Chimeric Japanese encephalitis virus/dengue 2 virus infectious clone: biological properties, immunogenicity and protection against dengue encephalitis in mice

Thomas J. Chambers,† Xiaoshan Jiang, Deborah A. Droll, Yan Liang, William S. M. Wold and Janice Nickells

A molecular clone of *Japanese encephalitis virus* (JE virus) was derived from the JE virus Nakayama strain and used to produce infectious JE virus in cell culture. The engineered JE virus resembled the parental JE virus in cell-culture properties and was related closely to other JE virus strains based on nucleotide sequence analysis. The JE virus clone was used as a genetic background for construction of a chimeric virus containing the structural proteins prM and E of *Dengue virus*, serotype 2. The chimeric JE/dengue 2 virus generated authentic dengue 2 structural proteins as assessed by immunoassays for the dengue E protein. It exhibited a small plaque size and less efficient growth in various cell lines than the parental JE virus. JE/dengue 2 virus was non-neuroinvasive for young adult mice, but displayed partial neurovirulence at doses up to 4 log p.f.u. given intracerebrally.

Immunization of 3-week-old mice with JE/dengue 2 virus yielded neutralizing-antibody titres against dengue 2 virus and conferred protection against dengue encephalitis caused by neuroadapted dengue 2 virus. A rise in post-challenge neutralizing-antibody titres against dengue 2 virus in surviving mice suggests that immunization is associated with establishment of a memory antibody response in this model. This study demonstrates the capacity of JE virus to serve as a vector for expression of heterologous flavivirus structural proteins. Similar to previous studies with other chimeric flaviviruses, this approach may be useful as a genetic system for engineering experimental vaccines against *Dengue virus* and other medically important flaviviruses.

INTRODUCTION

Within the family *Flaviviridae*, *Japanese encephalitis virus* (JE virus) and *Dengue virus* are important agents of acute encephalitis and haemorrhagic fever, respectively (Monath, 1986; Monath & Heinz, 1996). JE is a serious illness with a mortality rate as high as 30%, and can cause permanent neurological sequelae among survivors (Solomon et al., 2000). Although vaccine products are available for prevention of JE (Tsai, 1994), development of additional vaccines for worldwide use remains an important goal, because of suboptimal immunogenicity of live-attenuated vaccine (Hennessy et al., 1996) and safety issues with inactivated vaccine derived from mouse brain (Marfin et al., 2005; Monath, 2002; Takahashi et al., 2000).

There is an increasing worldwide burden of human disease due to dengue viruses (Gubler, 1997; Monath, 1994). Serotype-specific immunity does not confer cross-protection against secondary infections with heterologous serotypes (Halstead, 2003), which may contribute to the pathogenesis of dengue haemorrhagic fever and dengue shock syndrome as a result of antibody-dependent enhancement of infection mediated by cross-reactive, non-neutralizing antibodies. There is currently no licensed vaccine for dengue, despite intense efforts with a range of different approaches (Edelman, 2005; Hombach et al., 2005).

Molecular clone technology has been used to engineer chimeric flaviviruses as live-attenuated viral vaccines (Caufour et al., 2001; Huang et al., 2000; Mathenge et al., 2004; Pletnev & Men, 1998; Pletnev et al., 1992, 2001, 2002). We have used this approach to produce chimeric viruses composed of dengue or JE virus structural antigens within the backbone of the yellow fever virus 17D strain for the development of novel flavivirus vaccines (Chambers et al., 2002; Nickells et al., 2004).

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METHODS

Cells and viruses. Vero, BHK-21, SW-13 (human adrenocorticotrophic carcinoma) and C6/36 cells were originally obtained from the ATCC and maintained in alpha minimal essential medium (MEM) plus 10% fetal bovine serum (FBS). LLC-MK2 cells (ATCC) were maintained in M-199 medium plus 20% FBS. JE Nakayama virus was obtained from the ATCC and passed on LLC-MK2 cells. Virus was plaque-purified on Vero cells prior to molecular cloning. Dengue 2 virus (New Guinea C strain) and sucking mouse brain-passaged dengue 2 virus were obtained from Dr Jack Schlesinger (University of Rochester School of Medicine, Rochester, NY, USA) and passaged once on C6/36 cells at 28°C to produce working virus stocks. The dengue 2 virus New Guinea strain was used as a control virus for growth curves, plaque-reduction neutralization and immunoprecipitation experiments. Mouse brain-passaged dengue 2 virus was used for mouse-challenge experiments. Plaque assays were done for JE and dengue 2 viruses in Vero cells, using 1% ME agarose (Biowhittaker) overlay, in alpha MEM plus 5% FBS for 5 days at 37°C. Plaque assay for JE/dengue 2 virus was done in subconfluent monolayers of SW-13 cells using the same overlay and incubation conditions. Plaques were visualized by staining with 0.05% neutral red in PBS, followed by fixation in formalin and staining with 1% crystal violet.

