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NUCLEOTIDE AND AMINO ACID CHANGES IN WEST NILE VIRUS STRAINS EXHIBITING RENAL TROPISM IN HAMSTERS

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Abstract. Recent studies have shown that West Nile virus (WNV) can induce an asymptomatic persistent infection in the kidneys of experimentally infected hamsters. The chronically infected rodents shed virus in their urine for up to 8 months, despite the disappearance of viremia and the development of high levels of neutralizing antibodies. WNV, like most members of the Japanese encephalitis virus complex (*Flavivirus;* Flaviviridae), is assumed to be mainly neurotropic; little is known about the genetic basis for its renal tropism. In this study, complete sequence analyses were done to compare four WNV isolates from the urines of persistently infected hamsters with the wild-type parent virus (NY 385-99). Nucleotide changes, ranging from 0.05% to 0.09%, were identified in all of the WNV isolates from urine; most of the changes were in coding regions, causing amino acid substitutions in the E, NS1, NS2B, and NS5 proteins. The genetic changes associated with renal tropism were also accompanied by a loss of virulence for hamsters and a change in plaque morphology.

INTRODUCTION

West Nile virus (WNV), a member of the Japanese encephalitis serogroup of the genus *Flavivirus*, family Flaviviridae, first appeared in the Western Hemisphere in 1999, as a meningoencephalitis epidemic in New York City.[1] Since then, WNV has spread rapidly in North America, with a corresponding increase in the number of reported human cases.[2] Clinically, most human infections with WNV are either asymptomatic or result in a self-limited febrile illness (West Nile fever). In the United States, neuroinvasive disease occurs in only about 1:150 WNV cases, mainly the elderly and the immunosuppressed.[3–5] The long-term outcome of WNV infection in humans has not been well studied, but based on experience with Japanese encephalitis and St. Louis encephalitis virus infections,[6] it is assumed that most asymptomatic and mild cases recover without sequellae.

In the hamster model of WNV infection,[7,8] the outcome is also variable. Depending on the virus dose, route of infection, immune status and age of the animal, and previous experience with other flaviviruses, WNV infection in hamsters can result in at least three different clinical outcomes: asymptomatic infection; a brief (3–5 day) period of anorexia, somnolence, and muscle weakness, followed by recovery; or severe paralysis, encephalitic symptoms and death.[7–13]

In the hamster model, adult animals inoculated intraperitoneally (IP) with 10^4 infectious units of WNV strain NY385-99 develop a viremia of about 5–6 days.[7,11,13] WNV antibodies (IgM and HI) begin to appear in sera of the infected animals about Day 5, coinciding with the disappearance of infectious virus from their blood. During this acute phase of the infection, virus can also be cultured directly from the throat, urine, kidney, brain and other major organs.[11,12] Shortly after the appearance of humoral antibodies, WNV can no longer be recovered from blood, although it persists for various periods in spleen, kidney, lung and brain.[7,11,12] Eight days after infection, most of the hamsters appear ill; and about half of the animals develop severe encephalitis and die between Days 8 to 14. Usually, by the 15th day, no further deaths occur; and the survivors appear to fully recover, resuming their normal activity and growth.

Despite their clinical recovery and the presence of high levels of specific neutralizing antibodies in their sera, some of the surviving hamsters develop a persistent renal infection with chronic viruria.[11,12] Infectious WNV can be cultured directly from their urine for up to 8 months after the initial infection. By cocultivation, WNV can also be recovered from kidneys of the viruric hamsters; and by immunohistochemistry, WNV antigen can be detected in the distal renal tubules.

WNV strains isolated from urine of persistently infected animals have reduced virulence for hamsters and altered growth and plaque morphology in Vero cell cultures, compared with the original wild-type virus used to initiate the infection[11] (Tesh R, unpublished data). To better understand the genetic basis for the observed renal tropism and phenotypic changes, four WNV isolates from urine of persistently infected hamsters and the original parent virus were completely sequenced and compared. This paper reports the results of our findings.

MATERIALS AND METHODS

Virus. Five WNV strains were used in this study. One was the parent virus, WNV strain 385-99, which had been isolated from the liver of a snowy owl (*Nyctea scadiaca*) found dead in the Bronx Zoo in New York City, during the 1999 WNV outbreak.[7] This virus has been used extensively in development of our hamster model of WNV encephalitis[7,9–13]; the strain used in the current study had three previous passages in Vero cells. Henceforth it will be referred to as the "WNV parent strain." The other four WNV strains used were isolates from urine of persistently infected hamsters (see description below).

