SALIVARY GLAND MORPHOLOGY AND VIRUS TRANSMISSION DURING LONG-TERM CYTOPATHOLOGIC WEST NILE VIRUS INFECTION IN CULEX MOSQUITOES

YVETTE A. GIRARD, BRADLEY S. SCHNEIDER, CHARLES E. MCGEE, JULIE WEN, VIOLET C. HAN, VSEVOLOD POPOV, PETER W. MASON, AND STEPHEN HIGGS* 
Department of Pathology, University of Texas Medical Branch, Galveston, Texas

Abstract. The effect of long-term West Nile virus (WNV) infection on Culex salivary gland morphology and viability was evaluated by transmission electron microscopy during a four week period post-blood feeding. These studies showed that apoptosis and other cytopathologic changes occurred more frequently in WNV-infected mosquitoes compared with uninfected controls. The effect of long-term infection on WNV transmission was evaluated by titering virus in saliva over the same time period. Although the mean titer of WNV in mosquito saliva did not change significantly over time, the percentage of saliva samples containing WNV decreased. Because of the importance of saliva in blood meal acquisition and virus delivery, salivary gland pathology has the potential to affect mosquito feeding behavior and virus transmission. Results from this study add to a growing body of evidence that arbovirus infections in mosquito vectors can be cytopathic, and offer a potential mechanism for virus-induced cell death in mosquitoes.

INTRODUCTION

West Nile virus (WNV) is an emerging mosquito-borne virus in the family Flaviviridae, genus Flavivirus and is the etiologic agent of West Nile fever, West Nile encephalitis, and West Nile paralysis.1 Outbreaks of WNV-associated disease in humans, horses, and birds have become more frequent since the early 1990s, and have occurred in Africa, eastern Europe, the Mediterranean Basin, Russia, Australia, and the United States.2,3 In the United States, approximately 11,000 cases of West Nile fever and more than 8,000 cases of neuroinvasive West Nile disease in humans have been reported to the Centers for Disease Control and Prevention since the introduction of the virus into the Western Hemisphere in 1999.4 Susceptible avian hosts that may serve as virus-amplifying reservoirs typically become infected with WNV by the bite of a competent mosquito vector such as Culex pipiens quinquefasciatus. These mosquitoes are abundant in tropical and subtropical regions throughout the world, including the southern and western United States.5 In the United States in 2004, Cx. p. quinquefasciatus was the most commonly reported WNV-positive species during mosquito pool testing.6 Earlier immunohistochemical (IHC) and electron microscopic (EM) studies indicated that WNV infection is persistent in midgut, salivary gland, and nervous tissues of these vectors during a four-week period after an infectious artificial blood meal.7,8 What remains unknown is the nature of this long-term flavivirus infection including the effects of continuous WNV infection and replication on mosquito cell viability, tissue function, and virus transmission efficiency.

As with other vectors, Cx. p. quinquefasciatus ingest WNV whilst taking a blood meal from a viremic bird. A requisite for transmission of the virus, which can occur in Cx. pipiens as early as five days after ingestion of an infectious blood meal,9 is replication in salivary gland cells and secretion of the virus into the saliva cavity. WNV is inoculated into a host along with mosquito saliva that is secreted by the insect to facilitate blood feeding. Using transmission EM (TEM), we recently identified cytopathologic changes including cell death in the salivary glands of WNV-infected Cx. p. quinquefasciatus.8 As described herein, we performed additional ultrastructural studies of the salivary glands to differentiate age-related changes from virus-induced changes, and to elucidate the frequency and mechanism of salivary gland cell death associated with WNV replication. Because mosquito salivary gland cells support persistent WNV infection and are responsible for saliva production and secretion, we hypothesized that cytopathologic changes observed by TEM might impair saliva secretion, virus secretion, and virus maturation. Therefore, we assessed the quantity and infectivity of WNV secreted in saliva at time points corresponding to ultrastructural analysis.

Previously, we described WNV-induced proliferated membranes in infected Cx. p. quinquefasciatus salivary glands, including smooth membrane structures (SMS), convoluted membranes (CM), tubular proliferated membranes (TPM), and row-like parallel membrane structures (PMS).8 The detection of flaviviral genomic RNA, the replicative form of viral RNA, and nonstructural proteins involved in RNA replication (NS1, NS2A, NS3, NS4A, and NS5) in association with WNV infection of the salivary glands, displaying characteristics of apoptosis including shrunken and electron-dense nuclei with nuclear membrane blebbing and chromatin marginalization. In infected tissues, dying cells typically contained abundant SMS, CM, shrunken, electron-dense mitochondria, and non-compartmentalized WNV particles.

Our present study was designed to test the hypothesis that apoptotic cell death and other cytopathologies are spatially and temporally correlated with WNV infection of the salivary glands. Included in our analysis is an investigation of the frequency of newly-identified morphologies such as saliva pool-
ing, dense irregular saliva (DIS), and filamentous cytoplasmic membranes (FCM), as well as determination of the distribution, abundance, and morphology of proliferated membrane structures and paracrystalline arrays of WNV particles. Results from these studies suggest an increase in the frequency of apoptotic cell death may correspond to a decrease in WNV transmission. We conclude from these results that virus-induced changes in mosquito cells at late stages of infection could play a role in arbovirus transmission dynamics in nature.

