111. Tauber E, Kollaritsch H, von Sonnenburg F *et al.*: Randomized, double-blind, placebo-controlled Phase 3 trial of the safety and tolerability of IC51, an inactivated Japanese encephalitis vaccine. *J. Infect. Dis.* 198, 493–499 (2008).

112. Schuller E, Jilma B, Voicu V *et al.*: Long-term immunogenicity of the new Vero cell-derived, inactivated Japanese encephalitis virus vaccine IC51 six and 12 month results of a multicenter follow-up Phase 3 study. *Vaccine* 26, 4382–4386 (2008).

113. Schuller E, Klade CS, Wolfl G, Kaltenbock A, Dewasthaly S, Tauber E: Comparison of a single, high-dose vaccination regimen to the standard regimen for the investigational Japanese encephalitis vaccine, IC51: a randomized, observer-blind, controlled Phase 3 study. *Vaccine* 27, 2188–2193 (2009).

114. Kaltenbock A, Dubischar-Kastner K, Schuller E, Datla M, Klade CS, Kishore TS: Immunogenicity and safety of IXIARO((R)) (IC51) in a Phase II study in healthy Indian children between 1 and 3 years of age. *Vaccine* 28, 834–839 (2010).

- **Clinical trial of the IC51 vaccine in children. Since this new vaccine has been approved for adults only so far, this clinical trial to demonstrate its safety in children is important.**

115. Abe M, Shiosaki K, Hammar L *et al.*: Immunological equivalence between mouse brain-derived and Vero cell-derived Japanese encephalitis vaccines. *Virus. Res.* 121, 152–160 (2006).

116. Sugawara K, Nishiyama K, Ishikawa Y *et al.*: Development of Vero cell-derived inactivated Japanese encephalitis vaccine. *Biologicals* 30, 303–314 (2002).

117. Kuzuhara S, Nakamura H, Hayashida K *et al.*: Non-clinical and Phase I clinical trials of a Vero cell-derived inactivated Japanese encephalitis vaccine. *Vaccine* 21, 4519–4526 (2003).

118. Miyazaki C, Togashi T, Iribe K *et al.*: Phase III clinical trial of Vero cell derived inactivated vaccine against Japanese encephalitis. Presented at: *Proceeding of the 8th Annual Meeting of Japanese Society of Vaccinology* Osaka, Japan, 15–16 October (2005).

119. Ishikawa T: Development of new Japanese encephalitis vaccine. *Nippon. Rinsho.* 63, 2133–2137 (2005).

120. Instruction for use of Freeze-dried Japanese Encephalitis Vaccine (Cell Culture derived) JEBIK V. The Research Foundation for Microbial Diseases of Osaka University, Japan (2009).

121. Akechi M, Namazue J, Manabe S *et al.*: Clinical trials of the Freeze-dry Japanese encephalitis vaccine in children. Presented at: *Proceeding of the 13th annual meeting of Japanese Society for Vaccinology* Sapporo, Japan 26–27 September (2009).

122. Kikukawa A, Gomi Y, Akechi M *et al.*: Immunogenicity of freeze dried, cell culture-derived Japanese encephalitis vaccine (inactivated). Presented at: *Proceeeding of the 45th Annual Meeting of Ecology of Japanese Encephalitis Virus* Tokyo, Japan 28–29 May (2010).

123. Arai S, Matsunaga Y, Takasaki T *et al.*: Japanese encephalitis: surveillance and elimination effort in Japan from 1982 to 2004. *Jpn. J. Infect. Dis.* 61, 333–338 (2008).

124. Kim HC, Turell MJ, O'Guinn ML *et al.*: Historical review and surveillance of Japanese encephalitis, Republic of Korea, 2002–2004. *Entomol. Res.* 37, 267–274 (2007).

125. Wu YC, Huang YS, Chien LJ *et al.*: The epidemiology of Japanese encephalitis on Taiwan during 1966–1997. *Am. J. Trop. Med. Hyg.* 61, 78–84 (1999).

126. Huynh W, Cordato DJ, Kehdi E, Masters LT, Dedousis C: Post-vaccination encephalomyelitis: literature review and illustrative case. *J. Clin. Neurosci.* 15, 1315–1322 (2008).

127. Ohya T, Nagamitsu S, Yamashita Y, Matsuishi T: Serial magnetic resonance imaging and single photon emission computed tomography study of acute disseminated encephalomyelitis patient after Japanese encephalitis vaccination. *Kurume. Med. J.* 54, 95–99 (2007).

128. Okabe N: Background of recent JE vaccine issues. *Uirusu.* 55, 303–306 (2005).

129. Ferguson M, Kurane I, Wimalaratne O, Shin J, Wood D: WHO informal consultation on the scientific basis of specifications for production and control of inactivated Japanese encephalitis vaccines for human use, Geneva, Switzerland, 1–2 June 2006. *Vaccine* 25, 5233–5243 (2007).

130. Japanese encephalitis vaccines. *Wkly Epidemiol. Rec.* 81, 331–340 (2006).

131. Menge T, Kieseier BC, Nessler S, Hemmer B, Hartung HP, Stuve O: Acute disseminated encephalomyelitis: an acute hit against the brain. *Curr. Opin. Neurol.* 20, 247–254 (2007).

132. Sejvar JJ: Acute disseminated encephalomyelitis. *Curr. Infect. Dis. Rep.* 10, 307–314 (2008).

133. Hemachudha T, Griffin DE, Giffels JJ, Johnson RT, Moser AB, Phanuphak P: Myelin basic protein as an encephalitogen in encephalomyelitis and polyneuritis following rabies vaccination. *N. Engl. J. Med.* 316, 369–374 (1987).

134. Moore GR, Traugott U, Stone SH, Raine CS: Dose-dependency of MBP-induced demyelination in the guinea pig. *J. Neurol. Sci.* 70, 197–205 (1985).

135. Moore GR, McCarron RM, Traugott U, McFarlin DE, Raine CS: Critical threshold for dose of myelin basic protein in murine autoimmune encephalomyelitis. *J. Neurol. Sci.* 77, 173–184 (1987).

136. Momose H, Imai J, Hamaguchi I *et al.*: Induction of indistinguishable gene expression patterns in rats by Vero cell-derived and mouse brain-derived Japanese encephalitis vaccines. *Jpn. J. Infect. Dis.* 63, 25–30 (2010).

137. Chang KJ: Seasonal prevalence of anti-Japanese encephalitis virus antibody in pigs in different regions of Taiwan. *J. Microbiol. Immunol. Infect.* 35, 12–16 (2002).

138. Konishi E: Status of natural infection with Japanese encephalitis virus in Japan: prevalence of antibodies to the nonstructural 1 protein among humans and horses. *Vaccine* 27, 7129–7130 (2009).

139. Konishi E, Suzuki T: Ratios of subclinical to clinical Japanese encephalitis (JE) virus infections in vaccinated populations: evaluation of an inactivated JE vaccine by comparing the ratios with those in unvaccinated populations. *Vaccine* 21, 98–107 (2002).

140. Konishi E, Shoda M, Yamamoto S, Arai S, Tanaka-Taya K, Okabe N: Natural infection with Japanese encephalitis virus among inhabitants of Japan: a nationwide survey of antibodies against nonstructural 1 protein. *Vaccine* 24, 3054–3056 (2006).

141. Matsunaga T, Shoda M, Konishi E: Japanese encephalitis viral infection remains common in Japan. *Pediatr. Infect. Dis. J.* 27, 769–770 (2008).

142. 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).

143. Konishi E, Shoda M, Kondo T: Analysis of yearly changes in levels of antibodies to Japanese encephalitis virus nonstructural 1 protein in racehorses in central Japan shows high levels of natural virus activity still exist. *Vaccine* 24, 516–524 (2006).

144. Konishi E, Shoda M, Kondo T: Prevalence of antibody to Japanese encephalitis virus nonstructural 1 protein among racehorses in Japan: indication of natural infection and need for continuous vaccination. *Vaccine* 22, 1097–1103 (2004).

145. Das BP, Lal S, Saxena VK: Outdoor resting preference of *Culex tritaeniorhynchus*, the vector of Japanese encephalitis in Warangal and Karim Nagar districts, Andhra Pradesh. *J. Vector. Borne. Dis.* 41, 32–36 (2004).

146. Enserink M: What mosquitoes want: secrets of host attraction. *Science* 298, 90–92 (2002).

147. Steib BM, Geier M, Boeckh J: The effect of lactic acid on odour-related host preference of yellow fever mosquitoes. *Chem. Senses.* 26, 523–528 (2001).

148. Shimoda H, Okuda M, Iwata H, Mochizuki M, Maeda K: Seroprevalence of Japanese encephalitis virus infection in dogs. Presented at: *Proceeding of the 44th Annual Meeting of Ecology of Japanese encephalitis virus.* Chitose, Japan, 19–20 June (2009).

149. Yoshikawa A, Inoue S, Agoh M, Morita K: Serological surveillance on Japanese encephalitis virus infection of wild boars in Nagasaki. Presented at: *Proceeeding of the 44th Annual Meeting of Ecology of Japanese encephalitis virus.* Chitose, Japan, 19–20 June (2009).

150. Takasaki T, Kotaki A, Tajima S, Omatsu T, Lim CK, Kurane I: Isolation of Japanese encephalitis virus from wild boar and its characterization. Presented at: *Proceeding of the 57th Annual Meeting of Japanese Society for Virology.* Tokyo, Japan, 25–27 October (2009).

151. Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T: Bats: important reservoir hosts of emerging viruses. *Clin. Microbiol. Rev.* 19, 531–545 (2006).

152. van den Hurk AF, Smith CS, Field HE *et al.*: Transmission of Japanese Encephalitis virus from the black flying fox, *Pteropus alecto*, to *Culex annulirostris* mosquitoes, despite the absence of detectable viremia. *Am. J. Trop. Med. Hyg.* 81, 457–462 (2009).

153. Gratz NG: Critical review of the vector status of *Aedes albopictus*. *Med. Vet. Entomol.* 18, 215–227 (2004).

154. Paupy C, Delatte H, Bagny L, Corbel V, Fontenille D: *Aedes albopictus*, an arbovirus vector: from the darkness to the light. *Microbes. Infect.* 11, 1177–1185 (2009).

155. Tsuda Y, Kim KS: Sudden autumnal appearance of adult *Culex tritaeniorhynchus* (Diptera: *Culicidae*) at a park in urban Tokyo: first field evidence for prediapause migration. *J. Med. Entomol.* 45, 610–616 (2008).

156. Byrne K, Nichols RA: *Culex pipiens* in London Underground tunnels: differentiation between surface and subterranean populations. *Heredity* 82, 7–15 (1999).

157. Weng MH, Lien JC, Wang YM, Wu HL, Chin C: Susceptibility of three laboratory strains of *Aedes albopictus* (Diptera: Culicidae) to Japanese encephalitis virus from Taiwan. *J. Med. Entomol.* 34, 745–747 (1997).

158. Turell MJ, Mores CN, Dohm DJ *et al.*: Laboratory transmission of Japanese encephalitis and West Nile viruses by molestus form of *Culex pipiens* (Diptera: *Culicidae*) collected in Uzbekistan in 2004. *J. Med. Entomol.* 43, 296–300 (2006).

159. Weng MH, Lien JC, Wang YM, Lin CC, Lin HC, Chin C: Isolation of Japanese encephalitis virus from mosquitoes collected in Northern Taiwan between 1995 and 1996. *J. Microbiol. Immunol. Infect.* 32, 9–13 (1999).

160. Kuwayama M, Ito M, Takao S *et al.*: Japanese encephalitis virus in meningitis patients, Japan. *Emerg. Infect. Dis.* 11, 471–473 (2005).

161. Nerome R, Tajima S, Takasaki T *et al.*: Molecular epidemiological analyses of Japanese encephalitis virus isolates from swine in Japan from 2002 to 2004. *J. Gen. Virol.* 88, 2762–2768 (2007).

