Evaluation of RepliVAX WN, a Single-Cycle Flavivirus Vaccine, in a Non-Human Primate Model of West Nile Virus Infection

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Abstract. West Nile virus (WNV) causes serious neurologic disease, but no licensed vaccines are available to prevent this disease in humans. We have developed RepliVAX WN, a single-cycle flavivirus with an expected safety profile superior to other types of live-attenuated viral vaccines. In this report we describe studies examining RepliVAX WN safety, potency, and efficacy in a non-human primate model of WNV infection. A single immunization of four rhesus macaques with RepliVAX WN was safe and elicited detectable neutralizing antibody titers and IgM and IgG responses, and IgG titers were increased in two animals that received a second immunization. After challenge with WNV, three of four immunized animals were completely protected from viremia, and the remaining animal showed minimal viremia on one day. In contrast, the unvaccinated animal developed viremia that lasted six days. These results demonstrate the efficacy and safety of RepliVAX WN in this primate model of WNV infection.

INTRODUCTION

West Nile virus (WNV) was introduced into the United States in 1999 and has since become endemic in North America. Although WNV infection is usually asymptomatic, it can cause illness including WN encephalitis (WNE) and to date has resulted in more than 1,100 deaths in the United States.¹ Although a number of vaccines have been licensed to prevent WN disease in livestock,^{2–4} translation of these technologies into products for human use has proven difficult.⁵ Vaccine candidates based on chimeric live-attenuated viruses,⁶ protein subunits,⁷ and DNA preparations⁵ are currently in development, however none have been approved for use in humans. Thus, there remains a need for new vaccines.

West Nile virus is a member of the family *Flaviviridae* and the genus *Flavivirus* and has a single-stranded, positive-sense RNA genome that codes for three structural (capsid [C], pre-membrane/membrane [prM/M], and envelope [E]) and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. Although the structural proteins of WNV are required for production of viral particles, they are not necessary for genome replication. In addition to infectious virions, flavivirus-infected cells release sub-viral particles (SVPs). These particles are smaller than virions, but contain the antigenically important E protein and the prM/M protein, which is essential for correct folding and incorporation of the E protein into SVPs and viral particles.⁸ However, unlike virions, SVPs do not contain either the C protein or the viral genome, and are thus non-infectious. SVPs can be produced in a variety of systems by co-expression of the prM and E proteins,^{8,9} and have repeatedly been shown to stimulate protective immune responses against a number of flavivirus diseases.^{10–13}

We have previously described the construction of RepliVAX WN, a rationally attenuated, single-cycle virus vaccine to prevent WNE.^{14,15} The RepliVAX WN genome contains a large deletion in the gene encoding the C protein in an otherwise

complete WNV genome. RepliVAX WN can be propagated in cells expressing the WNV C protein,¹⁵ and when used for immunization each RepliVAX WN particle infects a single cell in which the genome undergoes multiple rounds of replication, resulting in the sustained production of WNV antigens (SVPs and NS1) without producing infectious progeny. Thus, RepliVAX WN demonstrates remarkable safety by producing a limited infection, yet is surprisingly potent. Vaccination with as little as 40,000 infectious units (IUs) completely protected mice¹⁵ and hamsters¹⁶ from WN disease. The T cell responses in RepliVAX WN-vaccinated mice are similar to those induced by WNV infection, and initial studies indicate immunization with RepliVAX WN can protect immunocompromised mice from lethal WNV challenge (Nikolich-Zugich, J. and others, unpublished data), suggesting that RepliVAX WN has the potential to be safe and effective in high-risk populations.

Here we report the initial evaluation of safety, potency, and efficacy of RepliVAX WN in a non-human primate (NHP) model of WNV infection. A single immunization with 10⁶ IU of RepliVAX WN was well-tolerated and induced antibody responses at levels known to correlate with protective immunity against flavivirus disease in humans.^{17–19} A second vaccination administered to half of the animals produced an enhanced WNV-specific antibody response, and upon challenge with WNV, three of four vaccinated animals were completely protected from WNV viremia. After challenge, immunized animals demonstrated a robust recall antibody response, and all animals displayed increased levels of activated dendritic cells (DCs) and T cells. These results demonstrate RepliVAX WN safety and efficacy in this NHP model of WNV infection.

