Journal of General Virology (2004), **85**, 2503–2513 DOI 10.1099/vir.0.80120-0

Fusion PCR generated Japanese encephalitis virus/dengue 4 virus chimera exhibits lack of neuroinvasiveness, attenuated neurovirulence, and a dual-flavi immune response in mice

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The first flavivirus chimera encoding dengue 4 virus (D4) PrM and E structural proteins in a Japanese encephalitis virus (JEV) backbone was successfully generated using the long-PCR based cDNA-fragment stitching (LPCRcFS) technique, demonstrating the technique's applicability for rapid preparation of flavivirus chimeras. The JEV/D4 chimera multiplied at levels equal to JEV and D4 in the mosquito cell line C6/36, while in a mouse neuronal cell line (N2a) JEV replicated efficiently, but JEV/D4 and D4 did not. In mouse challenge experiments, JEV/D4 showed a lack of neuroinvasiveness similar to D4 when inoculated intraperitoneally, but demonstrated attenuated neurovirulence ($LD_{50} = 3{\cdot}17 \times 10^4$ f.f.u.) when inoculated intracranially. It was also noted that mice receiving intraperitoneal challenge with JEV/D4 possessed D4-specific neutralization antibody and in addition clearly showed resistance to JEV intraperitoneal challenge (at $100 \times LD_{50}$). This suggests that immunity to anti-JEV non-structural protein(s) offers protection against JEV infection *in vivo*. Dengue secondary infection was also simulated by challenging mice pre-immunized with dengue 2 virus, with D4 or JEV/D4. Mice showed higher secondary antibody response to challenge with JEV/D4 than to D4, at 210 000 and 37 000 averaged ELISA units, respectively. Taken together, aside from demonstrating the LPCRcFS technique, it could be concluded that the PrM and E proteins are the major determinant of neuroinvasiveness for JEV. It is also expected that the JEV/D4 chimera with its pathogenicity in mice and atypical immune profile, could have applications in dengue prophylactic research, *in vivo* efficacy assessment of dengue vaccines and development of animal research on models of dengue secondary infection.

Received 19 March 2004
Accepted 19 May 2004

INTRODUCTION

The quartet of dengue virus serotypes (D1, D2, D3 and D4) are responsible for the steadily increasing number of dengue fever (DF) and dengue haemorrhagic fever (DHF) cases worldwide. Dengue is currently considered the most significant arthropod-borne virus disease, affecting tropical and subtropical regions, and it accounts for 20 million cases of infection and 24 000 deaths annually (WHO, 1997, 2002; Gubler, 2002). Japanese encephalitis virus (JEV) is one of the most important causes of viral encephalitis worldwide. It has an area of occurrence spanning most of east and south-east Asia, the Indian subcontinent down through Micronesia and as far south as northern Australia. It is estimated that JEV is responsible for 50 000 cases of illness and 15 000 deaths every year (Igarashi, 1992; Johansen *et al.*, 2001; Guirakhoo *et al.*, 1999; Solomon *et al.*, 2000a, b; WHO, 2002).

Both dengue and JEVs belong to the genus *Flaviviridae*, whose members possess a positive-sense non-segmented RNA genome of about 11 kb, which encodes a single polypeptide that is subsequently co- and post-translationally cleaved into three structural (C, PrM and E) and seven non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b and

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The GenBank/EMBL/DDBJ accession number for dengue 4 S-14 virus sequence described in this study is AY559316.

Supplementary table for primers used and electron microscopy figure are available in JGV Online.

NS5) proteins (Burke & Monath, 2001). However, the biological characteristics of these two viruses are somewhat different. JEV is mainly transmitted by mosquitoes of the genus *Culex* and infects a wide range of animals including equines, humans, swine and birds; while dengue virus is carried by mosquitoes of the genus *Aedes* and infects mainly humans and a limited number of wild primates. Clinical manifestations caused by the viruses are also different. While JEV causes encephalitic infection of the central nervous system, dengue virus infection presents as a self-limiting, though severe, influenza-like illness (DF) or acute haemorrhagic disease (DHF), which is most likely to occur when infection with one dengue serotype is followed by a second infection with a different serotype (Solomon *et al.*, 2000b).

There has been considerable research in recent years involving the construction of flavivirus chimeras using various reverse genetic technologies (Bray & Lai, 1991; Lai *et al.*, 1998; Falgout *et al.*, 1990; Liljeqvist & Stahl, 1999). To date, a number of flavivirus chimeras have been produced and characterized from stable infectious cDNA clones. These include chimeras combining dengue (D1, D2, D3 and D4), Tick-borne encephalitis, JE, yellow fever, Langat and West Nile viruses in various configurations (Pletnev *et al.*, 1992, 1993, 2002; Pletnev & Men, 1998; Monath *et al.*, 1999; Campbell & Pletnev, 2000; Guirakhoo *et al.*, 2000, 2001; Chambers *et al.*, 2003).

In this study, we aimed to generate a viable JEV/dengue chimera in order to determine viral genome areas (viral proteins) that govern the biological characteristics between the two viruses, with particular attention to PrM and E genes (proteins). To facilitate construction of the chimera virus, we utilized a long-PCR based cDNA-fragment stitching methodology (LPCRcFS), which we had previously applied in preparation of JEV/JEV chimera viruses (Morita *et al.*, 2001), where wild-type viral RNA was also used to directly generate the base cDNA for chimera construction. This is the first report of construction and characterization of a flavivirus chimera expressing D4 structural proteins (PrM and E) in a JEV backbone.

METHODS

Cell lines. The *Aedes albopictus* C6/36 cell line was grown at 28 °C in Eagle's minimal essential medium (E-MEM) with 10 % fetal calf serum (FCS) and 0·2 mM non-essential amino acids (Igarashi, 1978). African green monkey kidney (Vero) and porcine stable kidney cell lines (PS) were grown at 37 °C in E-MEM supplemented with 10 % FCS (Lad & Gupta, 2002). Murine neuroblastoma cell line, Neuro 2a (N2a), was grown at 37 °C in RPMI 1640 media (Gibco-BRL) supplemented with 10 % FCS in a 5 % CO_2 atmosphere.

