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Immunization of Flavivirus West Nile Recombinant Envelope Domain III Protein Induced Specific Immune Response and Protection against West Nile Virus Infection

Jang-Hann J. Chu, Cern-Cher S. Chiang, and Mah-Lee Ng

The domain III of the West Nile virus (WNV) envelope glycoprotein (E) was shown to serve as virus attachment domain to the cellular receptor, and neutralizing Abs have been mapped to this specific domain. In this study, domain III of the WNV E protein (WNV E DIII) was expressed as a recombinant protein and its potential as a subunit vaccine candidate was evaluated in BALB/C mice. Immunization of WNV E DIII protein with oligodeoxynucleotides (CpG-DNA) adjuvant by i.p. injection was conducted over a period of 3 wk. The immunized mice generated high titer of WNV-neutralizing Abs. Murine Ab against WNV E DIII protein was also capable of neutralizing Japanese encephalitis virus. The IgG isotypes generated were predominantly IgG2a in the murine sera against the recombinant protein. Splenocyte cultures from the mice coadministered with WNV E DIII protein and CpG secreted large amounts of IFN-γ and IL-2 and showed proliferation of T cells in the presence of WNV E DIII protein. Overall, this study highlighted that recombinant WNV E DIII protein delivered in combination with CpG adjuvant to mice generated a Th1 immune response type against WNV and can serve as a potential vaccine to prevent WNV infection. The Journal of Immunology, 2007, 178: 2699–2705.

West Nile virus (WNV) is a mosquito-transmitted, positive-stranded RNA virus grouped within the Japanese encephalitis virus (JEV) serocomplex of the genus Flavivirus in the family Flaviviridae. The WNV is the causative agent of the disease syndrome named West Nile Fever, including a spectrum of associated complications (meningo-encephalitis and acute flaccid paralysis) (1). This is a re-emerging mosquito-borne disease that is responsible for recent large outbreaks in the Western hemisphere. In 2005, there were 3000 human infections and 119 deaths reported in the United States (2). Currently, no specific treatment or licensed vaccine is available for prevention against this pathogenic virus. Therefore, there is an urgent need for the development of an effective prophylactic vaccine to prevent WNV infection.

The envelope (E) protein of flaviviruses is the virus attachment protein and is involved in interacting with the cellular receptor molecule(s). Crystallography data on the ectodomain of the flavivirus E protein revealed three distinct domains (DI, DII, and DIII) (3). The high antagonistic effect of recombinant WNV E DIII protein in blocking WNV infection in both mammalian and mosquito cells strongly suggested that WNV E DIII protein functions as the receptor-binding domain (4) and is responsible for the recognition and attachment to the cellular receptor (5–7). Importantly, a majority of the neutralizing epitopes have been mapped to the domain III region of the flavivirus E protein (8–11). Furthermore, the WNV E DIII protein can be expressed as a recombinant protein that independently folds as an individual functional fragment and is capable of eliciting neutralizing Abs against WNV (12). Hence, the potential of WNV E DIII protein to function as an effective recombinant subunit vaccine against WNV is assessed and is presented in this study.

Materials and Methods

Cells, viruses, and Abs

C6/36 cells, a continuous mosquito cell line (provided by Prof. A. Igarashi, University of Nagasaki, Nagasaki, Japan) derived from Aedes albopictus (Diptera: Culicidae) embryonic tissue were grown in L-15 medium (Invitrogen Life Technologies) containing 10% heat-inactivated FCS at 28°C. Vero cells (green monkey kidney cells; America Type Culture Collection) were maintained at 37°C in Medium 199 (Invitrogen Life Technologies) containing 10% inactivated FCS. WNV (Sarafend) and JEV (Nakayama) used in this study were provided by Prof. E. Westaway (Sir Albert Sackzowski Virus Research Laboratory, Queensland, Australia), and the virus was propagated in C6/36 cells throughout this study. Quantification of the viruses was performed on Vero cells using plaque assay as described previously (13). The Ab for the detection of WNV E protein was a monospecific polyclonal Ab provided by V. Deubel (Pasteur Institute, Paris, France).