MATERIALS AND METHODS

Cell lines and viruses. Vero(VEErep/Pac-Ubi-C*) used for RepliVAX WN production and Vero cells used for virus quantification have been described.¹⁵ The RepliVAX WN used for this study (RepliVAX WN.2 SP)¹⁵ was produced in Vero(VEErep/Pac-Ubi-C*) maintained in serum-free medium (SFM; OptiPro; Invitrogen, Carlsbad, CA, supplemented with 10 mM HEPES). Briefly, *in vitro* transcribed RepliVAX WN RNA was electroporated into BHK(VEErep/Pac-Ubi-C*)

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cells,¹⁵ and this RepliVAX WN harvest was used to infect Vero(VEErep/Pac-Ubi-C*) cells. The clarified RepliVAX WN harvest from Vero(VEErep/Pac-Ubi-C*) cells was enumerated on wild-type Vero cells,¹⁵ diluted in SFM to 10^6 IU/500 μ L, and used for immunization. Challenge studies were performed using WNV NY99 passaged once in Vero E6 cells. Virus neutralization assays were performed using a snowy owl isolate of WNV NY99.²⁰

Non-human primate manipulations. The NHP studies were conducted at the Southwest Foundation for Biomedical Research (SFBR, San Antonio, TX) and undertaken using protocols reviewed and approved by the Institutional Animal Care and Use Committee and the Institutional Biohazards Committee of the SFBR. Five male, 30–42-month-old rhesus macaques (*Macaca mulatta*) of Indian origin were selected from an SPF colony at SFBR based on results of hemagglutination inhibition (HAI) assays that demonstrated a lack of serological responses to WNV, yellow fever virus, Japanese encephalitis virus (JEV), St. Louis encephalitis virus, or any dengue virus serotype. Two weeks before vaccination, animals were transferred to an animal biosafety level 2 (ABSL-2) laboratory at SFBR. On day 0 of the study, four animals (26337, 20067, 20075, and 26506) were vaccinated with 10^6 IU of RepliVAX WN delivered in a volume of 500 μ L subcutaneously in the upper arm and the fifth animal (19854) received a mock vaccination of clarified SFM from uninfected Vero(VEErep/Pac-Ubi-C*) cells. To assess vaccine safety, vital signs, behavior, cognitive ability, hematologic parameters, and blood parameters of all animals were monitored after vaccination. On day 42, two animals (26337 and 20067) received a second vaccination with an identical dose of RepliVAX WN and the remaining three animals (20075, 26506, and 19854) were administered a mock vaccination. All animals were then transferred to the ABSL-3 facility at SFBR where they were challenged on study day 56 with 10^5 plaque-forming units of WNV NY99 delivered in a volume of 500 μ L subcutaneously in the upper arm. After virus challenge, animals were sedated and blood samples were collected at predetermined time intervals to determine cellular immune responses, serologic responses, and virus titers, which were used as the basis for determining vaccine potency and efficacy. On study day 89, all animals were humanely euthanized and tissues were harvested for histopathologic examination.

IgM and IgG ELISAs. West Nile virus-specific IgM and IgG levels in serum samples were analyzed by using the WNV IgM Capture DxSelect and IgG DxSelect ELISA kits (Focus Diagnostics, Cypress, CA) according to manufacturer's protocols. Serum samples collected from study animals before immunization (pre-immune); 7, 14, and 28 days post-primary vaccination; 14 days post-booster immunization (56 days post-primary vaccination); and 1, 4, 8, and 22 days post-challenge were tested at a 1:100 dilution. Results are reported as the average optical density (OD) observed at 450 nm in duplicate wells, and values are corrected for background activity detected from wells that received sample diluent containing no animal serum. Animals were considered to have detectable levels of antibody when titers exceeded those observed from pre-immune samples. An IgM positive control sample provided with the assay kit had an OD of 0.84, and the negative control sample had an OD of 0.01. For IgG, a positive control sample provided with the assay kit had an OD of 1.00, and the negative control sample had an OD of 0.07. All values for

vaccinated animals were above negative levels, but below ODs for positive control samples.

