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PERSISTENT SHEDDING OF WEST NILE VIRUS IN URINE OF EXPERIMENTALLY INFECTED HAMSTERS

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Abstract. Adult hamsters that survived experimental West Nile virus (WNV) infection developed persistent viruria. Infectious WNV could be cultured from their urine for up to 52 days. Immunohistochemical examination of kidneys of viruric animals showed foci of WNV antigen in renal tubular epithelial and vascular endothelial cells. These findings are compatible with virus replication and persistent infection of renal epithelial cells. The potential clinical and virologic significance of these findings as well as their possible epidemiologic importance are discussed.

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne flavivirus in the Japanese encephalitis serocomplex of the family Flaviviridae.[1] Although it is generally thought to be maintained primarily in a cycle involving *Culex* mosquitoes and birds,[2–4] WNV naturally infects a wide range of other vertebrates, including mammals and reptiles.[2,3,5] The outcome of WNV infection in vertebrates varies widely; in humans, for example, it can range from an asymptomatic infection to a brief febrile illness (West Nile fever) to meningoencephalitis, flaccid paralysis, and death, depending in part on the age, health, and immune status of the subject.[4,6] Studies of experimental WNV infection of equines,[7] hamsters,[8,9] mice,[10] and rhesus monkeys,[11] as well as observations of human cases where the precise time and mode of infection were known,[12–14] indicate that immunocompetent mammals respond to the virus in a similar manner. Following parenteral inoculation of WNV, most mammals develop a short period of viremia, followed by the development within 5–10 days of relatively high levels of humoral antibodies. If central nervous system (CNS) symptoms develop, they usually occur during the second week of infection, when the host often already has detectable humoral antibodies against the virus. If the host survives infection, complete recovery usually occurs, although some persons and animals who develop non-fatal CNS symptoms may continue to have sequellae (headache, muscle weakness, difficulty walking, and memory loss) for months after recovery from their acute illness.[6]

One of the important unanswered questions about WNV infection in vertebrates is whether the virus persists and chronic infection occurs in some people or animals. There are intriguing bits of evidence suggesting that chronic infection might occur. In a follow-up study of surviving WNV meningoencephalitis cases in New York, it was found that 60% of those tested still had detectable IgM antibodies in their sera 1.5 years later.[15] Likewise, Pogodina and others[16] reported that WNV can induce persistent CNS infection in experimentally infected rhesus monkeys, regardless of the route of inoculation or the symptoms (overt or asymptomatic) of the acute infection. These latter investigators showed that WNV could be detected by culture for up to 5.5 months in the CNS of experimentally infected macaques. Xiao and others[8] demonstrated a similar phenomenon in experimentally infected golden hamsters and were able to recover infectious virus from the CNS of some animals as long as 52 days after peripheral inoculation. Similar findings of persistent infection have been reported in humans and animals infected with tick-borne encephalitis virus[17,18] and with Japanese encephalitis virus,[19,20] two related flaviviruses that produce similar clinical manifestations to WNV.

To date, little is known about where WNV replication occurs in the vertebrate host. In an attempt to answer this question and to investigate the possibility of persistent infection, a series of experiments were carried out using a recently described hamster model of WNV encephalitis.[8,9] This paper reports some of our findings, which indicate that WNV persists in the brain and kidney of experimentally infected hamsters for several weeks after infection and that some animals develop a chronic renal infection and shed virus in their urine for prolonged periods of time.

MATERIALS AND METHODS

Virus. A second Vero cell passage of the 385-99 strain of WNV was used in the experiments. This strain was originally isolated from the liver of a dead snowy owl (*Nyctea scadiaca*) collected during an epizootic at the Bronx Zoo in New York City in the summer of 1999.[8] The dose of virus used to infect hamsters was $10^{4.0}$ 50% tissue culture infectious doses ($TCID_{50}$) units given intraperitoneally (ip).

