Konishi E, Kitai Y, Tabei Y, Nishimura K, Harada S	Natural Japanese encephalitis virus infection among humans in west and east Japan shows the need to continue a vaccination program	Vaccine	28	2664－2670	2010
Nidaira, M., Taira, K., Okano, S., Shinzato, T., Morikawa, T., Tokumine, M., Asato, Y., Tada, Y., Miyagi, K., Matsuda, S., Itokazu, K., Kudaka, J., Nakamura, M., Tamanaha, K.	Survey of Japanese Encephalitis Virus in Pigs on Miyako, Ishigaki, Kume, and Yonaguni Islands in Okinawa, Japan.	Jpn. J. Infect. Dis.	62	220-224	2009
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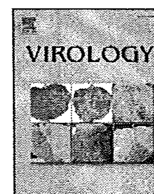

A single mutation in the Japanese encephalitis virus E protein (S123R) increases its growth rate in mouse neuroblastoma cells and its pathogenicity in mice

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ABSTRACT

We previously reported that the Japanese encephalitis virus (JEV) strain Mie/41/2002 has weak pathogenicity compared with the laboratory strain Beijing-1. To identify the determinants of its growth nature and pathogenicity, we produced intertypic viruses, rJEV(EB1-M41), rJEV(nEB1-M41) and rJEV(cEB1-M41), which contained the entire, the N-terminal, and the C-terminal half, respectively, of the Beijing-1 E region in the Mie/41/2002 background. The growth of rJEV(EB1-M41) in mouse neuroblastoma N18 cells and virulence in mice were similar to those of Beijing-1. rJEV(nEB1-M41) propagated in N18 cells to the same extent as did Beijing-1. Furthermore, we produced mutant viruses with single amino acid substitutions in the N-terminal half of the Mie/41/2002 E region. A Ser-123-Arg mutation in the Mie/41/2002 E protein exhibited significantly increased growth rate in N18 cells and virulence in mice. These results indicate that the position 123 in the E protein is responsible for determining the growth properties and pathogenicity of JEV.

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Introduction

Japanese encephalitis (JE) is a disease caused by Japanese encephalitis virus (JEV), which is transmitted to humans by mosquitoes. JEV causes serious nervous disorders encephalitis and meningitis. Approximately 1–3 of every 1000 JEV infections results in severe disease, and the fatality rate of JE is approximately 30%. Each year, 30,000–50,000 clinical cases of JE are reported with 10,000 deaths, mainly in China, South-East Asian countries, and India (Tsai, 2000). Most cases of JE occur in South, East, and South-East Asia (WHO, 1998). In recent decades, JE patients have been reported in northern area of Australia (Hanna et al., 1996). In Japan, more than 100 cases of JE were reported annually in the 1960s. After the mid-1960s, the incidence of JE has markedly decreased and less than 10 cases have been reported annually since the early 1990s. However, a high percentage of naïve pigs seroconvert to JEV every year in most regions of Japan, suggesting that JEV is still circulating in Japan. Although the reasons for the decrease in the number of JE cases after the mid-1960s are unclear, the establishment of a JE vaccination program, separation of pig farms and residential areas, and changes in rice farming procedures are likely important contributing factors.

JEV belongs to the genus *Flavivirus* within the family Flaviviridae and is now classified into five genotypes (genotype I–V) based on the sequence of its genomic RNA (Uchil and Satchidanandam, 2001; Solomon et al., 2003). JEV has a single-stranded, positive-sense RNA genome. The approximately 11-kb genome encodes three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) in one open reading frame. It also has non-translated regions (NTRs) in its 5′ and 3′ terminal ends (Lindenbach and Rice, 2001). The E (envelope) protein is the major structural protein that constitutes the surface structure of the flavivirus particles. This protein has a putative receptor-binding domain and neutralization epitopes and also plays major roles in determining viral pathogenicity by defining cell tropism and affecting penetration into susceptible cells (Lindenbach and Rice, 2001; Burke and Monath, 2001). The crystallographic structure of the E protein of a flavivirus tick-borne encephalitis virus revealed that the E protein forms head-to-tail homodimers and consists of three domains; domain I (central domain), II (dimerization domain), and III (immunoglobulin-like domain) (Rey et al., 1995).

Various approaches to clarify the molecular basis of JEV virulence have been made since the early 1990s by comparing the nucleotide sequences of virus strains with different degrees of virulence (Nitayaphan et al., 1990; Aihara et al., 1991; Cecilia and Gould, 1991; Hasegawa et al., 1992; Sumiyoshi et al., 1995; Chen et al., 1996; Ni and Barrett, 1996; Ni and Barrett, 1998; Arroyo et al., 2001; Wu et al., 2003; Lee et al., 2004; Chambers et al., 2007). Reports have suggested that some nucleotide substitutions in the E protein may correlate with the pathogenicity of JEV. A single amino acid substitution at position 138 (Glu to Lys) in the E protein is associated with attenuation of the JEV strain; this finding was demonstrated in a study using an infectious clone of JEV (Sumiyoshi et al., 1995; Zhao et al., 2005). It was also shown in a chimera of yellow fever virus and JEV that a single amino acid substitution at position 279 (Met to Lys) increases virulence in mice (Monath et al., 2002). On the other hand, recent reports showed that mutations in the 5′-NTR, C, and prM

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proteins are also critical for virus replication and pathogenesis in mice (Mori et al., 2005; Chambers et al., 2007; Kim et al., 2008).

Recently, we have isolated JEV from pigs and characterized the JEVs prevalent in Japan (Nerome et al., 2007). Of the new isolates, isolate Mie/41/2002 showed significantly weak virulence compared with that of genotype III strain Beijing-1 (Nerome et al., 2007). Genetic analysis indicated that there are some differences in the nucleotide sequence in E region between Mie/41/2002 and Beijing-1, raising the possibility that the amino acid residues in the E region of Mie/41/2002 may be related to the difference in virulence between Mie/41/2002 and Beijing-1. In the present study, we tested whether the E region of Beijing-1 can enhance the virulence of Mie/41/2002 in a mouse model by reconstituting three intertypic viruses containing the full or partial sequences of the E region of Beijing-1 and four single-missense mutant viruses in the Mie/41/2002 background. We also compared the growth properties of the viruses in vitro. Finally, we found a new molecular determinant for the growth properties and pathogenicity of JEV in the E protein.

Results

Growth properties of Mie/41/2002 and Beijing-1 in Vero, PK15 and C6/36 cells

Our previous study showed that the neurovirulence and neuroinvasiveness of Mie/41/2002 are significantly lower than those of Beijing-1 in mice (Nerome et al., 2007). This finding raises the possibility that Mie/41/2002 may replicate less efficiently than Beijing-1 in cultured cells. To further characterize the nature of Mie/41/2002 in vitro, we inoculated Vero cells, porcine kidney PK15 cells and mosquito C6/36 cells with these two strains, and the resulting plaque size and growth kinetics were compared. The plaque size of Mie/41/2002 was larger than that of Beijing-1 in Vero and PK15 cells (Fig. 1A and Table 1). Replication of Mie/41/2002 was faster than that of Beijing-1 in C6/36 cells (Fig. 1B). These results indicate that the ability of Mie/41/2002 to replicate in Vero, PK15 and C6/36 cells may be higher than that of Beijing-1 and that the virulence of JEV in mice is not necessarily correlated with its ability to replicate in these cell lines.

Growth properties of a intertypic JEV rJEV(EB1-M41) in Vero and N18 cells

It has been reported that the E protein is associated with the replication and virulence of JEV. Therefore, we hypothesized that the E region is involved in the different effects of Mie/41/2002 and Beijing-1. We compared the amino acid sequences of the E region of these two strains (Table 2). The identity of the amino acid sequence between these strains was 98.4%. Eight amino acids (positions 123, 129, 222, 227, 327, 366, 397, and 473) were different between Mie/41/2002 and Beijing-1. To investigate whether the differences in the amino acid sequences affect the growth properties of the JEV, we produced a recombinant intertypic JEV strain, rJEV(EB1-M41), which has the entire E region of Beijing-1 in the backbone of the Mie/41/2002 genome, as described in Materials and Methods. Plaque morphology and the growth rate of the recombinant virus in Vero cells were compared with those of Mie/41/2002 and Beijing-1. The plaques formed by rJEV(EB1-M41) were smaller than those formed by Mie/41/2002 and similar to those formed by Beijing-1 (Table 1). The growth kinetics of Mie/41/2002 was clearly faster than those of Beijing-1, and the kinetics of rJEV(EB1-M41) was between those of Mie/41/2002 and Beijing-1 (Fig. 2A). These data suggest that the E region of Beijing-1 is associated with small plaque size and is partially related to the slower growth rate of Beijing-1 in Vero cells.

Next, we examined the growth properties of the three JEV strains in mouse neuroblastoma-derived N18 cells. In contrast to the results in

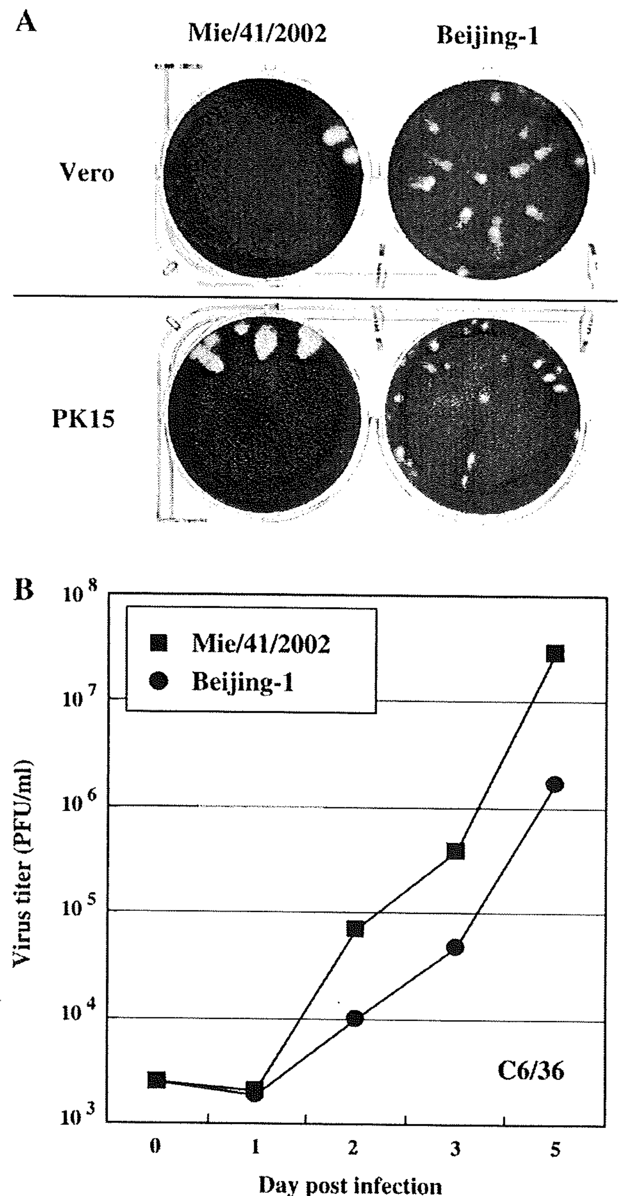

Fig. 1. (A) Plaque phenotypes of JEV Mie/41/2002 and Beijing-1 in Vero and porcine kidney PK15 cells. (B) Growth curves of Mie/41/2002 and Beijing-1 in mosquito C6/36 cells.

Vero cells, the growth rate of Beijing-1 was slightly faster than that of Mie/41/2002, and the steady state level of the number of infectious particles of Beijing-1 was significantly higher than that of Mie/41/2002 (Fig. 2B). Furthermore, the growth curve of rJEV(EB1-M41) in N18 cells was nearly equal to that of Beijing-1. These data suggest that Mie/41/2002 and Beijing-1 have different cell tropism and that the E region of Beijing-1 is involved in the nerve cell-tropic nature of the virus.

