Am. J. Trop. Med. Hyg., 72(3), 2005, pp. 325–329

ORAL TRANSMISSION OF WEST NILE VIRUS IN A HAMSTER MODEL

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Abstract. The results of experiments comparing the pathogenesis of West Nile virus (WNV) following infection by mosquito bite, needle inoculation, and ingestion are reported. Adult hamsters were readily infected by all three routes. The level and duration of viremia, clinical manifestations, pathology, and antibody response in the hamsters following mosquito infection and needle inoculation were similar; after oral infection, the onset of viremia was delayed and the mortality was lower, but the level and duration of viremia, histopathology, and antibody response were similar to the other routes. The results from this and previously published studies indicate that a wide variety of animal species are susceptible to oral infection with WNV and that orally infected animals develop a viremia and illness similar to that following the bite of infected mosquitoes. Oral infection appears to be an alternative transmission mechanism used by a number of different flaviviruses; its potential role in the natural history of WNV is discussed.

INTRODUCTION

West Nile virus (WNV) is a member of the Japanese encephalitis group of the genus *Flavivirus*, family Flaviviridae.[1] In 1999, WNV first appeared in the Americas during an outbreak in the New York City metropolitan area.[1,2] A bird/mosquito cycle appears to be the primary mechanism for virus transmission and maintenance, but this may be an overly simplistic model. Experimental and field data indicate that WNV transmission may occur by aerosol, ingestion, and direct contact among a wide variety of animal species (birds, mammals, and reptiles).[3–10] The relative importance of these non-vector modes of WNV transmission among animals in nature is difficult to ascertain, but they may be of importance in epizootic and enzootic situations or as a maintenance mechanism for the virus during periods when adult arthropod vectors are absent or inactive.

To evaluate the potential role of oral transmission in the natural cycle of WNV, we used a hamster model of West Nile encephalitis[11] to compare the efficiency and pathogenesis of oral infection with infection by mosquito bite and parenteral (needle) injection. This paper describes the results of our studies and discusses the potential role of oral infection in flavivirus ecology.

MATERIALS AND METHODS

Virus. A second Vero passage of the 385-99 strain of WNV was used in all experimental infections. This strain was originally isolated from the liver of a dead snowy owl *(Nyctea scadiaca*) collected at the Bronx Zoo in New York City during the summer of 1999.[11] Strain 385-99 was recently fully sequenced and differs by only a few nucleotides from the WNV New York 1999 prototype strain 382-99[12] (Xiao SY, unpublished data).

Animals. Adult female Syrian golden hamsters (*Mesocricetus auratus*) 8–12 weeks of age, obtained from Harlan Sprague Dawley (Indianapolis, IN), were used in the study. The animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council). All work with the infected animals was carried out in animal biosafety level 3 (ABSL-3) facilities under an approved University of Texas Medical Branch animal use protocol.

Virus assay. The presence and quantity of virus in daily blood samples from the infected hamsters were determined by plaque assay in monolayer cultures of Vero cells, as described previously.[13] Serial 10-fold dilutions from 10^{-1} to 10^{-7} of each sample were prepared in phosphate-buffered saline (PBS), pH 7.4, containing 10% fetal bovine serum (PBS diluent). Duplicate wells of 24-well microplate cultures of Vero cells were inoculated with each dilution. Cultures were incubated at 37°C, and plaques were counted four days later. Virus titers were calculated as the number of plaque-forming units (PFU) per milliliter of sample.

Antibody detection. Comparative studies of the antibody response of hamsters to WNV infection were done by a hemagglutination-inhibition (HI) test. A standard HI technique was used.[11,14] Antigens for the HI test were prepared from brains of WNV-infected baby mice by the sucrose-acetone extraction method.[14] Hamster sera were tested at serial twofold dilutions from 1:20 to 1:5,120 at pH 6.6, with four units of antigen and a 1:200 dilution of goose erythrocytes.

Mosquitoes. *Culex pipiens quinquefasciatus* (Sebring strain) were obtained from the Harris County Mosquito Control District (Houston, TX). The Sebring strain was originally collected in 1988 from Sebring County, Florida. The colony consisted of mosquitoes from > F_{30} generation and were maintained at 26°C, with a light:dark cycle of 14:10 with a one-hour crepuscular period to simulate dawn and dusk.