MATERIALS AND METHODS

**Virus.** The lineage I WNV isolate (strain 114) used in mosquito infections was derived from a blue jay brain/liver homogenate tested during surveillance in Houston, Texas in 2002. By sequence analysis (Genbank accession nos. AY185907 and AY187013), strain 114 clusters with the dominant genetic variant isolates that have emerged in the United States. Mouse virulence studies have shown the virus to be similar to WNV NY99 in phenotype. The virus was propagated in Vero cells and cell culture supernatant was harvested and stored at −80°C until used in mosquito blood meals.

**Mosquitoes.** Laboratory-reared, Sebring strain Cx. p. quinquefasciatus mosquitoes used for experiments were obtained from the Harris County Mosquito Control District in Houston, Texas and consisted of mosquitoes from F1 or later generations. Mosquitoes were maintained as previously described. Sebring strain mosquitoes are not significantly different from field-caught Cx. p. quinquefasciatus from Houston, Texas with respect to infection and dissemination rates of WNV strain 114 (Klingler KL and Higgs S, unpublished data).

**Mosquito infection.** Female mosquitoes were fed on infectious and non-infectious blood meals 5–7 days post-engorgement and were deprived of sucrose for 24 hours prior to feeding. For infectious blood meal preparation, a Vero cell monolayer was inoculated with WNV at a multiplicity of infection of 2, and cells and supernatant were harvested after three days, which coincides with peak virus titer. Blood meals consisted of either WNV-infected Vero cells and supernatant, or uninfected Vero cells and supernatant mixed in a 1:1 ratio with defibrinated sheep blood (Colorado Serum Company, Denver, CO), along with 2 mM adenosine triphosphate, which was used as a phagostimulant. Mosquitoes were exposed to warm (37°C) blood meals using a Hemotek feeding apparatus (Discovery Workshops, Accrington, United Kingdom) for one hour, after which fully engorged mosquitoes were sorted and kept in cartons at 28°C and fed 10% sucrose ad libitum. All experiments including mosquito infections and dissections were performed in Biohazard Safety Level-3 laboratories and insectaries.

**Mosquito and blood meal titration.** Infectious blood meals and three mosquitoes collected immediately after feeding on infectious blood (day 0) were titered in duplicate as serial 10-fold dilutions on Vero cells to determine the log10 infectious dose (TCID50/ml or mosquito). Mosquitoes were individually triturated in 1 mL of Leibovitz L-15 medium (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum, 1% tryptose phosphate broth, 100 U/mL of penicillin, and 100 μg/mL of streptomycin) and filtered through a 0.22-μm syringe filter prior to titration. Virus titers were calculated as tissue culture infectious dose 50% endpoints (TCID50) using the computer program ID50 version 5.0 (John L. Spouge; National Center for Biotechnology, National Institutes of Health, Bethesda, MD).

**Transmission electron microscopy.** Salivary glands were dissected from both WNV-infected and uninfected control mosquitoes on days 14, 21, and 28 post-blood feeding. Approximately 10–15 mosquitoes were aspirated from cartons and chilled on ice prior to dissection. Salivary glands were removed from the thorax in a drop of phosphate-buffered saline (PBS, pH 7.4) on a clean glass slide and transferred immediately into TEM fixative. Salivary glands were stored at 4°C for no longer than one month in the fixative composed of 2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% trinitrophenol, and 0.03% CaCl2 in 0.05 M cacodylate buffer (pH 7.3). Post-fixation was carried out in 1% OsO4 in 0.1M cacodylate buffer for one hour at room temperature, followed by en bloc staining in 1% uranyl acetate in 0.1 M maleate buffer for 20 minutes at 60°C. Mosquito salivary glands were dehydrated in an ethanol series and embedded in PolyBed 812 (Polysciences, Warrington, PA). Total sample sizes of 5–7 salivary glands were analyzed per time point per treatment group. Semi-thin (5 μm) sections were cut on a Reichert-Ultrapan 3 ultramicrotome (Leica Microsystems Inc., Bangcockburn, IL) and stained with toluidine blue for observation by light microscopy. When dark-staining cells or morphologic characteristics of the cytopathology were observed in 5-μm sections, ultra-thin sections (70 nm) were cut and placed on 2–3 copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips (Eindhoven, The Netherlands) CM-100 electron microscope at 60 kV. If dark-staining cells were absent by light microscopy, additional 5-μm sections were prepared, stained, and examined. Depending on salivary gland orientation, up to 12 levels were analyzed by light microscopy and up to 8 levels were analyzed by TEM. A total of 20 morphologic characteristics relating to cytoplasmic membranes and cell viability were evaluated by TEM in both infected and uninfected groups at each collection time point. The frequencies of each characteristic were tabulated and statistically analyzed between time points and between treatment groups using Fischer’s exact test using SPSS for Windows version 11.5.0 (SPSS Inc., Chicago, IL).

