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Japanese encephalitis virus vaccine candidates generated by chimerization with dengue virus type 4

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Abstract

Japanese encephalitis virus (JEV) is a leading cause of viral encephalitis worldwide and vaccination is one of the most effective ways to prevent disease. A suitable live-attenuated JEV vaccine could be formulated with a live-attenuated tetravalent dengue vaccine for the control of these viruses in endemic areas. Toward this goal, we generated chimeric virus vaccine candidates by replacing the precursor membrane (prM) and envelope (E) protein structural genes of recombinant dengue virus type 4 (rDEN4) or attenuated vaccine candidate rDEN4Δ30 with those of wild-type JEV strain India/78. Mutations were engineered in E, NS3 and NS4B protein genes to improve replication in Vero cells. The chimeric viruses were attenuated in mice and some elicited modest but protective levels of immunity after a single dose. One particular chimeric virus, bearing E protein mutation Q264H, replicated to higher titer in tissue culture and was significantly more immunogenic in mice. The results are compared with live-attenuated JEV vaccine strain SA14-14-2.

Keywords

Japanese encephalitis virus; Chimeric virus; Live-attenuated vaccine

1. Introduction

Japanese encephalitis virus (JEV), a member of the genus flavivirus, is a major cause of viral encephalitis in Asia. The virus has a single-strand, plus-sense, RNA genome of approximately 11 kb in length and a single ORF codes for a polyprotein that is processed by viral and host cellular proteases into 3 structural proteins (capsid [C], precursor-membrane [prM] and envelope [E]) and at least 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [1]. The E protein is responsible for cell binding and entry and is the major target of virus neutralizing antibodies that mediate protection from disease [2,3]. Although there exist five recognizable JEV genotypes, there is only a single JEV serotype [4]. Studies have demonstrated significant cross-neutralization and cross-protection between virus strains from different JEV genotypes [5–7], supporting the conclusion that immunity to

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one JEV strain would be sufficient to protect against strains of the other four genotypes [8,9].

Three billion people live in regions with endemic JEV transmission resulting in approximately 60,000 cases annually [10]. However, only a small proportion of human JEV infections lead to clinical disease, which ranges from a mild febrile illness to acute and potentially lethal meningoencephalomyelitis [11]. Typically, 20–40% of human cases are fatal and 45–70% of survivors have neurologic sequelae [12]. Thus, Japanese encephalitis continues to be a significant public health burden even though it is a vaccine preventable disease.

Inactivated JEV vaccines have been available for human use since the mid 1950s [8,13,14]. Vero cell culture-derived, inactivated vaccines have gradually replaced mouse brain and primary cell culture-derived vaccines and have proven to be safe and immunogenic [13,15]. The limitations of inactivated vaccines include a high production cost and the requirement for multiple doses, often making them impractical for mass vaccination in most JEV endemic areas. The only live-attenuated vaccine in widespread use is JEV SA14-14-2 (Chengdu Institute of Biological Products), which was licensed in China in 1988 and more recently in Nepal, Korea, and Sri Lanka [13] and is produced in primary hamster kidney cells. This vaccine recently gained WHO prequalification in October of 2013. Attempts to adapt JEV SA14-14-2 to other cell substrates, such as primary dog kidney (PDK) cells, have resulted in mutations in the virus and reduced vaccine efficacy [16,17]. This has led to alternative approaches to develop live-attenuated JEV vaccines such as IMOJEV (Acambis/Sanofi-Aventis), which incorporates the prM and E structural genes of JEV SA14-14-2 into the yellow fever virus (YFV) 17D vaccine strain, and has recently been licensed for use in Australia and Thailand [14].

JEV co-circulates with dengue virus (DENV) serotypes 1–4 in Asia, causing a significant amount of morbidity and mortality, especially in children [18]. There are currently no licensed DENV vaccines, although several are in various stages of clinical development [19,20]. The live-attenuated tetravalent DENV vaccine developed at NIH is safe and immunogenic in humans, and a single dose induces a trivalent or better neutralizing antibody response in the majority of vaccines [21]. This prompted us to design a compatible, live-attenuated JEV vaccine candidate for incorporation into the NIH tetravalent DENV vaccine, thus creating a pentavalent vaccine for use in Asia. To that end, we generated JEV vaccine candidates by replacing the structural genes of a recombinant DENV-4 (rDEN4) and vaccine candidate rDEN4Δ30 with those of JEV genotype III strain India/78. A similar strategy has been used to generate vaccine candidates for DENV-2, West Nile virus (WNV), St. Louis encephalitis virus (SLEV), and tick-borne encephalitis virus (TBEV) [22–25]. Here we report the construction, *in vitro* characterization, and evaluation in mice of these JEV chimeric virus vaccine candidates.

2. Materials and methods

2.1. Cells and viruses

Vero (African green monkey kidney) cells were maintained at 37 °C in OptiPRO- SFM (Gibco) supplemented with 4 mM L-glutamine (Gibco). C6/36 (*Aedes albopictus* mosquito) cells were maintained at 32 °C in EMEM (Lonza) supplemented with 10% FBS (HyClone), 2 mM L-glutamine, and 0.1 mM NEAA (Gibco). Raji (human B-cell lymphoblast) cells were maintained at 37 °C in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin (Gibco). HEK-293T (human kidney) cells were maintained at 37 °C in EMEM supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin.