Molecular cloning procedures. Total cellular RNA was extracted from JE Nakayama virus-infected Vero cells by using TRIzol RE agent (Gibco-BRL). The final RNA pellet was dissolved in RNase-free water. The primers for the reverse transcription and long PCR amplification were as follows (5’-3’). These primers were based on the partial JE Nakayama sequence (McAda et al., 1987) and the JaOAr5982 sequence (Sumiyoshi et al., 1987). Sense primers are indicated by (+) and antisense by (−): JE5’(+), GAGAATTTAGATTTGCTGG; JE’S+I-T-SP6(+), GCG-GGGCCATTTAAGTTGACACTATAGAGAAGTTTATCTGTGTGAAC; JE3443BspEI(+), GATCCTGGACAGAAAAATGGCTGTG; JE3463BspEI(−), GGACCCATTGTTGTTCTCCGAAATCG; JE3’(−), AGATCTCGGTTTCTTCCTACACC; JE3’KpolM(−), AGGTACGAGATCCTGGTTGTCTTCCCTACACC.

cDNA synthesis was done by using Superscript II RTase (Gibco-BRL) according to the manufacturer’s guidelines with slight modifications. In brief, 1–2 µg JE virus RNA was mixed with 10 pmol 3’-terminal primer [JE3’(−) or JE3463BspEI(−)], heated for 10 min at 70°C, cooled on ice and then incubated with Superscript II in the recommended buffer for 2–4 h at 42°C. Reactions were then treated with DNase-free ribonuclease H at 37°C for 20 min, followed by heating to 70°C for 10 min and then extraction with phenol/chloroform and ethanol precipitation of the cDNA reaction products. The final pellet was dissolved in water for PCR amplification.

PCR amplifications were done from cDNA by using the Expand High Fidelity PCR system (Roche). Reactions contained 10 pmol each of sense and antisense primers and were run for 30 cycles with the following program: 95°C for 30 s; 95°C for 1 min at 62°C and 3 min or 8 min at 72°C for the 5’ [JE5’(+)] plus [JE3463BspEI(−)] and 3’ [JE3’(−)] and [JE3463BspEI(−)] primer combinations, respectively. The final elongation step was 15 min at 72°C. PCR products were isolated on agarose gels, visualized by crystal violet staining, excised and recovered by using extraction with 6 M sodium iodide at 70°C. They were purified with the Wizard PCR Prep DNA purification system (Promega) and cloned into pCR-TOPO (Invitrogen). Clones were characterized by nucleotide sequencing.

To subclone the JE Nakayama virus genome from pCR-TOPO plasmids into plasmids suitable for generation of in vitro-ligation templates, the 5’-end PCR primer was modified to include a NotI restriction site followed by the Sp6 promoter sequence for cloning into a derivative of pYF5’3’IV (Rice et al., 1989) (see below). The 3’ PCR primer was modified to include a KpnI restriction site adjacent to the JE virus 3’ terminus to enable linearization of the transcription templates with KpnI. The JE genome was then constructed in two separate plasmids, similar to the construction of the YF5’2iv and other molecular clones (Chambers et al., 1999, 2003; Rice et al., 1989). Unique enzyme sites in the JE genome were predicted from the JaOAr5982 sequence (Sumiyoshi et al., 1987). Two PCR fragments (a 5’ fragment of approximately 3.4 kb and a 3’ fragment of approximately 7.6 kb), overlapping an authentic unique BspEI restriction site at nt 3445, formed the basis of the two-plasmid system.

The 5’-3’4 kb PCR fragment from the 5’ terminus to the BspEI site of JE virus (nt 3445) was cloned into pYF5’3’IVJE-E (a derivative of YF5’3’IV containing an engineered BspEI site; Chambers et al., 1999) by using the NotI (nt 6657) and BspI [nt 8579, yellow fever virus genome (YF) numbering] restriction sites. Several pJE5’3’B fragment clones harboring the 3.4 kb 5’-terminal PCR product were screened for infectivity together with clones of pCR-XL3’2B (see below), and one resulting clone, designated pJE5’NK25, was used to generate infectious JE virus.

The 7.6 kb 3’ fragment of the JE virus genome was engineered by PCR to contain the KpnI site at the 3’ terminus of the JE virus genome and cloned again into pCR-XL-TOPO (Invitrogen). Resulting plasmid clones were screened by partial nucleotide sequence analysis and restriction-enzyme profiles. Several clones were used for further construction of full-length cDNA templates. A working clone, designated pJE3’NK, was used for virus production.

The final plasmids pJE5’NB25 and pCR-XL3’2B, which yielded the two-plasmid system for regenerating JE virus, were used to determine the nucleotide sequence of the entire JE virus XJN infectious clone.

