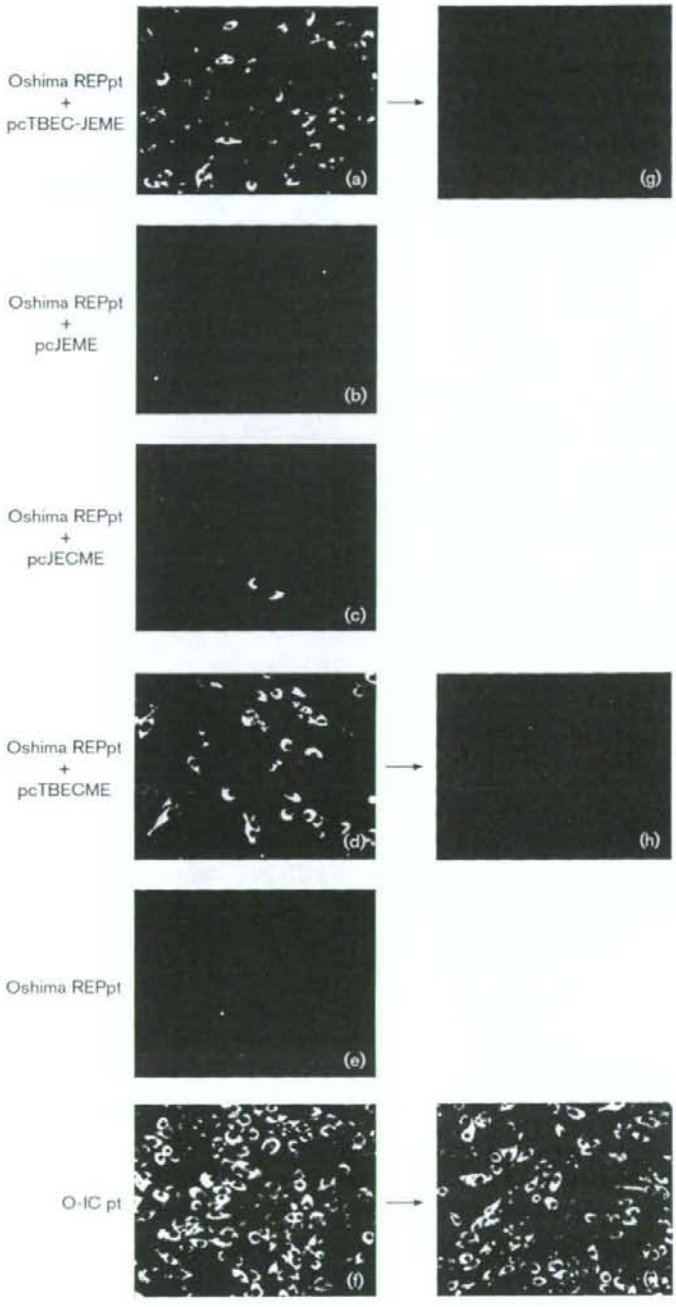

Fig. 2. Secretion of chimeric particles with single-round infectivity. (a–f) Fresh BHK-21 cells were infected with the culture supernatants of cells that were transfected with *in vitro*-synthesized Oshima REPpt RNA and (a) pcTBEC-JEME, (b) pcJEME, (c) pcJECME or (d) pcTBECME, or (e) Oshima REPpt alone. As a control (f), RNA from the full-length TBE virus infectious cDNA O-IC pt was used. (g–i) Reinfection of fresh BHK-21 cells with the culture supernatants: (a)→(g), (d)→(h) and (f)→(i). Expression of viral proteins was visualized by immunofluorescence using anti-NS3 antibodies.

E-specific antibodies for the JE virus prM and E protein bands in Oshima REPpt replicon-electroporated cells that were transfected sequentially with the pcTBEC-JEME, pcJECME and pcJEME plasmids. It has been reported previously that the C–prM junction is cleaved first by the NS3 protein on the cytoplasmic side, and then cleaved continuously by the ER signal peptidase on the ER-luminal side (Amberg *et al.*, 1994; Lobigs, 1993; Sato *et al.*, 1993).

In this case, prM protein bands were detected as independent forms from the C proteins and no C–prM polyprotein band was detected, which indicates that the C–prM junctions were cleaved by viral NS3 protease derived from the expression of the TBE virus replicon RNA. On the other hand, viral protein secretion was observed in the culture supernatants of replicon-electroporated cells that were transfected sequentially with the pcTBECME, pcTBEC-JEME and pcJEME plasmids. However, low levels of E protein were secreted from cells that were transfected with Oshima REPpt RNA and pcJECME. Furthermore, the harvested supernatant was used to infect BHK-21 cells for titration of infectious VLPs (Fig. 3b). A relatively high titre of infectious VLPs ($>10^6$ IU ml^{-1}) was secreted from cells that were transfected with both the pcTBECME and pcTBEC-JEME plasmids, whereas few VLPs (250 IU ml^{-1}) were secreted from cells that were transfected with Oshima REPpt RNA and pcJECME. Semiquantitative RT-PCR showed that the secretion levels of replicon RNAs also corresponded to the infectious titre of VLPs from cells transfected with each plasmid (Fig. 3c). On the other hand, pcJEME plasmid transfection into replicon-electroporated cells resulted in no secretion of infectious VLPs, despite the secretion of viral structural proteins. This viral protein secretion was due to the secretion of subviral particles (SPs), which consist of a viral envelope without nucleocapsid or genomic RNA, as reported in our previous study (Konishi *et al.*, 2001). These data indicate that the efficient packaging and incorporation of nucleocapsid require homologous interactions between flavivirus C proteins and non-structural proteins or genomic RNA, but not between C proteins and viral envelope proteins.

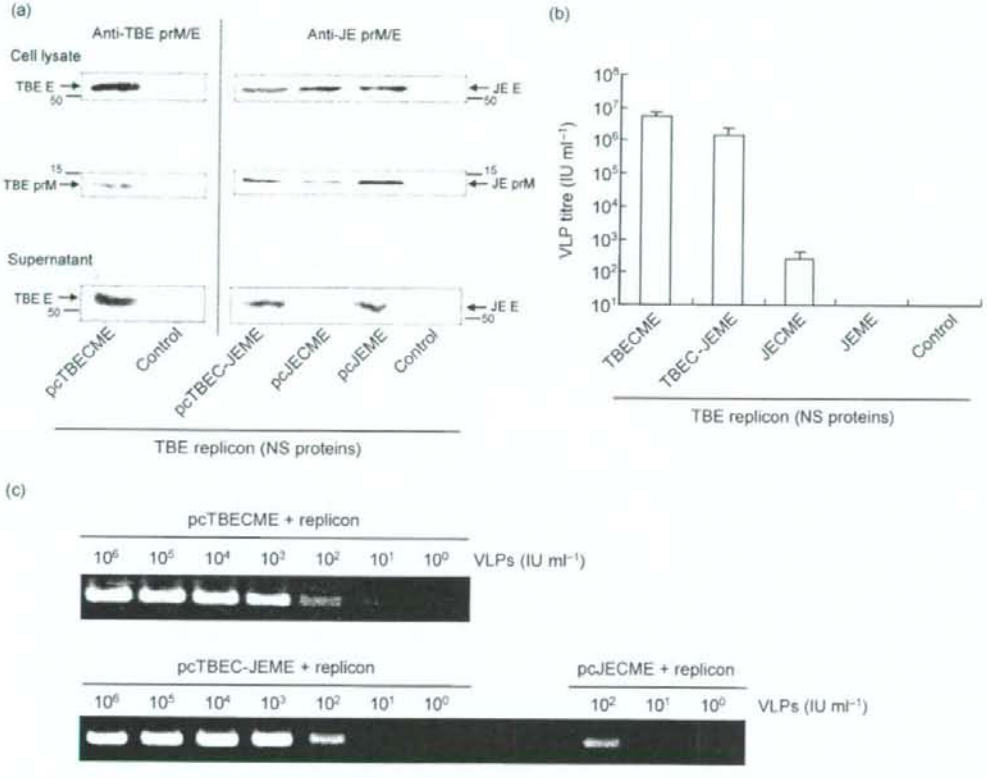

Fig. 3. Kinetics of VLP secretion. (a) Detection of flavivirus structural proteins. TBE virus replicon-electroporated BHK-21 cells were transfected sequentially with pcTBECME, pcTBEC-JEME, pcJECME, pcJEME or control plasmid. At 36 h post-transfection of the plasmid, cell lysate and supernatant samples were separated by SDS-PAGE under non-reducing conditions and subjected to Western blotting. Viral proteins were visualized by specific antibodies against the TBE virus prM/E (left panel) and JE virus prM/E (right panel) proteins. Positions of individual proteins are marked; molecular mass (in kDa) is indicated. (b) Titres (IU) contained in the culture fluids were determined by infectivity assay to BHK-21 cells. The data are means from four independent experiments (error bars indicate SEM). (c) Detection of replicon RNAs packaged in VLPs. Secreted VLPs were diluted serially and subjected to RT-PCR for the detection of TBE virus replicon RNA.

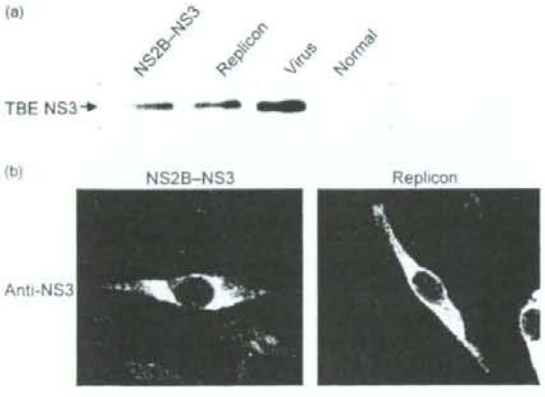

Fig. 4. (a) Expression of NS3 proteins in BHK-21 cells transfected with pcTBENS2B/3 plasmid and replicon RNAs. TBE virus-infected cells and untreated cells were used as a control. (b) Localization of NS3 proteins in BHK-21 cells transfected with pcTBENS2B/3 plasmid (left panel) and replicon RNAs (right panel). Expression of NS3 proteins was visualized by anti-NS3 antibodies at 36 h post-transfection.

