An inactivated Vero cell-grown Japanese encephalitis vaccine formulated with Advax, a novel inulin-based adjuvant, induces protective neutralizing antibody against homologous and heterologous flaviviruses

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Advax is a polysaccharide-based adjuvant that potently stimulates vaccine immunogenicity without the increased reactogenicity seen with other adjuvants. This study investigated the immunogenicity of a novel Advax-adjuvanted Vero cell culture candidate vaccine against Japanese encephalitis virus (JEV) in mice and horses. The results showed that, in mice, a two-immunization, low-dose (50 ng JEV antigen) regimen with adjuvanted vaccine produced solid neutralizing immunity comparable to that elicited with live ChimeriVax-JE immunization and superior to that elicited with tenfold higher doses of a traditional non-adjuvanted JEV vaccine (JE-VAX; Biken Institute) or a newly approved alum-adjuvanted vaccine (Jespect; Novartis). Mice vaccinated with the Advax-adjuvanted, but not the unadjuvanted vaccine, were protected against live JEV challenge. Equine immunizations against JEV with Advax-formulated vaccine similarly showed enhanced vaccine immunogenicity, confirming that the adjuvant effects of Advax are not restricted to rodent models. Advax-adjuvanted JEV vaccine elicited a balanced T-helper 1 (Th1)/Th2 immune response against JEV with protective levels of cross-neutralizing antibody against other viruses belonging to the JEV serocomplex, including Murray Valley encephalitis virus (MVEV). The adjuvanted JEV vaccine was well tolerated with minimal reactogenicity and no systemic toxicity in immunized animals. The cessation of manufacture of traditional mouse brain-derived unadjuvanted JEV vaccine in Japan has resulted in a JEV vaccine shortage internationally. There is also an ongoing lack of human vaccines against other JEV serocomplex flaviviruses, such as MVEV, making this adjuvanted, cell culture-grown JEV vaccine a promising candidate to address both needs with one vaccine.

INTRODUCTION

Japanese encephalitis (JE) is an acute central nervous system inflammatory disease caused by infection with the flavivirus Japanese encephalitis virus (JEV; reviewed by Solomon, 2004). It is the leading cause of viral encephalitis in South-East Asia, India and China. The geographical distribution of JEV is expanding, most recently in south-west India, the eastern Indonesian archipelago, New Guinea and the Torres Strait of Northern Australia (reviewed by Mackenzie et al., 2004). Annually, ~35 000 human cases of JE are reported, resulting in about 10 000 deaths and a high incidence of neuropsychiatric deficits among survivors. Children as well as non-immune adults are predominantly at risk of contracting the disease in endemic areas. JEV is an arbovirus and is transmitted by Culex mosquitoes to its vertebrate hosts (wild and domestic birds, and pigs); human infections with JEV do...
not result in a sufficiently high viraemia for maintenance of the transmission cycle. JE is also a veterinary disease with occasional fatal outcome in horses, and abortions and abnormal births in pigs.

Vaccination is the most effective means of preventing JEV infection and should be practised routinely in regions where the virus is responsible for human and veterinary disease (reviewed by Halstead & Tsai, 2004; Monath, 2002a). In the 1960s, an inactivated JE vaccine (JE-VAX; Biken Institute) produced from infected mouse brain was developed in Japan and licensed for use internationally. A three-dose vaccination regimen with this vaccine resulted in time-limited immunity in about 90% of immunized individuals (Hoke et al., 1988; Monath, 2002a). Whilst intermittent natural exposure to JEV in endemic areas probably gives rise to boosting of waning vaccine-induced immunity (Konishi & Suzuki, 2002), vaccine protection of individuals living in non-endemic regions requires regular vaccine boosters. In 2005, the Japanese government ceased its recommendation of the routine use of the mouse brain-grown JE vaccine, due to perceived safety problems and excess reactogenicity (Fischer et al., 2007). The motivation for this change in policy was anticipation that JE-VAX would be replaced by cell culture-grown inactivated JE (ccJE) vaccines under development by manufacturers in Japan, the USA, Europe and India. Several Vero cell-grown vaccines have undergone phase 2 and phase 3 trials to compare their safety and efficacy in humans with licensed mouse brain-grown vaccine (reviewed by Beasley et al., 2008). Non-inferiority to JE-VAX has been the primary end point for efficacy testing of new JE vaccine candidates (Beasley et al., 2008; Ferguson et al., 2007). Other JE vaccines that are in use or undergoing clinical trials are a live, attenuated vaccine (SA14-14-2 strain) used in China (reviewed by Halstead & Tsai, 2004; Monath, 2002a) and an as yet unapproved live, recombinant vaccine (ChimeriVax-JE) engineered by replacement of the immunogenic virion surface proteins, prM and E, in a yellow fever virus vaccine with those of JEV (Monath et al., 2003). Use of SA14-14-2 live JE vaccine in countries outside China is restricted by the uncertainty of quality control for adventitious agents in the uncharacterized cell line used for vaccine production.

