Non-structural proteins of dengue 2 virus offer limited protection to interferon-deficient mice after dengue 2 virus challenge

Amanda E. Calvert, Claire Y.-H. Huang, Richard M. Kinney and John T. Roehrig

Correspondence
Amanda E. Calvert
zpz0@cdc.gov

Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention (CDC), Public Health Service, US Department of Health and Human Services, PO Box 2087, Fort Collins, CO 80522, USA

Received 10 June 2005
Accepted 6 November 2005

Chimeric (D2/WN) viruses containing the pre-membrane (prM) and envelope (E) proteins of West Nile virus (WN virus) and the capsid (C) and non-structural proteins of dengue 2 (DEN2) virus were used to evaluate the protective immunity elicited by either the flaviviral E protein or non-structural proteins. AG129 interferon-deficient mice, previously shown to be protected against lethal DEN1 or DEN2 viral infection after vaccination with a wild-type or candidate vaccine strain of DEN1 or DEN2 virus, respectively, were immunized with chimeric D2/WN virus and then challenged with DEN2 virus. D2/WN chimeric viruses were non-pathogenic in AG129 mice. These viruses elicited little anti-DEN E antibody, high levels of anti-DEN NS1 antibody and no or very low levels of DEN2 virus-neutralizing antibodies. Only 15% of D2/WN-immunized mice survived challenge with DEN2 virus. However, their mean survival time increased by 11–14 days over non-immunized controls. These results suggest that, whilst the non-structural proteins were able to enhance mean survival times of AG129 mice, this protection was not as effective as protection mediated by the E protein.

INTRODUCTION

Dengue (DEN) viruses are important global pathogens, causing millions of cases of dengue fever and thousands of cases of dengue haemorrhagic fever (DHF) each year (Gubler, 1998; Monath, 1994). Currently, no vaccine exists against any of the four serotypes of DEN virus (DEN1–4). The production of a DEN virus vaccine is difficult, because infection with one serotype does not elicit long-term cross-protective immunity against the other serotypes and may, in fact, increase the severity of disease, causing DHF or dengue shock syndrome (DSS). This phenomenon of antibody-dependent enhancement (ADE) is thought to be mediated by immune complexes of non-neutralizing antibody and virus that attach to Fc receptor-bearing cells and increase virus infection directly or elicit the production of a variety of chemokines that modify the disease process (Halstead & O’Rourke, 1977; Littaua et al., 1990). A successful DEN vaccine will need to be tetravalent and capable of eliciting virus-neutralizing antibodies against all four serotypes to minimize the effects of non-neutralizing, virus-enhancing antibodies that may result in DHF or DSS.

Whilst a variety of experimental vaccine candidates have been investigated, most of these are either live-attenuated viruses or subunit vaccines utilizing the pre-membrane (prM) and envelope (E) proteins to produce high levels of virus-neutralizing antibody. The E protein, the major virion surface protein, is responsible for virus attachment to animal cells and virus-mediated cell-membrane fusion (Roehrig, 2003). Utilizing the E protein as a subunit vaccine is logical. However, including the E protein in any vaccine candidate could lead to the production of virus-enhancing antibodies that result in ADE as virus-neutralizing antibody wanes. In this case, regular reimmunization may be necessary to maintain protective levels of virus-neutralizing antibody.

The non-structural (NS) proteins, particularly NS1, are immunogenic and can elicit high-titre antibody. NS1, a glycoprotein, is also expressed on the surface of virus-infected cells. NS1 is not incorporated into the virion and therefore does not elicit virus-enhancing antibodies (Roehrig, 2003). The mechanism by which NS1 elicits protection is not fully understood; however, antibodies to NS1 bind surface-expressed NS1 and induce complement-mediated cytosis or antibody-dependent cellular cytotoxicity (Jacobs et al., 1994; Schlesinger et al., 1993). Immunization with recombinant or purified NS1 has been shown to be protective against flavivirus challenge (Falgout et al., 1990; Hall et al., 1996; Jacobs et al., 1992; Lin et al., 1998; Qu et al., 1993).