Secretion of subviral particles from cells that express chimeric structural proteins and NS2B–NS3 proteins

It is known that expression of the flavivirus envelope proteins prM and E leads to budding and secretion of SPs. To examine the mechanism involved in the differential secretion of infectious VLPs observed for pcTBEC-JEME and pcJECME transfection of replicon-electroporated cells, we investigated SP secretion from cells that expressed C–prM–E polyproteins with TBE virus NS2B–NS3 protease. The pcTBENS2B/3 plasmid, which expresses TBE virus NS2B–NS3 polyproteins, was prepared. The same amount of NS3 was expressed and similar cytoplasmic localization was observed in cells transfected with pcTBENS2B/3 compared with those transfected with the TBE virus replicon RNAs (Fig. 4).

Individual plasmids that express flavivirus C/prM/E structural proteins (pcTBECME, pcTBEC-JEME or pcJECME) were transfected alone or together with pcTBENS2B/3 into BHK-21 cells. When the C/prM/E viral structural proteins were expressed without NS2B–NS3 proteins, C–prM polyprotein bands larger than those seen for prM were detected by the prM-specific antibodies (Fig. 5; lower panel with cell lysate samples). When the

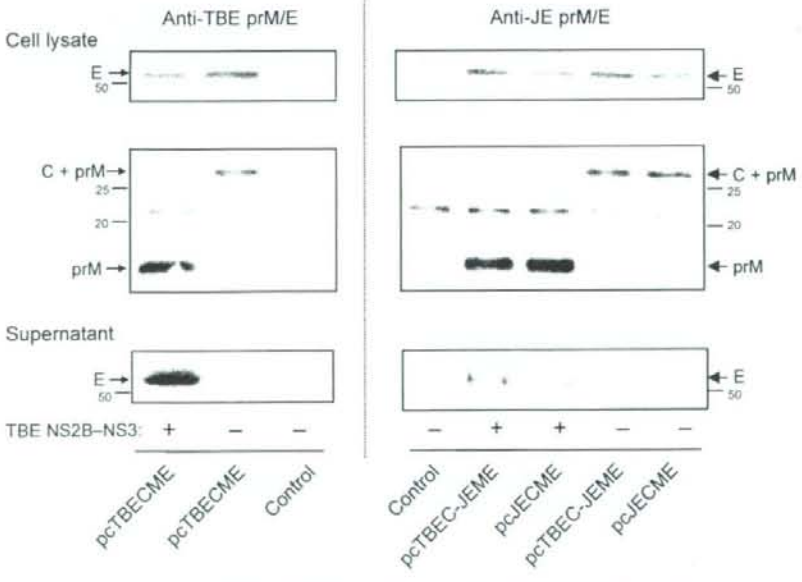

Fig. 5. Detection of flavivirus structural proteins in transfected BHK-21 cells. BHK-21 cells that were transfected with (+) or without (–) the pcTBENS2B/3 plasmid were co-transfected with pcTBECME, pcTBEC-JEME, pcJECME or control plasmid. At 36 h post-transfection, cell lysate and supernatant samples were separated by SDS-PAGE under non-reducing conditions and subjected to Western blotting. Viral proteins were visualized by using specific antibodies against the TBE virus prM/E (left panel) and JE virus prM/E (right panel) proteins. Positions of individual proteins are marked; molecular mass (in kDa) is indicated.

viral structural proteins were expressed with TBE virus NS2B–NS3 proteins, the prM protein bands were detected as separate forms from the C proteins and no C–prM polyprotein band was detected, which indicates that the C–prM junctions were cleaved by TBE virus NS3 protease, as observed for the co-expression of the TBE virus replicon RNA (Fig. 3). On the other hand, whilst no viral protein was secreted without NS2B–NS3 expression, viral protein secretion was observed in all supernatant samples of cells that expressed the flavivirus C/prM/E structural proteins together with TBE virus NS2B–NS3 proteins (Fig. 5; panel with supernatant samples), indicating that SPs are secreted by cleavage of the C–prM junction. This suggests that the low level of VLP secretion from cells that were transfected with pcJECME and TBE virus replicon RNA (Fig. 3) is not due to the budding property of the viral envelope prM/E proteins after processing by NS2B–NS3 protease, and that other factors, such as the mechanism for genome replication and packaging, are involved in the efficient secretion of infectious chimeric VLPs.

Neutralizing test for VLP infection of BHK-21 cells

To confirm the antigenic characteristics of the secreted VLPs, the reactivities of VLPs with anti-flavivirus antibodies were examined. The neutralization test for VLP infection of BHK-21 cells was performed by using the mouse mAbs and polyclonal antibodies against TBE virus, Langat virus (tick-borne flavivirus) and JE virus. As shown in Table 1, mAb 1H4, anti-TBE virus polyclonal antibodies and anti-Langat virus polyclonal antibodies neutralized TBE-envelope VLP infectivity at almost the same concentrations as they did native TBE virus infectivity. In addition, the anti-JE virus polyclonal antibodies neutralized JE-envelope VLP infectivity at almost the same concentration as they did JE virus infectivity. The cross-reactive mAb 4H8 showed similar reactivities for the VLPs and naïve viruses. These data show that the envelope glycoproteins of VLPs have the same antigenicity as those of authentic virus particles.

Table 1. Neutralizing titres of antibodies against infectious particles of flaviviruses

Data are reciprocal numbers of the highest serum dilution that reduced the virus focal count by 50%.

Antibody	VLPs*			
	TBE-envelope	JE-envelope	TBE virus	JE virus
mAbs†				
1H4	>640	<20	>640	<20
4H8	40	80	40	80
Polyclonal antibodies				
Anti-TBE virus	160	<20	320	<20
Anti-Langat virus	>640	<20	>640	<20
Anti-JE virus	<20	320	<20	640

*VLPs were prepared from BHK-21 cells transfected with Oshima REPpt replicon and pcTBEC-JEME (TBE-envelope VLPs) or pcJECME (JE-envelope VLPs).

†1H4, Specific for tick-borne flavivirus; 4H8, cross-reactive against tick-borne and mosquito-borne flavivirus.

Infectivities of TBE-envelope and JE-envelope VLPs for arthropod cells

The JE-envelope VLPs contain envelope proteins that are derived from the mosquito-borne JE virus and carry nucleocapsid and replicon RNA derived from the tick-borne TBE virus. Therefore, we examined the susceptibilities of cell lines derived from arthropods to infection with chimeric JE-envelope VLPs. ISE6 cells, derived from *I. scapularis*, and C6/36 cells, derived from *A. albopictus*, were infected with the TBE-envelope VLPs, JE-envelope VLPs, TBE virus or JE virus, and viral protein production was detected by IFA.

As shown in Fig. 6, the tick cell line ISE6 was susceptible to infection by the TBE virus and TBE-envelope VLPs and the viral proteins showed a cytoplasmic distribution. However, the ISE6 cells were not susceptible to infection by the JE virus or JE-envelope VLPs, although the JE-envelope VLPs contained replicon RNA derived from the TBE virus. On the other hand, the mosquito cell line C6/36 showed no signs of infection by the TBE virus or TBE-envelope VLPs. The C6/36 cells were positive for viral antigen after infection with the mosquito-borne JE virus. Interestingly, the C6/36 cells that were infected with JE-envelope VLPs were not positive by IFA.

In order to reveal the mechanism involved in the lack of susceptibility of tick and mosquito cells to JE-envelope VLPs, the internalization of VLPs was examined (Fig. 7). Following 1 h VLP absorption, replicon RNA was detected in ISE6 cells that were infected with TBE-envelope VLPs, whereas most of the JE-envelope VLPs remained in the supernatant and no replicon RNA was detected intracellularly. This indicates that the entry of JE-envelope VLPs into ISE6 cells is inefficient and, thus, no viral proteins are detected by IFA (Fig. 6). In contrast, the JE-envelope VLPs entered the C6/36 cells after 1 h VLP absorption, whereas the TBE-envelope VLPs remained in the supernatant (Fig. 7a). However, intracellular replicon RNAs disappeared at 8 h post-infection (Fig. 7b). Taken together with the IFA results (Fig. 6), this suggests that JE-envelope VLPs can enter C6/36 mosquito cells, but cannot replicate therein, due to fact that their genomic replicon RNA is derived from the tick-borne TBE virus. Direct transfection of C6/36 cells with TBE virus full-length RNA or replicon RNA resulted in neither viral protein production nor virus particle secretion, as reported previously (Mandl *et al.*, 1991).