Websites

201. WHO. Japanese encephalitis reported cases http://www.who.int/immunization_monitoring/en/globalsummary/timeseries/tsincidencejap.htm

202. CDC. Japanese encephalitis (JE) http://wwwnc.cdc.gov/travel/yellowbook/2010/chapter-2/japanese-encephalitis.aspx

203. WHO. WHO Vaccine Preventable Diseases Monitoring System http://www.who.int/immunization_monitoring/en/globalsummary/scheduleselect.cfm

204. Notification of the suspension of strong recommendation of the mouse brain derived Japanese encephalitis vaccine http://www.mhlw.go.jp/topics/2005/05/dl/tp0530-1a.pdf (in Japanese)

205. Standards for Biological products http://www.nih.go.jp/niid/MRBP/files/2009_jp.pdf (in Japanese)

206. Reports on immunization strategy for vaccine-preventable diseases http://mhlw-grants.niph.go.jp/niph/search/Download.do?nendo=2007&jigyoId=073091&bunkenNo=200726017A&pdf=20072601 7A0006.pdf. (in Japanese)

207. Reports on prevention of arthoropod-borne infectious diseases http://mhlw-grants.niph.go.jp/niph/search/Download.do?nendo=2008&jigyoId=083091&bunkenNo=200829008B&pdf=20082900 8B0005.pdf (in Japanese)

208. Prevalence of JE in Japan in 40's http://wwwhakusyo.mhlw.go.jp/wpdocs/hpaz196801/b0030.html (in Japanese)

209. The Ministry of Health, Labour and Welfare. Japanese encephalitis FAQ http://www.mhlw.go.jp/qa/kenkou/nouen/index.html (in Japanese)

210. Current status of JE vaccination in Japan http://idsc.nih.go.jp/iasr/30/352/dj3521.html

Journal of Virological Methods 171 (2011) 123–128

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Protocols

Non-structural protein 1 (NS1) antibody-based assays to differentiate West Nile (WN) virus from Japanese encephalitis virus infections in horses: Effects of WN virus NS1 antibodies induced by inactivated WN vaccine

Yoko Kitai[a], Takashi Kondo[b], Eiji Konishi[a,c,*]

[a] Division of Infectious Diseases, Department of International Health, Kobe University Graduate School of Health Sciences, Kobe, Japan
[b] Epizootic Research Center, Equine Research Institute, Japan Racing Association, Tochigi, Japan
[c] Division of Vaccinology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan

Article history:
Received 12 June 2010
Received in revised form 11 October 2010
Accepted 16 October 2010
Available online 23 October 2010

Keywords:
West Nile virus
Inactivated vaccine
Non-structural protein 1

ABSTRACT

Antibodies to non-structural protein 1 (NS1) of West Nile virus (WNV) have been used to differentiate WNV infection from infection by serologically cross-reactive flaviviruses, including Japanese encephalitis virus (JEV), in horses. However, since the inactivated West Nile (WN) vaccine has been reported to induce NS1 antibodies, there is concern about the reliability of using NS1-based assays for testing vaccinated horses. Therefore, the effect of inactivated WN vaccine-induced antibodies on an epitope-blocking ELISA and complement-dependent cytotoxicity (CDC) assay were investigated. Both assays are based on NS1 antibodies and were established previously to differentiate WNV from JEV infections in horses. Groups of three horses were vaccinated with two or three doses of a commercial inactivated WN vaccine and NS1 antibodies were detected by a conventional ELISA after the second vaccination. Vaccine-induced NS1 antibodies were also detected by blocking ELISA and a CDC assay and affected the ability of these assays to differentiate WNV from JEV infections. However, the effect was less significant in the CDC assay, where use of a low serum concentration ensured effective differentiation. The more efficient detection of infection-induced antibodies over vaccine-induced antibodies by the CDC assay was potentially attributable to the different IgG isotype profiles of these antibodies.

1. Introduction

West Nile (WN) fever and WN encephalitis are serious diseases in humans and horses. The causative agent is WN virus (WNV), a member of the *Flavivirus* genus, which belongs to the *Flaviviridae* family. WNV also belongs to the Japanese encephalitis (JE) serocomplex, which includes Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV) and Saint Louise encephalitis virus (SLEV) (Gubler et al., 2007). The rapid expansion of WNV in the Western hemisphere, following the invasion of this virus into the USA in 1999 (Petersen and Hayes, 2008), prompted the Japanese government to create manuals and guidelines for serological testing of WNV (Kobayashi and Kurane, 2003; Kurane, 2005; Ministry of Agriculture, Forestry and Fisheries and Ministry of Health, Labour and Welfare, Japan, 2003). An inactivated vaccine was approved in preparation for the arrival of WNV in Japan.

JEV is currently an infectious cause of disease in Japan. Since disease manifestations are similar between WNV and JEV infections (Castillo-Olivares and Wood, 2004; Endy and Nisalak, 2002; Gould and Solomon, 2008), laboratory tests are essential for differential diagnosis of WNV from JEV infections. Although virus isolation or viral RNA detection methods produce a firm diagnosis, the short time period of viremia or RNAemia limits the use of these methods (Sejvar and Marfin, 2006). Thus, antibody testing constitutes a more practicable alternative. However, diagnosis of WNV infection by detection of antibodies is complicated by the serological cross-reactivity among the JE serocomplex, even when using a neutralization test that provides high specificity (Kuno, 2003). For instance, when individuals preimmune to JEV were infected with WNV in animal models, including mice (Lim et al., 2008), horses (Shirafuji et al., 2009) and pigs (Williams et al., 2001), high levels of cross-reactive neutralizing antibodies to JEV were induced by anamnestic responses, which were equivalent to, or even higher than, antibody levels induced against WNV.

Antibody assays targeting the non-structural protein 1 (NS1) have been established to overcome the diagnostic problems caused by the cross-reactivity of flaviviruses (Blitvich et al., 2003; Hall et al., 1995; Kitai et al., 2007, 2010). Flavivirus NS1 contains more

* Corresponding author at: Department of International Health, Kobe University Graduate School of Health Sciences, 7-10-2, Tomogaoka, Suma-ku, Kobe 654-0142, Japan. Tel.: +81 78 796 4594; fax: +81 78 796 4594.
E-mail address: ekon@kobe-u.ac.jp (E. Konishi).

0166-0934/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2010.10.012

species-specific antigenic epitopes than the envelope (E) protein (Hall et al., 1990). In fact, western blot analyses using WNV-positive human plasma and chicken sera and dengue virus-positive human sera indicated that NS1 was more species-specific than the E protein (Oceguera et al., 2007). Using an NS1-based strategy, epitope-blocking ELISAs have been established to differentiate WNV infections from infections with SLEV (Blitvich et al., 2003) or MVEV (Hall et al., 1995). More recently, an epitope-blocking ELISA (Kitai et al., 2007) and a complement-dependent cytotoxicity (CDC) assay (Kitai et al., 2010) have been developed using this strategy to differentiate WNV from JEV infections in horses. The ability of these two assays to differentiate WNV from JEV infections has been demonstrated based on the assay cutoff that was obtained by using WNV antibody-negative control horses including those naturally infected with JEV. However, a drawback of the NS1-based strategy is that assays may be greatly affected by a vaccine that induces NS1 antibodies in the host. In horses, it was reported that the inactivated WN vaccine (Innovator, Fort Dodge Animal Health) induced antibodies to NS1 but a recombinant canarypox WN vaccine (Recombitek, Merial Limited) did not (Balasuriya et al., 2006). The present study aimed to investigate the effects of NS1 antibodies induced by the inactivated WN vaccine on the ability of the blocking ELISA and CDC assay to differentiate WNV from JEV infections.

2. Materials and methods

2.1. Virus strain

The NY99 strain of WNV, isolated from an infected horse, was obtained from the National Veterinary Services Laboratories, United States Department of Agriculture (Ames, IA, USA). This strain was passaged twice through Vero cells (JCRB9013: Japanese Collection of Research Bioresources, Osaka, Japan) and the infected culture fluids were used for neutralization tests.

2.2. Monoclonal antibodies

A monoclonal antibody, WN-2H4, specific for NS1 of WNV (Kitai et al., 2007) and monoclonal antibodies specific for equine IgGa, IgGb, IgGc and IgG(T) (Sugiura et al., 1998) have been described previously. These monoclonal antibodies were obtained in an ascites form from pristane-primed BALB/c mice and the IgG fraction was separated by precipitation with saturated ammonium sulfate followed by extensive dialysis against phosphate-buffered saline.

2.3. Serum samples

Six yearlings (Horses #1–6) were immunized intramuscularly with two or three doses of inactivated WN vaccine (Innovator, Fort Dodge Animal Health, Fort Dodge, IA, USA) at three-week intervals and bled until 16 weeks after vaccination. Sera collected previously from two yearlings at 28 days after experimental infection (Kitai et al., 2007) were used as a positive control in the conventional ELISA or for comparison in the IgG isotype profiles. For analyzing CDC activities in relation to IgG isotypes, an ICR mouse was immunized repeatedly by intraperitoneal inoculation with inactivated WN vaccine (Innovator, Fort Dodge Animal Health), while infected mouse sera collected previously from two mice 34 days after infection with WNV (Ishikawa et al., 2007) were pooled and used in this study. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University and the Equine Research Institute.

2.4. WNV NS1-expressing cells

The generation of cell lines transfected stably with the NS1 (2G2 cells) or NS1/NS2A genes (2G12 cells) of WNV has been described (Kitai et al., 2007). Briefly, CHO cells were transfected with a pcDNA3-based plasmid expressing the NS1 or NS1/NS2A gene of the WNV Eg101 strain, selected using G418-containing medium, and then cloned by limiting dilution. High percentages (80–100%) of 2G2 or 2G12 cells expressed the NS1 antigen, as determined by immunostaining. 2G2 cells were used as the antigen for the CDC assay, while the culture fluids of 2G2 or 2G12 cells containing secreted NS1 were used as the antigen for the conventional ELISA for detecting antibodies against NS1 of WNV, or the blocking ELISA, respectively.

2.5. Conventional ELISA to measure NS1 antibody levels

Antibody levels to WNV NS1 in horse and mouse sera were measured by a conventional ELISA, essentially following a method described previously (Kitai et al., 2007). Briefly, microplates sensitized with 3 ng of WNV NS1 antigen per well were incubated serially with test sera (1:100 dilution), conjugates and *p*-nitrophenyl phosphate. The WNV NS1 antigen was affinity-purified from culture fluids of 2G2 cells using the monoclonal antibody WN-2H4. The conjugates used in this study were alkaline phosphatase-conjugated rabbit anti-horse IgG (gamma chain-specific; Rockland Immunochemicals, Gilbertsville, PA, USA) and goat anti-mouse IgG (Fc fragment-specific; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). To minimize nonspecific reactions, a non-sensitized control plate was run in parallel, and the difference in absorbances from antigen-sensitized wells was recorded. Serum from a horse at 28 days after experimental infection with WNV (Horse #1; Kitai et al., 2007) was used as a positive control to minimize interplate variations in the ELISA for measuring antibody levels in horses.

2.6. Epitope-blocking ELISA

The epitope-blocking ELISA for differentiating WNV NS1 from JEV NS1 antibodies in horse sera was performed as described previously (Kitai et al., 2007). Briefly, microplates were incubated serially with: (i) rabbit serum hyperimmune to NS1 of WNV; (ii) culture fluids of 2G12 cells; (iii) test sera or ELISA diluent; (iv) WN-2H4 or affinity-purified mouse IgG1 (Bethyl Laboratories, Montgomery, TX, USA); (v) alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories); and (vi) *p*-nitrophenyl phosphate. Test sera were incubated in parallel with the ELISA diluent in step (iii), and WN-2H4 (isotype; IgG1) with mouse IgG1 (without any anti-WNV activity) in step (iv) to minimize nonspecific reactions. The percentage of inhibition of monoclonal antibody binding was calculated from the absorbances using the following formula: $100 - 100 \times [(A - B)/(C - D)]$, where A is the absorbance obtained with a combination of steps (iii) and (iv) with test sera and WN-2H4, B is obtained with the test sera and purified IgG1, C is obtained with ELISA diluent and WN-2H4, and D is obtained with ELISA diluent and purified IgG1, respectively. Sera with inhibition values of 27.6% or greater were determined to be positive for antibodies to WNV NS1.