JEV genotype III strain 7812474, designated “India/78” (Gen-Bank accession nos. EF688633 and U70387) [26,27] was obtained from the World Reference Center for Emerging Viruses and Arboviruses (UTMB, Galveston, TX), biologically cloned by terminal end-point dilution in Vero cells and sequenced. JEV strain SA14-14-2 was obtained from our laboratory archive and passaged once in Vero cells and sequenced. Although the previous passage history of our JEV strain SA14-14-2 was undocumented, the sequence matches that of Genbank accession no. JN604986 [28]. The rDEN4 and rDEN4Δ30 viruses were derived previously from cDNA clones [29].

2.2. Construction and recovery of chimeric viruses and rJEV

The cDNA clones of rDEN4 (p4) and rDEN4Δ30 (p4Δ30) were constructed previously [29]. A JEV-CME AscI-XhoI fragment containing the C, prM and E genes was synthesized and cloned into p4 and p4Δ30 to generate p4-JEV-CME and p4Δ30-JEV-CME (Fig. 1A). The JEV C gene sequence was exchanged for rDEN4 with a BglII-BstBI fragment, which generated p4-JEV-ME and p4Δ30-JEV-ME (Fig. 1A). The C-prM junction sequence was replaced using NheI-BstBI cDNA fragments (Fig. 1A and B). The cDNA gene segments encoding point mutations (Table 1) were cloned as BsiWI-XhoI, StuI-XmaI, and PmlI-XmaI cDNA fragments. Stability of chimeric virus cDNA constructs was improved by inserting a DNA stop codon linker (forward sequence 5′-TCGAGTGAGTTAACTTAGTCTAC-3′ and reverse sequence 5′-TCGAGTAGACTAAGTTAACTCAC-3′) into the XhoI restriction site.

A recombinant JEV (rJEV) India/78 was constructed from five synthesized cDNA segments and subcloned into an engineered AscI-SacI-FseI-EagI-EcoRI-Acc65I polycloning site within a modified pBR322 vector (Fig. 1C). Stability of the JEV cDNA construct was achieved by inserting a DNA stop codon linker (forward sequence 5′-CTGACTTAACTTAGACTAGAGCT-3′ and reverse sequence 5′-CTAGTCTAAGTTAAGTCAGAGCT-3′) into the SacI restriction site.

Recovery of the chimeric viruses and rJEV was accomplished by removing the stop codon linker sequence with restriction-enzyme digestion, followed by re-ligation and subsequent linearization with Acc65I. Full genome-length capped RNA transcripts were synthesized with the AmpliCap SP6 Message Maker Kit (Epicentre Biotechnologies). Purified transcripts were transfected into Vero or C6/36 cells and cell culture supernatant was harvested after 3–7 days and titered in Vero cells. Recovered viruses were purified by

multiple terminal dilutions, amplified in Vero cells to generate biological clones, and sequenced.

2.3. Mouse studies

Studies in mice were performed in accordance with the regulations and guidelines of the NIH (Bethesda, MD) and approved by the Animal Care and Use Committee of NIAID. Viruses were tested for neuroinvasiveness by intraperitoneal (IP) inoculation (0.1 mL) and neurovirulence by intracerebral (IC) inoculation (0.01 mL) of either 3-week-old (weanling) female or 3-day-old (suckling) Swiss Webster (SW) mice (Taconic). Groups of six weanling mice or ten suckling mice were inoculated with each virus dilution and monitored 21 days for signs of encephalitis leading to a moribund state, including tremors, seizure, and paralysis. Moribund mice were humanely euthanized to minimize pain and distress and LD_{50} values were determined [30].

To assess virus replication in the brain, groups of five weanling SW mice were inoculated IC with 10^3 PFU of virus. One group per virus was euthanized on day 1 and every other day for up to 11 days. Brains were homogenized individually as a 10% (w/v) suspension in L-15 medium, clarified by low-speed centrifugation, and frozen on dry ice. Virus titers were determined by plaque assay in Vero cells.

Immunogenicity and protection was evaluated in weanling mice. Groups of mice were immunized IP with 10^4 PFU of chimeric virus, rDEN4, or JEV SA14-14-2. In experiment #1, serum was collected on day 21 from groups of six mice for neutralization assays. To demonstrate protection, groups of ten immunized mice were challenged IP with 10^4 (experiment #2), 10^5 (experiment #3), or 10^6 (experiment #4) PFU of rJEV on day 28 or day 20 (experiment #4). All challenged mice were monitored for 21 days for signs of encephalitis and moribund mice were euthanized.

2.4. Tissue culture replication kinetics

Vero and C6/36 cells were infected at a multiplicity of infection (MOI) of 0.01 PFU/cell, washed three times with PBS and replenished with fresh medium. Infected Vero and C6/36 cells were incubated at 37 and 32 °C, respectively. Samples were taken at time 0 and every 24 h for 7 days and titered in Vero cells.

2.5. Production of JEV reporter virus particles (RVPs)

A cDNA fragment corresponding to the JEV India/78C, prM, and E genes was synthesized and cloned into mammalian expression vector pCMV6-AC (OriGene) to generate pJEV-CprME. A sub-genomic WNV replicon encoding GFP (WNIIrep-GFP) was provided by Dr. Ted Pierson (NIAID). JEV reporter virus particles (RVPs) were produced by co-transfecting HEK-293T cells, as described [31,32]. JEV RVP stocks were titered in Raji cells 2 days post-infection and the percentage of GFP expressing cells determined by flow cytometry.

