Development of chimaeric West Nile virus attenuated vaccine candidate based on the Japanese encephalitis vaccine strain SA14-14-2

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Mosquito-borne flaviviruses include a large group of important human medical pathogens. Several chimaeric flaviviruses have been constructed, and show potential for vaccine development. Although Japanese encephalitis virus (JEV) live vaccine SA14-14-2 has been widely used with ideal safety and efficacy profiles, no chimaeric flavivirus based on the JEV vaccine has been described to date. Based on the reverse genetic system of the JEV vaccine SA14-14-2, a novel live chimaeric flavivirus carrying the protective antigens of West Nile virus (WNV) was constructed and recovered in this study. The resulting chimaera (ChinWNV) replicated efficiently in both mammalian and mosquito cells and possessed genetic stability after in vitro serial passaging. ChinWNV exhibited a small-plaque phenotype, and its replication was significantly restricted in mouse peripheral blood and brain compared with parental WNV. Importantly, ChinWNV was highly attenuated with regard to both neurovirulence and neuroinvasiveness in mice. Furthermore, a single ChinWNV immunization stimulated robust WNV-specific adaptive immune responses in mice, conferring significant protection against lethal WNV infection. Our results demonstrate that chimaeric flaviviruses based on the JEV vaccine can serve as a powerful platform for vaccine development, and that ChinWNV represents a potential WNV vaccine candidate that merits further development.

INTRODUCTION

West Nile virus (WNV) is an emerging neurotropic arbovirus belonging to the genus Flavivirus, family Flaviviridae, which includes other important human pathogens, such as Japanese encephalitis virus (JEV), dengue virus (DENV), yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV). WNV cycles between mosquitoes and birds in nature, and bites by infected mosquitoes result in infection in humans. Patients infected with WNV develop a febrile illness, with a subset of cases progressing to a meningitis or encephalitis syndrome. Since its introduction into the western hemisphere in 1999, WNV has caused over 78 000 cases, with more than 30 000 confirmed cases in the USA alone (Petersen et al., 2012). Although the incidence of WNV infection in the USA decreased in 2008 and 2009, there was a robust increase in cases of WNV in 2012 (CDC, 2012). WNV has been recognized as the leading cause of arboviral neuroinvasive disease in the USA.

Flaviviruses contain a positive-sense RNA genome of ~11 kb that contains a single ORF flanked by a 5′ and a 3′ UTR. The genome encodes a single polyprotein, which is proteolytically processed into three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) by viral and host...
proteases. The E protein is the major target of the virus-neutralizing antibodies that confer protection against viral infection and requires the co-expression of prM to acquire its native conformation. The high conservation among flaviviruses with regard to genome organization, replicative strategy and gene expression enables the generation of viable chimaeric flaviviruses by interchanging genes among different flaviviruses through reverse genetics (Bray & Lai, 1991; Chambers et al., 1999; Pletnev et al., 1992).

Currently, there is neither an effective drug treatment nor a preventative vaccine against WNV infection, although several vaccines are licensed for use in horses and geese (Kahler, 2003; Ng et al., 2003; Samina et al., 2005). Numerous approaches for the development of a WNV vaccine for humans are being investigated, including inactivated vaccines (Lim et al., 2008; Posadas-Herrera et al., 2010), a RepliVAX vaccine based on single-cycle flavivirus particles (Nelson et al., 2010; Uhrlauf et al., 2011; Widman et al., 2009), viral vector vaccines (Coutant et al., 2008; Iyer et al., 2009; Martina et al., 2011; Minke et al., 2004; Scheppe-Berglind et al., 2007), recombinant subunit vaccines expressing E protein (Alonso-Padilla et al., 2011; Lieberman et al., 2009) and DNA vaccines (Chang et al., 2008; Davis et al., 2001; Despres et al., 2005; Ledgerwood et al., 2011; Prow et al., 2010). Live chimaeric WNV vaccine candidates based on attenuated DENV-4 and YFV vaccine strain YF-17D have recently been undergoing pre-clinical (Pletnev et al., 2006) and clinical (Biedenbender et al., 2011) trials.