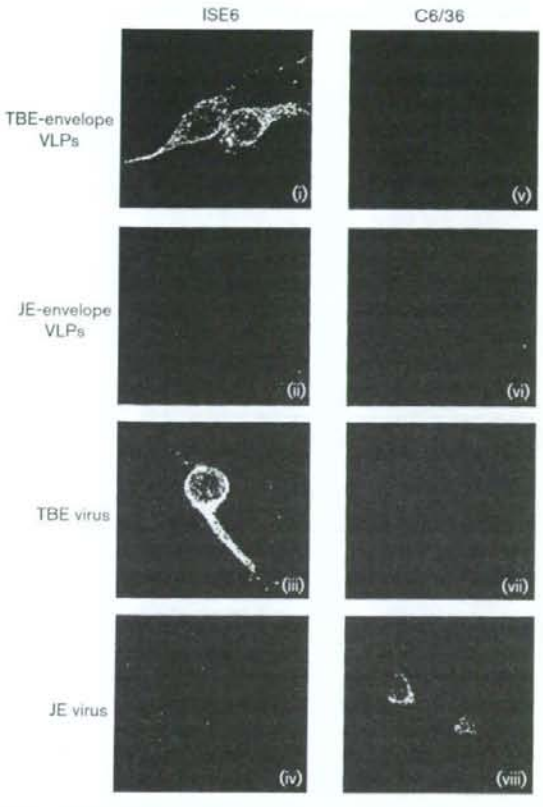

Fig. 6. Susceptibility of ISE6 (tick) and C6/36 (mosquito) cell lines to infectious flavivirus particles. ISE6 (i–iv) and C6/36 (v–viii) cells were infected with TBE-envelope VLPs (i, v), JE-envelope VLPs (ii, vi), TBE virus (iii, vii) or JE virus (iv, viii), prepared from the culture supernatants of BHK-21 cell cultures. Viral protein production was visualized by immunofluorescence using specific antibodies, as described in Methods.

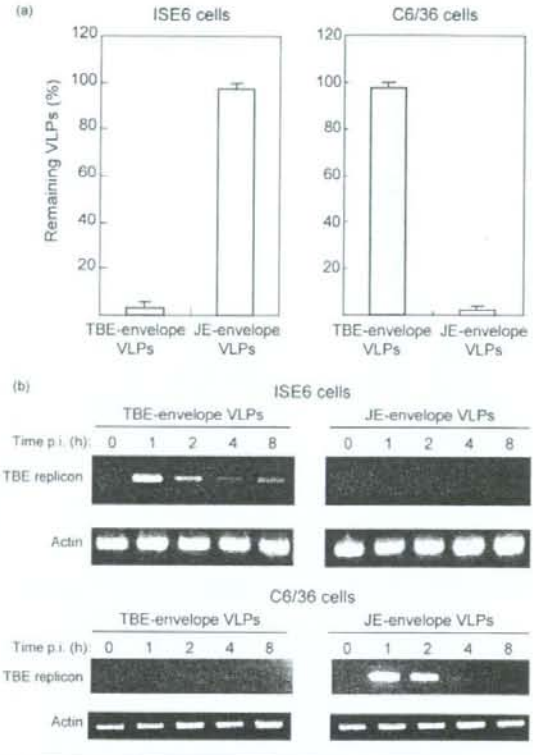

Fig. 7. (a) Internalization of TBE- or JE-envelope VLPs into arthropod cells. ISE6 and C6/36 cells were infected with VLPs. After 1 h absorption, the supernatants were harvested and remaining VLPs were titrated. Results are expressed as percentages of remaining VLPs compared with untreated controls. The data are means from three independent experiments (error bars indicate SEM). (b) Detection of replicon RNA from arthropod cells infected with TBE- or JE-envelope VLPs. ISE6 and C6/36 cells were infected with the VLPs. At each time point post-infection (p.i.), RNA samples were extracted and subjected to RT-PCR for the detection of TBE virus replicon RNA. Actin mRNA was used as an internal control.

DISCUSSION

In this study, we developed, for the first time, chimeric VLPs between mosquito-borne and tick-borne flaviviruses, using the *trans*-packaging system for TBE virus subgenomic replicons. In recent years, the development of reverse-genetic technologies has enabled the construction of flavivirus chimeras in various combinations (Caufour *et al.*, 2001; Guirakhoo *et al.*, 2000, 2001; Huang *et al.*, 2000; Mathenge *et al.*, 2004; Monath *et al.*, 1999; Pletnev & Men, 1998; Pletnev *et al.*, 1992, 2002). These chimeras may be useful tools for studying the biological features of flaviviruses and for vaccine development. However, several aspects of flavivirus chimeras require further study. The family *Flaviviridae* contains many important human pathogens that cause severe symptoms and high mortality (approx. 30 %). Thus, recent studies of flavivirus chimeras have tended to use less pathogenic or vaccine strains of flavivirus for the development of live-attenuated vaccines. On the other hand, flavivirus VLPs have the same antigenicity and virus-entry characteristics as the wild-type virus, and the single-round infectivity of VLPs allows infection experiments to be conducted under BSL-2 conditions (Gehrke *et al.*, 2003; Khromykh *et al.*, 1998; Scholle *et al.*, 2004; Yoshii *et al.*, 2005). Therefore, our strategy is that chimeric VLPs can substitute for chimeric viruses in investigations of the biological properties of flaviviruses.

Chimeric VLPs that packaged the TBE replicon were recovered only following *trans*-expression of the TBE virus

C and JE virus prM/E proteins. In many studies of flavivirus chimeras, chimeric viruses have been recovered successfully by replacement of the viral envelope protein prM and E genes by those from other flaviviruses. These data indicate that the C proteins (or nucleocapsid) interact non-specifically with viral envelope proteins and that this interaction is not important for the assembly of virus particles. On the other hand, in experiments using *trans*-expression of the JE virus C/prM/E proteins, we failed to recover chimeric VLPs. Similar results have been shown previously in several studies of chimeric viruses, in which substitution of the C/prM/E protein genes of a tick-borne flavivirus with those of mosquito-borne flaviviruses resulted in inefficient recovery of chimeric virus (Chambers *et al.*, 1999; Pletnev & Men, 1998; Pletnev *et al.*, 1992) and, in a study by Harvey *et al.* (2004), packaging efficiency of dengue type 2 virus replicon RNAs by *trans*-expression of the KUN virus C/prM/E proteins was lower than that of KUN virus replicon RNA; the detailed mechanism underlying this finding has not been analysed.

It is known that the C terminus of the C protein is processed by the activity of the viral NS2B–NS3 protease, and that the N terminus of prM is subsequently cleaved by the cellular signal peptidase (Amberg & Rice, 1999; Amberg *et al.*, 1994; Stocks & Lobigs, 1998; Yamshchikov & Compans, 1995; Yamshchikov *et al.*, 1997). The processed prM protein then forms a heterodimer with the E protein, which is essential for the maturation and assembly of infectious particles (Allison *et al.*, 1995; Konishi & Mason, 1993; Lorenz *et al.*, 2002). In cells that expressed the JE virus C/prM/E structural proteins and TBE virus replicon or NS2B–NS3, prM proteins were detected in the form cleaved from the C protein, which indicates that the JE virus C–prM junctions are cleaved by the TBE virus NS2B–NS3 protease (Figs 3 and 5). However, in spite of the processing of the C–prM junctions, the expression of JE virus C/prM/E proteins in cells that harboured the TBE virus replicon resulted in a low level of secretion of viral proteins compared with cells that expressed the TBE virus C and JE virus prM/E proteins or the TBE virus C/prM/E proteins, which secreted VLPs that packaged the replicon RNA. The difference between the *trans*-expressed polyproteins is a reflection of whether the respective C proteins are homologous to the genomic replicon RNA. Thus, in addition to the processing of C–prM junctions, other factors related to the homology between the C protein and genomic RNA regulate the assembly of VLPs that package replicon RNA.

Efficient secretion of E, in the form of non-infectious SPs, was observed by the expression of JE virus C/prM/E proteins and TBE virus NS2B–NS3 proteins, but few infectious VLPs and fewer E proteins were secreted by the expression of JE virus C/prM/E proteins in TBE replicon-transfected cells. This indicated that some factor involved in the replication of replicon RNA regulated the process of viral particle assembly and secretion and retained viral structural proteins intracellularly. Recent studies of flavivirus non-structural proteins have revealed the involvement of NS2A and NS3 in the assembly and/or release of infectious virus particles (Khromykh *et al.*, 2000; Kummerer & Rice, 2002; Liu *et al.*, 2002) and it has been reported that the packaging of flavivirus genomic RNA into infectious particles is coupled to genome replication (Khromykh *et al.*, 2001). From these studies, it has been concluded that homologous interaction between viral C and non-structural proteins or genomic RNA is important for the efficient assembly and secretion of infectious particles packaging genomic RNA. No signals or motifs in flavivirus RNA or C protein that determine the specificity of packaging have yet been defined. However, these findings can contribute to future studies of packaging signals and increase understanding of how flavivirus virions are assembled and secreted.

It has been shown that, in general, arthropod-borne flaviviruses infect either mosquito or tick cells (Lawrie *et al.*, 2004). However, it is not known whether the ability of certain flaviviruses to infect certain cells but not others is due to viral entry into the cells or to replication and subsequent release from infected cells. The chimeric VLPs developed in our study have viral envelopes that are derived from mosquito-borne JE virus, and nucleocapsid (C protein plus subgenomic replicon RNA) derived from tick-borne TBE virus. Virus particles of flaviviruses enter cells by receptor-mediated endocytosis, the viral envelope fuses with the endosomal membrane and then the genomic RNAs are uncoated from the nucleocapsid and replicate (Heinz *et al.*, 2004). Thus, our chimeric VLP system has the advantage that it can separate particle-internalization events from sequential viral-replication events. As shown in Figs 6 and 7, TBE virus and JE virus infected either ISE6 or C6/36 cells, and TBE-envelope VLPs infected only ISE6 cells. However, no TBE virus NS proteins were produced in either tick or mosquito cells that were infected with JE-envelope VLPs. RT-PCR revealed that the TBE- and JE-envelope VLPs could enter ISE6 or C6/36 cells, and that the TBE virus replicon RNAs could not replicate in C6/36 cells that were infected with JE-envelope VLPs. Similar results were reported in a study by Pletnev *et al.* (1992), in which a chimeric virus of TBE virus/dengue type 4 virus that contained prM/E protein genes from the TBE virus was restricted in its ability to enter C6/36 cells. It is not known whether JE virus genomic RNA can replicate in tick cells, but these data indicate that vector-specific factors in arthropod cells may be required for each step of virus entry and replication during flavivirus infection. Although the susceptibility of a tick- or mosquito-derived cell to a particular arbovirus does not always reflect vector association, it is a useful indicator.