Animals. Adult (8- to 10-week-old) female golden hamsters (*Mesocricetus auratus*), obtained from Harlan Sprague Dawley (Indianapolis, IN), were used in the experiments. The animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (In-

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stitute of Laboratory Animal Resources, National Research Council) under an animal use protocol approved by the University of Texas Medical Branch. All work with the infected animals was carried out in animal biosafety level 3 facilities.

Virus isolation and culture. Urine was collected at necropsy by direct needle aspiration from the bladder of infected hamsters, as described before.[11] Fresh urine (200 μL) was inoculated directly into 12.5 cm^2 flask cultures of Vero cells. After absorption for 1 hour at 37°C, maintenance medium, consisting of minimum essential medium with Earle's salts (Gibco, Grand Island, NY), 1.5% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin, was added; cultures were incubated at 37°C and observed daily for cytopathic effect (CPE). If CPE was observed within 10 days, a sample (125 μL) of the cell culture fluid was removed and tested for the presence of WNV, using the VecTest WNV antigen assay kit (Medical Analysis Systems, Camarillo, CA), following the manufacterer's instructions. If the sample tested positive for WNV, the remaining culture fluid was decanted and 5 mL of Trizol-LS reagent (Invitrogen, Carlsbad, CA) was added to the flask to lyse the cells in preparation for subsequent RNA extraction. The four virus isolates from hamster urine that were sequenced had only a single passage in Vero cells.

Experimental infection of hamsters and origin of WNV isolates from urine. Figure 1 shows a flow chart of the passage history of WNV strain NY385-99 in hamsters and the source and date of the urinary isolates that were examined in this study. Initially, the parent virus (NY385-99) was inoculated IP into 10 hamsters. Forty-eight days after infection, 3 of the animals (9317A, 9317B and 9317E) were killed and their urine was cultured in Vero cells for WNV. The 3 positive urine cultures were designated WNV strains 9317A, 9317B and 9317E (hamster passage 1).

Infectious urine from hamster 9317B was then inoculated IP into another uninfected adult hamster (T-35639B). Fifteen days after inoculation, this animal was killed and WNV was cultured from its urine (hamster passage 2).

Infectious urine from hamster T-35639B was inoculated IP into each of 60 uninfected hamsters. Twenty-six days after inoculation one of the animals in this group (H-8536) was killed and its urine was cultured. The virus isolate from this urine was designated as WNV strain TVP-9376 (hamster passage 3).

RNA extraction, RT-PCR, and sequencing. For total RNA extraction, 100 μL of the infected cell culture-Trizol mixture was mixed with an additional 900 μL of Trizol. This sample was subjected to RNA extraction using chloroform/isopropanol, as previously described.[14] Based on sequence information for the prototype New York WNV strain, 382-99 (AF196835), 22 primer pairs were designed for PCR amplification, with resultant fragments covering the full-length RNA genome (primer sequences available upon request). The PCR products were 500- to 600-bp in length, with 50- to 100-bp overlap between two contiguous target regions. For cDNA synthesis, the SuperScript II First-Strand Synthesis System for RT-PCR was used (Invitrogen, Carlsbad, CA), following the manufacture's protocol. The reaction mixture of 20 μL, contained 5 μL of total RNA, 1 μL of random hexamer primer (50 ng/μL), 1 μL of 10 mM dNTP mix, 3 μL of DEPC-treated water, 2 μL of 10x RT buffer, 4 μL of 25 mM $MgCl_2$, 2 μL of 0.1 M DTT, 1 μL of RNaseout (40 u/μL) and 1 μL of SuperScript II RT (200 u/μL). The reaction was stopped by heating at 94°C for 3 minutes, followed by the addition of 1 μL of RNaseH (2 u/μL) and incubation at 37°C for 20 minutes.