Comparison of the virulence of Mie/41/2002, Beijing-1, and rJEV (EB1-M41) in mice

To determine whether the E region of Beijing-1 is related to its virulence in vivo, mice were infected intraperitoneally with Mie/41/2002, Beijing-1, and rJEV(EB1-M41) and observed for 3 weeks (Table 3, experiment 1). We assessed the neuroinvasiveness by determining the ability of the viruses to replicate in peripheral tissues, invade the central nerve system, and cause encephalitis. Nine of the 10 mice infected with Beijing-1 had died, whereas 3 of the 10 mice

Table 1
Plaque size of recombinant JEVs in Vero cells.

Strain	Mean plaque size (mm) ± standard error[a]	P value (vs. Mie/41/2002)[b]	P value (vs. Beijing-1)[c]
Mie/41/2002	1.83 ± 0.04	–	<0.0001*
Beijing-1	0.92 ± 0.03	<0.0001*	–
rJEV(EB1-M41)	0.98 ± 0.03	<0.0001*	0.12
rJEV(nEB1-M41)	1.03 ± 0.04	<0.0001*	0.03*
rJEV(cEB1-M41)	1.78 ± 0.04	0.24	<0.0001*
rJEV(E123B1-M41)	1.03 ± 0.05	<0.0001*	0.04*
rJEV(E129B1-M41)	1.8 ± 0.04	0.31	<0.0001*
rJEV(E222B1-M41)	1.85 ± 0.04	0.37	<0.0001*
rJEV(E227B1-M41)	1.86 ± 0.03	0.27	<0.0001*

[a] Plaque diameters calculated for 15 plaques.
[b] P value relative to Mie/41/2002 by Welch's t-test. Asterisks indicate statistical significance.
[c] P value relative to Beijing-1 by Welch's t-test. Asterisks indicate statistical significance.

infected with Mie/41/2002 had died. Eight of the 10 mice inoculated with rJEV(EB1-M41) had died by 2 weeks after challenge, resembling the Beijing-1-inoculated group. This result suggests that the E protein contributes to the difference in virulence observed between the Mie/41/2002 and Beijing-1 strains in vivo and that there is a correlation between growth characteristics of JEV in N18 and virulence in vivo.

Effect of four single-missense mutations in E protein on the nature of Mie/41/2002 in vitro

Our results suggest that one or more of the eight amino acid sequence variances in the E region are associated with the growth properties of Mie/41/2002. To define the amino acid positions responsible for the virulence and growth properties, we produced two new intertypic recombinant viruses, rJEV(nEB1-M41) and rJEV (cEB1-M41), which have the N-terminal half (1–268) and the C-terminal half (269–500), respectively, of the E region of Beijing-1 in a Mie/41/2002 background (Table 2). We examined the growth properties of these viruses in Vero and N18 cells. In Vero cells, the plaques formed by rJEV(nEB1-M41) were clearly smaller than those formed by Mie/41/2002 and rJEV(cEB1-M41) but were slightly larger than those of Beijing-1 (Table 1). The growth kinetics of rJEV(nEB1-M41) and rJEV(cEB1-M41) in Vero cells were comparable to those of rJEV(EB1-M41) and Mie/41/2002, respectively (Fig. 3A). The growth rate of rJEV(nEB1-M41) was equivalent to that of Beijing-1 and rJEV (EB1-M41) in N18 cells (Fig. 3B). rJEV(cEB1-M41) and Mie/41/2002 showed similar growth rates in N18 cells, although the steady state level of the number of infectious particles of rJEV(cEB1-M41) was slightly lower as compared to that of Mie/41/2002. Our results suggest that the N-terminal half of the E region is responsible for the difference in the growth properties between Mie/41/2002 and Beijing-1.

To determine the amino acid in the E protein that is responsible for the Beijing-1-like phenotype, we produced four additional recombinant viruses, rJEV(E123B1-M41), rJEV(E129B1-M41), rJEV(E222B-M41) and rJEV(E227B1-M41), which each have single-missense mutations in the nE protein of Mie/41/2002 (Table 2). We examined the plaque size and growth characteristics of these viruses in vitro. In Vero cells, the mutant virus rJEV(E123B1-M41) formed smaller plaques than did Mie/41/2002 but its plaques were slightly larger than those of Beijing-1 (Table 1). The plaque size was similar than that of rJEV(nEB1-M41) (Table 1). However, growth kinetics analysis showed that the Ser-123-Arg (S123R) mutation did not affect the growth rate of Mie/41/2002 in Vero cells (Fig. 4A). In contrast, rJEV (E227B1-M41) grew slightly slower than Mie/41/2002 and the other three missense mutants, although the plaque morphology of rJEV (E227B1-M41) was similar to that of Mie/41/2002. Our data suggest that two different amino acid substitutions, S123R and Ser-227-Pro

Table 2
Difference of amino acid residues in the E region between Mie/41/2002 and Beijing-1.

Amino acid position in the E region	nE region[a]				cE region[b]			
	123	129	222	227	327	366	397	473
Mie/41/2002	S	M	S	S	T	S	H	V
Beijing-1	R	T	A	P	S	A	Y	I

[a] From amino acid positions 1–268 in the E region.
[b] From amino acid positions 269–500 in the E region.

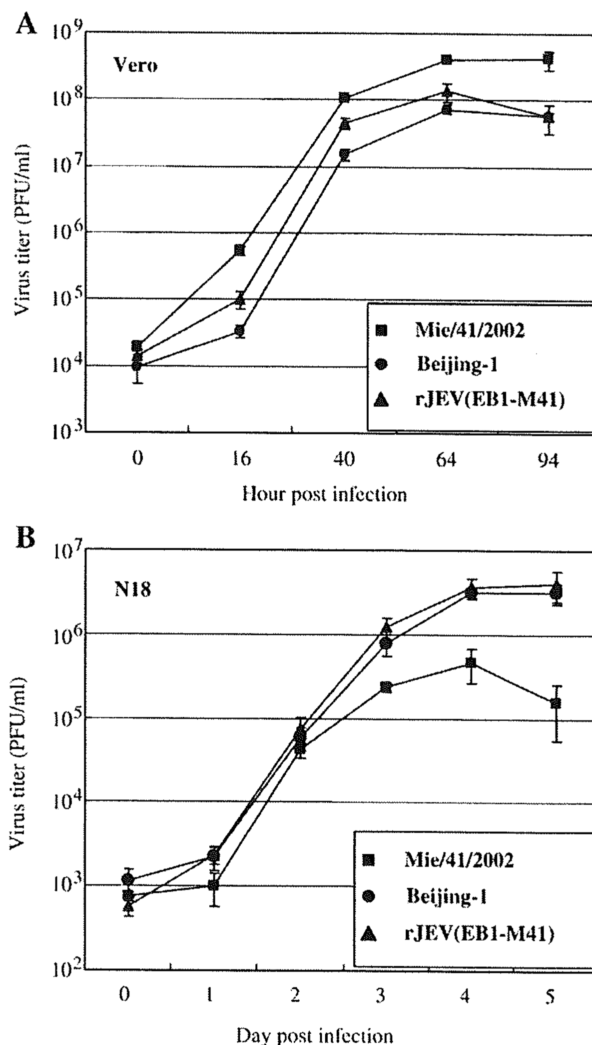

Fig. 2. Growth curves of Mie/41/2002, Beijing-1, and intertypic recombinant JEV rJEV (EB1-M41) in Vero cells (A) and in N18 cells (B). Values represent the mean and standard deviation (SD) for three independent tests.

Table 3
Mouse neuroinvasiveness of recombinant JEVs.

Virus	Experiment 1		Experiment 2		Experiment 3	
	Survival[a]	P value[b]	Survival[a]	P value[b]	Survival[a]	P value[b]
Mie/41/2002	7/10	–	9/10	–	7/10	–
Beijing-1	1/10	0.012*	0/10	<0.0001*	0/10	<0.0001*
rJEV(EB1-M41)	2/10	0.051				
rJEV(E123B1-M41)			3/10	0.004*	1/10	0.002*
rJEV(E129B1-M41)			9/10	0.97	7/10	0.86
rJEV(E222B1-M41)			9/10	1.00	6/10	0.48
rJEV(E227B1-M41)			7/10	0.27	7/10	0.85

[a] No. of mice surviving/no. of mice inoculated.
[b] P value relative to Mie/41/2002 by log-rank (Mantel–Cox) test. Asterisks indicate statistical significance.

(S227P), are independently related to the reduced plaque size and growth rate, respectively, in Vero cells.

In N18 cells, rJEV(E123B1-M41) and Beijing-1 had a similar growth curve; however, the growth kinetics of rJEV(E123B1-M41) was slightly higher than that of Beijing-1 (Fig. 4B). Mie/41/2002 and the other three recombinant viruses had similar growth patterns. These results suggest that only the S123R mutation increases the growth rate of Mie/41/2002 to the level of Beijing-1 in N18 cells and that Arg at position 123 in the E protein is a key factor in the nerve cell-tropic nature of Beijing-1.

Effect of missense mutations in E protein on the virulence of Mie/41/2002 in mice

To determine whether the single-missense mutations in Mie/41/2002 enhanced the virulence of Mie/41/2002 in vivo, mice were infected intraperitoneally with Mie/41/2002, Beijing-1, and the four missense mutant viruses (Table 3, experiments 2 and 3). Only 1 and 3 of the 10 mice infected with Mie/41/2002 had died, whereas all mice infected with Beijing-1 had died. In rJEV(E123B1-M41)-infected mice, 7 and 9 of the 10 mice had died, while 1 and 3 of the 10 mice infected with rJEV(E129B1-M41), 1 and 4 of the 10 mice infected with rJEV (E222B1-M41), and 3 and 3 of the 10 mice infected with rJEV(E227B1-

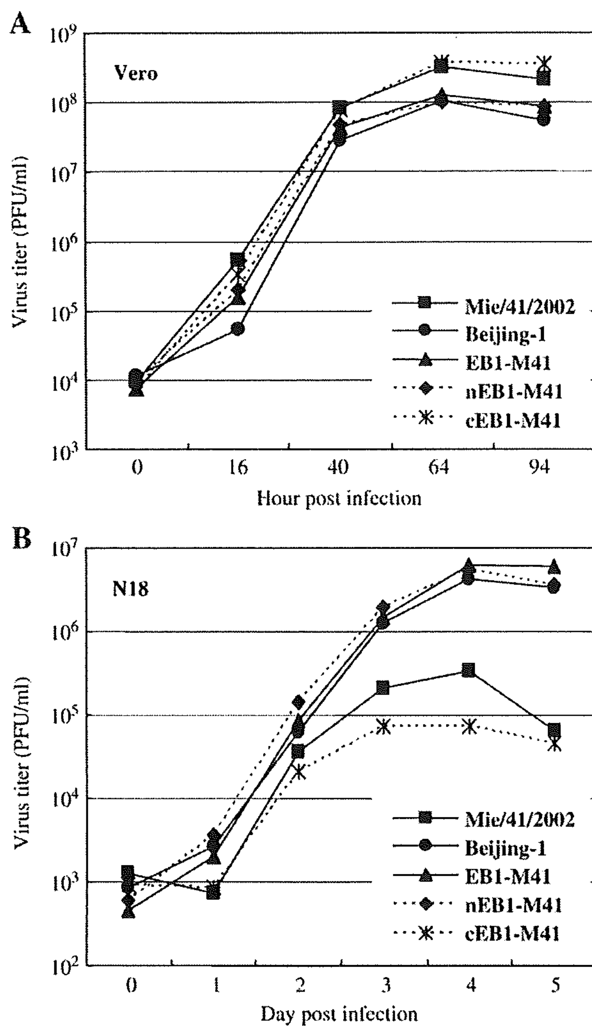

Fig. 3. Growth curves of Mie/41/2002, Beijing-1, rJEV(EB1-M41), rJEV(nEB1-M41) and rJEV(cEB1-M41) in Vero cells (A) and N18 cells (B).