The geographical expansion of JEV has resulted in endemic transmission of the virus in regions where antigenically closely related and medically important flaviviruses co-exist, i.e. West Nile virus (WNV) in India, and Murray Valley encephalitis virus (MVEV) and WNV in the Australasian region. In animal models, live infection with one of these viruses produces long-lived, cross-protective immunity against the others (Goverdhan et al., 1992; Hammon & Sather, 1956; Monath, 2002b; Tsai et al., 1998; Williams et al., 2001). However, in laboratory investigations, vaccination against JEV and related encephalitic flaviviruses has been associated with either cross-protective immunity (Chu et al., 2007; Lobigs et al., 2003, 2009; Martina et al., 2008; Takasaki et al., 2003) or disease-enhancing immunity (Broom et al., 2000; Lobigs et al., 2003, 2009; Wallace et al., 2003) against other viruses belonging to the JEV serocomplex. The latter is thought to be a consequence of the antibody-dependent enhancement phenomenon first described in studies with MVEV and WNV in vitro (Hawkes, 1964), and subsequently postulated to be the basis for the more severe disease associated with secondary dengue virus infections (reviewed by Halstead, 2003). The laboratory finding of instances of vaccine-induced enhancement of heterologous flavivirus infection is of some concern in relation to JE vaccine safety in humans, especially in regions where closely related flaviviruses are endemic. Vaccine efficacy in terms of magnitude and/or quality of the humoral immune response may be key to preventing infection enhancement (Lobigs et al., 2003, 2009).

The immunogenicity of inactivated vaccines can be significantly augmented by formulation with an appropriate adjuvant. A Vero cell-grown JE vaccine, IC51 (Jespect; Novartis) (Tauber et al., 2007), recently licensed in the USA and Australia, and other candidate ccJE vaccines (Appaiahgari & Vrati, 2004; Srivastava et al., 2001; Tauber et al., 2007) are formulated with aluminium hydroxide (alum), whilst other candidate vaccines have been left unadjuvanted (Kuzuhara et al., 2003; Sugawara et al., 2002; Toriniwa & Komiya, 2008). Alum adjuvants, whilst approved for human use, suffer from a number of disadvantages: the benefits of modest enhancement of antibody titres are often offset by increased injection-site pain and the potential for long-term granuloma formation and induction of eosinophilia (reviewed by Gupta, 1998). The ideal adjuvant for JE vaccines should enhance neutralizing antibody titres, have significant antigen-sparing potential and be well-tolerated and safe. Immunologically active polysaccharide particles such as delta inulin, on which the Advax family of adjuvants is based, have emerged as strong candidates for use as human vaccine adjuvants, combining potent adjuvant efficacy with good tolerability and safety (Petrovsky, 2006, 2008; Silva et al., 2004). Delta inulin adjuvants have been found to enhance neutralizing antibody and cellular immune responses and to provide antigen sparing when combined with a range of vaccine antigens including inactivated RNA viruses, e.g. seasonal or pandemic influenza vaccine (unpublished results). The purpose of this study was to examine the ability of Advax adjuvant to enhance the potency and provide dose sparing for a new ccJE vaccine under development in Japan (Toriniwa & Komiya, 2008). Given that natural infection with JEV is known to protect against related flaviviruses, our second objective was to assess the potential for an Advax-adjuvanted ccJE vaccine to provide JEV serocomplex cross-protective immunity.

**RESULTS**

**A delta inulin-based adjuvant potentiates virus-specific humoral immunity elicited with a Vero cell-grown inactivated JE vaccine**

Formalin-inactivated JEV derived from Vero cells cultured in serum-free medium (ccJE) was obtained from the
Kitasato Institute, Japan (Toriniwa & Komiya, 2008). To investigate whether the efficacy of this unadjuvanted ccJE formulation could be enhanced by inclusion of Advax, a delta inulin-based adjuvant, JEV-specific antibody responses elicited in mice by adjuvanted and non-adjuvanted ccJE given as a two-dose vaccination schedule were compared. For an additional immunogenicity comparison, a group of mice was also immunized with a comparable dose (0.5 µg) of JE-VAX, which corresponds to approximately one-tenth of the dose recommended for human use. Formulation of ccJE with Advax adjuvant potentiated the antibody response induced against JEV, measured by ELISA, by approximately 100-fold relative to the non-adjuvanted control groups in two independent experiments (P<0.001; Table 1). Unadjuvanted ccJE was marginally more immunogenic than JE-VAX (P=0.03, when data from experiments 1 and 2 in Table 1 were combined), as has been reported previously (Toriniwa & Komiya, 2008).

Virus-neutralizing antibody is key to protection against JE (reviewed by Mullbacher et al., 2003): a titre of ≥10 in a 50% plaque-reduction neutralization test (PRNT50) is indicative of protective immunity (Hombach et al., 2005). Formulation of ccJE with Advax resulted in potently increased neutralizing antibody responses against JEV in comparison with control groups (Table 1). Importantly, each recipient of the Advax-adjuvanted vaccine generated PRNT50 titres ≥40 in two independent experiments. This contrasted markedly with the marginal PRNT50 responses elicited by the unadjuvanted vaccines, which failed to induce detectable neutralizing antibody in 31 and 56% of recipients of JE-VAX and ccJE, respectively (combined results from experiments 1 and 2 in Table 1).

Immunization with JE-VAX gave marginally higher PRNT50 responses against JEV than ccJE, despite higher ELISA end-point titres obtained for ccJE than JE-VAX (Table 1). The probable explanation for this discrepancy is the effect of JEV strain differences on neutralization (Ferguson et al., 2008), as the JE-VAX and ccJE vaccines are produced from the Nakayama and Beijing-1 strains, respectively, and neutralization against the Nakayama strain was measured.