Virus strains. Viruses used in this study were: JEV (JaOArS982) strain isolated from a *Culex* mosquito pool in Osaka, Japan in 1982; dengue 4 (D4 S-14) strain isolated from a patient in Sri Lanka in 1978 and dengue 2 (ThNH07/93) strain, isolated from a dengue shock syndrome patient (DSS) in Thailand in 1993 (Hori *et al.*, 1986; Sumiyoshi *et al.*, 1987; Thant *et al.*, 1996). These viruses had been passaged in C6/36 cells more than 10 times since their isolation. Virus titres were determined by focus forming assay (f.f.a.) as described below.

Large-scale virus production and purification. Large-scale virus production was performed as previously described by Morita & Igarashi (1989). This procedure was essential in order to obtain sufficient amounts of template viral RNA necessary to generate the high quality cDNA used at the LPCRcFS stage. Briefly, 3·0 litres of C6/36 cells in culture ($1{\cdot}5 \times 10^6$ cells ml^{-1}) were infected with virus at a m.o.i. of 1·0 focus forming units (f.f.u.) per cell, and incubated in spinner bottle culture for 4–6 days. Harvested infected culture fluid (ICF) was clarified by centrifugation (8000 ***g*** for 30 min) and concentrated using sulfate cellulofine resin column chromatography followed by concentration-filtration using a centrifugal filter device as previously described (Morita *et al.*, 2001). Virus titre of the resulting concentrated virus fluid was approximately 10^{11} f.f.u. ml^{-1}.

Construction of chimeric cDNA. Construction of a full-length recombinant JEV/D4 cDNA was achieved according to the strategy delineated in Fig. 1, by means of high fidelity LPCRcFS (a modified fusion-PCR methodology) described earlier (Morita *et al.*, 2001). First, viral RNA was extracted from 500 µl concentrated virus fluid using Trizol LS reagent (Gibco-BRL) according to the manufacturer's instruction. Synthesis of first strand cDNA from both parental viral RNAs was performed using ReverTra Ace (Toyobo) and oligonucleotide primers (for primer list see Supplementary Table A online). Two pairs of chimeric complementary long-PCR primers (A^CF, D^CR and C^CF, B^CR), each 63–67 nt long were designed (Supplementary Table A). Approximately half the nucleotide length of each primer was derived from each of the parental viruses (JaOArS982 and D4 S-14). Primers A^CF and B^CR were used to generate the D4 PrM-E fragment with a 35 nt JEV overhang at each end. A JEV 5′ NCR primer (T7/JEV), which has 22 nt encoding the T7 RNA polymerase promoter sequence at its 5′ end was used in combination with D^CR to amplify the front end JEV cDNA fragment (JE-1). The two remaining downstream JEV cDNA fragments (JE-2 and JE-3) were synthesized using primers sets C^CF, JE1R and JE2F, JE3R. The end of each fragment consisted of a 35 nt overhang corresponding to the adjacent fragment (except at the 3′ end).

Each of these four fusion-ready cDNA fragments [100 ng (JE-1, D4-1, JE-2 and JE-3)] were first purified by using agarose gel electrophoresis and the Qiaex II gel extraction kit (Qiagen), and then progressively fused together in a series of low-cycle high fidelity 'stitching' long-PCRs, each using Platinum *Taq* polymerase kit (Gibco-BRL) as described previously (Morita *et al.*, 2001). These steps culminated in the generation of the full-length chimeric cDNA (Fig. 1).

***In vitro* transcription and transfection.** The full-length chimeric cDNA was *in vitro* transcribed using Ampliscribe T7 transcription kit (Epicentre Technologies) as described (Morita *et al.*, 2001). In short, 50 µl of transcription reaction mix containing 2·5 mM A-cap analogue 7mG(5′)ppp(5′) (NEB), 5 mM UTP, CTP, GTP and 0·5 mM ATP, RNase inhibitor (250 U), Ampliscribe T7 RNA polymerase (25 U), 250 ng cDNA (11 kb) and buffer supplied by the manufacturer were incubated at 37 °C for 150 min. *In vitro* transcription mix was immediately added to 1×10^7 C6/36 cells, which had been pre-washed with RNase-free-PBS, and resuspended in pre-chilled RNase-free-PBS (250 µl). The suspension was then placed in a 0·4 cm^2 gap Gene Pulser electroporation cuvette (BioRad) and pulsed at 350 V and 500 µF using BioRad's Gene pulser II, the cuvette was immediately placed on ice for 5 min and the content was aseptically transferred to a culture flask containing E-MEM with 10 % FCS. After 5 days incubation at 28 °C, total contents were passaged to a fresh flask of subconfluent C6/36 cells in E-MEM with 2 % FCS and incubated for 6 more days. This was repeated once,