In addition to their use in investigations of the biological properties of flaviviruses, chimeric VLPs can be applied to serological diagnosis as a substitute for neutralization testing, which uses infectious viruses. As shown in Table 1, infections with TBE- and JE-envelope VLPs were neutralized by mAbs and immune sera with titres similar to those

used to neutralize the TBE and JE viruses, indicating that the chimeric VLP system is an effective alternative to the use of native flaviviruses in neutralization tests. This chimeric VLP-based neutralization system does not require a high-level containment laboratory, as the subgenomic replicon RNA packaged in the VLPs does not have the genes that encode the viral structural proteins and thus is unable to produce infectious progeny viruses. Furthermore, by replacement of the prM/E genes with those from other flaviviruses in plasmids used for the *trans*-expression of viral structural proteins, the chimeric VLP system can be adjusted easily to develop neutralization tests for a variety of flaviviruses, as substitutes for tests involving the native viruses.

In summary, we have generated chimeric VLPs that comprise the JE virus envelope and TBE virus nucleocapsid, which contains the subgenomic replicon of the TBE virus. JE-envelope VLPs were secreted when the TBE virus C protein and JE virus prM/E proteins were expressed in TBE virus replicon-electroporated cells, but not when all of the structural proteins of JE virus were expressed. The TBE- and JE-envelope VLPs were neutralized by antibodies against flaviviruses with efficacies similar to those for the native TBE and JE viruses. The infectivities of the TBE- and JE-envelope VLPs for tick and mosquito cells suggest that vector host cell-specific factors are involved in each step of flavivirus entry and replication during arthropod infection.

ACKNOWLEDGEMENTS

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Epidemiology and Diagnosis of West Nile Virus Infection

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Abstract

West Nile (WN) virus produces a mosquito-borne infection manifesting in fever and, in serious cases, encephalitis. The virus that causes WN infections, WN virus, belongs to the family *Flaviviridae*, genus *Flavivirus*. The WN virus emerged in New York City in August, 1999, as the first case on the American continent, and seven human deaths were reported. Along with humans, deaths were observed among horses and various birds, such as crows. The WN virus epidemic expanded to the southern United States in 2000 and most of the rest of the country by 2004, extending as far as Argentina in 2007. Since there is a possibility of the WN virus spreading to Japan, where the Japanese encephalitis virus is also prevalent, the TaqMan assay was adapted to develop a sensitive, specific diagnostic test to differentiate WN virus from Japanese encephalitis virus.

Key words: epidemiology, diagnosis, West Nile virus infection

1. Epidemiology

1.1 Global epidemiology

The WN virus (WNV) was first isolated in 1937 from the blood of a febrile patient in the West Nile district of Uganda. WNV has since been found endemic over a wide range of areas in Africa, the Middle East, western Asia and Australia. Outbreaks of varying size occurred in Israel in 1941 and 1951-1954, and in Africa in 1974. After that, no large outbreaks were observed for 20 years, but from 1994 to 2000, WNV outbreaks occurred among humans and horses in Algeria in 1994, Morocco in 1996, Romania in 1996, Tunisia in 1997, the Czech Republic in 1997, the Congo in 1998, Italy in 1998, Israel in 1997-2000, Russia in 1999, France in 2000 and the United States in 1999-2002 (Marfin & Gubler, 2001).

1.2 Epidemiology on the American Continent

During the five years following the first diagnosis of WN encephalitis in August, 1999, in New York City, the WNV epidemic spread over 41 states. The total number of diagnosed patients was 26,997 and the total number of deaths was 1,008 (Table 1). (CDC (Centers for Disease Control and Prevention), 2008). Deaths in the United States were seen most often among persons older than 50 years and the average mortality rate was ca. 3.73%. Between 1999 and 2006, WNV was detected in 62 species of mosquitoes, with *Culex* species accounting for more than 98% of the total reported. Over the same period, 317 species of WNV-positive dead birds were reported, with American crows and blue jays accounting for more than 62% of the these cases. WNV outbreaks have also been reported among islands in the Caribbean Sea and in Argentina. The means of importation of the virus into the United States is unknown.

Table 1 Reported WNV disease cases in humans, United States, 1999-2007. (modified from CDC 2008)

Year	Total cases	Deaths
1999	62	7
2000	21	2
2001	66	9
2002	4,156	284
2003	9,862	264
2004	2,539	100
2005	3,000	119
2006	4,269	177
2007	3,022	76
Total	26,997	1,008

2. Causal Virus

WNV belongs to the family *Flaviviridae*, genus

Flavivirus and is included in the Japanese encephalitis (JE) virus serocomplex group. This group of viruses includes the St. Louis encephalitis virus of the United States and the Murray Valley encephalitis virus of Australia. The viral genome consists of single-stranded positive RNA and encodes three structural proteins (C, prM and E) and seven non-structural proteins.

WN virus isolates have been subjected to phylogenetic analysis, and can be subdivided into two major lineages. Lineage 1 includes most of the virus isolated since 1996, the American strains identified in 1999 and 2000, and the strains identified in Romania in 1996, Israel in 1999, and Volgograd, Russia in 1999. Lineage 2 includes most of the strains prevalent in Africa (Lanciotti *et al.*, 1999).

3. Transmission Cycle and Infection Route

The major transmission cycle of WNV occurs between mosquitoes and birds. The model of virus transmission proposed for the United States is shown in Fig. 1. Birds are the virus-amplifying hosts, and mosquitoes of the *Culex* species are the major vectors in many outbreaks. Humans and horses are infected by bites from virus-carrying mosquitoes, and while they show clinical symptoms such as fever and encephalitis, they are called 'dead-end hosts' as they do not serve as an infection source for mosquitoes, because the viremia in humans and horses is not high enough to infect the mosquitoes. Major vector species of mosquitoes in the United States are *Cx. pipiens, Cx. quinquefasciatus, Cx. restuans, Cx. salinarius* and *Cx. tarsalis*. Crows are often used as sentinel animals to monitor virus activity in the United States.

Routes of human infection other than mosquito bites include fetal infection through the placenta, infantile infection via breast feeding, infection due to blood transfusion or organ transplantation and laboratory-acquired infection during handling of the live virus or infected materials.

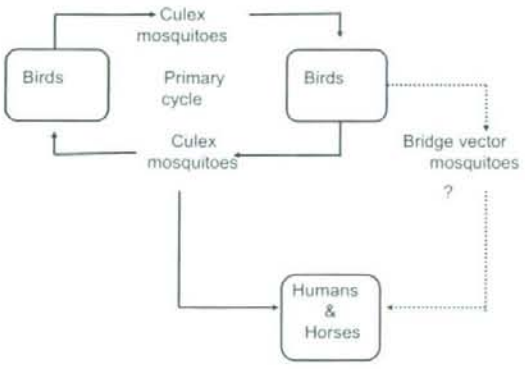

Fig. 1 Transmission cycle of WN virus. (modified from Campbell *et al.* 2002)

4. Clinical Symptoms

In humans, the incubation period of WNV is three to fifteen days following infection. Most cases are asymptomatic, but in some cases, fever and fatal encephalitis may develop. Mild cases show a febrile period of three to six days, headache, backache, myalgia, fatigue, rash and lymphadenopathy. Severe cases develop acute myelitis and encephalitis. The mortality rate of the outbreaks recorded in the United States has been approximately 3.73%.

In horses, the incubation period is five to ten days. Most infected horses are asymptomatic, but some show clinical symptoms. In the United States, reported symptoms include ataxia, weak legs, lying down, astasia, myospasm, fever, facial neuralgia, lip palsy, facial spasm, gnashing and blindness. The mortality rate, including euthanised cases, is 38%.

In birds, infection is asymptomatic, except in the United States and Israel. In the cases recorded in the United States, high mortality was observed among American crows and blue jays. Symptoms included depression, anorexia, weakness and loss of body weight. Severe cases manifest neurological signs such as ataxia, tremor, revolution and paresis. The clinical signs last for seven days.

5. Diagnosis

Diagnosis of WNV involves virus detection and serological examination. Virus isolation or virus genome detection is performed using sera or cerebrospinal fluids. IgM-captured ELISA, neutralization test, hemagglutination inhibition test and complement fixation test are used to detect WNV-specific antibodies. Acute phase sera, plasma, cerebrospinal fluids or post-mortem brain materials can all be used for virus isolation. For serological examination, paired sera from patients in the acute phase and convalescent phase should be collected and tested. Differential diagnosis should be considered for other flavivirus infections including JE, St. Louis encephalitis, tick-borne encephalitis, Murray Valley encephalitis and dengue. Other viral infections, such as alphavirus infection and herpesvirus infection, should be also considered.

5.1 Development of a sensitive diagnostic test for differentiation of WNV and Japanese encephalitis (JE) virus

Like WNV, JE virus is a mosquito-borne flavivirus from the JE virus (JEV) serocomplex, and causes encephalitis in humans and horses; it is widespread throughout most of Asia (Igarashi, 1992; Rosen, 1986). In areas where JEV is endemic, such as Japan, distinguishing between WNV and JEV is critical for correct identification of a WNV invasion. However, JE serocomplex flaviviruses cross-react antigenically and are thus not readily differentiated by serological methods (Martin, *et al.*, 2000). Molecular diagnostic methods

are therefore preferred, and the reverse transcriptase polymerase chain reaction (RT-PCR) has been used to develop sensitive and specific assays for the identification of WNV (Igarashi, *et al.*, 1994; Porter, *et al.*, 1993). Recently, more sensitive assays, such as fluorogenic real-time (TaqMan) PCR, SYBR Green-based real-time PCR and loop-mediated isothermal amplification (LAMP), have been developed for diagnostic detection of the WNV genome (Lanciotti, *et al.*, 2000; Papin, *et al.*, 2004; Parida *et al.*, 2004). In areas where JEV is endemic, it is necessary to perform specific assays for both WNV and JEV to make a definitive diagnosis. However, the diagnostic methods described above were designed to detect only WNV or, more specifically, the strain of WNV isolated in the United States. Here, we describe the development of a sensitive molecular diagnostic method that can detect and distinguish between WNV and JEV, using TaqMan RT-PCR analysis with a probe common to WNV and JEV strains (Sirato *et al.*, 2005).