### Adjuvanted ccJE protects mice against challenge with JEV

Groups of mice vaccinated with two doses of ccJE (0.5 µg) in the presence or absence of Advax adjuvant, or with comparable doses of JE-VAX, were challenged intranasally with the Nakayama strain of JEV at 6 weeks after completion of the vaccination schedule (Fig. 1). This challenge route is thought to result in entry of the virus into the olfactory lobe of the brain via the olfactory nerve and is less severe than direct intracranial infection (Barrett & Gould, 1986). Whilst 47% of naïve mice succumbed to the virus challenge, all animals immunized with the Advax-adjuvanted vaccine survived (P=0.026) without showing the characteristic signs of flaviviral encephalitis (ruffled fur, hunched posture, tremors and hind-leg paralysis). Interestingly, recipient groups of non-adjuvanted ccJE or JE-VAX showed an indication, although not significant (P=0.23 and P=0.67, respectively), towards increased mortality and reduced mean survival time relative to the untreated mice, which may reflect disease-enhancing immunity.

### Formulation of ccJE with Advax stimulates balanced T-helper cell 1 (Th1)/Th2 antibody responses

Live virus infection typically elicits a Th1-biased immune response characterized by the secretion of gamma interferon, efficient cellular immune responses and the

### Table 1. Antibody responses against JEV and related flaviviruses

<table>
<thead>
<tr>
<th>Vaccine*</th>
<th>Mean log_{10} antibody titre†</th>
<th>Mean PRNT_{50} titre‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JEV</td>
<td>MVEV</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JE-VAX</td>
<td>2.7 (&lt;2.0–3.5)</td>
<td>13 (&lt;10–40)</td>
</tr>
<tr>
<td>ccJE</td>
<td>3.4 (&lt;2.0–4.4)</td>
<td>7.5 (&lt;10–10)</td>
</tr>
<tr>
<td>ccJE + Advax</td>
<td>5.4 (5.1–5.7)</td>
<td>380 (40–2000)</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JE-VAX</td>
<td>3.0 (2.3–3.8)</td>
<td>21 (&lt;10–40)</td>
</tr>
<tr>
<td>ccJE</td>
<td>3.4 (2.3–4.1)</td>
<td>10 (&lt;10–40)</td>
</tr>
<tr>
<td>ccJE + Advax</td>
<td>5.3 (4.5–6.0)</td>
<td>250 (40–1000)</td>
</tr>
</tbody>
</table>

*Groups of 8-week-old C57Bl/6 mice (n=8 per group) were immunized with 0.5 µg JE-VAX or ccJE without or mixed with Advax adjuvant and boosted 3 weeks later. Sera were collected at 6 weeks after completion of the vaccination schedule.

†ELISA end-point titres of individual test sera were determined as described in Methods; mean titres and the range are given.

‡Plaque reduction neutralization by individual test sera against JEV (strain Nakayama) and pooled sera against MVEV (strain MVE-1-51) and WNV (strain Kunjin MRM61C) were determined as described in Methods; mean titres and the ranges are given.
production in mice of antibodies predominantly of the IgG2a/2b isotypes (Coutelier et al., 1987). IgG2a/2b isotypes have been associated with more efficient virus clearance in vivo in comparison with the IgG1 isotype, reflective of a Th2 immune response (see, for example, Huber et al., 2006; Markine-Goriaynoff & Coutelier, 2002; Nimmerjahn & Ravetch, 2005). The choice of vaccine adjuvant can bias the nature of the immune response and hence the antibody isotypes elicited; this is exemplified by alum, which promotes Th2 bias and elevated titres of IgG1 and IgE in vivo (reviewed by McKee et al., 2007). In contrast, inulin-based adjuvants provide balanced enhancement of both cellular (Th1) and humoral (Th2) immunity (Petrovsky, 2006). The results shown in Table 2 confirmed that formulation of ccJE with Advax elicited a balanced response in terms of the Th1/Th2 paradigm. High levels of JEV-specific IgG1 and IgG2b isotype antibodies were obtained in two independent experiments, with an IgG2b:IgG1 isotype ratio in the range of 1.1–2.1. This was comparable to the ratios found in recipients of unadjuvanted vaccines, although the titres for these were significantly lower, consistent with the lower ELISA endpoint titres relative to those elicited with the adjuvanted vaccine (Table 1). As C57Bl/6 mice lack the gene for IgG2a (Martin et al., 1998), stimulation of this subclass of Th1 type antibody could not be investigated in this mouse strain.

Dose-sparing effect of Advax adjuvant in formulation with ccJE

Our results showed that the magnitude of the humoral immune response induced with inactivated JE vaccine was significantly increased by mixing with the delta inulin adjuvant. To test whether inclusion of Advax adjuvant with ccJE would permit antigen sparing, the antigen dose was lowered to 50 ng in a two-dose vaccination regimen and JEV-specific antibody responses were measured. A significantly higher ELISA end-point (P=0.008) and PRNT50 titres (P=0.03) against JEV were achieved with the low antigen dose with Advax adjuvant compared with a tenfold higher antigen dose without adjuvant (Table 3). Remarkably, Advax-adjuvanted low-antigen-dose vaccinations elicited PRNT50 titres ≥10 against JEV in all mice, whereas six

Table 2. JEV–specific antibody isotypes elicited by immunization with inactivated JEV vaccines formulated with or without Advax adjuvant