An inexpensive JEV live attenuated vaccine, strain SA14-14-2, was developed in China and first licensed for human use in 1989; it has since been widely used in most JEV-endemic countries. Over 300 million doses have been produced and administered to approximately 120 million children, with no adverse events reported (Yu, 2010). Large-scale vaccination programmes have verified an effectiveness and efficacy of SA14-14-2, was developed in China and first licensed for human use in 1989; it has since been widely used in most JEV-endemic countries. Over 300 million doses have been produced and administered to approximately 120 million children, with no adverse events reported (Yu, 2010).

Results

**RESULTS**

**Design and characterization of ChinWNV**

The flavivirus prM protein plays a critical role in the proper folding of virion particles; thus, we engineered a chimaeric JEV/WNV virus (ChinWNV) cDNA in which the prM and E genes of JEV were replaced with the corresponding WNV genes using a subgenomic replicon of JEV as the genetic backbone (Fig. 1). To ensure efficient cleavage at the C–prM and E–NS1 junctions, the cDNA clone of ChinWNV retained the prM signal of JEV and the last three C-terminal amino acids of the JEV E protein. Plasmid DNA containing the genome of ChinWNV was sequenced and compared with that of the parental JEV and WNV. As shown in Table S1 (available in JGV Online), five nucleotides in the prM–E coding region of ChinWNV differed from those in WNV, three of which resulted in amino acid changes in the prM protein ([Ile128→Val] or E protein ([Ala247→Val and Glu465→Gly]). An A2496→C change causing the substitution Ile6→Leu in the NS1 protein was also identified.

After transfecting baby hamster kidney (BHK)-21 cells with in vitro-transcribed ChinWNV RNA, a typical cytopathic effect was observed. An indirect immunofluorescence assay (IFA) showed that the BHK-21 cells infected with ChinWNV or WNV were positive for antibodies specific to the WNV E protein but negative for JEV E protein-specific antibodies (Fig. 2a). As predicted, antibodies specific to the JEV NS1 protein detected the NS1 protein in the cells infected with the chimaera and JEV (Fig. 2a). The chimaeric genome structure of the rescued virus was then confirmed by full genome sequencing and was found to be identical to that encoded by the plasmid (data not shown).

A plaque assay of BHK-21 cells showed that ChinWNV produced a homogeneous population of plaques with a diameter of approximately 0.92 mm, which was significantly smaller than those of JEV or WNV (Fig. 2b). The growth efficiencies of ChinWNV and its parental viruses were compared in BHK-21 cells, with both ChinWNV and WNV reaching a production peak at approximately 48 h post-inoculation (p.i.) (Fig. 2d). Similar results were observed in Vero cells, and ChinWNV attained a peak titre of 10^6.8 p.f.u. ml^-1 at 48 h p.i. (Fig. 2d); all viruses attained peak titres at 72 h p.i. in C6/36 cells. ChinWNV reached the same peak titre of 10^6.7 p.f.u. ml^-1 as JEV at 72 h p.i., which was approximately 20-fold lower than that attained by WNV (Fig. 2d). Together, these observations demonstrated that ChinWNV possesses a chimaeric genomic structure, exhibits WNV-specific antigenicity, shows a small-plaque phenotype in BHK-21 cells and replicates efficiently in different cell lines.

**Genetic and plaque phenotypic stability of ChinWNV**

To assess the genetic stability of ChinWNV during sequential passaging in vitro, the chimaera recovered from BHK-21 cells (passage 0) was passaged up to eight times in Vero cells, the certified cell bank for vaccine production. Full genomic sequences of viruses were determined for passages 4 and 8. Compared with virus from passage 0, no amino acid change in the prM–E region or the JEV portion (UTR, C and NS regions) was found, even at passage 8.
(data not shown). Plaque assays showed that the small-plaque phenotype of ChinWNV in BHK-21 cells remained unchanged during in vitro passaging, indicating that there were no plaque variants overgrowing the original parental population (Fig. 2c). These data indicated that the chimaeric ChinWNV virus exhibits both genetic and plaque phenotypic stability during in vitro passaging.