Mosquito infection. Mosquitoes were infected with WNV by intrathoracic inoculation, as described previously.[15] After inoculation, the mosquitoes were held with 10% sucrose solution in a model 818 environmental chamber (Precision, Winchester, VA) at 26°C, with a 12 hour:12 hour light:dark cycle. After 12 days, the salivary glands were dissected from 10 mosquitoes and were examined by a standard immunofluorescence assay for evidence of WNV antigen.[16] The results indicated that 100% of the mosquitoes sampled were infected. On the following day, the remaining insects were allowed to feed on hamsters, as described below.

Virus transmission by mosquitoes. Thirteen days after WNV inoculation, 10 *Culex* females, deprived of sugar for 24 hours, were placed in each of 10 small fiberboard holding containers covered with fine nylon mesh. A single anesthetized hamster (Nembutal, 50 mg/kg given parenterally) was placed on the screening on the top of each mosquito container. Hamsters were exposed to the infected mosquitoes for

one hour in the dark and then were returned to their cages. The animals were examined daily for signs of illness, and a blood sample (200 μL) was obtained for seven consecutive days from the retro-orbital sinus for virus assay. Moribund animals were killed with Halothane (Halocarbon Laboratories, River Edge, NJ) and then perfused with 10% buffered formalin to fix the brain for histopathology. Surviving animals were bled one final time, 28 days after exposure to infected mosquitoes, and were then killed.

Parenteral infection. A total of 30 hamsters were inoculated intraperitoneally with $10^{4.0}$ 50% tissue culture infective doses ($TCID_{50}$) of WNV. Animals were monitored for signs of illness or death. A subset of 10 animals was bled daily for six consecutive days to quantify viremia. After 28 days, the surviving hamsters were bled for antibody determination.

Oral infection. Nine adult female hamsters were housed in individual cages and deprived of food for 12 hours. A single WNV-infected baby mouse was placed in the cage with each hamster. The mice had been inoculated intracerebrally with WNV 36 hours previously. When the baby mice were placed in the cages, the female hamsters quickly attacked and devoured them. The hamsters were subsequently observed for 28 days for signs of illness; they were also bled for 8 consecutive days after eating the mice, to determine if they developed viremia. Moribund hamsters were killed with Halothane and perfused with 10% buffered formalin. At the end of the observation period, the surviving animals were bled and tested for WNV antibodies by the HI test.

Histologic examination of hamster tissues. Samples of brain from the moribund hamsters in each of the three groups were fixed in 10% buffered formalin for 48 hours. After fixation, tissue samples were processed for routine paraffin embedding and sectioning. Tissue sections 4–5 μm in thickness, were made and stained by the hematoxylin and eosin and immunoperoxidase methods.[11] Immunohistochemical (IHC) staining methods used to detect the presence of WNV antigen in the hamster tissue have been previously described.[11] The primary antibody used for IHC straining was a mouse hyperimmune ascitic fluid prepared against the 385-99 strain of WNV.

RESULTS

Mosquito transmission. Groups of 10 WNV-infected *Cx. p. quinquefaciatus* were allowed to feed on each of 10 individual adult hamsters. All bitten hamsters became viremic (Figure 1). Viremia in the hamsters developed within 24 hours after being bitten by the infected mosquitoes. Peak virus titers (mean = $10^{5.5}$ PFU/mL of blood) occurred on day 2; the viremia lasted 5–6 days. Most hamsters displayed signs of illness (somnolence, anorexia, difficulty walking, tremors, limb paralysis), beginning about day 7 post-infection (pi). Four (40%) of the animals died or became gravely ill and were killed; the other six hamsters recovered. A survival curve for the animals is shown in Figure 2. The six survivors all had HI antibodies to WNV antigen when tested 28 days pi.

Parenteral infection. Thirty adult hamsters were inoculated intraperitoneally with 10^4 PFU of WNV. Ten animals were bled daily for six consecutive days. These hamsters also became viremic within 24 hours; peak virus titers (mean = $10^{5.2}$ PFU/mL) occurred on day 2 (Figure 1). The onset and duration of illness (7–14 days) and clinical symptoms in this group were similar to those observed in the mosquito infected group. Overall, 14 of the 30 animals (47%) in the inoculated group died or were killed. All of the survivors had WNV antibodies when tested 28 days pi.