**Saliva collection.** In two experiments, saliva was collected from mosquitoes infected with WNV at 14 (n = 20), 21 (n = 20), and either 25 (n = 12) or 28 (n = 16) days post-infection (dpi) as previously described. In one of the experiments, saliva was collected at 7 dpi (n = 20).

**Quantitative reverse transcription–polymerase chain reaction (qRT-PCR).** To thawed saliva samples, 105 μL of Leibovitz L-15 medium containing 1% fetal bovine serum (FBS) was added, and the sample was vortexed and centrifuged for one minute at 8,161 × g. A 50-μL subsample was added directly to 300 μL of RT buffer from the QIAamp viral RNA kit (Qiagen, Valencia, CA) and RNA was extracted within one hour following the manufacturer’s protocol. The total amount of RNA, expressed as the number of plaque-forming unit (PFU) equivalents of WNV in each saliva sample, was determined using a TaqMan One-Step RT-PCR (Applied Biosystems, Foster City, CA) as previously described. A standard curve was generated using three replicates of serial 10-fold dilutions of RNA extracted from virus stock with a known titer of 2 × 10^9 PFU/mL. Quantification of WNV PFU
equivalents in saliva samples was calculated by comparison of the threshold cycle (Ct) values of the samples to the standards using Cepheid software (Cepheid, Sunnyvale, CA). Values are expressed as the average of two wells multiplied by a dilution factor of 2 to represent the whole saliva sample. Data was visualized and statistically analyzed by Student’s unpaired t-test using SigmaPlot version 9.0 (SPSS Inc.).

Focus forming assay (FFA). The remaining 50/μL saliva subsample was added to 250/μL of Leibovitz L-15 medium containing 1% FBS, vortexed, and further serially diluted six-fold. A volume of 125 μL of each dilution was added in duplicate to a Vero cell monolayer in a 24-well tissue culture plate (Corning Inc., Corning, NY) and was incubated at room temperature for one hour on a rocker. After incubation, an overlay containing 1% FBS in one part 2×L-15 medium mixed with one part 1.2% Tragacanth (Sigma-Adrich, St. Louis, MO) solution was added to each well. Plates were incubated at 37°C for three days, at which time the overlay was aspirated, and wells were washed twice with PBS and allowed to dry in the biosafety cabinet. To each well, approximately 500 μL of a 1:1 (v/v) mixture of cold acetone/methanol was added and plates were kept at −20°C for a minimum of two hours and a maximum of two days. Cell monolayers were stained for the presence of foci of viral antigen by IHC as previously described. Foci of infection comprising antigen-positive cells were counted in each well and multiplied by dilution factors to determine the number of focus-forming units, i.e., infectious virus particles, per saliva sample.

RESULTS

Salivary gland morphology during WNV infection. In the current study, 10–15 salivary gland pairs were dissected from WNV-infected and uninfected control mosquitoes 14, 21, and 28 days after blood feeding. The infectious blood meal contained 7.9 log_{10} TCID_{50}/mL of WNV, and the mean ± SD titer of three mosquitoes collected immediately after blood feeding was 5.6 ± 0.6 log_{10} TCID_{50}/mosquito. After dissection into PBS, half of a salivary gland pair was fixed and processed for TEM. Tissues were evaluated for morphologic characteristics including cell viability, proliferated membrane morphology, virus distribution, and saliva morphology (Tables 1 and 2).

### Table 1

<table>
<thead>
<tr>
<th>Collection day/treatment group</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth membrane structures</td>
<td>6/6*</td>
<td>0/6</td>
<td>7/7*</td>
</tr>
<tr>
<td>Tubular proliferated membrane (TPM)</td>
<td>6/6*</td>
<td>0/6</td>
<td>7/7*</td>
</tr>
<tr>
<td>With parallel membrane structure</td>
<td>1/6</td>
<td>0/6</td>
<td>3/7</td>
</tr>
<tr>
<td>TFM/CM combination</td>
<td>4/6*</td>
<td>0/6</td>
<td>4/7</td>
</tr>
<tr>
<td>With disorganized morphology</td>
<td>0/6</td>
<td>0/6</td>
<td>0/7</td>
</tr>
<tr>
<td>Paracrystalline arrays of WNV in saliva</td>
<td>5/7*</td>
<td>0/6</td>
<td>6/7*</td>
</tr>
<tr>
<td>Vacularization</td>
<td>3/6</td>
<td>0/6</td>
<td>4/7</td>
</tr>
<tr>
<td>Phagolysosomal vacuoles</td>
<td>5/6</td>
<td>5/6</td>
<td>4/7</td>
</tr>
<tr>
<td>Dense irregular saliva</td>
<td>2/6</td>
<td>2/6</td>
<td>2/7</td>
</tr>
<tr>
<td>Filamentous cytoplasmic membranes</td>
<td>0/6</td>
<td>1/6</td>
<td>4/7†</td>
</tr>
<tr>
<td>Saliva pool</td>
<td>0/6</td>
<td>0/6</td>
<td>0/7</td>
</tr>
<tr>
<td>With apoptotic dead cells</td>
<td>0/0</td>
<td>0/0</td>
<td>3/4</td>
</tr>
<tr>
<td>Dense striated membranes</td>
<td>3/6</td>
<td>3/6</td>
<td>2/7</td>
</tr>
<tr>
<td>Electron transparency</td>
<td>3/6</td>
<td>1/6</td>
<td>2/7</td>
</tr>
</tbody>
</table>

