A Single Amino Acid Substitution in the NS2A Protein of Japanese Encephalitis Virus Affects Virus Propagation In Vitro but Not In Vivo

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We identified a unique amino acid of NS2A113, phenylalanine, that affects the efficient propagation of two Japanese encephalitis virus strains, JaTH160 and JaOArS982, in neuroblastoma Neuro-2a cells but not in cell lines of extraneural origin. This amino acid did not affect viral loads in the brain or survival curves in mice. These findings suggest that virus propagation in vitro may not reflect the level of virus neuroinvasiveness in vivo.

Japanese encephalitis virus (JEV) causes approximately 30,000 to 50,000 cases and 10,000 to 15,000 deaths in Asian countries annually (1, 2). JEV belongs to the family Flaviviridae, genus Flavivirus (3, 4), whose genomic RNA encodes one polyprotein, cleaved into three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (5). The clinical symptoms of JE vary from mild to severe and include a nonspecific febrile illness, meningitis, encephalitis, and meningoencephalitis (6, 7). The mechanism of severe central nervous system (CNS) disease is not fully understood.

To evaluate disease pathogenesis and virulence, mice have been employed as an infection model. Several viral and host factors affect disease severity during JEV infection. We previously suggested that the host response, resulting in immunopathological effects, contributes to fatal infections (8). Furthermore, we also demonstrated that the JaOArS982 and JaTH160 strains of JEV exhibited different virulence in mice (8). Therefore, a genetics-based comparison between these strains may provide an effective approach to identify viral factors contributing to severe disease.

Our previous results showed that following subcutaneous infection with 10⁴ PFU of JaTH160, mice showed 100% mortality, whereas JaOArS982 caused approximately 30% mortality in mice (8). We first constructed infectious cDNA clones harboring full-length genes of JaOArS982 and JaTH160 and produced infectious viruses S982-IC and JaTH-IC from the respective cDNAs (9). In the present study, subcutaneous infection with 10⁴ PFU of S982-IC and JaTH-IC viruses caused 40% and 100% mortality, respectively, in C57BL/6j (B6) mice (Japan SLC Cooperation, Japan CLEA Cooperation) (Fig. 1A), indicating that both JaTH-IC and S982-IC viruses possessed virulence potentials similar to those of their parent JaOArS982 and JaTH160 viruses. Our animal experimental protocols were approved by the Animal Care and Use Committee, Nagasaki University (approval numbers 091130-2/0912080807-9 and 100723-1-3/1008050873-3).

Our previous data showed that viral loads in the CNS of JaTH160-infected mice were significantly higher than those of JaOArS982-infected mice (8). This raised the possibility that virus propagation in neuronal cells is different between JaTH160 and

FIG 1 Virulence in mice and viral yields in cultured cells infected with the S982-IC and JaTH-IC viruses. (A and B) Survival curves (A) and weight ratios (B) of mice subcutaneously infected with 10⁴ PFU of each virus (n = 10). Statistical analysis was conducted by a log rank (Mantel-Cox) test. (C and D) Propagation of JaOArS982 (original virus) and S982-IC (derived from an infectious cDNA clone of JaOArS982), JaTH160 (original virus), and JaTH-IC (derived from an infectious cDNA clone of JaTH160) in N2a (C) and BHK (D) cells at 0, 24, 48, and 72 h postinfection. Error bars represent standard deviations. Statistical analysis was conducted by one-way analysis of variance.
JaOArS982. Thus, we next compared virus propagations in murine neuroblastoma Neuro-2a (N2a) cell lines.

N2a cells were infected with each JEV strain at a multiplicity of infection (MOI) of 0.1, and supernatants were harvested at 0, 24, 48, and 72 h postinfection. Virus titers were determined by plaque-forming assays on baby hamster kidney (BHK) cells (10). In N2a cells, JaTH-IC and the parent JaTH160 exhibited significantly higher virus yields than S982-IC and the parent JaOArS982 (Fig. 1B). However, there were no significant differences in virus yields between the four viruses in BHK cells (Fig. 1C), Vero (rhesus monkey kidney), PS (porcine kidney), and HeLa (human epithelial) cells (data not shown). These results suggest that replication in neuronal cells is different between JaTH160 and JaOArS982 viruses.

To determine the specific region of the viral gene affecting virus propagation in N2a cells, we constructed four chimeric JEV clones, S982_J1-IC, S982_J2-IC, S982_J3-IC, and S982_J4-IC showing the replacement of 5’UTR-NS1322, NS1322-NS335, NS335-NS5566, NS5566-3’UTR of S982-IC, respectively, with the corresponding region of JaTH-IC. (B) Genomic representation of single-amino-acid-substituted S982-IC and JaTH-IC. The white and black arrowheads indicate amino acids derived from S982-IC and JaTH-IC, respectively. S982-IC and JaTH-IC are also named S982_IA23LA113DB81 and JaTH_VA23FA113EB81, respectively.