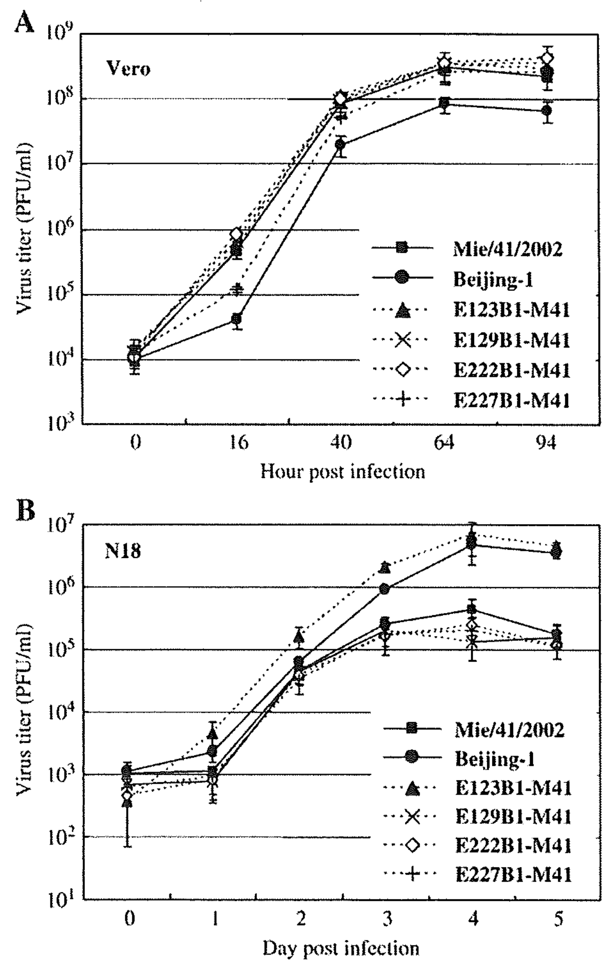

Fig. 4. Growth curves of Mie/41/2002, Beijing-1, rJEV(E123B1-M41), rJEV(E129B1-M41), rJEV(E222B1-M41), and rJEV(E227B1-M41) in Vero cells (A) and N18 cells (B). Values represent the mean and SD for three independent tests.

M41) had died. The survival curve of rJEV(E123B1-M41)-infected mice was similar to that of Beijing-1 (data not shown). This result suggests that the S123R mutation significantly enhances the virulence of Mie/41/2002 in vivo, indicating that the amino acid at position 123 in the E protein is responsible for determining the virulence of JEV in vivo.

Discussion

We previously reported that the JEV Mie/41/2002 strain has significantly weak virulence compared to that of the Beijing-1 strain (Nerome et al., 2007). In this paper we have attempted to identify the amino acid in the E protein that is responsible for the growth properties and pathogenicity of JEV. Our results showed that the virulence of Mie/41/2002 was increased by an amino acid substitution at position 123 (S123R). Previous reports have suggested many candidate sites in JEV that are involved in the attenuation of its virulence (Nitayaphan et al., 1990; Aihara et al., 1991; Cecilia and Gould, 1991; Hasegawa et al., 1992; Sumiyoshi et al., 1995; Chen et al., 1996, Ni and Barrett, 1996; Ni and Barrett, 1998; Arroyo et al., 2001; Wu et al., 2003; Lee et al., 2004; Chambers et al., 2007). However, only two sites in the E protein, 138 and 279, have been shown to be determinants of its viral pathogenicity (Sumiyoshi et al., 1995; Monath et al., 2002; Zhao et al., 2005). In the present study, we focused on the four sites – 123 (S123R), 129 (Met to Thr, M129T), 222 (Ser to Ala, S222A) and 227 (S227P) – in the E proteins. Our data demonstrate that the novel candidate position 123 is one of the molecular determinants of JEV virulence. The S123R mutation also increased the growth rate of Mie/41/2002 in mouse neuroblastoma N18 cells to the same level as observed with Beijing-1. The results of in vitro growth analysis in N18 cells were consistent with those of virulence experiment in vivo, suggesting that the increased virulence of the mutant Mie/41/2002 may attributed to increased growth activity of the virus caused by the S123R mutation in nerve cells. Previous report has indicated that virulence attenuation of JEV as a result of altered affinity for the cell surface glycosaminoglycan (GAG) occurs through at least one or two mutations in the E protein, suggesting that affinity for GAG is a key determinant for the pathogenicity of JEV (Lee et al., 2004). It is possible that the mutation S123R changes the affinity of the E protein for the surface molecule that is specifically expressed on nerve cells and that is required for attachment of JEV. On the basis of the crystallographic structure of the E protein from Tick-borne encephalitis virus and West Nile virus, the amino acid at position 123 is located in domain II, which is important for homodimerization of the E proteins (Rey et al., 1995; Kolaskar and Kulkarni-Kale, 1999; Nybakken et al., 2006). Single mutations responsible for the virulence and cell tropism of flaviviruses have been mapped on the E protein, and the sites cluster in three distinct regions: the distal face of domain III, the base of domain II, and the contact between the domain I and III (Rey et al., 1995). Position 123 is located in the second region. Amino acid substitutions in the second region are thought to influence virulence by affecting the low pH conformational transition, while mutations on the distal face of domain III are considered to influence cell attachment of flaviviruses (Rey et al., 1995; Lee et al., 2004). These findings suggest the possibility that the S123R mutation may alter the critical pH for the conformational change followed by the fusion process between the E protein and the endosomal membrane within the infected cells. Alternatively, an attenuating mutation at position 138 of the JEV E protein affects multiple steps of the viral life cycles and these changes may induce substantial attenuation of JEV (Zhao et al., 2005). Therefore, the mechanism for virulence enhancement by the S123R mutation may not be a simple process. Mutations M129T and S222A had no or weak effect on the growth properties in vitro and the virulence in mice, which suggests that these mutations may not be associated with the differences between Mie/41/2002 and Beijing-1. In Vero cells, the S227P mutant grew slightly slower than its parent Mie/41/2002, although the growth rates of the S123R, M129T, and S222A mutants were similar to that of Mie/41/2002. The growth kinetics of the S227P mutant in Vero cells was similar to those of the intertypic viruses rJEV(EB1-M41) and rJEV(nEB1-M41). These data suggest that one of the molecular determinants of efficient growth in Vero cells is the amino acid at position 227, whereas the major determinant of growth in N18 cells is the one at position 123. Interestingly, plaques induced by the S123R mutant were smaller than those of Mie/41/2002 and the other three mutants, which formed plaques of similar sizes in Vero cells. These observations imply that the plaque size of JEV is not necessarily correlated with the growth rate of the virus in Vero cells.

The Beijing-1 strain used in the study was obtained after 37 passages through suckling mouse brain and then 1 passage in Vero cells. A comparison of the amino acid sequence of this Beijing-1 strain and another Beijing-1 strain (GenBank accession L48961) (Hashimoto et al., 1988) showed two different amino acids in the E protein (positions 123 and 132), and the amino acid at position 123 of the Beijing-1 (L48961) E protein was Ser, which is the same as in Mie/41/2002. Alignment of the nucleotide sequences of JEV registered in the GenBank revealed that JEV strains with an Arg at position 123 (R123) in the E protein were the minority and that the great majority of JEV strains had a Ser residues at this position (S123) (data not shown). Flaviviruses usually exist as genetically heterogeneous populations and a specific variant may be easily selected according to the cells used for passage (Ni and Barrett, 1998; Wu et al., 2003; Chiou et al., 2005). It is possible that the R123 strain is suitable for replication of JEV in mouse nerve cells, and, therefore, had been selected for passages in suckling mouse brain. However, JEV strain GSS, which was isolated from the brain of a JE patient in China has an Arg at position 123 in the E protein, suggesting that the R123 type of JEV might be circulating in nature.

In the present study, we established a system for the production of recombinant JEV. Full-length infectious clones of flaviviruses have been used as powerful tools for studying replication, pathogenesis, and vaccine development. Several groups have already constructed full-length infectious clones for JEV (Sumiyoshi et al., 1992; Zhang et al., 2001; Mishin et al., 2001; Yun et al., 2003; Zeng et al., 2005; Zhao et al., 2005; Chambers et al., 2007; Liang et al., 2009). All strains used for the construction of the clones were genotype III JEV, whereas we used genotype I JEV Mie/41/2002. The major genotype of JEV isolated in Japan changed from genotype III to genotype I in the early 1990s (Ma et al., 2003; Yoshida et al., 2005). It has also been reported that a similar genotype shift occurred in Korea (Nam et al., 1996; Yang et al., 2004), northern Vietnam (Nga et al., 2004) and Thailand (Nitatpattana et al., 2008). In China, most JEV isolates were genotype III before 2001. However, in recent years, genotype I JEV has frequently been isolated in some areas in China (Wang et al., 2007; Zhang et al., 2009). These findings suggest that JEV genotype III has been replaced by genotype I in East and Southeast Asia. Thus, the findings in the present study are important for understanding the virulence of currently circulating genotype I JEV. Our infectious clones will be useful for studying growth properties and pathogenesis of genotype I JEV.

Materials and methods

Cell culture

Vero cells (9013 and NIBSC strains), porcine kidney PK15 cells and mosquito C6/36 cells were cultured at 37 °C for Vero and PK15 and 28 °C for C6/36, in 5% CO_2 in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and 100 U penicillin–streptomycin/ml. Mouse neuroblastoma N18 cells were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 100 U penicillin–streptomycin/ml.

Table 4
Primers used for construction of wild-type (Mie/41/2002) and mutant JEVcDNA clones.

Direction	Primer	Sequence (5′-3′)[a]
Forward (sense)	JEV.NotI-T7	GATCGCGGCCGC*TAATACGACTCACTATAG*AGAAG
	JEV.T7-5N	*TAATACGACTCACTATAG*AGAAGTTTATCTGTGTGAACTTC
	JEV.NotI-4665f	GATCGCGGCCGCCACAGGAGTTTACCGAATCATG
	JEV.6311f	CACGCACAAACGCTATACTAG
	JEV.8906f	CTCTCGGAGCAGTGTTCGCTG
	E123mF	GGAAGAATGATCCAACCAGAG
	E129mF	CCAACCAGAGAACATCAAGTACG
	E222mF	TCCCTGGACGTCCCCCTCAAG
	E227mF	CGCCCCCCTCAAGCACGGCATG
Reverse (antisense)	JEV.4786r	TCATGATGGCTGCTCCTCTAG
	JEV.7334r	GTTCTTCTCTGAGCAGCTCTG
	JEV.9418r	GACCACTTTGTGCCTGTACG
	JEV.BamHI-NsiI-3N	GCTGGATCCATGCATAGATCCTGTGTTCTTCCTCAC
	E123mR	AAT GGC CTT CCT GGT ACA AGA
	E129mR	ATTGTTCTTCCAATGGCCTTCG
	E222mR	AGAGCAAGGTCATGGAACCATTC
	E227mR	TCCAGGGAAGAGAAAGGTCATGG

[a] T7 polymerase promoter sequences in JEV.NotI-T7 and JEV.T7-5N primers are shown in *italic*. Nucleotides that are different from those of original Mie/41/2002 are indicated with underlines.

Viruses

Mie/41/2002 (GenBank accession AB241119) was isolated in Mie prefecture, Japan, in 2002 from swine serum and the virus was propagated in Vero cells (Nerome et al., 2007). Beijing-1 (accession AB510530) was obtained after 37 passages through suckling mouse brain and then 1 passage in Vero cells (NIBSC).