* Frequency of characteristic is significantly higher than the alternative treatment group from the same time point (P < 0.05).
† Frequency of characteristic is significantly higher than day 14 (P < 0.05).
‡ Frequency of characteristic is significantly higher than day 21 (P < 0.05).

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### Table 2

Features of apoptotic cell death in West Nile virus (WNV)–infected and uninfected salivary glands

<table>
<thead>
<tr>
<th>Collection day/treatment group</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of SG with apoptotic cells (nucleus observed)/total number of SG analyzed (%)</td>
<td>1/6 (14)</td>
<td>2/6 (33)</td>
<td>5/7 (71)</td>
</tr>
<tr>
<td>Apoptotic cells (all samples combined)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus observed</td>
<td>5</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>No nucleus observed</td>
<td>1</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>2</td>
<td>43</td>
</tr>
<tr>
<td>Apoptotic cell location in salivary gland</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central</td>
<td>Peripheral</td>
<td>Central</td>
<td>Peripheral</td>
</tr>
<tr>
<td>Number of SG with pre-apoptotic cells/total number of SG analyzed (%)</td>
<td>2/6 (33)</td>
<td>0/6 (0)</td>
<td>4/7 (57)</td>
</tr>
<tr>
<td>Pre-apoptotic cells (all samples combined)</td>
<td>2</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

* SG = salivary glands.
† Frequency of characteristic is significantly higher than day 14 (P < 0.05).
‡ Frequency of characteristic is significantly higher than the alternative treatment group, same time point (P < 0.05).
For each characteristic, the frequencies of occurrence were compared for WNV-infected and uninfected tissues within and between time points.

At all time points, SMS, CM, and TPM were present in infected tissues but were not observed in uninfected salivary gland cells (Table 1 and Figure 1A and B). The combination CM/TPM structures were less frequent at 14 dpi (4 of 6) and 21 dpi (4 of 7) compared with 28 dpi (6 of 6). The level of CM size and distribution did not vary over time; however, the frequency of CM/TPM disorganization increased significantly 28 dpi (4 of 6) compared with 14 and 21 dpi, when disorganization was not observed. Membranes within disorganized CM/TPM appeared loose, less compact, and less regular in conformation. The frequency of row-like parallel membrane structures (PMS) associated with TPM increased over time post-infection (1 of 3 at 14 dpi, 3 of 7 at 21 dpi, and 4 of 6 at 28 dpi). Endoplasmic reticulum vacuolization, defined as marked distention of the ER luminal space, was only observed in infected tissues (Table 1 and Figure 1A and C). Vacuolization increased over time in WNV-infected salivary...
glands (3 of 6 at 14 dpi, 4 of 7 at 21 dpi, and 5 of 6 at 28 dpi). As shown in Table 1, electron transparency of the salivary gland cell cytoplasm was associated with virus infection and the frequency of this characteristic increased over time post-infection. In infected tissues, 3 of 6, 2 of 7, and 4 of 6 salivary glands showed electron transparency at 14, 21, and 28 dpi, respectively, compared with 1 of 6 control salivary glands that displayed electron transparency only at 14 dpi.

Large accumulations of saliva (saliva pooling) occurred in the center of the lobe in both WNV-infected and uninfected salivary glands (Figure 1D). Cells surrounding the pool were flattened, suggesting that the acinar cells of the salivary gland had lost the normal concave shape that keeps extracellular saliva confined to the acinar cavity. In WNV-infected tissues, saliva pooling was only observed at 21 dpi (4 of 7) (Table 1). Saliva pooling was only observed in uninfected salivary glands on day 28 (2 of 7). Dead cell nuclei and cellular debris were observed in saliva pools in both infected (3 of 4) and uninfected (1 of 2) salivary glands (Table 1 and Figure 1D).

The term DIS describes the appearance of granular, electron-dense, spherical bodies and membranous material (possibly cellular in origin) within the saliva (Figure 2A). Similar frequencies of DIS, DSM and PhV were found in both infected and uninfected tissues throughout the study (Table 1). The frequency of occurrence of FCM, which was characterized by thin, wavy membranes abundant within the cytoplasmic space of some cells, was analyzed (Figure 2B). Cells containing FCW were only identified in 1 of 6 uninfected salivary gland samples on day 14, but were seen at a high frequency in WNV-infected mosquitoes at later time points (4 of 7 at 21 dpi and 5 of 6 at 28 dpi) (Table 1).