Animals. One hundred twenty adult (~12 weeks old) female golden hamsters (*Mesocricetus auratus*) were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Hamsters were housed three or four per cage under a 12:12 hours light/dark cycle with an unlimited food and water supply. Experiments were conducted according to a protocol approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee in an animal biosafety level 3 facility. All animal work was performed in accordance with guidelines outlined by the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC).

Experimental design. Initially, 120 hamsters were inoculated ip with 10^4 $TCID_{50}$ of WNV, as noted above. The animals were observed daily for signs of illness or death. On days 1, 3, 5, 7, 9, 11, 13, 21, 24, 31, 38, 45, 48, and 52 after inoculation, a sample of 2–5 of the infected animals was anesthetized with Halothane (Hydrocarbon Laboratories, River Edge, NJ), exsanguinated by cardiac puncture, and necropsied. Blood from each animal was saved for virus culture, and a sample of serum was collected for antibody determination. At necropsy, the abdomen was opened, and urine was aspirated directly from the bladder with a 1-mL syringe and 26-

gauge needle. Samples of spleen, kidney, and brain were also taken. One kidney was placed in 10% buffered formalin for histopathologic and immunohistochemical studies; the other kidney as well as samples of spleen, brain, blood, and urine were frozen in individual sterile vials at −80°C for subsequent virus assay.

Serologic test. Antibody determinations on the sera collected at necropsy were done by a hemagglutination inhibition (HI) test, as described previously.[8] The antigen used was prepared from brains of newborn mice injected intracerebrally with WNV; infected brains were treated by the sucrose-acetone extraction method.[21] Hamster sera were tested by HI at serial two-fold dilutions from 1:20 to 1:5,120 at pH 6.6 with four units of antigen and a 1:200 dilution of goose erythrocytes, following established protocols.[21]

Virus assay. Samples of spleen, kidney, and brain were triturated in sterile glass tissue homogenizers in phosphate-buffered saline (PBS), pH 7.4, containing 25% heat-inactivated (56°C for 30 minutes) fetal bovine serum (FBS) to prepare an approximate 10% (w/v) tissue suspension. After centrifugation, serial 10-fold dilutions of the supernatants were prepared in PBS containing 10% FBS; 100 μL of the undiluted tissue homogenate and dilutions from 10^{-1} to 10^{-6} were then inoculated into 24-well plastic tissue culture plates containing a complete monolayer of Vero cells, using two wells per dilution.[22] Samples of hamster urine and blood were diluted and titrated in the same manner. Cultures were incubated at 37°C and plaques were counted four days later. Virus titers were calculated as the number of plaque-forming units per milliliter of sample (urine or blood) or gram of tissue.

Histologic and immunohistochemical examination. After formalin fixation, one kidney from each animal was processed for routine embedding in paraffin. Sections (4–5 μm thick) sections were made and stained by the hematoxylin and eosin method or immunohistochemically.

Immunohistochemical staining for WNV antigen was performed as previously described.[8] A WNV mouse immune ascitic fluid was used as the primary antibody at a dilution of 1:80 and was incubated at 4°C overnight. A commercially available ISO-IHC immunostain kit (Inno-Genex, San Ramon, CA) was used to detect specifically bound primary antibodies and prevent nonspecific binding between species.[8,23]

RESULTS

Clinical observations. During the first six days after WNV infection, the hamsters appeared well, but beginning at about the seventh day, most of the animals became lethargic, somnolent, and anorexic. Between days 8 and 15, approximately 60% of the animals died or developed severe CNS symptoms and were killed. Their symptoms during this acute phase of the infection conformed to previous descriptions[8,9] of WNV infection in the hamster model.

By day 16, the surviving animals began to appear normal (active, curious, feeding well), although a few still had residual limb weakness and instability walking. These hamsters remained in good health throughout the duration of the experiment (52 days).

Antibody response. Table 1 shows the HI antibody titers in the animals' sera at the time they were killed and sampled. As observed previously,[8] HI antibodies to WNV antigen began to appear about day 5 and persisted for the duration of the experiment. In this study, only HI antibody titers were determined, but previous experiments[24] have demonstrated that hamsters also develop high levels of specific neutralizing and complement-fixing antibodies following WNV infection.

