Measles Vaccine Expressing the Secreted Form of West Nile VirusEnvelope GlycoproteinInduces Protective Immunity in SquirrelMonkeys, a New Model of West Nile VirusInfection

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West Nile virus (WNV) is a mosquito-borne flavivirus that emerged in North America and caused numerous cases of human encephalitis, thus urging the development of a vaccine. We previously demonstrated the efficacy of a recombinant measles vaccine (MV) expressing the secreted form of the envelope glycoprotein from WNV to prevent WNV encephalitis in mice. In the present study, we investigated the capacity of this vaccine candidate to control WNV infection in a primate model. We first established experimental WNV infection of squirrel monkeys (Saimiri sciureus). A high titer of virus was detected in plasma on day 2 after infection, and viremia persisted for 5 days. A single immunization of recombinant MV-WNV vaccine elicited anti-WNV neutralizing antibodies that strongly reduced WNV viremia at challenge. This study demonstrates for the first time the capacity of a recombinant live attenuated measles vector to protect nonhuman primates from a heterologous infectious challenge.

West Nile virus (WNV), an enveloped single-stranded RNA virus that belongs to the Flavivirus genus, circulates in natural transmission cycles that involve principally Culex mosquitoes and wild birds. Horses and humans are incidental hosts. Zoonotic WNV became a major health concern in North America, the Middle East, and Europe because of the emergence of a highly neurovirulent strain in North America in 1999, causing >1000 deaths [1]. Clinical manifestations range from uncomplicated fever to fatal meningoencephalitis. The emergence of virulent variants of WNV has been associated with a dramatic increase in the severity of infection in humans [2].

In the absence of treatment, a WNV vaccine is needed to control virus transmission and reduce disease in humans. A number of approaches are either already licensed for veterinary vaccines or under development for human vaccines. A recombinant WNV vaccine based on canarypox virus strategy has been licensed for use in horses [3, 4]. ChimeriVax-WN01, a live-attenuated WNV vaccine containing modified WNV premembrane (prM) and envelope (E) sequences inserted into the yellow fever 17D vaccine genome, has also been licensed for horse vaccination [5–7]. ChimeriVax-WN02, a vaccine designed for human use that contains 3 mutations introduced into the WN01 E gene, has been shown to be safe and immunogenic in phase II clinical trials [8]. RepliVAX, a vaccine based on single-cycle flavivirus particles induced durable protective immunity in rodents [9, 10]. A recombinant vesicular stomatitis virus (VSV)–based
WNV vaccine elicited humoral and cellular immune responses and protected mice from a WNV lethal challenge [11]. The protective efficacy of a recombinant subunit WNV vaccine based on the soluble form of the envelope E glycoprotein (WN.sE) has been demonstrated in rhesus monkeys challenged with WNV [12]. Nonintegrative lentiviral vectors expressing WN.sE also induced a robust protective humoral response in mice [13]. Other strategies include the production of the E or the recombinant EDIII of WNV antigens by insects cells that protected mice against WNV challenge [14].

In a previous study, we reported the efficacy of a recombinant measles vaccine (MV) expressing the secreted form of the envelope glycoprotein from the virulent IS-98-ST1 strain of WNV (MVSchw-sE<sub>WNV</sub>) to prevent WNV encephalitis in mice [15]. The vaccine induced a long-term protective memory composed of high levels of specific WNV neutralizing antibodies. The passive administration of antisera from immunized mice prevented WNV encephalitis in Balb/c mice, demonstrating the role of circulating neutralizing antibodies in protection. MV vaccine is a live-attenuated negative-stranded RNA virus. Produced on a large scale and distributed at low cost, this vaccine induces lifelong protective immunity after a single injection and has dramatically reduced measles childhood mortality by 90% during the last decades. Combined measles-arbovirus vaccines might be used to immunize both the pediatric and adult/adolescent populations in case of arboviral emergence. When given to the adult population, the MV vector would be an effective and safe carrier for the arbovirus vaccine, containing and preventing further such outbreaks. In this context, the use of recombinant measles vaccines might be valuable.

