Nonviremic transmission of West Nile virus

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West Nile virus (WNV) is now the predominant circulating arthropod-borne virus in the United States with >15,000 human cases and >600 fatalities since 1999. Conventionally, mosquitoes become infected when feeding on viremic birds and subsequently transmit the virus to susceptible hosts. Here, we demonstrate nonviremic transmission of WNV between cofeeding mosquitoes. Donor, *Culex pipiens quinquefasciatus* mosquitoes infected with WNV were fed simultaneously with uninfected ''recipient'' mosquitoes on naïve mice. At all times, donor and recipient mosquitoes were housed in separate sealed containers, precluding the possibility of mixing. Recipients became infected in all five trials, with infection rates as high as 5.8% and no detectable viremia in the hosts. Remarkably, a 2.3% infection rate was observed when 87 uninfected mosquitoes fed adjacent to a single infected mosquito. This phenomenon could potentially enhance virus survival, transmission, and dispersion and obviate the requirement for viremia. All vertebrates, including immune and insusceptible animals, might therefore facilitate mosquito infection. Our findings question the status of dead-end hosts in the WNV transmission cycle and may partly explain the success with which WNV established and rapidly dispersed throughout North America.

Culex | mosquito vector

The unexpected introduction of West Nile virus (WNV) into North America in 1999 has highlighted the importance of arthropod-borne viral diseases. As the number of fatal avian, equine, and human infections increased (refs. 1–3; www.cdc.gov/ncidod/dvbid/westnile), the rapidity and extent of the spread of WNV in North America, the Caribbean, and Central America exceeded all expectations and most predictions (4). Globally, WNV is found in an extraordinarily large and increasing number of arthropod species (refs. 2 and 5; www.cdc.gov/ncidod/dvbid/westnile), with 60 species of mosquito already implicated as potential vectors of the virus in the United States. As WNV continues to spread, it seems inevitable that more species of arthropod, with varying host preferences, will be used as vectors. Before we can predict the ecological consequences of WNV being introduced into fragile habitats, such as tropical rainforests with their endangered vertebrate species, we need to understand more precisely the dynamics of the natural transmission cycle. As described below, this cycle may be more complex than initially thought.

Nonsystemic or, more specifically with respect to viruses, nonviremic transmission (NVT) describes a phenomenon by which arthropod vectors become infected with a pathogen before its propagation in the host and its appearance in the circulatory system. The discovery of NVT challenged the paradigm that arboviruses are transmitted only by arthropods feeding on viremic hosts. NVT was first observed with ticks cofeeding on rodents (6–9), which was subsequently demonstrated by Mead *et al.* (10) for vesicular stomatitis virus (VSV) between cofeeding black flies, but has not previously been reported for mosquitoes. Our hypothesis for this study was that the quantity of WNV secreted in the saliva of infected mosquitoes is sufficiently high to infect adjacent cofeeding mosquitoes directly without the requirement for replication in the vertebrate host. This hypothesis seemed reasonable because relatively high titers of WNV are secreted in saliva (11). Moreover, hematophagous arthropod densities can reach high levels in many regions, and species-specific feeding preferences result in the aggregation of vectors feeding closely together with high attack rates over short time periods (12).

Materials and Methods

Mosquitoes. Laboratory-reared *Culex pipiens quinquefasciatus* (Sebring strain) were obtained from the Harris County Mosquito Control District, Houston. This species was chosen because it is an important vector for WNV in North America. The Sebring strain was collected in 1988 from Sebring County, Florida. The colony consists of mosquitoes from $>F_{30}$ generation and is maintained at 28°C with a light:dark cycle of 14 h:10 h with a 1-h crepuscular period to simulate dawn and dusk. Larvae were fed a 1:1 mixture of TetraMin fish flakes (Doctors Foster and Smith, Thinelander, WI) and crushed Prolab 2500 rodent diet (PMI Nutrition International, Brentwood, MI). Adults were provided with 10% sucrose ad libitum and fed weekly on anaesthetized hamsters according to National Institutes of Health guidelines for the humane use of laboratory animals.

Virus. A 2002 Houston isolate of WNV (lineage I) prepared as a mixed brain/liver homogenate from an infected Blue Jay (*Cyanocitta cristata*) and designated as strain 114 (GenBank accession no. AY187013) was used for all experiments (13). Stock virus was produced after a single passage in Vero (green monkey kidney) cell culture and harvested as tissue culture supernatant. Sequence analysis of this virus confirmed its homology with the 1999 New York strain (NY99 GenBank accession no. AF196835). Based on an optimized plaque assay technique (14), the titer of this frozen stock virus was 2×10^8 plaque forming units/ml.