Construction of JE/dengue 2 virus. To introduce the prM-E region of dengue 2 virus into the JE XJN clone, this region was engineered into pJE5’NB25 (described above) to replace the JE prM-E region. The plasmid pDVWS307 (Gualano et al., 1998), containing the dengue 2 prM-E region of the POU-218 strain, was used as the starting material. A fragment spanning the dengue 2 C/prM and E/NS1 junctions was amplified by PCR and cloned into pCR-TOPO to create pDEN25 (pPR-M-E-TOPO). Kdo sites were introduced immediately prior to the C/prM cleavage site in this plasmid, as well as pJE5’NB25, by silent mutagenesis. The C-terminal regions of the JE and dengue 2 E proteins were modified by introduction of an SpII site at the E/NS1 junction by site-directed mutagenesis, involving a single amino acid replacement (V-Q-A instead of V-H-A) at the signalase site. A redundant SpII site upstream in the dengue 2 E region was also eliminated by silent mutagenesis. The dengue 2 prM-E region was exchanged into pJE5’NB25 by using the KasI and SpII restriction sites to yield pJE5’NK/DEN2. This plasmid was sequenced through the engineered structural region to ensure the integrity of the clone.

RNA transcription and transformation. Full-length cDNA templates for infectious JE virus were assembled by using DNA fragments
from pJE5’NB25 (after digestion with NotI and BspEI) and pCRXL3’BK2 (after digestion with BspEI and KpnI). Fragments were isolated on low-melting-temperature agarose gels. Approximately 3-0 μg of the 3-4 kb NotI-BspEI fragment and 12-0 μg of the 7-6 kb BspEI-KpnI fragment were ligated in reactions containing 400 units T4 DNA ligase (New England Biolabs) for 12 h at 16°C. Reactions were then heat-inactivated, digested with KpnI, extracted with phenol/chloroform and precipitated with ethanol. The final pellet was dissolved in TE [10 mM Tris/HCl (pH 7.5), 1 mm EDTA] and the full-length ligation product was visualized by agarose-gel electrophoresis. Templates were transcribed by using Sp6 RNA polymerase (New England Biolabs), essentially as described previously (Rice et al., 1989). Transfection of confluent BHK cells was done by using Lipofectin (Gibco-BRL) and cells were incubated at 37°C in MEM plus 5% FBS. Medium was harvested at onset of cytopathic effects, approximately 4 days post-transfection. Infectious virus was titrated by plaque assay on Vero cells.

A similar protocol was used for recovery of transcription templates for JE/dengue 2 virus, except that pJE-5’NB25 was used instead of pJE-5’NB25. Transcription and transfection were done as described above.

**Virus growth curves.** Virus production in cell culture was analysed by using confluent monolayers of C6/36, LLC-MK2 or BHK-21 cells at an m.o.i. of 0-01 p.f.u. per cell. Triplicate samples were run for each virus in each cell line. After infection for 1 h at 37°C (BHK and LLC-MK2) or 28°C (C6/36 cells), media were replaced and cells were incubated at 37°C (BHK and LLC-MK2 cells) or 28°C (C6/36 cells). Media were harvested at 12 h (BHK and LLC-MK2 cells) or 24 h (C6/36 cells) intervals, followed by replacement with fresh medium (alpha MEM plus 5% FBS). Virus yields were quantified by plaque titration on Vero cells (JE Nakayama, JE-XJN and dengue 2 viruses) or SW-13 cells (JE/dengue 2 viruses), as described above.

**Nucleotide sequence analyses.** Plasmids encoding the JE Nakayama virus or JE/dengue 2 virus were sequenced by using Applied Biosystems BigDye sequence reactions and analysed on an ABI DNA sequencer.

**Plaque-reduction neutralization testing.** The plaque-reduction neutralization assay was done as described previously (Chambers et al., 1999, 2003). JE hyperimmune ascitic fluid and control non-immune ascitic fluids were obtained from the ATCC. Monoclonal antibody (mAb) to the dengue 2 envelope protein (cat. no. AS-22-99; clone 3H5) was obtained from Microbix Systems. Approximately 100 p.f.u. input virus was mixed with serial twofold dilutions of immune ascitic fluid, mAb or non-immune control ascitic fluid in various experiments. The reaction mixture was kept at 4°C for 6 h, followed by plaque assay on SW-13 cells (for JE/dengue 2 virus) or Vero cells (for JE and dengue 2 viruses) using conditions described earlier. Plaques were visualized as described earlier. Fifty and ninety per cent plaque-reduction end points were used for evaluation of neutralization titres.