FIG 2 Schematic representation of full-length chimeric and amino acid-substituted viruses derived from S982-IC and JaTH-IC. (A) Genome representations of S982_J1-IC, S982_J2-IC, S982_J3-IC, and S982_J4-IC showing the replacement of 5’UTR-NS1322, NS1322-NS335, NS335-NS5566, NS5566-3’UTR of S982-IC, respectively, with the corresponding region of JaTH-IC. (B) Genomic representation of single-amino-acid-substituted S982-IC and JaTH-IC. The white and black arrowheads indicate amino acids derived from S982-IC and JaTH-IC, respectively. S982-IC and JaTH-IC are also named S982_IA23LA113DB81 and JaTH_VA23FA113EB81, respectively.
S982_IA23LA113EB81 (Fig. 3B). Conversely, JaTH_VA23L113EB81 exhibited significantly lower virus yields than JaTH-IC, JaTH_IA23F113EB81, and JaTH_VA23F113DB81 (Fig. 3C). These results indicate that an amino acid substitution in NS2A113, F in JaTH-IC and L in S982-IC, is responsible for the difference in propagation in N2a cells.

To examine whether the amino acid substitution in NS2A113 contributes to virulence and virus propagation in vivo, B6 mice were subcutaneously inoculated with S982_IA23F113DB81 and JaTH_VA23F113EB81, and their mortality was observed. Viral loads in the brain were also compared as previously shown (8, 12). Unexpectedly, S982_IA23F113DB81 showed survival curves similar to that of the parent S982-IC virus (Fig. 4A), and there was no significant difference in viral loads in the brain between S982-IC- and S982_IA23F113DB81-infected mice (Fig. 4B). JaTH_VA23L113EB81 also showed survival curves and viral loads in the brain similar to those of the parent JaTH-IC virus (Fig. 4B). Thus, an amino acid substitution in NS2A113 did not explain the different viral loads and virulence in the brain between S982-IC and JaTH-IC viruses.

Flavivirus NS2A protein is a 22-kDa hydrophobic protein (13). Previous studies have shown that NS2A protein is involved in viral assembly/release, viral RNA synthesis, regulation of NS1 expression, and inhibition of type-I interferon response (14–20). These functions are affected by amino acid substitutions within NS2A, such as NS2A-G11A, -E20A, -P30A, -T33I, -L46H, -I59N, -D73H,
The influence of the NS2A-L113F substitution identified here in JEV infection has not been reported previously. NS2A has eight predicted transmembrane segments (pTMS), and NS2A113 appears to localize to pTMS4 (14). However, how NS2A113 substitution affects virus propagation in N2a cells remains unclear. Further investigation may provide information on the unknown function of NS2A.

Although a single amino acid substitution in NS2A113 alters viral propagation in N2a cells, this substitution did not affect viral loads in the brain or survival curves in mice. These findings suggest that virus propagation in vitro does not necessarily reflect virus replication in vivo. Further, other amino acid and/or nucleotide substitutions may affect host responses, such as antiviral activity. In this regard, this study helps to elucidate the mechanism of pathogenesis due to JEV infection in a mouse model.

Interestingly, our preliminary experiments showed that there were no significant differences in mortality following intracerebral inoculation between JaOArS982 and JaTH160. In our previous study of tick-borne encephalitis viruses, we suggested that the mechanism of fatal infection is fundamentally different between intracerebral and peripheral infection (10, 26). We further showed that immune responses were different between JaOArS982- and JaTH160-infected mice (8). From these observations, we attributed the differences in viral replication in the brains of JaOArS982 and JaTH160 to the peripherally induced host immune responses and the infiltration of immune cells into the brain. In addition, it appears that most of the volume of inoculum leaked from the brain due to intracranial pressure following intracerebral inoculation. Thus, we consider that intracerebral inoculation does not simply reflect virus infection and replication in neurons, and it appears that it is difficult to examine the differences in virulence mechanism between JaOArS982 and JaTH160.

We propose that actual virus propagation in the brain in vivo reflects a combined mechanism of viral replication properties in neuronal cells and the host antiviral immune response. Furthermore, we believe that the disease mechanisms of JEV in vivo involve a complex mechanism that includes the host immune response and neuronal infection in the CNS. Further investigations in a step-by-step fashion will provide clues to elucidate the precise pathogenic mechanisms of JEV infection and enable the development of effective treatment strategies for JE.
REFERENCES