TABLE 1

West Nile virus (WNV) titers obtained on samples of blood, urine, kidney, brain, and spleen taken from hamsters at various times (1–52 days) after experimental infection (PI) with the virus*

Animal no.	Day PI	HI titer	Blood	Urine	Kidney	Brain	Spleen
7000	1	0	4.6	Neg	2.9	Neg	4.0
7001	1	0	4.8	1.7	2.0	2.4	2.4
7002	1	0	4.7	NA	3.9	2.4	4.0
7003	1	0	4.2	2.7	2.9	Neg	3.8
7005	3	0	5.8	2.4	5.4	3.9	4.1
7006	3	0	6.3	4.9	6.2	4.7	5.7
7007	3	0	6.3	3.3	5.9	4.7	5.4
7008	3	0	5.8	NA	6.1	3.7	5.0
7009	3	NA	NA	4.2	NA	NA	NA
7010	5	1:80	3.4	NA	6.5	4.8	3.7
7011	5	1:80	6.4	4.2	9.0	8.3	7.2
7012	5	1:80	3.8	4.2	6.7	5.9	3.7
7013	5	1:80	2.6	3.7	7.0	7.1	3.0
7014	5	NA	NA	4.6	7.0	8.5	NA
7015	7	1:1,280	Neg	4.3	5.6	8.7	3.6
7016	7	1:640	Neg	NA	6.9	7.6	Neg
7017	7	1:640	Neg	3.2	6.2	Neg	Neg
7018	7	1:640	Neg	3.4	6.4	6.9	2.5
7019	7	NA	NA	4.3	NA	NA	NA
7020	9	1:640	Neg	Neg	5.2	4.2	Neg
7021	9	1:640	Neg	2.2	5.5	6.9	1.7
7022	9	1:1,280	Neg	3.9	6.9	6.3	Neg
7023	9	1:1,280	Neg	NA	5.9	5.0	Neg
7025	11	1:640	Neg	NA	5.2	7.0	Neg
7026	11	1:160	Neg	2.9	4.9	5.4	Neg
7027	11	1:640	Neg	2.6	5.3	7.1	Neg
7028	11	1:640	Neg	NA	4.7	4.3	Neg
7029	11	NA	NA	2.7	NA	NA	NA
7030	13	1:640	Neg	NA	4.3	8.2	Neg
7031	13	1:640	Neg	3.1	5.9	7.0	Neg
7032	13	1:640	Neg	1.7	4.6	3.7	2.9
7033	13	1:160	Neg	NA	6.2	4.9	Neg
7036	21	NA	Neg	Neg	Neg	Neg	Neg
7037	21	NA	Neg	Neg	Neg	2.0	Neg
7038	24	NA	Neg	NA	1.9	Neg	Neg
7039	24	1:640	Neg	3.1	Neg	Neg	Neg
7040	31	1:640	Neg	Neg	Neg	Neg	Neg
7041	31	1:320	Neg	Neg	Neg	Neg	Neg
7042	38	NA	Neg	Neg	Neg	Neg	Neg
7043	38	NA	Neg	Neg	Neg	Neg	Neg
7045	45	1:320	Neg	3.4	Neg	Neg	Neg
7046	45	1:320	Neg	3.3	Neg	Neg	Neg
5618-A	48	1:640	NA	1.8	NA	NA	NA
5618-B	48	1:320	NA	2.0	NA	NA	NA
5618-C	48	1:160	NA	Neg	NA	NA	NA
5618-D	48	1:320	NA	Neg	NA	NA	NA
5618-E	48	1:640	NA	1.8	NA	NA	NA
5618-F	48	1:2,560	NA	2.8	NA	NA	NA
7048	52	1:640	Neg	2.4	Neg	Neg	Neg
7049	52	1:1,280	Neg	2.1	Neg	Neg	Neg
7050	52	1:1,280	Neg	Neg	Neg	Neg	Neg

* WNV titers are expressed as $\log_{10}$ plaque-forming units (PFU) of virus/mL of sample or gram of tissue. WNV hemagglutination inhibition (HI) antibody titers in the animals' sera at the time of sampling are also shown. HI titer = WNV antibody titer in serum of animal at the time samples were taken (p = <1;20). PI = post-infection; Neg = $<10^{0.7}$ PFU/mL of sample; NA = sample not available or not tested.