**Attenuation of ChinWNV in mice**

To characterize further the virulence phenotypes of ChinWNV in mice, groups of mice were inoculated intraperitoneally (i.p.) with graded doses of ChinWNV or its parental viruses. Signs of illness or death were observed for at least 15 days. As shown in Table 1, no mice inoculated with ChinWNV at doses of up to $10^4$ p.f.u. exhibited encephalitis or died, and a $10^5$ p.f.u. dose of ChinWNV killed only one of seven mice, indicating that the LD$_{50}$ of ChinWNV should be higher than $10^5$ p.f.u. In contrast, the parental WNV LD$_{50}$ was calculated as 3.3 p.f.u. As expected, all JEV-infected mice survived during the 15-day observation period. Furthermore, virus replication in mouse brain was assessed by plaque assay following i.c. inoculation with each virus. The results showed that the replication of ChinWNV was significantly restricted in mouse brain in comparison with WNV at the indicated time points (Fig. 4c, d). Together, these data showed that the in vivo replication of ChinWNV was significantly restricted and that ChinWNV was significantly attenuated with regard to both its neuroinvasiveness and neurovirulence.

**Immunogenicity of ChinWNV in mice**

To evaluate the immunogenicity of ChinWNV in mice, groups of mice were immunized with the chimaera by the subcutaneous (s.c.) route; PBS served as the control. At 2 and 4 weeks post-immunization, the mouse sera were tested by IFA for IgG antibody titre and by a 50% plaque reduction neutralization test (PRNT$_{50}$) for neutralizing antibody titre. Mice inoculated with $10^4$ or $10^5$ p.f.u. of ChinWNV showed moderate levels of anti-WNV IgG...
Fig. 2. Recovery and in vitro characterization of ChinWNV. (a) Immunofluorescence staining of JEV, ChinWNV, WNV and mock-infected BHK-21 cells with specific anti-JEV-E, anti-WNV-E and JEV-NS1 antibodies. (b) Plaque morphology of JEV, ChinWNV and WNV in BHK-21 cells at 3 days post-inoculation (p.i.). (c) Plaque morphology of the chimaeric virus ChinWNV after serial passages in Vero cells. ChinWNV was passaged in Vero cells up to eight times. The plaque phenotypes of viruses at passages 4 (P4) and 8 (P8) were examined using BHK-21 cells at 3 days p.i. Mean plaque sizes (mean diameter ± SD, mm) were estimated by counting 40 representative plaques. (d) Growth curves of JEV, ChinWNV and WNV in mammalian and mosquito cell lines. Monolayers of BHK-21, Vero and C6/36 cells were infected with the indicated viruses at an m.o.i. of 0.01. At each time point, the medium was removed and the virus titres in the medium determined in BHK-21 cells by plaque assay.

Table 1. Neuroinvasiveness of JEV, ChinWNV and WNV in mice

Groups of 4-week-old BALB/c female mice were i.p. inoculated with the indicated doses of viruses. The mortality was monitored for 15 days after inoculation.

<table>
<thead>
<tr>
<th>Virus (strain)</th>
<th>Dose (p.f.u.)</th>
<th>Mortality (%) (no. dead/no. tested)</th>
<th>MST (day)*</th>
<th>LD$_{50}$ (p.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChinWNV</td>
<td>$10^5$</td>
<td>14.3 (1/7)</td>
<td>9</td>
<td>$&gt;10^5$</td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>0 (0/6)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$10^3$</td>
<td>0 (0/7)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$10^2$</td>
<td>0 (0/6)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>WNV (Chin-01)</td>
<td>$10^{1.1}$</td>
<td>100 (7/7)</td>
<td>8</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>$10^{1.4}$</td>
<td>85.7 (6/7)</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^{0.7}$</td>
<td>66.7 (4/6)</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^0$</td>
<td>0 (0/6)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>JEV (SA14-14-2)</td>
<td>$10^6$</td>
<td>0 (0/6)</td>
<td>NA</td>
<td>$&gt;10^6$</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>0 (0/7)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Mean survival time (MST) for mice that died; NA, not applicable.
antibodies at 14 days post-immunization, and the antibody levels rapidly increased 4 weeks post-immunization (Fig. 5a). The PRNT<sub>50</sub> showed that neutralizing antibodies against WNV were successfully induced on day 14 post-immunization, peaking at titres of approximately 1 : 80 and 1 : 40 in the 10<sup>4</sup> and 10<sup>5</sup> p.f.u. groups, respectively (Fig. 5b). No anti-WNV IgG or neutralizing antibodies were detected in the serum from any control mouse. These data demonstrated that a single immunization with ChinWNV stimulated a robust WNV-specific antibody response.

