Potentiation of West Nile Encephalitis by Mosquito Feeding

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ABSTRACT

Mosquitoes infect human beings with arboviruses while taking a blood meal, inoculating virus with their saliva. Mosquito saliva contains compounds that counter host hemostatic, inflammatory, and immune responses. Modulation of these crucial defensive responses may facilitate virus infection. Using a murine model we explored the potential for mosquitoes to impact the course of West Nile virus (WNV) disease by determining whether differences in pathogenesis occurred in the presence or absence of mosquito saliva. Mice inoculated intradermally with 10⁴ pfu of WNV subsequent to the feeding of mosquitoes developed more progressive infection, higher viremia, and accelerated neuroinvasion than the mice inoculated with WNV alone. At a lower dose of WNV (10² pfu), mice fed upon by mosquitoes had a lower survival rate. This study suggests that mosquito feeding and factors in mosquito saliva can potentiate WNV infection, and offers a possible mechanism for this effect via accelerated infection of the brain.

INTRODUCTION

The introduction and rapid spread of West Nile virus (WNV) in the United States, affecting human beings, birds, and domestic animals, has highlighted the importance of arthropod-borne (arbo-) viruses to public health. Clinically, human infection can range from asymptomatic, with seroconversion to severe meningitis, encephalitis, acute flaccid paralysis, or death (18). The determinants of disease development and severity, however, are not well defined. Human infection occurs when an infectious mosquito takes a blood meal, injecting WNV with its saliva. Mosquito saliva contains factors that actively modulate host hemostatic, inflammatory, and immune responses (30). WNV is maintained in nature via transmission between susceptible vertebrate hosts by mosquitoes, including those of the genera Aedes, Anopheles, Culex, and Ochlerotatus (5). In particular, Aedes aegypti are well adapted to feed on mammals, and previous work has demonstrated that their saliva has immunomodulatory activity (37). Although these mosquitoes are not the principal WNV vector, this species has been found to be infected with this virus in the field; they are competent vectors of WNV (35); and they may act as bridging vectors between the avian–Culex cycle and mammalian hosts (34).

Enhanced infection attributable to components of arthropod saliva is a recognized phenomenon, and mosquito saliva can affect both immune responses and virus pathogenesis (33). Saliva of Ochlerotatus triseriatus potentiates vesicular stomatitis New Jersey virus infection in mice, causing resistant mice to become infected (23); and treatment of mouse fibroblast cells with Oc. triseriatus salivary gland extract (SGE) significantly increases viral replication compared to untreated controls (24). Studies with other vectors are insightful with respect to vector-induced disease potentiation. Zeidner et al. (46) observed enhanced spirochete loads in target organs

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when *Borrelia burgdorferi* spirochetes were co-inoculated with tick saliva. Correspondingly, the percentage of guinea pigs with detectable uremia more than doubled when the inoculation of tick-borne encephalitis contained tick SGE (22a). Co-injection of *Leishmania* parasites with sandfly SGE significantly enhances parasite burden, lesion size, and disease progression (3,26,33).

Previous work concerning mosquito saliva potentiation of viral infection has compared two routes of infection: needle inoculation and mosquito inoculation (23,28,29). Such comparisons are subject to variation in virus concentration and phenotype generated by the divergent sources of virus. Mosquitoes transmit a range of viral titers (9,19,32,36); for example, individual mosquitoes transmit from 1.0 to 5.0 log0 pfu of WNV (36). Studies that use mosquitoes to transmit virus may therefore be difficult to interpret because of differences in the virus titer delivered. Despite the fact that the selective differences in the environmental conditions provided by insect and mammalian cells have been shown to produce incongruent viral phenotypes (e.g., virulence or tropism) (22,27,43), previous studies on vector potentiation of disease have ignored the potential influence of different inoculation methods. Therefore, one cannot rule out that the differences noted in these studies are caused by intrinsic differences of the viral inoculum. To determine whether mosquito saliva or feeding has an effect on WNV pathogenesis, we performed studies to compare disease development and progression after infection with known titers of well-characterized WNV infection in the presence or absence of mosquito saliva.

