DIFFERENCES IN HAEMAGGLUTINATION ACTIVITY AND ELECTROPHORETIC MOBILITY OF E PROTEIN BETWEEN THE PARENT SA14 AND ATTENUATED VACCINE SA14-14-2 STRAINS OF JAPANESE ENCEPHALITIS VIRUS

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Summary. – E protein characteristics of the attenuated Japanese encephalitis (JE) virus strain SA(A) derived from the live vaccine strain SA14-14-2 were compared with those of the virulent strain SA(V). SA(A) showed lower haemagglutination (HA) activity with broader optimum pH range of HA reaction than SA(V), and the E protein of SA(A) had slower electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) than that of SA(V). These properties of SA(A) E protein appear to be caused by eight amino acid changes found in the E protein coding region of the SA(A) virus genome.

Key words: Japanese encephalitis virus; attenuated vaccine strain; E protein; haemagglutination activity; electrophoretic mobility; amino acid changes

JE virus, a member of the family *Flaviviridae*, is an important mosquito-borne virus associated with a serious human illness. In the People's Republic of China, the live attenuated JE vaccine strain SA14-14-2 was developed from the virulent SA14 strain by multiple passages in primary hamster kidney (PHK) cells and mice (Ao *et al.*, 1983; Yu *et al.*, 1981, 1988). The live vaccine strain SA14-14-2 was markedly attenuated in comparison with the parent SA14. The vaccine strain SA14-14-2 grown in PHK cells has been used exclusively in People's Republic of China in both humans and pigs.

We compared the nucleotide sequences of the SA(V) and SA(A) strains derived from parental strains SA14 and SA14-14-2, respectively, after plaque purification in BHK-21 cells and growth in C6/36 cells (Aihara *et al.*, 1991). We found 57 nucleotide differences resulting in 24 amino acid substitutions between the two strains in the entire 10,976 nucleotides long genome. However, the genome changes responsible for the virulence or attenuation cannot be defined yet. There are eight amino acid substitutions which originate from 11 nucleotide differences in the E coding region. Many missense mutations are located in this region in contrast to the rest of the coding region where 43 nucleotide differences lead to 16 amino acid substitutions. The large number of amino acid changes found in the E coding region may affect E protein characteristics of the two strains. In this study, we examined the HA activity and electrophoretic mobility of E proteins derived from SA(V)- and SA(A)-infected mouse brains and demonstrated differences in the properties of these two E proteins.

Abbreviations: BSA = bovine serum albumin; FCS = foetal calf serum; HA = haemagglutination; HBSS = Hanks' balanced salt solution; ic = intracerebral; JE = Japanese encephalitis; MEM = Eagle's Minimum Essential Medium; PAGE = polyacrylamide gel electrophoresis; PHK = primary hamster kidney; p.i. = post inoculation; sc = subcutaneous; SDS = sodium dodecyl sulfate; TPB = tryptose phosphate broth region of the SA(A) virus genome.

BHK-21 cells were grown at 37 °C in Eagle's Minimum Essential Medium (MEM) supplemented with 5% foetal calf serum (FCS) and 0.3% tryptose phosphate broth (TPB). C6/36 cells were grown at 28 °C in MEM supplemented with 10% FCS and seven non-essential amino acids at a concentration of 0.2 mmol/l each (Igarashi, 1978). The virulent JE virus strain SA14 and its attenuated vaccine strain SA14-14-2 were obtained from Dr. Y.X. Yu, the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, People's Republic of China.

Both SA14 and SA14-14-2 strains were plaque purified in BHK-21 cells and then passaged three times in C6/36 cells. The strains derived from SA14 and SA14-14-2 were designated SA(V) and SA(A), respectively, and were studied here.

Two to three day-old newborn mice (ICR strain) were inoculated ic with 10^6 PFU of SA(V) or SA(A) strains in 0.02 ml of Hanks' balanced salt solution (HBSS) containing 0.5% bovine serum albumin (BSA). Brains of infected mice were homogenized (10% w/v) with HBSS containing 0.5% BSA, centrifuged, and the supernatants were collected. The latter were assayed for virus infectivity by plaque test in BHK-21 cells (Aihara *et al.*, 1991).

HA antigen was prepared from SA(V)- or SA(A)-infected newborn mouse brains by sucrose-acetone extraction. HA test was performed according to the protocol of Clarke and Casals (1958) using goose erythrocytes at pH from 6.0 to 7.0.

Western blot analysis was performed using an E-specific rabbit antiserum. Sucrose-acetone extracts from SA(V)- or SA(A)-infected mouse brains were separated by SDS-8% PAGE (Laemmli, 1970) and blotted onto nitrocellulose membranes (Towbin *et al.*, 1979). The latter were incubated overnight at 4 °C with 1:1000 dilution of rabbit antiserum against JE virus E protein. This antiserum was prepared by repeated sc injection of E protein recovered from SDS-PAGE. The membranes were washed with PBS containing 0.1% Tween 20, incubated for 1 hr at room temperature with biotinylated anti-rabbit IgG (Vectastain), then washed and incubated for 1 hr at room temperature with Vectastain ABC reagent. After washing, the membranes were stained with diaminobenzidine and hydrogen peroxide.