Virus assay. Table 1 summarizes the results of virus titrations done on samples of blood, urine, kidney, brain, and spleen taken at necropsy from the hamsters at various time (1–52 days) after experimental infection. As described previously,[8] WNV was detected in the animals' blood during the

first five days. Subsequently, no infectious virus was detected in blood. The pattern of virus recovery from the spleen was similar, except that WNV was detected in two of five hamsters on day 7, one of four hamsters on day 9, and one of four animals on day 13.

Infectious WNV persisted in the kidney and the brain for 13 days after infection. One of two hamsters sampled on day 21 had detectable virus in its brain, and one of two animals sampled on day 24 had detectable virus in its kidney. All subsequent cultures of kidney and brain were negative.

Most interesting was the persistence of infectious WNV in the urine aspirated from bladder. Urine was not always available for testing, since some animals had an empty bladder when they were dissected. However, infectious virus was present in the urine of approximately 60% of the animals for as long as 52 days after infection.

Pathologic and immunohistochemical findings in kidneys. No prominent pathology was seen in kidneys of the infected hamsters, although focal peri-tubular inflammation was intermittently observed in some animals. The inflammation usually consisted of a mixture of lymphocytes and macrophages, without overt tubular necrosis (Figures 1 and 2A). Immunohistochemically, WNV antigen was first noted in the kidneys on day 7; it was single or multifocal in distribution and located within the epithelial cells of the tubules (Figures 2B and 2C). In most of the positive samples, the antigen-positive foci were in the medulla, particularly toward the renal papillae. In some of the chronically viruric hamsters, WNV antigen was also noted in the lumen of the tubules, as proteinaceous casts with macrophages, surrounding antigen-positive tubular epithelium and endothelial cells in the same foci (Figure 2D). The histologic and virologic findings (presence of WNV antigen in the kidneys and absence of infectious virus from blood) are compatible with viral replication in the renal epithelial cells. However, the presence of antigen-positive macrophages might also indicate passive absorption of WNV-immune complexes from the blood or urine.

DISCUSSION

The results of this experiment confirm those of two earlier reports[8,9] indicating that adult hamsters experimentally infected with WNV develop a brief viremia of about five-days duration. Specific antibodies (IgM and HI) begin to appear in the sera of infected animals about day 5, coinciding with the disappearance of infectious virus from the blood. During this acute phase of infection, virus can also be cultured directly from the throat, urine, kidney, brain, and other major organs (Table 1 and Tesh RB, Siriin M, unpublished data). Shortly after the appearance of humoral antibodies, WNV can no longer be recovered from blood or from tissue homogenates of most organs (i.e., liver, spleen, lung), but it persists in urine, kidney and brain. Significant quantities of virus can still be cultured directly from homogenates of kidney and brain of infected hamsters for up to 14–21 days, even though the animals appear to have recovered from their acute infection and illness[8,9] (Table 1 and Tesh RB, Siriin M, unpublished data). After approximately 21 days, WNV cannot be cultured directly from the blood or tissues of the infected animals.