**Protective efficacy of ChinWNV in mice**

To determine the *in vivo* efficacy of ChinWNV protection against WNV infection, all mice immunized with ChinWNV were i.p. challenged with 100 LD<sub>50</sub> WNV at 28 days post-immunization. The survival curve showed that immunization with ChinWNV conferred 87.5 and 75 % protection against lethal WNV challenge in the 10<sup>4</sup>

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![Figure 3](image-url)  
**Fig. 3.** Viraemia profile of ChinWNV in mice. Four-week-old BALB/c female mice were inoculated i.p. with 100 p.f.u. of the indicated viruses. Sera were collected at 1, 2 and 3 days p.i. The virus titre was determined by plaque assay using BHK-21 cells.

![Figure 4](image-url)  
**Fig. 4.** Neurovirulence of ChinWNV in mice. (a, b) Survival data for BALB/c mice inoculated with JEV, ChinWNV and WNV. Groups of 4-week-old BALB/c mice (n=6–8) were i.c. inoculated with the indicated dose of each virus. The significance of the differences in mortality and mean survival time between the ChinWNV- and WNV-inoculated groups was determined using a log-rank test with GraphPad Prism 5.0. **P<0.01, ***P<0.001. (c, d) Replication of JEV, ChinWNV and WNV in mouse brain. Groups of 4-week-old BALB/c mice were i.c. inoculated with the indicated dose of each virus. The brain tissues of three mice in each group at the indicated time points were harvested, homogenized and titrated by plaque assay using BHK-21 cells. Dashed lines represent the limit of sensitivity of the plaque assay for each mouse brain. *P<0.05, **P<0.01, ***P<0.001.
and 10^5 p.f.u. groups, respectively, whereas all control mice developed symptoms of central nervous system infection and died 6–11 days after challenge, as expected (Fig. 5c). The viraemia profiles were also assayed within 3 days post-challenge. The control mice developed a high level of viraemia as early as day 1 post-challenge, and attained a peak titre of 10^4.5 p.f.u. ml\(^{-1}\) on day 2 post-challenge (Fig. 5d). Conversely, the ChinWNV-immunized mice developed only a transient, low-level viraemia on day 2 post-challenge. The peak titres of viraemia of the ChinWNV-immunized mice were approximately 100-fold lower than those of the control mice. These results showed that ChinWNV immunization is able to significantly reduce the peripheral replication of WNV in mice and indicated that a single immunization with ChinWNV conferred solid protection from WNV-induced viraemia and death in mice.

**DISCUSSION**

Here, we described a novel rationally designed WNV vaccine candidate based on the well-known live attenuated JEV vaccine strain SA14-14-2. We demonstrated that ChinWNV replicated well in cell culture, displayed genetic stability and exhibited an excellent safety profile with regard to a significant attenuation of neuroinvasiveness and neurovirulence. Most importantly, a single immunization of ChinWNV elicited a protective immune response against a lethal challenge of WNV in mice.

Chimaeric flavivirus technology has been used successfully to create attenuated chimaeric live viruses with vaccine potential, including JEV, DENV, TBEV and WNV, based on YF-17D or an attenuated DENV-4 (Arroyo et al., 2001; Chambers et al., 1999; Guirakhoo et al., 2000; Pletnev et al., 2001, 2003). The JEV vaccine strain SA14-14-2 has a remarkable record of safety and efficacy and an excellent genetic stability. These features indicate that the vaccine strain is an ideal alternative for delivering structural genes that encode protective antigens to produce live attenuated candidate vaccines. Recently, an infectious cDNA clone of JEV vaccine strain SA14-14-2 was obtained (Ye et al., 2012), enabling the generation of a chimaeric JEV–WNV chimera using the vaccine strain as the genetic backbone. Indeed, we succeeded in constructing and recovering viable ChinWNV by substituting the prM and E genes of JEV with the corresponding WNV genes. To ensure proper

![Fig. 5. Immunogenicity and protective efficacy of ChinWNV in mice.](http://vir.sgmjournals.org)
processing of the prM and E proteins of WNV, the signal sequence of JEV between C and prM and the last three C-terminal amino acids of the JE V E protein were retained in ChinWNV (Fig. 1). As expected, the WNV E protein from the chimaera appeared to possess the appropriate antigenic properties. This finding is consistent with previous observations that the signal sequence between the C and prM proteins influences viability (Lee et al., 2000; Pletnev et al., 2002). We found that ChinWNV exhibited a higher growth efficiency than the parental JE V in mammalian cells (BHK-21 and Vero cells) and produced significantly smaller plaques than its parental viruses in BHK-21 cells. Additionally, ChinWNV exhibited a high genetic stability and retained the small-plaque phenotype after passage 8 in Vero cells, suggesting that JE V strain SA14-14-2 is an excellent vector for maintaining the attenuated virulence phenotype of the chimaera.