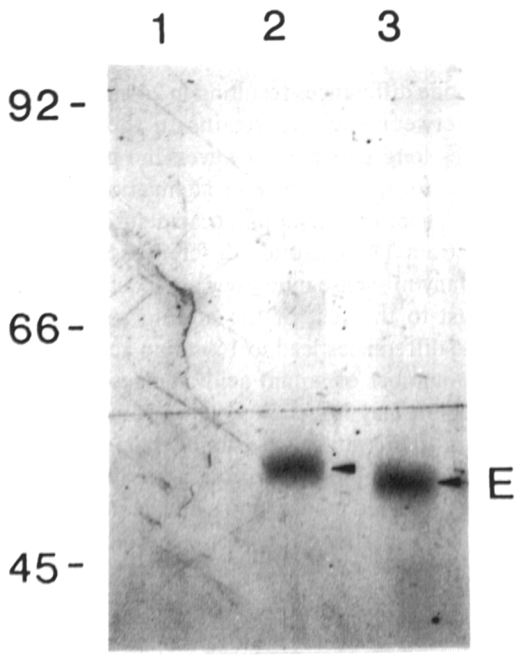

Fig. 1
Western blot analysis of SA(V) and SA(A) E proteins
Mock - (lane 1), SA(A) - (lane 2), and SA(V) - (lane 3) infected mouse brain preparations were separated by SDS-8% PAGE and probed with antiserum against JE virus E protein. E proteins (E, arrows) and molecular size markers (M_r in K) are indicated.

Table 1. Virus infectivity and HA activity in brains of mice infected with SA(V) or SA(A) virus strains

Virus strain	Infectivity (log PFU/ml)	HA	
		Titer[a]	Optimum pH
SA(V)[b]	7.0	160	6.7
SA(A)[c]	7.0	20	6.3 - 6.7

[a]HA titers are expressed as reciprocals of the highest positive dilutions of virus materials. HA reaction was performed at a pH range from 6.0 - 7.0.
[b]Virus material obtained from brains of mice 1 day p.i.
[c]Virus material obtained from brains of mice 6 days p.i.

Newborn mice were inoculated ic with 10^6 PFU of SA(V) or SA(A) strain. The SA(V) infectivity titers increased rapidly after inoculation and reached values of 10^{10} PFU/g in newborn mouse brains already on day 3 before death. In contrast, SA(A) grew much more slowly than SA(V). The virus peak titer of the SA(A) strain was about 100-fold lower (10^8 PFU/g) and was obtained much later (day 6) in the newborn mouse brains before death (data not shown). In another type of experiment we attempted to determine the relationship of the infectivity to the HA activity for the SA(V) and SA(A) strains.

Table 1 shows the virus infectivity and HA activity in the newborn mouse brains infected with SA(V) or SA(A) on day 1 or 6 post ic inoculation, respectively. The virus infectivity titers of both SA(V) and SA(A) preparations were 10^7 PFU/ml. The HA test showed that SA(V) produced a titer of 160 at the optimum pH 6.7, while SA(A) titer was 20 in the broad pH range 6.3 – 6.7. The HA titer of SA(A) was 8-fold lower than that of SA(V), though the viral infectivity of both was the same in *in vitro* assay. SA(A) showed lower HA activity with broader optimum pH range than SA(V).

The relative E protein contents in both preparations were then estimated by Western blot analysis using an E-specific rabbit antiserum. The antiserum was found to react equally well with both SA(V) and SA(A) E proteins. The E level of SA(A) was the same as that of SA(V), as shown in Fig. 1. This was confirmed by densitometric scanning. Fig. 1 showed also that in the SA(V) preparation there was a band of 51 K (lane 3), whereas in the SA(A) preparation there was a band of 52 K (lane 2). Thus, the mobility in SDS-PAGE of the SA(A) E protein was slower than that of SA(V).

Previously, we reported the comparison of genome sequences of the strains SA(V) and SA(A) (Aihara *et al.*, 1991). The amino acid changes in the E protein of the strains SA(V) and SA(A) were found at positions 107 (Leu to Phe), 138 (Glu to Lys), 176 (Ile to Val), 177 (Thr to Ala), 264 (Gln to His), 279 (Lys to Met), 315 (Ala to Val), and 439 (Lys to Arg) (Table 2). The amino acid changes probably led to the above described altered HA activity of SA(A)

Table 2. Amino acid differences in the E protein between the SA(V) and SA(A) virus strains

Amino acid position[a]	Amino acid residue	
	SA(V)	SA(A)
107	Leu	Phe
138	Glu	Lys
176	Ile	Val
177	Thr	Ala
264	Gln	His
279	Lys	Met
315	Ala	Val
439	Lys	Arg

[a]Position numbering starts at the first amino acid of the E protein.

as well as the slower mobility of SA(A) E protein in comparison with SA(V). No change in the single potential N-glycosylation site in the E coding region was found. Three of eight amino acid changes at positions 138 (Glu to Lys), 177 (Thr to Ala), and 279 (Lys to Met) affect the charge or hydrophobicity. These amino acid changes may alter the electrophoretic mobility of SA(A) E protein (Panyim and Chalkley, 1971; De Jong *et al.*, 1978).

The Leu to Phe substitution at position 107 occurred in flavivirus highly conserved sequence, residues 98-110. This region has been suggested to be part of the receptor binding site, based on analysis of E protein antigenic structure (Mandl *et al.*, 1989; Roehrig *et al.*, 1989). Roehrig *et al.* (1990) have also suggested spatial overlap of residues 225-249 within R2 domain into residues 98-110. The mutations of SA(A) at positions 138 and 279 are proximal to this overlapping region. The substitution at position 138 (Glu to Lys) could cause a change in alpha-helix propensity and affect the secondary structure of the E protein due to the change from a negatively to positively charged residue. The substitution of hydrophobic for hydrophilic residue (Lys to Met) at position 279 could change the beta-sheet propensity. The amino acid changes at positions 138 and 279 presumably introduce conformational change in the receptor binding site on the E protein and lead to the altered HA activity of SA(A). The predicted conformational change in the E protein may affect virus binding to the receptors on neural cells, leading to the decreased virulence or attenuation of SA(A).

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