Production of recombinant JEV

RNA from JEV Mie/41/2002 was extracted from the culture supernatant fluid by use of High Pure Viral RNA Kit (Roche Diagnostics), and it was used for the synthesis of viral cDNA using the SuperScript III Reverse Transcriptase (Invitrogen). Primers used for construction of the recombinant clone are listed in Table 4. The 5′ terminal-NS3 region of the JEV genome (region 1) was amplified with primers JEV.T7-5N and JEV.4786r for the first PCR and primers JEV.NotI-T7 and JEV.4786r for the second PCR. The primers JEV.T7-5N and JEV.NotI-T7 contain the complete T7 polymerase promoter sequence (TAATACGACTCACTATAG). The NS2B-NS4B region of the JEV genome (region 2) was amplified with the primers JEV.NotI-4665f and JEV.7334r. The NS3–NS5 region of the JEV genome (region 3) was amplified with the primers JEV.6311f and JEV.9418r, and the NS5-3′ terminal region of the JEV genome (region 4) was amplified with the primers JEV.8906f and JEV.BamHI-NsiI-3N. All PCR reactions were done using a thermostable high-fidelity DNA polymerase KOD-plus (Toyobo). The PCR product of the region 2 was first subcloned into the low-copy-number plasmid pMW119 (Nippon Gene) at an EcoRI–KpnI site (M41R2/pMW119) using competent-cell Stbl2 (Invitrogen). In this process the EcoRI site in the pMW119 plasmid was disrupted. The PCR fragment of the region 1 was subcloned into NotI–EcoRI site of M41R2/pMW119 (M41R12/pMW119), and then the region 4 fragment was subcloned into KpnI–BamHI site of M41R12/pMW119 (M41R124/pMW119). The complete JEV clone (rJEV(Mie/41/2002)/pMW119) was constructed by insertion of the region 3 into KpnI site of M41R124/pMW119. The nucleotide sequence of the viral genome region of the recombinant clones were checked after amplification of the plasmids in *Eschrichia coli*. To construct the molecular clones of the intertypic viruses rJEV(EB1-M41), rJEV(nEB1-M41) and rJEV(cEB1-M41), the E region (MluI-AgeI region), nE region (N-terminal side of E region) and cE region (C-terminal side of E region), respectively, of rJEV(Mie/41/2002)/pMW119 were replaced with the corresponding region of Beijing-1(smb37v1).

To construct four clones of missense mutant viruses rJEV(E123B1-M41), rJEV(E129B1-M41), rJEV(E222B1-M41) and rJEV(E227B1-M41), single-missense mutations (S123R, M129T, S222A and S227P) were introduced into rJEV(Mie/41/2002)/pMW119 by inverse PCR-based site-directed mutagenesis (Tajima et al., 2006) by use of the following primer sets: E123mF and E123mR for S123R; E129mF and E129mR for M129T; E222mF and E222mR for S222A; and E227mF and E227mR for S227P (Table 4). The rJEV clones were digested at the 3′ end of viral genome with NsiI, and the linearized DNA was transcribed by using the mMASSAGE mMACHINE T7 kit (Invitrogen). Recombinant viruses were recovered by transfection with in vitro-transcribed RNA into Vero cells as described previously (Tajima et al., 2006).

Analysis of growth kinetics and plaque size

For the growth kinetics analysis cells were plated into 6-well culture plate (3×10^5 for Vero and 6×10^5 for N18 and C6/36 cells) and infected with original and mutant JEVs at a multiplicity of infection of 0.1 (Vero cells) or 0.01 (N18 and C6/36 cells) plaque forming units (PFU)/cell. Small aliquots of the media were recovered periodically, and the titer of the aliquots was determined by a plaque assay on Vero cells grown in 12-well culture plates. To evaluate the plaque size, Vero and PK-15 cells (3×10^5) were plated in six-well plates and inoculated with the viruses. Five days after inoculation, cells were fixed with a 3.7% (v/v) formaldehyde solution in phosphate-buffer saline for 1 h, then the methylcellulose overlay was removed and the cells were stained with methylene blue solution for 2 h. The diameters of 15 plaques were measured and the mean plaque size in mm + standard error was calculated. Differences in mean plaque sizes were analyzed using Welch's *t*-test.

Mouse challenge

Female ddY mice (3 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were maintained in a specific-pathogen-free environment. Groups of mice ($n=10$) were intraperitoneally inoculated with 1×10^4 PFU (100 μl) of recombinant virus solution diluted with 0.9% NaCl solution. The mice were observed for 3 weeks after inoculation to determine survival rates. All experiments were conducted in accordance with the Fundamental Rules for Animal Experiments of our institute. Survival curve comparisons were performed using Prism software (GraphPad software) statistical analysis that uses the log-rank (Mantel–Cox) test.

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Prevalence of Antibodies to Japanese Encephalitis Virus among Inhabitants in Java Island, Indonesia, with a Small Pig Population

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Abstract. Japanese encephalitis virus (JEV) is maintained through a transmission cycle between amplifier swine and vector mosquitoes in a peridomestic environment. Thus, studies on natural JEV activities in an environment with a small size of pig population have been limited. Here, we surveyed antibodies against JEV in inhabitants of Jakarta and Surabaya located in Java Island (Indonesia), which has a small swine population. Overall, 2.2% of 1,211 sera collected in Jakarta and 1.8% of 1,751 sera collected in Surabaya had neutralizing antibody titers of ≥ 1:160 (90% plaque reduction). All the samples with titers of ≥ 1:160 against JEV were also examined for neutralizing antibodies against each of four dengue viruses to confirm that JEV antibody prevalences obtained in the present survey were not attributable to serologic cross-reactivities among flaviviruses distributed in Java. These results indicated that people in Java Island are exposed to natural JEV infections despite a small swine population.

INTRODUCTION

Japanese encephalitis (JE) is the most important cause of viral encephalitis in Asia.[1] Although the majority of humans infected with JE virus are asymptomatic, some develop acute encephalitis at a ratio of subclinical to clinical infections in the range of 25:1 to 1,000:1.[2] Mortality is approximately 20% and half of the survivors have severe neuropsychiatric sequelae. Fifty thousand cases are reported annually with one billion people at risk of infection. Japanese encephalitis is a vaccine-preventable disease. The number of patients dramatically reduced after introduction of vaccination in some countries, including Japan, South Korea, and Taiwan.[3] Therefore, most of the patients currently occur in tropical and subtropical regions of Asia.

Swine are a major amplifying host of Japanese encephalitis virus (JEV) in a peridomestic environment. Mosquitoes acquire the virus from viremic swine and may transmit it to humans.[4,5] In most swine-raising countries in Asia, antibodies against JEV have been detected in humans and animals, indicating active JEV circulation in nature, for instance as reported in India[6] and Nepal.[7] This is also the case even in countries where JE is controlled by vaccination in humans: recent surveys in Korea revealed prevalences of 12.1% in goats[8] and 51.3% in cattle.[9] However, relatively high antibody prevalences in animals were also reported in Singapore,[10,11] where pig farming has been eliminated and human cases have been rare since the early 1990s.[12]

Java, one of the main islands of the Indonesian archipelago, constitutes an environment with a small size of pig population: it is difficult to raise pigs in this island because of the religious precepts and laws of the Muslim community. Consistent with the small swine population, laboratory-confirmed JE patients are rare in Java Island, if any.[13] However, studies on natural JEV activities, though limited, reported virus isolation from mosquitoes[14–18] and swine[15] in and around Jakarta, West Java. Some of these studies also determined *Culex tritaeniorhynchus* and *Culex gelidus* as the predominant vectors.[17,18]

Antibody surveys have also been limited in Java Island. In contrast to the small swine population, relatively high prevalence rates have been reported: for humans 8.4% in Madjalengka, West Java[19] and approximately 10%[20] or 2%[21] in Surabaya, East Java; for pigs approximately 90% at a slaughterhouse in Jakarta[22]; and for horses 50% near Jakarta.[23] However, the antibody titers in these studies were determined mainly by a hemagglutination-inhibiting (HAI) test that shows high serologic cross-reactions against dengue viruses, other flavivirus members distributed in Indonesia. Although some studies[21,23] used neutralization tests, which are more specific than HAI tests for differentiation from anti-dengue antibodies, the cross-reactivity between JEV and each of four dengue viruses in their neutralization test systems, and neutralizing antibody titers against dengue viruses in samples positive for antibodies to JEV, were not seen. Even using neutralization tests, it is highly probable that significant levels of cross-reactivity against dengue viruses may cause false-positive results for JEV antibodies, particularly in areas where dengue is highly endemic, like Indonesia.[24]

The present study was carried out to survey antibodies to JEV in inhabitants of Jakarta and Surabaya. A total of 2,962 sera were examined for JEV antibodies by a neutralization test. To eliminate potential false-positive results in the neutralization test because of serologic cross-reactivities against dengue viruses, all the samples found positive for neutralizing antibodies against JEV were examined for neutralizing antibodies against any of four types of dengue viruses. The results indicated that 2.2% and 1.8% of populations in Jakarta and Surabaya, respectively, had neutralizing antibody titers of ≥ 1:160 in a 90% plaque reduction assay, indicating that people were exposed to natural JEV infections in Java Island, an area with a small swine population.

MATERIALS AND METHODS

Study subjects. All the sera used in this survey were also used in our earlier surveys of antibodies to a different infectious agent.[25,26] A total of 1,211 serum samples were collected from patients at general practitioners and hospitals

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in Jakarta in 2001, and 1,751 sera at the Emergency Unit of Doctor Soetomo Hospital in Surabaya from 1999 to 2000. The survey populations were mostly the inhabitants of Jakarta or Surabaya City and were randomly selected without any bias relating to JE. The Jakarta City had a population of 7,423,379 people in 661.52 km^2 in 2001,[27] whereas the Surabaya City had a population of 2,709,334 people in 326.36 km^2 in 2000[28]; thus, the present survey populations corresponded to 0.016% and 0.065% of the total populations of Jakarta and Surabaya, respectively. The survey subjects ranged from 20 to 85 years of age in the Jakarta samples and from 0 to 100 years of age in the Surabaya samples. Mean ages (±standard deviation) in the Jakarta samples were 39.9 (±12.3) years in males, 42.3 (±14.1) years in females, and 41.2 (±13.4) years in total, whereas those in the Surabaya samples were 44.7 (±21.2) years in males, 45.1 (±19.6) years in females, and 44.9 (±20.4) years in total: there were no significant differences between genders ($P > 0.05$ by the Student's two-sample t test) in both samples (see Tables 3 and 4 for age and gender compositions). The gender (male: female) compositions in the present survey populations were 46%:54% in Jakarta and 52%:48% in Surabaya, whereas those of the general populations were 52%:48% in Jakarta and 50%:50% in Surabaya. Serum samples from babies < 6 months of age, which may contain maternally transferred antibodies, were not used in this survey. For transportation of sera at room temperature from Indonesia to Japan, sodium azide was added to the serum samples at a final concentration of 0.1%. The study protocol was reviewed and approved by the Ethical Committee of Kobe University Graduate School of Medicine (Ethical Committee Approval Number 561).

Antibodies. Hyperimmune mouse ascitic fluids (HMAFs) against the Nakayama strain of JEV, the Mochizuki strain of dengue type 1 virus (DENV1), the New Guinea C (NGC) strain of dengue type 2 virus (DENV2), the H87 strain of dengue type 3 virus (DENV3), and the H241 strain of dengue type 4 virus (DENV4) have been described previously.[29] Briefly, these were collected from adult ICR mice, which were repeatedly immunized with each of the JEV and DENV1–4 in a form of 10% homogenate of suckling mouse brain, followed by the inoculation with sarcoma 180 cells.

