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A plant-produced vaccine protects mice against lethal West Nile virus infection without enhancing Zika or dengue virus infectivity

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ABSTRACT

West Nile virus (WNV) has caused multiple global outbreaks with increased frequency of neuroinvasive disease in recent years. Despite many years of research, there are no licensed therapeutics or vaccines available for human use. One of the major impediments of vaccine development against WNV is the potential enhancement of infection by related flaviviruses in vaccinated subjects through the mechanism of antibody-dependent enhancement of infection (ADE). For instance, the recent finding of enhancement of Zika virus (ZIKV) infection by pre-exposure to WNV further complicates the development of WNV vaccines. Epidemics of WNV and the potential risk of ADE by current vaccine candidates demand the development of effective and safe vaccines. We have previously reported that the domain III (DIII) of the WNV envelope protein can be readily expressed in *Nicotiana benthamiana* leaves, purified to homogeneity, and promote antigen-specific antibody response in mice. Herein, we further investigated the *in vivo* potency of a plant-made DIII (plant-DIII) in providing protective immunity against WNV infection. Furthermore, we examined if vaccination with plant-DIII would enhance the risk of a subsequent infection by ZIKV and Dengue virus (DENV). Plant-DIII vaccination evoked antigen-specific cellular immune responses as well as humoral responses. DIII-specific antibodies were neutralizing and the neutralization titers met the threshold correlated with protective immunity by vaccines against multiple flaviviruses. Furthermore, passive administration of anti-plant DIII mouse serum provided full protection against a lethal challenge of WNV infection in mice. Notably, plant DIII-induced antibodies did not enhance ZIKV and DENV infection in Fc gamma receptor-expressing cells, addressing the concern of WNV vaccines in inducing cross-reactive antibodies and sensitizing subjects to subsequent infection by heterologous flavivirus. This study provides the first report of a WNV subunit vaccine that induces protective immunity, while circumventing induction of antibodies with enhancing activity for ZIKV and DENV infection.

1. Introduction

West Nile virus (WNV) is a member of the genus *Flavivirus* in the family *Flaviviridae,* and shares a high degree of sequence similarity to dengue virus (DENV), Zika virus (ZIKV), tick-borne encephalitis virus (TBEV), and yellow fever virus (YFV) [1]. For example, WNV shares an overall genome structure with these flavivirus and 84%, 66%, 59%, and 52.3% nucleotide sequence identity with TBEV, DENV-2, ZIKV, and YFV, respectively [2,3]. WNV entered into the Western hemisphere in the United States (US) in 1999, with cases also described in Canada, the Caribbean and Latin American regions [1]. Majority of WNV infection in humans is asymptomatic. Symptomatic WNV infection can cause malaise, fever, and a maculopapular rash, while neuroinvasive disease symptoms include encephalitis, meningitis, and/or possible death [1]. The elderly, individuals who are immunocompromised, or those who carry certain genetic factors are at a higher risk of developing life-threatening neurological diseases [4,5]. In recent years, outbreaks of WNV have become more frequent and severe with higher instance of patients with neuroinvasive complications [6]. However, currently there is no approved WNV vaccine for human use.

One of the challenges for WNV vaccine development is the increased risk of infection by related flaviviruses in vaccinated subjects due to the phenomenon of antibody-dependent enhancement of infection (ADE). ADE may occur between WNV and related flaviviruses such as DENV and ZIKV due to their high degree of

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genetic similarity and co-circulation in many parts of the world [7]. As a result, WNV vaccines based on conserved epitopes among related flaviviruses would have the potential to induce cross-reactive antibodies that augment entry and replication of DENV and ZIKV in Fc gamma receptor (FcγR)-expressing cells and lead to DENV or ZIKV infection in vaccinated subjects [8]. Indeed, mutual enhancement between WNV and ZIKV infections has been recently observed [7]. Thus, there is an urgent call for the development of WNV vaccines that are not only effective but also safe with a minimal risk of ADE to combat the threat of WNV infection on a global scale.

WNV Envelope (E) glycoprotein is a major target for the host antibody response and its domain III (DIII) contains the majority of type-specific neutralizing epitopes that elicit a strong host antibody response and/or protective immunity [9]. For approved human vaccines against flaviviruses YFV and TBEV, a neutralizing antibody response has been found to correlate with protection [10,11]. Neutralizing antibodies have also been demonstrated to play important roles in the protection against infection by other flaviviruses [12]. As a result, DIII has been explored as a promising WNV vaccine candidate and has been expressed in insect and bacterial cell cultures [13,14]. However, bacterial cell-produced DIII is insoluble and demands a solubilization and refolding process to be effective, which is not only cumbersome but also inconsistent in producing a recombinant DIII protein with native epitopes [14].