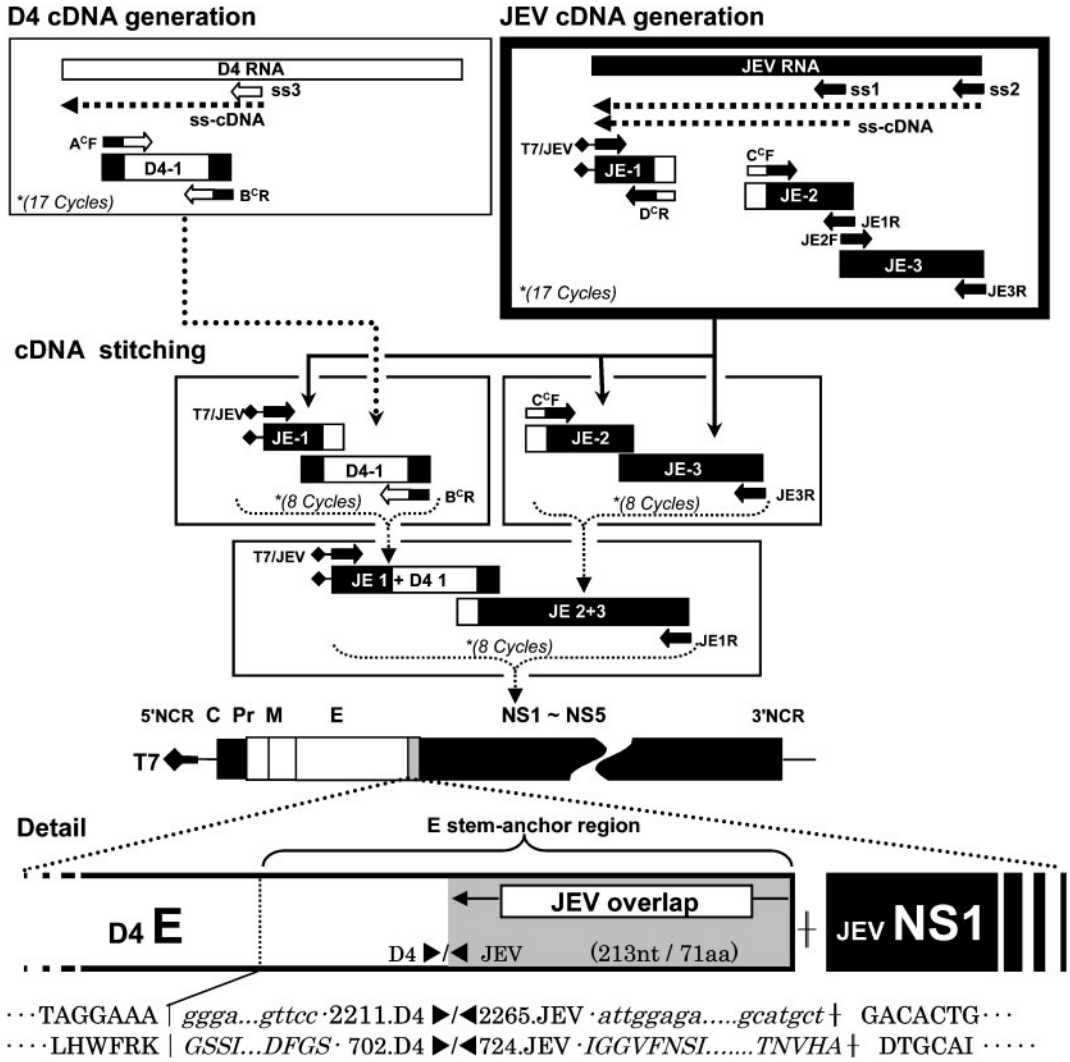

Fig. 1. High fidelity LPCRcFS chimerization strategy. Generation of JEV/D4 infectious cDNA with T7 promoter at 5′ end and D4 structural proteins. The cDNA fragments generated from extracted ssRNA are subsequently fused together in a series of high fidelity long-PCR cDNA fragment stitching reactions. Bottom detail, shows the 71 aa JEV overlap into the E gene's stem-anchor region. Asterisk (*), number of long-PCR cycles used in cDNA generation and stitching reactions.

this time transferring only ICF to the fresh flask. The ICF recovered was aliquoted and frozen at −80 °C for later use in comparative characterization against both parental viruses.

Determination of nucleotide sequence of viral RNA. To determine the nucleotide sequence of original and chimeric viruses, PCR fragments were amplified by RT-PCR from viral RNAs extracted from virus-infected fluids, purified and sequenced using BigDye terminator cycle sequencing reaction kit in an ABI 3100 automated sequencer (Applied Biosystems). The results were analysed using DNASIS version 3.7 software (Hitachi software engineering).

Virus infection. C6/36, PS and N2a cells grown to confluence in 6-well plates were each infected with chimera or parent viruses at an m.o.i. of 0·05 f.f.u. per cell. The overlay was discarded after incubation for 2 h at 28 or 37 °C and 1 ml of fresh E-MEM with 2 % FCS was added to each well. Contents from each well were harvested every 24 h. Infected cells were collected, centrifuged (3000 r.p.m. for 15 min) and used for flow cytometry analysis. Aliquots of ICF were collected for virus quantification by f.f.a. and antigen-capture ELISA.

Flow cytometry. Harvested cells were fixed with 2 % formaldehyde in PBS for 20 min and then washed twice with PBS. Permeablization was done using IC-Perm (Biosource International), and then cells were stained using an Alexa Fluor 488-labelled anti-flavivirus group-specific monoclonal antibody (FITC-6b6c-1) (Mathews & Roehrig, 1984). The green fluorescence of individual cells was measured using a FacScan flow cytometer (Becton Dickinson).

Focus and plaque forming assay. For virus quantification by f.f.a., 10-fold serial dilutions of the ICF samples were inoculated in duplicate into confluent C6/36 cells in a 96-well plate, incubated for 1 h and overlaid with 100 µl E-MEM with 2 % FCS containing

0·5 % methyl cellulose 4000. After incubation at 28 °C for 2 days (JEV and JEV/D4) and 3 days (D4), infection foci were visualized using the immuno-peroxidase staining procedure described before, counted and viral titre determined (Morita *et al.*, 2001).

Plaque forming ability and size for each virus was studied in three different cell lines, C6/36, PS and Vero. Cells were grown to confluence in a 12-well plate, the media was then aspirated. Virus fluids containing 100, 50, 25 and 5 f.f.u. of the parental and chimeric viruses in 300 µl of dilution media (E-MEM with 2 % FCS) were prepared and added to duplicate wells. After the incubation for 1 h at 28 or 37 °C with swirling every 20 min, 1 ml of E-MEM with 2 % FCS containing 1 % methyl cellulose 4000 was overlaid. After 4 days incubation, cells were fixed by addition of 1 ml of 5 % formaldehyde and incubated for 30 min. The plaques were then visualized by means of immuno-peroxidase staining using a panel of monoclonal antibodies specific for JEV or D4 E protein through a procedure described elsewhere (Blaney *et al.*, 2003).

The plates were photographed using a HAD camera (Sony Electronics) and the image was converted to the actual size maintaining aspect ratio. Mean plaque diameters for 20 non-overlapping plaques for each virus were then determined.

Plaque reduction virus neutralizing test (PRNT). To determine the virus neutralizing titre of sera collected from mice infected with the chimera and parental viruses (see mouse experiment section below), PRNT was performed against the two parental viruses, JEV (JaOrS982) and D4 (S-14). Briefly, sera were heat-inactivated at 56 °C for 30 min and then serially diluted in a twofold manner from 1 : 10 to 1 : 1280. To 100 µl of each serum dilution, an equal volume of virus ICF with a titre of 50 f.f.u. was added and incubated for 2 h at 37 °C. Each dilution was then added to duplicate wells on a 12-well culture plate with PS cells previously grown to confluence. After 4 days for JEV and 6 days for D4, the cells were fixed using 5 % formaldehyde for 1 h and stained with 0·1 % crystal violet in 20 % ethanol, rinsed with several washes of water and the result interpreted. More than 50 % reduction in plaques was designated PRNT-positive.