Serology. Neutralizing antibodies were titrated using plaque reduction assays performed with the Nakayama strain of JEV in the absence of complement, as previously described.[30] Briefly, 2-fold serial dilutions of test specimens starting from 1:10 were mixed with JEV and incubated overnight on ice. The antibody-virus mixture was then titrated on Vero cells. The neutralization titer was expressed as the maximum serum dilution yielding a 90% reduction in plaque number. For testing human sera, the serum dilution started from 1:160, because sodium azide contained in the sera affected most of the Vero cell monolayers at serum dilutions of ≤ 1:80. Antibodies to each of DENV1–4 were titrated by neutralization tests, as described previously for JEV antibody titration, except for the inclusion of rabbit complement (Low-Tox-M Rabbit Complement; Cedarlane, Hornby, Canada) in the virus-antibody mixture at a final concentration of 5% and the use of immunochemical staining to count foci. The viruses used for the tests were DENV1 (Mochizuki), DENV2 (NGC), DENV3 (H87), and DENV4 (H241).[29] The addition of complement usually showed 4- to 8-fold increases in neutralizing antibody titer in our test system. For immunostaining, cells were fixed, blocked with phosphate-buffered saline (PBS) containing normal horse serum at 1%, and then incubated serially with HMAF specific for each dengue virus, biotinylated anti-mouse IgG, the avidin-biotin complex (ABC) reagents, and the VIP substrate (Vector Laboratories, Burlingame, CA).

Statistical analysis. The significance of differences in antibody prevalence was evaluated by the χ^2 test with the Yates' correction factor. Significance of difference in geometric mean neutralizing antibody titers was evaluated by the Student's two-sample t test. Probability levels (P) of < 0.05 were considered significant.

RESULTS

Pig populations. The pig populations in Java Island obtained from statistics from 1990 to 2006, available in literature,[31–34] were 126,000–432,000 heads with an average of 232,353 per year; calculated to be 0.99–3.39 with an average of 1.82 heads/km^2 as adjusted by the land area. These numbers were considerably smaller than the corresponding numbers in a pig-raising area in Indonesia (Bali Island, neighboring Java)[31–34] and another country (Japan).[35] Specifically, the average pig populations and their densities were, respectively, 965,294 heads and 171.36 heads/km^2 in Bali Island and 10,222,062 heads and 27.05 heads/km^2 in Japan.

Levels of cross-neutralizing antibodies. Our neutralization test system showed small levels of cross-reactivity among JEV and DENV1–4, as previously described.[29] Specifically, HMAF against each of DENV1–4 showed neutralizing antibody titers of 1:5,120, or 1:10,240 against the homologous virus, but ≤ 1:10 against JEV, indicating that the cross-neutralization titers were ≤ 1/512 of the specific titer. More specifically, HMAF against DENV1 showed titers of 1:5,120 against DENV1 and < 1:10 against JEV, indicating a difference of > 512-fold. Similarly, 512-, > 1,024-, and 512-fold differences were shown between homologous virus and JEV in titers of HMAFs against DENV2, DENV3, and DENV4, respectively. Although all the neutralization tests for the previous comparisons were performed in the presence of complement to increase the test sensitivity,[29] we included complement only in the test for dengue antibodies but not for JEV antibodies in the present survey; thus, the difference in titers against JEV and dengue viruses seems to be larger than those described previously. Although different assay systems were used for this comparison (focus-forming method for DENV1–4 but plaque-forming method for JEV), previous experiments in our laboratory have shown that equivalent neutralizing antibody titers were obtained with both methods.[36]

To further evaluate the effect of cross-reactivity of dengue virus antibodies on neutralizing antibody titers against JEV, we examined a mixture of HMAFs against DENV1–4 for neutralizing antibodies against JEV. For this experiment, the mixture was prepared with an equal volume of HMAF and complement was included in the virus-antibody mixture. Although data are not shown, all 11 different combinations of any of the 4 HMAFs (against DENV1–4) showed undetectable antibody titers (< 1:10), suggesting that antibodies to one type of dengue virus do not synergistically increase their titers against JEV in the presence of antibodies to other type(s) of dengue virus. In addition, to examine if the presence of dengue virus antibodies would increase a titer against JEV, serial 2-fold dilutions of HMAF against JEV were mixed with the mixture of 4 HMAFs (against DENV1–4). The results showed

no increase in JEV antibody titers at any dilutions of HMAF against JEV within the range from < 1:10 to 1:160 even in the presence of complement in the neutralization test system (data not shown). Finally, we assessed the effect of HMAFs against DENV1–4 on titers of JEV antibodies included in human sera. A human serum sample that had a titer of 1:1280 against JEV (see Table 2, a male sample aged 39 years from Surabaya) was diluted 128-fold with each of HMAFs against DENV1–4 or the mixture of 4 HMAFs, as well as PBS as a control. The complement was not included in the virus-antibody mixture in this experiment. The result showed that the neutralizing antibody titer against JEV was ≤ 1:10 in all serum-HMAF and serum-PBS mixtures without any increase resulting from the presence of HMAF at titers of 1:5,120–1:10,240 or 1:1,280–1:2,560 (when 4 HMAFs were mixed) against dengue viruses (data not shown). Although these comparisons have limitations because of the use of HMAFs prepared with standard dengue virus strains, these results support the data of the previous experiment showing the cross-neutralization titers within ≤ 1/512 of the specific titer and indicate that the cross-reactivity did not increase even under the condition where antibodies against more than one type of dengue virus were included.

Neutralizing antibody titers against dengue viruses. Examination of 2,962 sera for neutralizing antibodies against JEV provided titers of ≥ 1:160 in 27 samples collected in Jakarta and 31 samples collected in Surabaya. Tables 1 and 2 show the gender and age of these samples, as well as the neutralizing antibody titers against JEV and DENV1–4. The maximum titers against dengue viruses were 1:5,120 for DENV1 and DENV3, and 1:10,240 for DENV2 and DENV4. On the basis of the previous cross-neutralization experiment using HMAF, these results indicated that all the present samples showing neutralizing titers of ≥ 1:160 against JEV possessed specific JEV antibodies: our system does not seem to show these high JEV antibody titers only because of cross-reactivities of dengue antibodies contained in these samples. Although sodium azide was included in human sera at 0.1%, we confirmed no effect of this chemical on neutralizing antibody titers by demonstrating that titers in 10 selected human sera were not altered after extensive dialysis against PBS to remove sodium azide from the sera (data not shown).

Prevalence of antibodies to JEV. Serum samples collected in Jakarta and Surabaya were grouped in 10-year increments, except for those over 60 (for Jakarta) and 80 (for Surabaya) years of age, which were grouped in one age group. As shown in Tables 3 and 4, the overall prevalence of antibodies showing titers of ≥ 1:160 against JEV was 2.2% in Jakarta and 1.8% in Surabaya.

No significant differences in JEV antibody prevalence were detected between males and females and between ages in Jakarta populations ($P > 0.05$). On the other hand, JEV antibody prevalence in Surabaya populations showed significant differences between age groups of ≤ 9 and 50–59 years (4.2% versus 0.4%, $P < 0.05$) and between age groups of 10–19 and 40–49 years (4.9% versus 0.4%, $P < 0.05$) or 50–59 years (4.9% versus 0.4%, $P < 0.01$). The prevalence in males was not significantly different from those in females in each age group and the total age group of the Surabaya population ($P > 0.05$). Comparisons between Jakarta and Surabaya populations of corresponding ages/genders showed no significant differences, except for total populations in the age group of 40–49 years (3.5% versus 0.4%, $P < 0.05$).

TABLE 1
Neutralizing antibody titers against Japanese encephalitis virus (JEV) and DENV1–4 in Jakarta samples*

Gender	Age (years)	JEV	Neutralizing antibody titer against			
			DENV1	DENV2	DENV3	DENV4
Male	27	1:160	< 1:160	< 1:160	1:320	1:320
	28	1:160	1:160	1:2,560	1:320	1:160
	30	1:320	< 1:160	< 1:160	< 1:160	< 1:160
	31	1:320	1:1,280	< 1:160	1:160	< 1:160
	32	1:320	< 1:160	1:160	1:160	< 1:160
	37	1:320	< 1:160	1:160	1:320	< 1:160
	40	1:320	< 1:160	< 1:160	1:320	1:320
	44	1:320	1:320	< 1:160	1:160	< 1:160
	48	1:160	1:320	1:640	1:320	< 1:160
	48	1:320	1:320	1:160	1:320	1:160
	49	1:320	< 1:160	< 1:160	1:320	< 1:160
	56	1:640	1:320	1:640	1:640	< 1:160
	67	1:320	1:1,280	1:640	1:2,560	1:160
Female	23	1:160	< 1:160	1:160	1:160	< 1:160
	26	1:160	< 1:160	1:160	1:640	< 1:160
	26	1:160	< 1:160	1:160	1:320	< 1:160
	30	1:320	1:1,280	1:160	1:160	< 1:160
	32	1:160	< 1:160	< 1:160	< 1:160	1:160
	40	1:320	1:1,280	1:640	1:160	1:640
	40	1:160	< 1:160	1:320	1:160	< 1:160
	43	1:640	< 1:160	< 1:160	< 1:160	< 1:160
	43	1:160	1:5,120	1:640	1:640	1:320
	45	1:320	1:160	1:640	1:320	1:320
	46	1:160	1:320	1:160	1:160	< 1:160
	48	1:160	1:160	1:160	1:320	< 1:160
	50	1:320	1:160	1:160	< 1:160	1:320
	50	1:160	1:160	1:1,280	1:160	1:160

* Because cells were affected by sodium azide that was added in sera for their transportation from Indonesia to Japan, only neutralizing antibody titers of ≥ 1:160 were determined.

TABLE 2
Neutralizing antibody titers against Japanese encephalitis virus (JEV) and DENV1–4 in Surabaya samples*

Gender	Age (years)	JEV	Neutralizing antibody titer against DENV1	DENV2	DENV3	DENV4
Male	2	1:160	1:5,120	1:1,280	1:2,560	< 1:160
	4	1:160	< 1:160	1:160	1:640	1:640
	12	1:320	< 1:160	1:1,280	1:1,280	1:640
	13	1:640	1:640	1:5,120	1:2,560	1:10,240
	14	1:160	< 1:160	1:160	< 1:160	< 1:160
	15	1:160	< 1:160	< 1:160	< 1:160	< 1:160
	15	1:160	< 1:160	1:2,560	< 1:160	1:320
	18	1:320	< 1:160	1:1,280	1:2,560	1:160
	20	1:160	< 1:160	< 1:160	1:1,280	1:320
	29	1:160	1:640	< 1:160	1:5,120	1:320
	31	1:320	< 1:160	1:1,280	< 1:160	1:640
	38	1:160	< 1:160	1:320	1:160	< 1:160
	38	1:640	1:320	1:160	< 1:160	< 1:160
	39	1:320	< 1:160	1:160	1:160	< 1:160
	39	1:1,280	< 1:160	1:320	1:640	1:320
	39	1:640	< 1:160	1:320	1:160	< 1:160
	54	1:320	1:640	1:640	1:5,120	1:320
	70	1:160	1:160	1:10,240	1:2,560	1:1,280
	71	1:160	< 1:160	< 1:160	< 1:160	< 1:160
	76	1:320	< 1:160	< 1:160	1:160	< 1:160
	76	1:160	1:160	1:160	< 1:160	< 1:160
	81	1:320	< 1:160	1:160	< 1:160	< 1:160
Female	9	1:320	< 1:160	1:1,280	1:2,560	1:320
	22	1:160	1:320	1:160	1:160	< 1:160
	26	1:160	1:160	1:320	1:160	1:160
	42	1:320	1:160	< 1:160	< 1:160	1:320
	63	1:160	< 1:160	1:640	< 1:160	< 1:160
	63	1:160	1:640	1:160	1:160	< 1:160
	63	1:320	1:320	1:320	< 1:160	< 1:160
	70	1:320	1:160	< 1:160	1:160	1:320
	78	1:640	< 1:160	1:640	1:640	1:320

* Because cells were affected by sodium azide that was added in sera for their transportation from Indonesia to Japan, only neutralizing antibody titers of ≥ 1:160 were determined.