<table>
<thead>
<tr>
<th>Vaccine*</th>
<th>Anti-JEV antibody isotype titre†</th>
<th>Isotype ratio (IgG2b/IgG1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG1</td>
<td>IgG2b</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JE-VAX</td>
<td>1.5 (0.2)</td>
<td>0.9 (0.3)</td>
</tr>
<tr>
<td>ccJE</td>
<td>2.9 (1.1)</td>
<td>7.5 (3.1)</td>
</tr>
<tr>
<td>ccJE + Advax</td>
<td>7.3 (0.7)</td>
<td>15.2 (1.6)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JE-VAX</td>
<td>1.4 (0.4)</td>
<td>2.6 (1.5)</td>
</tr>
<tr>
<td>ccJE</td>
<td>3.3 (0.6)</td>
<td>4.2 (1.2)</td>
</tr>
<tr>
<td>ccJE + Advax</td>
<td>9.2 (1.7)</td>
<td>10.3 (2.5)</td>
</tr>
</tbody>
</table>

*Groups of 8-week-old C57Bl/6 mice (n=8 per group) were immunized with 0.5 μg JE-VAX or ccJE without or mixed with Advax adjuvant and boosted 3 weeks later. Sera were collected at 6 weeks after completion of the vaccination schedule. For JEV-specific ELISA antibody titres, see Table 1.
†The relative antibody isotypes of pooled test sera were calculated as described in Methods; the mean of two determinations (SEM in parentheses) is given.
out of seven and two out of six mice in the unadjuvanted
groups immunized with 50 ng or even 0.5 μg ccJE,
respectively, failed to produce detectable neutralizing anti-
body. For comparison, the live, recombinant ChimeriVax-JE
vaccine was also included in the dose-sparing study. Prime
and booster vaccinations with 10^5 p.f.u. ChimeriVax-JE
elicted a level of humoral immunity against JEV comparable
to that achieved with the Advax-adjuvanted 50 ng low-
antigen-dose immunization, but was clearly inferior to that
induced with the adjuvanted 0.5 μg ccJE dose (Table 3).

Heterologous prime–boost with Advax-
adjuvanted ccJE and ChimeriVax-JE

Heterologous prime–boost vaccination with live followed by
inactivated vaccines can result in remarkable augmentation
of immune responses in comparison with homologous
prime–boost with either of the vaccines alone (reviewed
by Ranasinghe & Ramshaw, 2009). To investigate whether
the humoral immune responses induced with Advax-
adjuvanted ccJE could be further enhanced by heterologous
prime–boost with ChimeriVax-JE, different homologous
and heterologous prime–boost combinations with the two
vaccines were tested (Table 4). As shown above (Table 3), a
two-dose vaccination schedule with Advax-adjuvanted ccJE
provided significantly higher JEV-specific ELISA (P=0.01)
and PRNT_{50} (P=0.05) antibody titres than ChimeriVax-JE.
Notably, neither combination of heterologous prime–boost
with the two vaccines augmented the JEV-specific antibody
responses relative to mice immunized with two doses of
Advax-adjuvanted ccJE, although improved responses were
obtained in comparison with homologous prime–boost with
ChimeriVax-JE.

Comparison of the immune-potentiating value
of Advax and alum adjuvants in formulation with JE
vaccines

The recently licensed Vero cell-grown JE vaccine IC51
(Jespect) is adjuvanted with alum (Tauber et al., 2007).
Hence, it was of interest to compare the immune-
enhancing effect of Advax and alum when used to adjuvant
cJE vaccines. Experiments conducted in BALB/c mice
again confirmed the potent adjuvant effect of Advax in
augmenting JEV-specific antibody responses elicited with
the ccJE vaccine relative to unadjuvanted vaccine delivery
when measured by ELISA (P=0.004) and neutralization
assay (P=0.01; Table 5, experiment 1). Notably, all animals
in the ccJE + Advax group showed PRNT_{50} titres ≥20
against JEV, whilst three out of five mice in the
unadjuvanted ccJE group failed to produce detectable
titres. Alum was poorer at immune potentiating the ccJE
vaccine relative to Advax with a significant difference
found in the PRNT_{50} titres against JEV (P=0.01).

Table 3. Dose-sparing potency of Advax adjuvant in formulation with ccJE vaccine

<table>
<thead>
<tr>
<th>Vaccine*</th>
<th>Dose</th>
<th>No. of mice per group</th>
<th>Mean log_{10} antibody titre</th>
<th>Mean PRNT_{50} titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>ccJE</td>
<td>0.5 μg</td>
<td>6</td>
<td>3.5 (2.9–4.4)</td>
<td>12 (&lt;10–20)</td>
</tr>
<tr>
<td></td>
<td>0.05 μg</td>
<td>7</td>
<td>2.4 (&lt;2.0–3.2)</td>
<td>6 (&lt;10–10)</td>
</tr>
<tr>
<td>ccJE + Advax</td>
<td>0.5 μg</td>
<td>6</td>
<td>≥5.5 (5.1–5.7)</td>
<td>160 (pool only)</td>
</tr>
<tr>
<td></td>
<td>0.05 μg</td>
<td>7</td>
<td>4.6 (3.9–5.4)</td>
<td>49 (10–80)</td>
</tr>
<tr>
<td>ChimeriVax-JE</td>
<td>10^5 p.f.u.</td>
<td>6</td>
<td>4.6 (3.6–5.4)</td>
<td>40 (pool only)</td>
</tr>
</tbody>
</table>

*Groups of 8-week-old C57Bl/6 mice were immunized subcutaneously with tenfold-different amounts of ccJE without or mixed with Advax
adjuvant, or with ChimeriVax-JE vaccine, and boosted 3 weeks later with the same doses that were used for priming. At 6 weeks after completion of
the vaccination schedule, sera were collected and mean JEV-specific ELISA end-point and PRNT_{50} titres (ranges in parentheses) were determined.