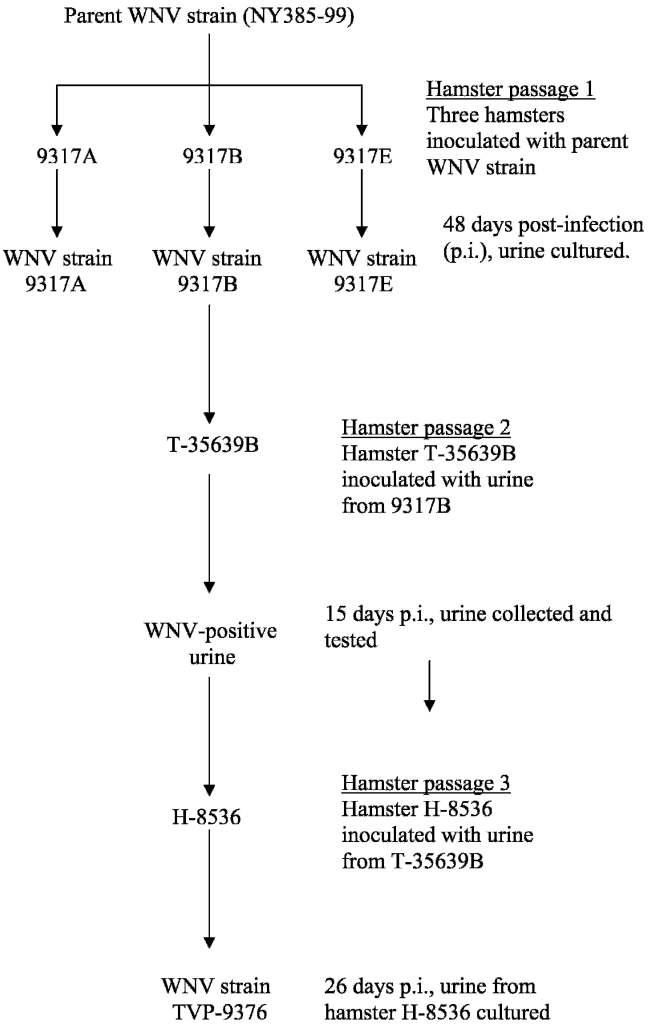

FIGURE 1. Passage history of WNV strains sequenced.

For the PCR reaction, 2 μL of cDNA was added to 45 μL of the PCR reaction mixture containing 0.4 mmol each of the forward and reverse primers, 10 mM Tris HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 400 μM each of dNTP and 0.1% Triton X-100, and 2.0 units Taq DNA polymerase (Promega, Madison, WI). The PCR reaction was carried out in a PTC200 thermocycler (MJ Research, Ramsey, MN). Initial denaturing was at 94°C for 3 minutes, then 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, for 35 cycles, with an extension at 72°C for 10 minutes after the last cycle. The PCR products were screened by 1.5% agarose gel electrophoresis, and purified from the gel using the QIAquick kit (Qiagen Inc., Valencia, CA). The resulting templates were directly sequenced in both directions with the amplifying primers, by using the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the University of Texas Medical Branch Biomolecular Resource Facility/Protein Chemistry Laboratory.

Sequence analysis. Initial editing and assembly of sequence data were performed using the SeqMan program of the DNAStar software package (DNASTAR, Madison, WI). Nucleotide and deduced amino acid sequences of the com-

plete genome of each isolate were aligned using the MegAlign program (DNASTAR), and ClustalW in the MacVector program (Accelrys, Madison, WI).

RESULTS

Change in hamster virulence associated with persistent infection. In the initial hamster infection with the parent WNV strain (hamster passage 1), a total of 10 animals were inoculated IP with 10^4 tissue culture infectious $dose_{50}$ ($TCID_{50}$) of virus (Figure 1). Four of the animals died of encephalitis; and six hamsters survived. The mortality rate (40%) in this group was consistent with previous studies[7,11,13] which indicated that 10^4 $TCID_{50}$ of WNV strain NY385-99 given IP to adult hamsters results in a mortality of approximately 50%. Four of the six surviving animals in this group had persistent renal infections and viruria when tested 48 days after infection (p.i.).[12] Infectious urine (titer = $10^{2.0}$ PFU/μL) from one of these hamsters (9317B) was inoculated IP into another uninfected hamster (T-35639B; hamster passage 2). Fifteen days later, infectious urine from hamster T-35639B was diluted 10-fold in phosphate-buffered saline, containing 30% fetal bovine serum to increase the total volume; and 100 μL of the mixture was inoculated IP into a group of 60 uninfected hamsters (hamster passage 3). The virus dose of the inoculum was $10^{2.8}$ PFU/mL. Urine from one of these animals (H-8536) was cultured 26 days p.i. and sequenced (strain TVP-9376).