2.7. CDC assay

The CDC assay for measuring antibodies to WNV NS1 was performed as described previously (Kitai et al., 2010). Briefly, 50 μl of serum-free minimal essential medium (SF-MEM) containing 5×10^4 2G2 cells was mixed with an equal volume of heat-inactivated test serum diluted in SF-MEM and incubated

on ice for 30 min. Then, 11 μl of rabbit complement (Low-Tox-M Rabbit Complement; Cedarlane, Hornby, Canada) were added (final concentration, 10%) and incubated at 37 °C for 2 h. Following centrifugation, 50 μl of the supernatant was mixed with 50 μl of a lactose dehydrogenase (LDH) substrate (Cytotoxicity Detection Kit Plus [LDH]; Roche, Mannheim, Germany) and incubated at room temperature for 15 min, followed by spectrophotometry at 490 nm. The percentage of specific cell lysis was calculated according to the manufacturer's instructions using the following formula: $100 \times [(A - C)/(B - C)]$, where A represents the absorbance obtained with the test serum (experimental release), B represents the absorbance obtained by lysing all of the target cells with 1% Triton X-100 (maximum release), and C represents the absorbance obtained with target cells incubated in SF-MEM containing rabbit complement at 10% (minimum release). The cutoff for specific lysis to differentiate positive from negative sera for WNV NS1 antibodies was 19.8%. In the one-dilution method, the percentage of specific cell lysis obtained in 1:10–1:80 dilutions of sera was used as the WNV NS1 antibody level. In the endpoint method, the WNV NS1 antibody titer was expressed as the highest serum dilution giving greater than 19.8% specific lysis.

2.8. IgG isotyping

The IgG isotypes of WNV-specific antibodies were determined by conventional ELISA, using monoclonal antibodies specific for each equine IgG isotype prepared in our laboratory (Sugiura et al., 1998), commercial horseradish peroxidase-conjugated goat anti-horse IgGa, IgGc or IgG(T) or sheep anti-horse IgGb (Bethyl Laboratories), or alkaline phosphatase-conjugated goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (Southern Biotech, Birmingham, AL, USA). The antibody titer was expressed as the maximum serum dilution showing an absorbance greater than an arbitrary cutoff of 0.2.

2.9. Neutralization test

Neutralizing antibody titers were determined by a plaque reduction assay using the NY99 strain of WNV and Vero cells. The neutralizing antibody titer was expressed as the highest serum dilution yielding a 50% reduction in plaque number.

3. Results

3.1. Time course of WNV NS1 antibodies in sera from horses vaccinated with inactivated WN vaccine

Two groups of three yearlings were immunized with two or three doses of the inactivated WN vaccine and bled until 16 weeks after vaccination (Fig. 1). Prior to evaluation of the blocking ELISA and the CDC assay, sera were tested by the neutralizing test and a conventional ELISA to measure WNV NS1 antibodies (Fig. 1, upper panels). Neutralizing antibody titers began to increase after the second dose in all horses, demonstrating the effectiveness of the vaccine. Overall, three doses induced higher titers than two doses. The time courses of NS1 antibodies paralleled those of neutralizing antibodies in all horses. These results indicated that NS1 antibodies were induced by the inactivated WN vaccine in horses after the second dose, and suggested that NS1 antigen was contained in the vaccine preparation along with E antigen, the main component of the inactivated WN vaccine relevant to the induction of neutralizing antibodies.

3.2. Effect of NS1 antibodies on the blocking ELISA

In the blocking ELISA (Fig. 1, middle panels), the time courses of the inhibition percentages were similar to those obtained using the conventional ELISA. Specifically, all horses became positive after the second dose and remained so until the end of the experimental period (week 16), except for Horse #4 in which the NS1 antibodies (determined by the conventional ELISA) were at the lowest level among the six horses. This result indicated that NS1 antibodies induced by the inactivated WN vaccine were detected by the blocking ELISA.

3.3. Effect of NS1 antibodies on the CDC assay

The vaccine-induced NS1 antibodies were also detected by the end-point method of the CDC assay (Fig. 1, middle panels). However, two of the five horses (excluding Horse #4, which was negative throughout the experimental period) became negative at the end of the experimental period. Furthermore, when sera were tested at a 1:80 dilution in the one-dilution method where specific lysis of 19.8% is set as a cutoff value (Fig. 1, lower panels), almost all samples showed low percentages of specific lysis and only two samples were determined as positive and these were only positive for a limited period after the booster dose (Horse #1 was positive at weeks 6 and 8, and Horse #6 at week 5). These results indicated that although vaccine-induced NS1 antibodies were detectable in the CDC assay, the time period of positive detection was not as long as that shown by the blocking ELISA and was shortest when a 1:80 dilution of serum samples was used in the one-dilution method.

3.4. Comparison between IgG isotypes induced by infection and vaccination in mice and horses

To investigate the mechanism underlying the low NS1 antibody levels shown by the CDC assay, the IgG isotypes of NS1 antibodies induced by the inactivated WN vaccine were analyzed and compared with those induced by infection (Fig. 2A–C). Using a mouse model, the NS1 antibody titers of vaccinated or infected mice were found to be 1:400 or 1:1600, respectively, with an approximately 4-fold lower titer induced by vaccination than by infection, as determined by the conventional ELISA (Fig. 2A, upper panel; open and closed circles). By contrast, the CDC assay provided equivalent percentages of specific lysis (approximately 15%) in vaccinated and infected mice at serum dilutions of 1:100 and 1:1,600, respectively, with a 16-fold difference between vaccinated and infected mice (Fig. 2A, lower panel; open and closed circles). Thus, CDC activity relative to the ELISA antibody level was approximately 4-fold higher in infected than vaccinated mice. For IgG isotyping, ELISA antibody titers obtained with vaccinated and infected mice for each isotype were normalized against those obtained for whole IgG. As shown in Fig. 2B, IgG1 was induced at a higher level by vaccination than by infection, whereas other isotypes (IgG2a, IgG2b and IgG3) showed the opposite profile. Furthermore, a monoclonal antibody of the IgG1 isotype (WN-2H4) showed little CDC activity, in contrast to the high reactivities observed in the conventional ELISA (Fig. 2A; diamonds). These results indicated that the CDC assay showed lower antibody levels in vaccinated than infected mice, and this difference was related to the fact that the IgG1 antibodies predominantly induced by vaccination did not show CDC activity in mice.

Next, the IgG isotypes induced by infection or vaccination were compared in horses (Fig. 2C). Sera selected for this comparison were from Horse #2 at six weeks after the first dose of inactivated WN vaccine, which was obtained in the present study, and from Horse #2 at 28 days after experimental infection with WNV, which was obtained in a previous study (Kitai et al., 2007). These sera

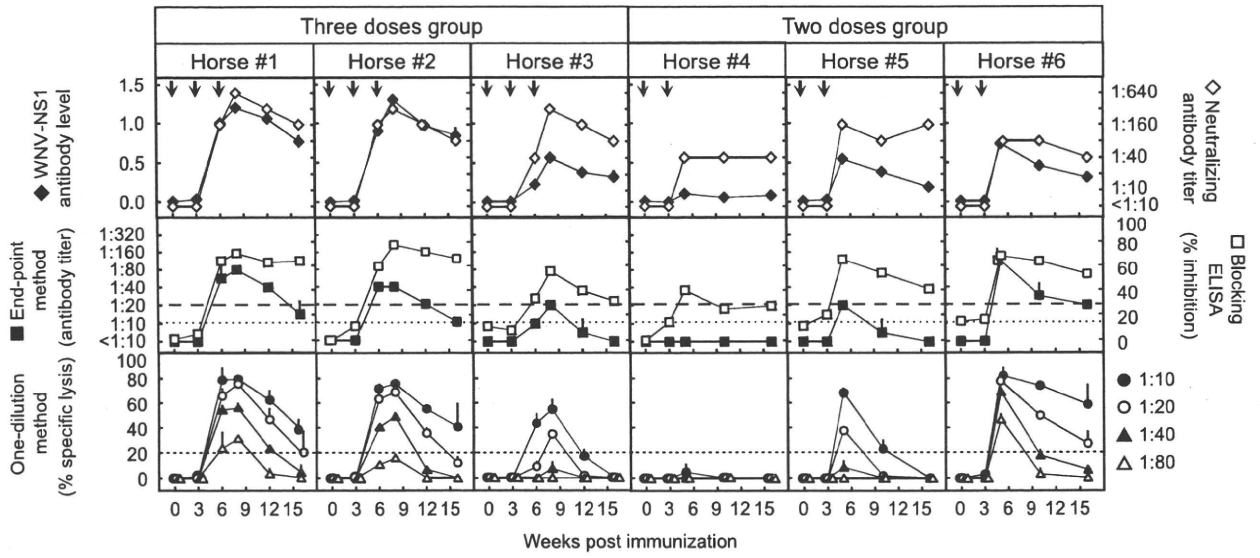

Fig. 1. Time courses of NS1 and neutralizing antibodies in sera serially collected from horses vaccinated with inactivated WN vaccine. Sera from horses immunized with three or two doses of inactivated WN vaccine were tested by the neutralization test (open diamond), conventional ELISA (closed diamond), blocking ELISA (open square), and the CDC assay to measure WNV NS1 antibody. In the CDC assay, sera were tested by the one-dilution method at serum dilutions of 1:10 (closed circles), 1:20 (open circles), 1:40 (closed triangles) and 1:80 (open triangles), and by the end-point method (closed squares). Each datum of the conventional ELISA and CDC assay represents the average value obtained in two separate experiments (standard deviations are indicated by bars). Dotted lines indicate the cutoff values using the one-dilution (19.8%) or end-point (1:10) methods of the CDC assay. Dashed lines indicate the cutoff value in the blocking ELISA (27.6%). Arrows indicate the time of vaccination.