Infectious Blood Meal Preparation. Before feeding mosquitoes, fresh virus was propagated by inoculating a monolayer of Vero cells in T25 tissue culture flasks (Nalge Nunc International, Rochester, NY) with stock virus at a multiplicity of infection of 2.0 by using 1 ml of Leibovitz L-15 medium supplemented with 10% FBS, 10% tryptose phosphate broth, and 100 units/ml penicillin and 100 μg/ml streptomycin. After rocking for 1 h, 4 ml of medium was added and cells were incubated at 37°C. At 3 days postinfection (p.i.), tissue culture supernatant medium was harvested from the flasks and mixed with an equal volume of defibrinated sheep blood (Colorado Serum, Denver). Adenosine triphosphate was added as a phagostimulant to a final concentration of 2 mM.

Infection and Maintenance of Donor Mosquitoes. To infect donor mosquitoes, the infectious blood meal was heated to 37°C and transferred to the chamber of a Hemotek feeding apparatus (ref. 15; Discovery Workshops, Accrington, Lancashire, U.K.) housed in an isolation glove box within a Biosafety Level 3 insectary. The chamber was placed on top of the mesh of 1-quart

Abbreviations: WNV, West Nile virus; NVT, nonviremic transmission; VSV, vesicular stomatitis virus; p.i., postinfection; $TCID_{50}$, tissue culture infectious dose 50 percent.

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Table 1. Nonviremic transmission of WNV between *Cx. p. quinquefasciatus* mosquitoes

Days p.i. of donor mosquitoes (experiment)	Infectious blood meal virus titer/ml	Mean donor whole body virus titer*	No. of donors engorged	No. of recipients infected/*n* (%)†	Mean recipient whole body titer (*n*)†
7 (1a)	7.95	5.49 ± 0.88	48	2/36 (5.6)	4.29 ± 4.57 (2)
14 (1b)	7.95	5.67 ± 1.66	39	10/173 (5.8)	4.55 ± 2.27 (10)
14 (2)	8.52	6.70 ± 0.62	7	1/50 (2.0)	4.52 ± 0.00 (1)
14 (3)	8.95	6.09 ± 0.55	57	3/124 (2.4)	5.47 ± 0.50 (3)
14 (4)	7.95	5.95	1	2/87 (2.3)	4.74 ± 0.30 (2)

All titers are expressed as tissue culture infectious dose endpoints ($\log_{10}$ $TCID_{50}$ per ml or per mosquito).

*Mean whole body titer based on $TCID_{50}$ of 20 donor mosquitoes with blood in gut from cofeed (except for experiments 2 and 4, which had only 7 and 1 infected mosquitoes engorged on the mouse, respectively).

†Recipients tested on day 14 subsequent to cofeeding with donors.

cartons containing up to 100 5- to 7-day-old female mosquitoes that had been deprived of sucrose for 12 h before presentation of the blood meal. After a 1-h feeding period, cartons of mosquitoes were chilled and fully engorged females were transferred to new cartons. Cartons containing the experimental mosquitoes were placed in a sealed, humidified plastic box and maintained with 10% sucrose in a Precision model 818 environmental chamber (Precision, Winchester, VA) at 28°C and a 12 h:12 h light:dark cycle. A sample of the blood meal was collected and stored at −80°C for later titration.

Feeding of Recipient Mosquitoes. Five experimental feeds were performed. The basic scheme to test for NVT was based on allowing the donor mosquitoes, previously infected by artificial blood meal, to begin refeeding on an anaesthetized mouse as approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee. Recipient mosquitoes were then provided with access to the mouse. At all times, donor and recipient mosquitoes were housed in separate sealed containers, precluding the possibility of mixing the two groups.

For the first experiment, donor mosquitoes were allowed to feed at both day 7 and day 14 p.i. on an anaesthetized mouse placed on top of the cage. In subsequent experiments, all donor mosquitoes were held for 14 days before feeding on the anaesthetized mouse. After the donor mosquitoes had probed or fed for 5 min, a sealed carton containing recipient mosquitoes was placed next to that housing the donors. The mouse was repositioned to rest across the two adjacent cartons for 1 h, thus providing donors and recipients simultaneous access to the uninfected rodent. After 1 h, the mouse was euthanized and mosquitoes were chilled. In the fourth experiment, serum was collected from the mouse, and 100-μl aliquots were titrated in duplicate on Vero cells as described below. Donor mosquitoes were frozen immediately after feeding for subsequent analysis to determine the whole body viral titer. Recipient mosquitoes were chilled, and engorged females were held for 14 days at 28°C as described above. On day 14 p.i., all donor mosquitoes were frozen at −80°C for later analysis.