To determine the sensitivity of the TaqMan assay, the assay was performed with serially-diluted cDNA from the NY99-6922 strain of WNV (Fig. 2). It was established that a cut-off (Ct) value of <40 and ΔRn signal of >0.5 indicated that WNV was present. The specificity of the primer sets was tested using various flaviviruses (Table 2). The TaqMan assay was performed using the indicated volume of cDNA synthesized from total RNA. The results indicated that the primer set for WNV could detect only WNV strains, including both lineage 1 and 2 viruses. The primer set for JEV could detect only JEV strains, including genotypes 1 (Ishikawa) and 3 (JaGAr01, Nakayama, and Beijing). The MVE virus, which is one of the JE serocomplex viruses, was not detected by either primer set. Flaviviruses of other serocomplexes, such as TBE virus, Dengue virus, Langat virus, and Powassan virus, were also not detected by either primer set.

To measure the sensitivity of the TaqMan assay for other WNV and JEV strains, the assay was performed

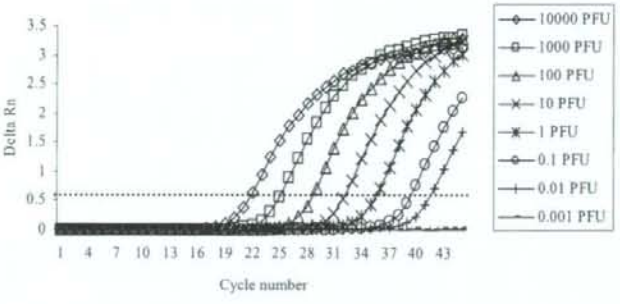

Fig. 2 Determination of the cut-off values for the TaqMan assay. The assay was performed with serially diluted cDNA from the NY99-6922 strain of WNV. The dotted line is drawn at ΔRn = 0.5. (Data from Shirato *et al.* 2005.)

Table 2 Specificity of the multi-probe TaqMan assay. (modified from Shirato *et al.* 2005)

		Primer set used			
		WNV primers		JEV primers	
Virus	Amount of template	Ct*	Result**	Ct	Result
WNV					
Lineage 1					
NY99-6922	1 μg of total RNA	16.8 ± 0.18	Pos	> 40.0	Neg
NY99-A301	1 μg of total RNA	17.7 ± 0.15	Pos	> 40.0	Neg
BC787	1 μg of total RNA	17.6 ± 0.03	Pos	> 40.0	Neg
6-LP	1 PFU	33.6 ± 0.00	Pos	> 40.0	Neg
6-SP	1 PFU	31.2 ± 0.07	Pos	> 40.0	Neg
Eg101	1 μg of total RNA	17.0 ± 0.04	Pos	> 40.0	Neg
Kunjin (OP393)	100 PFU	34.7 ± 0.20	Pos	> 40.0	Neg
Lineage 2					
FCG	1 μg of total RNA	17.2 ± 0.03	Pos	> 40.0	Neg
JEV					
Genotype 1					
Ishikawa	1,000 PFU	> 40.0	Neg	23.5 ± 0.03	Pos
Genotype 3					
Nakayama	1 μg of total RNA	> 40.0	Neg	20.1 ± 0.19	Pos
Beijing	1 μg of total RNA	> 40.0	Neg	15.8 ± 0.2	Pos
JaGAr01	1 μg of total RNA	> 40.0	Neg	16.3 ± 0.11	Pos

Pos=positive; Neg=negative
* Data are represented as the mean ± standard deviation.
** Ct values < 40 with ΔRn signals of > 0.5 were considered positive.

using serially diluted cDNA samples from titrated virus stocks (Table 3). At least 10^{-1} plaque-forming units (PFU) of virus was required for fluorescence detection by the TaqMan assay with WNV or JEV primer sets. However, the sensitivity for detection of Kunjin virus was lower than that of other viruses; at least 10 PFU of Kunjin virus were required for detection. Although the TaqMan assay was first performed using a 50 µl reaction volume, there was no change in sensitivity when the assay was performed using 25 µl or 12.5 µl reaction volumes. The TaqMan assay could detect 10^{-1} PFU or more of virus even in these smaller reaction volumes (data not shown). Therefore, all TaqMan assays were performed using a 25 µl reaction volume thereafter.

We also examined whether the primer sets used in this study could detect viruses in animal tissues. As Japan is a WNV-free country, we were unable to obtain archived clinical samples from animals naturally infected with WNV. Therefore, the TaqMan assay was performed using tissues from experimentally-infected mice. BALB/c mice were infected with 10^6 PFU of the NY99-6922 strain of WNV via the intraperitoneal route, and tissue samples were collected on the indicated days post-infection (p.i.). Total RNA was extracted, and cDNA was synthesized and tested using the TaqMan assay. The assay detected viral RNA in blood, spleen and brain samples (Table 4). Viruses were detected at both the early (three days p.i.) and late (eight days p.i.) stages of infection. Plaque assays were also performed to detect viruses in infected animal tissues.

This paper describes a highly sensitive genetic diagnostic method that can detect WNV and JEV using TaqMan RT-PCR analysis with a single probe that is common to both WNV and JEV strains. WNV strains of both lineage 1 and 2 were successfully detected when using the primer set for WNV, and genotype 1 and 3 JEV were detected when using the primer set for JEV. Viral RNA was detected in experimentally-infected animal tissues. Therefore, it is considered likely that the method described here is sufficiently sensitive and specific for detecting WNV and JEV strains in both human and animal samples.

Table 3 Sensitivity of the multi-probe the TaqMan assay. (modified from Shirato *et al.* 2005)

	Virus dose (PFU)					
Virus	10^3	10^2	10^1	10^0	10^{-1}	10^{-2}
WNV						
Lineage 1						
NY99-6922	Pos*	Pos	Pos	Pos	Pos	Neg
6-LP	Pos	Pos	Pos	Pos	Pos	Neg
6-SP	Pos	Pos	Pos	Pos	Pos	Neg
Eg101	Pos	Pos	Pos	Pos	Pos	Neg
Kunjin (OP393)	Pos	Pos	Pos	Neg	Neg	Neg
Lineage 2						
FCG	Pos	Pos	Pos	Pos	Pos	Pos
JEV						
Genotype 1						
Ishikawa	Pos	Pos	Pos	Pos	Pos	Neg
Genotype 3						
JaGAr01	Pos	Pos	Pos	Pos	Pos	Neg

Pos=positive; Neg=negative
Ct values < 40 with ΔRn signals of > 0.5 were considered positive.

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Table 4 Detection of WNV and JEV in experimentally infected mouse tissues using the multi-probe TaqMan assay. (modified from Shirato, 2005)

						Primer set			
						WNV		JEV	
Infecting virus	Number	Days p. i.	Clinical signs	Tissue	Virus titer*	Ct	Result**	Ct	Result
NY99-6922	1	3	No signs	Blood	1.3	37.5	Pos	> 40.0	Neg
				Spleen	3.6	30.2	Pos	> 40.0	Neg
	2	3	No signs	Blood	1.0	39.9	Pos	> 40.0	Neg
				Spleen	3.8	30.6	Pos	> 40.0	Neg
	3	3	No signs	Blood	1.0	37.1	Pos	> 40.0	Neg
				Spleen	3.6	31.1	Pos	> 40.0	Neg
	4	8	Dead	Spleen	2.3	32.0	Pos	> 40.0	Neg
				Brain	> 7.0	14.4	Pos	> 40.0	Neg
	5	8	Moderate encephalitis	Blood	1.3	38.3	Pos	> 40.0	Neg
				Spleen	2.8	30.2	Pos	> 40.0	Neg
				Brain	5.6	24.5	Pos	> 40.0	Neg

Pos=positive; Neg, negative; n.d.=not detected
* The viral titer in blood samples is expressed as log PFU/ml and that of tissue samples is expressed as log PFU/g. The detection limits were 10 PFU/ml (blood) and 100 PFU/g (spleen and brain).
** Ct values < 40 with ΔRn signals of > 0.5 were considered positive.

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Construction and evaluation of a chimeric pseudoinfectious virus vaccine to prevent Japanese encephalitis

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Summary Multiple vaccines exist to control Japanese encephalitis (JE), but all suffer from problems. We have developed a new type of flavivirus vaccine, a pseudoinfectious virus (RepliVAX WN) that prevents West Nile virus (WNV)-induced disease. Here, we describe production of a chimeric RepliVAX (RepliVAX JE) that expresses the JE virus (JEV) prM and E proteins. Our prototype RepliVAX JE replicated poorly in cells, but blind passage produced a better-growing derivative, and analyses of this derivative allowed us to engineer a second-generation RepliVAX (RepliVAX JE.2) that grew to high titers. RepliVAX JE.2 elicited neutralizing antibodies in both mice and hamsters and provided 100% protection from a lethal challenge with JEV or WNV, respectively. These results demonstrate the utility our RepliVAX platform for producing a JE vaccine.