Cloning, expression, and purification of recombinant WNV E DIII protein

WNV (Sarafend) viral RNA was extracted from WNV-infected Vero cells using RNeasy (Qiagen) according to the manufacturer’s instructions. Approximately 5 μg of viral RNA was resuspended in a final volume of 40 μl of RNase-free water. RT-PCR was used to amplify the gene encoding the E protein using the Superscript one-step RT-PCR system (Invitrogen Life Technologies). The gene sequence corresponding to aa 299–401 of the C-terminal of WNV E DIII protein was amplified by PCR from the viral cDNA using Advantage II polymerase enzyme (BD Clontech) using the following set of primers: forward primer, 5′-GGAACTCCGAACAA CATATGGTGTTATGCTC-3′, and reverse primer, 5′-ACCGGATCT TACCCGATTGTGCGCCAGTG-3′. The EcoRI and BamHI restriction sites are underlined. A PCR product of 440 nt was obtained after PCR

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3 Abbreviations used in this paper: WNV, West Nile virus; E, envelope; IPTG, isopropyl β-D-thiogalactoside; JEV, Japanese encephalitis virus.

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amplification under the following conditions: 33 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1.5 min. The PCR product was then purified, digested with the restriction enzymes EcoRI and BamHI, and followed by ligation into the corresponding restriction sites of the pET32 vector (Novagen). The nucleotide sequence was confirmed by using DNA sequence analysis. Escherichia coli cells (strain BL21(DE3)) was transformed with the pET32 vector containing the inserted fragment. The recombinant bacteria were grown until OD600 of the culture reached 0.6, and protein expression was induced by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM at 30°C for 4–6 h. Cells were harvested by centrifugation at 5,000 × g for 20 min at 4°C and washed twice with a buffer containing 1% Nonidet P-40, 20 μg · ml−1 DNAse, and 1 mM PMSF. Cell lysis was performed by sonication and followed by centrifugation at 48,000 × g for 30 min at 4°C (Beckman Coulter). The recombinant WNV E DIII protein was expressed in both soluble and insoluble fractions. The soluble WNV E DIII protein was further purified and concentrated as previously described in Ref. 4. Cleavage of the thioredoxin tag was conducted using enterokinase (Invitrogen Life Technologies) following the manufacturer’s recommendations. Electrophoresis was performed using a PhastGel system (Amersham Biosciences). For the native gel, PhastGel native buffer strips (0.08 1-alanine and 0.25 M Tris (pH 8.8), made of 3% agarose) were used. Protein samples were mixed with native gel running buffer (0.1 M Tris-HCl (pH 8.8), 20% glycerol, and 0.0025% bromophenol blue) to a final concentration of ~1 mg/ml. For the SDS-PAGE gel, PhastGel SDS buffer strips (0.20 M tricine, 0.20 M Tris, and 0.55% SDS (pH 7.5), made of 3% agarose) were used. Protein samples were mixed with running buffer (0.1 M Tris-HCl (pH 8.8), 20% glycerol, 1% DTT, and 3% SDS and 0.0025% bromophenol blue) to a final concentration of ~1 mg/ml. Six microliters of each sample was loaded onto a homogeneous 12.5% polyacrylamide gel. Western blot analysis was performed using anti-WNV E protein monospecific polyclonal Ab. In brief, the membranes were incubated with the anti-WNV E protein polyclonal Ab (1/500 dilution) at room temperature for 1 h, followed by three washes in TBST. An alkaline phosphatase-conjugated goat anti-rabbit IgG (Chemicon International) at a dilution of 1/1000 was used to detect the primary Ab. Membrane blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate from Chemicon International. The presence of endotoxin contamination in the purified recombinant WNV E DIII protein was measured using commercially available chromogenic assay (Toxicolor system; Seikagaku Industries).

**Immunization procedures**

Specific pathogen-free BALB/C mice were obtained from Laboratory Animal Center (National University of Singapore) and maintained strictly according to the approved Institutional Animal Care and Use Committee protocols. Four groups of 6-wk-old female BALB/C mice (six mice per group) were immunized i.p. three times at weekly interval. The first group of mice received 100 μg of the recombinant WNV E DIII protein diluted in PBS; a second group received 100 μg of the recombinant WNV E DIII protein diluted in PBS with 50 μg of Cpg adjuvant (30 μl); a third group received 130 μl of PBS alone and the final group received 50 μg of Cpg adjuvant (30 μl) alone. The Cpg adjuvant was purchased from Qiagen, and it contains immunostimulatory unmethylated Cpg oligonucleotides (14). Serum samples were collected from the central tail vein before immunization and 2 wk after the final immunization. The specificity of the murine sera for WNV E DIII protein was determined using the Western blot analysis, immunofluorescence assay, and virus protection assay. Two weeks after the final immunization, mice were sacrificed, and the spleens aseptically removed for in vitro splenocyte cultures.