**Radiolabelling of infected cells.** Cells were infected at an m.o.i. of 5 p.f.u. per cell and labelled in medium containing 20 μCi (740 kBq) [35S]methionine ml⁻¹ (ICN) at approximately 20 h post-infection for 4–6 h. Cells were lysed in Triton lysis solution [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% BSA, 40 μg PMSF ml⁻¹]. Immunoprecipitation was done by using 3 μl antiserum (anti-JE virus hyperimmune ascitic fluid, non-immune ascitic fluid or anti-dengue 2 hyperimmune ascitic fluid) with samples kept at 4°C overnight. Immunoprecipitated proteins were recovered by using Pansorbin (Calbiochem), followed by washing the reactions and preparation and analysis of the recovered proteins on 13% SDS-PAGE gels as described previously (Chambers et al., 1999, 2003). Proteins were visualized by fluorography.

**Mouse experiments.** ICR mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN, USA) and handled in accordance with institutional guidelines on the care and use of laboratory animals. Mice were used for immunization at 3 weeks of age, using intraperitoneal inoculation of JE/dengue 2 virus. For immunization/challenge experiments, mice were inoculated with JE/dengue 2 viruses at 3 weeks of age and challenged at 2–3 weeks post-immunization by intracerebral injection of neuroadapted dengue 2 virus, as described previously (Chambers et al., 2003). Mice were observed until moribund and then euthanized, or otherwise sacrificed after 4 or 8 weeks, and sera were collected for measurement of neutralizing-antibody titres. Neuroviremia testing was done in mice at 4 weeks of age by using intracerebral inoculation.

## RESULTS

### Recovery of infectious JE and JE/dengue 2 viruses

Fig. 1 indicates the structure of the plasmids engineered to encode infectious JE Nakayama and JE/dengue 2 viruses. Yields of infectious virus after transfection with the JE-XJN clone were approximately 5 × 10⁷ p.f.u. (ml medium)⁻¹ and the plaque size on Vero cells was identical to that of parental JE Nakayama virus (approx. 3–4 mm after 4–5 days incubation). Transfection harvests of JE/dengue 2 virus yielded much lower concentrations of virus (approx. 5 × 10⁶ p.f.u. ml⁻¹). The plaques were only detectable by plaque assay on SW-13 cells and were very small (approx. 1 mm in size after 5 days incubation at 37°C).

### Nucleotide sequence analysis

The complete nucleotide sequence of the JE-XJN clone was determined from the plasmid DNAs used for construction of transcription templates. Comparison of the nucleotide and deduced amino acid sequences of this virus with those of other JE virus strains exhibiting a virulent phenotype (SA14 (Ni et al., 1995), JaOArS982 (Sumiyoshi et al., 1987), Beijing-1 (Hashimoto et al., 1988), P3 (Ni & Barrett, 1996), JaGar01 (Mangada & Takegami, 1999) and Vellore P20778 (Vrati, 2000)] revealed an overall level of sequence similarity of between 97 and 98% at the nucleotide level and 98 and 99% at the amino acid level (data not shown). The 5’ untranslated region (UTR) was identical to those of four other JE virus strains, whereas similarity of the 3’ UTR was between 97 and 98%. Among the viral structural proteins, the level of similarity varied from 97 to 99% at the nucleotide level and from 97 to 99% at the amino acid level. Among the viral non-structural proteins, levels of nucleotide similarity ranged between 97 and 100%, and amino acid similarity varied from 95 to 100%.  

### Antigenic properties of recovered viruses

In tests of neutralization specificity (Table 1), both the JE Nakayama parental virus and the JE-XJN clone were neutralized by JE virus hyperimmune ascitic fluid at high dilutions. These viruses were not neutralized by non-immune ascitic fluid and only weakly by neutralizing mAb
against dengue 2 virus. Dengue 2 virus was neutralized efficiently by the dengue 2-specific mAb, but not by non-immune ascitic fluid or JE hyperimmune ascitic fluid. Two clones of JE/dengue 2 virus were neutralized by anti-dengue 2 mAb. End-point dilutions for 90% plaque reduction were lower than those observed for dengue 2 virus, although 50% plaque-reduction end points were similar. Sequence variations between the dengue 2 and JE/dengue 2 viruses may be responsible for the difference in neutralization activity of the mAb observed here, as the E proteins represent different strains of dengue 2 virus. The JE/dengue 2 viruses were not neutralized efficiently by non-immune ascitic fluid or by anti-JE virus hyperimmune ascitic fluid.