2.6. JEV RVP serum neutralization assay

JEV RVPs were diluted in the linear range of infectivity and incubated for 1 h at 37 °C with an equal volume of serially diluted mouse serum or JEV hyperimmune mouse positive

control ascitic fluid (American Type Culture Collection). RVP-serum mixtures were combined with an equal volume of Raji cells, incubated at 37 °C for two days, fixed with paraformaldehyde, and counted by flow cytometry to determine the percentage of GFP-expressing cells. The data were fit by a dose–response curve (variable slope) to determine the effective concentration of serum where 50% neutralization occurred (EC_{50}) and titers are reported as the reciprocal geometric mean EC_{50} titer (GMT) of mice that seroconverted (detectable EC_{50}).

2.7. Statistical analysis

Comparisons of virus titer in tissue culture were analyzed using an unpaired t-test with statistical significance ($p < 0.05$) determined by the Holm-Sidak method. Mouse survival analyses were done using the Log-rank (Mantel-Cox) test, with $p < 0.05$ required for significance.

3. Results

3.1. Recovery and characterization of first-generation chimeric viruses

Replacing the C, prM and E structural genes of rDEN4 and rDENΔ30 with those of JEV did not produce viable chimeric viruses on multiple attempts and this strategy was abandoned. However, replacing just the prM and E genes generated viable chimeric viruses (Table 1). JEV/DEN4 and JEV/DEN4Δ30 ME chimeric viruses containing three different C-prM junctions (Fig. 1B) were recovered in either C6/36 or Vero cells. The chimeric viruses replicated in Vero cells to titers of 5.3–6.6 $\log_{10}$PFU/mL and initially had small plaque morphologies after recovery while some developed larger plaques following Vero cell adaptation (Table 1). The biologically cloned chimeric viruses were sequenced and all had acquired multiple mutations (Table 1).

The six chimeric viruses, rDEN4, rDEN4Δ30, and JEV SA14-14-2 did not cause mortality in weanling mice by IC or IP routes of inoculation of a dose up to 10^4 PFU (Table 2). By comparison, wild-type (wt) JEV was neurovirulent and neuroinvasive, having relatively low IC and IP LD_{50} levels similar to the recombinant-derived rJEV that was recovered in both Vero and C6/36 cells (Table 2).

3.2. Recovery and characterization of second-generation chimeric viruses

Combinations of mutations that were identified in first-generation chimeric viruses were introduced into second-generation cDNA clones (Table 1). Together the NS4B-P101L and Δ30 UTR deletion mutation produced a virus that replicated to low titer and was not tested further. However, six other chimeric viruses were recovered and replicated to peak titers of 3.9–6.6 $\log_{10}$PFU/mL and had small plaque morphologies after recovery (except JEV/DEN4 AC-7a), and in some cases developed larger plaques following Vero cell adaptation (Table 1). The chimeric viruses were sequenced and most had acquired additional mutations (generally in the structural-protein regions), with the exception of JEV/DEN4 AC-7a, which was genetically stable (Table 1).

3.3. Virulence of second-generation chimeric viruses for mice

The second-generation chimeric viruses along with rDEN4, rDEN4Δ30, and JEV SA14-14-2 were screened for IP or IC virulence in 3-week-old mice at both a high (up to 10^4 PFU) and low (10^2 PFU) dose. Mortality was not observed for these viruses, which confirmed their attenuated phenotypes (Table 2). Wild-type JEV and rJEV were tested at a 10^2 PFU dose IP and IC and caused >50% IP mortality and 100% IC mortality (data not shown).

Virulence of the second-generation chimeric viruses was subsequently tested in the more sensitive suckling mouse model. Both wt JEV and rJEV were highly virulent, having LD_{50} levels of ~1 PFU and median survival times of 4 days (Table 2). JEV SA14-14-2 and JEV/DEN4 AC-7a had LD_{50} levels similar to wt JEV, but had significantly longer median survival times (Table 2). JEV/DEN4 clones B-1a, C-14 and JEV/DEN4Δ30 D-6a had significantly higher LD_{50} levels and longer median survival times (Table 2). JEV/DEN4Δ30 D-6a mortality did not follow a clear dose response, with 60%, 60%, and 40% mortality at 10^1, 10^2, and 10^3 PFU doses, respectively. The remaining chimeric viruses caused little or no mortality in suckling mice, similar to rDEN4 and rDEN4Δ30 (Table 2).

The genomes of JEV/DEN4 clones C-14 and AC-7a differed by a single nucleotide (Q264H amino acid substitution) in the E protein (Table 1). JEV/DEN4 AC-7a, bearing E mutation Q264H, had an ~10-fold lower IC LD_{50} and a 4-day reduction in median survival time (Table 2 and Fig. 2A), indicating that this mutation significantly increases virulence in suckling mice.