Until recently, comparing the relative protective capacity of the E protein and the NS proteins following virus immunization has been difficult, largely because there has
been no system available for direct comparisons. Animals vaccinated with live-attenuated viruses elicit both anti-E and anti-NS protein antibodies, and protection is largely mediated by the anti-E protein virus-neutralizing antibodies. Protection of animals immunized individually with either purified E protein or NS1 protein is also difficult to compare, because neither antigen is administered in the context of replicating virus. Differences in protein immunogenicity might be overcome by increasing the dose of one or the other protein in the administered vaccine.

Chimeric flaviviruses are excellent tools to compare protective immunity elicited by virus structural or NS proteins during virus replication. A wide variety of heterotypic chimeric flaviviruses, containing a backbone from one flavivirus that has had its surface proteins replaced with the surface proteins from another flavivirus, have been shown to replicate and induce protective immunity in mice and/or non-human primates (Arroyo et al., 2001; Caufour et al., 1996; Chambers et al., 2003; Guirakhoo et al., 2000; Huang et al., 2003, 2005; Johnson & Roehrig, 1999; Pletnev et al., 2001, 2003; van der Most et al., 2000).

In earlier studies, we demonstrated that adult AG129 mice were susceptible to a peripheral infection with mouse-adapted DEN2 virus strain New Guinea C (NGC) (Johnson & Roehrig, 1999). These mice produced neutralizing antibody when immunized with either DEN2 wild-type 16681 virus or its vaccine strain PDK-53, both of which protected the animals against lethal DEN2 NGC virus challenge (Johnson & Roehrig, 1999). In similar studies, the alpha/beta interferon (IFN-α/β)-receptor pathway was found to play an essential role in inhibiting initial virus replication in extraneural sites and controlling subsequent viral spread to the central nervous system (CNS) (Shresta et al., 2004). However, this interferon pathway is not required for protection from disease (Johnson & Roehrig, 1999; Shresta et al., 2004).

Here, we have used infectious cDNA clones of DEN2 16681 virus to produce fully infectious chimeric viruses that have had the DEN2 prM and E proteins replaced by the prM and E proteins of West Nile virus (WN virus), strain NY99. By inoculating DEN2 NGC virus-susceptible AG129 mice with these chimeric viruses and challenging them with the NGC virus, we have been able to determine directly protective effects contributed by the DEN2 viral NS proteins following immunization with live virus in this interferon-deficient mouse model.

The DEN2 mouse-adapted NGC virus has been passaged numerous times in suckling mouse brain to increase its neurovirulence (a kind gift from Kenneth Eckels, Walter Reed Army Medical Center, Washington DC, USA). A stock of this virus was grown in C6/36 cells and titrated at $2.5 \times 10^{6}$ p.f.u. ml$^{-1}$.

Construction and characterization of the chimeric D2/WN viruses has been described previously (Huang et al., 2005). These chimeric viruses were derived from infectious cDNA clones pD2/WN-P1 and pD2/WN-E1-112. The D2/WN-P1 chimera contains the prM-E genes of WN NY99 virus in the wild-type DEN2 16681 genomic background, whilst the D2/WN-E1-112 chimera contains the WN NY99 prM-E genes in the genomic background of the candidate DEN2 PDK-53 vaccine virus. Genomic sequence analysis revealed the expected sequence for the D2/WN-P1 chimera. However, two mutations resulted in amino acid substitutions at NS2A-22 (Met to Val/Met mix) and NS2B-93 (Gln to Gln/Arg mix) in the D2/WN-E1-112 chimera (Huang et al., 2005).

**Mouse experiments.** AG129 mice deficient in IFN-α/β and γ responses (van den Broek et al., 1995) were bred in-house. The mice were handled as specified by institutional guidelines for care and use in accordance with the Institutional Animal Care and Use Committee recommendations. Both of the D2/WN chimeras, WN NY99 virus and DEN2 NGC virus were evaluated for neuroinvasiveness in 5–8-week-old AG129 mice. Seven groups of mice (n = 5) were inoculated intraperitoneally (i.p.) with 10-fold dilutions of each virus (0–10,000 p.f.u.). Animals were monitored for 4 weeks after inoculation and 50% intraperitoneal lethal-dose end points (i.p., LD$_{50}$) were calculated (Reed & Muench, 1938).