**Evaluation of immune responses in mice**

Indirect immunofluorescence detection of viral Ag in WNV-infected vero cells using the immunized murine sera was performed as essentially described in Ref. 13. The murine sera were diluted at 1/100 dilution, and the secondary goat anti-mouse Ab conjugated to FITC was used at a dilution of 1/500. Direct ELISA for quantifying Ag-specific IgG and the IgG subclasses were conducted using plates coated with recombinant WNV E DIII protein. WNV E DIII is located at the surface of the virus particles based on the structural analysis of the E protein and represents a significant portion of the neutralizing Ab response (7, 8, 10, 15). Microtiter plates were coated with recombinant WNV E DIII protein overnight at 4°C at 100 ng/well in coating buffer (0.015 M Na2CO3, 0.03 M NaHCO3, and 0.003 M NaN3 (pH 9.6)). Sera from immunized mice were diluted from 1/50 through 1/10,000 in PBS with 2% BSA and added to the duplicate wells before being incubated for 1 h at room temperature. Plates were washed three times with PBST. HRP-conjugated goat anti-mouse IgG (Zymed Laboratories) at a dilution of 1/1,000 in PBS with Tween 20 (PBST; Sigma-Aldrich) was added for 1 h at room temperature. After washing three times with PBST, color development was achieved using O-phenylene diamine dihydrochloride (Sigma-Aldrich) as substrate, and the plate was read using an ELISA TouchScreen plate reader (Tecan), running in Magellan2 software, at wavelength of 490 nm. To determine the relative levels of different IgG subclasses, anti-murine IgG1 and IgG2a conjugated with HRP (Zymed Laboratories) were used as secondary Abs at a dilution of 1/1,000 in PBST (Sigma-Aldrich).

Single-cell suspensions of the spleens were prepared by mechanical dissociation. Splenocytes were resuspended and adjusted to 4 × 10^6 cells/ml in RPMI 1640 medium with 10% heat-inactivated FCS. The murine splenocytes were cultured in the presence of 5 μg of WNV E DIII protein. T cell mitogen Con A (5 μg/ml; Vector Laboratories) was added to positive control wells, whereas an equal volume of RPMI 1640 medium was added to the negative control wells. To quantitate T cell proliferation, the commercial cell proliferation assay kit from Promega was used accordingly to manufacturer’s instructions. Proliferation of the T lymphocyte proliferation was expressed as stimulation index, which is the ratio of OD (OD 570 nm of stimulated wells (stimulated cells) to OD 570 nm of unstimulated ones (16)). The splenocyte culture supernatants were also harvested on days 2, 3, 4, and 5 after restimulation to determine the kinetics of different cytokine production. Cytokine analysis was performed with ELISA using OptEIA mouse IL-2, IL-4, IL-6, and IFN-γ ELISA kits (BD Pharmingen) as instructed by the manufacturer’s protocol. Experiments were performed in duplicates.

**Virus protection assay in cells and suckling mice**

Pooled sera from mice immunized with WNV E DIII protein together with the controls were assayed for neutralizing Abs against WNV using cell culture and mouse protection assay. For the virus plaque neutralization cell culture assay, 50 μl of the pooled sera (500 μg · ml−1) collected as described above was diluted in 2-fold serial dilutions (from 1/2 to 1/8192).
Both the WNV and JEV were adjusted to 500 PFU in 50 μl of virus diluent (HBSS) and added to different tubes containing serial diluted Abs. The mixed Abs and virus were incubated at 37°C for 1 h and subsequently added to a Vero cell monolayer at a density of 5 × 10⁴ cells and further incubated at 37°C for 1 h. After incubation, the inocula were removed, and the cell monolayer was rinsed once with virus diluent. Overlay medium was added and the plates were incubated at 37°C for an additional 4 days, and virus plaques were stained with 0.5% crystal violet. Three independent experiments were conducted.