Paracrystalline arrays of WNV particles in saliva were observed in most WNV-infected salivary glands at all time points; 5 of 7 salivary glands at 14 dpi, 6 of 7 at 21 dpi, and 4 of 6 at 28 dpi. Arrays were sometimes extremely large (Figure 3A). Within salivary acinar cavities, WNV particles associated with an unknown saliva component that itself crystallized (Figure 3A). Paracrystalline arrays were commonly seen in salivary gland ducts as shown in Figure 3B.

**Frequency of cell death in WNV-infected and uninfected salivary glands.** Morphologic features of apoptotic cells observed in this study included abnormally shaped, electron-dense nuclei with chromatin marginalization. These cells were termed apoptotic-like by investigators in a previous report. Cells containing these features are herefore termed apoptotic based on extensive morphologic examination, although biochemical analysis is needed to further confirm this assignment. In infected tissues, apoptotic cells were generally located centrally in the gland (Table 2) and had increased cytoplasmic electron-density and overall distorted cell shape. Shrunken and electron-dense mitochondria, SMS either free or within ER vesicle packets, and non–membrane-bound WNV particles were observed in infected, but not control, tissues (Figure 4A). In some instances, apoptotic cells showed evidence of membrane permeability, or disruption, which is suggestive of secondary necrosis. In those instances, cellular contents, including nuclei, were observed within the saliva cavity (Figure 4B). In uninfected salivary glands, apoptotic cells were generally located peripherally in the gland and were shrunken with an electron-dense cytoplasm, enlarged mitochondria, and an electron-dense and/or distorted nucleus with condensed chromatin (Figure 4C). Compared with apoptotic cells in WNV-infected mosquitoes, the nuclear envelope of dead cells in uninfected control salivary glands was typically well-defined.

In WNV-infected mosquitoes, the percentage of salivary glands containing apoptotic cells increased over time from 14% to 71% and finally to 83% at 14, 21, and 28 dpi, respectively (Table 2). This frequency was significantly greater in tissues collected 28 dpi compared with 14 dpi ($P < 0.05$). In
contrast, the frequency of apoptotic cells never exceeded 33% in uninfected salivary glands. The total number of apoptotic cells with visible nuclei in WNV-infected salivary glands increased from 5 at 14 dpi to 36 at 21 dpi, but decreased to 13 at 28 dpi. In contrast, in infected tissues, apoptotic cells lacking an observable nucleus increased in number from 1 at 14 dpi to 7 at 21 dpi and 26 at 28 dpi. When combined, the overall number of apoptotic cells (with and without a nucleus observed) in infected tissues plateaued between 21 and 28 dpi at approximately 40 cells. In tissues collected at 28 dpi, apoptotic cells were found both centrally and peripherally within the gland (Table 2). In uninfected salivary glands, the total number of apoptotic cells (nucleus and no nucleus observed) was low, but increased slightly over time: 2 on day 14 and day 21, and 10 (6 with nuclei observed) on day 28 (Table 2).

Pre-apoptotic cells were defined as cells displaying increased cytoplasmic condensation or cell shrinkage, mild chromatin condensation or nuclear distortion, and/or cytopathology such as vacuolization or abnormal cell shape (Figure 4D). The frequency of pre-apoptotic cells increased over time in WNV-infected salivary glands, from 33% to 57% and 66% at 14, 21, and 28 dpi, respectively (Table 2), although the total number of pre-apoptotic cells in salivary glands was low (2 at 14 dpi, 8 at 21 dpi, and 6 at 28 dpi). Pre-apoptotic cells were not seen in uninfected tissues.

Effect of long-term salivary gland infection on WNV transmission. As described above, salivary gland cells infected with WNV undergo progressive cytopathologic changes during long-term infection. Many of these changes increased in frequency over time suggesting they are associated with ongoing WNV replication. We hypothesized that these pathologic changes would impair virus maturation and saliva secretion (and therefore virus secretion). To test this, we collected saliva from WNV-infected mosquitoes and determined both virus titer and infectivity in saliva over time post-infection. Two groups of Cx. p. quinquefasciatus mosquitoes were orally infected during two separate experiments using blood meals containing 7.5 log_{10} TCID_{50}/mL of WNV. The mean ± SD titer of mosquitoes (n = 3) collected immediately after blood-feeding was 5.6 ± 0.6 and 5.0 ± 0.5 log_{10} TCID_{50}/mosquito. Blood meal and day 0 values were not significantly different (P > 0.05). Each saliva sample was divided into two for testing by FFA to determine the amount of infectious virus per sample, and by qRT-PCR to determine the relative levels of WNV RNA per sample (expressed as PFU equivalents).