Antigen and antibody ELISA. Antigen-capture ELISA was performed as previously described by Igarashi *et al.* (1995). Indirect IgG ELISA was performed on sera collected at 3 weeks post-infection and 3 weeks post-secondary challenge as previously described by Bundo *et al.* (1981). Optical density was determined using a Multiscan LX photospectrometer. ELISA titres were calculated from standardized reciprocal dilution values using Thermo-Labsystem's Ascent photospectrometric data analysis software version 2.6.

Mouse experiments. Male C57BL/6N mice (Charles River) at 3- and 4-week old were used. Inoculations were performed intracranially (i.c.) and intraperitoneally (i.p.) with the requisite virus dilutions, and observation was maintained over a 28-day period, during which morbidity and mortality were recorded. Mice were injected with 0·05 ml i.c. or 0·5 ml i.p. of the requisite concentration of virus diluted in E-MEM with 2 % FCS. Where JEV mortality levels prevented sera collection, 50 µl of JE vaccine (JE-Vax; Biken) was used instead, at one tenth of the human child dose (500 µl). The vaccine was diluted in E-MEM with 2 % FCS and inoculated per mouse. Control groups received an equivalent volume of E-MEM with 2 % FCS via the same route. LD_{50} was calculated according to the method described by Reed & Muench (1938). Animals were handled according to the regulations of the Nagasaki University Medical School animal experimentation facility. Inoculations and retro-orbital bleeding were carried out under diethyl ether anaesthesia. Replication of JEV/D4 in brain tissue of dead mice was confirmed by RT-PCR assay and virus isolation from filtered tissue homogenate.

RESULTS

Generation of JEV/D4 infectious chimera virus

The flavivirus chimera (JEV/D4) encodes dengue 4 virus PrM and E structural proteins in a JEV backbone. The chimera virus was designed to have a 71 aa JEV overlap at the C terminus of the E protein so as to have the chimeric junction occur in the flexible stem-anchor region of the protein (Fig. 1; detail). Within days of viral RNA extraction, assembly of the full-length chimera cDNA was successfully achieved by applying a LPCRcFS method according to the construction strategy diagram in Fig. 1.

In vitro transcription of JEV/D4 chimera RNA and transfection of the resultant RNA into C6/36 cells were carried out as described in Methods. Successful recovery of the infectious JEV/D4 chimera virus was confirmed on day 5 post-transfection by immuno-staining, antigen ELISA and negative-staining electron microscopy (see Supplementary Fig. A online). Its post-transfection recovery duration was on a par with that of its JEV parent. The recovered virus was then passaged twice in C6/36 cells, the final passage having a titre of 10^7 f.f.u. ml^{-1}.

To confirm further generation of the chimera virus, virus-infected C6/36 cells were immuno-stained, as described earlier in Methods. The JEV/D4 chimera-infected C6/36 cells reacted with JE-NS1-specific and D4-E-specific monoclonal antibodies but not with JE-E-specific monoclonal antibodies.

Comparison of parental and chimeric C, PrM, E and NS1 genes

The nucleotide sequence of C, PrM, E and NS1 gene regions of the chimera and the parental viruses were determined to confirm that chimerization was achieved as designed, and to check for changes to the genome that could result in amino acid differences in the viral structural and NS1 proteins, believed to be the major targets of immune response. It was confirmed that the chimerization was successfully achieved. The differences observed are presented in Table 1.

Biological characterization of chimera virus in culture cells

We compared virus growth kinetics and plaque size of the JEV/D4 chimera in C6/36, PS, N2a and/or Vero cells with its parental viruses, JEV (JaOArS982) and D4 (S-14) as shown in Fig. 2.

In the mosquito cell line C6/36, JEV/D4's growth kinetics measured in the form of virus titre, antigen ELISA titres and percentage of cells infected, showed a profile similar to the parental viruses, both of which multiplied efficiently in the cell line (Fig. 2a). Virus titre, antigen ELISA titre and percentage cell infection of the chimera virus were as high as the two parental viruses, suggesting that chimerization

Table 1. Changes in chimera genome from parental viruses

sil, Silent amino acid change.

Region	Nucleotide change	Amino acid change
C*	400 A-G	Q102R
Pr†	–	–
M†	–	–
E†	1167 A-G	*sil*
	2152 C-T‡	T405I‡§
NS1*	–	–

*Position based on JEV JaOrS982 (M18370).
†Position based on D4 (M14931).
‡Stem-anchor region, E protein.
§Amino acid position based on E protein of D4-14.

did not hamper virus replication in mosquito cells. It is noted that those values of JEV/D4 together with JEV reached a maximal plateau level earlier than D4. No temperature sensitivity was observed among the chimera or parental viruses (data not shown).

In mammalian cells, the growth kinetics for JEV/D4 were atypical in relation to its parental viruses. Fig. 2(b) shows virus titres of JEV/D4 and two parental viruses in PS and N2a cell lines. JEV multiplied well in PS and N2a cells, and virus titres reached 10^7 f.f.u. ml^{-1} for both cell lines. However, D4 did not replicate in N2a cells and did so poorly in PS cells where its virus titre reached 10^2 f.f.u. ml^{-1}. While JEV/D4, like D4, did not replicate in N2a cells, it did so in PS cells reaching 10^4 f.f.u. ml^{-1}, a level 100 times higher than those recorded for D4. While the D4 S-14 strain did not infect Vero cells, JEV/D4 did. Fig. 2(a) and (c) highlight the differences in the antigen production level of JEV/D4 in Vero and C6/36 cells.

Virus plaque sizes were also compared in C6/36, PS and Vero cells (Fig. 3a and b). JEV produced the largest plaques in all cell lines assayed followed by the JEV/D4 chimera, which produced plaques intermediate in size in both mammalian cell lines, but plaques smaller than both parental viruses in C6/36.

Pathogenicity of JEV/D4 in mice

Weaned mice are known to be a good animal encephalitis model for JEV (Monath *et al.*, 2003). Pathogenicity of JEV/D4 was evaluated in this animal model and compared with that of its parental wild-type viruses.