Oral infection. Nine adult hamsters each consumed a WNV-infected baby mouse. These animals were bled for

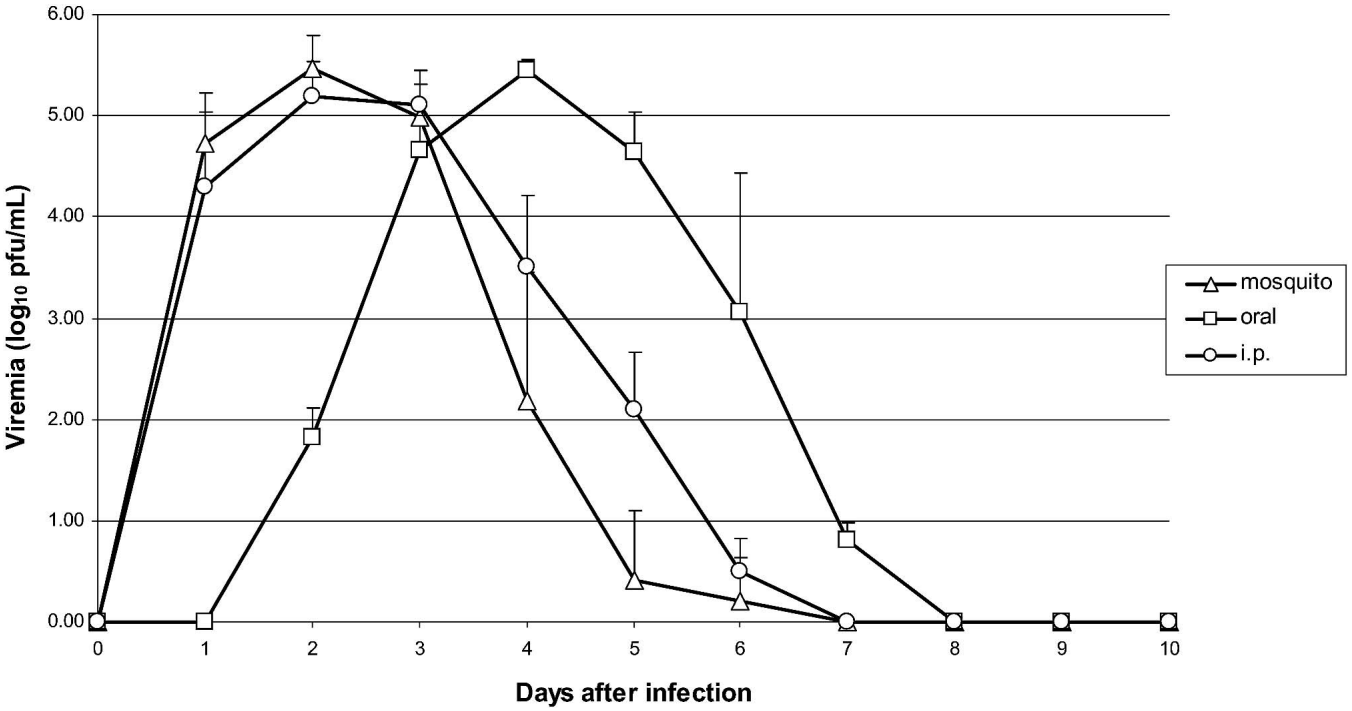

FIGURE 1. Daily mean + SD viremia in hamsters following mosquito, parenteral, or oral infection with West Nile virus. pfu = plaque-forming units; i.p. = intraperitoneally.