Mouse protection assay were conducted using newborn BALB/c mice (2 days old). The WNV or JEV was adjusted to 500 PFU in virus diluent (HBSS) and added to the tube containing undiluted pooled sera (obtained as described above) in a 1/1 mixture. The virus-serum mixture was incubated for 1 h at 37°C and inoculated intracerebrally into the mice. Mouse groups were observed daily and mortality was recorded. Brains and spleens were harvested from mice at death or on the day of killing for virus titration using plaque assay and histopathology analyses. All mice were euthanized by CO₂. The brains were removed and sectioned into equal parts for immunohistochemistry and viral isolation. The former was fixed in 4% paraformaldehyde (Merck) for at least 48 h before processing and embedding in paraffin. Four-micrometer-thick sections were fished onto silane-coated slides and dried at 37°C overnight. Immunohistochemistry detection of the presence of WNV E protein in the infected cells.

Statistical analysis

All in vitro assays were repeated twice or three times, and error bars drawn represent one SE from the mean. Figures consist of a representative experiment conducted at least two to three with similar results. Statistical analysis was done using the ANOVA test (Analyze IT). Procedures were based on nonparametric analyses (Mann-Whitney U test); comparisons between the different groups were made using a nonparametric Wilcoxon signed test. Values of p > 0.05 were considered to be significant. Statistical analysis was performed using the SPSS statistical package (SPSS).

### Results

Expression and purification of the recombinant WNV E domain III protein

Recombinant WNV E III protein (amino acid residues from E-299 to E-401) was expressed from the pET32a expression vector in recombinant E. coli cells after IPTG induction. The WNV E III protein was expressed as a fusion protein with thioredoxin at the N-terminal of the protein. The recombinant WNV E III protein (33 kDa) was detected in both the soluble fraction (supernatant; Fig. 1a, lane 3) and insoluble (pellet; Fig. 1a, lane 2) fraction after sonication (Fig. 1a). The soluble WNV E III protein was then subjected to the removal of the N-terminal thioredoxin by enterokinase cleavage, and the soluble WNV E III (13 kDa; Fig. 1a, lane 4) protein was further purified and concentrated to ~5 mg/ml. The authenticity and proper folding of the purified recombinant WNV E III protein was further confirmed by the detection of the WNV E III protein (13 kDa) using the anti-WNV E protein polyclonal Ab (the Ab is generated from the full-length WNV E protein) via Western blot analysis (Fig. 1b, lanes 2 (native gel) and 3).

Immune responses elicited by immunization of the recombinant WNV E DIII protein

Groups of BALB/c mice (n = 6) were immunized with the purified recombinant WNV E DIII protein alone, the purified recombinant

### Table I. WNV E DIII protein Ab titers and IgG subclasses

<table>
<thead>
<tr>
<th>Mice Immunized with the following Combinations</th>
<th>End Point Ab Titer Causing 50% Virus Reduction</th>
<th>IgG1 (Th2) (mg/ml)</th>
<th>IgG2a (Th1) (mg/ml)</th>
<th>IgG1 IgG2a</th>
<th>IgG2a IgG1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS alone</td>
<td>NA</td>
<td>0.08 ± 0.005</td>
<td>0.09 ± 0.003</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CpG alone</td>
<td>NA</td>
<td>0.07 ± 0.008</td>
<td>0.06 ± 0.005</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>WNV E DIII alone</td>
<td>1/1000</td>
<td>3.20 ± 0.015</td>
<td>5.40 ± 0.030</td>
<td>0.7</td>
<td>1.7</td>
</tr>
<tr>
<td>WNV E DIII plus CpG</td>
<td>1/5000</td>
<td>3.05 ± 0.016</td>
<td>5.90 ± 0.035</td>
<td>0.6</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*ELISA was performed using recombinant WNV E DIII protein coated onto 96-well microtiter plates as described in Materials and Methods. Number of animals used in this experiments to obtain SD was n = 6. Similar results were observed in multiple sets of experiments. NA, Not applicable.*
of the number of mice surviving to number of mice inoculated with the suckling mice. Survival rate of suckling mice represents the percentage effective although not complete protection from JEV infections are seen in mice immunized with WNV E DIII protein and WNV E DIII protein with CpG were mixed with WNV and incubated for 1 h before inoculation into suckling mice. The mice are kept until 24 days postinoculation to record the survival rate. When the concentrations of the antisera are adequate, the mice survived. WNV mixed with PBS or CpG adjuvant were negative controls and offer no protection. a. The pooled sera collected from mice immunized with the different combination of Ags (PBS alone, CpG alone, WNV E DIII protein, and WNV E DIII protein with CpG) were analyzed for their neutralizing ability as described in Materials and Methods. Sera from mice immunized with WNV E DIII protein alone or WNV E DIII protein in the presence of CpG produce potent neutralizing Ab against WNV. In contrast, sera from mice immunized with PBS alone or CpG alone are not capable of neutralizing WNV even at high Ab titer. Experiments were repeated three times. b. Dose-response analyses of sera from mice immunized with WNV E DIII protein or coadministrated with CpG can detect viral Ag (E protein) in WNV-infected Vero cells, indicating that the Ab against WNV E DIII protein recognizes the native viral E protein (Fig. 2b). Virus E protein was not detected by sera from mice that were immunized with PBS, CpG adjuvant, or preimmune sera (Fig. 2b).