Detection of viremia. Virus in serum samples was quantified by using a modification of a focus-formation assay previously described.²¹ This modified protocol was optimized and validated by demonstrating that at the dilutions tested in this study, normal macaque serum did not inhibit focus formation. Briefly, duplicate Vero cell monolayers in 12-well plates were overlaid with 0.2 mL of 1:3 dilutions of serum in culture fluid. After a one-hour adsorption, cells were overlaid with minimal essential medium containing 0.6% tragacanth (MP Biomedicals, Solon, OH), 1% fetal bovine serum, and 10 mM HEPES and incubated for 48 hours at 37°C. After fixation with 50% acetone–50% methanol, foci were visualized by immunostaining, counted, and the infectious titers were calculated and expressed in focus forming units per milliliter (FFU/mL) as previously described.¹⁵

Neutralization assays. Neutralizing antibody titers were determined on Vero cells by using a focus-reduction assay.¹⁴ Briefly, serial two-fold dilutions (ranging from 1:8 to 1:128) of heat-inactivated serum were incubated with approximately 150 FFU of WNV NY99 for one hour at 37°C and then inoculated onto Vero cell monolayers in 24-well plates. Serum samples from each study animal obtained before immunization (pre-immune) were also tested to assess levels of non-specific neutralization. As with detection of viremia, after a one-hour adsorption, cells were overlaid with minimal essential medium/tragacanth and incubated for 40 hours at 37°C. After fixation, foci were visualized by immunostaining as previously described.¹⁵ Neutralizing antibody titers for each animal were reported as the highest serum dilution yielding 50% or 80% reduction in focus number compared with those obtained from virus incubated with pre-immune serum of the corresponding animal.²² Serum from an animal eliminated from our study because of prior exposure to WNV was included as a positive control in these assays, and was observed to have a 50% neutralizing titer of 1:1,024, which is in good agreement with post-WNV challenge neutralization titers observed in other animals (see Results).

Hemagglutination inhibition assays. The hemagglutination inhibition (HAI) titers were determined by a modification of the Clarke and Casals method²³ using gander erythrocytes (Lampire Biological Laboratories, Pipersville, PA). Serum samples collected before immunization (pre-immune); 7, 14, and 28 days post-primary vaccination; 14 days post-booster immunization (56 days post-primary vaccination); and 22 days post-challenge were tested at serial two-fold dilutions ranging from 1:8 to 1:1,024. To evaluate the relative contribution of IgM to HAI titers, tests on 7 and 14 day post-vaccination samples were run in parallel with a portion of the serum treated with an equal amount of 0.2M 2-mercaptoethanol (2-ME) for one hour at 37°C to destroy pentameric IgM molecules.²⁴

Flow cytometry. Antibodies used for six-color flow cytometry assays (Supplementary Table 1, available at www.ajtmh.org) were specific for human antigens, but were determined to cross-react with rhesus macaque cell surface proteins. Whole blood in EDTA was incubated with a panel of fluorochrome-conjugated monoclonal antibodies for 30 minutes at room temperature. Erythrocytes were eliminated by a standard whole-blood lysis method (BD PharmLyse Solution; BD Biosciences, San Jose, CA), and the remaining cells were

washed twice with phosphate-buffered saline and fixed with 1.6% methanol free-formaldehyde/phosphate-buffered saline (Polysciences Inc., Warrington, PA). Cells were refrigerated until data were acquired by using a CyAn™ ADP instrument (Beckman Coulter Inc., Fullerton, CA) equipped with lasers of 405, 488, and 635 nm excitation lines. Electronic compensation and analyses were done using Summit version 4.3 software. For general phenotypes, 10,000 events were acquired, but for DC analyses, up to 100,000 events were recorded. The DCs were identified as lineage (CD2, CD3, CD14, and CD20)[−] HLA-DR⁺ cells, and further divided into myeloid (CD11c⁺) or plasmacytoid (CD123⁺); T cells were identified as CD3⁺ and further divided into CD4⁺ or CD8⁺ cells.