Quantitative analysis of neutralizing antibody titers against JEV. Individual neutralizing antibody titers against JEV ranged from < 1:160 to 1:640 and < 1:160 to 1:1,280 in the Jakarta and Surabaya populations, respectively (Tables 1 and 2). Comparisons of geometric mean neutralizing antibody titers using samples that showed titers of ≥ 1:160 indicated no statistically significant differences between Jakarta (1:248) and Surabaya (1:256) populations ($P > 0.05$). In addition, no differences were detected between genders or between age groups in each of the Jakarta and Surabaya populations that showed titers of ≥ 1:160 ($P > 0.05$; data not shown).

DISCUSSION

The present study revealed that 2.2% and 1.8% of Jakarta and Surabaya populations, respectively, had neutralizing antibody titers of ≥ 1:160 against JEV. Because there should be populations showing titers of < 1:160, the real prevalence of JEV antibodies is considered to be higher than these percentages. In Indonesia where regular mass vaccination programs against JE are not used, the presence of neutralizing antibodies indicate previous exposure(s) to JEV infection. Because the present study eliminated the possibility to increase JE seropositivity resulting from high serologic cross-reactivity against dengue viruses, the results indicate a relatively high prevalence of antibodies to JEV among inhabitants in Jakarta and Surabaya. On the other hand, there remains the possibility that some of the present subjects possessing neutralizing antibodies against JEV had been infected with JEV outside Java Island where JE might be endemic.

TABLE 3
Prevalence of Japanese encephalitis virus (JEV) antibodies in the Jakarta population

Age	% Prevalence (number of positive/total number)* Male	Female	Total
20–29	1.4 (2/145)	2.0 (3/148)	1.7 (5/293)
30–39	2.9 (4/137)	1.6 (2/129)	2.3 (6/266)
40–49	3.0 (5/167)	3.9 (7/178)	3.5 (12/345)
50–59	1.5 (1/67)	2.0 (2/102)	1.8 (3/169)
≥ 60	2.3 (1/44)	0.0 (0/94)	0.7 (1/138)
Total	2.3 (13/560)	2.1 (14/651)	2.2 (27/1,211)

* Prevalence of antibodies showing neutralization titers of ≥ 1:160. See text for results of statistical evaluation for significant differences.

TABLE 4
Prevalence of Japanese encephalitis virus (JEV) antibodies in the Surabaya population

Age	% Prevalence (number of positive/total number)* Male	Female	Total
≥ 9	4.9 (2/41)	3.2 (1/31)	4.2 (3/72)
10–19	7.8 (6/77)	0.0 (0/45)	4.9 (6/122)
20–29	1.6 (2/125)	1.7 (2/120)	1.6 (4/245)
30–39	3.8 (6/157)	0.0 (0/130)	2.1 (6/287)
40–49	0.0 (0/105)	0.7 (1/138)	0.4 (1/243)
50–59	0.8 (1/126)	0.0 (0/129)	0.4 (1/255)
60–69	0.0 (0/122)	1.9 (3/155)	1.1 (3/277)
70–79	3.0 (4/132)	2.9 (2/69)	3.0 (6/201)
≥ 80	3.8 (1/26)	0.0 (0/23)	2.0 (1/49)
Total	2.4 (22/911)	1.1 (9/840)	1.8 (31/1,751)

* Prevalence of antibodies showing neutralization titers of ≥ 1:160. See text for results of statistical evaluation for significant differences.

The number and density of pigs were related to JE incidence, when these factors in Java Island were compared with those in Bali Island during and around the period of serum collection in Jakarta (2001) and Surabaya (1999–2000). Specifically, information from the statistics[31–34] indicates the pig populations in Java were considerably smaller than those in Bali. Although the numbers of laboratory-confirmed JE patients were not reported from the Indonesian government, an earlier literature reported no or few, if any, JE patients in Java Island.[13] On the other hand, JE cases have been reported in Bali[37,38] and in travelers returning from Bali.[39–42] One study revealed that the annual incidence per 100,000 children less than 10 years of age in Bali was 7.1.[38] In Japan, more than a thousand JE cases occurred annually in the past, but following the wide distribution of an inactivated vaccine in 1967, the annual number of cases dramatically reduced, staying below 10 out of approximately 100 million people since 1992,[43] even though pigs have been raised in this country.[35]

In contrast, the prevalence of JEV antibodies does not seem to be significantly related to the pig population and the number of patients, when comparison was made in Java, Bali, and Japan. In Japan, the national JE surveillance program reported that 2 of 227 (0.9%) people 40 to 49 years of age had neutralizing antibody titers of ≥ 1:160 in 2004[43]: the effect of vaccination carried out before 15 years of age is considered negligible in this age group and thus the neutralizing antibodies possessed by this population would represent natural exposure to JEV infection. This percentage (0.9%) was approximately a half of those obtained in the present study for populations of Jakarta (2.2%) and Surabaya (1.8%) who showed corresponding antibody titers (≥ 1:160). Although seroprevalence data in Balinese were not available, 70% of pigs in Bali possessed HAI antibodies.[38] Because the national JE surveillance program in Japan reported 50–100% seropositivities in pigs in most of the southern and western areas of Japan,[43] the natural JEV activity would be comparable in Bali and Japan. Thus, it should be emphasized that in Java, JEV transmission still continues despite a relatively small number of pigs and no (or few) human JE cases. This situation resembles that in Singapore where pigs are not raised.[10–12]

Low JE incidence in Japan is considered to be a result of vaccination, whereas that in Java is probably related to a small pig population. One of the potential reasons for low incidence under continued JEV transmission in Java may be the inoculum size per exposed person, which is closely related to the number and density of viremic pigs. It is speculated that the ratio of subclinical to clinical infections decreases with decreased inoculum size. Because the tropical climate and the style of pig farming are similar in neighboring islands of the same country, the difference in the number of JE cases between Java and Bali seems to be mostly attributed to the difference in pig population. Further studies are needed to elucidate other epidemiologic factors involved in natural JEV activities in Indonesia. Although JEV activities in an environment with a small swine population are poorly understood, they would provide important indications and implications for the ecology of JEV and epidemiology of JE.

Comparative statistical analyses of JEV antibody prevalences between genders, age groups, or areas detected significant differences in a few populations. A possible explanation for the difference would be a difference in the opportunities to acquire natural exposure to JEV infections, but factor(s) involved in the opportunities could not be identified. Because the annual JE incidence was almost constant during several years in other endemic countries in Asia without introduction of vaccination,[3] and therefore natural JEV activities are considered similar in each year also in Java, we consider at this moment that the significant difference is a random variation and people are equally exposed to infective mosquito bites. Jakarta is more urbanized and has a wider city area than Surabaya. Although no recent reports on seasonal abundance of vector (*Culex*) mosquitoes are available, exposure to infected mosquito bites seems equally frequent in Jakarta and Surabaya inhabitants, because *Culex* mosquitoes are generally believed to have the ability to fly for long distances and in this case infected mosquitoes are considered to move from rural area surrounding these cities.

In conclusion, people in Jakarta and Surabaya are still exposed to natural JEV infections, despite a relatively small number of pigs. On the other hand, the JE incidence in Java is very few, if any, to the best of our knowledge. Thus, the introduction of mass vaccination against JE may not be urgently needed in this region. However, considering relatively high antibody prevalence, JEV is circulated in nature and people are exposed to infective mosquito bites: the transmission cycle through vector mosquitoes seems to be established. Therefore, it would be required to continuously monitor the incidence of JE and to be prepared for a potential increase in JE patients.

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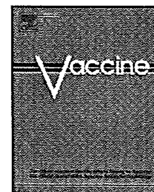

Detection by ELISA of antibodies to Japanese encephalitis virus nonstructural 1 protein induced in subclinically infected humans

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ABSTRACT

Japanese encephalitis (JE) is a fatal mosquito-borne disease that is vaccine-preventable. The natural infection rate is a critical factor for evaluations of the necessity for vaccination. Detection of antibodies to virus nonstructural (NS) proteins is a theoretical strategy to survey natural infections among populations vaccinated with an inactivated JE vaccine consisting of only structural proteins. Here, we present our development of an enzyme-linked immunosorbent assay (ELISA) to detect low levels of NS1 antibodies induced in humans with subclinical infections. We used a casein-based ELISA diluent to minimize nonspecific reactions. A tentative cut-off value (0.185) was statistically calculated from NS1 antibody levels obtained with healthy American individuals negative for antibodies to JE virus. Comparison with our previously developed immunostaining method provided a significant correlation coefficient (0.764; $P < 0.001$) and high qualitative agreement (82.5%). The presence of NS1 antibodies in sera was confirmed by Western blotting analysis. Using serially collected sera, we estimated the duration of NS1 antibodies between seroconversion and seroreversion to be 4.2 years.

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1. Introduction

Japanese encephalitis (JE) is a mosquito-borne disease with a high mortality rate of approximately 20% [1]. Japanese encephalitis virus (JEV), a member of the genus *Flavivirus* in the family *Flaviviridae*, is the causative agent, and is distributed in many areas of Asia and parts of Oceania [2]. Although the introduction of vaccination reduced the incidence of JE in some countries, approximately 50,000 cases still occur annually, among which 10,000 cases die [3]. In Japan, more than 1000 cases have been reported every year [4]. Following the wide distribution of the inactivated JE vaccine in 1967, the number of JE cases was dramatically reduced and stayed below 10 after 1992; indicating the contribution of the vaccination program [5]. However, the recommendation for JE vaccination ceased in 2005 after the occurrence of a case with severe side effects in which the involvement of the vaccination could not be completely ruled out [6,7].

Asymptomatic infections frequently occur following natural exposure to JEV infection, although some develop severe acute encephalitis [8]. The ratio of subclinical to clinical infections reported to date ranges from 25:1 to 1000:1 [9–12], greatly depending on factors such as the virus strain distributed in the area surveyed, the environmental factors including the abundance of vector mosquitoes, and the nutritional status of the local population. Although Japan has seen only a small number of such patients, it is not known how many natural or subclinical infections exist. Therefore, to evaluate the significance of vaccination, the natural infection rate is a critical factor. However, the natural infection rate among vaccinated populations is difficult to determine using conventional serological tests such as neutralization and hemagglutination-inhibiting (HAI) tests, which exclusively measure antibodies to the structural proteins of JEV.

Antibodies to JEV nonstructural proteins constitute a marker of natural infection among vaccinated populations [13]. Inactivated JE vaccine, made up of a purified virion fraction [14], only induces antibodies to structural proteins; thereby antibodies to nonstructural proteins induced by infection can be used to differentiate infected from vaccinated individuals. We have established an immunochemical staining method for detecting antibodies to the nonstructural protein 1 (NS1) of JEV [15]. Although this method is sensitive enough to detect low levels of antibodies induced in subclinically infected humans, the assay procedure is somewhat cumbersome and results are visually judged by the naked eye.

Enzyme-linked immunosorbent assay (ELISA) is simpler and more objective than an immunostaining method, and thus more suitable for testing large numbers of specimens within a limited time period. We have been able to establish an ELISA to detect NS1 antibodies in horse sera [16]: horses also fall victim to JEV

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infection. However, most human sera cause nonspecific reactions in ELISA that are too high to reliably measure low levels of antibodies induced by subclinical infections; although an ELISA has been reported to measure NS1 antibodies induced in sera of clinical cases [17]. As a different strategy to measure low-level antibodies, we also developed a complement-dependent cytotoxicity (CDC) assay [18]. Although this assay was successful with horse sera, it was difficult to determine an appropriate cell line for the examination of human sera: target cells that can be used in CDC assay to measure NS1 antibodies in horse sera were nonspecifically lysed by sera from some healthy humans negative for NS1 antibodies.