3.4. Tissue culture replication of JEV/DEN4 clones C-14 and AC-7a

Tissue culture replication experiments were performed for JEV/DEN4 clones C-14 and AC-7a to determine if the E-Q264H mutation that increased virulence for suckling mice also increased replication. In Vero cells, JEV/DEN4 AC-7a had a modest but significant increase in titer on days 3–7 post-infection and an increase in peak titer of 0.6 $\log_{10}$PFU/mL compared to JEV/DEN4 C-14 (Fig. 2B). The difference in replication between viruses was more pronounced in C6/36 cells, with JEV/DEN4 AC-7a reaching significantly higher titers on days 1–7 post-infection, with an increase in peak titer of 1.5 $\log_{10}$PFU/mL (Fig. 2C) indicating that the E-Q264H mutation enhances replication in mammalian and mosquito cells.

3.5. Replication of chimeric viruses and controls in weanling mouse brain

rJEV replicated in the brain of weanling mice with a mean peak titer greater than 8 $\log_{10}$PFU/g by day 5 post-infection, at which point complete mortality was observed (Fig. 3). JEV SA14-14-2 replicated to greater than 4 $\log_{10}$PFU/g of brain by day 5 post-infection followed by a drop in titer to below the limit of detection and all mice survived with no signs of disease (Fig. 3). All six, second-generation chimeric viruses behaved like rDEN4, having virus titers at or below the limit of detection through day 11 post-infection and showing no signs of disease (Fig. 3).

3.6. Immunogenicity and protection from wild-type rJEV challenge

Several experiments were performed in weanling mice to determine if the chimeric viruses were immunogenic. In experiment #1, immunization with JEV/DEN4 clones AC-7a, D-5a, and JEV/DEN4Δ30 B-2a resulted in relatively low seroconversion frequencies and mean neutralization titers (GMT) of 10–50 (Table 3). Mice immunized with the other three candidates did not induce detectable JEV neutralizing antibody titers, similar to mock and rDEN4 immunized control mice. In comparison, all mice immunized with JEV SA14-14-2 seroconverted, with a GMT of 267 (Table 3).

In experiment #2, immunized mice were challenged with 10^4 PFU of rJEV. Mock and rDEN4 immunized groups of mice only had 20% and 30% mortality, respectively, so statistically significant levels of protection could not be established (Table 3). However, all mice immunized with JEV/DEN4 AC-7a and JEV SA14-14-2 survived, while those immunized with JEV/DEN4 clones D-5a, B-1a and C-14 experienced some mortality (Table 3).

In experiment #3, immunized mice were challenged with a higher, 10^5 PFU dose of rJEV that resulted in 40% mortality in mock and rDEN4 immunized groups of mice. All of the mice immunized with JEV/DEN4Δ30 D-6a and JEV SA14-14-2 survived challenge, and this was statistically significant (Table 3). JEV/DEN4Δ30 B-2a immunization resulted in 90% survival, but this was not statistically different from mock or rDEN4 immunized mice.

Experiment #4 was designed to correlate the frequency of seroconversion with protection among immunized mice. Immunization with JEV/DEN4 AC-7a and JEV SA14-14-2 resulted in 60% seroconversion and neutralizing GMTs of 80 and 41, respectively. All mice immunized with JEV SA14-14-2 survived challenge, compared to 90% for JEV/DEN4 AC-7a (Table 3). Mice immunized with JEV/DEN4 clones C-14, D-5a or JEV/DEN4Δ30 D-6a each had one mouse seroconvert with relatively low neutralizing antibody titers and only mice immunized with JEV/DEN^{4}C–^{14} had significant protection from challenge with rJEV (Table 3). Mice immunized with the remaining two chimeric viruses did not induce detectable neutralizing antibody, but JEV/DEN4 B-1a immunization did improve survival (Table 3). Overall, JEV/DEN4 AC-7a was the only candidate to demonstrate protection in conjunction with a substantial level of seroconversion.

4. Discussion

Since JEV co-circulates with DENV 1–4 in Asia, it would be ideal to produce an inexpensive, live-attenuated, pentavalent vaccine to protect against both diseases. With this goal in mind, we developed a set of chimeric viruses by replacing the structural protein genes of rDEN4 and rDEN4Δ30 with those of wt JEV India/78 to generate vaccine candidates that were compatible with the NIH tetravalent DENV vaccine [21]. Replacing the C, prM and E genes of rDEN4 or rDEN4Δ30 with those of JEV did not result in viable chimeric viruses, similar to results of comparable strategies with other flaviviruses [22,33,34]. However, we were able to recover viable chimeric viruses by exchanging only the prM and E protein genes, inferring that the C protein gene may be incompatible between

flaviviruses due to its lack of sequence conservation, which may hinder genome cyclization and encapsidation of viral RNA [35–43].

The first-generation ME chimeric viruses accumulated a number of mutations throughout the genome during passaging and adaptation to Vero cells. All six first-generation candidates contained at least one NS4B mutation (P101L, T105I, V109A, L112S, A240V) known to improve DENV replication in Vero cells [44]. The NS4B-P101L mutation has been shown to decrease replication in mosquitoes or mosquito cell lines and enhance replication in mammalian cell lines [45]. The importance of mutations that were identified outside of the NS4B gene has not been defined. The combinations of mutations E-I430T/NS4B-P101L or NS3-S158L/NS4B-T105I were each found in two separate chimeric viruses, which may indicate that they work well in concert. Additionally, utilization of JEV, rDEN4 and WNV C-prM junctions produced viable chimeric viruses with similar *in vitro* and *in vivo* phenotypes, and the lack of mutation within this site indicated that they were genetically stable and interchangeable.