Statistical analyses. Statistical analyses were carried out using Graphpad (San Diego, CA) Prism Analysis Software. Data were analyzed using one-way analysis of variance with Bonferroni's post-test (antibodies) or unpaired *t*-test (viremia).

RESULTS

Safety of RepliVAX WN vaccination in non-human primates.

The rhesus macaque model of WNV infection^{25–27} was chosen to assess the safety and efficacy of RepliVAX WN in NHPs. Although this model does not produce clinically apparent disease, sustained levels of WNV viremia are detectable in the sera of infected animals for up to five days post-challenge,²⁵ and animals develop detectable immune responses to vaccination and challenge. Five male rhesus macaques (30–42 months of age) were chosen for study based on preliminary HAI serologic analysis that confirmed lack of previous exposure to medically important mosquito-borne flaviviruses. Four of these animals (26337, 20067, 20075, and 26506) were injected subcutaneously in the upper arm with 10⁶ IU of RepliVAX WN and one animal (19854) received a mock injection by the same route. No significant fluctuations in body weight or temperature were observed in any of the animals as a result of vaccination (Supplementary Figure 1, available at www.ajtmh.org). Clinical observation showed no changes in food or water consumption, appearance, or general attitude in any animals after RepliVAX WN vaccination. Heart rates and respiration rates of all animals remained normal, all blood chemistry tests yielded results within normal limits, and no clinical signs of cognitive or neural impairment were observed in any of the vaccinated animals. These results indicate that RepliVAX WN vaccination is safe and well-tolerated by NHPs.

Induction of WNV-specific IgM and IgG responses by one dose of RepliVAX WN. Humoral immunity is believed to play a primary role in clearing flavivirus infections^{28–31} and provides an excellent predictor of vaccine efficacy.^{17,32,33} To assess the potency of RepliVAX WN in NHPs and determine the kinetics of RepliVAX-induced antibody production, WNV-specific IgM and IgG levels were analyzed throughout the study. The IgM antibody responses measured by ELISA or HAI were detectable in all four vaccinated animals by day 7, and these responses generally increased by day 14 post-vaccination (Figure 1A and Table 1). In three of the four vaccinated animals (26337, 26506, and 20075), WNV-specific IgM responses were significantly greater ($P < 0.05$) on day 14 than on day 7 (Figure 1A). The presence of WNV-specific IgM was also apparent from the presence of 2-mercaptoethanol-sensitive

HAI activity in serum (Table 1). By day 14 post-vaccination, low but detectable levels of IgG were observed in 2 of these animals by detection of 2-mercaptoethanol-resistant HAI antibody titers of 1:8 (Table 1). At 28 days post-vaccination, 50% neutralizing antibody and HAI titers were 1:32–1:64 in all vaccinated animals, and these primates demonstrated detectable 80% neutralization titers (Table 1). Similar kinetics of antibody response were obtained using an ELISA to detect WNV-specific IgG (Figure 1B). The induction of WNV-specific antibody responses after one immunization with RepliVAX WN indicates its potential utility as a single-dose vaccine.