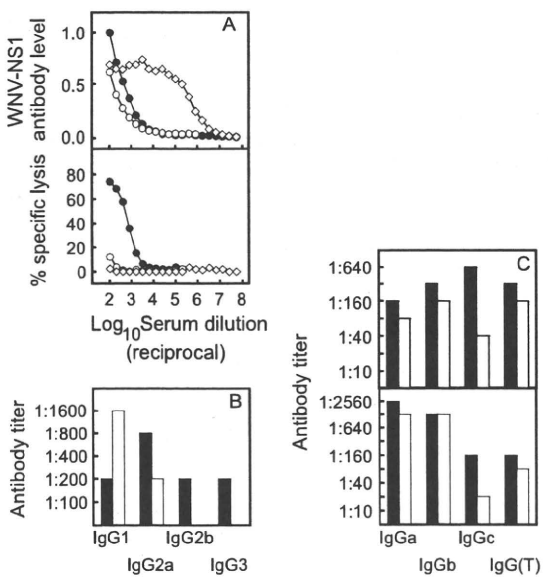

Fig. 2. Isotype analysis of NS1 antibodies induced by vaccination with the inactivated WN vaccine in comparison with those induced by infection. (A) Comparison of the dose–response curves obtained with sera from WNV-infected mice (closed circle), a mouse vaccinated with the inactivated WN vaccine (open circle), and a monoclonal antibody to WNV NS1 (WN-2H4, open diamond), in a conventional ELISA to measure antibodies to WNV NS1 (upper panel) and the CDC assay (lower panel). (B) Comparison of ELISA antibody titers of each IgG isotype in sera from infected (closed bar) or vaccinated (open bar) mice. Titers obtained with infected and vaccinated mice were normalized with antibody titers obtained with whole IgG in the conventional ELISA: the antibody titer of serum pooled from infected mice was 4-fold higher than that of a vaccinated mouse using the conventional ELISA. (C) Comparison of ELISA antibody titers of each IgG isotype induced in infected or vaccinated horses. Sera from an infected horse (Horse #2; Kitai et al., 2007) at 28 days after experimental infection with WNV (closed bar) and from Horse #2 (in the present study) at six weeks after the first vaccination (open bar) were used. Results were obtained using monoclonal antibodies to equine IgG isotypes (upper panel) or commercial conjugates (lower panel) as detector antibodies.

were selected because they showed equivalent ELISA antibody levels (0.993 and 1.101) at a serum dilution of 1:100 and the same ELISA antibody titer (1:800), whereas the geometric mean CDC antibody titers were 1:40 and 1:226 in vaccinated and infected horses, respectively. Two different panels of specific antibodies, in-house monoclonals and commercial polyclonals, were used to detect each isotype in this ELISA for more reliable isotyping of horse IgG. Although IgGa, IgGb and IgG(T) antibodies, which have complement fixing activities, showed lower titers in vaccinated than infected horses for most isotypes, the differences in the isotypes of induced antibodies between vaccinated and infected horses were not as clear as those in mice.

4. Discussion

The findings of this study confirmed those of a previous study by Balasuriya et al. (2006), which reported that inactivated WN vaccine could induce NS1 antibodies in horses: the same vaccine (Innovator, Fort Dodge Animal Health) was used in the present study as had been employed in the study by Balasuriya et al. (2006). The phenomenon was not observed with inactivated JE vaccines that did not induce NS1 antibodies in horses (Konishi et al., 2004). Although attempts to detect NS1 antigens in the inactivated WN vaccine failed (data not shown), it is highly probable that NS1 antigens are present with this vaccine preparation: horses vaccinated with three doses induced higher NS1 antibody levels than those vaccinated with two doses. The process of vaccine preparation probably differs between WN and JE vaccines in terms of the residual NS1 antigens. Although an earlier report showed the presence of NS1 antibodies in horses vaccinated with the inactivated WN vaccine in a particular time point (Balasuriya et al., 2006), the present study investigated the time course of NS1 antibody responses, in relation to their effects on NS1 antibody-based assays.

The ability of the blocking ELISA and the CDC assay to differentiate WNV from JEV infections is based on significant differences in assay results between WNV- and JEV-infected horses, and is therefore disturbed by NS1 antibodies induced in horses vaccinated with inactivated WN vaccine. The present study demonstrated that

inactivated WN vaccine-induced NS1 antibodies were detected by both assays. However, positive results were obtained in a shorter time period in the CDC assay (especially in the one-dilution method using a 1:80 dilution of serum samples) than the blocking ELISA. The 1:80 dilution in the CDC assay is a stringent criterion by which false-positive results by JEV NS1 antibodies are unlikely to be obtained in horses including those vaccinated with inactivated JE vaccine or naturally infected with JEV (Kitai et al., 2010). Using this cutoff point (1:80 dilution), four of the six vaccinated horses were negative for WNV NS1 antibodies throughout the experimental period, while the other two horses were positive for WNV NS1 antibodies for a short period after the booster immunization. On the other hand, horses infected experimentally with WNV that became positive for CDC antibody levels/titers 12–18 days post-infection remained positive until the end of the experimental period (35 days post-infection; Kitai et al., 2010). In addition, quantitative comparisons between the results of the present study and those of a previous study (Kitai et al., 2010) indicated that CDC antibody levels relative to those obtained by a conventional ELISA were lower in vaccinated than infected horses (data not shown). Therefore, the CDC assay is useful for differentiating WNV from JEV infections even in horses vaccinated with the inactivated WN vaccine, provided that this stringent cutoff point (1:80 dilution) is applied.

The IgG isotypes induced and their involvement in CDC activity may be one explanation for the differences in CDC antibody responses induced by infection or vaccination. In general, complement-fixing activities differ between IgG isotypes in mice (Frank and Fries, 1989), humans (Burton and Woof, 1992; Frank and Fries, 1989), or horses (Lewis et al., 2008). Also, the IgG isotype profiles differ between antibodies induced by infection and by protein-based vaccines in horses (Nelson et al., 1998; Sheoran et al., 1997), mice (Simmons et al., 2001) or humans (Giammanco et al., 2003; Huang et al., 2006). In the present study, the relationship between CDC antibody levels and IgG isotypes was shown clearly in a mouse model (Fig. 2A and B). However, isotype analyses in horses did not show differences between infected and vaccinated animals, using sera with equivalent antibody levels in a conventional ELISA (Fig. 2C). The complement fixing activity of equine IgG isotypes remains debatable, since the descriptions in earlier studies with IgGa, IgGb, IgGc and IgG(T) isotypes are different from a recent report that regrouped them as IgG1 to IgG7 (Wagner, 2006; Wagner et al., 2004). Complement-fixing activity has been associated with IgGa and IgGb isotypes according to a report by McGuire et al. (1973). However, IgG(T) may also exhibit this type of activity, since complement-fixing activity is shown by the recently described IgG1, IgG3, IgG4 and IgG7 isotype categories and IgG(T) is related to IgG3 (Lewis et al., 2008). Since horses infected experimentally used in the previous study (Kitai et al., 2007) did not develop any clinical manifestations, symptomatic horses seem to show higher NS1 antibody levels in the CDC assay, providing greater differences in CDC antibody levels from vaccinated horses.

It is assumed that blocking ELISAs based on antibodies to NS1 that are used to differentiate WNV/SLEV (Blitvich et al., 2003) or Kunjin virus/MVEV (Hall et al., 1995) infections are also affected by WNV NS1 antibodies induced by inactivated WN vaccine. In the USA, three WN vaccines for horses, in addition to inactivated vaccines, have been licensed (Kramer et al., 2008). A yellow fever virus (YFV) 17D-based chimeric virus vaccine (Monath, 2001) can induce antibodies to NS1 of YFV, which may cross-react with WNV NS1; however, the canarypox virus-based recombinant and DNA vaccines that lack any flavivirus NS1 gene do not appear to induce antibodies to NS1. In fact, no horses vaccinated with the canarypox virus-based recombinant vaccine induced NS1 antibodies (Balasuriya et al., 2006). In Japan, an inactivated WN vaccine has been licensed but has not yet been employed, since WNV is still absent from Japan. Therefore, for now both assays are useful for differentiating WNV from JEV infections. Under conditions where an inactivated vaccine is in general use, the CDC assay would be a preferable method for differential diagnosis of these viruses.

In conclusion, NS1 antibody-based assays for differentiating WNV from JEV infections are affected by NS1 antibodies induced by inactivated WN vaccine, compromising an effective diagnosis. However, the findings of the present study show that a CDC assay may provide a solution to this problem. A functional assay, like a CDC assay, was found to be less significantly affected by vaccine-induced NS1 antibodies than a binding assay, like a blocking ELISA. Nevertheless, it is still important to confirm the vaccine history prior to differentiation of WNV from other flavivirus infections by NS1 antibody-based assays.

Conflicts of interest

None of the authors have any conflict of interest in relation to the content of the present work.

Acknowledgments

This study was supported in part by grants from the Japan Racing Association, from the Research on Emerging and Re-emerging Infectious Diseases, the Ministry of Health, Labour and Welfare of Japan (H20-Shinkou-ippan-003), and from the Program of Founding Research Centers for Emerging and Re-emerging Infectious Diseases, the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

References

Balasuriya, U.B., Shi, P.Y., Wong, S.J., Demarest, V.L., Gardner, I.A., Hullinger, P.J., Ferraro, G.L., Boone, J.D., De Cino, C.L., Glaser, A.L., Renshaw, R.W., Ledizet, M., Koski, R.A., MacLachlan, N.J., 2006. Detection of antibodies to West Nile virus in equine sera using microsphere immunoassay. J. Vet. Diagn. Invest. 18, 392–395.

Blitvich, B.J., Marlenee, N.L., Hall, R.A., Calisher, C.H., Bowen, R.A., Roehrig, J.T., Komar, N., Langevin, S.A., Beaty, B.J., 2003. Epitope-blocking enzyme-linked immunosorbent assays for the detection of serum antibodies to West Nile virus in multiple avian species. J. Clin. Microbiol. 41, 1041–1047.

Burton, D.R., Woof, J.M., 1992. Human antibody effector function. Adv. Immunol. 51, 1–84.

Castillo-Olivares, J., Wood, J., 2004. West Nile virus infection of horses. Vet. Res. 35, 467–483.

Endy, T.P., Nisalak, A., 2002. Japanese encephalitis virus: ecology and epidemiology. Curr. Top. Microbiol. Immunol. 267, 11–48.

Frank, M.M., Fries, L.F., 1989. Complement. In: Paul, W.E. (Ed.), Fundamental Immunology, 2nd ed. Raven Press, New York, pp. 679–701.

Giammanco, A.S., Taormina, A., Chiarini, G., Dardanoni, G., Stefanelli, P., Salmaso, S., Mastrantonio, P., 2003. Analogous IgG subclass response to pertussis toxin in vaccinated children, healthy or affected by whooping cough. Vaccine 21, 1924–1931.

Gould, E.A., Solomon, T., 2008. Pathogenic flaviviruses. Lancet 371, 500–509.

Gubler, D.J., Kuno, G., Markoff, L., 2007. Flaviviruses. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, pp. 1153–1252.

Hall, R.A., Broom, A.K., Hartnett, A.C., Howard, M.J., Mackenzie, J.S., 1995. Immunodominant epitopes on the NS1 protein of MVE and KUN viruses serve as targets for a blocking ELISA to detect virus-specific antibodies in sentinel animal serum. J. Virol. Methods 51, 201–210.

Hall, R.A., Kay, B.H., Burgess, G.W., Clancy, P., Fanning, I.D., 1990. Epitope analysis of the envelope and non-structural glycoproteins of Murray Valley encephalitis virus. J. Gen. Virol. 71, 2923–2930.

Huang, C.F., Lin, S.S., Ho, Y.C., Chen, F.L., Yang, C.C., 2006. The immune response induced by hepatitis B virus principal antigens. Cell. Mol. Immunol. 3, 97–106.

Ishikawa, T., Takasaki, T., Kurane, I., Nukuzuma, S., Kondo, T., Konishi, E., 2007. Co-immunization with West Nile DNA and inactivated vaccines provides synergistic increases in their immunogenicities in mice. Microbes Infect. 9, 1089–1095.

Kitai, Y., Kondo, T., Konishi, E., 2010. Complement-dependent cytotoxicity assay for differentiating West Nile virus from Japanese encephalitis virus infections in horses. Clin. Vaccine Immunol. 17, 875–878.

Kitai, Y., Shoda, M., Kondo, T., Konishi, E., 2007. Epitope-blocking enzyme-linked immunosorbent assay to differentiate West Nile virus from Japanese encephalitis virus infections in equine sera. Clin. Vaccine Immunol. 14, 1024–1031.

Kobayashi, M., Kurane, I., 2003. Guidelines for the Countermeasure to Vector Mosquitoes of West Nile Fever. National Institute of Infectious Diseases, Tokyo, Japan (in Japanese).

Konishi, E., Shoda, M., Ajiro, N., Kondo, T., 2004. Development and evaluation of an enzyme-linked immunosorbent assay for quantifying antibodies to Japanese encephalitis virus nonstructural 1 protein to detect subclinical infections in vaccinated horses. J. Clin. Microbiol. 42, 5087–5093.