We have previously used the Schwarz strain of MV to develop a human immunodeficiency virus (HIV) vaccine that is currently in phase I clinical trials [16–18]. This vector is also used to develop a dengue vaccine, a closely related Flavivirus [19–21]. In the present work, we evaluated the protective efficacy of recombinant MVSchw-sE<sub>WNV</sub> to protect nonhuman primates (NHPs) from WNV infection. We first established experimental infection with WNV in squirrel monkeys (Saimiri sciureus). The data presented here show that WNV replicates in squirrel monkeys, showing that they can be used as a new NHP model for WNV infection. We then report the immunogenicity and efficacy of MVSchw-sE<sub>WNV</sub> to protect these animals from experimental WNV challenge.

**MATERIALS AND METHODS**

**Viruses**

WNV strain IS-98-ST1 (GenBank accession number AF 481864) [22], a closely related variant of NY99 strain [23], was propagated in AP61 cell monolayers of Aedes pseudoscutellaris mosquitoes [22]. Purification on sucrose gradients and virus titration on AP61 cells by focus immunodetection assay were performed as previously described [22, 24]. The recombinant MVSchw-sE<sub>WNV</sub> vaccine virus was prepared as previously described [15] and viral titer was determined by endpoint dilution assay on Vero cells.

**Animals**

Squirrel monkeys (S. sciureus) were purchased from Bioprim (Baziège, France). They were housed in the BSL-4 animal care facility in Lyon. The Région Rhône Alpes ethics committee approved experimental methods. The animals were male and female adults (0.8–1.4 kg). For the establishment of WNV challenge, 6 animals (IV1, IV2, IV3, IV4, IV5, IV6) were anaesthetized with ketamine hydrochloride (300 mg/kg) and infected by intravenous administration (saphenous vein) of 1.0 × 10<sup>5</sup> plaque-forming units (pfu) of WNV IS-98-ST1, delivered in a volume of 200 μL phosphate-buffered saline (PBS). Two control animals (M1, M2) were mock infected with the same volume of PBS. After infection, the animals were evaluated for changes in clinical signs and body weight. Blood samples were obtained on the following days from the anesthetized monkeys: days 0, 2, and 5 for IV1 and IV2; days 0, 3, 6, and 11 for IV3 and IV4; and days 0, 4, 9, and 12 for IV5 and IV6. Monkeys were euthanized at day 7 for IV1 and IV2, at day 14 for IV3 and IV4, and at day 15 for IV5 and IV6. Samples of blood, urine, spleen, kidneys, liver, intestine, heart, lung, salivary gland, inguinal lymph nodes (LNs), mesenteric LN, spinal cord (abdominal, cervical, thoracic), brain (frontal cortex, midbrain, distal cortex), cerebellum, and meninges were collected, and tissues were harvested for histopathologic examination and stored at −70°C.

To evaluate the immunogenicity of the vaccine candidate, 8 animals (3, 4, 5, 6, 9, 10, 11, 12) were inoculated intramuscularly with a single 200-μL dose containing 3.0 × 10<sup>5</sup> median tissue-culture infective doses (TCID<sub>50</sub>) of MVSchw-sE<sub>WNV</sub> vaccine virus. Two control animals (1, 2) received 200 μL of sterile PBS and two other control animals (7, 8) received empty MVSchw vaccine. After 15 days (9, 10, 11, 12) or 30 days (3, 4, 5, 6), animals were challenged by intravenous inoculation of 1.0 × 10<sup>5</sup> pfu of wild-type WNV IS-98-ST1 suspended in 200 μL PBS. Monkeys were monitored for viremia, clinical signs, and antibody response. Euthanasia was performed at day 10 for animals 1, 2, and 4; at day 11 for animals 3 and 5; at day 14 for animal 9, and at day 15 for animal 11. Samples of blood, spleen, liver, and inguinal and mesenteric LNs were collected, and tissues were harvested for histopathologic examination and stored at −70°C.