For immunogenicity studies, 3–5-week-old AG129 mice (n = 10) were inoculated i.p. with 1000 p.f.u. in 0.1 ml D2/WN-E1-112 or D2/WN-P1 chimeric virus. Mice were boosted 2 weeks later with an equivalent dose. Naïve mice were inoculated in the same manner with medium. Mice were bled 2 weeks after each vaccination. Challenge was performed by i.p. inoculation with 100 p.f.u. WN NY99 virus or 1000 p.f.u. DEN2 NGC virus, diluted in medium, at 14 days after the booster immunization. Challenged mice that showed signs of morbidity were euthanized. Sera were collected from surviving mice for antibody analysis.

**Production of virus cell lysate.** C6/36 cell monolayers were grown in 150 cm$^2$ culture flasks. Confluent monolayers were inoculated with either WN NY99 virus or DEN2 16681 virus at an m.o.i. of 0–1. WN and DEN2 virus-infected cells were harvested on day 4 or 7 post-infection, respectively. Cell lysate was produced as described previously (Bliotich et al., 2003). Control antigen was prepared similarly with uninfected C6/36 cells.

**Virus purification.** WN NY99 and DEN2 16681 viruses were grown in C6/36 cells and purified on glycerol tartrate gradients as described by Obijeski et al. (1976). These purified stocks were used in subsequent serological assays.

**Standardization of viral proteins for use in serological assays.** DEN2 and WN E proteins were quantified for ELISA by end-point titration in an antigen-capture ELISA to standardize the amounts of the E protein present in virus-infected cell lysate and purified virus. WN virus antigen was captured with monoclonal antibody (mAb) 3.91D and detected with mAb 6B6C-1 and goat anti-mouse Ab conjugated to alkaline phosphatase. As indicated by the ELISA end-point titrations of WN antigens, purified WN virus was used at a concentration of 0.07 μg per well and WN viral lysate was diluted to 1:400 in the experimental ELISA. DEN2 virus antigen was captured with mAb 4G2 and detected with mAb 3H5 and goat anti-mouse Ab conjugated to alkaline phosphatase. In the experimental assays, DEN2 purified virus was used at a concentration of 0.03 μg per well and DEN2 viral lysate was diluted to 1:100.
DEN2 antigen was quantified for Western blotting by running dilutions of lysate on a 4–12 % Bis/Tris SDS-PAGE gel and staining with anti-E protein mAb 1A6A-8 to DEN2 Jamaica strain 1409. E protein bands from lysate were detected with a Bio-Rad densitometer and compared in order to quantify relative amounts of E protein. DEN2 viral lysate antigen was used at a dilution of 1:10.

ELISA. The inner 60 wells of a 96-well microtitre plate (Immulon 2HB; Thermo Lab Systems) were coated with viral lysate or purified virus at an appropriate dilution in carbonate/bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4 °C. Plates were washed five times with PBS/0.1 % Tween wash buffer with an automatic plate washer. Non-specific binding sites were blocked with 3 % goat serum in PBS (100 μl per well) for 1 h at 37 °C. Blocking solution was discarded and sera or mAb were added in twofold serial dilutions (50 μl per well) and incubated at 37 °C for 1 h. Plates were washed five times before the addition of goat anti-mouse antibody conjugated to alkaline phosphatase (50 μl per well), diluted 1:100 in PBS. After an incubation period of 1 h at 37 °C, plates were washed again five times. Alkaline phosphatase substrate (Sigma) was added to each well of the plate (100 μl per well) and incubated in the dark at room temperature for 30 min. OD405 was read on an automatic plate reader. Test samples for each dilution of sera were analysed in duplicate. End points were determined as an OD405 reading of at least twice that of the mean OD405 from normal sera reacting to antigen. Titres were expressed as the geometric mean of the reciprocal of the end points.

Plaque-reduction neutralization test (PRNT). The PRNT was performed in six-well plates of Vero cells as described previously (Huang et al., 2005). The mouse sera were heat-inactivated (56 °C for 30 min) and the tests were performed without addition of exogenous complement. End-point titres were expressed as the geometric mean of the serum dilution yielding at least 80 % reduction in the number of plaques (PRNT80).