The present study aims to establish an ELISA for measuring low-level antibodies to JEV NS1 in human sera. Substitution of the conventionally used ELISA diluent containing bovine serum albumin (BSA) to a casein-based diluent resulted in the reduction of nonspecific reactions; thus the ELISA could measure NS1 antibodies induced in subclinically infected humans.

2. Materials and methods

2.1. Antibodies

Monoclonals, JE-2D5 [16] and JE-6H4 [19] specific for JEV NS1 and JE-10B4 specific for JEV E [20], were described previously. These monoclonals were obtained in an ascites form from pristane-primed BALB/c mice for the present study.

2.2. Immunoaffinity purification

A 3G8 cell line stably transfected with the *NS1* and *NS2A* genes of JEV was described previously [16]. NS1 antigen contained in culture fluids were affinity-purified with a monoclonal specific for NS1 (JE-2D5) coupled to Sepharose 4B beads (NHS-activated Sepharose 4B Fast Flow; GE healthcare UK Ltd., Buckinghamshire, England) as described previously [16]. To check purity, the proteins were heated at 100 °C for 2 min under non-reducing conditions and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by detection by silver staining (Silver Staining Kit; GE healthcare UK Ltd.). The purity of NS1 was >95% of the total protein.

2.3. ELISA for quantification of NS1 antigen

NS1 antigen was quantified using a sandwich ELISA as described previously [16]. Briefly, microplates sensitized with rabbit anti-NS1 hyperimmune sera were serially incubated with test samples, a monoclonal antibody to NS1 (JE-2D5), alkaline phosphatase-conjugated anti-mouse IgG, and *p*-nitrophenyl phosphate. Antigen levels were calculated from absorbance values obtained with the sample and a reference standard, and then expressed as the NS1 protein amount in nanogram per millilitre. The reference standard was prepared with affinity-purified NS1 obtained from culture fluids of JEV-infected Vero cells using JE-2D5 antibody. The NS1 protein amount contained in the standard NS1 preparation was estimated by comparison with bovine serum albumin (BSA) samples in silver-stained gels.

2.4. Western blot analyses

Analyses were performed essentially as previously described [21]. Briefly, an affinity-purified NS1 antigen was run on the standard Laemmli gels under non-reducing conditions. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA) and incubated with test specimens (human sera or monoclonal JE-6H4 as a control), with alkaline phosphatase-conjugated anti-human IgG or anti-mouse IgG and then with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate. For reference, the E antigen purified from culture fluids of CHO cells stably transfected with the *premembrane* (*prM*) and *envelope* (*E*) genes of JEV [21] was used for preparing Western blots. This purification was done by polyethylene glycol precipitation and sucrose density gradient centrifugation as described previously [21].

2.5. ELISA for quantifying NS1 antibodies in human sera

A conventional ELISA was performed for quantifying antibodies to NS1. Microplates (Maxisorp: Nunc A/S, Roskilde, Denmark) were sensitized by incubation at 4 °C overnight with purified NS1 antigens at 10 ng/well in 0.1 M sodium carbonate buffer (pH 9.6), followed by incubation at 37 °C for 30 min with the ELISA diluent. The ELISA diluent was 0.05 M Tris–HCl (pH 8.0) containing 0.2% casein (Sigma–Aldrich Corporation, St. Louis, MO), 0.05% Tween 20, 1 mM EDTA and 0.15 M NaCl, essentially as previously described [22]. Sensitized plates were incubated serially with test sera at a 1:100 dilution unless otherwise specified, alkaline phosphatase-conjugated goat anti-human IgG (Biosource, Camarillo, CA) at a 1:5000 dilution, and *p*-nitrophenyl phosphate at 1 mg/ml. The ELISA diluent was used for preparing dilutions of test sera and the conjugate. Tests were done in duplicate. The same protocol was adopted in ELISA using monkey sera, except for the use of alkaline phosphatase-conjugated goat anti-monkey IgG (Bethyl Laboratories, Montgomery, TX).

To eliminate nonspecific reactions, a non-sensitized control plate incubated only with the sodium carbonate buffer (followed by incubation with ELISA diluent) at the sensitization process was run in parallel. The difference between absorbances obtained with antigen-sensitized and non-sensitized wells was regarded as reaction specific for NS1. When the subtraction provided a minus value, we assigned 0.000 to the result. To minimize interplate variations, a constant positive control serum prepared from a 1:1000 dilution of patient serum was included in every plate, and absorbances obtained with test samples were adjusted with the value for the positive control as 1.0. The adjusted absorbances were expressed as ELISA values. ELISA values of 0.185 or higher were determined as positive for NS1 antibodies.

2.6. Human samples

Sera from three JE patients were supplied by the Department of Virology, National Institute of Health, Korea, through Dr. Robert E. Shope, Yale Arbovirus Research Unit, Yale University School of Medicine, CT [23]. Plasmas from seven JE patients were supplied by the Department of Virology, Armed Forces Research Institute of Medical Sciences, Thailand, through Dr. Ichiro Kurane, Department of Medicine, University of Massachusetts Medical Center, MA [24]. Negative control sera used for determination of the cut-off value differentiating positive from negative samples were obtained from 40 healthy American volunteers who were recruited for evaluation of inactivated JE vaccine in a clinical trial [25] and who had no history of yellow fever vaccination and no detectable neutralizing antibodies against JE virus: these were supplied by the Walter Reed Army Institute of Research through Dr. Robert E. Shope [23]. Serum specimens collected from 1982 through 1983 from 40 healthy people at the Miki Health Center, Hyogo Prefecture in Japan, were the same as those used in our previous study to establish an immunostaining method to measure NS1 antibodies [15]. Their NS1 antibody titers were thus known: these sera were used for comparison between the ELISA and immunostaining methods. A total of 363 pairs of sera collected from 1982 through 1985 at 1-year intervals from 162 individuals (45 males and 117 females) at the Miki Health Center [15] were used for estimating the duration of anti-

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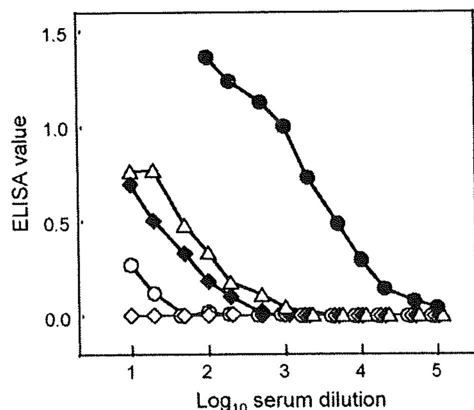

Fig. 1. Dose–response absorbance curves obtained with ELISA to measure antibodies to JEV NS1. Serum samples used were from one JE patient (closed circle) and two healthy American volunteers who were negative for neutralizing antibodies against JEV (open circle and diamond), as well as two Japanese who were positive for NS1 antibodies in the immunostaining method (open triangle and closed diamond). This patient serum was used as a positive control for the present ELISA.

body responses. The use of all the human samples in the present study was approved by the Ethical Committee of the Kobe University School of Medicine (Ethical Committee Approval Number 739).

2.7. Monkey sera

Sera of cynomolgus monkeys experimentally infected with JEV were collected and stored for our earlier study [26]. Briefly, sera used in the present study were those serially collected from two monkeys inoculated intranasally with 1×10^9 PFU of the JaTH160 strain of JEV; these developed NS1 antibodies as determined by the immunostaining method (animals #21 and #27 in reference [26]). Although these monkeys were inoculated with a plasmid pNGVL4a prior to the JEV infection, the pNGVL4a is a control vector that did not contain any JEV-related genes and we regarded these monkeys as unimmunized.

2.8. Statistical analysis

Calculations of correlation coefficients and evaluations of statistical significance were done using Microsoft Excel 2003. Probability levels (P) of less than 0.05 were considered significant.

3. Results

3.1. Dose-dependent antibody–response curve

To evaluate the present ELISA system, dose–response curves were obtained using sera from one patient, two subclinically infected human subjects and two others who had resided in a non-endemic area (Fig. 1). The subclinically infected humans were determined by the immunostaining method presented in our earlier study [15]. Patient serum showed a linear dose response within the dilution range used for this experiment (1:10^2 to 1:10^5), whereas no positive ELISA values were obtained in most dilutions of the two negative sera. Sera from the two subclinically infected subjects showed ELISA values higher than those of negative sera and lower than those of patient serum. Since one negative sera showed ELISA values of 0.121–0.267 at dilutions of 1:10–1:20, we decided to use a 1:100 dilution of test sera for the present ELISA, one which is generally used in conventional ELISA for antibody quantification.

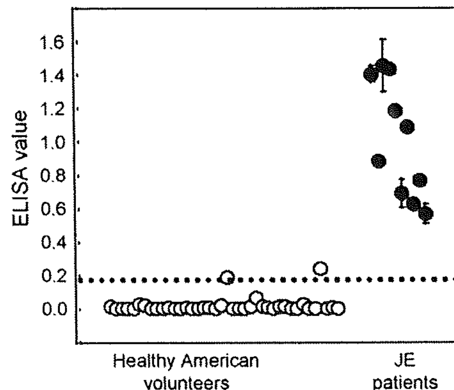

Fig. 2. Comparison of ELISA values obtained from 40 healthy American volunteers and 10 JE patients. Each datum represents an average obtained in two separate experiments with standard deviations (indicated by bars). A dotted line indicates the cut-off value calculated from ELISA values obtained from the American volunteers.

3.2. Determination of the cut-off value

To determine the cut-off value for differentiating positive from negative results, 40 negative control sera were tested. For comparison, 10 patient derived samples were also run in parallel (Fig. 2). Almost all negative sera (95%) showed ELISA values below 0.065, but two sera showed a relatively high value of 0.188 and 0.237. On the other hand, ELISA values obtained with patient samples ranged from 0.566 to 1.453. The mean ELISA value obtained with 40 negative controls was 0.0167 with a standard deviation of 0.0473. The confidence limit calculated from the mean and standard deviation at a probability level of 0.1% was 0.185. This value was tentatively decided as the cut-off value between positive and negative results. The results of this experiment also showed the reproducibility of the present ELISA: small standard deviation (SD) values were obtained from two separate experiments in almost all samples.

3.3. Comparison of ELISA and immunostaining methods

The present ELISA system was evaluated by comparing it with the previously developed immunostaining method. For this evaluation, we used the human sera used in our earlier study to develop the immunostaining method to measure NS1 antibody titers: 20 positive and 20 negative sera were selected. As shown in Fig. 3, the ELISA values significantly correlated with the immunostaining NS1 antibody titers with correlation coefficients of 0.764 ($P < 0.001$). A

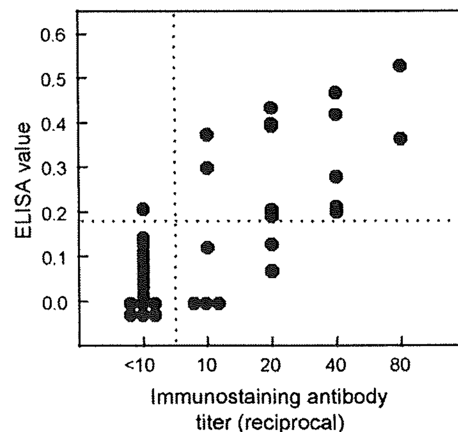

Fig. 3. Comparison of the ELISA and immunostaining methods using human sera positive or negative for NS1 antibodies by immunostaining (20 samples for each). A dotted line indicates the cut-off values for ELISA (0.185) and immunostaining (1:10).

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Table 1
Qualitative comparisons between ELISA and immunostaining methods for detection of antibodies to JEV NS1, using 40 human serum samples.