Introduction

Japanese encephalitis virus (JEV) is an emerging/re-emerging threat throughout Southern and Southeast Asia and Australia [1,2]. Although most JEV infections are asymptomatic, the estimated 0.3% that lead to disease result in over 35,000 cases including 10,000 deaths annually worldwide [2], and many of the remaining cases produce permanent sequelae.

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Currently the most commonly used JE vaccines are a mouse brain-derived formalin-inactivated viral (INV) vaccine used in Japan, South Korea, Taiwan, Thailand, United States and Europe and a live-attenuated virus (LAV) vaccine that is used in China. Although the INV vaccine has been used extensively, it requires three doses and so is too expensive for routine use in many countries with high-risks of disease. Further, several vaccine-associated adverse events have been reported for this product [3,4]. The Chinese LAV vaccine [5] has not yet been approved for sale in first-world markets, and there remain concerns about both the quality of its manufacture and its potential for reversion to virulence. More recently, cell culture-derived INV vaccines [6,7], subunit vaccines [8–12], an adenovirus vector-based vaccine [13], and a chimeric vaccine based on another flavivirus [14] have been developed as new JE vaccine candidates.

JEV, a member of the genus *Flavivirus* of the family *Flaviviridae*, belongs to the JE serocomplex which also includes West Nile virus (WNV) [15]. The flavivirus virion contains a capsid (C) protein complexed with the positive-strand RNA genome within a lipid bilayer that contains the envelope (E) and membrane (M) proteins; the latter is synthesized as a precursor protein (prM) in infected cells. The flavivirus genome encodes these three structural proteins as well as seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) as part of a single translational open-reading frame. The E protein plays important roles in receptor binding and cell fusion and possesses most of the virus-neutralizing epitopes [16]. Cells expressing the flavivirus prM and E proteins in the absence of other viral components are known to secrete subviral particles (SVPs) which are known to be excellent immunogens [17–20].

Recently, it was shown that C-deleted flavivirus genomes engineered from tick-borne encephalitis virus could be used as RNA vaccines [21]. Based on this observation, we developed and evaluated vaccines that consist of pseudoinfectious flaviviruses with a similar deletion in C [22]. These pseudoinfectious viruses (named RepliVAX) can be propagated in C protein-expressing cells, where the trans-expressed C permits them to form infectious particles that are structurally identical to live virus. When RepliVAX infect normal cells, their genomes replicate and the RepliVAX-infected cells release SVPs and secreted forms of NS1, but these cells cannot produce infectious particles; consistent with these properties we have shown RepliVAX WN to be a safe and effective flavivirus vaccine candidate [22,23].

In the present study, we developed and evaluated a new JE vaccine candidate based on RepliVAX technology. To achieve this, a chimeric RepliVAX (encoding the JEV *prM-E* genes in place of the WNV *prM-E* genes; hereinafter referred to as RepliVAX JE) was produced. RepliVAX JE initially grew poorly in C-expressing cells, but quickly acquired a point mutation in the prM signal peptide that enhanced its growth. This mutation was used to produce a second-generation RepliVAX (RepliVAX JE.2) that was evaluated in mice and hamsters. A single immunization with RepliVAX JE.2 induced neutralizing antibodies in both species, and the immunized mice and hamsters were protected from lethal JEV and WNV challenge, respectively.

Materials and methods

Cells, viruses and virus-like particles (VLP)

Vero cells [22] were grown at 37 °C in Eagle's minimal essential medium (MEM) containing 6% fetal bovine serum (FBS). BHK cells expressing noncytopathic Venezuelan equine encephalitis virus (VEEV) replicons (VEErep) [24] encoding various flavivirus proteins were grown at 37 °C as previously described [25]. BHK cells expressing WNV C {BHK(VEErep/Pac-Ubi-C*)}, BHK(VEErep/WNprMtrE/Pac) cells expressing WNV prM and 80% of WNV E, and BHK(VEErep/Pac-Ubi-WNNS1NS2A) expressing WNV NS1-NS2A are described in the accompanying paper [23]. BHK(VEErep/WNC*-JEprM-E/Pac) expressing WNV C and JEV prM-E and BHK(VEErep/JEprMtrE/Pac) cells expressing JEV prM and 80% of JEV E are described below.

JE-WN chimeric virus containing JE prM-E (see below) used for neutralization tests was prepared in BHK cells by electroporation. WNV VLPs (WNVLP) containing a subgenomic WNV replicon with the firefly *luciferase* gene (WNR C-Luc2A NS1-5) and enveloped in WNV E were produced using BHK(VEErep/C*-E/Pac) cells (see below) [25]. JEV VLPs (JEVLP) containing the same WNR C-Luc2A NS1-5 genome and enveloped in JEV E were prepared in BHK(VEErep/WNC*-JEprM-E/Pac) cells. RepliVAX JE was grown in MEM containing 1% FBS and 10 mM HEPES (maintenance-media) on BHK(VEErep/Pac-Ubi-C*) cells. JEV Beijing P3 [26] and WNV NY 99 [22] were prepared in suckling-mouse brain and Vero cells, respectively. Both viruses were titrated on Vero cells using focus-formation assays (see below).

Plasmids

A previously described RepliVAX WN cDNA [22], that was introduced into a bacterial artificial chromosome (BAC) plasmid to enhance its stability [27], was used to produce a RepliVAX JE cDNA by replacing the WNV *prM-E* gene of RepliVAX WN with the *prM-E* gene of JEV Nakayama [28]. The resulting RepliVAX JE cDNA consisted of a truncated WNV *C* [22], JEV *prM-E* and WNV nonstructural protein genes.

To construct the JE-WN chimeric virus cDNA, the WNV gene of a WNV infectious cDNA clone [29] was replaced by the same *prM-E* gene of JEV Nakayama (see above).

To obtain a cell line that would permit packaging of a WNR into a JEV envelope, a VEErep (VEErepC*-JEprM-E/Pac) was created by substituting the JEV *prM-E* gene (see above) for the WNV *prM-E* gene in VEErep/C*-E/Pac. For production of antigens for ELISA, a VEErep encoding JEV *prM* followed by the first 416 codons of the *E* gene (80% of full length E) fused to a 6xhistidine-tag at its C-terminus was constructed.

Electroporation

VEErep plasmids were used to establish VEErep-harboring cell lines as previously described [25].

The RepliVAX JE plasmid, the JE-WN chimeric infectious cDNA plasmid, and WNR C-Luc2A NS1-5 were used to

produce infectious particles in BHK cells (JE-WN) or the required packaging cell lines (RepliVAX and WNR), as previously described [25,29].

Infective titration and immunostaining

For *in vitro* growth curves and to evaluate focus morphologies, RepliVAX JE was serially diluted and inoculated into BHK(VEErep/Pac-Ubi-C*) cell monolayers. Following absorption for 2 h, cells were overlaid with 0.6% tragacanth (MP Biomedicals, Solon, OH) containing 1% FBS and 10 mM HEPES (overlay-media), and incubated for 72 h. The resulting infectious foci were visualized by immunostaining as described previously [29].

To determine the titer of RepliVAX JE stocks for use in animals, dilutions of RepliVAX JE were inoculated into Vero cells and the monolayers were immunostained as described above, and individual cells (or pairs of cells resulting from cell-division post-infection) were enumerated to determine the number of infectious units (IU).

Sequencing analysis

RepliVAX JE preparations were inoculated into Vero cells at a multiplicity of infection (moi) of 10. Following incubation for 48 h, RNA was extracted from the cells using TRIzol (Invitrogen, Carlsbad, CA), and fragments of cDNA spanning the genome were reverse transcribed, PCR-amplified, gel purified and sequenced at as previously described [29].

Western blotting

RepliVAX JE were inoculated into Vero cell cultures at a moi of 10 and incubated with 10% VP-SFM (Invitrogen)-90% MEM. At 72 h, culture fluids (CF) were collected and cell lysates were prepared using 0.1% Triton X-100, 300 mM NaCl, 50 mM Tris–HCl pH7.6. For normalization, parallel monolayers were fixed with 4% paraformaldehyde and the number of infected cells was determined using immunofluorescence assays. Briefly, fixed cells were permeabilized with 0.5% Triton X-100, then incubated with goat anti-WNV NS3 antibody (R&D Systems, Minneapolis, MN) followed by Alexa Fluor 488 anti-goat IgG (Invitrogen). Protein concentrations of cell lysates were determined by DC protein assay kit (BioRad, Hercules, CA), and samples normalized to contain equal amounts of protein and equal number of positive cells were resolved on 4–12% gradient polyacrylamide gels (Invitrogen), transferred to polyvinylidine difluoride membranes, and then incubated with either a mixture of goat anti-WNV NS3, mouse anti-WNV E (monoclonal antibody 7H2; BioReliance, Rockville, MD) and mouse anti-actin (mixture of monoclonal antibodies; Sigma, Saint Louis, MO), or mouse anti-WNV NS1 antibody from hybridoma 6H4 [30] and mouse anti-actin. Following washing, the membranes were incubated with peroxidase-conjugated anti goat IgG and peroxidase-conjugated anti mouse IgG (KPL), and bound peroxidase was visualized by using ECL Plus System (GE healthcare, Pittsburgh, PA).

Animal experiments

Groups of 10 females 5-week-old Swiss Webster mice or 5-week-old golden Syrian hamsters (Harlan Sprague–Dawley, Indianapolis, IN) were immunized with 100 µl of RepliVAX JE (diluted in maintenance-media) via the intraperitoneal (i.p.) route. Groups of 10 control animals were inoculated with 100 µl of maintenance-media. All animals were weighed daily from 2 days prior to immunization until 7 days post-vaccination to monitor RepliVAX JE safety. At 21 days post-vaccination, serum was collected from animals by retro orbital puncture. Sera from animals in each group were heat inactivated (30 min at 56 °C) and pooled for determination of Neutralizing antibody titers (Neut-titers) and individual sera were tested for antibodies specific for E and NS1 proteins by using ELISA (see below).

