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MicroRNA reduction of neuronal West Nile virus replication attenuates and affords a protective immune response in mice

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ABSTRACT

West Nile virus (WNV) is an important agent of human encephalitis that has quickly become endemic across much of the United States since its identification in North America in 1999. While the majority (~75%) of infections are subclinical, neurologic disease can occur in a subset of cases, with outcomes including permanent neurologic damage and death. Currently, there are no WNV vaccines approved for use in humans. This study introduces a novel vaccine platform for WNV to reduce viral replication in the central nervous system while maintaining peripheral replication to elicit strong neutralizing antibody titers. Vaccine candidates were engineered to incorporate microRNA (miRNA) target sequences for a cognate miRNA expressed only in neurons, allowing the host miRNAs to target viral transcription through endogenous RNA silencing. To maintain stability, these targets were incorporated in multiple locations within the 3′-untranslated region, flanking sequences essential for viral replication without affecting the viral open reading frame. All candidates replicated comparably to wild type WNV *in vitro* within cells that did not express the cognate miRNA. Insertional control viruses were also capable of neuroinvasion and neurovirulence *in vivo* in CD-1 mice. Vaccine viruses were safe at all doses tested and did not demonstrate mutations associated with a reversion to virulence when serially passaged in mice. All vaccine constructs were protective from lethal challenge in mice, producing 93–100% protection at the highest dose tested. Overall, this is a safe and effective attenuation strategy with broad potential application for vaccine development.

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1. Introduction

Since first identified in North America in 1999, West Nile virus (WNV) has become endemic and the leading cause of arboviral encephalitis in the United States [1,2]. Approximately 70–80% of WNV infections are asymptomatic but can cause acute encephalitis resulting in permanent neurological damage or death in approximately 0.1% of infections [3–9]. WNV has been responsible for ~40,000 cases of human disease and ~1700 deaths in the U.S. (http://www.cdc.gov/westnile/statsmaps/index.html). While the mechanism(s) of viral entry into the central nervous system (CNS) are not completely understood [10], replication in neurons of the CNS is critical for the virus to elicit neuropathology. Despite the high prevalence of the WNV in endemic areas, there are currently no vaccines or targeted therapeutics for humans.

A member of the family *Flaviviridae*, genus *Flavivirus*, WNV is an enveloped virus with a positive-sense single-stranded RNA genome of ~11 kb encoding a single open reading frame flanked by 5′ and 3′-untranslated regions (UTRs). The genome strongly resembles a mammalian messenger RNA (mRNA) including a 5′ capped RNA with a 3′-UTR lacking 3′-polyadenylation. Both UTRs contain conserved structural elements that are essential for viral replication and viral RNA synthesis [11–14].

MicroRNAs (miRNAs) are small (~23 nt) RNAs that bind to target sequences in the 3′-UTR of eukaryote mRNAs, thus serving an important role in regulating post-transcriptional gene expression [15]. miRNAs with perfect or near-perfect complementarity for their targets, especially in the 5′ "seed" sequence, facilitate direct cleavage of target mRNAs [15] and miRNA targets spaced in short intervals within the mRNA (13–35 nt apart) have been shown to promote optimal silencing of protein expression. By using strategically placed target sequences for the neuron-specific mature miRNA (miR)-124a within the 3′-UTR of WNV, this study

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investigated the capacity for using miRNA-mediated WNV silencing in neurons as a strategy for WNV vaccination [16]. This strategy has been previously described for other viruses but has not been applied to WNV [17,18].

2. Materials and methods

2.1. Plasmids, viruses, and cells

Stocks of recombinant WNVs containing miRNA target sequences were derived as previously described from cDNA clones, pWN-AB and pWN-CG, containing the 5′ and 3′ portions of the NY99 strain (WT WNV), respectively [19]. A schematic and descriptions are presented in Fig. 1 and Table 1. For construction of vaccine (WNVΔVax-1, -2a, -2b, and -3) and control (WNVΔCtrl-1, -2a, -2b, and -3) viruses, cassettes containing miRNA target sequences specific for mammalian neurons (miR-124a) and for insect cell cycle regulation (miR-14) were synthesized and inserted in the 3′-UTR in several combinations and expressing multiple copies [20,21]. Control viruses were generated using the miR-1175 target sequence, a highly expressed miRNA identified in the mosquito midgut [22]. Unlike miR-14, miR-1175 is not expressed in C6/36 cells, therefore WNVΔCtrl viruses served as insertional controls for C6/36 replication. All miR-1175 targets were inserted in identical locations in control viruses vs. vaccine constructs (miR-124a/miR-14 targets). Finally, to assess specificity of miR-124a on peripheral replication and neuroinvasiveness, viruses were generated using 3 copies of only either miR-14 and miR-124a in a cassette in the same position as the WNVΔCtrl/Vax-1 constructs. Transcription of viral RNA from ligated, linearized cDNA template was performed as previously described [19]. Transcribed RNA was then electroporated into BHK-21 cells [19].