Proteins produced by the JE and the JE/dengue 2 viruses in Vero cells were analysed by immunoprecipitation of [35S]methionine-labelled proteins with JE- and dengue-specific antisera and SDS-PAGE. Fig. 2(a) shows that the JE-XJN virus clone and JE Nakayama virus generated a similar profile of proteins reactive with antisera to JE virus and consistent in size with the JE NS5, NS3, E, NS1, prM and NS2B proteins. JE/dengue 2 virus generated proteins reactive with antisera to JE virus and consistent in size with the JE NS5, NS3, NS1 and NS2B proteins. The dengue 2 envelope protein [molecular mass (MM) slightly greater than that for the JE envelope protein (MM 55 kDa)] was also detected; however, the dengue 2 prM protein was not detected. JE/dengue 2 virus generated proteins reactive with antisera to dengue 2 virus and consistent in size with the dengue envelope (MM 55 kDa) and prM (MM 22 kDa) proteins (Fig. 2b). These two proteins were also produced by dengue 2 virus; however, the apparent MMs were slightly greater than those of the JE/dengue 2 virus proteins. Differences in glycosylation resulting from growth of the dengue virus in C6/36 cells may explain this phenomenon. JE virus produced proteins reactive with antisera to dengue 2 virus and consistent in size with the JE NS5, NS3, E, NS1, prM and NS2B proteins. The JE envelope protein, but not prM, was also detected. The viral proteins immunoprecipitated with antisera to JE and dengue 2 viruses were not detected in mock-infected cells and not with the use of non-immune ascitic fluid, except for some minor reactivity with the JE NS5 protein.

### Table 1. Plaque-reduction neutralization testing

All titres are reciprocals of serum dilution.

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<th></th>
<th>NIAS*</th>
<th>JE†</th>
<th>DEN-2‡</th>
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<tr>
<td><strong>JE-NAK</strong></td>
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<tr>
<td>50%</td>
<td>20</td>
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<td>90%</td>
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<td><strong>JE-XJN</strong></td>
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<tr>
<td>50%</td>
<td>20</td>
<td>2560–5000</td>
<td>80</td>
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<tr>
<td>90%</td>
<td>20</td>
<td>1600–2560</td>
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<td><strong>DEN2</strong></td>
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<td>50%</td>
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<td>90%</td>
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*Non-immune ascitic fluid.
†JE hyperimmune ascitic fluid.
‡Dengue 2-specific neutralizing mAb.

### Growth properties of JE and JE/dengue 2 viruses

The growth kinetics of the JE-XJN virus, two clones of the JE/dengue 2 virus and the parental dengue 2 virus were

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**Fig. 1.** Plasmid templates for the JE Nakayama virus and JE/dengue 2 chimeric virus. (a) JE-XJN clone. BspEI and KpnI restriction sites are used for production of in vitro-ligated transcription templates. Bold regions depict JE virus sequence. The 7.6 kb JE 3′-terminal sequence is in reverse orientation in pCR JE3′NK. (b) JE/dengue 2 virus. The JE-XJN backbone is shown in dark shading and the prM–E structural region derived from the dengue 2 virus is shown in light shading. Amino acid sequences at the chimeric C/prM and E/NS1 boundaries are shown. For clarity, the non-structural region is not drawn to scale.
compared in LLC-MK₂, BHK and C6/36 cells (Fig. 3). In separate experiments, the JE Nakayama virus and JE-XJN virus were compared for growth properties in these cell lines, and no significant differences in growth properties between these two viruses were observed (data not shown).

In BHK cells (Fig. 3a), the peak titre of JE-XJN virus was 6·8 log p.f.u. ml⁻¹ at 60 h. The two JE/dengue 2 clones produced virus less efficiently than JE virus, and peak titres of approximately 5·5 log p.f.u. ml⁻¹ were reached at 60 h post-infection. Dengue 2 virus generated virus with the same kinetics as for the JE/dengue 2 viruses and reached a similar peak titre at 60 h post-infection.

In LLC-MK₂ cells (Fig. 3b), the JE-XJN virus reached a peak titre of 6·25 log p.f.u. ml⁻¹ at 60 h post-infection. The JE/dengue 2 clones produced virus less rapidly than JE-XJN and reached lower peak titres of only 5 log p.f.u. ml⁻¹ between 48 and 60 h post-infection. Dengue 2 virus exhibited a slower rate of virus production than JE/dengue 2 and JE-XJN viruses, but eventually generated a peak virus titre intermediate between those of the other viruses (5·5 p.f.u. ml⁻¹ at 84 h post-infection).

In C6/36 cells (Fig. 3c), the growth kinetics of JE virus and the JE/dengue 2 viruses were similar to one another over the first 72 h post-infection, but JE virus eventually reached a peak titre of 7 log p.f.u. ml⁻¹ at 120 h, whereas the JE/dengue 2 virus did not reach more than 6·25 log p.f.u. ml⁻¹ at 72 h. Dengue 2 virus yields were lower than those of the other viruses over the initial 72 h and the maximum virus yield detected (6·5 log p.f.u. ml⁻¹) occurred at the last time point tested. Differences observed in virus production among the viruses tested in these experiments were judged to be significant based on the very small variability observed for mean and standard deviations determined for each sample.