To assess efficacy, mice were challenged at 28 days post-vaccination with 5×10^6 focus forming units (ffu) of JEV Beijing P3 (calculated to be 30 50% lethal doses (LD_{50}) in 9-week-old mice (Mason and Bourne, Unpublished)) diluted to 100 µl in PBS containing 10% FBS via the i.p. route. Hamsters were challenged at 28 days post-vaccination with 1×10^7 ffu of WNV NY99 in 100 µl diluted in PBS containing 10% FBS via the i.p. route. All animals were weighed daily for 14 days post-challenge and examined daily until day 21 post-challenge for signs of infection. Animals that appeared to be unable to survive until the next day (based on lethargy, weight-loss, difficulty remaining upright, or hind-limb paralysis) were humanely euthanized and recorded as "dead" the following day, in compliance with Animal Care and Use requirements. All surviving animals were euthanized and bled at 21 days post-challenge.

Neutralization tests

Neut-titers were determined on Vero cells using a focus-reduction assay [22] and/or luciferase activity-reduction assay. For the focus-reduction assay, serially diluted heat-inactivated sera were incubated with 60 ffu of JE-WN chimeric virus for 1 h at 37 °C, and then inoculated onto Vero cell monolayers prepared in 24-well plates. Following a 2 h absorption, the cells were overlaid with overlay-media. At 24 h, the cells were immunostained as described above. Neut-titers are reported as the highest serum dilution yielding 90% reduction in focus number.

For the luciferase activity-reduction assay, serially diluted heat-inactivated sera were incubated with JEVLPs or WNVLPs diluted with maintenance-media for 1 h at 37 °C, and then 50 µl of this mixture was inoculated into Vero monolayers prepared in black-wall 96-well plates (Greiner Bio-One, Monroe, NC). Following a 2 h absorption, the inoculum was aspirated and cells were rinsed with PBS twice and incubated in maintenance-media. At 24 h, the VLP-encoded luciferase activities were measured by addition of Steady-Glo Luciferase Assay System reagent (Promega) diluted in lysis buffer and light measurement was performed as previously described [25]. The Neut-titer was expressed as the highest serum dilution yielding a 90% reduction in light detected.

ELISA to determine antibody levels against E and NS1 proteins

Ninety-six well Immulon 2HB plates (Thermo Labsystems, Franklin, MA) were sensitized with truncated JEV E, truncated WNV E or WNV NS1 at 4 °C overnight in carbonate buffer. The protein preparations consisted of clarified culture fluids harvested from the appropriate VEErep-bearing BHK cells grown in 10% VP-SFM-90% MEM. Antigens were pretitrated for reactivity, and a 1:4 dilution of truncated JEV E and WNV E, and a 1:128 dilution of WNV NS1 were selected for use. The plates were sequentially incubated with sera at a dilution of 1:100, and peroxidase-conjugated anti mouse IgG or anti hamster IgG (KPL) using standard techniques. After approximately 5 min incubation with TMB substrate (Sigma), the reaction was stopped by adding equal volume of 1 M HCl. Absorbance at 450 nm was measured on a VERSA max spectrophotometer (Molecular Devices, Sunnyvale, CA) and expressed in optical density (O.D.) units.

Statistical analysis

Growth kinetics and potency were compared by Student's *t*-test. Survival incidence data were compared by Fisher's exact test. All comparisons were two tailed.

Results

Blind passage of RepliVAX JE in BHK cells expressing WNV C improved their growth

To make a RepliVAX JE–encoding cDNA plasmid, the cDNA corresponding to the *prM-E* genes of our RepliVAX WN plasmid was replaced by a cDNA encoding JEV *prM-E*. Insertion was accomplished to produce in-frame fusions at the signalase cleavage sites between WNV C and JEV prM, and JEV E and WNV NS1. To obtain RepliVAX JE, *in vitro* transcribed RNA obtained from this cDNA was electroporated into BHK(VEErep/Pac-Ubi-C*) cells (see methods). Surprisingly, the RepliVAX JE yields were over 20-times lower than RepliVAX WN [22], although the live chimera of WNV encoding the JEV prM/E genes grew to high titers (data not shown).

Since JEV-WNV chimerization might have affected the replication of RepliVAX JE, blind passage of RepliVAX JE in BHK(VEErep/Pac-Ubi-C*) cells was used to obtain a better-growing RepliVAX JE. The third blind passage RepliVAX JE (RepliVAX JE p3) produced larger foci on BHK(VEErep/Pac-Ubi-C*) cells than the original RepliVAX JE (RepliVAX JE p0), and the size of these foci did not change after an additional seven passages (data not shown). Side-by-side comparisons of the growth of RepliVAX JE p0, p3 and p10 revealed that p3 and p10 were markedly improved compared to p0 (Fig. 1).

Sequence analyses were used to determine how RepliVAX JE had changed during blind passage. For this purpose, RepliVAX JE p3 was inoculated to Vero cells to obtain RNA free of contamination with the BHK(VEErep/Pac-Ubi-C*) *C* gene, and 48 h following infection, RNA was extracted and sequenced as described in the methods. Analyses of these sequence data revealed that RepliVAX JE p3 had only two nucleotide changes relative to

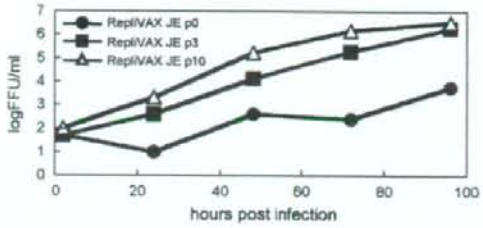

Figure 1 Comparison of growth of original and blind-passed RepliVAX JE. RepliVAX JE p0 (circle), RepliVAX JE p3 (square) and RepliVAX JE p10 (triangle) were inoculated on BHK(VEErep/Pac-Ubi-C*) cells at an equal moi, and then CF were harvested at 2 h, 24 h, 48 h, 72 h and 96 h post-infection and the titers were determined on BHK(VEErep/Pac-Ubi-C*) cells. These cells were used for titrations so that we could also visualize focus sizes (see text), even though these cells display titers 50-times lower than those obtained on Vero cells.

the parental RepliVAX JE. The first change, found in the third codon downstream of the NS2B/NS3 cleavage site that separates truncated C from the signal sequence for prM, was a G to U mutation, which resulted in a K to N codon-change (QKKR/GGKT → QKKR/GGNT). The second nucleotide change, an A to U in the *prM* gene, did not produce an amino acid change. Sequence analyses of the full-length genome of RepliVAX JE p10 failed to detect any additional changes, indicating that the sequence attained in RepliVAX JE p3 become the predominant, stable sequence early in the passaging series.

Construction of a second-generation of RepliVAX JE

Although two nucleotide changes were found in RepliVAX JE p3 genome, we focused on the single coding change near the NS2B/NS3 cleavage site. To assess whether the mutation was responsible for the growth improvement, the mutation was introduced into the original RepliVAX JE genome (producing a construct referred to as RepliVAX JE.2).

Comparison of the focus sizes of RepliVAX JE and RepliVAX JE.2 on BHK(VEErep/Pac-Ubi-C*) cells revealed that RepliVAX JE.2 produced larger foci that were indistinguishable in size from foci produced by RepliVAX JE p3 (data not shown). Titers obtained from RepliVAX JE.2 electroporated BHK(VEErep/Pac-Ubi-C*) cells at 96 h were significantly higher than those of RepliVAX JE (by Student's *t*-test, $p < 0.01$; Fig. 2). Although these titers were lower than those shown for RepliVAX JE p3 in Figure 1, the lower titers obtained in Fig. 2 were likely due to the effect of electroporation on the cells, since BHK(VEErep/Pac-Ubi-C*) cells infected with RepliVAX JE.2 routinely produced titers of greater than 10^7 IU/ml on Vero cells (results not shown).

To address the possibility that alteration in the prM signal sequence of RepliVAX JE could result in an altered ability to produce SVPs in infected cells lacking C, we compared the synthesis and secretion of E, NS1 and NS3 in RepliVAX JE and RepliVAX JE.2-infected Vero cells. Visual inspection of the Western blot shown in Fig. 3 failed to detect any differences in the intracellular proteins produced by cells infected with these two RepliVAX JE. Further, cells infected

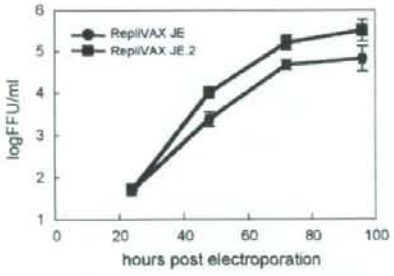

Figure 2 Comparison of recovery of RepliVAX JE from BHK(VEErep/Pac-Ubi-C*) cells electroporated with similar amounts of RNA from RepliVAX JE (circle) and RepliVAX JE.2 (square). CF were harvested from cell layers at 24 h, 48 h, 72 h and 96 h post-electroporation and titers were determined on BHK(VEErep/Pac-Ubi-C*) cells. These cells were used for titrations so that we could also visualize focus sizes (see text), even though these cells display titers 50-times lower than those obtained on Vero cells.

with either RepliVAX JE released similar amounts of NS1 into the CF as shown by these same Western analyses (Fig. 3) and ELISA (results not shown). In contrast, this Western shows that E was present in higher amounts in CF of RepliVAX JE.2-infected Vero cells than in the CF from cells infected with RepliVAX JE (Fig. 3), and ELISA data demonstrated 2.5-fold more extracellular E in the CF harvested from RepliVAX JE.2-infected Vero cells (results not shown). Taken together, these data show that the NS2B/NS3 cleavage site mutation in RepliVAX JE.2 improves RepliVAX JE growth and enhances E secretion, two properties that should make the second-generation RepliVAX JE a superior vaccine.