Vero and BHK-21 cells were maintained in Dulbecco's minimal essential medium (DMEM) with 5% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO_2 (Life Technologies). SH-SY5Y neuroblastoma cells (gift of D. Beckham) were maintained in DMEM/F12 with 10% FBS and antibiotics, temperature, and CO_2 as above [23]. *Aedes albopictus* mosquito (C6/36) cells were maintained as Vero and BHK-21 cells with 10% FBS at 28 °C.

2.2. Viral growth profiles and quantification assays

Vero and C6/36 cells were plated in 6-well plates and inoculated with viruses in triplicate (3 wells/virus) at a multiplicity of infection of 0.1. Viral adsorption was performed for 1 h at 37 °C (Vero) or 28 °C (C6/36), then cells were washed three times with PBS and 2 mL of media added to the culture. At 0, 24, 48, and 72 h post-inoculation (h.p.i.), 50 μL supernatant was removed from each well, diluted 1:10 in media containing 20% FBS, and stored at −80 °C. Viral titers were determined by plaque assay or 50% tissue culture infectious dose ($TCID_{50}$) on Vero cells as described [24]. No difference in titer was observed for viruses utilized between plaque [25] or $TCID_{50}$ [26] assay in this cell line; thus, all titers presented herein are reported as infectious units (IU). Plaque size was determined by measuring the size in mm of 10 well-isolated plaques. All plaque size measurements were made during the same experiment to preclude any intra-experimental variation in agarose concentration and cell density. SH-SY5Y cells were plated in 12-well plates and inoculated with virus and titrated as above.

2.3. Mouse viremia, neurovirulence, and neuroinvasiveness assessments

All animal procedures were reviewed and approved by the CDC IACUC.

2.3.1. Viremia study

Groups of 21 5-week-old female CD-1 outbred mice (Charles River Laboratories) were inoculated intraperitoneally (IP) with [100 μL] of viral inoculum (1000 IU) in PBS using WT, miR-14, and miR-124a viruses. Three animals were bled by cardiac puncture and euthanized daily through dpi 7 for serum viremia titers. Blood was spun in serum separator tubes, and viruses from sera were titrated by plaque assay as described above on Vero cells. An additional 15 3-week-old CD-1 females were inoculated IP with 1000 IU using the same three viruses in order to independently assess induction of morbidity/mortality.

2.3.2. Neuroinvasiveness/neurovirulence study

Groups of 5 3-week-old female CD-1 outbred mice (Charles River Laboratories) were inoculated intracranially (IC) or by the IP route with 10 or 100 μL, respectively, of viral inoculum in PBS. Doses of 0.1, 1, 10, and 100 IU (IC) or 1, 10, 100, and 1000 IU (IP) were used for WT and miRNA insertional mutants. IC inoculations were performed with a 29-gauge needle with an affixed stopper to limit penetration to 2 mm while IP inoculations were performed with a 27-gauge needle. Mice were monitored for clinical signs of encephalitis, including hunched posture, ataxia, hind limb paralysis, or recumbency, and euthanized when signs of encephalitis or paralysis were apparent. Groups of 9 additional 3-week-old CD-1 mice were inoculated by the IC route and three processed daily at dpi 1, 3 and 5 for histology and immunohistochemistry (IHC) (see below).

2.4. Histology and immunohistochemistry

Mice were euthanized as described above and brains were immediately fixed in 10% buffered formalin for at least 48 h and then embedded in paraffin. 4-μm sections were processed by standard histologic techniques, and side-by-side sections were stained with either hematoxylin and eosin (H&E) or were processed for IHC. Slides for IHC were stained with polyclonal rabbit serum primed for the WNV NS5 protein, using Dako Invision + system-HRP labeled polymer anti-rabbit (Dako) and NovaRED peroxidase substrate (Vector Laboratories), and counterstained with Mayer's hematoxylin.

2.5. Assay of viral stability in mice

Groups of 3 5-week-old CD-1 mice were inoculated with 1000 IU IP of each WNVΔVax with a 27-gauge needle. Mice were bled by cardiac puncture and euthanized at 3 days post infection (d.p.i.). Virus from sera was titrated, and new cohorts of 3 5-week-old mice inoculated with passaged virus at the same dose. This was repeated for five total passages. Viruses were sequenced retrospectively until the first passage where a mutation arose was determined.

2.6. Mouse protection studies

Groups of 15 5-week-old CD-1 mice were immunized IP with 100 μL of each WNVΔVax virus at 100/1000 IU doses with 16 PBS-inoculated mice serving as a control group. No morbidity or mortality was observed for 21 days post-immunization. At this point, whole blood was collected in a serum separator tube, spun at 5000*g* for 5 min, and sera collected for quantification of neutralizing antibody by 90% plaque reduction neutralization test ($PRNT_{90}$) (see below). Following blood collection, mice were challenged with an estimated 100,000 LD_{50} doses of WNV strain NY99 (10,000 IU) IP and monitored for clinical signs of encephalitis as above. At 21 days post-challenge, blood was collected for $PRNT_{90}$ as described above.

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