Based on known immunomodulatory activity of mosquito saliva (31,37,42) and the potentiation of diseases by the saliva of other vectors (20,33), we postulated that the dysregulation of key immune mediators by mosquito saliva could have an impact on WNV disease. A murine model of infection (2) was used in which a low-passage WNV isolate was inoculated intradermally (i.d.) to mimic natural infection. Treatment groups were evaluated by comparing viremia, display of symptoms, and viral titer in selected organs.

**MATERIALS AND METHODS**

**Viruses.** West Nile virus strain 114 (GenBank accession nos. AY187013 and AY185907) (14) is genetically and phenotypically identical to WNV-NY99 (10). The virus was inoculated i.d. at a concentration of either 10^2 or 10^4 plaque-forming units (pfu), levels that are within the range of virus naturally secreted by mosquitoes (9,36).

**Mice.** Female, 4-week-old, Swiss Webster mice were obtained from Harlan (Indianapolis, IN), and housed in a biosafety level-3 animal facility. Mice were initially divided into four groups (n = 6) by the inoculum they received: PBS (negative control), WNV alone (WNV<sub>alone</sub>), WNV with SGE (WNV<sub>SGE</sub>), and WNV after the feeding of uninfected mosquitoes (WNV<sub>mos</sub>). Later experiments lacked the group concomitantly injected with WNV and SGE because of the greater deviation of the group inoculated with WNV after the feeding of mosquitoes in the first trial of this study. An inoculation containing either 10^2 or 10^4 pfu of WNV in a volume of 20 µL, was administered intradermally (i.d.) in the lower abdomen. Mice were observed twice daily for progression of symptoms. Three mice per group were alternately bled every other day for 9 days, and blood was allowed to clot at room temperature (30 min) before centrifugation (8 min at 850 × g). After separation of serum, samples were stored at −80°C until serum viremia was determined via titration.

**Virus titration.** Serum samples and inoculums were titrated as serial 10-fold dilutions on Vero cells as previously described (17). Wells were scored for cytopathic effect (cpe) to calculate the tissue culture infectious dose 50% endpoint titers (log<sub>10</sub>TCD<sub>50</sub>) by using the method of Karber (21).

**Neutralization assay.** Neutralization tests were performed by mixing 3-fold dilutions of sera, beginning at a dilution of 1/50, with WNV virus-like particles (VLPs), generated by a modification of the methods of Scholle et al. (47), encoding firefly luciferase gene between the coding regions of the C and NS1 proteins (details of construction to be published elsewhere). After a 60-min incubation at 37°C, the antibody/VLP mixtures were then allowed to infect Vero cells grown in a black-walled, 96-well plate for 24 h. The supernatant fluid was then removed from the wells, and the cell layers were lysed in a luciferase reporter buffer and read in a luminometer. Values are reported as the serum dilution that reduced the yield of photons by 80%.

**Organs.** Four-week-old female Swiss Webster mice were divided into groups (n = 6 per group) receiving injections of WNV<sub>alone</sub> or WNV<sub>mos</sub>. On 4 and 7 days post-inoculation (dpi), under sedation, three of the mice from each group were euthanized and perfused with PBS (pH 7.4). The organs were surgically removed and half of each organ placed in RNAlater (Qiagen, Valencia, CA) until RNA isolation. As described elsewhere, RNA was isolated (31) and RNA levels of WNV were quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) (36), normalized to a constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then multiplied by 10,000 to assist in interpretation of results. Viral titers resulting from this quantitative method are expressed as equivalent pfu ± standard error. The remaining half of each organ was fixed, embedded, and processed by immunohistochemistry for detection of...
WNV antigen using VECTASTAIN Elite Avidin/Biotinylated Enzyme Complex Kit (Vector Laboratories, Burlingame, CA) and primary polyclonal antibody reactive against WNV as previously described (14).