**Analysis of Humoral Responses**

Anti-WNV antibodies were detected in heat-inactivated sera by use of an enzyme-linked immunosorbant assay (ELISA) as
previously described [22]. Sera were considered positive when the optical density (OD) was twice the OD of sera from control animals. Anti-WNV neutralizing antibodies were detected using a plaque reduction neutralization test (PRNT) as previously described [15]. The end point neutralization titer was calculated as the highest serum dilution tested that reduced the number of plaques by at least 50% (PRNT50).

Detection of Viral RNA and Virus Titration
Sera (25 µL) were diluted in 0.5 mL Dulbecco’s modified Eagle’s medium (DMEM)/5% fetal calf serum (FCS), and organs were homogenized with glass beads in the same volume. Viral RNA was extracted (QIAamp viral RNA extraction kit,Qiagen) and analyzed by 1-step WNV real-time polymerase chain reaction (PCR) by using high fidelity enzyme (Roche, Mannheim, Germany) as described elsewhere [25]. Real-time quantitative amplification of WNV RNA was performed with RealArtTM WNV LC real-time PCR kit (Qiagen). For virus titration, samples were diluted in 250 µL medium, and infectivity of serial dilutions was assayed on Vero cells overlaid with DMEM Glutamax/2% FCS containing 0.8% final (weight/volume) carboxy methylcellulose. After 4 days of incubation, cells were fixed and crystal violet stained for plaque count determination.

RESULTS

WN IS-98-ST1 Virus Replicates in Squirrel Monkeys
Several NHP models have been evaluated for WNV infection. Among them, rhesus macaques and baboons develop WNV viremia without symptoms, except after intracranial inoculation [5, 9, 26–29]. To be in compliance with specific French recommendation for manipulating WNV in vivo in highly confined facilities, we developed a new NHP model for WNV infection based on a small animal that can be easily housed in small cages confined in BSL4 facilities. We choose the New World squirrel monkey (S. sciureus), weighing less than a kilo. This monkey was previously used as a model to study infections with Plasmodium falciparum and Plasmodium vivax [30], human T-cell leukemia virus [31], or Nipah virus [32].
We infected 6 animals (IV1, IV2, IV3, IV4, IV5, IV6) by intravenous administration of $1.0 \times 10^5$ pfu of WNV (IS-98-st1). The animals were clinically observed and blood samples were collected during the following 2 weeks. Animals were sacrificed at different time points to monitor the virus biodistribution in organs. No morbidity or mortality was observed. A slight increase of body temperature was observed in infected animals concomitant with the peak of viremia between days 2 and 3 and lasting maximum 2 days (mean temperature of 39.31°C at day 0, which is the average temperature of these monkeys, and mean temperature of 40.39°C at day 2 of infection). All blood chemistry analysis yielded results within normal limits. The presence of WNV RNA in serum samples was detected by quantitative real-time PCR. All monkeys became viremic after inoculation with WNV IS-98-ST1. The magnitude and presence of viremia are shown in Figure 1A. Viral RNA was detected in all monkeys on days 2, 3, and 4 after infection. The maximum amount of viral RNA in blood was detected on day 2 ($2.0 \times 10^6$ viral genomic RNA copies/mL). The presence of infectious virus in serum samples that tested positive with quantitative real-time PCR was assessed by plaque formation assay on Vero cells. The peak virus titer was detected on day 2 at $3.6 \times 10^4$ pfu/mL, and the viremia persisted until day 5 after infection (Figure 1B).