Mosquito Analysis. For titration, donor mosquitoes were collected at either 7 days p.i. (experiment 1a) or at 14 days p.i. (experiments 1b, 2, 3, and 4). Recipient mosquitoes were collected at 14 days p.i. To determine the whole-body viral titer, individual mosquitoes were triturated in 1 ml of L-15 medium, filtered through a 0.22 μM syringe filter (Millipore, Cork, Ireland), and titrated as serial 10-fold dilutions on Vero cells as described in ref. 16. Wells were scored for cytopathic effect (cpe) to calculate the tissue culture infectious dose 50% endpoint titers ($\log_{10}TCID_{50}$) by using the method of Karber (17). To confirm that the observed cpe corresponded with the replication of virus and presence of flavivirus antigen, randomly selected plates were processed as described by Schoepp and Beaty (18) and stained with a mouse hyperimmune antibody raised against WNV (T3 4876) by using a standard indirect immunofluorescence assay (IFA) as described in refs. 16, 19, and 20. In all cases, IFA detection of antigen exactly corresponded with wells in which cpe was observed, and no antigens were detected in the uninfected control and healthy wells.

Results and Discussion

Donor *Cx. p. quinquefasciatus* mosquitoes were infected with WNV by presentation with an infectious blood meal. After 1 h, engorged mosquitoes were transferred into a new carton and maintained at 28°C. The infectious titers of WNV in the blood meals ranged from 7.95 to 8.95 $\log_{10}$ $TCID_{50}$/ml with mosquitoes ingesting 5.66–6.47 $\log_{10}$ $TCID_{50}$ per mosquito as determined by virus titration in Vero cells. Previous experiments have demonstrated that this dosage range results in the infection of salivary glands as early as 5 days p.i. and a 97–100% WNV dissemination rate within 14 days (11, 21).

In the first experiment, donor mosquitoes were permitted to feed on an anaesthetized uninfected mouse at 7 days p.i. and again at 14 days p.i. Mean whole-body titers for 20 of these donors from the first experiment, sampled at 7 and 14 days p.i., were 5.49 ± 0.88 and 5.67 ± 1.66 $\log_{10}$ $TCID_{50}$ per mosquito, respectively. In the subsequent experiments, when donors were fed only at 14 days p.i., the mean individual mosquito WNV titers were 6.70 ± 0.62, 6.09 ± 0.55, and 5.95. Similar titers have been observed in wild-caught *Cx. p. quinquefasciatus* mosquitoes orally infected with WNV (S.H., unpublished data).

After experimental infection of susceptible cells by flaviviruses, there is an eclipse period during which no infectious virus can be detected (22, 23). In our first three transmission experiments with mice, the sera were not tested for the presence of virus after feeding with infected mosquitoes. However, in the fourth experiment, serum was collected from the mouse immediately after being fed, but no virus was detected. Moreover, viremia was not detected in subsequent tests of sera collected from two other mice immediately after being fed infected mosquitoes (14 days p.i., mean WNV titer 5.32 ± 0.14 $\log_{10}TCID_{50}$ per mosquito, $n = 4$). In mice inoculated with WNV, viremia is not detected before 8 h p.i., and, moreover, there is no detectable virus in serum samples collected immediately after inoculation (A.D.T. Barrett, personal communication). The 1-h mosquito feeding period would therefore be insufficient for the virus to complete a cycle of infection and replication and to produce viremia in the mouse. Nevertheless, in all five cofeeding experiments, recipient mosquitoes became infected with WNV (Table 1). The experiment therefore satisfies our definition of the NVT phenomenon. A total of 470 recipient mosquitoes were tested for virus, of which 18 were positive for WNV (3.8%), giving an average infection rate of 3.1 ± 1.8. Transmission to recipient mosquitoes was observed from donors held at both 7 and 14 days p.i. Whole-body WNV titers for

recipients exceeded 4 $\log_{10}$TCID$_{50}$ per mosquito for 14 of 18 of the infected mosquitoes. Eight recipients had titers >5 $\log_{10}$ TCID$_{50}$ per mosquito, and five had >6 $\log_{10}$ TCID$_{50}$ per mosquito. The mean WNV titer in the recipients was 4.70 ± 2.0 $\log_{10}$ TCID$_{50}$ per mosquito. Because this result is similar to titers attained in orally infected mosquitoes at 14 days p.i., a time when virus is observed in saliva (24) and when 93% of females can transmit up to 5.29 $\log_{10}$ plaque-forming units of WNV (11), we believe that many of the infected recipient mosquitoes would have been capable of transmitting WNV. Infections with WNV can be generated by inoculation of very low titers of virus, for example, crows can be infected by mosquitoes that probe but fail to engorge (25).