Table 4. Homologous and heterologous prime–boost with Advax-adjuvanted ccJE and ChimeriVax-JE

<table>
<thead>
<tr>
<th>Vaccine*</th>
<th>Boost</th>
<th>Mean log_{10} antibody titre</th>
<th>Mean PRNT_{50} titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>ccJE + Advax</td>
<td>ccJE + Advax</td>
<td>4.9 (4.5–5.4)</td>
<td>76 (20–160)</td>
</tr>
<tr>
<td>ChimeriVax-JE</td>
<td>ChimeriVax-JE</td>
<td>3.4 (2.7–3.9)</td>
<td>21 (&lt;10–80)</td>
</tr>
<tr>
<td>ChimeriVax-JE</td>
<td>ccJE + Advax</td>
<td>4.8 (4.5–5.4)</td>
<td>60 (20–80)</td>
</tr>
<tr>
<td>ccJE + Advax</td>
<td>ChimeriVax-JE</td>
<td>4.5 (4.2–4.8)</td>
<td>40 (10–80)</td>
</tr>
</tbody>
</table>

*Advax-adjuvanted ccJE (0.5 μg) and ChimeriVax-JE (10^5 p.f.u.) were used for immunizations. Groups of 8-
week-old C57Bl/6 mice (n=5 per group) were primed and boosted 2 weeks later as indicated. Sera were
collected 3 weeks after completion of the vaccination schedules and mean JEV-specific ELISA end-point and
PRNT_{50} titres (ranges in parentheses) of individual sera were determined.
Next, we compared the immunogenicity of Advax-adjuvanted ccJE with that of the Jespect JE vaccine (Tauber et al., 2007) in a dose-sparing experiment (Table 5, experiment 2). Whilst comparable levels of virus-specific antibody responses were elicited with 0.5 μg antigen, a tenfold reduction in antigen dose revealed that Advax-adjuvanted ccJE was significantly more potent than Jespect in inducing protective humoral immunity against JEV (P<0.001 and P<0.03 in ELISA and PRNT50, respectively).

**Advax adjuvant stimulates protective neutralizing antibody responses against heterologous viruses belonging to the JEV serocomplex**

Neutralizing antibody against MVEV and WNV elicited with Advax-adjuvanted ccJE in C57Bl/6 mice was measured in two independent experiments (Table 1). MVEV-specific PRNT50 titres of pooled sera ranging from 40 to 160 were observed; these results suggested that solid cross-protective immunity against MVEV was achieved with the adjuvanted ccJE vaccine. Consistent with the greater genetic distance between WNV and JEV compared with MVEV and JEV, the WNV-specific PRNT50 titres were lower (10–20) but nevertheless predictive of cross-protection. Notably, the neutralization titres against the heterologous viruses achieved with Advax-adjuvanted ccJE were equal to or greater than those against the homologous virus obtained with unadjuvanted ccJE or JE-VAX; the latter vaccinations gave no detectable cross-neutralizing activity against either MVEV or WNV. Similar results were obtained in BALB/c mice (Table 5). Importantly, all animals responded with PRNT50 titres ≥10 against MVEV when immunized with Advax-adjuvanted ccJE. However, a comparable level of cross-protective immunity against MVEV was not achieved in Jespect-immunized mice (Table 5).

To expand on these findings, the cross-neutralization activity against MVEV of immune responses elicited with Advax-adjuvanted or unadjuvanted JE-VAX was compared with that induced with ChimeriVax-JE (Fig. 2). Vaccination with Advax-adjuvanted JE-VAX induced marginally greater anti-JEV ELISA titres than ChimeriVax-JE (P=0.06) and were 50-fold higher in magnitude than those achieved with unadjuvanted JE-VAX. However, the anti-MVEV PRNT50 titres in Advax-adjuvanted JE-VAX immunized mice were significantly higher than in ChimeriVax-JE vaccine recipients (P=0.03) and yet again exceeded the threshold considered to be required for protective immunity (PRNT50 ≥10) in all animals. Unadjuvanted JE-VAX failed to elicit detectable neutralizing antibodies against MVEV. JEV-specific PRNT50 titres of pooled sera from the unadjuvanted and Advax-adjuvanted JE-VAX and ChimeriVax-JE vaccine recipients were 20, 200 and 80, respectively, consistent with the observation throughout this study that the magnitude of the neutralizing antibody response against the homologous virus correlates with that against other flaviviruses belonging to the JEV serocomplex.

**Efficacy of Advax-adjuvanted ccJE vaccine in horses**

Horses are susceptible to infection with JEV and are vaccinated in endemic regions against the virus to protect them from a potentially fatal encephalitis. Accordingly, they are a suitable large-animal model for pre-clinical JE vaccine trials. Horses were immunized with a standard human dose of JE-VAX (6 μg JEV antigen) or an

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**Table 5. Comparison of the immunogenicity of Advax- and alum-adjuvanted cell culture-grown JE vaccines in BALB/c mice**