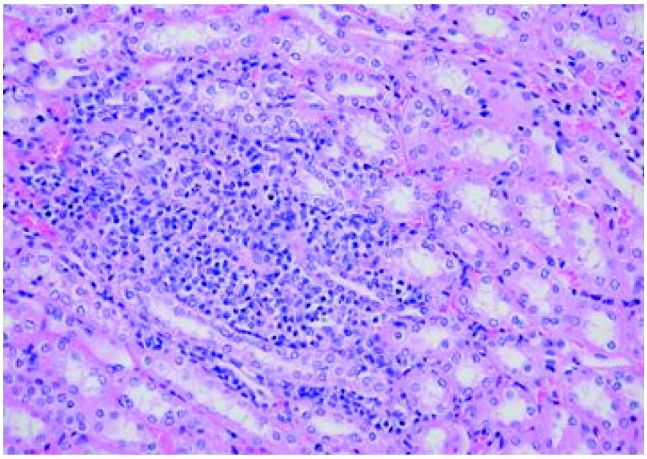

FIGURE 1. Kidney from a hamster persistently infected (48 days postinfection) with West Nile virus showing focal infiltration by lymphocytes and macrophages forming a small nodule (hematoxylin and eosin stained, magnification × 100).

In the present study, WNV was recovered from the urine of some hamsters for up to 52 days after infection; at that point, the experiment ended since we had used up all of the surviving animals. However, in a second larger and still ongoing experiment, we have recovered WNV from serial urine samples from experimentally infected hamsters for up to 170 days after infection (Tesh RB, Guzman H, unpublished data). Furthermore, we have found that infectious WNV can be recovered consistently from the kidneys of hamsters with viruria, if the renal tissue is co-cultivated on a monlayer of Vero cells,[25] instead of trituration of the kidney, followed by inoculation and culture of the homogenate, which is our usual procedure for isolating the virus (Tesh RB, Siirin M, unpublished data). Presumably, antibodies present in the sera and interstitial fluids of the chronically infected hamsters inactivate WNV released from the renal tissue during trituration.

The demonstration of chronic renal infection and persistent shedding of WNV in hamster urine in our experiment is very similar to observations made more than 30 years ago in animal studies with another flavivirus (Modoc). Modoc virus (MODV) naturally infects deer mice (*Peromyscus maniculatus*) in the western United States.[26] The available information suggests that MODV is not vector-borne, but that it is maintained naturally in deer mouse populations by horizontal and possibly vertical transmission.[26–29] Experimental studies[26–28] have demonstrated that both deer mice and hamsters develop persistent MODV infection with chronic viruria. Davis and Hardy[27] showed that hamsters experimentally infected with MODV developed a brief viremia lasting 2–6 days, followed by the development of HI and neutralizing antibodies by day 7. Virus was chronically shed in the urine of the infected animals for at least 12 weeks. During this period, virus could not be isolated directly from organ homogenates of the persistently infected hamsters. However, by co-cultivating the tissues on monolayer cultures of Vero cells, it was possible to recover MODV from organs (kidney and lung) of the chronically infected hamsters for up to 32 weeks after the initial infection.[27,29] This is analogous to what we observed in the WNV-infected hamsters.

Because the clinical manifestations of severe WNV infection usually involve the CNS, most histopathologic studies of infected vertebrates have focused on the brain and spinal cord. However, the kidney is also involved in West Nile virus infection.[3,5,9,30–34] Furthermore, experimental studies of WNV infection in birds and alligators indicate that these ani-

