and contribute to the pathogenesis. In the case of poliovirus type 3 revertant, a point mutation in the 5' non-coding region was associated with an increase in neurovirulence of the virus (Evans *et al*, 1985). A significant reduction in neurovirulence in mice of chimeric TBEV/dengue 4 virus was detected in mutants with defects either at the TBEV pre-M cleavage site, the TBEV E glycosylation site, or the first dengue NS1 glycosylation site (Pletnev *et al*, 1993). These findings necessitate more thorough study of the sequence variation of strain TH91-CMJE 6A in parallel with functional studies, possibly through the use of full-length cDNA clone for the introduction and testing for the significance of individual changes (Lai *et al*, 1991; Pletnev *et al*, 1992, 1993).

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