To identify the sites of virus replication in vivo, the biodistribution of WNV IS-98-ST1 was assessed in various organs at day 7, 14, or 15 after infection. Viral RNA was detected in the lymphoid organs of all infected animals (at day 7, 14, or 15 after infection): spleen (6 of 6 animals; $4.8 \times 10^5$ to $8.8 \times 10^6$ viral RNA copies/mL), inguinal LN (6 of 6 animals; $3.8 \times 10^4$ to $3.4 \times 10^5$ viral RNA copies/mL), and mesenteric LN (6 of 6 animals; $1.8 \times 10^4$ to $5.6 \times 10^4$ viral RNA copies/mL). Viral RNA was found in the liver only at day 7 after infection (2 of 6 animals; $4.4 \times 10^4$ to $7.0 \times 10^4$ viral RNA copies/mL), in the kidneys (1 of 4 animals; $9.2 \times 10^4$ viral RNA copies/mL), and in the lungs (1 of 4 animals; $1.2 \times 10^5$ viral RNA copies/mL). This biodistribution reflects the blood viremia and demonstrates a short viral persistence in these organs. No other organ was found positive; specifically, the brain, spinal chord, meninges, gut, and salivary glands remained negative. Unfortunately, because of the very short period allotted to us for use of the BSL4 facility, the complete viral clearance from lymphoid organs could not be followed over the long term. The same organs underwent histopathologic analysis. Some foci of infection were observed in the liver, with disorganization of the hepatic parenchyma, immune cells infiltration, and endothelial inflammation. The other histological sections appeared normal. WNV was shed in the urine (2 of 4 animals; $1.6 \times 10^6$ to $3.0 \times 10^6$ viral RNA copies/mL) detected at day 7.

To assess the induction of immunity to the virus, we determined the kinetics of WNV-specific immunoglobulin G (IgG) antibody production in sera from infected monkeys. The ELISA IgG antibody response was detectable in all infected animals. Specific WNV IgG was detected at significant levels as soon as 5 days after infection, and the antibody titers continued to increase over the next 10 days, while mock-infected animals remained negative (Figure 1C). All animals demonstrated 50% neutralizing antibody titers above 1:160 on the day of euthanasia (Table 1).

MVSchw-sE WNV Vaccine Candidate Is Immunogenic and Protects Squirrel Monkeys From WNV Challenge
To establish the preclinical proof-of-concept of the efficacy of recombinant MVSchw-sE WNV vaccine candidate in NHPs, we
immunized a group of 8 squirrel monkeys by administrating 1 intramuscular dose of MVSchw-sEWNV ($3.0 \times 10^6$ TCID50). As controls, 2 monkeys were immunized with the same dose of standard MVSchw vaccine, and 2 monkeys were mock immunized with PBS. Because WNV is a zoonotic infection that may emerge suddenly, we evaluated whether vaccination in an emergency setting would rapidly be protective. To this end, the 12 animals were divided in 2 groups of 6 (4 vaccinated, 2 controls) that were challenged early with WNV IS-98-st1 at 15 or 30 days after immunization.

No vaccine-related clinical signs, changes in body weight, or changes in blood chemistry were observed among immunized animals. At challenge, a slight increase in body temperature was recorded in control animals (39.9°C mean temperature at
day 3 after challenge) but not in MVSchw-sEWNV immunized animals (39.1°C mean temperature at day 3 after challenge). The vaccine immunogenicity was assessed by determining the presence of WNV-specific antibodies in monkey sera measured by ELISA and PRNT$_{50}$ at 15 or 30 days after vaccination with a single dose of MVSchw-sEWNV (Figure 2A and B, Tables 2 and 3). Low levels of WNV-specific IgG were induced early at day 15 after immunization in 6 of 8 vaccinated animals, and levels increased on day 30, while control animals remained negative (Figure 2A). Interestingly, levels of neutralizing antibodies were raised in all immunized animals at days 15 and 30 after immunization (PRNT$_{50}$ titers, 1:20 to 1:40). After challenge, the levels of antibodies remained high and unchanged in monkeys that were challenged at day 30 and were boosted in monkeys that were challenged at day 15. On the day of euthanasia, both control and vaccinated monkeys had developed high IgG titers (Figure 2A and B). In the vaccinated animals, the postchallenge neutralizing antibody titer was 1 log higher than in control animals (1/500–1/2000, compared with 1/200), indicating a boosted recall response to WNV in immunized animals, even shortly after immunization (Tables 2 and 3).