Second-generation ME chimeric viruses were engineered to contain a set of mutations based on observations from the first generation. After recovery and adaptation to Vero cells, only JEV/DEN4 AC-7a bearing the NS4B-P101L/E-I430T/E-Q264H combination was genetically stable. Mutation E-I430T was found in three independently- isolated large-plaque chimeric viruses suggesting that it alleviates an incompatibility created by chimerization and might stabilize the virus genome when combined with NS4B-P101L.

In weanling mice, wt JEV and rJEV were neuroinvasive and highly neurovirulent, replicating to high titer in the brain and causing uniform mortality. The JEV/DEN4 chimeric viruses, by contrast, behaved like the rDEN4 and rDEN4Δ30 parent viruses, which were avirulent and had little or no detectable replication in the brain. In comparison, the JEV SA14-14-2 vaccine strain was also avirulent in this mouse model, but surprisingly replicated to greater than 4 $\log_{10}$PFU/g of brain.

In the more permissive suckling mouse model, wt JEV, rJEV, and JEV SA14-14-2 were highly neurovirulent, whereas rDEN4 and rDEN4Δ30 were avirulent, consistent with their lack of neurovirulence. Various levels of attenuation were observed for the chimeric viruses in these mice. The most attenuated chimeric virus, JEV/DEN4Δ30 B-2a, bearing a 3′ UTR deletion mutation that attenuates rDEN4 [29,46], caused no mortality. The least attenuated chimeric virus in suckling mice, JEV/DEN4 AC-7a, had a virulence phenotype similar to the JEV SA14-14-2 vaccine strain.

JEV/DEN4 clones C-14 and AC-7a differed by a single Q264H amino acid substitution in the E protein that increased replication in mammalian (Vero) and mosquito (C6/36) cells, virulence for suckling mice, and immunogenicity in weanling mice. The ability of mutation NS4B-P101L, which is shared by JEV/DEN4 clones C-14 and AC-7a, to enhance replication in mammalian cells [45] likely masks the true magnitude of the phenotype conferred by E-Q264H. This E protein mutation is rare among wt JEV strains and was one of many mutations that arose during efforts to improve immunogenicity of the JEV SA14-14-2 vaccine strain, which included subcutaneous passage in suckling mice [17]. Our

data indicate that E-Q264H might improve immunogenicity by increasing virus replication *in vivo*, as it does *in vitro*. It is notable that E-Q264H did not increase replication of JEV/DEN4 AC-7a to detectable levels in weanling mouse brain, indicating that the enhanced replication may only occur in peripheral tissues. E protein residue 264 is located in domain II at the dimer interface, adjacent to histidine (H) 263, which is conserved among JEV serocomplex viruses [47]. Protonation of histidine residues at acidic pH is proposed to drive structural rearrangements of the E protein necessary for flavivirus membrane fusion [48] and the E-Q264H mutation could have an impact on this mechanism.

The rDEN4 and rDEN4Δ30 clones have been used as a platform to generate chimeric virus vaccine candidates for WNV and SLEV, both JEV-serocomplex viruses, with mixed results [23,24]. Like SLEV, chimerization of JEV with rDEN4 or rDEN4Δ30 produced several vaccine candidates that were over-attenuated in mice. However, a single vaccine candidate (JEV/DEN4 AC-7a) bearing the replication enhancing E-Q264H mutation was genetically stable, attenuated, and immunogenic in mice, suggesting it is a suitable candidate for further development. We are currently investigating this possibility along with alternative live-attenuated JEV vaccine options that would be appropriate for combination with the NIH tetravalent DENV vaccine.