Kramer, L.D., Styer, L.M., Ebel, G.D., 2008. A global perspective on the epidemiology of West Nile virus. Annu. Rev. Entomol. 53, 61–81.

Kuno, G., 2003. Serodiagnosis of flaviviral infections and vaccinations in humans. Adv. Virus Res. 61, 3–65.

Kurane, I., 2005. West Nile fever. In: Japan Medical Association, Ministry of Health, Labour and Welfare (Eds.), Guidelines for Diagnosis and Treatments of Infectious Diseases 2004. Igakushoin, Tokyo, Japan, pp. 104–105 (in Japanese).

Lewis, M.J., Wagner, B., Woof, J.M., 2008. The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies. Mol. Immunol. 45, 818–827.

Lim, C.K., Takasaki, T., Kotaki, A., Kurane, I., 2008. Vero cell-derived inactivated West Nile (WN) vaccine induces protective immunity against lethal WN virus infection in mice and shows a facilitated neutralizing antibody response in mice previously immunized with Japanese encephalitis vaccine. Virology 374, 60–70.

McGuire, T.C., Crawford, T.B., Henson, J.B., 1973. The isolation, characterization and functional properties of equine immunoglobulin classes and subclasses. In: Bryans, J.T., Gerber, H. (Eds.), Proceedings of the Third International Conference on Equine Infectious diseases, Paris 1972. Karger, Basel, pp. 364–381.

Ministry of Agriculture, Forestry and Fisheries, Ministry of Health, Labour and Welfares, Japan, 2003. Manuals for Prevention from West Nile Virus Infections. Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan (in Japanese).

Monath, T.P., 2001. Prospects for development of a vaccine against the West Nile virus. Ann. N. Y. Acad. Sci. 951, 1–12.

Nelson, K.M., Schram, B.R., McGregor, M.W., Sheoran, A.S., Olsen, C.W., Lunn, D.P., 1998. Local and systemic isotype-specific antibody responses to equine influenza virus infection versus conventional vaccination. Vaccine 16, 1306–1313.

Oceguera III, L.F., Patiris, P.J., Chiles, R.E., Busch, M.P., Tobler, L.H., Hanson, C.V., 2007. Flavivirus serology by Western blot analysis. Am. J. Trop. Med. Hyg. 77, 159–163.

Petersen, L.R., Hayes, E.B., 2008. West Nile virus in the Americas. Med. Clin. North Am. 92, 1307–1322.

Sejvar, J.J., Marfin, A.A., 2006. Manifestations of West Nile neuroinvasive disease. Rev. Med. Virol. 16, 209–224.

Sheoran, A.S., Sponseller, B.T., Holmes, M.A., Timoney, J.F., 1997. Serum and mucosal antibody isotype responses to M-like protein (SeM) of *Streptococcus equi* in convalescent and vaccinated horses. Vet. Immunol. Immunopathol. 59, 239–251.

Shirafuji, H., Kanehira, K., Kamio, T., Kubo, M., Shibahara, T., Konishi, M., Murakami, K., Nakamura, Y., Yamanaka, T., Kondo, T., Matsumura, T., Muranaka, M., Katayama, Y., 2009. Antibody responses induced by experimental West Nile virus infection with or without previous immunization with inactivated Japanese encephalitis vaccine in horses. J. Vet. Med. Sci. 71, 969–974.

Simmons, M., Murphy, G.S., Kochel, T., Raviprakash, K., Hayes, C.G., 2001. Characterization of antibody responses to combinations of a dengue-2 DNA and dengue-2 recombinant subunit vaccine. Am. J. Trop. Med. Hyg. 65, 420–426.

Sugiura, T., Kondo, T., Imagawa, H., Kamada, M., 1998. Production of monoclonal antibodies to six isotypes of horse immunoglobulin. Vet. Immunol. Immunopathol. 62, 145–151.

Wagner, B., 2006. Immunoglobulins and immunoglobulin genes of the horse. Dev. Comp. Immunol. 30, 155–164.

Wagner, B., Miller, D.C., Lear, T.L., Antczak, D.F., 2004. The complete map of the Ig heavy chain constant gene region reveals evidence for seven IgG isotypes and for IgD in the horse. J. Immunol. 173, 3230–3242.

Williams, D.T., Daniels, P.W., Lunt, R.A., Wang, L.F., Newberry, K.M., Mackenzie, J.S., 2001. Experimental infections of pigs with Japanese encephalitis virus and closely related Australian flaviviruses. Am. J. Trop. Med. Hyg. 65, 379–387.

Virology 396 (2010) 298–304

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

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

Shigeru Tajima *, Reiko Nerome, Yoko Nukui, Fumihiro Kato, Tomohiko Takasaki, Ichiro Kurane *

Department of Virology 1, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan

ARTICLE INFO

Article history:
Received 3 September 2009
Returned to author for revision
13 October 2009
Accepted 21 October 2009
Available online 14 November 2009

Keywords:
Japanese encephalitis virus
Infections clone
E protein
Pathogenesis

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.

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

* Corresponding authors. Fax: +81 3 5285 1188.
E-mail addresses: stajima@nih.go.jp (S. Tajima), kurane@nih.go.jp (I. Kurane).

0042-6822/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2009.10.035

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

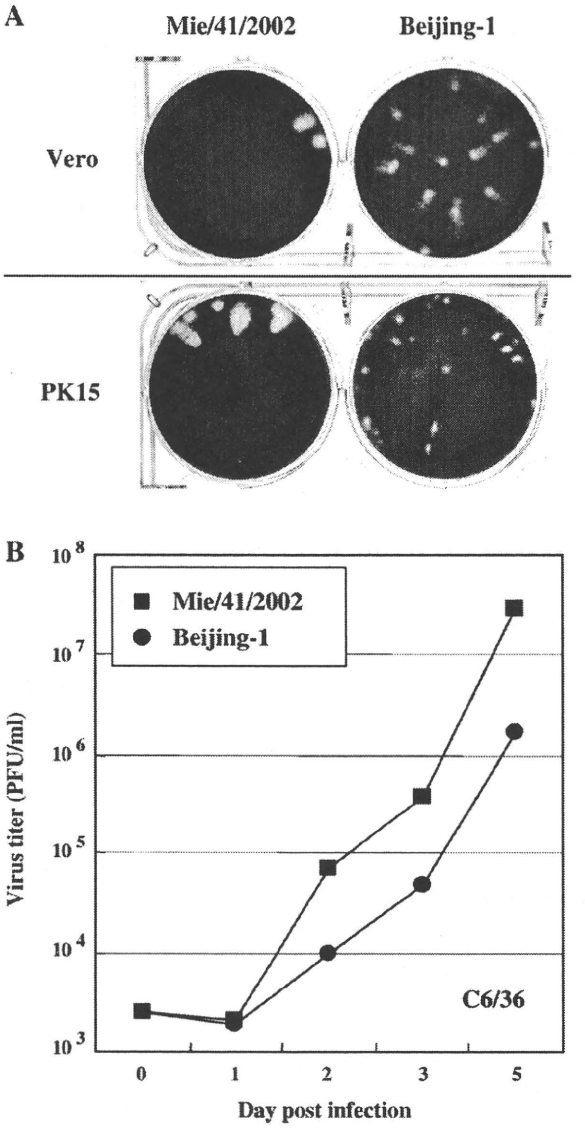

Fig. 1. (A) Plaque phenotypes of JEV Mie/41/2002 and Beijing-1 in Vero and porcine kidney PK15 cells. (B) Growth curves of Mie/41/2002 and Beijing-1 in mosquito C6/36 cells.

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.

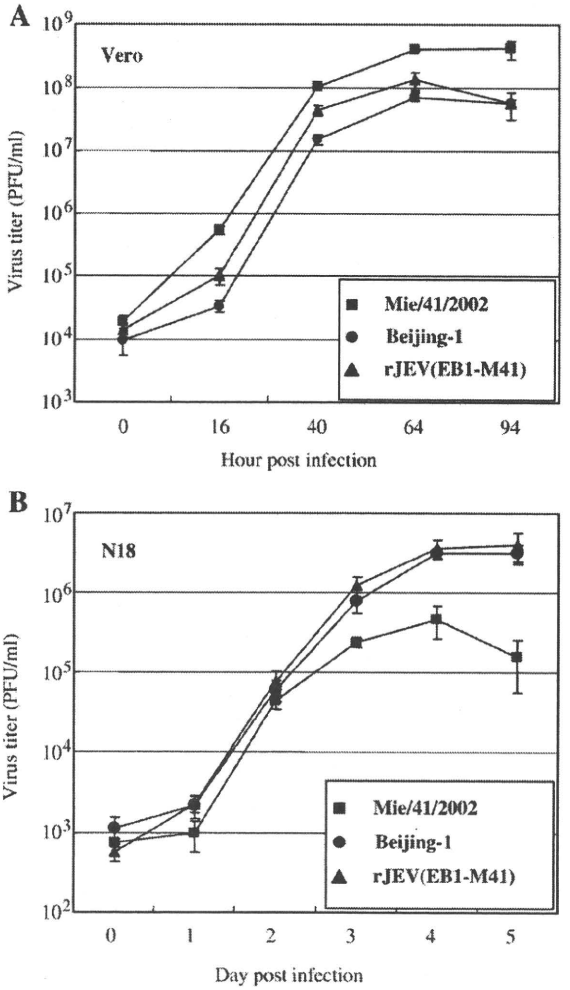

Fig. 2. Growth curves of Mie/41/2002, Beijing-1, and intertypic recombinant JEV rJEV (EB1-M41) in Vero cells (A) and in N18 cells (B). Values represent the mean and standard deviation (SD) for three independent tests.

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-

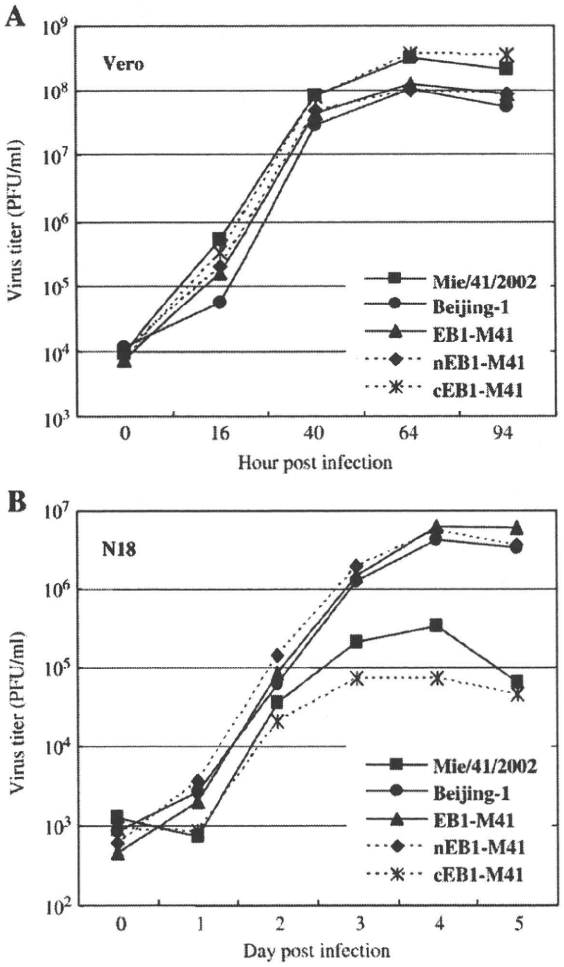

Fig. 3. Growth curves of Mie/41/2002, Beijing-1, rJEV(EB1-M41), rJEV(nEB1-M41) and rJEV(cEB1-M41) in Vero cells (A) and N18 cells (B).