ELISA antibodies	No. of samples with "immunostaining" antibodies		Total
	Positive	Negative	
Positive	14	1	15
Negative	6	19	25
Total	20	20	40

Based on the results shown in Fig. 3.

qualitative comparison (Table 1) indicated that the results obtained by ELISA were consistent with those obtained by the immunostaining method for 82.5% of the samples (33 of 40) with a sensitivity of 70.0% (14 of 20) and a specificity of 95.0% (19 of 20).

3.4. Reaction of NS1-positive sera on Western blot

To confirm the presence of NS1 antibodies in human sera found positive in the present ELISA, positive or negative sera were examined by Western blotting analysis. This analysis was done using purified NS1 antigen used for sensitization of microplates. For reference, the analysis was also done using purified E antigen. For control, these antigens were incubated with monoclonals to NS1 (JE-6H4) or E (JE-10B4). Fig. 4 shows representative results: one patient's serum, as along with those of two persons who had stayed in an endemic country and possessed neutralizing antibodies with or without NS1 antibodies in ELISA. As shown, the results obtained by the ELISA correlated with those obtained on Western blots. NS1-positive samples showed the major band in the position corresponding to the band obtained with a monoclonal to NS1, as well as faint bands corresponding to NS1′, a product of the expression of the *NS1* and *NS2A* genes [27], and the NS1 dimmers, a general form secreted from infected cells. These results confirmed that NS1 antibodies in human sera were correctly detected in the present ELISA.

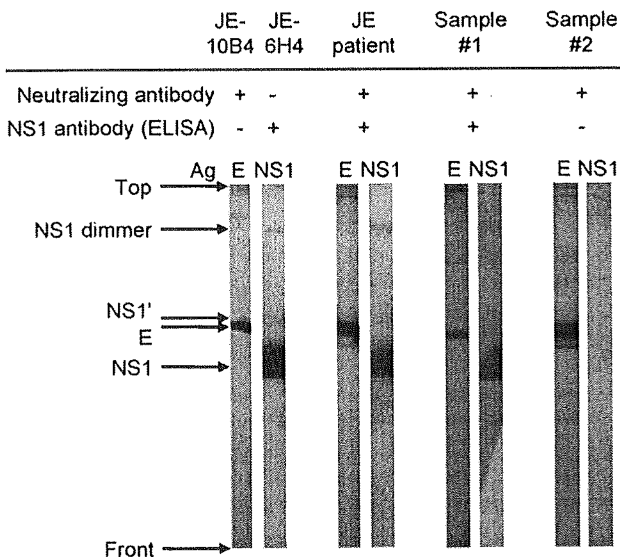

Fig. 4. Western blot analyses of sera from a JE patient and two neutralizing antibody-positive human subjects with (Sample #1) or without (Sample #2) NS1 antibodies as determined by the present ELISA: neutralizing antibodies in human samples were tested in our earlier study [15]. The affinity-purified NS1 antigen was used with the purified E antigen as a reference. Samples were run on a 10% polyacrylamide gel. For control, the blots were stained with monoclonal to E (JE-10B4) or NS1 (JE-6H4). NS1′ is an elongated form of NS1, which is produced by expression of the *NS1* and *NS2A* genes in JEV-infected mammalian cells [27].

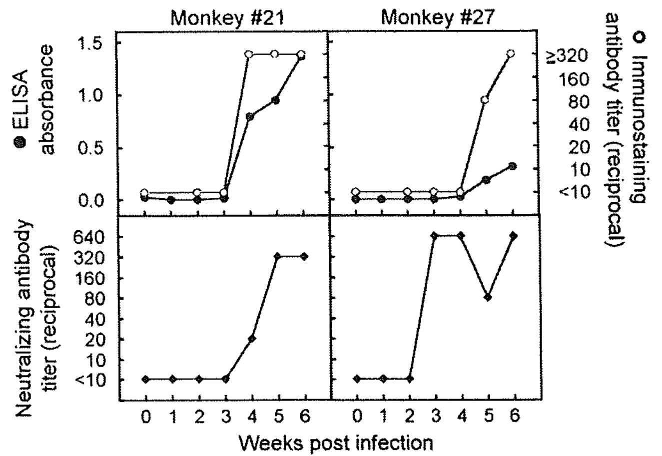

Fig. 5. Time-course of NS1 antibody levels in sera serially collected from two monkeys following experimental infection with JEV. NS1 antibody levels (closed circle) obtained by the present ELISA were compared with immunostaining antibody titers (open circle) and neutralizing antibody titers (closed diamond) that were determined by a 90% plaque reduction assay in our earlier study [26].

3.5. Time course of NS1 antibody levels in experimentally infected monkeys

To further evaluate the present ELISA, we tested sera serially collected from experimentally infected monkeys; for which NS1 antibody titers obtained by the immunostaining method and neutralizing antibody titers have been reported [26]. As shown in Fig. 5, NS1 antibody levels obtained by ELISA increased at 4 or 5 weeks post-infection in monkeys #21 or #27, respectively, consistent with NS1 antibody titers obtained by the immunostaining method. By contrast, neutralizing antibodies appeared earlier in monkey #27 than #21. These results indicated that NS1 antibodies developed after the infection of monkeys were also detected in the present ELISA and suggested that there are individual variations in the timing for detecting antibodies to NS1 or E and in the level of NS1 antibodies.

3.6. Duration of NS1 antibodies

The duration for NS1 antibodies between seroconversion and seroreversion is an important factor involved in NS1 antibody surveys: the annual infection rate can be estimated from the antibody prevalence at a particular time point by dividing it by the duration. To obtain the duration, 363 pairs of sera collected from the same individuals at an interval of 1 year were used. First, the cut-off value to differentiate significant from non-significant increases in ELISA values was obtained from the difference in ELISA values between the first and the second serum samples of these pairs. The mean difference was 0.00571 with a standard deviation of 0.0750 (data not shown). The confidence limit at a probability level of 1% was used for the cut-off value, calculated as 0.181. Second, the mean increase in paired sera that seroconverted and showed increases of 0.181 or more was obtained. Seven of the 363 pairs fulfilled these conditions (Fig. 6, left panel): their mean increase was 0.405. This value is considered to represent the increase in NS1 antibody level caused by natural infection with JEV. Third, the mean decrease in the paired sera in which the first sera were positive for NS1 antibodies and showed a decrease within 1 year was obtained. There were 23 pairs that met these conditions (Fig. 6, right panel); their mean decrease was 0.0976. This value is considered to represent the decrease in NS1 antibody level over the period of 1 year. Finally, the duration was obtained by dividing the mean increase (0.405) by the mean decrease (0.0976); calculated to be 4.2. Thus, we estimated 4.2 years

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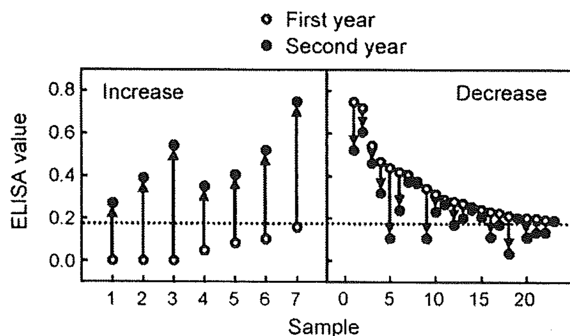

Fig. 6. Difference in ELISA values between two paired sera collected at a 1-year interval. ELISA values in the first year (open circle) were compared with those in the second year (closed circle) in 7 pairs showing seroconversion and increase of 0.181 or more in ELISA value (left panel) and 23 pairs whose first sera were positive and showed a decrease (right panel).

as the duration for NS1 antibodies between seroconversion and seroreversion.

4. Discussion

The NS1 protein is an antigen well suited for use for antibody testing to distinguish individuals naturally infected with JEV from uninfected ones in a population vaccinated with an inactivated JE vaccine [13]. The NS1 protein is the only nonstructural protein secreted from infected mammalian cells, among the seven nonstructural proteins of flaviviruses [28]. It has been shown to be an effective immunogen for inducing protective antibodies in flavivirus-infected animals [29,30]. Thus, higher levels of antibody induction are expected to be found against NS1 than other nonstructural proteins. In addition, the secreted form of NS1 can be readily used as an antigen for antibody assays. The absence of NS1 antigen in the inactivated JE vaccine preparation, as well as there being no induction of NS1 antibodies in vaccinated American volunteers recruited in a clinical trial, has been confirmed elsewhere (data not shown).

Measurement of NS1 antibodies has been important for estimating how frequently humans and horses have acquired natural exposure to JEV infections. Seroepidemiologic surveys in the early 1980s and mid 1990s among inhabitants in Japan using previously established methods to measure NS1 antibodies have revealed annual infection rates of 5–10% in and around Kobe, west-central Japan [15]; 0.2–3.4% in 8 selected prefectures across Japan in 2001 [31]; and an average of 2.8% in Tokyo during 2001–2004 [32]. Furthermore, surveys in 1998–2000 among racehorses revealed annual infection rates of 15–67% in 5 prefectures in central and south Japan [33], and an average of 18% in Shiga Prefecture (mid-central) in 1998–2003, and Ibaraki Prefecture (east-central) in 1999–2003 [34]. Thus, relatively high percentages of humans and horses presented with NS1 antibodies.

The reduction of nonspecific reactions is critical for the detection of low levels of antibodies. The success of the immunostaining method for measurements of NS1 antibodies induced by asymptomatic infections is based on the principle of this assay in which both NS1-expressing and non-expressing cell colonies are observed for differences in stain intensity in a single microscopic field [15]. Although the levels of nonspecific reactions differ according to the individual, the difference in stain intensity implied the presence of NS1-specific antibodies in test specimens. Thus determination of the specific reaction was not hampered by nonspecific reactions. In ELISA, nonspecific reactions are generally reduced by running non-sensitized plates in parallel, and by obtaining the difference in absorbances from antigen-sensitized plates. This strategy is useful for measuring NS1 antibodies in horse sera [16], probably because of the relatively high levels of NS1 antibodies induced in this animal species, which seems to acquire larger numbers of infective mosquito bites in nature than do individual humans. However, most of the human sera provided high absorbances in both sensitized and non-sensitized plates. The experimental variations occurring in the plates are an obstacle to consistent detection of low levels of NS1 antibodies. Although several attempts to reduce nonspecific reactions failed, we finally detected low levels of NS1 antibodies by using a casein-based ELISA diluent that has been described in an epitope-blocking ELISA [22].

One advantage of the present ELISA method over the previous immunostaining method is its objectivity, since the results are obtained in numeral form. Quantitative and qualitative comparisons between ELISA and immunostaining provided a correlation coefficient of 0.764 and agreement of 82.5%, which were similar to the correlation coefficient of 0.799 and agreement of 85.3% between these assays for measurement of NS1 antibodies in horse sera [16]. In horses, a previously established CDC assay that can also provide numeral data showed a higher correlation coefficient and agreement with ELISA (0.848 and 95.0%) than the immunostaining method (0.784 and 87.5%) [18]. These differences are probably attributable to the fact that the ELISA is more objective than the immunostaining method. In the present comparison using human sera, 6 of 20 sera positive for immunostaining were negative for ELISA and 1 of 20 sera positive for ELISA was negative for immunostaining; showing that the immunostaining method tended to produce more false-positive results than did ELISA. This was demonstrated in part by Western blotting analysis by which two serum samples showing an immunostaining antibody titer of 1:20 but negative in ELISA were determined to be negative (data not shown).

In conclusion, the present ELISA using a casein-based diluent was able to detect low levels of antibodies to JEV NS1 induced by subclinical infections in vaccinated humans. The ELISA is a powerful tool for seroepidemiology: thus, this method can contribute to NS1 antibody surveys and the debate about the necessity to continue a vaccination program for humans.

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