Increased antibody titers in animals given booster immunizations with a second dose of RepliVAX WN. Two of the four vaccinated animals (26337 and 20067) were chosen at random to receive a second vaccination subcutaneously with 10⁶ IU of RepliVAX WN 42 days after primary vaccination. The remaining three animals (20075, 26506, and 19854) received a mock vaccination. As with primary vaccination, no adverse clinical responses were observed after the second vaccination. Fourteen days after these inoculations, WNV-specific IgG levels were significantly higher (3–4-fold; $P < 0.01$) in animals given a booster immunization relative to those animals that received only one vaccination (Figure 1B). However, the effect of the booster immunization was not accompanied by a detectable increase in neutralizing antibody titers (Table 1). The 50% neutralizing antibody titers of the two animals given booster immunizations were 1:32 and 1:128 (compared with 1:32 and 1:64 28 days after primary vaccination), and titers of the animals that received one dose of RepliVAX WN decreased slightly from 1:32 on day 28 to 1:16 by day 56 (Table 1), a trend corroborated by measurement of total WNV-specific IgG levels (Figure 1B). Although these results indicate that a second dose of RepliVAX WN induces higher levels of IgG, this did not appear to correlate with a detectable increase in neutralizing activity.

Protection of rhesus macaques from WNV viremia by one or two immunizations with RepliVAX WN. Fifty-six days after primary vaccination (14 days after booster immunization in animals that received two vaccinations), all NHPs in this study were challenged with a subcutaneous inoculation of 10⁵ PFU of WNV NY99. Clinical observations were performed daily after challenge, and as expected from previous studies using this model,^{25,26,34} no clinical signs of WNV disease were detected in any animals during the 33 day post-challenge observation period. However, data from viremia analyses confirmed the protective efficacy of RepliVAX WN vaccination. The unvaccinated animal (19854) developed a WNV viremia of greater than 100 FFU/mL that persisted for four days (Figure 2), which was consistent with data obtained from a cohort of four unvaccinated rhesus macaques previously challenged under conditions identical to those used in this study (Carrión Jr, R. and others, unpublished data). Analysis of WNV viremia showed that in both animals that received two doses of RepliVAX WN (26337 and 20067), and in one receiving a single dose (20075), infectious WNV was not detectable in the serum at any point in time after challenge (Figure 2). Furthermore, the single vaccinated animal in which viremia was detected (26506) demonstrated an extremely low level of virus (7.5 FFU/mL) for only one day, immediately after challenge. Thus, the viremia detected in this animal appeared to be caused by the inoculum, and the overall viral load in this animal was significantly lower ($P < 0.02$) than that

FIGURE 1. West Nile virus-specific antibody levels determined by enzyme-linked immunosorbent assay. **A**, IgM levels in the serum of non-human primates (NHPs) on the day of vaccination (pre-immune), days 7 and 14 post-vaccination, and days 1, 4, and 8 post-challenge. **B**, IgG levels in the serum of NHPs on the day of vaccination (pre-immune), day 28 post-vaccination, day 14 post-boost immunization, and days 8 and 22 post-challenge. Bars indicate average optical density values and extended bars indicate standard deviation between technical replicates.

observed in the unvaccinated animal in this study (19854; Figure 2), and also lower than that in four similarly challenged unvaccinated animals (Carrion Jr, R. and others, unpublished data).

Serum IgM and IgG levels were analyzed post-challenge to assess the ability of vaccinated animals to mount a humoral recall response upon challenge. When measured by ELISA, WNV-specific IgM was found to be relatively high in animals receiving two vaccinations and remained consistent throughout the eight days after WNV challenge. In contrast, animals receiving one dose of RepliVAX WN had relatively low levels of WNV-specific IgM one day after WNV challenge, but these levels increased on days 4 and 8 post-challenge, a trend not observed in the unvaccinated animal. All vaccinated animals, regardless of dose schedule, demonstrated robust levels of IgG against WNV at 8 days post-challenge (Figure 1B). These levels were significantly higher ($P < 0.001$) than that observed in the unvaccinated primate. The increase in IgG levels observed in the singly vaccinated animals, similar to the observed increases in IgM levels over the same post-challenge period, likely indicate that these levels represent a humoral recall response to RepliVAX WN vaccination. However, by 22 days post-challenge, the unvaccinated primate had developed IgG titers comparable to those observed in vaccinated animals (Figure 1B), and all animals demonstrated similar 50% neutralizing antibody titers (Table 1). Interestingly, the vaccinated animal that showed a detectable viremia had a post-challenge neutralizing antibody titer of 1:1,024, which was in excess of all other study animals.