Mice inoculated with the recombinant WNV E DIII protein (without the CpG adjuvant) developed anti-WNV E DIII Ab after the third booster while immunization of WNV E DIII protein in the presence of CpG adjuvant further enhanced the anti-WNV E DIII Ab titer significantly from 1/1000 to 1/5000 as shown in Table I. The inoculation of mice with either PBS alone or CpG alone did not produce any specific Ab against the recombinant WNV E DIII protein as shown by ELISA (Table I). In addition, the specific serum IgG isotype was determined by ELISA, and the results are shown in Table I. Mice immunized with either WNV E DIII protein alone or in the presence of CpG adjuvant induced more IgG2a than IgG1. The ratios (IgG2a to IgG1) were 1.7 and 2.0, respectively. The results of the serum isotype analysis suggested that WNV E DIII protein induced a more Th1 immune response.

Next, the cell-based and suckling mouse protection assays were conducted to analyze the titer and efficacy of the neutralizing Abs in preventing WNV infection. The ability of the induced Abs to confer protection against WNV infection was first determined via plaque reduction assay. As shown in Fig. 3a, incubation of anti-WNV E DIII with WNV conferred potent neutralizing effects. The dilutions of the antisera capable of reducing WNV infection by 90% were determined to be 1/64 for mice immunized with the recombinant WNV E DIII protein alone and 1/128 for mice coimmunized with WNV E DIII protein and CpG adjuvant. No neutralizing activity was detectable from the preimmune sera, and sera from mice inoculated with PBS or CpG adjuvant.

In the mouse protection assay, sera from mice immunized with the recombinant WNV E DIII protein alone and WNV E DIII protein in the presence of CpG adjuvant conferred mice with full virus (with or without antiserum). d. Photomicrographs of brains from mice challenged with WNV in either the presence or absence of WNV E DIII protein-neutralizing Ab. Viral Ags (at the perinuclear region of the cells) are detected in the brain cells of mice challenged with WNV in the absence of WNV E DIII protein-neutralizing Ab (left image with arrows), indicating the presence of virus replication. In contrast, viral Ag is not detected in the brain cells of mice in the presence of WNV E DIII protein-neutralizing Ab (right image).
FIGURE 4. T lymphocyte proliferation of immunized murine splenocyte cultures in response to the inoculation of recombinant WNV E DIII protein. Isolated T lymphocytes from mice immunized with PBS alone, CpG alone, WNV E DIII protein, and WNV E DIII protein with CpG were stimulated with WNV E DIII protein. The stimulation index is defined as the ratio of stimulated cell to unstimulated ones. Variation of T cell proliferation is observed among all the groups. The highest proliferation level is noted from mice immunized with WNV E DIII protein + CpG, followed by WNV E DIII protein alone. Readings from these two groups are both significantly higher ($p > 0.05$) than the other two groups. *, significant differences.