The remaining 59 animals in this latter group (hamster passage 3) were maintained and sampled periodically for persistent WNV infection and chronic viruria, for 8 months. The procedure and results were described in another publication.[11] Although the hamsters in this group developed a viremia and antibody response similar to those observed in hamsters infected with the parent WNV strain, none of the 60 hamster passage 3 animals became ill or died.[11] Thus it appeared that the virus from urine had lost neurovirulence for hamsters after only two serial passages.

To test this hypothesis, urine was collected from two more animals (H-8535 and H-8537) in the hamster passage 3 cohort at 170 and 172 days p.i., respectively.[11] The urine samples from these two animals were WNV-positive and were cultured once in Vero cells to amplify the virus. The titers of the resulting WNV stocks, designated strains H-8535 and H-8537, were $10^{7.7}$ and $10^{6.5}$ PFU/mL, respectively. Two groups of 11 clean hamsters each were subsequently inoculated IP with $10^{6.7}$ PFU of WNV strain H-8535 and $10^{5.5}$ PFU of WNV strain H-8537. None of the inoculated animals ($N = 22$) from this fourth hamster passage became ill or died, and all seroconverted. Eight (73%) of the hamsters inoculated with WNV strain H-8535 and 9 (82%) of the animals inoculated with WNV strain H-8537 developed chronic infection and viruria. These results confirmed that the urine-passaged virus had lost neurovirulence and had developed increased renal tropism, compared with the parent WNV strain.

Nucleotide sequence changes associated with persistent infection. Full length nucleotide sequences were deposited for the parent WNV strain (GenBank accession no. AY842931), the 3 first hamster passage urine isolates, WNV strains 9317A (AY848695), 9317B (DQ066423), and 9317E (AY848696), and the third hamster passage urine isolate, WNV strain TVP9376 (AY848697) (Figure 1). When nucleotide changes were found, the RT-PCR and sequencing were repeated from the original Trizol lysate to confirm the changes.

The genome of WNV strain NY 385-99 (GenBank accession no. AY842931) exhibited standard flavivirus genomic organization. A short 5′ noncoding region of 96 nucleotides is followed by an ATG initiation codon at position 97 and a single open reading frame of 10,302 nucleotides, followed by a 3′ noncoding region of 631 nucleotides. The sequences were aligned without introduction of gaps or deletions, and nucleotide changes were identified.

When compared with the parent virus, nucleotide changes ranging from 0.045% to 0.091% were present in each of the 4 urine isolates (Table 1). The mutations involved genomic regions of the envelope protein (E), nonstructural proteins (NS1, NS2A, NS2B, NS3, NS5), and the 3′ noncoding region. For WNV strain 9317B, five of the changes resulted in amino acid substitutions; the others were silent mutations. Two of the mutations, a C to T at nucleotide 1465, and T to C at nucleotide 6405, were consensus among the 4 virus strains

TABLE 1
Nucleic acid changes in four WNV strains isolated from urine of chronically infected hamsters compared to the parent strain (NY395-99)

Nucleotides	NY385-99	9317A	9317E	9317B	TVP-9376	Genome region
1027	G	G	G	A	A	E*
1465	C	T	T	T	T	E*
3017	T	T	T	C	C	NS1*
3922	T	C	T	T	T	NS2A
4515	G	G	G	A	A	NS2B*
4539	T	T	C	T	T	NS2B
5658	C	T	C	C	C	NS3
6405	T	C	C	C	C	NS3
8181	G	G	A	G	G	NS5*
8235	T	T	T	C	C	NS5
8849	A	A	A	G	G	NS5*
10338	C	C	T	C	C	NS5
10393	C	T	C	C	C	NS5
10545	C	C	C	T	T	3′-NCR
10655	C	C	C	T	T	3′-NCR
10777	T	T	T	T	C	3′-NCR
Total nucleotide changes	5	5	9	10		
Divergence (%)		0.045%	0.045%	0.082%	0.091%	

* Results in amino acid substitution.

from urine. Also noteworthy was the fact that only one additional new mutation occurred in WNV strain TVP-9376, after two more hamster passages.

Changes in amino acid sequence of the hamster-passaged viral isolates. The amino acid sequences translated from the open reading frames of the five WNV strains were aligned. When compared with the parent WNV, the four hamster-passaged urine isolates exhibited amino acid substitutions at the six sites listed in Table 2. Two of these substitutions were in the E protein: V to M in WNV strains 9317B and TVP-9376 at position E-21; and L to F in all four hamster-strains at position E-167. All of the other substitutions were in nonstructural proteins. There were no additional amino acid changes between WNV strain 9317B and its progeny, WNV strain TVP-9376, after two further hamster passages. The predicted hydrophilicity of the latter two WNV strains was significantly increased due to the amino acid substitutions at regions E-21 and NS1-183 (Table 2).