Immunoblotting. DEN2 16681 viral proteins from the lysate of virus-infected C6/36 cells were separated by SDS-PAGE on 4–12 % Bis/Tris gels (Invitrogen). Proteins were blotted electrophoretically from the gels onto nitrocellulose membranes and washed for 15 min in PBS/0.1 % Tween wash buffer. Non-specific binding sites were blocked with 3 % goat serum in PBS overnight at 4 °C. Sera taken from mice vaccinated with the D2/WN-E1-112 or D2/WN-P1 chimeric virus were pooled and diluted 1:50 in PBS. Anti-DEN2 E protein mAb 1A6A-8, anti-DEN2 NS1 mAb 9A9 (generously provided by Jacob Schlesinger, University of Rochester Medical Center, NY, USA) and anti-DEN2 NGC mouse hyperimmune ascitic fluid were also tested. Diluted sera were incubated with the membrane for 1 h with gentle rocking. Membranes were washed again in PBS/0.1 % Tween wash buffer three times for 5 min each. goat anti-mouse antibody conjugated to alkaline phosphatase was diluted 1:200 and incubated on the membrane for 1 h with gentle rocking. Membranes were washed as described previously and BCIP/NBT phosphatase substrate (KPL) was added to the membrane until a colour change appeared. The reaction was stopped by the addition of water.

RESULTS

Infection of AG129 mice with D2/WN chimeras

As expected, wild-type WN NY99 virus was highly virulent in AG129 mice, with an i.p. LD50 of 0.15 p.f.u. (Fig. 1). As shown previously (Johnson & Roehrig, 1999), neuro-adapted DEN2 NGC virus was also highly virulent, with an i.p. LD50 of 2.1 p.f.u. Mice receiving the D2/WN-P1 or D2/WN-E1-112 chimeric virus (0.01–105 p.f.u.) did not exhibit morbidity or mortality (Fig. 1). Thus, the D2/WN chimeras were highly attenuated in AG129 mice. Mice were bled at 4 weeks after viral inoculation to assess the antibody response to the D2/WN chimeras. Mice inoculated with more than 10 p.f.u. D2/WN chimeric virus produced measurable amounts of ELISA antibody to WN and DEN2 virus, low neutralizing antibody to WN virus and no detectable neutralizing antibody to DEN2 virus (data not shown).

Immunogenicity of D2/WN chimeras in AG129 mice

Antibody responses in AG129 mice immunized i.p. with 1000 p.f.u. of each D2/WN chimeric virus were evaluated by ELISA, PRNT and immunoblot (Table 1; Fig. 2). Three sets of sera were pooled for these analyses: (i) sera obtained 2 weeks after the primary immunization, (ii) sera obtained 2 weeks after the secondary immunization and (iii) sera obtained 47 days after challenge of boosted mice with DEN2 NGC virus.

The D2/WN chimeras produced antibody responses to WN structural and DEN2 non-structural viral proteins. Anti-DEN2 ELISA titres were minimal (<1.7 log10) against gradient-purified DEN2 16681 virus, but were significant (2.03–2.18 log10) against the DEN2 16681 virus-infected C6/36 cell lysate, following primary or secondary immunization of AG129 mice with each of the D2/WN chimeras (Table 1). Correspondingly, pooled D2/WN-immune sera reacted strongly with DEN2 NS1, but minimally with DEN2 E protein, by Western blotting (Fig. 2, lanes 1–2) and

Fig. 1. Virulence of D2/WN chimeras and wild-type viruses in AG129 mice. Mice (n = 8) were inoculated i.p. with 10-fold serial dilutions of WN NY99, DEN2 NGC, D2/WN-E1-112 and D2/WN-P1 viruses as described in Methods. ▲, D2/WN-P1;◆, D2/WN-E1-112; △, WN NY99; ■, DEN2 NGC.
these sera showed minimal or no neutralizing activity against DEN2 virus (log_10 titres of \(\leq 1.0\) in Table 1) following primary or secondary immunization. The identity of the DEN2 NS1 band in the Western blot was confirmed by detection with anti-DEN2 NS1-specific mAb 9A9 (Fig. 2, lane 4). A band of about 92 kDa appeared to be a non-viral protein in the C6/36 lysate, because sera reacted with this protein in the uninfected C6/36 lysate control (Fig. 2, control lane 7). Although this band was approximately the expected size of dimeric NS1, the band was not detected by DEN2 NS1-specific mAb 9A9 (Fig. 2, lane 4). The broader E protein band in Fig. 2, lane 5, might be explained by a probably higher concentration of DEN2 E-specific antibody in mAb 1A6A-8, relative to sera obtained from D2/WN-immunized AG129 mice (Fig. 2, lanes 3 and 6).