Serially diluted viruses were inoculated into 4-week old C57BL/6N mice via i.c. and i.p. routes as described in Methods and the mice were observed for 28 days. Mortality and LD_{50} for each virus and infection-route are shown in Table 2. Values of LD_{50} for JEV (JaOArS981) through i.c. and i.p. were 2·67 f.f.u. and $2{\cdot}36 \times 10^3$ f.f.u., respectively. As expected, D4 S-14 did not show any mortality in either i.c. or i.p. challenge even at maximum virus load of 10^6 and 10^7 f.f.u., respectively. JEV/D4 did not show mortality in i.p. inoculation (maximum virus load, $1{\cdot}1 \times 10^7$ f.f.u.), but did show mortality in i.c. inoculation. The value of LD_{50} (i.c.) for JEV/D4 was $3{\cdot}17 \times 10^4$ f.f.u.

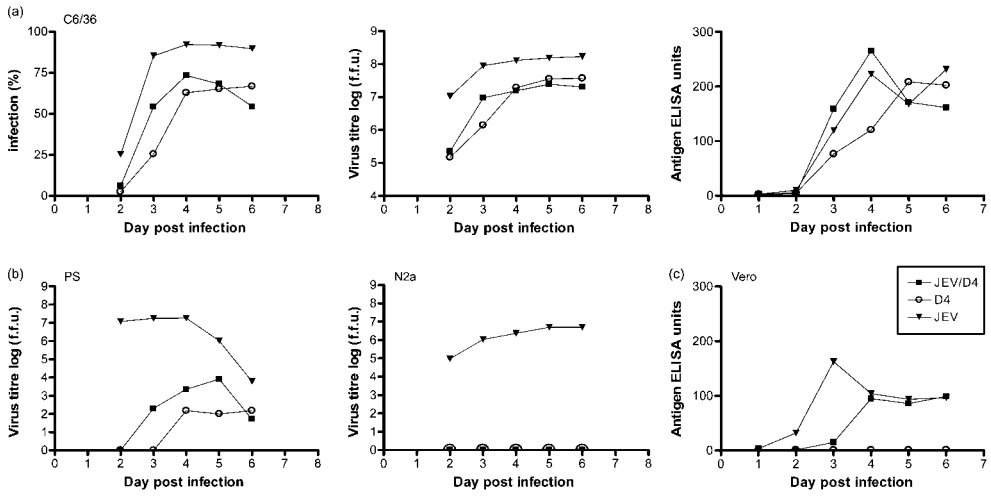

Fig. 2. Infection and growth kinetics *in vitro*. The viruses were each inoculated at an m.o.i. of 0·05, and over a 6-day period ICF was sampled for virus growth and antigen ELISA assessment while cells were harvested and processed for percentage infection analysis using flow cytometry (performed with 10 000 events for each sample). (a) Percentage infection, virus growth rates and antigen ELISA levels for chimera and parental viruses in C6/36 cells. (b) Virus growth rates in PS, N2a for chimera and parental viruses. Detection limit for the f.f.a. is 10 f.f.u. ml^{-1}. (c) Levels of viral antigen by ELISA in Vero cells for chimera and parental viruses.

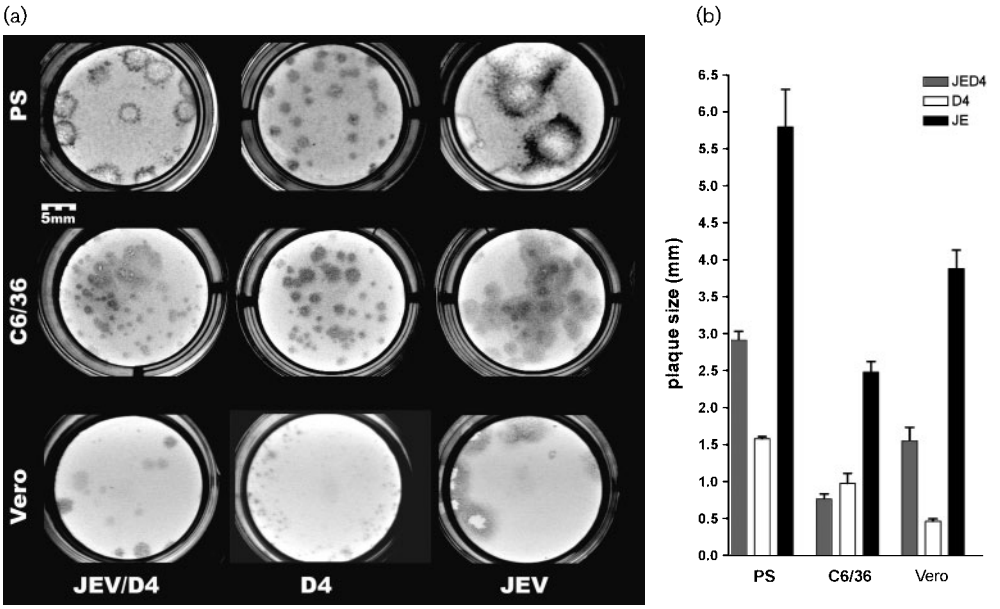

Fig. 3. Plaque morphology assay. (a) Photographic images of plaques produced by inoculation of the chimera and parental viruses in different cell lines. (b) Bar graph comparison of mean plaque size. Error bars display standard error.

The above-mentioned pathogenicity in mice of all the three viruses were confirmed in another set of experiments, in which 4-week old C57BL/6N mice were inoculated with 10^5 f.f.u. or 10^7 f.f.u. of each virus by i.c. and i.p., respectively. Results are presented in Fig. 4(a) and (b) as Kaplan–Meier percentage survival graphs. D4 S-14 inoculated mice demonstrated no mortality under both i.c. and i.p. challenge. JEV (JaOArS982) showed 100 % mortality by day 7 in i.c. and 75 % by day 14 in i.p. The JEV/D4 i.p. inoculated mice did not show mortality. However, 50 % mortality was recorded when the chimera virus was introduced by i.c. route.