Mosquitoes. *Aedes aegypti* mosquitoes were reared and maintained in an insectary at 27°C and 80% relative humidity, as previously described (16). Uninfected female *Ae. aegypti* mosquitoes 8–12 days post-eclosion were allowed to feed on the specified groups of mice as approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch. Individual mice were ventrally shaved and placed on top of mesh covering 1-quart cartons containing 15 mosquitoes that had been deprived of sucrose for 12 h to encourage feeding. The site of feeding was restricted to a circular area (1.0 cm in diameter) on the lower abdomen of an anesthetized mouse using an appropriately sized cardboard template, which was located between the mouse and mosquitoes. After a 30-min feeding period, WNV was inoculated i.d. into the center of the feeding site. Mosquitoes from each mouse were chilled and the number of engorged females was noted; an average of 11.5 (±1.5) mosquitoes fed on each mouse.

Salivary gland extract. To obtain SGE, salivary glands were dissected from female *Ae. aegypti* mosquitoes (7–10 days post-eclosion), as previously described (31), and was mixed with WNV (see above) to create a 20-μL inoculum containing an equivalent of 1.0 salivary gland pair.

Statistical analysis. The Student unpaired t test was used to assess significance between tissue titers of WNV. Analysis was performed using SigmaPlot (Systat Software, Inc., Point Richmond, CA). Wilcoxon signed ranks test was performed, using Analyse-It General 1.71 (Analyse-It Software, Ltd., Leeds, England), and two-tailed p values were generated, to compare survival curves. Fisher exact tests were used to compare the proportion of tissues with detectable WNV. In all statistical tests, values of p < 0.05 were considered to be significant.

RESULTS

Mosquito saliva was found to enhance progress of disease and mortality. To determine whether mosquito saliva alters the course of WNV infection, mice were inoculated i.d. with WNV alone (WNV alone) or concomitantly with mosquito saliva, which was provided either via addition of SGE to viral inoculum (WNV SGE) or via direct feeding of mosquitoes on mice (WNV mos). Survival curves between groups varied (Figs. 1A–1C). The inoculum of 10^2 pfu of WNV resulted in similar survival curves between groups, but differences in overall survival rates. All WNV mos mice died, but on average, 24.9% of mice injected with WNV alone survived (Fig. 1B). This suggests that when low amounts of virus were inoculated, factors in mosquito saliva altered the disease in a manner that enhanced the likelihood of a fatal outcome.

Infection with 10^4 pfu of WNV resulted in 100% mortality in all experimental groups (Fig. 1A,C). Mice inoculated with 10^4 pfu of WNV subsequent to the feeding of mosquitoes (WNV mos) succumbed earliest to the infection [mean survival time (MST): 7.5 days], followed by the WNV SGE (MST: 8.6 days) and finally the WN-
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Valne group (MST: 10.6 days). These findings support the hypothesis that mosquito feeding accelerates the progression of viral infection. The intermediate MST of the WNVSGE group suggests that this modulation is caused by saliva constituents rather than by the mechanical act of mosquito feeding.

The reduced potentiation activity of SGE may be an effect of the extraction process or the inclusion of components absent from saliva. Certainly, during isolation of SGE, it is possible that enzymes are released that could compromise the potency of the saliva. The effect of saliva in SGE may also be diluted by the presence of cellular proteins. Interestingly, at 9 dpi, all of the WNVmos mice had died, whereas all of the WNValone mice remained alive (Fig. 1A). All trials with 10^4 pfu of WNV demonstrated parallel observations (Fig. 1B). At both WNV doses, the WNVmos and WNValone curves were significantly different (p = 0.03). Survival curves did not differ significantly between the WNValone group inoculated with 10^4 pfu and the WNVmos group inoculated with 10^2 pfu, suggesting that an inoculum of 10^2 pfu of WNV with mosquito saliva was equivalent to an inoculum of 10^4 pfu of WNValone with respect to pathogenesis.

Mosquito feeding prior to virus inoculation increased viremia. To investigate the mechanism by which mosquito feeding enhanced WNV disease progression, we measured viremia levels for 7 dpi. Virus levels peaked at 2 and 3 dpi. On 2 dpi, mice treated with 10^2 and 10^4 pfu of WNValone had comparatively low titers of 1.3 ± 0.42 and 1.8 ± 0.56 log_{10}TCID_{50}/mL, respectively (Fig. 2A). In contrast, WNVmos mice inoculated with 10^2 and 10^4 pfu of WNV had relatively high titers of 2.9 ± 0.33 and 3.3 ± 0.33 log_{10}TCID_{50}/mL (Fig. 2A). Mice in the WNVmos groups generally had higher viremias compared to other treatment groups (2 dpi, p = 0.003), as well as longer durations of viremia (3.0 ± 0.3 days) compared to the WNValone groups (2.1 ± 0.2 days).