<table>
<thead>
<tr>
<th>Vaccine*</th>
<th>Dose (μg)</th>
<th>Mean log₁₀ antibody titre</th>
<th>Mean PRNT₅₀ titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>JEV</td>
<td>MVEV</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ccJE</td>
<td>0.5</td>
<td>4.0 (3.6–4.2)</td>
<td>13 (&lt;10–40)</td>
</tr>
<tr>
<td>ccJE + Advax</td>
<td>0.5</td>
<td>4.9 (4.5–5.4)</td>
<td>77 (20–160)</td>
</tr>
<tr>
<td>ccJE + alum</td>
<td>0.5</td>
<td>4.3 (3.6–5.1)</td>
<td>16 (&lt;10–20)</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ccJE + Advax</td>
<td>0.5</td>
<td>≥4.8 (3.9–5.1)</td>
<td>124 (20–320)</td>
</tr>
<tr>
<td>0.05</td>
<td>4.6 (4.2–4.8)</td>
<td>74 (10–160)</td>
<td>NT</td>
</tr>
<tr>
<td>Jespect</td>
<td>0.5</td>
<td>3.6 (3.2–4.1)</td>
<td>100 (20–320)</td>
</tr>
<tr>
<td>0.05</td>
<td>2.7 (&lt;2.3–4.1)</td>
<td>6 (&lt;10–10)</td>
<td>NT</td>
</tr>
</tbody>
</table>

*In experiment 1, groups of 8-week-old BALB/c mice (n=6 per group) were immunized subcutaneously with ccJE without or mixed with Advax or alum adjuvant and boosted 3 weeks later. In experiment 2, groups of mice (n=10 per group) were immunized with tenfold different amounts of Advax-adjuvanted ccJE or with (Jespect and boosted 3 weeks later. At 4 weeks after completion of the vaccination schedules, sera were collected) and mean JEV-specific ELISA end-point and PRNT₅₀ titres (ranges in parentheses) were determined.

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equivalent dose of ccJE formulated with or without Advax and boosted once (JE-VAX group) or twice (ccJE groups) at 5-week intervals. Comparison of PRNT\textsubscript{50} titres against JEV after two doses of vaccine showed that ccJE gave approximately threefold higher responses than JE-VAX (Table 6). When ccJE was formulated with Advax, the neutralizing antibody responses were augmented four- and sixfold relative to the unadjuvanted ccJE group after two and three immunizations, respectively. A single booster immunization was critically required to achieve high levels of neutralizing antibody; however, the second boosters did not result in significant further increases in titres.

Neutralizing antibody titres against MVEV and WNV were also measured (Table 6). The data showed that formulation of ccJE with Advax increased the cross-protective immunity against both viruses. Accordingly, in this large-animal model, the cross-protective value of inactivated JE vaccines also strictly correlated with the magnitude of the homologous responses.

**DISCUSSION**

Advax is a novel polysaccharide adjuvant based on microparticles of delta inulin that is under development for a range of vaccine applications (P. D. Cooper and N. Petrovsky, patent application WO 2006/02410, PCT/AU2005/001328: new polymorphic form of inulin and uses thereof; in preparation). Here, we showed in mice, as well as in a pre-clinical vaccination study in horses, that formulation of inactivated JE vaccines with Advax greatly enhances virus-specific immunity relative to unadjuvanted forms of the same vaccines. Advax-mediated immune

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**Table 6.** Neutralizing antibody titres in horses immunized with JE-VAX or ccJE without or in the presence of different Advax formulations

<table>
<thead>
<tr>
<th>Vaccine*</th>
<th>Horse</th>
<th>Post-prime</th>
<th>Post-1st boost</th>
<th>Post-2nd boost</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>JEV</td>
<td>JEV; MVEV</td>
<td>JEV; MVEV</td>
</tr>
<tr>
<td>JE-VAX</td>
<td>#1</td>
<td>&lt;10</td>
<td>90; 10</td>
<td>&lt;10; NT</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>&lt;10</td>
<td>30; 10</td>
<td>10; NT</td>
</tr>
<tr>
<td></td>
<td>GMT</td>
<td>&lt;10</td>
<td>52; 10</td>
<td>&lt;10; NT</td>
</tr>
<tr>
<td>ccJE</td>
<td>#3</td>
<td>30</td>
<td>100; 80</td>
<td>100; 40</td>
</tr>
<tr>
<td></td>
<td>#4</td>
<td>10</td>
<td>200; 20</td>
<td>100; 20</td>
</tr>
<tr>
<td></td>
<td>#5</td>
<td>&lt;10</td>
<td>200; 40</td>
<td>200; &lt;10</td>
</tr>
<tr>
<td></td>
<td>#6</td>
<td>10</td>
<td>200; 40</td>
<td>100; 20</td>
</tr>
<tr>
<td></td>
<td>GMT</td>
<td>11</td>
<td>168; 40</td>
<td>141; 17</td>
</tr>
<tr>
<td>ccJE + Advax</td>
<td>#7</td>
<td>10</td>
<td>800; 40</td>
<td>1600; 80</td>
</tr>
<tr>
<td></td>
<td>#8</td>
<td>30</td>
<td>1600; 30</td>
<td>1600; 320</td>
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<tr>
<td></td>
<td>#9</td>
<td>10</td>
<td>200; 20</td>
<td>200; 20</td>
</tr>
<tr>
<td></td>
<td>GMT</td>
<td>14</td>
<td>635; 50</td>
<td>800; 80</td>
</tr>
</tbody>
</table>