Nonsystemic transmission (NST) has been reported before for several pathogens, including spirochetes (26), tick-borne viruses (6–9), and VSV by black flies (10). As discussed by Lord and Tabachnick (27) and Norman *et al.* (28), the epidemiological consequences of NST for these pathogens are considerable, but the demonstration of NVT by WNV is especially important for several reasons. Compared with the other nonsystemically transmitted pathogens, WNV is an important emerging virus, is widely distributed in the world, is transmitted by numerous species of vectors, and is pathogenic for a variety of vertebrates including humans.

Surveillance plans and control strategies implemented by national and local agencies in an effort to minimize the impact of the disease in the United States (www.cdc.gov/ncidod/dvbid/westnile/resources/wnv-guidelines-aug-2003.pdf) have assumed a conventional vertebrate to mosquito to vertebrate virus transmission cycle involving viremia. Vertebrates that do not become viremic, or in which viremia is below a threshold required for vector infection, are regarded as dead-end hosts. Because most mammals fit this definition, monitoring and plotting bird mortality has been an important tool to map the distribution of WNV and to target vector control efforts. Although WNV has killed 234 different bird species in the United States (www.cdc.gov/ncidod/dvbid/westnile), surveillance has focused on corvids, notably blue jays (*Cyanocitta cristata*) and American crows (*Corvus brachyrhynchos*), species that produce a relatively high viremia and have a high mortality rate (ref. 25; www.cdc.gov/ncidod/dvbid/westnile). Corvids represent only a small proportion of the total bird population present in most areas, but, for practical reasons, this bias toward the collection of moribund or dead corvids (29) and targeting of mosquito control efforts to areas where these birds are collected, has been widely implemented.

Two parameters that will influence the probability of NVT occurring in nature are mosquito population densities and WNV infections rates. These factors are impacted by numerous factors and are difficult to determine precisely from trapping data. However, in certain habitats, the conditions may be permissive for NVT. Mosquito densities may be very high in some regions. For example, mosquito traps operated in Florida often catch tens of thousands of mosquitoes in a few hours and, recently, one CDC miniature light trap baited with dry ice set in Desoto County captured an estimated 82,000 mosquitoes in one night (R. Nasci, personal communication). In areas of Texas, 13.9% of the *Cx. p. quinquefasciatus* pools have tested positive for WNV, and minimum field infection rates of 3.3 per 1,000 have been recorded (29). There is now year-round WNV activity in Texas (30), a region with large bird populations and 86 species of mosquitoes (31, 32). In areas of Canada where infection rates of 41.37 and 78.78 per 1,000 have been reported for *Cx. pipiens* and *Cx. restuans*, respectively (33), one can speculate that the potential for NVT to occur naturally and to exert a significant impact on WNV epidemiology could be relatively high.

Nonviremic transmission of tick-borne encephalitis virus and Louping ill virus is the major transmission mechanism by which these viruses survive and are dispersed clinally across the forests and hillsides of the northern hemisphere (34). Because ticks also may be vectors for WNV (5), direct transmission between adjacent vectors cofeeding on either susceptible or insusceptible immune hosts or nonimmune hosts has implications for WNV evolution, survival, epidemiology, and dispersion. Recently, NVT of WNV has been demonstrated to occur between infected and uninfected *Ornithodorus moubata* ticks (35). Although the authors concluded that ticks are unlikely to play a major role in WNV transmission, they suggested that some species have the potential to act as reservoirs. NVT would provide a long-term survival strategy because pathogens survive for many months in these long-lived arthropods. Furthermore, long-term survival by direct transmission between vectors, without replication in the vertebrate, might result in the selection of virus variants with different pathogenicity phenotypes from the conventionally transmitted parental virus.

Lord and Tabachnick (27) developed a mathematical model for NVT by using VSV as an example. This model seems equally applicable to WNV transmitted by mosquitoes, which, like black flies, feed rapidly. These authors considered that NVT would probably occur most efficiently between flies feeding closely to each other rather than between flies cofeeding at a distance, either temporally or spatially. Biting flies have a nonrandom distribution among hosts and, like black fly vectors of VSV, mosquitoes may aggregate as a result of behavioral responses to host cues so that a host may be fed on by many mosquitoes at the same time (12). The demonstration of WNV NVT between mosquitoes cofeeding on mice therefore mimics feeding patterns that occur in nature. A mammal host was used in preference to an avian one, because the importance of birds in WNV transmission is well established. Mammals are generally regarded as a dead end host for WNV but, as discussed below, are known to be fed on by large numbers of hematophagous flies. Our data show that under certain conditions, the frequency of NVT of WNV may exceed 5% in the mouse model, but there are no obvious reasons why NVT should not occur when mosquitoes feed on avian hosts.