In contrast to the non-structural protein specificity of anti-DEN2 antibodies, D2/WN-immune AG129 sera recognized the WN E protein. After the primary immunization, D2/WN-P1- and D2/WN-E1-112-immune mouse sera reacted to somewhat higher titres against gradient-purified WN virus (log_10 titres of 3.72 and 3.55, respectively) than against WN-infected C6/36 cell lysate (log_10 titres of 3.05 and 2.99, respectively) by ELISA. Following secondary immunization, ELISA antibody titres versus gradient-purified WN virus (D2/WN-P1, 3.54 log_10; D2/WN-E1-112, 3.48 log_10) and WN viral lysate (D2/WN-P1, 3.51 log_10; D2/WN-E1-112, 3.44 log_10) were equivalent. The WN E protein would be expected to be present in both purified WN virus and lysates of WN virus-infected C6/36 cells. The D2/WN chimeric

![Fig. 2. Reactivity of mouse sera, pooled from both of the experimental groups immunized with either D2/WN-P1 or D2/WN-E1-112 virus, with DEN2 16681 viral proteins. A Western blot of DEN2 virus-infected (lanes 1–6) and uninfected (lane 7) C6/36 cell lysates was prepared as described in Methods. Lane 1, mouse sera after the primary immunization; lane 2, mouse sera after the secondary immunization; lane 3, mouse sera after challenge with DEN2 NGC virus; lane 4, anti-DEN2 NS1 mAb 9A9; lane 5, anti-DEN2 E protein mAb 1A6A-8; lane 6, anti-DEN2 NGC mouse hyperimmune ascitic fluid; lane 7, mouse sera after challenge with DEN2 NGC virus, uninfected C6/36 lysate; lane 8, Bio-Rad low-molecular-mass marker (sizes are shown in kDa).](image_url)

### Table 1. DEN2 and WN virus-specific antibody responses in AG129 mice following primary and secondary immunizations with D2/WN chimeras and after challenge with DEN2 virus

<table>
<thead>
<tr>
<th>Vaccine*</th>
<th>Geometric mean reciprocal ELISA titre (log_{10})</th>
<th>Geometric mean reciprocal PRNT_{80} (log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>WN purified virus</td>
<td>DEN2 purified virus</td>
<td>WN virus lysate</td>
</tr>
<tr>
<td>DEN2 virus</td>
<td>DEN2 virus lysate</td>
<td>WN virus</td>
</tr>
<tr>
<td>1+</td>
<td>2-</td>
<td>p.c.</td>
</tr>
<tr>
<td>D2/WN-P1</td>
<td>3.72</td>
<td>3.54</td>
</tr>
<tr>
<td>D2/WN-E1-112</td>
<td>3.35</td>
<td>3.48</td>
</tr>
<tr>
<td>BA-1</td>
<td>0.69</td>
<td>0.70</td>
</tr>
</tbody>
</table>

*AG129 mice were immunized i.p. with 1000 p.f.u. D2/WN chimeras or with BA-1 diluent.

1+ Sera obtained 2 weeks after the primary immunization.
2+ Sera obtained 2 weeks after the secondary immunization.
3+ Sera taken from mice surviving challenge with DEN2 NGC virus.
4+ Sera from three of 10 mice that survived challenge with DEN2 NGC virus.
      §Sera obtained 2 weeks after the primary immunization.
      §| NT, Not tested. No mice survived challenge with DEN2 NGC virus.