Induction of D4-specific virus neutralizing antibody by JEV/D4 in mice

We examined the level and specificity of antibodies elicited by JEV/D4 infection in mice. Sera from two groups of five

Table 2. LD_{50} results

Determination of LD_{50} (f.f.u.) for chimeric and parental viruses for inoculation via intracranial and intraperitoneal routes in 4-week-old C57BL/CN mice. LD_{50} (f.f.u.) value was calculated according to the method reported by Reed & Muench (1938).

Virus	Intracranial			Intraperitoneal		
	Amount (f.f.u.)	Killed/ inoculated	LD_{50} (f.f.u.)	Amount (f.f.u.)	Killed/ inoculated	LD_{50} (f.f.u.)
JEV/D4	$1{\cdot}10\times10^6$	4/5		$1{\cdot}10\times10^7$	0/5	
	$1{\cdot}10\times10^5$	4/5	$3{\cdot}17\times10^4$	$1{\cdot}10\times10^6$	0/5	$>1{\cdot}10\times10^7$
	$1{\cdot}10\times10^4$	2/5		$1{\cdot}10\times10^5$	0/5	
JEV	$1{\cdot}50\times10^3$	5/5		$1{\cdot}50\times10^6$	5/5	
	$1{\cdot}50\times10^2$	5/5		$1{\cdot}50\times10^5$	3/5	
	$1{\cdot}50\times10^1$	5/5	2·67	$1{\cdot}50\times10^4$	4/5	$2{\cdot}36\times10^3$
	1·50	1/5		$1{\cdot}50\times10^3$	3/5	
	0·15	1/5		$1{\cdot}50\times10^2$	1/5	
D4	$1{\cdot}00\times10^6$	0/5		$1{\cdot}00\times10^7$	0/5	
	$1{\cdot}00\times10^5$	0/5	$>1{\cdot}00\times10^6$	$1{\cdot}00\times10^6$	0/5	$>1{\cdot}00\times10^7$
	$1{\cdot}00\times10^4$	0/5		$1{\cdot}00\times10^5$	0/5	

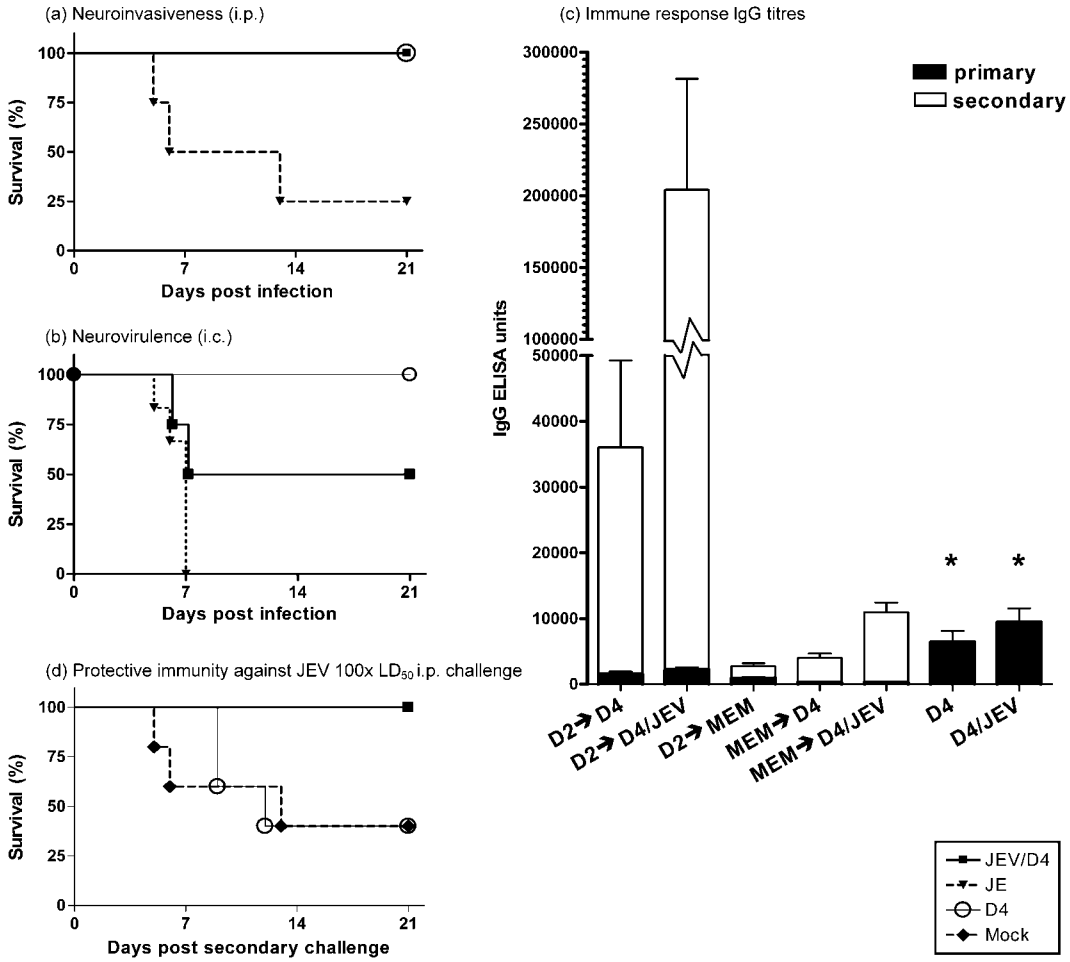

Fig. 4. Mouse inoculations, survival rates and immune responses. (a) Neuroinvasiveness (peripheral neurovirulence); survival rates following i.p. inoculation of 4-week old C57BL/CN mice with 10^7 f.f.u. of parental and chimera virus. Mice were observed for paralysis and death for 4 weeks post-inoculation. (b) Neurovirulence; survival rates of 4-week old C57BL/CN mice following i.c. inoculation with 10^5 f.f.u. of parental and chimera virus. Mice were observed for 4 weeks post-inoculation. (c) Secondary immune response IgG ELISA titres. Graph for antibody ELISA titres showing variation in antibody levels in 3-week old C57BL/6N mice after primary inoculation with one dengue serotype, the chimera or a mock solution and subsequent secondary inoculation with D4 JEV/D4 chimera or the mock solution. Asterisk (*), the last two bars display titre results after primary inoculation only. By Fisher's exact test the variation in secondary antibody titres between D4 and JEV/D4 was found to be highly significant ($P<0{\cdot}0001$). (d) Protective immunity to JEV $100\times$ i.p. LD_{50} challenge. Graph showing percentage survival for three groups of 4-week old mice primarily inoculated with JEV/D4, D4 and EMEM-2 % FCS (control), via the intraperitoneal route. After 3 weeks, all three groups of mice were challenged with JEV at $10\times$ i.p. LD_{50} and observed for the 4 ensuing weeks.