Analyses of cellular responses to RepliVAX WN vaccination and WNV infection. Flow cytometry was performed to identify changes in DC and T cell number and activation status after vaccination with RepliVAX WN and challenge with WNV. In general, vaccination did not induce detectable changes in the levels of circulating DC subsets (Figure 3A). There were marked transient changes in the levels of DCs immediately after WNV challenge with myeloid DC (MDC), and in particular plasmacytoid DC (PDC) numbers increasing (Figure 3A, left). Furthermore, MDCs and PDCs demonstrated a marked increase in the levels of CD86 expression after challenge with WNV, with PDCs showing a greater fluctuation compared with pre-challenge levels (Figure 3A). Circulating numbers of these cells and their levels of activation returned to pre-challenge values by one week post-challenge.

Similarly, vaccination failed to elicit detectable changes in overall T cell numbers or their activation status (Figure 3B), but circulating levels of T cell subsets changed as a result of challenge with WNV, although these fluctuations were not as dramatic as those observed for DCs. Moderate increases in CD4⁺ and CD8⁺ T cells were observed after WNV challenge (Figure 3B), whereas changes for natural killer cells and B cells were minimal. Activation of T cells, as measured by expression of CD69, was evident after challenge, particularly in the case of CD4⁺ T cells (Figure 3B). Interestingly, although the unvaccinated primate (19854) and animals that received one RepliVAX WN vaccination (26506 and 20075) showed similar levels of CD8⁺ T cell activation after challenge, animals that received two doses of RepliVAX WN (20067 and 26337)

TABLE 1
Comparison of RepliVAX WN and West Nile virus-induced antibody titers*

Animal	Vaccination schedule (booster immunization)†	Antibody titer									
		Pre-immune		Day 7 post-immunization		Day 14 post-immunization		Day 28 post-immunization		Day 14 post-booster immunization	
		HAI		HAI	HAI + 2-ME‡	HAI	HAI + 2-ME‡	50% Neut (80% Neut)	HAI	50% Neut (80% Neut)	HAI
26337	RepliVAX WN/RepliVAX WN	< 1:8	1:8	< 1:8	< 1:8	1:32	< 1:8	1:64 (1:32)	1:64	1:128 (1:64)	1:64
20067	RepliVAX WN/RepliVAX WN	< 1:8	1:16	< 1:8	< 1:8	1:32	1:8	1:32 (1:16)	1:32	1:32 (1:8)	1:32
20075	RepliVAX WN/mock	1:8	1:16	< 1:8	< 1:8	1:64	1:8	1:32 (1:8)	1:64	1:16 (< 1:8)	1:16
26506	RepliVAX WN/mock	< 1:8	1:8	< 1:8	< 1:8	1:32	1:8	1:32 (1:8)	1:64	1:16 (< 1:8)	1:16
19854	Mock/mock	1:8	1:8	< 1:8	< 1:8	< 1:8	< 1:8	< 1:8 (< 1:8)	1:8	< 1:8 (< 1:8)	1:8

* HAI = hemagglutination inhibition; 2-ME = 2-mercaptoethanol; Neut = neutralization.
† All RepliVAX WN inoculations were 10⁶ infectious units, culture fluid, used as a mock inoculum, was recovered from uninfected Vero (VEErep/Pac-Ubi-C*), booster immunization was performed at 42 days post-immunization (see text).
‡ Differences between HAI titer obtained in the presence and absence of 2-ME can be used to infer West Nile virus-specific IgM titers (see text).

FIGURE 2. Serum viremia levels after challenge with West Nile virus (WNV). A focus formation assay was used to assess the amount of circulating infectious WNV in the serum of challenged animals at the indicated days after challenge with 10⁵ plaque-forming units of WNV NY99. Animals 26337 and 20067 received two vaccinations with RepliVAX WN before challenge, animal 26506 and 20075 received one dose of RepliVAX WN, and animal 19854 received a mock vaccination.

did not show generalized activation of CD8⁺ T cells after challenge (Figure 3B).