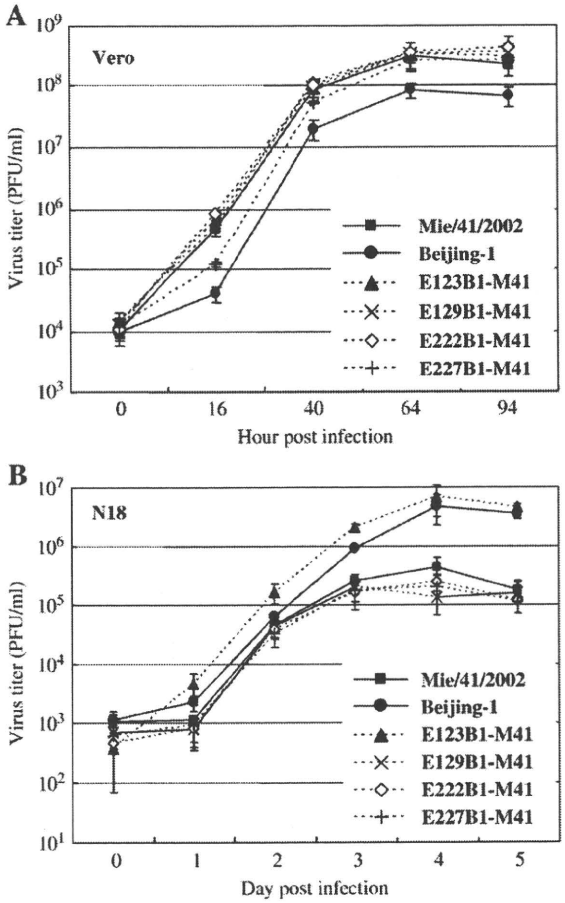

Fig. 4. Growth curves of Mie/41/2002, Beijing-1, rJEV(E123B1-M41), rJEV(E129B1-M41), rJEV(E222B1-M41), and rJEV(E227B1-M41) in Vero cells (A) and N18 cells (B). Values represent the mean and SD for three independent tests.

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	GATCGCGGCCGCCCACAGGAGTTTACCGAATCATG
	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.

Acknowledgments

We thank Dr. Yoshio Mori (Osaka University) for providing N18 cells and PK15 cells. This work was partly supported by the grant for the Research on Emerging and Re-emerging Infectious Diseases from Japan Health Science Foundation (H20-Shinkou-ippan-003, H20-Shinkou-ippan 015, and H20-Iyaku-ippan-077).

References

Aihara, S., Rao, C.M., Yu, Y.X., Lee, T., Watanabe, K., Komiya, T., Sumiyoshi, H., Hashimoto, H., Nomoto, A., 1991. Identification of mutations that occurred on the genome of Japanese encephalitis virus during the attenuation process. Virus Genes 5, 95–109.

Arroyo, J., Guirakhoo, F., Fenner, S., Zhang, Z.X., Monath, T.P., Chambers, T.J., 2001. Molecular basis for attenuation of neurovirulence of a yellow fever virus/Japanese encephalitis virus chimera vaccine (ChimeriVax-JE). J. Virol. 75, 934–942.

Burke, D.S., Monath, T.P., 2001. Flaviviruses, In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th ed. Lippincott Willams and Wilkins, Philadelphia, pp. 1043–1125.

Cecilia, D., Gould, E.A., 1991. Nucleotide changes responsible for loss of neuroinvasiveness in Japanese encephalitis virus neutralization-resistant mutants. Virology 181, 70–77.

Chambers, T.J., Droll, D.A., Jiang, X., Wold, W.S., Nickells, J.A., 2007. JE Nakayama/JE SA14-14-2 virus structural region intertypic viruses: biological properties in the mouse model of neuroinvasive disease. Virology 366, 51–61.

Chen, L.K., Liao, C.L., Lin, C.G., Lai, S.C., Liu, C.I., Ma, S.H., Huang, Y.Y., Lin, Y.L., 1996. Persistence of Japanese encephalitis virus is associated with abnormal expression of the nonstructural protein NS1 in host cells. Virology 217, 220–229.

Chiou, S.S., Liu, H., Chuang, C.K., Lin, C.C., Chen, W.J., 2005. Fitness of Japanese encephalitis virus to Neuro-2a cells is determined by interactions of the viral envelope protein with highly sulfated glycosaminoglycans on the cell surface. J. Med. Virol. 76, 583–592.

Hanna, J.N., Ritchie, S.A., Phillips, D.A., Shield, J., Bailey, M.C., Mackenzie, J.S., Poidinger, M., McCall, B.J., Mills, P.J., 1996. An outbreak of Japanese encephalitis in the Torres Strait, Australia, 1995. Med. J. Aust. 165, 256–260.

Hasegawa, H., Yoshida, M., Shiosaka, T., Fujita, S., Kobayashi, Y., 1992. Mutations in the envelope protein of Japanese encephalitis virus affect entry into cultured cells and virulence in mice. Virology 191, 158–165.

Hashimoto, H., Nomoto, A., Watanabe, K., Mori, T., Takezawa, T., Aizawa, C., Takegami, T., Hiramatsu, K., 1988. Molecular cloning and complete nucleotide sequence of the genome of Japanese encephalitis virus Beijing-1 strain. Virus Genes 1, 305–317.

Kim, J.M., Yun, S.I., Song, B.H., Hahn, Y.S., Lee, C.H., Oh, H.W., Lee, Y.M., 2008. A single N-linked glycosylation site in the Japanese encephalitis virus prM protein is critical for cell type-specific prM protein biogenesis, virus particle release, and pathogenicity in mice. J. Virol. 82, 7846–7862.

Kolaskar, A.S., Kulkarni-Kale, U., 1999. Prediction of three-dimensional structure and mapping of conformational epitopes of envelope glycoprotein of Japanese encephalitis virus. Virology 261, 31–42.

Lee, E., Hall, R.A., Lobigs, M., 2004. Common E protein determinants for attenuation of glycosaminoglycan-binding variants of Japanese encephalitis and West Nile viruses. J. Virol. 78, 8271–8280.

Liang, J.J., Liao, C.L., Liao, J.T., Lee, Y.L., Lin, Y.L., 2009. A Japanese encephalitis virus vaccine candidate strain is attenuated by decreasing its interferon antagonistic ability. Vaccine 27, 2746–2754.

Lindenbach, B.D., Rice, C.M., 2001. Flaviviridae: the viruses and their replication, In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th ed. Lippincott Willams and Wilkins, Philadelphia, pp. 991–1041.

Ma, S.P., Yoshida, Y., Makino, Y., Tadano, M., Ono, T., Ogawa, M., 2003. Short report: a major genotype of Japanese encephalitis virus currently circulating in Japan. Am. J. Trop. Med. Hyg. 69, 151–154.

Mishin, V.P., Cominelli, F., Yamshchikov, V.F., 2001. A 'minimal' approach in design of flavivirus infectious DNA. Virus. Res. 81, 113–123.

Monath, T.P., Arroyo, J., Levenbook, I., Zhang, Z.X., Catalan, J., Draper, K., Guirakhoo, F., 2002. Single mutation in the flavivirus envelope protein hinge region increases neurovirulence for mice and monkeys but decreases viscerotropism for monkeys: relevance to development and safety testing of live, attenuated vaccines. J. Virol. 76, 1932–1943.

Mori, Y., Okabayashi, T., Yamashita, T., Zhao, Z., Wakita, T., Yasui, K., Hasebe, F., Tadano, M., Konishi, E., Moriishi, K., Matsuura, Y., 2005. Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. J. Virol. 79, 3448–3458.

Nam, J.H., Chung, Y.J., Ban, S.J., Kim, E.J., Park, Y.K., Cho, H.W., 1996. Envelope gene sequence variation among Japanese encephalitis viruses isolated in Korea. Acta Virol. 40, 303–309.

Nerome, R., Tajima, S., Takasaki, T., Yoshida, T., Kotaki, A., Lim, C.K., Ito, M., Sugiyama, A., Yamauchi, A., Yano, T., Kameyama, T., Morishita, I., Kuwayama, M., Ogawa, T., Sahara, K., Ikegaya, A., Kanda, M., Hosoya, Y., Itokazu, K., Onishi, H., Chiya, S., Yoshida, Y., Tabei, Y., Katsuki, K., Tabata, K., Harada, S., Kurane, I., 2007. Molecular epidemiological analyses of Japanese encephalitis virus isolates from swine in Japan from 2002 to 2004. J. Gen. Virol. 88, 2762–2768.

Nga, P.T., del Carmen Parquet, M., Cuong, V.D., Ma, S.P., Hasebe, F., Inoue, S., Makino, Y., Takagi, M., Nam, V.S., Morita, K., 2004. Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from Southeast Asia to East Asia. J. Gen. Virol. 85, 1625–1631.

Ni, H., Barrett, A.D., 1996. Molecular differences between wild-type Japanese encephalitis virus strains of high and low mouse neuroinvasiveness. J. Gen. Virol. 77, 1449–1455.

Ni, H., Barrett, A.D., 1998. Attenuation of Japanese encephalitis virus by selection of its mouse brain membrane receptor preparation escape variants. Virology 241, 30–36.

Nitatpattana, N., Dubot-Peres, A., Gouilh, M.A., Souris, M., Barbazan, P., Yoksan, S., de Lamballerie, X., Gonzalez, J.P., 2008. Change in Japanese encephalitis virus distribution. Thailand Emerg. Infect. Dis. 14, 1762–1765.

Nitayaphan, S., Grant, J.A., Chang, G.J., Trent, D.W., 1990. Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA-14-14-2. Virology 177, 541–552.

Nybakken, G.E., Nelson, C.A., Chen, B.R., Diamond, M.S., Fremont, D.H., 2006. Crystal structure of the West Nile virus envelope glycoprotein. J. Virol. 80, 11467–11474.

Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., Harrison, S.C., 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature 375, 291–298.

Solomon, T., Ni, H., Beasley, D.W., Ekkelenkamp, M., Cardosa, M.J., Barrett, A.D., 2003. Origin and evolution of Japanese encephalitis virus in southeast Asia. J. Virol. 77, 3091–3098.

Sumiyoshi, H., Hoke, C.H., Trent, D.W., 1992. Infectious Japanese encephalitis virus RNA can be synthesized from in vitro-ligated cDNA templates. J. Virol. 66, 5425–5431.

Sumiyoshi, H., Tignor, G.H., Shope, R.E., 1995. Characterization of a highly attenuated Japanese encephalitis virus generated from molecularly cloned cDNA. J. Infect. Dis. 171, 1144–1151.

Tajima, S., Nukui, Y., Ito, M., Takasaki, T., Kurane, I., 2006. Nineteen nucleotides in the variable region of 3′ non-translated region are dispensable for the replication of dengue type 1 virus in vitro. Virus Res. 116, 38–44.

Tsai, T.F., 2000. New initiatives for the control of Japanese encephalitis by vaccination: minutes of a WHO/CVI meeting, Bangkok, Thailand, 13–15 October 1998. Vaccine 18, 1–25.

Uchil, P.D., Satchidanandam, V., 2001. Phylogenetic analysis of Japanese encephalitis virus: envelope gene based analysis reveals a fifth genotype, geographic clustering, and multiple introductions of the virus into the Indian subcontinent. Am. J. Trop. Med. Hyg. 65, 242–251.

Wang, H.Y., Takasaki, T., Fu, S.H., Sun, X.H., Zhang, H.L., Wang, Z.X., Hao, Z.Y., Zhang, J.K., Tang, Q., Kotaki, A., Tajima, S., Liang, X.F., Yang, W.Z., Kurane, I., Liang, G.D., 2007. Molecular epidemiological analysis of Japanese encephalitis virus in China. J. Gen. Virol. 88, 885–894.

WHO, 1998. Japanese encephalitis vaccine. Wkly. Epidemiol. Rec. 73, 337–344.

Wu, S.C., Lin, C.W., Lee, S.C., Lian, W.C., 2003. Phenotypic and genotypic characterization of the neurovirulence and neuroinvasiveness of a large-plaque attenuated Japanese encephalitis virus isolate. Microbes Infect. 5, 475–480.