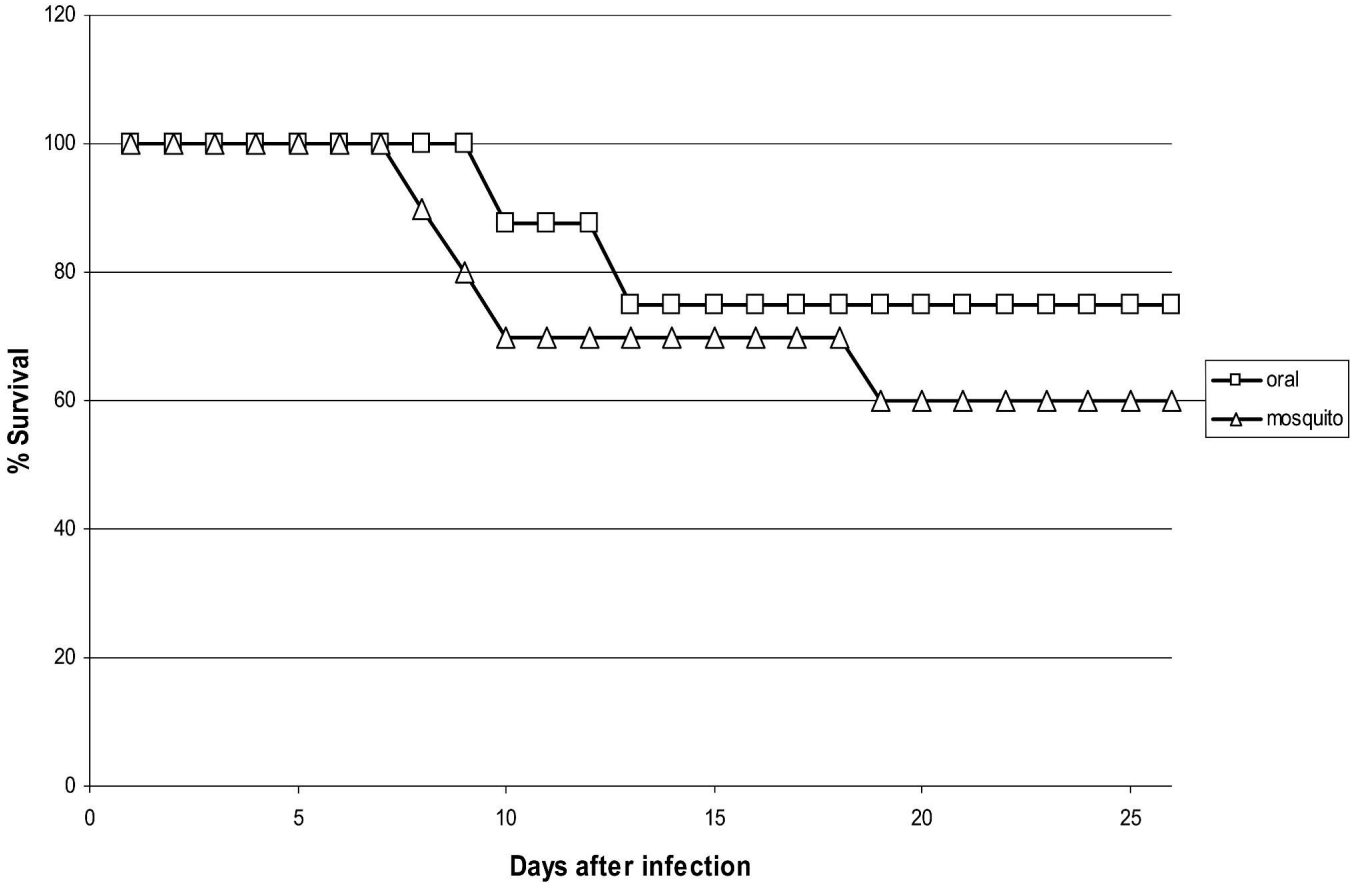

FIGURE 2. Survival curve in hamsters following mosquito and oral infection with West Nile virus.

eight consecutive days after feeding (af). Only one hamster (#3194) did not develop viremia or seroconvert; it apparently was not infected (Table 1). A second animal (#3196) developed viremia, but was found dead on day 4 af, so it was excluded from the final calculations. The seven remaining hamsters had a delayed viremia, compared with the mosquito-infected and parenterally infected groups. Four of the seven animals had detectable viremia on the second day af, and six of seven were viremic by day 3. One hamster (#3198) did not develop a detectable viremia until day 7 af. The daily mean levels of viremia for six orally infected hamsters (#3192, 3193, 3195, 3197, 3199, and 3200) are shown in Figure 1. The highest mean titers for this group occurred on day 4 (mean = $10^{5.5}$ PFU/mL). Although delayed by about two days, the mean titers and duration of viremia in the latter group were similar to results obtained with the mosquito-infected and parenterally infected animals. Excluding animal #3196, two (28.6%) of the seven orally infected hamsters died. A survival curve of the orally infected animals is shown in Figure 2; hamster #3198 was included in this calculation.

TABLE 1
Pattern of viremia in hamsters after ingestion of West Nile virus*

Hamster number	Day after West Nile virus ingestion							
	D-1	D-2	D-3	D-4	D-5	D-6	D-7	D-8
3192	0	0	4.5	5.5	5.0	5.0	1.7	0
3193	0	0	3.7	5.6	5.0	4.4	0	0
3194	0	0	0	0	0	0	0	0
3195	0	3.9	5.4	5.3	4.2	3.6	1.4	0
3196	0	3.2	5.0	Dead	–	–	–	–
3197	0	3.3	5.0	5.5	4.3	1.4	0	0
3198	0	0	0	0	0	0	3.2	5.2
3199	0	3.7	5.2	5.4	4.3	0	0	0
3200	0	0	4.2	5.4	5.0	4.0	1.7	0

* Virus titer in blood expressed as $\log_{10}$ plaque-forming units (PFU) per milliliter of blood. 0 = < 1.4.