In contrast to the non-structural protein specificity of anti-DEN2 antibodies, D2/WN-immune AG129 sera recognized the WN E protein. After the primary immunization, D2/WN-P1- and D2/WN-E1-112-immune mouse sera reacted to somewhat higher titres against gradient-purified WN virus (log_{10} titres of 3.72 and 3.35, respectively) than against WN-infected C6/36 cell lysate (log_{10} titres of 3.05 and 2.99, respectively) by ELISA. Following secondary immunization, ELISA antibody titres versus gradient-purified WN virus (D2/WN-P1, 3.54 log_{10}; D2/WN-E1-112, 3.48 log_{10}) and WN viral lysate (D2/WN-P1, 3.51 log_{10}; D2/WN-E1-112, 3.44 log_{10}) were equivalent. The WN E protein would be expected to be present in both purified WN virus and lysates of WN virus-infected C6/36 cells. The D2/WN chimeric
viruses elicited significant levels of neutralizing antibody against WN virus after the primary (D2/WN-P1, log_{10} titre 2-32; D2/WN-E1-112, log_{10} titre 2-04) and the secondary (D2/WN-P1, log_{10} titre 2-41; D2/WN-E1-112, log_{10} titre 2-04) immunizations. These data demonstrated that the D2/WN chimeras elicited WN E protein-reactive neutralizing antibodies in AG129 mice. Pool D2/WN-immune AG129 mouse sera also reacted with the WN E protein by Western blot analysis (data not shown).

These results indicated that AG129 mice immunized with the D2/WN chimeric viruses developed antiviral antibodies that were specific for the E and prM proteins of WN virus and the non-structural proteins (primarily NS1, as indicated by Western blot analysis) of the DEN2 16681 chimeric vector.

**Protective efficacy of D2/WN chimeric viruses**

Two weeks after the secondary vaccination, immunized and control (BA-1) mice were challenged i.p. with WN NY99 or DEN2 NGC virus. No D2/WN-immunized mice (0/20) survived WN virus challenge and only 15% (3/20), all in the D2/WN-E1-112 group, survived challenge with DEN2 virus (Table 2). However, mean survival times (MSTs) of the D2/WN chimera-vaccinated mice challenged with WN virus or DEN2 virus were all significantly longer than those of the BA-1 control group (P<0.011). Relative to the non-vaccinated control mice, immunization with the D2/WN-E1-112 or D2/WN-P1 chimera increased mouse MST by 2 or 4 days following WN viral challenge, or by 14 or 11 days following DEN2 NGC viral challenge, respectively (Table 2). High ELISA antibody titres to WN (≥4-03) and DEN2 (2-99 or ≥3-50 for purified virus or virus-infected cell lysate, respectively) viral antigen were detected in the sera from the three D2/WN-E1-112 mice that survived DEN2 NGC virus challenge (Table 1). High levels of neutralizing antibodies to WN virus (≥2-51) and moderate levels of neutralizing antibodies to DEN2 virus (1-68) were produced after DEN2 virus challenge in these three mice (Table 1). Western blot analysis revealed antibody responses to the E protein as well as the NS1 protein of DEN2 virus following DEN2 virus challenge (Fig. 2).

**DISCUSSION**

Previously, we have shown that, unlike normal mice, AG129 mice, deficient in IFN-α, -β and -γ responses, are susceptible to a peripheral challenge with DEN virus. These mice serve as a reliable model for DEN virus vaccination-challenge studies (Johnson & Roehrig, 1999). AG129 mice demonstrated 100% mortality when challenged with the neuro-adapted NGC strain of DEN2 virus or the Mochizuki strain of DEN1 virus (Johnson & Roehrig, 1999; Huang et al., 2003). By utilizing this small-animal model, we showed previously that vaccination with wild-type DEN2 16681, its PDK-53 vaccine derivative or chimeric DEN2/DEN1 virus elicited high levels of virus-neutralizing antibody and protected all animals from subsequent i.p. challenge with lethal DEN2 NGC or DEN1 Mochizuki virus (Johnson & Roehrig, 1999; Huang et al., 2003). Here, we have used D2/WN chimeric viruses to evaluate their ability to (i) replicate in AG129 interferon-deficient mice, (ii) produce antiviral and neutralizing-antibody responses to DEN and WN viruses and (iii) induce a protective antibody response against peripheral challenge with DEN2 NGC virus. Both the wild-type DEN2 16681 virus-vectored D2/WN-P1 chimera and the attenuated DEN2 PDK-53 virus-vectored D2/WN-E1-112 chimera were non-pathogenic in AG129 mice and elicited high anti-WN virus antibody titres. Not surprisingly, the D2/WN chimeric vaccines elicited little anti-DEN E protein antibody, high levels of anti-DEN NS1 antibody and no or very low levels of DEN2 virus-neutralizing antibodies. Whilst the MST of mice immunized with D2/WN-P1 or D2/WN-E1-112 chimera increased by 11 and 14 days, respectively, over non-vaccinated controls, only 15% (three of 20, all three in the D2/WN-E1-112 group) of the D2/WN-immunized mice survived DEN2 NGC virus challenge after two vaccinations. These results indicated that, when tested in replicating chimeric viruses containing the heterologous WN prM-E genes, the DEN2 non-structural proteins were able to significantly increase the MSTs of DEN2 NGC virus-challenged AG129 mice. However, this protection was not as effective as the complete protection afforded by the DEN2 virus-specific E protein following immunization of AG129 mice with DEN2 16681 or PDK-53 virus (Johnson & Roehrig, 1999).