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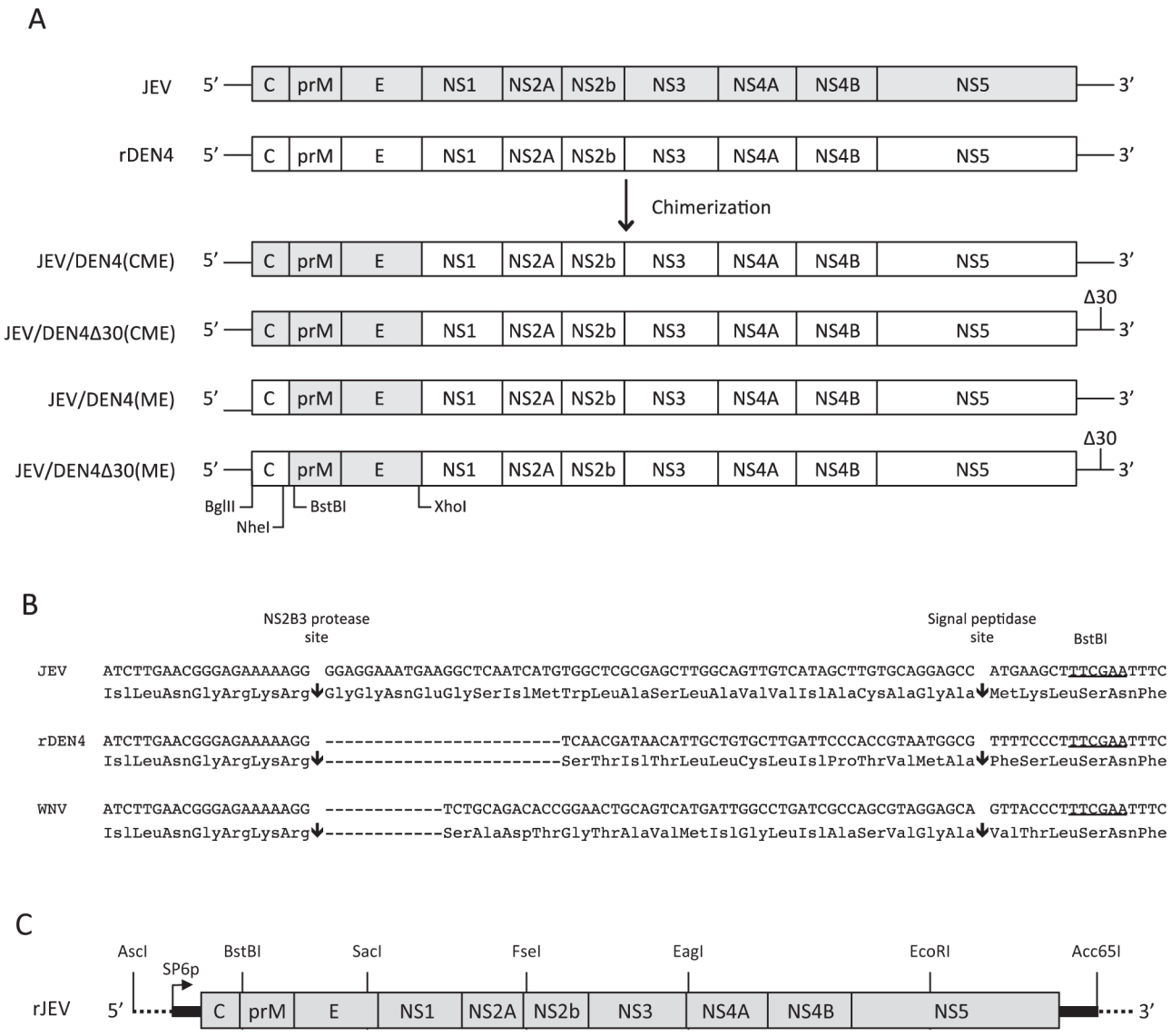

Fig. 1.

Construction of recombinant chimeric virus and wild-type JEV cDNA clones. (A) For chimeric viruses, the C, prM and E genes, or just the prM and E genes of rDEN4 and rDEN4Δ30 cDNA clones (p4 and p4Δ30, respectively) were replaced with those of JEV strain India/78. The locations of restriction enzyme recognition sites used for cloning are indicated. (B) Three C-prM junction sequences, corresponding to JEV, rDEN4 and WNV, were used for constructing the JEV/DEN4 (ME) clones. The nucleotide and amino acid sequences surrounding the NS2B3 protease and signal peptidase cleavages sites (arrows) at the C-prM junction are shown. The BstBI restriction enzyme recognition site used for cloning is underlined. The other restriction enzyme site used for cloning (NheI) is located further upstream and not depicted in this figure. (C) Construction of a recombinant JEV India/78 cDNA clone. Restriction enzyme recognition sites used for cloning of the six, synthesized cDNA segments are indicated. UTR sequence is indicated in black and flanking

vector sequence is indicated by a dotted line. The SP6 polymerase start site (SP6p) for generation of infectious transcripts is shown.

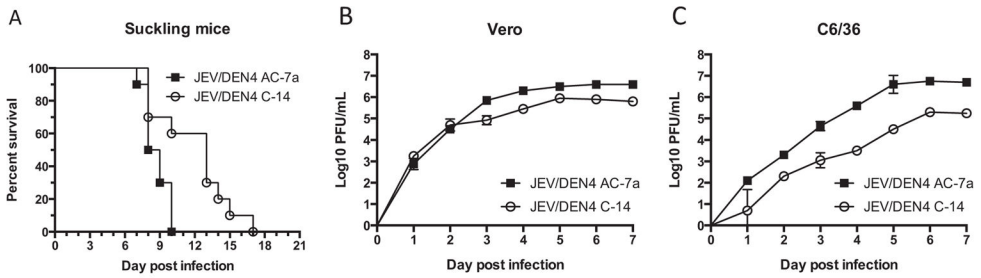

Fig. 2.

The impact of JEV/DEN4 AC-7a envelope protein mutation Q264H on virulence in suckling mice and replication in tissue culture. (A) Survival curves for suckling mice injected IC with 10^3 PFU of JEV/DEN4 clones C-14 and AC-7a were significantly different by Log-rank test ($p = 0.0076$). Replication of JEV/DEN4 clones C-14 and AC-7a in Vero (B) and C6/36 (C) cells infected at an MOI of 0.01 PFU/cell. Samples were taken daily and titered in Vero cells. Error bars represent the standard error of the mean (SEM) from two independent experiments.