The safety of ChinWNV, which is a principal issue for all live viral vaccines, was characterized by a lack of neuroinvasiveness and significantly reduced viraemia compared with the parental WNV in mice. Previous studies have shown that chimaerization of WNV with YF-17D or DENV-4 nearly eliminates the peripheral virulence of its WNV parent (Arroyo et al., 2004; Pletnev et al., 2002, 2003). The decreased efficiency of gene product interactions was thought to be the basis for the marked attenuation exhibited by these chimaeras. Additionally, the presence of attenuating mutations in the genetic background of the chimaeric virus also leads to attenuation (Chambers et al., 1999; Durbin et al., 2001; Huang et al., 2000). Previously, a novel attenuation mutation within the NS2A gene of SA14-14-2 was found to confer restricted replication of the vaccine virus in mouse brain (Ye et al., 2012). We infer that chimaerization and potential attenuation mutations in the genetic backbone confer the attenuation phenotype of ChinWNV in vivo. ChinWNV remarkably enhanced the survival rate of i.c.-route ChinWNV-infected mice, and its replication was greatly restricted in mouse brain compared with the same dose of i.c.-infected WNV (Fig. 3), whilst retaining a low level of neurovirulence. These findings are unsurprising, as chimaeric WNV viruses based on YF-17D or DENV-4 also retain a degree of neurovirulence in adult mice (Arroyo et al., 2004; Pletnev et al., 2003). Currently, we are constructing a series of mutant ChinWNV viruses containing amino acid substitutions within the neurovirulence determinants of the WNV prM–E proteins to evaluate the in vivo effects of these mutations on the virulence phenotype of ChinWNV.

Although highly attenuated, ChinWNV rapidly stimulated a moderate to high level of adaptive immune response to highly virulent WNV. A restriction of virus replication of WNV in ChinWNV-immunized mice was observed, as suggested by the significant reduction in viraemia. The IgG antibody response in mice immunized with ChinWNV showed a dose–response effect, whilst the neutralizing antibody response was lower at a higher dose of ChinWNV (Fig. 5b). This ‘prozone effect’ has also been observed in previous studies (Guirakhoo et al., 1999; Monath et al., 2003). The neutralizing antibody response in the ChinWNV-immunized mice correlated well with protection. In our study, a 75–87.5% protection against lethal WNV challenge was attained when a single dose of ChinWNV was administrated. In contrast, chimaeric WNV based on YF-17D and DENV-4 required a single dose as high as 104 and 106 plaque-forming units, respectively, to provide full protection against lethal WNV infection in mice (Arroyo et al., 2004; Pletnev et al., 2002). We speculate that this difference in protective efficacy could be attributed to the challenge virus. In our study, the i.p. LD50 of challenge WNV was 3.3 plaque-forming units, which was approximately three times more virulent than the WNV strain NY99 used in a previous study (Pletnev et al., 2002). Note that even a single immunization with 104 plaque-forming units, ChinWNV induced a high level of neutralizing antibodies (1:80) against parental WNV. In general, a neutralizing antibody titre over 1:10 is recognized as an indicator of protection.

In summary, using the JE V vaccine strain SA14-14-2 as the genetic backbone, we developed a novel live attenuated chimaeric virus, ChinWNV, possessing WNV prM–E genes. ChinWNV exhibited a high replication rate in Vero cells, in vitro genetic stability and an attenuation profile in mice. A single dose of ChinWNV rapidly elicited strong humoral immune responses in mice that provided solid protection from lethal WNV challenge. These excellent profiles of ChinWNV make it a promising live attenuated vaccine candidate against WNV for evaluation in monkeys.

METHODS

Viruses and cells. WNV strain Chin-01 (GenBank accession no. AY490240.2) and JE V vaccine strain SA14-14-2 (GenBank accession no. D90195) were prepared and titrated in BHK-21 cells, as described previously (Li et al., 2010; Ye et al., 2012). BHK-21 cells and Vero cells were maintained in Dulbecco’s minimal essential medium (Invitrogen) supplemented with 10% heat-inactivated FBS at 37°C. Aedes albopictus C6/36 cells were cultured in RPMI 1640 supplemented with non-essential amino acids (Invitrogen) and 10% FBS at 28°C.