At early time points, mice in the WNVmos group had consistently higher viremias than did the WNValone group (Fig. 2A), suggesting rapid amplification of viral replication. To evaluate localized replication, at 1 and 3 dpi, RNA was isolated from skin samples at the inoculation site and from the draining lymph nodes. Mice in the WNVmos group consistently had more WNV in the skin (6.4-fold and 15.8-fold more at 1 and 3 dpi, respectively) and in the draining lymph node (25.9-fold and 3.4-fold more at 1 and 3 dpi, respectively) compared to mice inoculated with WNValone. Although these results were not significant (p = 0.07), they were consistent with the trend of enhanced viral replication in the presence of mosquito saliva. These data suggest that mosquito saliva promotes WNV replication at the primary infection sites, thereby accelerating disease development.

Levels of neutralizing antibody to WNV were determined to ascertain whether the observed in vitro inhibition of B cells by mosquito saliva (37) correlated with effects on the in vivo humoral response to WNV. Low neutralizing antibody titers detected in all groups at 7 dpi
were not statistically significantly different (WNV\textsubscript{alone}, 180 ± 11; WNV\textsubscript{SGE}, 530 ± 130; WNV\textsubscript{mos}, 260 ± 78).

Mosquito feeding accelerated neuroinvasion. Because of the significance of central nervous system (CNS) infection in disease outcome, viral titers in the brain were determined at two time points to investigate whether mosquito saliva influences viral entry or replication in the brain. At 4 dpi, significantly more virus was detected real-time RT-PCR in brains of WNV\textsubscript{mos} mice as compared to WNV\textsubscript{alone} mice ($p = 0.01$). WNV was only detected in the brains of WNV\textsubscript{mos} mice (three of six mice; average of 410 equivalent pfu) (Fig. 2B), suggesting that mosquito saliva accelerates infection of the brain. Pathologic changes were not observed in hematoxylin and eosin–stained sections of brain, and WNV antigen was undetectable by immunohistochemistry. The absence of viral antigen and pathology at this early time point is consistent with previous reports (44).

At 7 dpi, brain tissues of WNV\textsubscript{mos} mice had an average viral titer 350 times higher than the WNV\textsubscript{alone} group ($8.4 \times 10^4 ± 7.9 \times 10^4$ equivalent pfu vs. $2.5 \times 10^2 ± 1.7 \times 10^2$ equivalent pfu) by real-time RT-PCR (Fig. 2C). Of the WNV\textsubscript{mos} mice, 100% (10/10) had detectable WNV in brain tissue, compared to only 80% (8/10) of WNV\textsubscript{alone} brain tissue samples, again supporting the proposition that mosquito saliva enhances CNS infection. As previously reported (44), we observed minimal perivascular cuffing and contracted large neurons in deep layers of the cerebral cortex at 7 dpi in both groups (data not shown). WNV antigen was detected by immunohistochemistry in the brains of 2 of 6 mice in the WNV\textsubscript{alone} group. Antigen distribution was limited to small foci of neurons within the striatum and cerebral cortex. Staining was intense and widespread compared to the WNV\textsubscript{alone} group (Fig. 3). More than 10 foci were observed in two of the brain samples, whereas in the other two samples, staining was too extensive to enumerate foci. In contrast, only one focus per antigen-positive brain was identified in day 7 samples from the WNV\textsubscript{alone} group.