**Fig. 2.** Immunoprecipitation of [³⁵S]methionine-labelled viral proteins. MM sizes (kDa) are indicated on the left. (a) Mock indicates mock-infected cells, JEV-J indicates JE-XJN virus clone, JE/D2 indicates JE/dengue 2 virus and JEV-N indicates parental JE Nakayama virus. NI and JE indicate non-immune sera and hyperimmune mouse antisera to JE virus, respectively. Arrowheads indicate the JE virus E (upper) and prM (lower) proteins. Asterisk indicates the dengue 2 E protein. (b) JEV-J and JE/D2 are as indicated in (a) and DEN-2 indicates dengue 2 virus. D2 indicates hyperimmune sera to dengue 2 virus. Arrowhead is as described in (a); asterisks mark the dengue 2 E (upper) and prM (lower) proteins.

**Fig. 3.** Growth curves of JE-XJN, JE/DEN2 and dengue 2 viruses. (a), (b) and (c) show results for BHK cells, LLC-MK₂ cells and C6/36 cells, respectively. Values represent the mean ± SD for three independent samples for each time point. In almost all cases, the deviations were small and error bars are not visible.
Mouse neurovirulence testing

Neurovirulence of the JE/dengue 2 virus was initially evaluated by intracerebral inoculation of 4-week-old ICR mice. Three different plaque-purified isolates (1.1, 3.1 and 4.1) were tested in these experiments (Table 2). At doses in the range of 3–4 log p.f.u., there was only subtotal mortality, with 70, 60–64 and 83% mortality for clones 1.1, 4.1 and 3.1, respectively. Clones 3.1 and 4.1 were tested in dose-ranging experiments down to as low as 1 log p.f.u. Mortality varied between 20 and 60% over the dose range tested. At the lowest dose (1–4 p.f.u.), mortality rates of between 20 and 40% were observed for each clone. Mean survival times of mice succumbing to these infections ranged from 5 to 7 days among various doses, with an overall range of 4–11 days.

Immunogenicity of chimeric JE/dengue 2 virus

To determine antibody responses elicited by JE/dengue 2 virus, 3-week-old mice were inoculated by the intraperitoneal route with JE/dengue 2 viruses (either clone 1.1 or 4.1) at doses of 4·5 log p.f.u. A total of 25 mice inoculated in this manner did not exhibit any mortality or signs of illness over the period of observation of these experiments. Mice were sacrificed beginning at 7 weeks post-inoculation for measurement of neutralizing antibodies against dengue 2 virus (Table 3). Only three of 25 mice inoculated with JE/dengue 2 virus failed to seroconvert to anti-dengue antibody, based on a cut-off value of 1:10, the lowest dilution tested in mock-infected mice. Data for the two JE/dengue 2 clones tested were very similar (range of neutralization titres was from 1:10 to 1:1280 for both clones 1.1 and 4.1). Results from these two groups were therefore pooled to calculate geometric mean titres (GMTs) for each time point at which sera were obtained. Titres remained low between 7 and 10 weeks post-immunization (1:20 to 1:88, except for one sample of 1:1280) and differences among GMTs over this interval were not statistically significant. GMTs rose to 403 and 320 at weeks 12 and 14 post-inoculation, respectively. The differences in GMTs were significant when comparing the mice from 9 weeks, but not 7 weeks, with those from either 12 or 14 weeks (the GMT at 7 weeks was unexpectedly high, due to one sample with a titre of 1280). Although the difference in GMT between weeks 12 and 14 was not significant, the differences between these later time points combined and the combination of weeks 7, 9 and 10 post-inoculation were all significantly different. The maximum titre observed in these experiments (1:1280) occurred at 14 weeks, except for the single mouse at 7 weeks.

Protection against dengue 2 encephalitis

Because JE/dengue 2 virus elicited anti-dengue 2 neutralizing antibody in mice, the level of protection against encephalitis caused by neuroadapted dengue 2 virus was then tested. Mice were mock-immunized or immunized with JE/dengue 2 virus (clone 4.1, 2·5 × 10^3 p.f.u. intraperitoneal) at 3 weeks of age and challenged at 5 weeks of age with 3·8 × 10^3 p.f.u. neuroadapted dengue 2 virus by intracerebral inoculation. In this protocol, fatal encephalitis occurs prior to the onset of age-related resistance to dengue 2 virus, at approximately 5 weeks of age (Chambers et al., 2003). Fig. 4 shows that all 18 mice immunized with JE/dengue 2 survived challenge. None of these manifested any signs of illness for a 1 month period following the challenge. In contrast, 13/17 mock-immunized mice succumbed to the challenge, with a mean survival time of 6·8 days (range 5–11). Remaining mice exhibited illness signs, including moderate ruffling and decreased mobility. The difference in mortality rates between the two groups was highly significant (P<0·001).