Plasmid construction. All plasmids were constructed using standard molecular biology protocols and confirmed by DNA sequencing. The genetic construction of the full-length infectious clone of JE V has been described previously (Ye et al., 2012). The cloning sites were engineered to permit the replacement of the prM and E coding sequences of JE V with the corresponding sequences of WNV. The resulting plasmid contained the full-length cDNA of the JE V–WNV chimaera (pChinWNV).

Recovery and characterization of ChinWNV. The XhoI-linearized plasmid pChinWNV was subjected to in vitro transcription using a RiboMAX Large Scale RNA Production System (Promega) in the presence of the m7GpppA cap analogue (Promega). The RNA transcripts were transfected into BHK-21 cells using Lipofectamine 2000 (Invitrogen), and the rescued virus was then harvested at approximately day 5 post-transfection as the working virus stock.
An indirect IFA was performed, as described previously (Deng et al., 2011). Briefly, confluent BHK-21 cells in six-well plates (with coverslips in the wells) were infected with viruses at an m.o.i. of 0.01. At 48 h p.i., the cells were fixed with ice-cold acetone and incubated with primary antibodies (anti-JEV E protein, anti-WNV E protein and anti-JEV NS1 protein), followed by incubation with secondary FITC-conjugated antibody. Positive cells were detected using fluorescence microscopy. Growth curves were generated by infecting confluent BHK-21, Vero and C6/36 cells in a 12-well plate (m.o.i.=0.01). The cell supernatants were collected at successive 24 h intervals p.i. and the virus yield in each sample was then quantified using a plaque assay.

**Genetic stability assay.** ChinWNV was serially passaged in Vero cells (m.o.i.=0.01). Viral RNA was extracted from the supernatant of passages 4 and 8, and cDNA was synthesized and sequenced. The plaque phenotypes of the virus at passages 4 and 8 were also assessed by plaque assay.

**Neutralization assay.** Neutralizing antibody titres were determined using a standard PRNT50. Briefly, a 1:10 dilution of serum was heat inactivated for 30 min at 56 °C, and serial twofold dilutions of the inactivated serum were mixed with equal volumes of WNV suspension, followed by incubation at 37 °C for 1 h. The virus/antibody mixtures were then added to BHK-21 cells grown in 12-well plates. The concentration of infectious virus was determined by plaque assay.

**Virulence in mice.** All animal experiments in this study were performed in strict accordance with the guidelines of the Experimental Animal Ethics Committee of Beijing Institute of Microbiology and Epidemiology. Female 4-week-old BALB/c mice were housed in a conventional animal facility. All mice were maintained in a reverse-flow clean room, and were provided with sterile food and water ad libitum. Mice were randomly divided into two groups. One group was inoculated intracerebrally (i.c.) with Confluent BHK-21 cells infected with WNV (m.o.i. = 0.1) by the i.p. route. On days 1, 2 and 3 p.i., sera were collected to analyse virus titre and the virus yield was determined by plaque assay. All animal experiments in this study were performed in strict accordance with the guidelines of the Experimental Animal Ethics Committee of Beijing Institute of Microbiology and Epidemiology. All animal experiments in this study were performed in strict accordance with the guidelines of the Experimental Animal Ethics Committee of Beijing Institute of Microbiology and Epidemiology.

**Immunoactivation programme.** Immunogenicity was assessed by s.c. inoculation of ChinWNV or PBS in mice. The serum was collected 1 day prior to immunization and at 2 and 4 weeks post-immunization, to determine the titres of WNV IgG antibodies using IFA and WNV neutralizing antibodies with neutralization assays.

**Protection assay.** For the protection assay, all ChinWNV-immunized mice were i.p. challenged with 10^5.6 p.f.u. (approx. 100 LD50) of WNV at 4 weeks post-immunization. On days 1, 2 and 3 p.i., the sera were collected for the viraemia analysis. Signs of illness and death were observed daily for at least 15 days.

**Statistical analysis.** For the survival analysis, Kaplan–Meier survival curves were analysed using a log-rank test and standard GraphPad Prism software 5.0. Mean values were obtained from at least three independent experiments, and Student’s t-test was used to assess the significant differences (P<0.05).

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