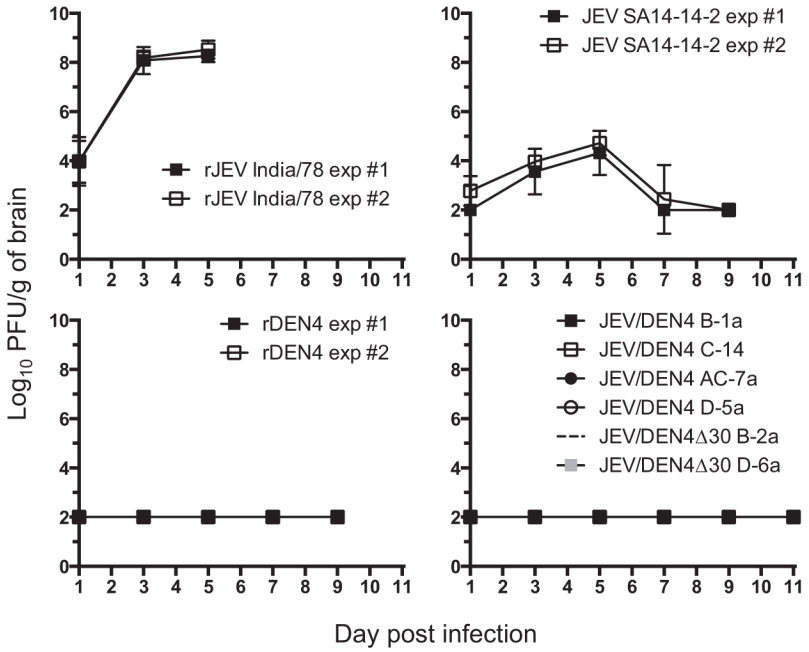

Fig. 3.

Replication of chimeric viruses and controls in the brain of weanling mice. Groups of five-, three-week-old, SW mice per time-point were infected IC with 10^3 PFU of virus. Beginning on day 1 post-infection, and then every other day thereafter, groups of animals were euthanized and brains were harvested and homogenized. Virus titers were determined in Vero cells, with a limit of detection of 2 $\log_{10}$ PFU/g of brain. Data for rJEV India/78, JEV SA14-14-2 and rDEN4 are shown for two independent experiments (#1 and #2). The six, second-generation chimeric viruses and rDEN4 had titers at or below the limit of detection. Error bars represent the standard deviation (SD).

Table 1

Properties of chimeric viruses.

Virus[a] (clone)	C-prM junction	Gene[b]	Nucleotide change (chimeric virus numbering)[b]	Amino acid change (individual protein numbering)[b]	Phenotype in vero cells		
					Mean peak titer (log_{10} PFU/mL)	Plaque size prior to adaptation[c,d]	Plaque size after adaptation[c]
First-generation							
JEV/DEN4 (CF-ME-0-13)	JEV	E	T1465C	F167S	6.0	Small	Large
		E	A1900G	K312R			
		E	T2254C	I430T			
		NS4B	C7171T	P101L			
JEV/DEN4 (GG-ME-1-17)	DENV-4	E	G1733C	Q264H	6.6	Small	Large
		E	T2230C	I430T			
		NS4B	C7147T	P101L			
		3′UTR	10,485 A insert	N/A			
JEV/DEN4 (GG-ME-2-21)	WNV	E	A1671C	M240L	6.1	Small	Medium
		E	A2391G	M480V			
		NS3	C5026T	S158L			
		NS4B	C7171T	T105I			
JEV/DEN4Δ30 (CF-ME-0-14)	JEV	E	G1722T	V253F	5.5	Small	Small
		NS2A	A4023G	M168V			
		NS3	G5170T	R202I			
		NS4B	T7195C	V109A			
		NS4B	C7588T	A240V			
JEV/DEN4Δ30 (GG-ME-1-24)	DENV-4	NS3	C5014T	S158L	5.6	Small	Small
		NS4B	C7159T	T105I			
JEV/DEN4Δ30 (CF-ME-2-18)	WNV	E	T2043C	S364P	5.3	Small	Small
		E	G2190A	G413R			
		NS4B	T7192C	L112S			
Second-generation							
JEV/DEN4 (B-1a)	DENV-4	prM	G765A	E109K	5.8	Small	Small
		NS3	**C5014T**	**S158L**			

Virus[a] (clone)	C-prM junction	Gene[b]	Nucleotide change (chimeric virus numbering)[b]	Amino acid change (individual protein numbering)[b]	Phenotype in vero cells		
					Mean peak titer (log_{10} PFU/mL)	Plaque size prior to adaptation[c,d]	Plaque size after adaptation[c]
		NS4B	**C7159T**	**T105I**			
JEV/DEN4 (C-14)	DENV-4	E	T2230C	I430T	6.0	Small	Large
		NS4B	**C7147T**	**P101L**			
JEV/DEN4 (AC-7a)	DENV-4	**E**	**G1733C**	**Q264H**	6.6	Large	Large
		E	**T2230C**	**I430T**			
		NS4B	**C7147T**	**P101L**			
JEV/DEN4 (D-5a)	DENV-4	E	A1567G	K209R	6.0	Small	Medium
		NS4B	**T7180C**	**L112S**			
JEV/DEN4Δ30 (B-2a)	DENV-4	prM	T936C	Y166H	5.0	Small	Small
		NS3	**C5014T**	**S158L**			
		NS4B	**C7159T**	**T105I**			
JEV/DEN4Δ30 (D-6a)	DENV-4	NS3	G4667T	M42I	3.9	Small	Small
		NS4B	**T7180C**	**L112S**			

[a] All viruses are ME chimeric viruses (CME chimeric viruses were not viable). ME has been omitted from the virus names.