*Horses were immunized subcutaneously with 6 μg doses of JE-VAX, ccJE or Advax-adjuvanted ccJE. The vaccination schedule was as follows: week 0, first dose; week 5, second dose; week 10, third dose for ccJE ± Advax adjuvant group only. Sera were tested for antibody prior to commencement of immunizations and found to be negative (data not shown). Serum collection was on the day of immunization at weeks 5 and 10, and at week 17.
potentiation had functional value in terms of increasing the magnitude of neutralizing antibody responses and provided complete protection of mice from intranasal challenge with JEV. The Advax adjuvant enhanced the immunogenicity of both mouse brain- and cell culture-grown JE vaccine antigens to a comparable degree: in both cases, JEV-specific ELISA antibody titres were augmented by up to 100-fold and neutralization titres by 10–50-fold when approximately one-tenth of a human dose of vaccine was used in a two-dose immunization schedule in the mouse studies. Importantly, this vaccination regimen resulted in all mice (five separate experiments, two inbred mouse strains) responding with PRNT50 titres >20, which is indicative of solid protective immunity against JEV infection. This contrasted with a sizeable incidence of vaccine failure (PRNT50 <10) in recipients of the unadjuvanted vaccines. In human immunizations, a two-dose vaccination schedule with JE-VAX failed to generate detectable neutralizing antibody in ~20% of vaccine recipients (Poland et al., 1990) and three doses were required for adequate humoral immunity in >90% of immunized individuals (reviewed by Halstead & Tsai, 2004). Accordingly, the mouse model resembles immunization in humans with JE-VAX with respect to the relatively inefficient induction of neutralizing antibody. Given the potent immune stimulation of inactivated JE vaccines with Advax adjuvant in mice, and in view of recent results from trials using hepatitis B and influenza virus antigens, which showed that Advax is fully effective in human subjects (unpublished results), we anticipate that Advax will similarly augment immunity in human vaccination against JEV.

A remarkable property of Advax when formulated with ccJE was the level of dose sparing of antigen that could be achieved. Thus, a two-dose immunization schedule of mice with a 100-fold lower amount of antigen (50 ng) than recommended for use in humans elicited protective levels of neutralizing antibody that were comparable to those recommended for use in humans elicited protective levels with a 100-fold lower amount of antigen (50 ng) than currently used in formulations with ccJE. This was significantly less effective following a tenfold dose reduction. This side-by-side comparison highlights the greater immune-potentiating value of Advax relative to alum in formulations with ccJE. It is an important clinical consideration and may be indicative of a reduced risk of vaccine failure with Advax- relative to alum-adjuvanted JE vaccines. Furthermore, whereas alum adjuvant is recognized to impart major Th2 bias to vaccine responses, as demonstrated by predominant induction of IgG1 and IgE antibody isotypes in mice, Advax adjuvant provided a balanced enhancement of both Th1 and Th2 responses, as demonstrated by induction of JEV-specific IgG2b as well as IgG1 in C57Bl/6 mice.

Equine vaccinations with Advax-adjuvanted ccJE confirmed the efficient immune-potentiating property of Advax in a large-animal model. Fourfold increases in neutralizing antibody responses against JEV were achieved in comparison with unadjuvanted ccJE, in a two-dose vaccination schedule. A third dose of either adjuvanted or unadjuvanted ccJE did not boost the humoral immune responses further. These results have significant implications in terms of improved JE vaccine efficacy and potential antigen dose sparing for veterinary and human immunization.

A second key finding of this study was the demonstration of protective levels of cross-neutralizing antibody against related viruses belonging to the JEV serocomplex in mice and horses vaccinated with ccJE vaccine only when formulated with Advax adjuvant. This may be important in terms of human health when vaccination against JEV is considered for a population residing in endemic areas or for travellers to regions where related flaviviruses such as WNV or MVEV coexist with JEV. We and others have shown in animal models that, in these circumstances, the induction of cross-neutralizing antibody is a desirable property of a JE vaccine not only for the benefit of protection against the other pathogens, but also to reduce the potential risk of immune enhancement of infection with a heterologous flavivirus (Broom et al., 2000; Lobigs et al., 2003, 2009). In the absence of adjuvant, the inactivated JE vaccines induced low and often undetectable levels of neutralizing antibody against MVEV and WNV. A similar observation has been made in humans immunized with JE-VAX (Kanesa-Thasan et al., 2002) or live SA14-14-2 JE vaccine (Tang et al., 2008). In contrast, we demonstrated that the inclusion of Advax with JE-VAX or ccJE consistently induced and boosted JE serocomplex cross-neutralizing immunity in mice and horses, suggesting that protection against multiple related flaviviruses can be achieved using a single Advax-adjuvanted inactivated JE vaccine.

The mechanism by which Advax adjuvants stimulate vaccine immunogenicity remains under investigation. This and other investigations (Petrovsky, 2006; Silva et al., 2004) have shown that the adjuvant does not give rise to a polarization of the CD4+ T-cell response against the vaccine antigen. Balanced cellular and humoral immunity elicited with Advax-adjuvanted JE vaccines in terms of the Th1/Th2 paradigm may be a factor, in addition to the potent enhancement of the magnitude of the antibody response, that contributes to stimulation of cross-neutralizing immunity and thereby reduces the risk of vaccine-induced enhanced susceptibility to heterologous flaviviruses. Notably, a Th2 bias in the immune response induced by vaccination has been associated with disease enhancement (see, for example, Huisman et al., 2009; Moghaddam et al., 2006; Polack, 2007; Yang et al., 2001). If this were also the case in infections with JEV and related flaviviruses, adjuvants that produce a strong Th2 bias, such as alum salts (McKee et al., 2007), could compromise vaccine safety and may be better replaced in human vaccines by adjuvants such as Advax that provide a more balanced immune enhancement.
METHODS

Animals. C57Bl/6 and BALB/c mice were bred under specific-pathogen-free conditions and supplied by the Animal Breeding Facility at the John Curtin School of Medical Research, Australian National University (ANU), Canberra, Australia. Female mice were used in all experiments. All animal experiments were approved by the ANU or the University of Queensland animal ethics committees.