The WNV titers in tissues were higher in mice exposed to mosquitoes. The virus titers of selected tissues were determined at two time-points to investigate the influence of mosquito saliva on virus tropisms and/or tissue-specific replication (Figs. 2B, 2C). By real-time RT-PCR, no differences between treatment groups were found in virus titers of the spleen, liver, heart, and kidney samples at 4 dpi (Fig. 2B), although virus was detected in all tissues. At 7 dpi, tissues from the WNV\textsubscript{mos} mice tended to have higher viral loads than WNV\textsubscript{alone} mice (Fig. 2C). The use of an inbred mouse strain may have reduced variability between individuals; however, our preference for outbred mice more accurately represents the diversity of natural populations. The greater variation may have reduced the power to detect significant differences between groups; thus reproducible, albeit not statistically significant, differences must be considered carefully. All heart samples contained detectable levels of WNV, yet the average titer of WNV in the WNV\textsubscript{mos} group was 3.4 times higher than that of the WNV\textsubscript{alone} group. Correspondingly, in kidneys, titers in the WNV\textsubscript{mos} group were 4.1 times greater than the average titers in the WNV\textsubscript{alone} group. Similar differences were observed in spleens (Fig. 2C), although only 43% (3/7) of WNV\textsubscript{alone} spleens had detectable WNV as compared to 100% (10/10) of WNV\textsubscript{mos} spleens ($p = 0.02$). Overall, these observations suggest that exposure to mosquito feeding promotes infection of specific tissues, and/or impairs the ability of tissues to clear the viral infection.

**FIG. 3.** Amplified levels of West Nile virus (WNV) antigen in the brains of WNV\textsubscript{mos} mice at 7 dpi as detected by immunohistochemistry in the forebrain. The WNV\textsubscript{alone} section (left panel) contains a single small foci, whereas the WNV\textsubscript{mos} section (right panel) displays widespread staining. Scale bar = 50 μm.
higher viremia during early stages of infection. These dif-
ferences may be explained by mosquito feeding–induced 
or saliva-induced accelerated neuroinvasion of WNV and 
amplified average virus titers within the brain.

This study clarifies and extends previous studies that have 
described mosquito-associated potentiation of arbo-
virus infection. Limesand et al. (23) demonstrated that 
94% of the 3-week-old mice fed on by mosquitoes 
infected with vesicular stomatitis New Jersey virus devel-
oped antibody, whereas antibody was detected in only 
13% of needle-inoculated mice. In a related study, mice 
did not become infected after injection of Cache Valley 
mosquito alone, however, injection of the virus into sites of 
mosquito feeding resulted in detectable viremia and pro-
duction of anti-Cache Valley antibody (13). Previously, 
La Crosse virus caused high levels of mortality in mice 
when injected by infected mosquitoes or inoculated into 
the site of intense mosquito feeding, whereas needle-in-
oculated virus, even at high titer, rarely caused mortality 
(S. Higgs and B. Beaty, unpublished data). In addition, 
a dose effect was noted: as the number of mosquitoes fed 
per mouse increased, mice developed clinical symptoms 
and succumbed to infection more rapidly.

The mechanisms of WNV pathogenesis are not com-
pletely understood; of what is known, possible targets of 
salivary modulation can be inferred. In mice, WNV in-
duces a systemic infection, resulting in encephalitis and 
death (38). Type-1 interferons (IFNs) and humoral im-
muinity provide immediate defence against the dissemi-
nation of WNV (1,6,12,25). Recent studies have shown 
that co-inoculation of Ae. aegypti saliva with an arbovirus 
leads to >50% inhibition of the local type-1 and type-2 
IFN response of the host at crucial early time points in 
infection, compared to inoculation of the virus alone (31). 
This saliva-induced suppression of interferon corre-
sponds with other studies (15,24,45). Because IFN-α and 
IFN-β have demonstrated protective activity against 
WNV (1), their suppression could impair the ability of 
the innate immune response to resist viral infection while 
the adaptive response establishes, thereby permitting 
replication and dissemination of the virus early in infec-
tion. Cellular immunity also participates in recovery of 
the host from WNV infection (39,41), and cytotoxic T 
lymphocytes and a robust Th1 response have proved to 
be important in host defense against flaviviruses infec-
tion (8). Indirect inhibition of antiviral cytokines and the 
Th1 response may be precipitated by a substantial in-
crease in Th2 cytokines observed after mosquito feed-
ing which have an inhibitory effect on natural killer 
cells, IFN-γ production, and antigen presentation (31,45). 
Mosquito saliva has the potential to affect both innate 
and adaptive immune defences against WNV infection, 
and these modulations may, at least in part, determine the 
course of viral pathogenesis. It is important to note that 
effects of mosquito saliva on systemic host immune re-
ponse have been observed for up to 14 days after mos-
quito feeding (45). Differences observed in this study be-
tween experimental groups as early as 1 and 2 dpi suggest 
that the effect of mosquito saliva may be caused by mod-
ulation of host innate immune responses, although pre-
vious studies have also suggested effects of saliva on 
adaptive immune response (37,45).