[b] Engineered mutations introduced into the second generation viruses are shown in bold.

[c] Plaque size was measured on day 6 post-infection: small is <1 mm, medium is 1–2 mm, and large is >2 mm. Vero-adapted rJEV India/78 (3 days post-infection) and rDEN4 produce large plaques. Vero-adapted rDENΔ30 produces medium plaques. JEV SA14-14-2 (3 days post-infection) produces small plaques.

[d] Plaque size on Vero cells directly after recovery of virus and prior to terminal dilutions on Vero cells.

Table 2

Virulence in weanling and suckling mice.

Virus	Clone	C-prM junction	Weanling mice (n = 6/dose)		Suckling mice (n = 10/dose)	
			IP LD_{50} (PFU)	IC LD_{50} (PFU)	IC LD_{50} (PFU)	Median survival time (days)[a]
JEV India/78	Wild-type	–	40	0.6	1.5	4
rJEV India/78	Recombinant	–	10	0.4	0.3	4
JEV SA14-14-2	Vaccine Strain	–	>10,000[b]	>10,000	0.3	7
rDEN4	Recombinant	–	>10,000	>10,000	>100	>21[c,d,e]
rDEN4Δ30	Vaccine strain	–	>10,000	>10,000	>100	>21[d,e]
First-generation						
JEV/DEN4	CF-ME-0-13	JEV	>10,000	>10,000	NT	NT[g]
JEV/DEN4	GG-ME-1-17	DENV-4	>10,000	>10,000	NT	NT
JEV/DEN4	GG-ME-2-21	WNV	>10,000	>10,000	NT	NT
JEV/DEN4Δ30	CF-ME-0-14	JEV	>10,000	>3000	NT	NT
JEV/DEN4Δ30	GG-ME-1-24	DENV-4	>10,000	>4000	NT	NT
JEV/DEN4Δ30	CF-ME-2-18	WNV	>10,000	>2000	NT	NT
Second-generation						
JEV/DEN4	B-1a	DENV-4	>10,000	>3200	22	10[c,d,e]
JEV/DEN4	C-14	DENV-4	>10,000	>5000	7	13[d,e]
JEV/DEN4	AC-7a	DENV-4	>10,000	>10,000	0.8	8.5[d,e,f]
JEV/DEN4	D-5a	DENV-4	>10,000	>10,000	≥1000	18.5[d,e]
JEV/DEN4Δ30	B-2a	DENV-4	>10,000	>1000	>1000	>21[d,e]
JEV/DEN4Δ30	D-6a	DENV-4	>1600	>160	17[h]	>21[d,e]

[a] Reported for the high dose IC (10^3 PFU).

[b] All values shown with ">" sign indicate the highest dose administered.

[c] >21 days is reported if the majority of mice survived until the end of the study.

[d] Survival time is statistically different from rJEV India/78 by Log-rank (Mantel-Cox) test.

[e] Survival time is statistically different from JEV SA14-14-2 by Log-rank (Mantel-Cox) test.

[f] Survival time is statistically different from JEV/DEN4 C-14 by Log-rank (Mantel-Cox) test.

[g] Not tested (NT).

[h] Mortality did not follow a clear dose response.

Table 3

Immunogenicity and protection in mice.

Virus	Clone	Experiment #1 (n = 6)		Experiment #2 (n = 10)	Experiment #3 (n = 10)	Experiment #4 (n = 10)		
		Seroconversion	GMT ($1/EC_{50}$)[a]	Percent survival (10^4 PFU rJEV)	Percent survival (10^5 PFU rJEV)	Seroconversion	GMT ($1/EC_{50}$)[a]	Percent survival (10^6 PFU rJEV)
JEV/DEN4	B-1a	0/6	<8	80	_[b]	0/10	<8	80
JEV/DEN4	C-14	0/6	<8	80	–	1/10	8	100[c]
JEV/DEN4	AC-7a	2/6	50	100	–	6/10	80	90[e]
JEV/DEN4	D-5a	2/6	28	90	–	1/10	8	60
JEV/DEN4Δ30	B-2a	1/6	10	–	90	0/10	<8	50[e]
JEV/DEN4Δ30	D-6a	0/6	<8	–	100[c,d]	1/10	28	30[e]
JEV SA14-14-2	Vaccine strain	6/6	267	100	100[c,d]	6/10	41	100[c]
rDEN4	Recombinant	0/6	<8	70	60	0/10	<8	70
Mock	Not applicable	0/6	<8	80	60	0/10	<8	60

[a] Geometric mean reciprocal EC_{50} titers for mice that seroconverted (limit of detection: <8).

[b] A dash indicates the virus was not tested.

[c] Survival curve is statistically different from mock immunized mice by Log-rank (Mantel-Cox) test.

[d] Survival curve is statistically different from rDEN4 immunized mice by Log-rank (Mantel-Cox) test.

[e] These groups contained mice that were significantly undersized for their reported age (AC-7a = 2/10 mice, B-2a = 9/10 mice, D-6a = 4/10 mice) at the time of immunization and this may have affected the experimental outcomes.