mice each (3-week old) were collected 3 weeks after being i.p. inoculated with 10^6 f.f.u. of D4 or JEV/D4, and subjected to indirect IgG-antibody ELISA (using purified D4 antigen) and to PRNT. Individual sera from D4 and JEV/D4-infected mice were assayed by antibody ELISA. All the 10 mice demonstrated elevation of antibodies detected by the ELISA system, though the chimera induced a marginally higher titre (less than double) than that observed for an equivalent primary challenge with the parental D4 virus (Fig. 4c).

Sera for each virus group were pooled and examined further by PRNT as described in Methods. JE vaccine immunized mouse serum was used as a positive control of JEV antibody. Results for the PRNT are shown in Table 3. These indicate that the chimera induced D4-specific virus neutralizing antibody 1 : 320. The level of PRNT titre induced JEV/D4 in mice was at a level equivalent to that recorded for D4-challenged mice, 1 : 320.

Secondary immune response in mice

To determine whether JEV/D4 chimera exhibits unique infection characteristics in a dengue secondary infection

Table 3. PRNT results

Results for 50 % plaque neutralizing titres of pooled sera from mice groups inoculated with the chimera virus, D4 and JEV, assayed against JEV/D4, JE vaccine (JE-Vax; Biken) and D4 viruses.

Virus	Sera from mice inoculated with:			
	Mock	JED4	JE-Vax	D4
JEV	<1:10	<1:10	1:320	<1:10
D4	<1:10	1:320	<1:10	1:320

scenario, we pre-immunized mice with D2 virus and later challenged them with D4 (a heterologous dengue serotype) or the JEV/D4 chimera virus, and recorded the antibody responses.

Seven groups of five 3-week old C57BL/6N mice were inoculated via the i.p. route and bled according to the following schedule as illustrated in Fig. 4(c). Three groups received primary inoculation of 10^6 f.f.u. of D2 virus, the fourth and fifth received mock inoculation (E-MEM with 2 % FCS), the sixth group with 10^6 f.f.u. of D4, while the seventh was inoculated with 10^6 f.f.u. of JEV/D4 chimera. After three weeks, the mice were bled retro-orbitally, and sera stored at −20 °C. After 3 days convalescence, the mice received secondary challenge as follows. The first group was challenged with 10^6 f.f.u. of D4, the second with 10^6 f.f.u. of JEV/D4 chimera, the third with 0·6 ml E-MEM with 2 % FCS (mock), the fourth group with 10^6 f.f.u. of D4 and the fifth with 10^6 f.f.u. of JEV/D4. Groups six and seven were not included in the secondary challenge experiment. After three weeks, the mice were again bled retro-orbitally and sera, including those previously stored at −20 °C, were subjected to indirect IgG-ELISA to determine anti-D4 antibody levels.

As shown in Fig. 4(c), the secondary immune response was significantly higher in mice receiving secondary challenge with JEV/D4 than with D4 in the groups of mice primarily immunized with D2. The immune response end point titres averaged at 210 000 for JEV/D4 compared with a mean of 37 000 for D4; this represents an approximate sixfold difference.

Cross protection against JEV challenge

To determine whether the JEV/D4 chimera offered protective immunity against JEV, three groups of five 4-week old mice were inoculated i.p. with 10^7 f.f.u. of D4 virus and JEV/D4, and mock inoculum (an equivalent volume of E-MEM with 2 % FCS). Two weeks post-primary inoculation, they received a JEV secondary i.p. challenge ($1{\cdot}5 \times 10^6$ f.f.u. for each mouse in all groups, approximately 100 times JEV i.p. LD_{50} established in 4-week old mice), the mice were then observed for 28 days, and deaths recorded. A Kaplan–Meier graph shows the results of protective immunity against JEV induced by parental D4 and the JEV/D4 chimera (Fig. 4d). While mice primarily inoculated with JEV/D4 had no incidence of mortality, the groups of mice inoculated with D4 and those mock inoculated with E-MEM-2 % FCS, both displayed a 66 % mortality rate, demonstrating cross protection against JEV by an immune response elicited by JEV/D4 infection.

DISCUSSION

The first chimera encoding D4 structural proteins (PrM and E) within a JEV backbone was constructed by means of the LPCRcFS methodology (Gritsun & Gould, 1995; Morita *et al.*, 2001). Previously, a number of flavivirus chimeras have been constructed from vector cloned cDNA (Falgout *et al.*, 1990; Bray & Lai, 1991; Lai *et al.*, 1998; Pletnev & Men, 1998; Liljeqvist & Stahl, 1999; Monath *et al.*, 1999; Campbell & Pletnev, 2000; Guirakhoo *et al.*, 2000, 2001; Pletnev *et al.*, 1992, 1993, 2002; Chambers *et al.*, 2003; Huang *et al.*, 2003). Here, we demonstrate successful assembly of an infectious JEV/D4 chimeric cDNA by the LPCRcFS methodology, indicating the technique's applicability for rapid flavivirus chimera assembly.

The PrM and E genes from D4 were inserted into the JEV backbone, with the JEV C gene and the D4 PrM gene meeting end to end with no lag or overlap. The opposite end of the D4 insert was designed to allow the junction between D4 and JEV to occur within a well-conserved stretch of the flexible stem-anchor of the E region. It was hoped that by doing so NS1 would be processed normally and any adverse impact on both E and NS1's structural conformations avoided.