DISCUSSION

We have previously demonstrated that RepliVAX WN, a novel single-cycle live-attenuated virus vaccine, can be produced using technology compatible to human vaccine production.¹⁵ In the current study, we report initial evaluation of RepliVAX WN produced using these methods in an NHP model of WNV infection. The model selected, parenterally challenged rhesus macaques, displays signs of infection consistent with those that occur in most humans infected with WNV. Specifically, WNV infection of rhesus macaques is not associated with symptomatic disease or death. However, this model provides important tests of primate safety and potency, and these animals display a WNV viremia that persists for multiple days,²⁵ which enables viremia to be used as an endpoint for evaluating vaccine efficacy.^{26,34}

In our studies, vaccination with one or two doses of RepliVAX WN was well-tolerated by all of the animals with no adverse clinical or physiologic signs. These findings were not surprising because of the high level of attenuation of RepliVAX WN,¹⁴ which is consistent with its inability to produce a spreading infection. Moreover, this genetically restricted ability to produce spreading infection makes markers of attenuation used to evaluate traditional live-attenuated viral vaccine candidates such as vaccine viremia and neurovirulence largely unnecessary for RepliVAX WN. Nevertheless, no pathologic changes consistent with encephalitic flavivirus infection were detected in neural tissues examined post-challenge, which suggests that neither RepliVAX WN nor challenge virus were able to cause any serious neurologic problems.

The potency of RepliVAX WN was evaluated in single-dose and multi-dose vaccination regimens. After one vaccination with 10⁶ IU of RepliVAX WN, all NHPs developed WNV-specific IgM responses by 7 days, which increased sharply in three of four animals by 14 days post-vaccination. The kinetics of IgM appearance suggests a durable presentation of WNV

FIGURE 3. **A**, Flow cytometry analyses of circulating dendritic cells in rhesus macaques vaccinated with RepliVAX WN and challenged with West Nile virus (WNV). Four rhesus macaques were vaccinated with RepliVAX WN (solid arrows) on day 0, two animals received an additional RepliVAX WN vaccination on day 42 (20067 and 26337, dashed lines), and two other animals received a mock vaccination on day 42 (20075 and 26506, solid lines). A fifth macaque (19854) was mock vaccinated on days 0 and 42. All animals were challenged with WNV on day 56 (dashed arrow). Levels of circulating myeloid dendritic cells (MDCs) (top left) and plasmacytoid dendritic cells (PDCs) (bottom left) were determined, and activated MDCs (top right) and PDCs (bottom right) were determined using expression of CD86 as the indicator of DC activation. **B**, Flow cytometry analyses of circulating T lymphocytes in rhesus macaques vaccinated with RepliVAX WN and challenged with WNV. Levels of circulating CD4⁺ (top left) and CD8⁺ (bottom left) T cells, and the levels of activated CD4⁺ (top right) and CD8⁺ (bottom right) T cells were determined by flow cytometry using expression of CD69 was used as the indicator of cell activation.

antigens by RepliVAX WN-infected cells, and closely mirrored the kinetics of IgM responses observed in WNV-infected rhesus macaques.²⁵ The presence of 2-mercaptoethanol-resistant HAI activity by day 14 post-RepliVAX WN vaccination demonstrated the presence of WNV-specific IgG, and HAI and 50% neutralizing antibody titers increased by day 28 to levels (1:32–1:64) that exceed those believed to correlate with pro-

tective immunity against JEV in humans.^{17–19} Although high levels of circulating antibodies were elicited by RepliVAX WN vaccination, global activation and proliferation of DCs and T cells in the blood were not detected at the time points we sampled after primary or secondary vaccination.