Protection against WNV infection at neat concentration (Fig. 3b). Subsequent dilution of the antisera at 1/100 from mice inoculated with WNV E DIII protein with CpG can protect up of 50% of the sucking mice from WNV infection, whereas the anti-WNV E DIII protein sera (without CpG adjuvant) protected only 33.3% of the sucking mice at the same dilution. In contrast, the PBS or CpG adjuvant-inoculated mice did not survive the infection with WNV (Fig. 3b). The murine anti-WNV E DIII Ab was also capable of protecting mice from JEV infection, although complete protection was not observed (Fig. 3c). Mice inoculated with JEV in the presence of anti-WNV E DIII Ab generated from the mice inoculated with WNV E DIII protein alone or in combination with the CpG adjuvant was able to confer a survival rate of 67 and 83%, respectively (Fig. 3c).

All the mice inoculated with WNV (from the mouse protection experiment) were also examined for pathological abnormalities in the brain at the time of sacrifice. H&E-stained sections showed no significant neuropathological damage in mice inoculated with WNV in the presence of WNV E DIII-specific neutralizing Ab when compared with samples in the absence of anti-WNV E DIII Ab (data not shown). The result was further confirmed by staining the viral Ags in the brain sections of the experimental mice. Viral Ags were detected in the brain of those mice that were inoculated with WNV without the presence of WNV E DIII-specific neutralizing Ab (Fig. 3d, left image indicated by arrows). The Ags were not detected in the brains of mice that were inoculated with WNV and WNV E DIII-specific neutralizing Ab (Fig. 3d, right image). The mice were sacrificed at day 2 postinoculation for both sets of experiments.

Proliferation of T cell in splenocytes is generally related to the cell-mediated immunity; therefore, WNV E DIII protein-specific T cell proliferation assay was performed (Fig. 4). The level of WNV E DIII protein-specific T cell proliferation response varied among all the groups. The highest level of proliferation was observed in the splenocytes of mice administrated with WNV E DIII protein in the presence of CpG followed by mice inoculated with WNV E DIII protein alone. In contrast, mice inoculated with PBS alone or CpG alone did not show significant proliferation of the T cells upon stimulation with WNV E DIII protein.

Furthermore, Th1- and Th2-type cytokine production by fresh splenocytes from all the immunized mice were measured. Supernatant from splenocyte cultures stimulated with the recombinant WNV E DIII protein in vitro were harvested, and the release of IL-2, IL-4, IL-6, and IFN-γ was assessed at different times after restimulation. The capacity of splenocytes to be stimulated in vitro and to produce cytokines was confirmed by production of cytokines in the presence of Con A (data not shown). Residual production from in vivo priming was evaluated in wells incubated with RPMI 1640 medium only (data not shown).

Splenocytes from mice receiving PBS alone or CpG adjuvant alone did not produce any significant cytokine titers after in vitro restimulation with WNV E DIII protein (Fig. 5). Splenocytes from mice immunized with recombinant WNV E DIII protein alone induced predominantly Th1-type cellular immune responses, as the levels of Th1 (IFN-γ and IL-2)-type cytokines (Fig. 5, a and b) were higher than that of Th2 (IL-4 and IL-6)-type cytokines (Fig. 5, c and d). IFN-γ production was the highest among the four cytokines being studied, reaching a concentration of 1402.67 ± 13.32 pg/ml on day 4 after restimulation (Fig. 5a), whereas IL-2 production was found to peak on day 4 with a concentration of 336.11 ± 32.52 pg/ml (Fig. 5b). Similarly, the splenocyte cultures

FIGURE 5. Cytokine profiling of splenic cells from immunized mice. Quantification of IFN-α (a), IL-2 (b), IL-4 (c), and IL-6 (d) production by splenic cells from mice immunized with PBS alone, CpG alone, WNV E DIII protein, or WNV E DIII protein with CpG upon stimulation with WNV E DIII protein for a period of 5 days. Splenic cells from mice immunized with WNV E DIII protein and WNV E DIII protein + CpG produced significantly higher concentration of both IFN-α and IL-2 in the presence of recombinant WNV E DIII protein stimulation. Compared with the control mice inoculated with PBS, significant differences in the induction of the cytokines are observed.
of mice that received the recombinant WNV E DIII protein in combination with CpG adjuvant also produced a more Th1-like cytokine profile. However, much higher concentrations of IL-2 and IFN-γ (Fig. 5, a and b) were observed within 3 days after restimulation with the recombinant WNV E DIII protein. In addition, low levels of Th2 cytokines, IL-4, or IL-6 (Fig. 5, c and d) were detected on days 4 and 5 after restimulation from the splenocyte cultures of WNV E DIII protein alone or WNV E DIII protein and CpG.