Lord and Tabachnick (27) were unable to assess quantitatively the relative importance of viremic and nonviremic transmission, but concluded that the implications of NVT are likely to be far reaching and should be considered when developing strategies for controlling vector-borne diseases. A simplistic evaluation for WNV validates their concern. Although our NVT rates for WNV are lower than those observed for VSV (10), the scale of the WNV epidemic and the intensity of transmission necessitate its consideration as a potentially important component of the life cycle. Given that 2,000 flies may feed on a vertebrate per hour (W.J. Tabachnick and E. Schmidtmann, personal communication) and an infection rate of 78 of 1,000 has been reported for WNV (33), it follows that several infected mosquitoes could feed on a large animal during a 1-h period. In our least successful experiment in which seven donors fed in 1 h, we observed a NVT rate of 2%. Extrapolating to a situation with an attack rate of 2,000 per h, a 2% NVT rate would conservatively result in the infection of 40 recipient mosquitoes, a 5.71-fold amplification in the number of infected vectors. Remarkably, a single infected mosquito was associated with a 2.3% NVT rate. Considering the scale of the United States WNV epidemic with thousands of avian, equine, and human cases, millions of mosquitoes must be involved in the transmission of the virus. Because wild-caught mosquitoes infected through NVT cannot be differentiated from mosquitoes infected through vertebrate viremia, a critical component of the WNV transmission cycle may have been overlooked. NVT influences virus transmission rates, accelerates virus transmission times, and permits insusceptible and immune vertebrates to play a key role in the infection and transmission

process, thereby altering the dynamics of virus spread in both vector and vertebrate populations.

WNV has been isolated from a broader range of vectors than any other arbovirus. In North America, the major mosquito vectors of WNV vary according to region, for example *Cx. p. pipiens* in the Northeast, *Cx. p. quinquefasciatus* in the South, and *Cx. tarsalis* in California (36). These and other arthropod species have been tested to determine their relative susceptibility to WNV infection and competence to transmit the virus. These evaluations are based on the viremic mode of infection, but it is possible that susceptibility to direct infection by NVT and competence to transmit to cofeeding vectors may influence the role of certain species currently believed to be unimportant as vectors. For example, species that rarely, if ever, feed on birds have largely been ignored. Because they are not feeding on avian hosts that produce high viremias, it has been assumed that they are unlikely to become infected. With NVT, this assumption should be reevaluated.

Given our demonstration of NVT by mosquitoes, the poorly understood role of ticks as vectors of WNV (5, 35) but their well-documented capacity for NVT of other arboviruses, and the recently demonstrated capacity for NVT of WNV (35), the potential importance of this mode of horizontal transmission for WNV cannot be overstated. Because susceptible, insusceptible, and immune hosts can support NVT (6–8), the population of vertebrates that may contribute to the WNV transmission cycle is probably much greater than was previously realized. Furthermore, with no requirement for a latent incubation period in the vertebrate, because virus can be directly transmitted from one mosquito to another, the transmission process is accelerated. Based on our experimental data and considering the demonstration of NVT by a single infected mosquito that was cofeeding with just 87 others, we believe that NVT is a plausible component of the WNV transmission cycle that may have contributed to its rapid spread in North America. Moreover, the importance of so-called dead-end hosts and vaccinated animals may have to be reconsidered. The numerous equine infections (www.cdc.gov/ncidod/dvbid/westnile) and attraction of large numbers of hematophagous arthropods to such animals (12) supports the idea that, through NVT, they may be playing an important, but hitherto unappreciated, role in amplifying the number of infected vectors.

As a consequence of our findings, the assessment of epidemic risk and effective implementation of suitable vaccination strategies and vector control measures may need to be reassessed for this increasingly important arbovirus and, perhaps, for other mosquito-borne arboviruses, including alphaviruses such as Venezuelan equine encephalitis virus that produce relatively high titer infections in the vector but for which the threshold for vector infection may be relatively low (37). Experimentally, lower thresholds are required to infect mosquitoes feeding on an infected vertebrate than when fed on artificial blood meals, but the mechanism underlying this difference is unknown. Because in our study mosquitoes became infected in the apparent absence of WNV viremia, NVT challenges the traditional concept of the infectious threshold. Indeed, NVT might even play a role in the transmission cycle of viruses such as dengue that, despite causing relatively low viremias, cause millions of dengue fever cases annually.

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