To evaluate the efficacy of protection afforded by immunization with MVSchw-sEWNV, we monitored WNV replication and infectivity titers in the sera of challenged monkeys. On the basis of the kinetics of WNV replication in squirrel monkeys observed in the previous study, we collected the sera at days 0, 2, 4, 5, 7, 9, 12, and 14 after challenge and assessed the presence of WNV by quantitative real-time PCR at all time points and by virus plaque titration assay at the peak of replication on days 2 and 4 after challenge. All animals were PCR negative after day 4; no delay in viral replication was observed. Both WNV genomic RNA and infectious titers were lower at the peak of replication on day 2 after challenge in the sera of vaccinated animals, compared with titers in controls (Figure 2C), demonstrating the efficacy of immunization to control challenge viremia. In animals challenged 15 days after immunization, the WNV RNA copy number and infectious titer were 10-fold lower. In animals challenged 30 days after immunization, the viral copy number was reduced by 3 logs, and the infectious viremia was undetectable in all animals (Figure 2D). When WNV genomic RNA was assessed in animal organs at euthanasia 11 or 14 days after challenge, it was found reduced by 1 log in mesenteric LNs of vaccinated animals as compared to control monkeys. A small amount of persisting viral RNA was also found in other organs. Unfortunately, because of the very short time allotted for use of the BSL4 facility, complete viral clearance could not be followed over the long term.

### DISCUSSION

In this work, we first established experimental WNV infection of New World squirrel monkeys, *S. sciureus*, which are easy to handle in a BSL4 facility. We evaluated the level and duration of WNV viremia, the viral biodistribution, and the kinetics of the humoral immune response. Infected animals did not develop clinical or physiologic changes after infection, except for a slight and transitory body temperature increase. A clear viremia was detected with high titers at 2 days after inoculation, declining at day 5 and clearing at day 7. At days 7 and 14 after infection, the virus was still present in lymphoid organs and liver, whereas the central nervous system (CNS; brain and spinal chord) remained uninfected. WNV neutralizing antibodies were elicited in all infected monkeys. In rhesus macaques previously infected with 10$^3$ pfu of WNV, viremia persisted for 5 days at 10$^2$ pfu/mL in the serum, and humoral response was elicited, with no deaths or clinical symptoms observed [9, 26, 27]. Only intracranial inoculation of rhesus macaques generated WNV encephalitis symptoms [5, 28]. A similar lack of clinical symptoms with induction of immune response was observed in baboons infected with WNV [29]. Thus, as already observed with other NHPs, no productive infection of the CNS was evidenced in infected squirrel monkeys. Presumably, as in humans, in which only a small number of infected individuals develop CNS disease, infection of a larger number of animals must occur to observe these events.

We then evaluated the immunogenicity and efficacy of a recombinant live attenuated measles vaccine expressing the sE of WNV (MVSchw-sEWNV) to protect these animals from WNV challenge. As early as 15 days after immunization with a single dose, WNV-specific IgG was detectable in 6 of 8 animals. Neutralizing antibodies were induced with a PRNT$_{50}$ of 1:20 in all vaccinated animals by day 15 after vaccination. The titers increased sharply in all vaccinated animals by day 30 after immunization. After challenge by intravenous
administration of $10^5$ pfu of WNV, a strong reduction of viremia was observed in both groups of animals immunized either 15 or 30 days before challenge. The copy number of viral genome was reduced by 85% (1 log$_{10}$) in the sera of animals vaccinated 15 days prior to challenge and by 99.8% (3 log$_{10}$) in animals vaccinated 30 days before challenge. In the latter group, no infectious viremia was detected, indicating an effective control of virus replication. However, approximately $10^4$ copies/mL of viral RNA were still detected in sera at day 2 after challenge, and smaller amounts were detected in lymph nodes 11 days after challenge at euthanasia. Calibration of our quantitative real-time PCR indicates that 1 pfu of virus contains approximately $10^4$ copies of viral genomic RNA, which is a typical ratio. Therefore, the detection of small amounts of viral RNA may either indicate a low level of WNV dissemination despite immunization, or it may represent the remaining inoculum ($10^5$ pfu) still detectable by a sensitive PCR. Nevertheless, the blood of animals vaccinated 30 days before challenge remained noninfectious after challenge, as no infectious virus was detected by plaque assay. Whether this degree of efficacy would be human protective or would prevent CNS dissemination cannot be established in this model.

