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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Running title

A unique amino acid substitution in NS2A of JEV

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Abstract

We identified a unique amino acid of NS2A$_{113}$ 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 nor survival curves in mice. These findings suggest that virus propagation in vitro may not reflect the level of virus neuroinvasiveness in vivo.
Japanese encephalitis (JE) virus (JEV) causes approximately 30,000 to 50,000 cases and 10,000-15,000 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 non-specific 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 genetic-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 $10^4$ 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 of S982-IC and JaTH-IC from each cDNA, respectively (9). In the present study, subcutaneous infection with $10^4$ 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) (Figure 1A), indicating that both JaTH-IC and S982-IC viruses possessed virulence potentials similar to their parent JaOArS982 and JaTH160 viruses. Our animal experimental protocols were approved by the Animal Care and Use Committee, Nagasaki University (approval number: 091130-2-7 /0912080807-9, 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 JaOArS982. Thus, we next compared virus propagations in murine neuroblastoma Neuro-2a (N2a) cell lines.

N2a cells were infected with each JEV strain at an m.o.i. of 0.1 and supernatants were harvested at 0, 24, 48 and 72 hours post-infection (pi). Virus titers were determined by plaque forming assays on Baby Hamster Kidney (BHK) cells (10). In N2a cells, JaTH-IC and the parent JaTH160 viruses exhibited significantly higher virus
yields compared with S982-IC and the parent JaOArS982 viruses (Figure 1B). However, there were no significant differences of virus yields between the four viruses in BHK cells (Figure 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, as shown in Figure 2A. S982_J2-IC exhibited a similar growth curve to JaTH-IC and produced significantly higher virus titers than S982-IC, S982_J1-IC, S982_J3-IC and S982_J4-IC (Figure 3A). Thus, the viral genome sequence in the region of NS1322 to NS335 affects virus propagation in N2a cells.

There are three amino acid differences in this region between JaOArS982 and JaTH160. Thus, we inserted amino acid substitutes into S982-IC and JaTH-IC, as shown in Figure 2B. Site-directed mutagenesis was used, as described previously (11). S982_IA23FA113DB81 showed significantly higher virus production in N2a cells compared with S982-IC, S982_VA23LA113DB81 and S982_IA23LA113EB81 (Figure 3B). Conversely, JaTH_VA23LA113EB81 exhibited significantly lower virus yields than JaTH-IC, JaTH_IA23FA113EB81 and JaTH_VA23FA113DB81 (Figure 3C). These results indicate that an
amino acid substitution in NS2A_{113}, 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 NS2A_{113} contributes to the virulence and virus propagation in vivo, B6 mice were subcutaneously inoculated with S982\_I_{A23}F_{A113}D_{B81} and JaTH\_V_{A23}L_{A113}E_{B81}, and their mortality was observed. Viral loads in the brain were also compared as previously shown (8, 12). Unexpectedly, S982\_I_{A23}F_{A113}D_{B81} showed similar survival curves to the parent S982-IC virus (Figure 4A) and there was no significant difference in viral loads in the brain between S982-IC- and S982\_I_{A23}F_{A113}D_{B81}-infected mice (Figure 4B). JaTH\_V_{A23}L_{A113}E_{B81} also showed similar survival curves and similar viral loads in the brain to the parent JaTH-IC virus (Figure 4B). Thus, an amino acid substitution in NS2A_{113} 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,\,-R84S/A/E,\,-E100A,\,M108K,\,D125A,\,-Q187A,\,-K188A,\,-K190S, \,and\,-G200A (14, 16-25).
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. 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 nor 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 of 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 assumed that different viral replications in the brains between JaOArS982 and JaTH160
attributes to the peripherally induced host immune responses and those immune cells
infiltrating in the brains. 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 different
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.

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References


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Figure Legends

FIG 1  Virulence in mice and viral yields in cultured cells infected with the S982-IC and JaTH-IC viruses. (A) Survival curves and (B) Weight ratios of mice subcutaneously
infected with $10^4$ PFU of each virus (n=10). P: Log-rank (Mantel-Cox) Test.

Propagation of JaOArS982 (original virus) and S982-IC (derived from infectious cDNA clone of JaOArS982), JaTH160 (original virus) and JaTH-IC (derived from infectious cDNA clone of JaTH160) viruses in N2a (C) and BHK cells (D) at 0, 24, 48 and 72 hours post-infection. Error bars represent standard deviations. p: One-way analysis of variance.

**FIG 2** Schematic representation of full-length chimeric and amino acid substituted-viruses derived from S982-IC and JaTH-IC. (A) A genome representations of S982_J1-IC, S982_J2-IC, S982_J3-IC and S982_J4-IC showing the replacement of 5'UTR-NS1322, NS1323-NS335, NS336-NS5566, NS5567-3'UTR of S982-IC, respectively, with the corresponding region of JaTH-IC. (B) A genome representation of a 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 3** Growth curves for virus propagation of the chimeric and amino acid substituted-viruses in N2a cells at 0, 24, 48 and 72 hours post-infection. (A) Viral yields
of S982_J1-IC, S982_J2-IC, S982_J3-IC and S982_J4-IC compared with S982-IC and
JaTH-IC viruses. Viral yields of JaTH-IC and S982-IC are the same data as FIG 1C.
Viral yields of amino acid substituted-S982-IC (B) and JaTH-IC (C) viruses. p:
One-way analysis of variance. The white and black arrowheads indicate amino acids
derived from S982-IC and JaTH-IC, respectively.

**FIG 4** Virulence in mice and viral loads in the brains of mice following subcutaneous
infection with $10^4$ PFU of S982_IA23L_A113DB81 (S982-IC), S982_IA23FA113DB81,
JaTH_VA23FA113EB81 (JaTH-IC), and JaTH_VA23LA113EB81. (A) Survival curves (n=10)
P: Gehan-Breslow-Wilcoxon Test. (B) Viral loads in the brain (n=6). P: Kruskal-Wallis
test, *: p<0.05 by Dunn’s Multiple Comparison Test.
FIG 2

A

S982-IC

JaTH-IC

S982_J1-IC

S982_J2-IC

S982_J3-IC

S982_J4-IC

B

S982_IA_23IA_113DB1 (S982-IC)

S982_VA_23IA_113DB1

S982_IA_23FA_113DB1

S982_IA_23FA_113DB1

JaTH_VA_23FA_113EB1 (JaTH-IC)

JaTH_IA_23FA_113EB1

JaTH_VA_23IA_113EB1

JaTH_VA_23IA_113EB1

JaTH_VA_23FA_113EB1
FIG 4

A

Survival rate (%) vs. days post inoculation

- S982_I\textsubscript{A23}\_L\textsubscript{A113}D\textsubscript{B81}
- S982_I\textsubscript{A23}\_F\textsubscript{A113}D\textsubscript{B81}
- JaTH\_V\textsubscript{A23}\_F\textsubscript{A113}E\textsubscript{B81}
- JaTH\_V\textsubscript{A23}\_L\textsubscript{A113}E\textsubscript{B81}

P = 0.0004

B

Log\textsubscript{10} PFU / g tissue

- S982_I\textsubscript{A23}\_L\textsubscript{A113}D\textsubscript{B81}
- S982_I\textsubscript{A23}\_F\textsubscript{A113}D\textsubscript{B81}
- JaTH\_V\textsubscript{A23}\_F\textsubscript{A113}E\textsubscript{B81}
- JaTH\_V\textsubscript{A23}\_L\textsubscript{A113}E\textsubscript{B81}

P = 0.0012