The accelerated and increased death rate in mice in-
fected with WNV after the feeding of mosquitoes may 
be attributable to earlier and enhanced infection of the 
brain, especially considering that fatal arboviral infection 
in vertebrates involves CNS pathology including leth-
argy, decreased responsiveness, confusion, and paralysis 
(7). The enhancement of neurological infection observed 
in WNV motorists mice may be explained by direct effects of 
mosquito feeding or indirectly via immune response to 
mosquito saliva (31) and enhanced viremia. Previous re-
search demonstrated that tumor necrosis factor–α (TNF-
α) levels were enhanced ~6-fold by mosquito feeding 
(11). Wang et al. (40) showed that TNF-α receptor 1 sig-
naling is instrumental in enhancement of blood–brain 
barrier permeability to WNV infection. The experimen-
tal effect of mosquito feeding on disease development is 
also particularly significant, given that, in a natural set-
ting, hosts are continually fed upon by mosquitoes and 
often at high densities.

Higher viremia early in infection could also contribute 
toward accelerated pathogenesis, because a high viremia 
enhances the potential for the brain to become infected 
(4). Higher virus levels in the serum of WNV motorists 
 mice at 2 dpi may facilitate viral dissemination and entry into the 
CNS before establishment of a protective immune re-
sponse. This early divergence between groups suggests 
that the innate immune response is affected by mosquito 
feeding. The finding of enhanced WNV viremia in mos-
quito-exposed mice concurs with a study that demon-
strated higher and longer viremias after exposure to La 
Crosse virus if infection was via Oc. triseriatus mosqui-
toes versus a needle (28). Edwards et al. (13) also ob-
served enhancement of Cache Valley viremia caused by 
mosquito feeding. These studies along with the present 
research suggest a complex role for mosquito saliva not 
only in disease pathogenesis, but also in the natural cy-
cle of arboviruses.

Neutralizing antibody titer did not vary significantly 
between groups at 7 dpi, despite higher viremia in the
groups of mice exposed to mosquitoes. The higher viral titers in the WNV\textsuperscript{mos} group might be expected to stimulate the production of elevated serum antibody titers (31a); therefore, our data may indicate suppression of antibody response by mosquito saliva. Previous studies that reported an inhibitory effect of saliva on B-cell proliferation were conducted in vitro and did not investigate the response during an active viral infection. Additional research is needed to determine whether differences exist at earlier time points or in the CNS.

The study results suggest that the potentiation of WNV infection induced by mosquito feeding is linked to factors in mosquito saliva, as opposed to the mechanical act of feeding. Potentiation of infection in the WNV\textsuperscript{mos} group cannot be explained by enhanced hematogenous virus dissemination due to the physical puncturing of capillaries by mosquito probing at the site of virus inoculation, given that WNV infection was similarly potentiated in the WNV\textsuperscript{SHE} group.

Our data demonstrate that mosquito feeding can potentiate infection of an important emerging arbovirus, and suggest that this potentiation is caused by factors in mosquito saliva. Importantly, this potentiation can lead to differences in the survival rates and disease course of the host. The effect of mosquito saliva alters early pathogenesis, such as amplified viral titer proximal to the inoculation site and serum, and later pathogenesis, including accelerated neuroinvasion and the time and rate of death. Further work in this area will allow for determination of specific factors in vector saliva that affect disease course and may thereby lead to enhancements in prophylactics for arthropod-borne diseases.

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