Recently, we showed that the D2/WN chimeras based on the DEN2 PDK-53 vaccine retain the attenuated phenotypic characteristics of the candidate PDK-53 vaccine virus, and induce in Swiss Webster mice a protective immune response against WN NY99 virus challenge (Huang et al., 2005). We

### Table 2. Mean survival times of AG129 mice vaccinated with D2/WN chimeras and challenged with WN or DEN2 virus

<table>
<thead>
<tr>
<th>Group*</th>
<th>Challenge virus</th>
<th>Survivors/total</th>
<th>MST</th>
<th>Survivors/total</th>
<th>MST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WN virus†</td>
<td>D2/WN virus‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2/WN-E1-112</td>
<td>0/10</td>
<td>8 (1-3)§</td>
<td>3/10</td>
<td>32 (5-1)</td>
<td></td>
</tr>
<tr>
<td>D2/WN-P1</td>
<td>0/10</td>
<td>10 (4-1)</td>
<td>0/10</td>
<td>29 (11-6)</td>
<td></td>
</tr>
<tr>
<td>BA-1</td>
<td>0/10</td>
<td>6 (0-4)</td>
<td>0/10</td>
<td>18 (2-8)</td>
<td></td>
</tr>
</tbody>
</table>

*AG129 mice immunized i.p. with 1000 p.f.u. D2/WN chimera or BA-1 diluent. WN prM-E gene was expressed in the DEN2 16681 (P1) or PDK-53 (E1-112) background.
†Immunized mice were challenged i.p. with 100 p.f.u. WN NY99 virus.
‡Immunized mice were challenged i.p. with 1000 p.f.u. DEN2 NGC virus.
§Mean survival time (SD in parentheses) in days.
have also reported previously that chimeric DEN2/DEN1 viruses containing prM-E of the DEN1 virus in the DEN2 genomic background were able to protect AG129 mice against lethal DEN1 Mochizuki virus challenge (Huang et al., 2003). In the present study, the attenuated D2/WN viruses induced ELISA antibodies and neutralizing antibodies against WN virus. However, this response was not sufficient to protect the immunized AG129 mice against peripheral challenge with the neurotropic WN virus. This may be due to the major role that IFN-α/β plays in controlling virus replication. Whilst AG129 mice have normal-functioning immune systems, it is evident that they are unable to clear overloading or long-term viral infections. G129 mice lacking the IFN-γ receptor were able to clear a lymphocytic choriomeningitis virus (LCMV) infection, whereas A129 mice lacking the IFN-α/β receptor were unable to do so even after surviving viral infection for 20 days (van den Broek et al., 1995). Similarly, IFN-α/β receptor-deficient mice infected with Semliki Forest virus (SFV) died within 4–10 days, whilst wild-type and IFN-γ receptor-deficient mice survived SFV infection (Muller et al., 1994).

IFN-α/β also plays a major role in regulating the cytotoxic T-cell response. This may be an important component for clearance of overwhelming virus load and exhaustion of cytotoxic T-lymphocyte (CTL) precursors (van den Broek et al., 1995). This has been shown to occur in AG129 mice with slow-replicating strains of LCMV. The CTLs and CTL precursors were depleted quickly in AG129 mice when infected with the LCMV strain ARM. This led to lifelong viral persistence in the mice because of overwhelming virus replication and exhaustion of the CTLs, resulting in a lack of virus-specific CTL precursors (van den Broek et al., 1995). This indicates that the IFN-α/β receptor is essential for antiviral clearance, especially in highly virulent encephalitic and meningitic viruses such as SFV and LCMV, and may be the reason that AG129 mice succumbed to WN virus challenge in our study. From this study and previous reports, WN NY99 virus replicates more robustly in AG129 mice, resulting in significantly lower i.p. LD₅₀ values, than either DEN2 NGC or DEN1 Mochizuki virus (Johnson & Roehrig, 1999; Huang et al., 2003).