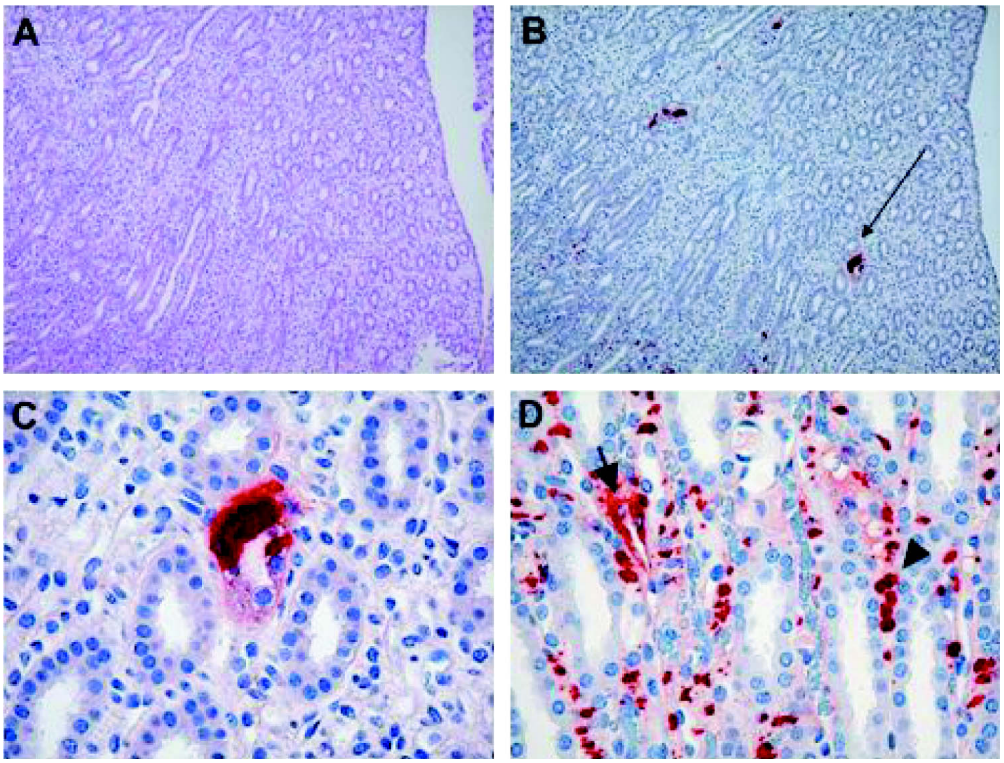

FIGURE 2. Distribution of West Nile virus antigen in the kidneys of hamsters on day 48. **A**, Focal non-specific lymphocytic infiltration was observed between the tubules; otherwise, there was no evidence of degeneration or necrosis (hematoxylin and eosin stained, magnification × 30). **B**, Same field as **A** with positive antigen staining present in a few foci of tubular epithelium (organ color) (immunohistochemical stained, magnification × 30). **C**, Higher magnification of area indicated by the **arrow** in **B**, showing tubular epithelial cells positive for viral antigen. In this area, the epithelial cells appear slightly enlarged, with mild cytoplasmic vacuolation (immunohistochemical stained, magnification × 120). **D**, Many antigen positive cells, including vascular endothelium (**arrow**) and macrophages between the renal tubules (**arrowhead**) (immunohistochemical stained, magnification × 120).

mals shed virus in their cloacal contents.[3,5,31,32] Thus, our finding of WNV shedding in the urine of infected hamsters is probably not limited to these rodents; it may occur in other vertebrate species as well.

The epidemiologic significance of chronic renal infection and shedding of WNV in urine of the infected host is uncertain. Theoretically, transmission of the virus could occur to another animal by aerosol or by ingestion of infectious urine. Alternatively, the infected host could be eaten by a predator. Aerosol infection seems least likely because of the relatively low levels of virus present in urine or cloacal contents of infected animals[31] (Table 1). However, a variety of vertebrate species are susceptible to oral infection with WNV.[3,5,32,35–37] Thus, oral transmission of WNV directly from one infected vertebrate to another, without a mosquito vector, may be an alternative mode of WNV transmission in nature.[37]

The prolonged excretion of WNV in the urine of infected hamsters, even after the appearance of humoral antibodies, is interesting for another reason. If a similar phenomenon occurs in other mammals, then it might be used as a diagnostic tool. Most human cases of WNV encephalitis do not manifest CNS symptoms or seek medical care until the second week after infection. Consequently, many patients already have IgM antibodies at the time of hospital admission, and virus isolation from blood or spinal fluid is uncommon.[4] Based on our findings in hamsters, urine might be a source of virus for diagnostic purposes long after it is no longer detectable in a patient's blood or spinal fluid.

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