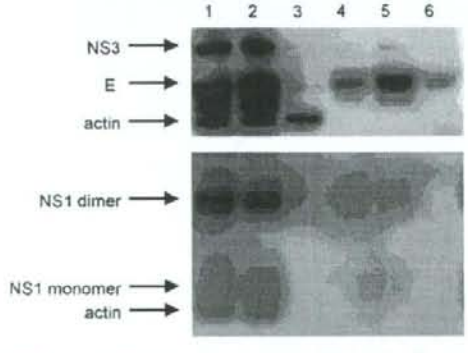

Figure 3 Comparison of antigen production by Vero cells infected with RepliVAX JE or RepliVAX JE.2. CF and cell lysates were collected at 72 h post-infection and equivalent amounts of proteins containing the same amount of infected cells were subjected to Western blot analysis, and E, NS1, NS3 and actin proteins were visualized as described in methods. (lane 1) RepliVAX JE-infected cell lysate; (lane 2) RepliVAX JE.2-infected cell lysate; (lane 3) mock-infected cell lysate; (lane 4) RepliVAX JE-infected cell CF; (lane 5) RepliVAX JE.2-infected cell CF; (lane 6) mock-infected cell CF.

Evaluation of RepliVAX JE in mice

To assess RepliVAX JE safety, potency and efficacy, we used a well-established JEV Beijing P3 strain murine model [26]. Groups of mice were vaccinated with 1×10^6, 2×10^5 and 4×10^4 IU of RepliVAX JE.2, 1×10^6 IU of RepliVAX JE or MEM. Since RepliVAX JE.2 grew better (Fig. 2) and produced more soluble E than RepliVAX JE (Fig. 3), most studies were performed with this second-generation vaccine candidate. Daily observations (including weighing) failed to reveal any adverse effects of any RepliVAX JE consistent with previous safety data obtained with RepliVAX WN [22].

To evaluate the humoral immune responses elicited by vaccination, sera collected at day-21 post-vaccination were tested for presence of JEV-neutralizing antibodies using two methods. The first method utilized a JE-WN chimeric virus in a classical prevention-of-viral-foci-formation test. Using a chimeric flavivirus in place of the actual agent of interest has been shown to be useful by others [31], and application of chimeras is particularly helpful in this case, since JEV is a USDA Select Agent, severely restricting its use. The focus reduction-based neutralization test performed with the JE-WN chimera revealed that pooled sera obtained from groups of mice immunized with any tested dose of RepliVAX JE or RepliVAX JE.2 produced neutralizing antibodies (Table 1). Consistent with these data, our luciferase activity-based neutralization test, which measures the ability of sera to prevent infection by JEVLPs containing Luc-expressing WNV replicons showed similar neutralizing titers (Table 1). In both assays, no neutralizing antibodies were detected in MEM-immunized mice (Table 1). Since the results obtained by the luciferase activity-based neutralization test were consistent with those obtained by the focus reduction-based test, the luciferase activity-based test was used in all subsequent experiments.

In addition to Neut-titers obtained from serum pools, antibody levels against JEV E and WNV NS1 were measured for individual mice by ELISA. Mice immunized with 1×10^6 IU of RepliVAX JE.2 showed the highest levels of antibodies against JEV E protein, but strong responses against JEV E protein were detected at all RepliVAX JE.2 doses (Fig. 4). Animals immunized with 1×10^6 IU of RepliVAX JE.2 had more serum antibodies against both JEV E and WNV NS1 than animals immunized with the same dose of RepliVAX JE although

Table 1 Immunogenicity of RepliVAX JE.2 in mice[a]

Immunogen	Dose (IU)	Traditional Neut-titer[b]	Luc Neut-titer[c]
RepliVAX JE.2	1×10^6	1:160	1:160
RepliVAX JE.2	2×10^5	1:80	1:80
RepliVAX JE.2	4×10^4	1:40	1:40
RepliVAX JE	1×10^6	1:80	1:160
MEM	–	<1:40	<1:40

[a] Titers were determined at 21 days post-vaccination.
[b] 90% focus reduction neutralizing antibody titer (Neut-titer) was determined with JE-WN chimeric virus.
[c] 90% Luc activity reduction Neut-titer was determined with the JEVLP containing the *Luc* gene.

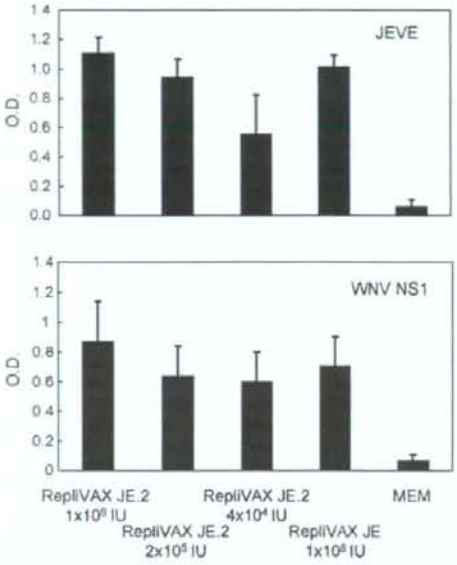

Figure 4 Serum ELISA antibody levels detected in vaccinated mice. Antibody levels to the JEV E and WNV NS1 proteins were detected in mouse sera collected at 21 days post-vaccination with the indicated preparations. The bars show the mean O.D. (450 nm) values obtained with sera from individual animals and the extended bars show the standard deviation among these samples.

this increase was not statistically significant (by Student's *t*-test, $p > 0.01$; Fig. 4).

The protective efficacy of RepliVAX JE vaccination was determined by challenging vaccinated mice with 30 LD_{50} of JEV Beijing P3 strain at 28 days post-vaccination. This challenge was lethal in all control mice, with the final animal succumbing to disease on day 17 post-challenge. In contrast, all mice immunized with RepliVAX JE.2 or RepliVAX JE survived challenge (Fig. 5; each $p < 0.001$ vs. control), and no mice immunized with RepliVAX JE.2 or RepliVAX JE showed any clinical signs of JE such as ruffling and abnormal gait, changes in behavior, or loss of weight. Analysis of sera collected at 21 days post-challenge demonstrated that all mice developed high titers of neutralizing antibodies (>1:2560, data not shown), indicating efficient challenge and inability of vaccination to prevent viral replication. Nevertheless, these studies demonstrate that a single immunization with as few as 4×10^4 IU of RepliVAX JE.2-induced protective immunity.

Evaluation of RepliVAX JE in hamsters

RepliVAX JE was tested in a second animal model by inoculating groups of ten 5-week-old hamsters with two different doses of RepliVAX JE.2 (Table 2 and Fig. 6). Tests on sera collected at 21 days post-vaccination with either dose demonstrated that the pooled sera samples contained similar high-levels of neutralizing antibodies against JEV

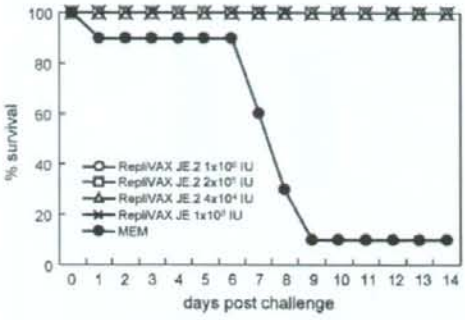

Figure 5 Survival data following JEV challenge of vaccinated mice. At 28 days post-vaccination, mice were challenged with 30 LD_{50} of JEV Beijing P3 strain, and survival was monitored for 14 days. The single MEM-inoculated mouse that survived to day 14 died 3 days later and all other mice remained disease-free until 21 days post-challenge. Open circle, open square, open triangle, cross and closed circle indicated mice inoculated with 1×10^6 IU of RepliVAX JE.2, 2×10^5 IU of RepliVAX JE.2, 4×10^4 IU of RepliVAX JE.2, 1×10^6 IU of RepliVAX JE and MEM, respectively.

(Table 2). Since we planned to challenge these hamsters with WNV (see below), neutralizing antibodies against WNV were also examined, and found to be much lower than those specific for JEV (Table 2). Neutralizing antibodies against JEV or WNV were not detected in MEM-immunized hamsters (Table 2).

Interestingly, similar levels of antibodies to JEV E protein were detected in individual animals immunized with either dose of RepliVAX JE.2 (Fig. 6). However, dose-dependent antibody responses to WNV E and WNV NS1 proteins were detected in these animals (Fig. 6).

Preliminary studies showed that our mouse-virulent JEV strain failed to cause any signs of illness in hamsters (results not shown). Therefore, based on the work of Tesh et al. showing that a live-attenuated JE vaccine could cross-protect hamsters from WNV challenge [32], the RepliVAX JE.2-vaccinated hamsters were challenged at 28 days post-vaccination with WNV NY99. Eight of 10 control hamsters immunized with MEM died by day 14 post-challenge (Fig. 7) and the remaining two control animals showed clinical signs of WN encephalitis including ruffling and abnormal gait, and both animals lost over 10% of their body weight during the observation period. In contrast, both doses of RepliVAX JE.2

Table 2 Immunogenicity of RepliVAX JE.2 in hamsters[a]

Immunogen	Dose (IU)	Neut-titer JEV[b]	Neut-titer WNV[c]
RepliVAX JE.2	1×10^6	1:160	1:40
RepliVAX JE.2	2×10^5	1:160	1:40
MEM	–	<1:40	<1:40

[a] Titers were determined at 21 days post-vaccination.
[b] 90%Luc activity reduction Neut-titer was determined with the JEVLP containing the *Luc* gene.
[c] 90%Luc activity reduction Neut-titer was determined with the WNVLP containing the *Luc* gene.