Sera from the mice in these experiments were tested for post-challenge neutralizing responses against dengue 2 virus. The GMT of mock-immunized mice that did not survive dengue virus challenge and were moribund after

<table>
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<tr>
<th>Virus*</th>
<th>Dose†</th>
<th>No. mice</th>
<th>Mortality (%)</th>
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<tbody>
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<td>3-6</td>
<td>10</td>
<td>7/10 (70)</td>
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<td>7/11 (64)</td>
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<td>1-0</td>
<td>5</td>
<td>2/5 (40)</td>
</tr>
<tr>
<td></td>
<td>0-0</td>
<td>5</td>
<td>2/5 (40)</td>
</tr>
<tr>
<td>JE/DEN2 clone 4.1</td>
<td>3-6</td>
<td>5</td>
<td>3/5 (60)</td>
</tr>
<tr>
<td></td>
<td>2-6</td>
<td>5</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td></td>
<td>1-6</td>
<td>5</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td></td>
<td>0-6</td>
<td>5</td>
<td>1/5 (20)</td>
</tr>
</tbody>
</table>

*Virus indicates plaque-purified virus clones.
†Dose in log p.f.u. by the intracerebral route in 3-week-old ICR mice.

Table 3. Neutralizing-antibody titres in JE/dengue 2-immunized mice

<table>
<thead>
<tr>
<th>Group*</th>
<th>n</th>
<th>GMT</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-immunized</td>
<td>3</td>
<td>&lt;10-00</td>
<td>10</td>
</tr>
<tr>
<td>Immunized, 7 weeks</td>
<td>7</td>
<td>88-33</td>
<td>10–1280</td>
</tr>
<tr>
<td>Immunized, 9 weeks</td>
<td>8</td>
<td>20-00</td>
<td>10–80</td>
</tr>
<tr>
<td>Immunized, 10 weeks</td>
<td>5</td>
<td>80-00</td>
<td>10–310</td>
</tr>
<tr>
<td>Immunized, 12 weeks</td>
<td>3</td>
<td>403-17</td>
<td>160–1280</td>
</tr>
<tr>
<td>Immunized, 14 weeks</td>
<td>2</td>
<td>320-00</td>
<td>320</td>
</tr>
<tr>
<td>Immunized (total)</td>
<td>25</td>
<td>71-60</td>
<td>10–1280</td>
</tr>
</tbody>
</table>

*Statistical significance for pairwise comparisons between mock-immunized and immunized groups is as follows: aP=0·034; bP=NS; cP=0·037; dP=0·046; fP=0·03. Immunized (total) represents GMT of entire immunized group. All comparisons among immunized groups were not significant (NS) except for 9 weeks vs 12 weeks (P=0·012) and 9 weeks vs 14 weeks (P=0·030).
significant groups. Differences between all other groups were not although the non-survivors were bled earlier than the other

JE/dengue 2-immunized mice that survived virus challenge and were bled at 8 weeks was 871, ranging from 320 to 2560 JE/dengue 2 virus containing the structural proteins prM and E of the JE Nakayama background. JE/dengue 2 virus exhibited small plaque phenotype of approximately 1 mm in size, only detectable on SW-13 cells, and growth properties differed among various cell lines. JE/dengue 2 virus replicated less efficiently than the parental JE virus in all cell lines, but generally similarly to dengue 2 virus. In C6/36 cells, JE/dengue 2 virus yielded lower peak titres than JE and dengue 2 viruses. This suggests that the presence of the dengue prM and E proteins contributes to less efficient growth of the JE/dengue 2 viruses compared with JE virus, although the viruses were still able to replicate to peak titres of between 5 and 6 log p.f.u. ml\(^{-1}\). Studies with other chimeric viruses have shown some reduction in replication efficiency in C6/36 cells, although the degree of impairment is variable (Caufour et al., 2001; Mathenge et al., 2004; Pletnev & Men, 1998; Pletnev et al., 1992, 2001, 2002). In LLC-MK\(_2\) cells, dengue 2 virus exhibited reduced kinetics of virus production compared with JE-XJN and JE/dengue 2 viruses. The dengue 2 prM and E proteins may contribute to less efficient virus production, due to effects on virus entry and spread at the low multiplicities used in these experiments. The less efficient virus production for JE/dengue 2 virus compared with JE-XJN is consistent with this hypothesis, although virus-specific factors contributed by JE-XJN compensate for the deleterious effects of the dengue structural proteins during the early stages of JE/dengue 2 infection in this cell line. Overall, the results with JE/dengue 2 are generally similar to these reports and do not indicate any unexpected cell-culture properties with this chimeric virus, other than the small plaque size.