CTLs are primarily generated against the non-structural protein 3 (NS3) of flaviviruses (Lobigs et al., 1994). Spaulding et al. (1999) demonstrated that a primary dengue infection induces flavivirus cross-reactive NS3-specific CTLs. These cross-reactive CTLs may play a protective role in limiting virus replication, but they may also contribute to an increased risk of DHF during secondary infections. Children suffering from DHF were found to have higher levels of soluble CD8⁺ in their serum than children with dengue fever (Kurane et al., 1991). Although CTL responses are important in protective immunity, antibody-mediated protection, especially neutralizing antibody, plays a key role in vaccine-induced immunity and may be a more relevant focus than the CTL response. The CTL response of these mice to the D2/WN chimeras is of interest and, whilst it is beyond the scope of this study, it should be considered for future investigations.

In earlier studies, we showed that AG129 mice produce neutralizing antibody to DEN2 virus and are protected completely against DEN2 NGC peripheral virus challenge when immunized with the candidate DEN2 PDK-53 vaccine strain (Johnson & Roehrig, 1999). Shresta et al. (2004) showed that the IFN-α/β receptor-mediated activity limited the initial DEN2 replication in extraneural sites and controlled viral spread into the CNS. They also showed that the IFN-γ pathway later acted to restrict virus replication in the periphery and eliminate DEN virus from the CNS (Shresta et al., 2004). We hypothesize that AG129 mice in this study may not have been able to clear a DEN2 virus infection after vaccination, due to a lack of neutralizing antibody to DEN2 virus and the absence of a functioning IFN response to clear DEN2 virus from extraneural sites and the CNS.

NS1, a major immunogen in flavivirus infection, is an attractive choice for vaccination studies because it avoids the risk of incorporating anti-virion antibodies that might lead to ADE. NS1 is secreted from and expressed on the surface of infected cells and induces complement-mediated cytolysis for clearance of the virus infection (Schlesinger et al., 1993). NS1 has been expressed in a variety of ways to induce protective immunity in mice. Studies expressing NS1 along with the structural proteins in recombinant viruses, such as Vaccinia virus and baculoviruses, have shown that NS1 does not produce a high-titre antibody response to DEN virus (Bray et al., 1989; Zhang et al., 1988; Zhao et al., 1987). However, immunization with recombinant or purified NS1 has been shown to provide protection against flavivirus challenge (Fal-gout et al., 1990; Hall et al., 1996; Jacobs et al., 1992; Qu et al., 1993). Other studies have shown that the amino acid sequence context of flaviviral NS1 is vital to its correct expression and secretion. DNA constructs expressing NS1 and portions of NS2A do not secrete NS1 as well as constructs expressing NS1 alone, and do not induce protective immunity in mice (Konishi et al., 1991; Lin et al., 1998; Pugachev et al., 1995).

In this study, the DEN2 virus-specific non-structural proteins, including NS1, were expressed in a replicating chimeric D2/WN virus system to determine the degree of protection against DEN2 NGC virus challenge conferred by the non-structural proteins of DEN2 virus alone. The results showed that, whilst some increase in the MSTs was afforded to D2/WN-immunized, DEN2 virus-challenged AG129 mice, the DEN2 non-structural proteins alone were not adequate to induce protective immunity in these mice. Even though a few mice survived DEN-2 virus challenge and MSTs increased without pre-existing neutralizing antibody to DEN-2 virus, the anti-NS1 antibody response mounted against DEN-2 virus was not sufficient to protect mice from lethal challenge. These results are not consistent with other studies that have found anti-NS1 antibodies to be sufficient to protect mice from lethal virus challenge following direct immunization with NS1. However, given that the previous
studies were performed in mice with a functioning interferon response, such comparisons might not be valid. Further investigation into the immunogenic characteristics of NS1 should be conducted before recommending its use as the sole component of a vaccine against DEN viral infection.

REFERENCES


zation of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. *J Virol* 64, 4356–4363.


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