Despite a clear trend of decreasing saliva virus titers over time in both assays, mean virus titers in saliva as determined by FFA and qRT-PCR were not significantly different between time points (P > 0.05) (Figure 5A and 5B). Mean ± SD WNV titers detected by FFA in mosquito saliva were 2.5 ± 1.4 \times 10^{3}, 7.1 ± 3.2 \times 10^{3}, 8.1 ± 3.6 \times 10^{3}, and 1.9 ± 1.3 \times 10^{3} focus-forming units at 7, 14, 21, and 25–28 dpi, respectively. Mean WNV titers detected by qRT-PCR in mosquito saliva were 1.1 ± 1.0 \times 10^{4}, 5.1 ± 1.8 \times 10^{3}, 4.9 ± 1.6 \times 10^{3}, and 1.1 ± 0.5 \times 10^{3} PFU equivalents at 7, 14, 21, and 25–28 dpi, respectively. When the ratio of focus-forming units to PFU equivalents was taken for each saliva sample, there was an overall decreasing trend in ratios from 14 to 25–28 dpi, although there was no significant difference in ratios over time (P > 0.05) (Figure 5C). The mean ± SD ratios were 7.1 ± 6.7, 2.0 ± 0.5, 0.9 ± 0.2, and 0.5 ± 0.2 on days 7, 14, 21, and 25–28, respectively.

The frequency of WNV-positive saliva samples was determined at each time point of collection. We found that the number of WNV-positive saliva samples decreased significantly over time post-infection by both FFA and qRT-PCR methods (Figure 6). By qRT-PCR, WNV RNA was detected in 88% of saliva samples at 14 dpi, 70% at 21 dpi, and 64% at
25–28 dpi (P < 0.05 compared with 14 dpi). By FFA, the frequency of WNV-positive saliva samples decreased significantly from 83% at 14 dpi to 53% (P < 0.05) at 21 dpi and 39% at 25–28 dpi (P < 0.05 compared with 14 dpi).

**Titration of WNV in mosquito bodies and salivary glands during long-term infection.** Whole body titers were determined for 3 mosquitoes at 0 and 3 dpi and 10 mosquitoes at 7, 10, 14, 17, 21, 25, and 28 dpi after a blood meal containing 6.5 log_{10} TCID_{50}/mL of WNV. Mosquito body titers remained constant at approximately 6 log_{10} TCID_{50}/mosquito between 10 dpi and 28 dpi, and 100% of mosquitoes were WNV-positive at each time point (Figure 7). A qRT-PCR assay was performed on salivary gland pairs dissected from 8 WNV-infected *Cx. p. quinquefasciatus* mosquitoes collected every other day between 1 and 21 dpi after a blood meal containing 7.5 log_{10} TCID_{50}/mL of WNV. Salivary gland titers ranged between 1.5 x 10^6 and 6 x 10^6 PFU equivalents/mosquito from 11 dpi to 21 dpi, with no significant change over time (P > 0.05) (Figure 7).

**DISCUSSION**

This study details the morphologic and cytopathologic changes that occur in mosquito vector salivary glands during WNV infection, and evaluates the effects of long-term salivary gland infection on WNV transmission. Using age- and generation-matched uninfected control mosquitoes in our TEM analysis, we were able to distinguish age-related from infection-related changes in the salivary gland cell cytoplasm and nucleus during a four-week period after blood feeding. Our findings identified a correlation between long-term WNV infection and both increased frequency of salivary
gland apoptosis and decreased WNV transmission. Mosquitoes in these studies were exposed to blood meal titers similar to, and sometimes lower than, those seen in laboratory infections of natural bird reservoirs. Future studies that analyze the frequency of salivary gland cytopathology in mosquitoes exposed to a range of blood meal titers, and performed in other important mosquito vectors such as *Cx. tarsalis* and *Cx. p. pipiens*, are necessary to better understand the importance of this phenomenon in nature. It is likely that both the dose of virus to which salivary glands are exposed and the susceptibility of the salivary gland cells to virus replication determines the outcome of infection including the timing, the mechanism, and the frequency of cell death. Since the early 1980s, light microscopy and TEM studies, feeding behavior studies, and survivorship and reproductive effort studies of arbovirus-vector interactions have challenged the notion that mosquitoes are unharmed by arbovirus infections. This report adds to the growing body of evidence that arboviruses cause pathologic changes in vector mosquitoes and, impor-

**FIGURE 5.** A, Quantification of West Nile virus (WNV) in saliva by focus forming assay (FFA). B, Relative levels of WNV plaque-forming unit (PFU) equivalents in individual saliva detected by quantitative reverse transcription–polymerase chain reaction. C, Ratio of focus-forming units to viral PFU equivalents of WNV in individual mosquito saliva samples. Dotted horizontal lines represent mean values. Solid horizontal lines within boxes represent median values. Shaded boxes contain values between the 25th and 75th quartiles. Dots represent outliers. Vertical bars represent 95% confidence intervals.

**FIGURE 6.** Detection of West Nile virus (WNV) in mosquito saliva by focus-forming assay and quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Numbers above bars represent percentages. Number of mosquitoes tested per time point = 7 days post-infection (dpi), n = 20; 14 dpi, n = 40; 21 dpi, n = 40; 25–28 dpi, n = 28.