Histologic examination of the brain. Fresh brain tissue, suitable for hematoxylin and eosin and IHC examination, was only available from the few moribund hamsters in each group. However, the type and degree of pathology in brain sections of these animals were similar to that described in an earlier hamster study,[11] regardless of the mode of infection. Essentially all brain sections showed neuronal degeneration in the cerebral cortex and spongiform changes in the basal ganglia. West Nile virus antigen-positive cytoplasmic staining was observed in neurons of the cerebral cortex, hippocampus, and basal ganglia of all animals.

DISCUSSION

The results of this study demonstrate that hamsters can be readily infected with WNV by mosquito bite, needle inoculation, or ingestion. Furthermore, the resulting levels of viremia, clinical manifestations, and pathology were similar regardless of the mode of infection. The titers of HI antibodies in the surviving hamsters in the three groups, when tested one

month after infection, were also similar. In the orally infected hamsters, the onset of viremia and of disease were slower than in the mosquito-infected and parenterally infected groups; the mortality was also lower. Similar results have been observed in studies of experimental WNV infection of cats,[17] birds,[5] and alligators.[8] Collectively, these findings suggest that a wide range of vertebrate species are susceptible to oral infection with WNV.

In other studies with experimentally (parenterally) infected hamsters, we have demonstrated that many of the surviving animals develop a chronic renal infection and shed WNV in their urine for up to five months after infection.[13] Hamsters in the present study were not tested for viruria, but based on the similar pathogenesis of the virus following oral, parenteral, and mosquito infection, we assume that viruria would occur, irrespective of the mode of infection. Persistent renal infection and chronic viruria have been demonstrated with another flavivirus (Modoc) in naturally infected white-footed mice (*Peromyscus maniculatus*) and in experimentally infected hamsters.[18,19] Within the genus *Flavivirus*, there are two clades of non-vectored viruses: the bat salivary gland viruses (Rio Bravo, Dakar bat, Montana myotis leukoencephalitis, Phnom-Penh bat, Batu Cave, Bukalasa bat, and Carey Island) and the rodent-associated viruses (Modoc, Cowbone Ridge, Sal Vieja, San Perlita, Jutiapa, and Apoi).[19–21] Experimental evidence[18,19,22,23] suggests that these viruses are maintained in nature by animal-to-animal transmission through infected saliva, in the case of the bat salivary gland viruses, or via infectious urine, in the case of the rodent-associated viruses. In addition, several viruses in the tick-borne encephalitis (TBE) clade of flaviviruses (louping-ill, Powassan, and TBE) can be transmitted by ingestion of infected milk.[24–27] Collectively, these observations on the behavior and ecology of a number of disparate flaviviruses seem to indicate a pattern, namely that the flaviviruses as a group are not restricted to a single mode of transmission. Oral infection appears to be a fairly common transmission mechanism among members of the *Flavivirus* genus. Oral transmission clearly occurs with WNV, although its epidemiologic importance is still uncertain. However, it serves as a reminder of how little we really understand about the natural history and maintenance of WNV. To date, most of the emphasis of WNV control activities in North America has been on killing mosquitoes or avoiding their bites. The evidence presented suggests that this approach may be too simplistic and that further research is needed to identify the full range of reservoir hosts, and the relative importance of different transmission modes and maintenance mechanisms for WNV in nature.

Interestingly, Ramakrishna and others[28] recently reported that mice could be infected orally with Japanese encephalitis virus (JEV). Following oral administration of live JEV, the animals developed HI and neutralizing antibodies and were subsequently protected against intracerebral challenge with a lethal dose of JEV. These investigators proposed that oral immunization with a live avirulent JEV immunogen might be a cheap and simple method to immunize human populations against Japanese encephalitis. Based on the results of our study, such an approach would seem risky, especially in immunocompromised individuals. Nonetheless, their experimental results[28] illustrate the ease of oral infection with another flavivirus that is closely related to WNV.

Received July 20, 2004. Accepted for publication August 11, 2004.

Acknowledgments: We thank Marina Siirin and Hilda Guzman for technical support and Dora Salinas for help in preparing the manuscript.

Financial support: This work was supported by contracts N01-AI25489 and N01-AI30027 from the National Institutes of Health and cooperative agreements U50/CCU 62054103 and U50/CCU620539 from the Centers for Disease Control and Prevention.

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