Nevertheless, the cytokine profiling study has shown that the administration of mice with recombinant WNV E DIII protein resulted in the induction of specific and potent Th1-type cellular immune responses in the spleens of immunized mice. This specific cellular response was further enhanced in the presence of CpG adjuvant.

**Discussion**

The current WNV epidemic is predicted to continue over the next few years as the virus expands its geographic distribution. This significantly impacts both human and veterinary concerns. With no specific treatment against WNV infection, precedence is given to developing an effective vaccine against it. A number of WNV vaccine candidates had been evaluated in animal models and approved for use on horses (17–22). These include an inactivated WNV horse vaccine and a recombinant vaccine using the canarypox virus to express WNV Ags (18, 19). Another study used a chimeric live virus incorporating the NY99 WNV prM (precursor membrane protein), and the E gene inserted into the infectious clone backbone of the yellow fever or dengue virus serotype 4 have been constructed and serotype 4 and is now pending evaluation in clinical trials (21–22). However, the risk of immune enhancement of heterologous flavivirus infection with inactivated virus and the legitimate safety concerns with the chimeric flaviviruses are highly debatable. Recently, an elegant study by Oliphant et al. (10) demonstrated the feasibility of using humanized mAb with therapeutic potential against WNV infection in mice. The humanized mAb was mapped to an epitope localized to the E protein or any virus particle. Domain III of the WNV E protein has been documented recently to serve as the domain of the E protein that binds to cellular receptor integrin αvβ3 (6). Neutralizing Abs against WNV have been mapped to epitopes localized on the WNV E DIII (7–11). Therapeutic trials in mice with humanized anti-WNV E DIII Ab have also shown to be highly effective in preventing WNV infection (9, 10), suggesting that the recombinant WNV E DIII protein is an attractive vaccine candidate. In this study, WNV E DIII protein was easily expressed in large quantity as a soluble protein in E. coli and maintains proper folding of the native structure. Furthermore, it induced the production of neutralizing Abs and protective immunity in mice against WNV infection and encephalitis. Cross-protective immunity was also generated against JEV infection with the recombinant WNV E DIII protein. This result is not surprising and is consistent with previous studies that showed the existence of cross-protection given the high similarity in the structure between WNV and JEV (17, 23).

The use of recombinant WNV E DIII protein as a WNV vaccine candidate also offers other advantages over existing WNV immunization strategies. First, the recombinant WNV DIII protein does not involve the use of attenuated or chimeric viruses, hence excluding the risk of the virus undergoing recombination and changing of tropism, which could result in increased pathogenicity. However, the potential risk of endotoxin contamination has to be taken into serious consideration with bacterial-based vaccine. To ensure that the purified WNV E DIII protein was not contaminated by the presence of endotoxin, a chromogenic assay for detecting endotoxin in the recombinant viral protein preparation was conducted. No significant level of endotoxin contamination was detected in the purified recombinant WNV E DIII protein used for immunization (data not shown). Therefore, the safety issue using recombinant WNV E DIII protein for immunization is addressed.

The suitability of using CpG DNA as an adjuvant to enhance the protective effectiveness of the recombinant WNV subunit vaccine was also evaluated in this study. Immunostimulatory CpG DNA has been well documented to improve the functional activity of professional APCs by triggering the production of cytokines and chemokines that supports the development of adaptive immune responses (24). Our experiments show that coadministering CpG adjuvant with WNV E DIII protein boosted Ab production significantly and triggered the preferential production of IgG2a. The coadministration also increased the production of Th1 cytokines (IFN-γ and IL-2) as compared with mice immunized with WNV E DIII protein alone. The increased magnitude of immune response contributed by the CpG adjuvant is of particular importance for the development of Ag-specific CTL that protects vaccinated individuals from WNV infection. Ongoing clinical studies to evaluate the safety and activity of CpG adjuvant in humans are being conducted. Currently, available results from the clinical trials suggest that the CpG adjuvant is safe and useful in boosting the immunogenicity of coadministered vaccines (24). The approach of coadministering recombinant flavivirus E domain III protein with CpG adjuvant may hold promise in the future development of an effective vaccine for flaviviruses.

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**Disclosures**

The authors have no financial conflict of interest.

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