**FIGURE 7.** Whole body titration of West Nile virus (WNV)–infected *Culex pipiens quinquefasciatus* mosquitoes over a 28-day course of infection (●). qRT-PCR of individual salivary glands dissected over a 21-day course of infection (△). TCID<sub>50</sub> = 50% tissue culture infectious dose; PFU = plaque-forming units. Error bars show the SD.
tantly, indicates a need for more studies of transmission dynamics during long-term arbovirus infection in mosquitoes.

Salivary gland analysis by TEM showed that proliferated membranes associated with WNV infection did not differ dramatically in abundance over time post-infection; however, there were detectable changes in the morphology of these membranes. Disorganization of CM/TPM structures increased over time, particularly after four weeks of infection. Row-like PMS within TPM also increased in frequency over time. It is possible that PMS result from the disruption or loosening of CM/TPM at late time points of infection. Further research is necessary to determine if the morphologic changes in CM/TPM correspond to a decrease in membrane usage for viral genome replication or proteolytic processing. Increasing electron transparency of the salivary gland cell cytoplasm and vacuolization of the ER observed at late time points during WNV infection are possibly because of the intense involvement of ER membranes and ribosomes in WNV replication. Increasing in frequency over time in WNV-infected tissues, FCM morphology and location suggest that these membranes represent degenerating elements of the ER and Golgi apparatus. Elucidation of the role of these membranes in virus-associated cell death and salivary gland cell turnover or senescence requires further study.

Saliva pooling in our study was seen in WNV-infected salivary glands one week earlier than in uninfected tissues. In salivary glands from both infected and uninfected mosquitoes, pooling was associated with cell death and the appearance of cytoplasmic contents in saliva. Although saliva pooling appears to be a normal, age-related phenomenon, the process may be accelerated by WNV infection. Within saliva, paracrystalline arrays of WNV were abundant in frequency and distribution throughout the four-week course of analysis. These arrays were found within the acinar cavity, within the salivary gland duct, and within saliva pools. In contrast, arrays of WNV were not found within the cytoplasm of infected cells, suggesting that the phenomenon requires the particular milieu of mosquito saliva for their formation. Paracrystalline arrays of similar size, morphology, and location have been identified in studies of St. Louis encephalitis virus by Whitfield and others in salivary glands of Cx. p. pippens.27 These investigators identified paracrystalline arrays at later time points than in our study, namely 25 and 32 dpi. Structural similarities between these two Japanese encephalitis serocomplex relatives28 may explain their common array formations. However, the earlier occurrence of paracrystalline arrays in WNV-infected Cx. p. quinquefasciatus may also reflect differences in the replication dynamics of these two viruses. Paracrystalline arrays of the flavivirus Japanese encephalitis virus (JEV) have also been identified in Cx. tritaeniorhynchus, although these arrays were small, not reportedly abundant, and occurred at 26 dpi in the saliva cavity.29 The significance of paracrystalline arrays in transmission has thus far been overlooked. These arrays, presumably maintained in saliva secretions during mosquito feeding, could increase the dose of virus delivered to a host and explain the wide range of titers detected in mosquito saliva in our study and other studies quantifying arboviruses in saliva.16,30,31 Future research into salivary or viral factors that contribute to the formation of paracrystalline arrays, as well as the significance of array formation in host infection, are likely to contribute to our understanding of arbovirus transmission and pathogenesis.

In mammalian tissues, apoptotic cell death is not uncommon during infections with mosquito-borne flaviviruses such as WNV, JEV, Langat virus, and dengue virus (DENV).32-41 In vitro experiments indicate that both apoptotic and necrotic cell death pathways occur during WNV infections, and these outcomes may be dose dependent.32,42-44 In our study, the number of mosquitoes with apoptotic cell death and the total number of dead cells per gland increased over time. In contrast, the percentage and total number of dead cells in uninfected mosquitoes remained relatively constant over a four-week period. These data suggest that although age-related salivary gland cell death is a natural phenomenon, long-term WNV infection increases cell death. Our TEM analysis showed that dead cells in WNV-infected salivary glands contain morphologies suggestive of apoptosis similar to those identified in apoptotic mammalian cells infected with WNV.32,33 Comparable morphologies indicative of apoptosis include cell shrinkage, extensive vacuolization, chromatin condensation, nuclear blebbing and degeneration, and a loss of plasma membrane integrity.32,33 Interestingly, in our study, the number of dead salivary gland cells containing observable nuclei decreased between 21 and 28 dpi. It is possible that these nucleus-free figures represent apoptotic bodies.