In our previous publication, we reported using a plant-based expression system to overcome these challenges, for a robust and scalable production of DIII as a promising WNV vaccine candidate [15]. We demonstrated that DIII was expressed at high levels in *Nicotiana benthamiana* plants within 4 days post-introduction of the DIII expression cassette. In contrast to *E. coli*-produced DIII, plant-produced DIII (plant-DIII) was soluble, and can be readily purified to >95% homogeneity without labor-intensive solubilizing and refolding processes [15]. We also demonstrated that immunization of plant-DIII elicited a potent antigen-specific antibody response in mice.

Here, we report a follow-up study of the efficacy of plant-DIII as a promising vaccine against WNV. In this study, we reveal that plant-DIII can also elicit antigen-specific cellular and humoral immune responses, while demonstrating DIII-specific antibodies (anti-plant DIII) neutralized WNV with a neutralization titer threshold that correlated with protective immunity of other known flavivirus vaccines. Importantly, passive transfer of anti-plant DIII serum protected 100% of mice against a lethal WNV challenge. Notably, anti-plant DIII antibodies did not enhance infection of ZIKV and DENV in Fc gamma receptor (FcγR)-expressing cells, offsetting the concern of WNV vaccines in inducing cross-reactive antibodies and sensitizing people to subsequent infection by heterologous flaviviruses. In brief, our plant-DIII based vaccine can effectively prevent WNV infection, along with offering improved safety, and purification efficiency compared to alternative vaccine candidates in development.

2. Material and methods

2.1. Ethics statement and biosafety

All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees at The University of Southern Mississippi (USM). All the *in vitro* experiments and animal studies involving live WNV were performed by the certified personnel in biosafety level 3 (BSL3) laboratories following standard biosafety protocols approved by the USM Institutional Biosafety Committees (IBC). Experiments with ZIKV and DENV were conducted with standard biosafety protocols approved by the IBC of Arizona State University by the certified personnel in biosafety level 2 (BSL2) laboratories.

2.2. Production of WNV envelope protein DIIII in N. Benthamiana plants

Plant expression vectors for WNV E DIII [15] was agroinfiltrated into leaves of *N. benthamiana* plants as described previously [16–22]. Leaves were harvested 4 days post agroinfiltration (dpi) and DIII was extracted and purified with Ni^{2+} immobilized metal affinity chromatography (IMAC) as previously described [15]. Details of these methods are provided in Supplementary material.

2.3. Mouse immunization

Five-week old female BALB/c mice were divided into 2 groups (n = 6 per group). Group 1 received saline buffer (PBS) with aluminum hydroxide gel (alum, InvivoGen, CA) as mock immunized controls and groups 2 received 25 μg of plant-DIII per dosage. On day 0, each mouse was injected subcutaneously with 100 μl PBS (Group 1) or 100 μl material containing 25 μg purified DIII protein (Group 2) in PBS with alum as adjuvant (DIII protein solution: alum volume ratio = 1:1). Mice were boosted three times (days 21, 42 and 63 post-immunization) with the same dosage and immune protocol as in the 1st immunization. Retro-orbital blood samples were collected on day 0 before the immunization (pre-immune sample) and on days 14 (week 2), 35 (week 5), and 56 (week 8) after the 1st immunization. Mice were humanely euthanized on day 77 (week 11), final blood samples were collected, and the spleens were aseptically removed for *in vitro* splenocyte cultures.

2.4. In vivo passive antibody transfer protection experiments

Serum isolated from PBS or plant DIII-immunized mice at week 11 was heat-inactivated for 30 min at 56 °C and stored at −80 °C. 5-week old female BALB/c mice were divided into 3 groups (n = 10 per group). Groups 1 and 2 received 50 μl of serum from PBS or plant DIII-immunized mice, respectively. Group 3 received 10 μg of the monoclonal antibody (mAb) E16 [23] as a positive control. Mice anesthetized with 30% isoflurane were passively administered serum or E16 mAb via r.o. injection 1 h before intraperitoneal (i.p.) inoculation with 10^2 plaque-forming units (PFU) of WNV (CT2741, kindly provided by Dr. John F. Anderson at the Connecticut Agricultural Experiment Station) in 1% gelatin. Mice were monitored for survival for 25 days post infection. The survival curves were constructed using data from two independent experiments.

2.5. Splenocyte culture and cytokine production

Spleens were isolated and mechanically dissociated to prepare single-cell from immunized mice and supernatants from splenocyte cultures were then collected to determine cytokine production as described previously [24]. Details of these methods are provided in Supplementary material.

2.6. Plaque reduction neutralization test (PRNT) assay

WNV-specific neutralizing antibodies were measured with a PRNT assay according to our previous report with minor modifications [25–27]. Neutralizing antibody titers were expressed as the reciprocal of the highest dilution of serum that neutralized ≥50% of WNV. Details of the PRNT method are provided in the Supplementary material.

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