Yang, D.K., Kim, B.H., Kweon, C.H., Kwon, J.H., Lim, S.I., Han, H.R., 2004. Molecular characterization of full-length genome of Japanese encephalitis virus (KV1899) isolated from pigs in Korea. J. Vet. Sci. 5, 197–205.

Yoshida, Y., Tabei, Y., Hasegawa, M., Nagashima, M., Morozumi, S., 2005. Genotypic analysis of Japanese encephalitis virus strains isolated from swine in Tokyo. Japan Jpn. J. Infect. Dis. 58, 259–261.

Yun, S.I., Kim, S.Y., Rice, C.M., Lee, Y.M., 2003. Development and application of a reverse genetics system for Japanese encephalitis virus. J. Virol. 77, 6450–6465.

Zeng, M., Jia, L.L., Yu, Y.X., Dong, G.M., Liu, W.X., Wang, Z.W., Li, D.F., 2005. Construction of infectious Japanese encephalitis virus clone based on the cDNA template of the attenuated live vaccine production strain SA14-14-2. Zhonghua Shi Yan He Lin Chuang Bing Du Xue ZaZhi 19, 9–11.

Zhang, F., Huang, Q., Ma, W., Jiang, S., Fan, Y., Zhang, H., 2001. Amplification and cloning of the full-length genome of Japanese encephalitis virus by a novel long RT-PCR protocol in a cosmid vector. J. Virol. Methods 96, 171–182.

Zhang, J.S., Zhao, Q.M., Zhang, P.H., Jia, N., Cao, W.C., 2009. Genomic sequence of a Japanese encephalitis virus isolate from southern China. Arch. Virol. 154, 1177–1180.

Zhao, Z., Date, T., Li, Y., Kato, T., Miyamoto, M., Yasui, K., Wakita, T., 2005. Characterization of the E-138 (Glu/Lys) mutation in Japanese encephalitis virus by using a stable, full-length, infectious cDNA clone. J. Gen. Virol. 86, 2209–2220.

Dogs as Sentinels for Human Infection with Japanese Encephalitis Virus

Hiroshi Shimoda, Yoshito Ohno, Masami Mochizuki, Hiroyuki Iwata, Masaru Okuda, and Ken Maeda

Because serosurveys of Japanese encephalitis virus (JEV) among wild animals and pigs may not accurately reflect risk for humans in urban/residential areas, we examined seroprevalence among dogs and cats. We found that JEV-infected mosquitoes have spread throughout Japan and that dogs, but not cats, might be good sentinels for monitoring JEV infection in urban/residential areas.

Japanese encephalitis virus (JEV), a common cause of serious acute encephalitis in humans, is primarily transmitted by *Culex tritaeniorhynchus* mosquitoes and is widely endemic to Southeast Asia and the Western Pacific region (*1*). Annual incidence of Japanese encephalitis (JE) is ≈50,000 cases,with 10,000 deaths (*2*). In Japan during the 1950s, several thousand JE cases occurred each year. However, as a result of a JEV vaccination program, isolation of pig farms, and reduction of mosquitoes, the number of JE cases in Japan has decreased markedly, to <10 cases per year since 1992 (*3*). In 2005, the strong recommendation for JE vaccination was halted because of a severe vaccine-associated side effect in 1 person; however, since 2009, a newly developed JE vaccine has been available and vaccination has been resumed.

Annual serosurveys for JEV antibodies in pigs, the main amplifiers of JEV, tend to show high seropositivity in western Japan (*3*). Our previous study of JEV in wild animals in the Kinki district also showed high seroprevalence: 83% among wild boars and 59% among raccoons (*4*). These data indicate that JEV remains endemic to Japan.

However, serosurveys of wild animals and pigs may not accurately reflect risk for humans because these animals remain separate from human populations and thus may not indicate the prevalence of JEV in urban/residential areas of Japan. Therefore, additional monitoring of the risk for JEV infection in humans in these areas, in addition to annual surveillance of pigs, is needed. To determine seroprevalence in family-owned dogs and cats, which share living space with humans, we conducted serosurveys of JEV in these species.

Author affiliations: Yamaguchi University, Yoshida, Yamaguchi, Japan (H. Shimoda, Y. Ohno, H. Iwata, M. Okuda, K. Maeda); and Kyoritsu Seiyaku Corporation, Kudankita, Chiyoda-ku, Tokyo, Japan (M. Mochizuki)

DOI: 10.3201/eid1607.091757

The Study

First, to examine whether dogs and cats were infected with JEV, we analyzed serum samples from 100 dogs and 292 cats in Yamaguchi Prefecture, which is in the western part of Honshu, Japan. Dog samples were collected during 2006–2007, and cat samples were collected during 1997–1999 and 2004–2005. An 80% plaque-reduction neutralizing test using virus JEV/sw/Chiba/88/2002 was performed as described (*4*). Virus JEV/sw/Chiba/88/2002 is genetically classified as genotype I (*5*). To analyze the results statistically, we performed χ^2 and Fisher exact probability tests. The significance level was $p<0.05$.

Results showed that 17% of dogs and 1% of cats were seropositive for JEV; thus, seropositivity was ≈10-fold higher among dogs than among cats (Table 1). In addition, outdoor-only dogs (38%) were 3.7-fold more likely to be seropositive than were indoor-only dogs (10%) ($p<0.05$). Antibody prevalence did not differ significantly between male (14%) and female (20%) dogs ($p>0.05$; data not shown).

Next, serum samples from 652 dogs in every district in Japan during 2006–2007 were examined for seroprevalence of JEV. The results showed that 25% of dogs had virus-neutralizing antibodies against JEV. In northern Japan, 0% and 9% of dogs from the Hokkaido and Tohoku districts, respectively, were seropositive; these levels were significantly lower than those for other districts ($p<0.05$). In contrast, in southern Japan, 61% and 47% of dogs in the Shikoku and Kyushu districts, respectively, were seropositive; these levels were significantly higher than those for other districts ($p<0.05$). Seropositivity to JEV in the Kanto (17%), Chubu (18%), Kinki (23%), and Chugoku (26%) districts showed no significant differences ($p>0.05$) (Figure). In addition, 45% of outdoor-only dogs and 8% of indoor-only dogs were seropositive for JEV, thus confirming that outdoor-only dogs were 5.5-fold more likely than indoor-only dogs to be seropositive ($p<0.05$) (Table 2). Regarding the areas of residence, 21% of dogs in urban/residential areas and 43% of dogs in rural areas were seropositive; the results for rural areas were significantly higher than those for urban/residential areas (Table 2). No significant correlation was found between ages of dogs and JEV seropositivity (data not shown).

Discussion and Conclusions

Our findings of significantly higher JEV seropositivity among dogs than cats are similar to those found in a 1954–

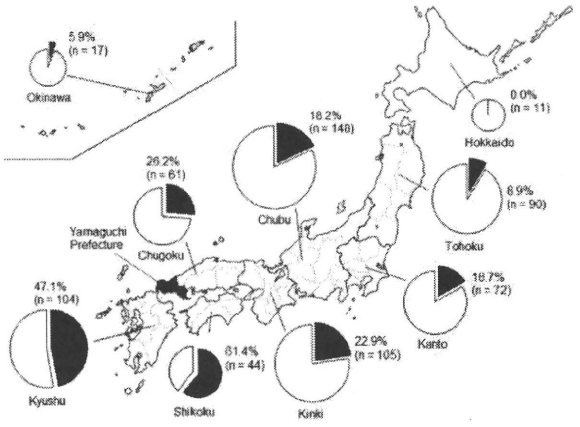

Figure. Seropositivity for Japanese encephalitis virus among dogs in 9 districts of Japan, 2006–2007. Numbers in parenthesis indicate number of dogs tested. The size of each circle indicates the number of samples. Black pie chart segments indicate the proportion of seropositive dogs; white segments indicate proportion of seronegative dogs.

1955 study in which 55% of dogs and 10% of cats were seropositive for JEV (*6*). Studies of another mosquito-vectored virus found 26% of dogs and 9% of cats in Louisiana and 5% of dogs and no cats in New York to be seropositive for West Nile virus (WNV) (*7,8*). Previous reports on host feeding patterns of JEV and WNV vectors showed that although *Culex* spp. mosquitoes feed on various mammals, including dogs, cats, and humans, they tend to feed more on dogs than on cats or humans (*9,10*). These reports are consistent with our finding that seropositivity was higher among dogs than among cats and humans and indicate that some JEV vectors do occasionally feed on humans.

Our nationwide serosurvey indicated that JEV prevalence was significantly lower in the Hokkaido and Tohoku districts and significantly higher in the Kyushu and Shikoku districts (Figure). Annual serosurveys of pigs have also shown that JEV seropositivity rates are higher for pigs in western than in northern Japan. In addition, during 2005–2007 in Japan, 24 JE cases in humans were reported, most of which occurred in western Japan (*11*). This finding is consistent with our data, suggesting that serosurveys in dogs accurately reflect JEV infection risk for humans in Japan.

Our finding that 45% of outdoor-only dogs were seropositive for JEV is similar to the finding of a previous study, conducted during a WNV epidemic among humans, that 69% of outdoor-only dogs were seropositive for WNV (*7*). A serosurvey in the Kanto district of Tokyo during 1954–1955 showed that 49% of stray dogs were seropositive for JEV (*6*). Results of these studies are similar to our results, indicating that risk for JEV infection remains high in Japan, particularly in the western part. In addition, confirmation of seropositivity among indoor-only dogs (8%) indicates that JEV-infected mosquitoes may enter houses; thus, infants and elderly persons, who tend to go outside less frequently, might also be at risk for JEV infection.

Table 1. Seroprevalence of Japanese encephalitis virus among dogs (2006–2007) and cats (1997–2005), Yamaguchi, Japan*

Animals	No. examined	No. (%) positive
Dogs		
Indoor	58	6 (10)
Outdoor	21	8 (38)
Both or unknown	21	3 (14)
Total	100	17 (17)
Cats	292	3 (1)

*Housing information obtained by questionnaire.

That seropositivity in rural areas (43%) was significantly higher than that in urban/residential areas (21%) suggests that pig farms and rice paddies in rural areas are associated with JEV. However, the relatively high seropositivity in urban/residential areas suggests that JEV infection risk for humans remains high, even in areas with few pig farms and rice paddies. Because pigs are housed away from humans, serosurveys of pigs in urban/residential areas are limited. Therefore, dogs, which are found in all areas of Japan, may be better sentinels for JEV infection in these areas. However, information such as whether dogs become viremic after JEV infection or how long anti-JEV antibodies last in them remains unclear.

In conclusion, using dogs as sentinels indicated that the risk for human infection with JEV remains high, even in urban/residential areas. Therefore, to assess the continuing risk for JEV infection in humans in urban/residential areas of Japan, we recommend JEV surveillance among pigs every year and surveillance among dogs every several years.

Acknowledgments

We thank Tomohiko Takasaki for kindly providing virus JEV/sw/Chiba/88/2002.

This work was supported by a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of

Table 2. Seroprevalence of Japanese encephalitis virus among dogs throughout Japan, 2006–2007*

Location	No. examined	No. (%) positive
Where dog stays		
Indoors	222	18 (8)
Outdoors	234	105 (45)
Both or unknown	196	41 (21)
Type of area		
Urban/residential	405	86 (21)
Rural	152	65 (43)
Unknown	95	13 (14)

*Housing information obtained by questionnaire.

Health, Labour and Welfare of Japan (H20-Shinko-Ippan-003) and by a grant from The Morinaga Foundation.

Mr Shimoda is a student in the Department of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan. His research interest is zoonoses.