Despite the robust immune response elicited by primary RepliVAX WN vaccination, two animals were selected to

receive a second identical RepliVAX WN dose to assess the usefulness of such a vaccination schedule. The ELISAs for WNV-specific IgG showed that this booster immunization increased antibody reactivity by 3–7-fold over pre-booster values, but neutralizing antibody assays did not detect a corresponding increase in neutralizing antibody titers. This finding could be a result of the induction of a population of non-neutralizing or weakly neutralizing antibodies resulting from administration of a booster dose. Although these antibodies may not be detected in virus neutralization *in vitro*, they may play a role in virus opsonization *in vivo* and are thus important in the context of protective immunity. Because of the small scale of this study, these results do not strongly support or contradict the usefulness of a second dose of RepliVAX WN, and therefore the optimal administration schedule for RepliVAX WN vaccination needs further investigation. Interestingly, the unvaccinated animal in this study demonstrated a robust 50% neutralizing antibody titer of 1:512 after WNV challenge, a substantially higher titer than that elicited 28 days after a single immunization with RepliVAX WN (1:32–1:64). This difference in antibody response, along with a relative lack of large-scale DC or T cell activation, can be attributed to the inability of the single-cycle RepliVAX WN to produce viremia that is observed in WNV infection, and highlights a key safety feature of RepliVAX WN over traditional live-attenuated viral vaccines.

After challenge with WNV, three of four vaccinated animals were completely protected from viremia, and the lone vaccinated animal that developed a detectable viremia displayed a titer of only 7.5 FFU/mL of serum at day 1 post-challenge, and WNV was undetectable in this animal on all other days tested. In contrast, WNV was detected in the serum of the unvaccinated animal at 48 times this level on day 1 post-challenge, remained at elevated levels (≥ 100 FFU/mL) through day 4, and was still detectable 6 days post-challenge. The results obtained with this unvaccinated animal closely mirror those observed previously in a cohort of four rhesus macaques challenged with an identical WNV preparation and provide further evidence of the reproducibility of this model and the effectiveness of RepliVAX WN in preventing WN viremia in NHPs.

Animals receiving one vaccination displayed a sharp increase in WNV-specific IgM and IgG levels eight days after WNV challenge that was not observed in the unvaccinated animal, which indicated that vaccination with RepliVAX WN resulted in formation of a memory B cell population capable of rapid activation by WNV infection. Flow cytometry was performed on blood samples collected during the post-challenge study period to examine the cellular response to acute WNV infection, and what role if any vaccination played in shaping these responses. Challenge with WNV resulted in an increase in the numbers of activated DCs and T cells, in particular PDCs, which produce high levels of interferon alpha when exposed to WNV.³⁵ Sharp increases in activated CD4⁺ and CD8⁺ T cells also were observed in nearly all animals. Interestingly, animals receiving two doses of RepliVAX WN did not show an increase in activated CD8⁺ T cells that was seen in all other animals. If this finding represents a repeatable observation, it may indicate that high levels of vaccine-induced circulating antibody along with innate immune responses could limit WNV infection enough to prevent large-scale activation of CD8⁺ T cells.

In conclusion, we have demonstrated that vaccination of rhesus macaques with RepliVAX WN was safe and well-tolerated,

and that one dose elicited humoral immune responses within ranges known to correlate with flavivirus vaccine efficacy.¹⁹ Administration of a second dose of RepliVAX WN to a subset of NHPs appeared to increase WNV-specific IgG levels compared with animals receiving a single dose. However, the overall benefit of a second vaccination was not clear. Upon challenge with WNV, three of four vaccinated primates were completely protected from WNV viremia, and all vaccinated animals demonstrated a strong anamnestic IgG response to WNV challenge. These findings were in sharp contrast to those detected in the challenged unvaccinated animal. This animal developed a sustained WNV viremia and displayed delayed IgM and IgG responses to challenge. Taken together with our previous studies demonstrating safety and efficacy of RepliVAX WN in two rodent models of WNV disease,¹⁵ the findings presented in this small-scale NHP study support the development of RepliVAX WN as a vaccine for use in humans.

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