Viruses. Working stocks of JEV (strain Nakayama), MVEV (strain MVE-1-51) and WNV (strain Kunjin MRM61C/60) were 10% suckling mouse brain homogenates in Hank’s balanced salt solution containing 20 mM HEPES buffer (pH 8.0) and 0.2% BSA (HBSS-BSA). Virus titres were determined by plaque formation on Vero cells, as described previously (Licon Luna et al., 2002).

Vaccines and adjuvant. JE-VAX (Sanofi Pasteur Inc.) was prepared prior to immunizations as recommended by the supplier. A human dose of this vaccine contains ~6 µg purified, formalin-inactivated JEV (Nakayama strain). Vero cell culture-grown inactivated ccJE vaccine (Beijing-1 strain) (Toriniwa & Komiya, 2008) was obtained from the Kitasato Institute, Japan. ChimeriVax-JE (Monath et al., 2003) was propagated for one passage in Vero cells grown in M199 medium (Invitrogen) supplemented with 5% fetal bovine serum and antibiotics. The clarified infected culture supernatant had a titre of 1 x 10^7 p.f.u. ml^-1 in Vero cells and was frozen in single-use aliquots. Immunization with each of the vaccines was by the subcutaneous route. Advax D adjuvant, which is based on microparticulate delta inulin, was obtained from Vaxine Pty Ltd, Adelaide, Australia. Advax is supplied as a sterile, preservative-free, fine particulate suspension of delta inulin particles in a phosphate buffer at neutral pH and has a milky white appearance similar to alum. The adjuvant is prepared from inulin (β-D-[2-1]polyfructofuranosyl β-D-glucose), a natural plant-derived polysaccharide found in nature mainly as a storage polymer backbone does not pass through a sugar residue but comprises a chain of fructose units originating from a single glucose unit. It has a structure unusual in a polysaccharide in that the polymer backbone does not pass through a sugar residue but comprises a hydrophobic polyoxyethylene chain of -C-C-O- linkages. Inulin’s relatively hydrophobic, polyoxyethylene-like backbone plus its non-ionized nature allow inulin to be crystallized into various isomorphic forms, of which delta inulin is the most temperature-stable and adjuvant-active isomer. Advax adjuvant was combined with antigen by simple admixture immediately prior to immunization. Unlike aluminium salt adjuvant, Advax adjuvant does not adsorb the antigen when mixed and hence the combined formulation can be injected immediately after mixing. Vaccines (ccJE or JE-VAX) were diluted in PBS to the required antigen concentration, as indicated, with or without formulation with Advax (10 mg ml^-1 final concentration) or alum (aluminium phosphate, 20 mg ml^-1 final concentration) prior to injection of a volume of 0.1 ml of the adjuvanted vaccines. JEV was purchased from CSL Ltd, Australia; a 0.5 ml dose of the vaccine contains 6 µg inactivated JEV (SA14-14-2 strain). The vaccine was diluted in PBS containing 0.1% aluminium hydroxide to obtain the required antigen doses, as indicated.

Horse immunization. Groups of standard bred, female horses, 4–8 years of age and seronegative for JEV, MVEV and WNV, were immunized with JE vaccines by subcutaneous injections. Horses were boosted once or twice at 5-week intervals, as indicated, and sera were collected at 5 weeks after the first and second, and 7 weeks after the third immunization.

Serological tests. For titration of JEV-reactive antibody in mouse serum, ELISAs were performed with horseradish peroxidase-conjugated goat anti-mouse Ig and the peroxidase substrate 2,2'-azino-di(3-ethyl-benzthiazoline sulfonate) as described previously (Colombage et al., 1998). The JEV Nakayama strain was used for ELISA antigen production. For determination of ELISA end-point titres, absorbance cut-off values were established as the mean absorbance of eight negative-control wells containing sera of naive mice plus 3 SD. Absorbance values of test sera were considered positive if they were equal to or greater than the absorbance cut-off and end-point titres calculated as log_{10} of the reciprocal of the last dilution giving a positive absorbance value.

A PRNT_{50} was performed by incubating ~400 p.f.u. JEV (Nakayama strain), MVEV (MVE-1-51 strain) or WNV (Kunjin MRM61C strain) in 110 µl HBSS-BSA with serial twofold dilutions of antisera in the same buffer in a 96-well tray at 37 °C for 1 h. Complement was inactivated by heating the sera at 56 °C for 0.5 h before use. Duplicate 0.1 ml aliquots were assayed for infective virus by plaque formation on Vero cell monolayers grown in six-well tissue culture trays. The percentage plaque reduction was calculated relative to virus controls incubated with naive serum from the same mouse strain. Controls yielded 50–100 p.f.u. per well. PRNT_{50} titres are given as the reciprocal of serum dilutions that resulted in a ≥50% reduction in the number of plaques.

The isotypes of JEV-specific antibodies in serum samples were determined using an ELISA-based Mouse Typer Subtyping kit (Bio-Rad) according to the supplier’s instructions. JEV-coated ELISA trays were used and serum samples were diluted 100-fold in Blotto/ Tween 20 and assayed in duplicate. Three naive control sera were included in each test. To determine the relative isotype titres, the mean absorbance values of test sera were divided by twice the corresponding mean absorbance value of the control sera.

Statistics. Differences in survival ratios in mouse challenge experiments were assessed using Fisher’s exact test, and a Wilcoxon signed-rank test was used to assess differences in antibody titres for significance. Samples with titres below the detection limit of the serological assays were given titres of half that of the detection limit for calculations.

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REFERENCES