Apoptotic nuclei, virus-induced membranes, WNV particles, and other cellular contents were identified at late time points of infection within saliva cavities, which suggested that membrane disruption occurred in some instances after WNV-induced apoptosis. Loss of plasma membrane integrity and cell lysis in Vero cells infected with high doses of WNV was observed by Chu and Ng to correspond to a necrotic mechanism of cell death.32 We hypothesize that either secondary necrosis can occur after apoptosis during long-term salivary gland cell infection with WNV, or infected salivary gland cells may die by a recently described mechanism of cell death called aponecrosis.45,46 During studies of cytopathology associated with Sindbis virus (Togaviridae, Alphavirus, SINV), Karpf and Brown reported that cell death in a vertebrate cell line (baby hamster kidney cells) corresponded clearly to a large induction of apoptosis, but the virus caused only slight induction of apoptosis in cytopathic Aedes albopictus C7-10 cells.47 In addition, cytopathic C7-10 cells infected with SINV were reported to display morphologic characteristics of necrosis by TEM, which suggested to the investigators that both cell death mechanisms may explain the observed mosquito cell death. Molecular-based methods would further contribute to our understanding of the mechanism of salivary gland cell death caused by WNV.

Previous IHC and TEM studies from our laboratory have shown that 100% of Cx. p. quinquefasciatus salivary gland cells are infected with WNV 14 dpi, i.e., approximately 6-9 days after initial salivary gland infection.7,8 As shown in our current study, cell death frequently occurred in salivary glands 21 and 28 dpi, equating to approximately 14 days of viral replication in the glands. This late timing of cell death may indicate that the mechanism of apoptosis is unrelated to expression of viral genes such as capsid and NS2B-NS3, as proposed by other investigators studying WNV-induced apoptosis in mammalian cells.33,35 We propose that apoptotic cell death in Culex salivary glands is due to a shift in the relative abundance of pro-apoptotic and anti-apoptotic proteins during continuous virus replication. Apoptosis may be a response to the physiologic disturbances caused by persis-
tently high levels of WNV replication and the decrease or cessation of host cell gene transcription and protein synthesis. Considering the extensive ER membrane proliferation and vacuolization that occurs in salivary gland cells during WNV replication, ER malfunctioning or stress may induce an unfolded protein response similar to that seen during JEV infection.\textsuperscript{43} Investigation into the role of viral gene products, host membranes, and host apoptotic and anti-apoptotic enzymes in salivary gland cell death during long-term WNV infection is warranted.

Since effective saliva secretion is essential for WNV transmission by mosquitoes, we investigated the transmission of WNV during long-term infection of \textit{Cx. p. quinquefasciatus}. Although WNV whole body and salivary gland titers did not decrease over time post-infection, fewer mosquitoes secreted saliva with detectable levels of WNV at 21 and 25–28 dpi compared with 14 dpi. One plausible explanation for the temporal decrease in the proportion of mosquitoes with WNV-positive saliva is that the normal mosquito feeding behaviors are impaired during WNV infection of the mosquito nervous system, as suggested by Platt and others studying DENV-3.\textsuperscript{23} In the laboratory, cerebral and suboesophageal ganglia of \textit{Cx. p. quinquefasciatus} are infected with WNV between 8 and 10 dpi.\textsuperscript{7} These ganglia receive signals from sensilla found on cibarial and pharyngeal pumps, and may regulate the dilator muscles of these structures.\textsuperscript{45} Virus infection of these nervous tissues could disrupt pump and valve function, thereby affecting mosquito feeding and salivation. Virus transmission, virus tropism, and mosquito behavior studies are necessary to elucidate the role of nervous tissue infection in mosquito blood feeding and saliva output.

Studies using TEM are particularly useful for detailed investigation of the underlying mechanisms of virus-vector interactions that determine vector competence. In this report, we successfully used TEM to understand morphologic aspects of the \textit{Culex} mosquito cellular response to long-term WNV infection. Further investigation of \textit{Culex} feeding behavior and WNV transmission to vertebrate hosts during long-term infection would greatly complement this work. Additionally, research on the inter-relation between WNV-induced salivary gland cytopathology and saliva production and secretion will improve our understanding of WNV-vector and vector-host interactions, particularly as they relate to transmission dynamics in nature and the risk of human and avian WNV infection.

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Authors’ addresses: Yvette A. Girard, Bradley S. Schneider, Charles E. McGee, and Stephen Higgs, Department of Pathology, University of Texas Medical Branch, 2,104 Keiller, 301 University Boulevard, Galveston, TX 77555-0609, Telephone: 409-747-2426, Fax: 409-747-2436, E-mails: ygirard@utmb.edu, bbschnee@utmb.edu, cemcgee@utmb.edu, and shtiggs@utmb.edu. Julie Wen, Violet C. Han, and Vsevolod Popov, Department of Pathology, University of Texas Medical Branch, 2,102 Keiller, 301 University Boulevard, Galveston, TX 77555-0609, Telephone: 409-747-2423, Fax: 409-747-2437, E-mails: jwen@utmb.edu, vchan@utmb.edu, and vpopov@utmb.edu. Peter W. Mason, Department of Pathology, University of Texas Medical Branch, Sealy Center for Vaccine Research, 3,200 Mary Moody Northern Pavilion, 301 University Boulevard, Galveston, TX 77555-0436; Telephone: 409-747-8143, Fax: 409-747-8150, E-mail: pwmsnson@utmb.edu.

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