Despite the procedure advantages in speed and the reduction in procedural complexity entailed in chimera production, the application of the long-PCR amplification procedure increases the likelihood of a chance mutation occurring. When compared with its parental viruses, JEV/D4 showed three nucleotide alternations that corresponded to two amino acid substitutions along JEV/D4 chimera's C, PrM, E and NS1 gene regions (spanning 3483 nt) as shown in Table 1, suggesting that seven more nucleotide changes could be anticipated for the remainder of the genome. This limitation could be overcome in the future by the development of higher replication fidelity DNA polymerases. Of the amino acid changes recorded, one (Q102R) occurred in the C protein representing minor hydrophobicity change. Literature would suggest that little or no impact is expected to result from this change (Lobigs, 1993; Amberg *et al.*, 1994; Kofler *et al.*, 2002). The other change (T405I) occurred in the D4 portion, in the first helix of the E protein stem-anchor region. The resultant increase in hydrophobicity could slightly improve the E protein trimer's quaternary stability in the fusogenic state, when the E protein's stem-anchor helices are expected to become intercalated in the inter-trimer clefts. However, little alternate impact is

anticipated (Hopp & Woods, 1981; Kyte & Doolittle, 1982; Zhang *et al.*, 2003; Modis *et al.*, 2003, 2004).

Infection and growth profiles of JEV/D4 demonstrated a clear tendency to infect and grow in mosquito cells at levels similar to its parental viruses, indicating both the structural and non-structural proteins of the chimera were properly processed in the infected cells and expressed biologic function equal to the wild-type parental viruses. However, growth of the chimera in N2a cells was as poor as D4, while JEV multiplied efficiently. PrM-E proteins of D4 are considered to be a major determinant of the cell tropism (Kawano *et al.*, 1993). Growth of the chimera in PS cells was at its peak, being 100 times greater than the D4 parental, though much lower than JEV parental (Fig. 2), suggesting that poor multiplication of D4 virus in these cells compared with JEV is attributed to biological characteristics of both the structural and non-structural proteins of D4.

Though neuroinvasiveness (peripheral neurovirulence, post-i.p. challenge) was not observed, neurovirulence post-i.c. challenge was recognized *in vivo*, for JEV/D4 chimera. The fact that the chimera is capable of causing JE-like symptoms in the mouse model despite lacking the JEV structural proteins (PrM and E), while still lacking infectivity in a murine neuroblastoma cell line (N2a), highlights the differences between *in vivo* and *in vitro* systems. This discrepancy suggests that when neurovirulence is being evaluated, where possible, *in vivo* assays should not be replaced wholly by *in vitro* assays.

It is noted that D4-specific virus neutralizing antibody was elicited in chimera-infected mice. The virus neutralization profile of the chimera-generated antibody was the same as that induced by D4 parental virus infection (Table 3), suggesting immunogenic, mostly conformational epitopes of authentic E protein of D4 were well conserved in the E protein of JEV/D4. This supports our earlier supposition that the amino acid substitution (T684I) in the stem-anchor of the chimera E protein had little impact.

Dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) often occur in cases of secondary infection of dengue. Antibody-dependent enhancement (ADE) in secondary dengue infection is one of the hypothesized triggers for activating a subsequent cascade of hyper-immunopathological events that result in DHF and DSS (Solomon *et al.*, 2000b). We determined that after primary mice challenge with dengue 2 (D2), the secondary immune response to JEV/D4 challenge was significantly different from that recorded for a D4 secondary challenge. Antibody response after secondary infection with JEV/D4 was up to 285 000 units compared with 200 000–$1{\cdot}2 \times 10^6$ ELISA units among human DHF/DSS patients (our unpublished data). We ascribe this to the JEV component of the JEV/D4 chimera increasing the rate of virus replication and expression of viral proteins resulting in an elevated immune response. At present, we have no direct evidence that ADE is accompanied by this secondary hyper-immune reaction. However, it would be worthwhile to investigate the possibility of developing a system that could study the aetiology of DHF in an animal model, by means of chimeric viruses.

A number of studies have proposed that the flavivirus NS1 protein can elicit protective immunity *in vivo* (Falgout *et al.*, 1990; Hall *et al.*, 1996; Timofeev *et al.*, 1998; Schlesinger *et al.*, 1985, 1986, 1987, 1990; Khoretonenko *et al.*, 2003). These findings pave the way to the development of new flavivirus vaccines that do not induce ADE. Notably, mice primarily inoculated with the JEV/D4 chimera resisted secondary i.p. challenge with $100 \times LD_{50}$ JEV, while those inoculated with D4 did not (Fig. 4d). This indicates that an immune response against JEV's non-structural proteins, most possibly NS1, is likely to be responsible for the observed resistance.

In addition to its use in neurovirulence genetic-determinant mapping, a neurovirulent chimera carrying D4 virus antigenic determinants has primary application as a challenge virus in experimental prophylaxis studies carried out using mice, such as evaluation of D4 monovalent or a tetravalent vaccine efficacy in a non-primate *in vivo* system. Despite smaller plaques observed for JEV/D4 in C6/36 cells, antigen capture ELISA results indicate that JEV/D4 had an antigen production level and profile comparable to JEV. This offers the possibility of using the chimera in C6/36 cell culture as an improved source of high titre D4 E antigen for subsequent use in dengue diagnostic assays. It is known that many D4 strains can infect Vero cells, but our D4 S-14 strain did not. While the precise reason for this is unclear, this characteristic provided an interesting pointer to the possible role of non-structural proteins in infectivity.

The generation of JEV/D4 adds to a growing collection of flavi-in-flavi chimeras and, together with the LPCRcFS method, opens new possibilities for wider, more rapid chimera experimentation in vaccine development and flavivirus pathogenesis research. Further studies involving chimerization of JEV to other dengue serotypes, to produce additional 'Jengue' chimeras, are under way to determine whether these characters are pervasive.

ACKNOWLEDGEMENTS

The first author has been a recipient of the Monbusho Scholarship from the Ministry of Education, Science Sport and Culture of Japan during the period 1999–2004. The authors thank Mr Paresh S. Shah of the National Institute of Virology, Pune, India, for the gift of the porcine kidney cell line (PS^{2001}) used in this study. This study was supported in part by Grant-in-Aid for scientific research (B) (No. 13576016 and No. 15406020) from the Ministry of Education, Science Sport and Culture of Japan, the Grant for Research on Emerging and Reemerging Infectious Diseases (H15-Shinko-19) from the Ministry of Health and Welfare and Labour of Japan and the 21st century Centers of Excellence [COE] program on 'Global Strategies for Control of Tropical and Emerging Infectious Diseases' Nagasaki University.

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