The neutralizing antibodies elicited 30 days after immunization (PRNT 1/20–1/40) were sufficient to reduce by 3 logs the challenge viremia within 2 days. On the day of euthanasia, the PRNT titers were 10 times higher in vaccinated animals than in controls, indicating that the anamnestic immunity elicited by immunization was boosted by challenge. Boosting the vaccine anamnestic immunity to higher than protective levels...
is expected in vaccinated individuals encountering the infectious virus. Because of BSL4 constraints, we could not evaluate the long-term protection. However, lifelong immunity is a hallmark of live attenuated MV vaccination, and we previously demonstrated that recombinant MV vector induces long-term immunity [18, 19, 33]. A combined MV-WNV vaccine should be administered to adolescent and adult populations who are already preimmune to MV because of their childhood vaccination, which might prevent or reduce the efficacy of a recombinant MV. However, numerous studies have shown that vaccination of previously immunized individuals results in a boost of anti-MV antibodies, indicating that the live vaccine replicated despite preexisting immunity [34–36]. Moreover, we previously demonstrated that a recombinant MV vector expressing HIV antigens induced antibodies to HIV in mice and macaques with MV preexisting immunity, provided that 2 injections with a higher dose are given [17]. Yet, this point needs to be further evaluated in the case of immunization with MVSchw-sE<sub>WNV</sub>.

In conclusion, we have demonstrated that vaccination of squirrel monkeys with a single dose of a recombinant measles Schwarz vaccine expressing the sE protein of WNV (MVSchw-sE<sub>WNV</sub>) was safe and elicited WNV neutralizing antibodies. In animals challenged as early as 15 days after vaccination, infection was reduced 10-fold. In animals challenged 30 days after immunization, no infectious WNV viremia was detected, and viral RNA was reduced by 3 logs. In contrast, control animals developed a strong WNV viremia. We could not document the cellular immune responses induced by this vaccination; therefore, we cannot draw conclusions about the correlates of protection. However, in our previous study in mice, we demonstrated that protection correlated with neutralizing antibodies, as passive transfer of immune sera from immunized mice in naive animals conferred protection [15]. Although this NHP model does not allow assessment of protection from clinical disease, except from mild fever, we can assume that a significant reduction in viral load at challenge should help avoid neurological complications. Therefore, this study supports the protective efficacy of the MVSchw-sE<sub>WNV</sub> candidate vaccine and its potential as a human vaccine to be used in the case of epidemic WNV infection. This is also the first study that demonstrates the capacity of a recombinant measles vector to protect NHPs from an infectious challenge.

Table 2. WNV Neutralizing Antibody Titers Determined by Plaque Reduction Neutralization Test (PRNT<sub>50</sub>) in the Serum of Infected Animals 30 Days After Immunization and on the Day of Euthanasia

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<tr>
<th>Animals&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PRNT&lt;sub&gt;50&lt;/sub&gt; Titer&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> Animals #1 and #2 were mock-immunized with PBS; Animals #3, #4, #5, #6 were immunized with MVSchw-sE<sub>WNV</sub>; Animals #1 and #2 were euthanized on day 10 post-challenge, animals #3, #5, #6 were euthanized on day 11 post-challenge.

<sup>b</sup> Highest serum dilution tested that reduced the number of plaques by at least 50%.

Abbreviation: PBS, phosphate-buffered saline.

Table 3. WNV Neutralizing Antibody Titers Determined by Plaque Reduction Neutralization Tests (PRNT<sub>50</sub>) in the Serum of Infected Animals 15 Days After Immunization and on the Day of Euthanasia

<table>
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<tr>
<th>Animals&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> Animals #7 and #8 were immunized with empty MV; Animals #9, #10, #11, #12 were immunized with MVSchw-sE<sub>WNV</sub>; Animals #7, #8, #10 and #12 were euthanized on day 14 post-challenge, animals #9 and #11 were euthanized on day 15 post-challenge.

<sup>b</sup> Reciprocal of the highest serum dilution tested that reduced the number of plaques by at least 50%.

Abbreviation: MV, empty measles vaccine.

References