In neurovirulence testing in 4-week outbred mice, independent plaque isolates of the JE/dengue 2 virus exhibited similar profiles, characterized by only a moderate level of neurovirulence, with subtotal mortality observed at doses as high as 3-6-4.0 log p.f.u. and dose-dependent effects on mortality at lower doses. Lack of uniform mortality in mice over this dose range is notable and suggests an attenuated phenotype of JE/dengue 2 virus that could be related to restricted replicative capacity of this virus in mouse tissues. Attenuating effects on neurovirulence have been observed for other flavivirus chimeras in which the prM–E regions of dengue virus strains have been substituted into the genome of otherwise neurovirulent viruses, such as Yellow fever virus or JE virus (Caufour et al., 2001; Chambers et al., 1999, 2003; Mathenge et al., 2004). In the case of the chimeric yellow fever/dengue viruses, the degree of attenuation is profound. However, similarly to results with JE/dengue 2 virus (Mathenge et al., 2004), we observed that the prM–E region of dengue 2 is inadequate to attenuate JE virus fully for neurovirulence in young adult mice. This may be due to the higher intrinsic level of virulence associated with JE virus in this particular model system. There is not expected to be any contribution of the dengue 2 prM–E genes to the neurovirulence properties of the JE/dengue 2 virus, as the parental dengue 2 strain (PUO-218) is highly attenuated for neurovirulence in young adult mice. This may be due to the higher intrinsic level of virulence associated with JE virus in this particular model system. There is not expected to be any contribution of the dengue 2 prM–E genes to the neurovirulence properties of the JE/dengue 2 virus, as the parental dengue 2 strain (PUO-218) is highly attenuated for neurovirulence, even in 3-day-old mice (Gualano et al., 1998). In contrast, JE virus is highly neurovirulent in adult mice, with LD\(_{50}\) values often well below 1 p.f.u. (Ni &

DISCUSSION

In this study, a molecular clone of the JE Nakayama virus was constructed and the recovered virus (JE-XJN) was found to resemble the JE parental virus, based on plaque size, virus yield and antigenic properties in cell-culture experiments. Nucleotide sequence data indicated that the JE-XJN virus contained a high level of sequence similarity to the genomes of other virulent JE viruses, i.e. between 97 and 99 % at the nucleotide level. These results are similar to those of other studies that have reported recovery of infectious JE virus from plasmid templates (Mishin et al., 2001; Sumiyoshi et al., 1992; Yun et al., 2003; Zhao et al., 2005).

The JE-XJN clone was used to construct a chimeric JE/dengue 2 virus containing the structural proteins prM and E of dengue 2 virus in the JE Nakayama background. JE/dengue 2 virus exhibited antigenic properties characteristic of the dengue 2 E protein. Plaque-purified clones of this virus exhibited a small plaque phenotype of approximately

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**Fig. 4.** Protection of ICR mice against dengue 2 encephalitis after immunization with the JE/dengue 2 virus. Mice were immunized or mock-immunized, challenged by intracerebral inoculation with neuroadapted dengue 2 virus and observed until moribund.

5 days was 37-0, ranging from 10 to 160 (n = 8). The GMT of mock-immunized mice that did survive challenge was 404 at 4 weeks, ranging from 320 to 2560 (n = 8). The GMT of a subgroup of JE/dengue 2-immunized mice that survived virus challenge and were bled at 4 weeks was 691, ranging from 80 to 2560 (n = 18). The GMT of a second subgroup of JE/dengue 2-immunized mice that survived virus challenge and were bled at 8 weeks was 871, ranging from 320 to 2560 (n = 9). Differences between mock-immunized non-surviving mice and all other groups were highly significant, although the non-survivors were bled earlier than the other groups. Differences between all other groups were not significant (P > 0.05).
Peripheral inoculation of mice with JE/dengue 2 virus elicited protection against encephalitis caused by neuroadapted dengue 2 virus. This occurred despite the slow appearance of neutralizing antibodies (Table 3). Mock-immunized survivors also generated significant levels of neutralizing antibodies. The role of these responses in protecting mock-infected mice from fatal encephalitis is unclear, as age-related factors also contribute to resistance against dengue. However, the levels of post-challenge neutralizing antibodies detected in surviving JE/dengue 2-immunized mice were greater than those of mock-immunized survivors and continued to rise between 4 and 8 weeks post-challenge. This suggests an induction of memory B-cell responses by the immunization as early as 3 or 4 weeks following infection, even though neutralizing-antibody responses were low for up to 7 weeks post-inoculation (Table 3). The exact role of this antibody response versus a cellular immune response in providing protection against dengue virus in this model remains to be determined.

JE virus causes an acute encephalitis that can be prevented by vaccination with live-attenuated JE virus [JE-SA14-14-2; (Chinese vaccine strain; Eckels et al., 1995; Lee & Lobigs, 2002; Ni & Barrett, 1996). The neutralizing-antibody responses induced by JE/dengue 2 virus were variable in magnitude and did not reach peak levels until 12 weeks post-inoculation. The long interval required for the development of this peak response in mice inoculated with JE/dengue 2 virus may result from inefficient virus replication in these mice, resulting in a low antigen load and delayed induction of the peak immune response.