References

1. Mackenzie JS, Chua KB, Daniels PW, Eaton BT, Field HE, Hall RA, et al. Emerging viral diseases of Southeast Asia and the Western Pacific. Emerg Infect Dis. 2001;7:497–504. DOI: 10.3201/eid0703.010303
2. Erlanger TE, Weiss S, Keiser J, Utzinger J, Wiedenmayer K. Past, present, and future of Japanese encephalitis. Emerg Infect Dis. 2009;15:1–7. DOI: 10.3201/eid1501.080311
3. Arai S, Matsunaga Y, Takasaki T, Tanaka-Taya K, Taniguchi K, Okabe N, et al.; Vaccine Preventable Diseases Surveillance Program of Japan. Japanese encephalitis: surveillance and elimination effort in Japan from 1982 to 2004. Jpn J Infect Dis. 2008;61:333–8.
4. Ohno Y, Sato H, Suzuki K, Yokoyama M, Uni S, Shibasaki T, et al. Detection of antibodies against Japanese encephalitis virus in raccoons, raccoon dogs and wild boars in Japan. J Vet Med Sci. 2009;71:1035–9. DOI: 10.1292/jvms.71.1035
5. Nerome R, Tajima S, Takasaki T, Yoshida T, Kotaki A, Lim CK, et al. Molecular epidemiological analyses of Japanese encephalitis virus isolates from swine in Japan from 2002 to 2004. J Gen Virol. 2007;88:2762–8. DOI: 10.1099/vir.0.82941-0
6. Nakamura J. Japanese encephalitis in animals [in Japanese]. Shinkei Kenkyu No Shinpo. 1967;11:19–29.
7. Kile JC, Panella NA, Komar N, Chow CC, MacNeil A, Robbins B, et al. Serologic survey of cats and dogs during an epidemic of West Nile virus infection in humans. J Am Vet Med Assoc. 2005;226:1349–53. DOI: 10.2460/javma.2005.226.1349
8. Komar N, Panella NA, Boyce E. Exposure of domestic mammals to West Nile virus during an outbreak of human encephalitis, New York City, 1999. Emerg Infect Dis. 2001;7:736–8. DOI: 10.3201/eid0704.010424
9. Mitchell CJ, Chen PS, Boreham PF. Host-feeding patterns and behavior of 4 *Culex* species in an endemic area of Japanese encephalitis. Bull World Health Organ. 1973;49:293–9.
10. Molaei G, Andreadis TG, Armstrong PM, Bueno R Jr, Dennett JA, Real SV, et al. Host feeding pattern of *Culex quinquefasciatus* (Diptera: Culicidae) and its role in transmission of West Nile virus in Harris County, Texas. Am J Trop Med Hyg. 2007;77:73–81.
11. Japanese encephalitis, Japan, 2003–2008. Infectious Agents Surveillance Report. 2009;30:147–8.

Address for correspondence: Ken Maeda, Laboratory of Veterinary Microbiology, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan; email: kmaeda@yamaguchi-u.ac.jp

Arch Virol (2011) 156:63–69
DOI 10.1007/s00705-010-0816-8

ORIGINAL ARTICLE

Restoration of replication-defective dengue type 1 virus bearing mutations in the N-terminal cytoplasmic portion of NS4A by additional mutations in NS4B

Shigeru Tajima · Tomohiko Takasaki · Ichiro Kurane

Received: 27 April 2010 / Accepted: 26 August 2010 / Published online: 30 September 2010

Abstract Flavivirus NS4A has an N-terminal hydrophilic cytoplasmic portion; however, the role of this portion remains poorly understood. In this study, we show that a recombinant dengue type 1 virus (DENV-1) in which a subportion (amino acids 27–34) of the N-terminal portion of NS4A is replaced by the corresponding region from Japanese encephalitis virus (JEV) is defective in replication. Using the defective mutant clone NS4A(27–34^{JEV}), we recovered suppressor mutant viruses that carry various non-synonymous mutations. Site-directed mutational analysis indicated that a single non-synonymous mutation in NS4B that is found in the suppressor viruses is sufficient to restore NS4A(27–34^{JEV}). Recombinant DENV-1 with single mutations in NS4B had increased growth properties as compared to the wild-type virus and NS4A(27–34^{JEV}) virus bearing the same NS4B mutation. Collectively, our results suggest that the NS4B mutation enhanced the growth of DENV-1, irrespective of the sequence of the 27–34 subportion NS4A.

Introduction

Dengue viruses (DENV-1 to 4) are members of the family *Flaviviridae* and contain a single-stranded, positive-sense RNA genome of approximately 11 kb. The viruses are etiologic agents of dengue fever (DF), dengue haemorrhagic fever (DHF), and dengue shock syndrome (DSS). The viruses are transmitted to humans by *Aedes* mosquitoes [3, 5]. The presence of heterotypic dengue virus antibodies is a risk factor for developing DHF and DSS in secondary infections [5, 8]. On the other hand, genotypic differences also appear to be associated with differences in virulence [22]. The DENV genome encodes three structural proteins (C, prM, and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) in one open reading frame, as well as non-translated regions (NTRs) at its 5′ and 3′ termini [12]. Two of these proteins, NS3 and NS5, have been identified as the viral protease/helicase and polymerase, respectively. Although the roles of the other NS proteins remain to be determined, it is known that these proteins are colocalized with NS3 and NS5, and accordingly, they are considered likely to participate in viral replication [7, 13, 15]. Furthermore, recent studies have indicated that some flaviviral NS proteins act as antagonists of the interferon (IFN) signal pathway [2, 4, 9, 10, 14, 18–20]. NS4A is a small hydrophobic protein and is among the least studied of the NS proteins. NS4A localizes to the sites of viral RNA replication and interacts with NS1, which is also a member of the replication complex [11, 15, 17, 27]. These findings indicate that NS4A is a component of the flaviviral replication complex and plays a role in viral RNA replication, perhaps by anchoring replicase components to cellular membranes [12]. Recent findings suggest that NS4A induces membrane rearrangements to form the viral replication complex [17, 21]. DENV-2 NS4A also has weak inhibitory activity against the IFN signaling pathway [18]. DEAD-box RNA helicase DDX42, which interacts with Japanese encephalitis virus (JEV) NS4A, is able to overcome the JEV-induced antagonism of IFN responses,

Electronic supplementary material The online version of this article (doi:10.1007/s00705-010-0816-8) contains supplementary material, which is available to authorized users.

S. Tajima (✉) · T. Takasaki · I. Kurane
Department of Virology 1, National Institute of Infectious Diseases (NIID), 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan
e-mail: stajima@nih.go.jp

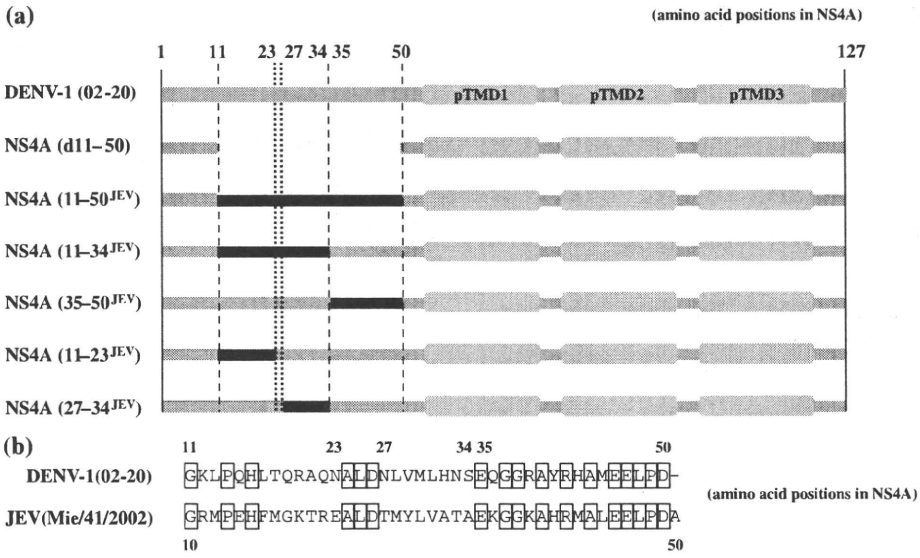

Fig. 1 **a** Schematic representation of wild-type (02–20 strain) and deletion/replacement mutants of DENV-1 NS4A. The *black boxes* indicate the regions that were replaced by the corresponding regions of JEV(Mie/41/2002). pTMD, predicted transmembrane domain [17]. *Numbers* indicate the amino acid positions in the NS4A protein. **b** Sequence alignment of the N-terminal portion (11–50) of the DENV-1(02–20) and JEV(Mie/41/2002) NS4A proteins. *Boxes* indicate identical amino acid residues

supporting the idea that NS4A is associated with modulation of the IFN pathway [9]. NS4A consists of an N-terminal portion (amino acid residues 1–50) and four internal hydrophobic regions (amino acid residues 51–127; Fig. 1). The hydrophobic regions associate with cellular membranes (pTMD in Fig. 1), whereas the hydrophilic N-terminal portion is exposed to the cytoplasm [17]. Using West Nile virus (WNV) NS3 helicase protein linked to the N-terminal portion of NS4A, Shiryaev et al. [23] recently showed that the N-terminal portion functions as a cofactor of NS3 and regulates the ATPase activity of the NS3 helicase. However, no viral or cellular factors that bind to the N-terminal portion of NS4A were identified, and the role of the N-terminal portion in the lifecycle of flaviviruses remains to be determined. Flavivirus NS4B is the largest of the small hydrophobic NS proteins and may act as an anchor protein in the replication complex [12, 27]. In addition, recent findings demonstrate that NS4B blocks the interferon response in host cells by interfering with the STAT-1 pathway [2, 18, 19]. NS4B also interacts with NS3 and dissociates NS3 from single-stranded RNA [26]. Thus, the nature of NS4B is similar to that of NS4A, and therefore the two proteins may possibly function cooperatively in viral replication and the anti-host response. It has also been suggested that the cleavage of the NS4A–NS4B polyprotein intermediate by the viral NS2B–NS3 protease plays a major role in cytoplasmic membrane rearrangement, which apparently facilitates efficient replication of viral RNA [17, 21].

To gain insight into the nature of the N-terminal cytoplasmic portion of DENV-1 NS4A, DENV-1 clones in which subportions of the N-terminal portion were replaced by the corresponding regions of the Japanese encephalitis virus (JEV) were constructed. Using these clones, we found that the amino acid 27–34 subportion of DENV-1 NS4A could not be replaced by the subportion of JEV. We then recovered suppressor mutant viruses of the DENV-1 27–34 replacement mutant bearing additional missense mutations. Finally, the relationship between the subportion mutation and the additional mutations and their role in viral replication are examined and discussed.

Materials and methods

Cells and viruses

Vero cells (NIBSC strain) were cultured at 37°C in 5% CO_2 in Eagle's minimum essential medium (MEM) (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U of penicillin–streptomycin per ml. Recombinant DENV-1, rDENV-1(02–20), derived from DENV-1 isolate NIID02-20 (accession no. AB178040), was prepared in our laboratory as described previously [24].

Construction of recombinant mutant DENV-1 clones and recovery of the recombinant viruses

Wild-type (original) recombinant DENV-1 (rDENV-1) clone rDENV-1(02–20)/pMW119 [24] was used for the construction of deletion and replacement mutant DENV-1 clones. Primers and oligonucleotides used for constructing mutant clones are listed in Table S1. We constructed twelve recombinant clones of mutant viruses, NS4A(d11–50), NS4A(11–50^{JEV}), NS4A(11–34^{JEV}), NS4A(35–50^{JEV}), NS4A(11–23^{JEV}), NS4A(27–34^{JEV}), NS4A(27–34^{JEV}-A3225U), NS4A(27–34^{JEV}-A5628C), NS4A(27–34^{JEV}-A7714U), NS4A(27–34^{JEV}-G7165U), C7165U, and C7152U, by introducing