DISCUSSION

The prototype WNV strain for the United States (382-99) was isolated from a Chilean flamingo (*Phoenicopterus chilensis*) at the Bronx Zoo during the New York City encephalitis outbreak in 1999.[15,16] It belongs to WNV genetic lineage I.[15] The WNV strain (NY385-99) used in the current study and in our previously described experiments with the hamster model[5,7,9–13] was isolated from a dead owl at the Bronx Zoo during the same epizootic.[16] WNV isolate NY385-99 has only nine nucleotide differences and one amino acid substitution when compared with the New York 382-99 prototype strain (Xiao SY, unpublished data). The 2 WNV strains are genetically and phenotypically very similar.

The clinical significance of WNV infection is mainly because of its ability to cause neuroinvasive disease in humans, equines and birds. Consequently, our initial studies with the hamster model were focused on the neuropathogenesis and neurotropism of the virus.[5,7,9,10,13] However, subsequent studies revealed that some of the surviving animals developed a persistent renal infection with chronic viruria.[11,12] Immunohistochemical studies of the persistently infected hamsters demonstrated that WNV antigen was localized to the distal tubular epithelial cells and interstitium of the kidney.[11] Infectious WNV could be recovered by direct culture from urine and by cocultivation of kidney tissue from persistently infected hamsters.[11] It was also observed that WNV strains isolated from the chronically infected animals had reduced neurovirulence for hamsters and that the plaque morphology and growth characteristics of these strains were different from those of the WNV parent.[11] Collectively, these observations suggested that genetic changes had occurred in the parent WNV strain during its serial passage in hamster kidneys and urine.

TABLE 2

Amino acid substitutions in four WNV strains isolated from urine of chronically infected hamsters compared to the parent strain (NY385-99)

Amino acids	385-99	9317A	9317E	9317B	TVP-9376	Region
311	V	V	V	M	M	E-21
457	L	F	F	F	F	E-167
974	I	I	I	T	T	NS1-183
1473	M	M	M	I	I	NS2B-99
2695	M	M	I	M	M	NS5-167
2918	E	E	E	G	G	NS5-390

In the present study, 4 of 10 animals inoculated with the WNV parent strain at the first hamster passage level died. Of the 6 survivors, 4 hamsters had infectious WNV in their urine when tested 48 days p.i. The fatality (40%) and survival (60%) rates with WNV infection in this initial group were similar to what we observed in previous experiments with WNV strain NY385-99.[7,9,12,13] However, by the third hamster passage, the virus had lost neurovirulence for hamsters and had developed increased renal tropism, as 90% of the animals ($N = 60$) developed persistent infection and viruria for varying periods of time.[11] To detect possible genetic changes, we selected 3 WNV isolates (strains 9317A, 9317B, and 9317E) from urine of chronically infected hamsters at the first hamster passage level and one urine isolate (strain TVP-9376) from the third hamster passage level for PCR amplification and direct sequencing (Figure 1). After the first hamster passage, genetic changes were observed in WNV strains isolated from urine of each of the 3 hamsters (Tables 1 and 2). Some of the changes were unique to isolates from different hamsters, but two of the mutations (C to T at nucleotide 1465, and T to C at nucleotide 6405) were common to all three isolates (Table 1). After two further hamster passages of WNV strain 9317B, only one additional mutation was detected in WNV strain TVP-9376 (Figure 1).

The differences found in the nucleotide sequences of the hamster-passaged viruses may have resulted from *de novo* mutations, or simply by selection from a pool of existing quasispecies in the parent NY385-99 virus stock. To address this question, we resequenced the parent virus at these various sites of difference, and did not find alternative nucleotides. Furthermore, additional mutations have been identified in WNV urine isolates from the fourth hamster passage (Wu XY and others, unpublished data), suggesting that at least some *de novo* changes are responsible for the nucleotide differences. However, the possibility remains that the sequences obtained could have came from another nonprevailing WNV genotype in the pool that had become dominant through renal selection.

Another less likely explanation for the observed nucleotide changes in the WNV urine isolates could be that the mutations occurred during passage in Vero cells. The infectious urine from the hamsters was passaged once in Vero cell culture into order to amplify the virus and to obtain sufficient RNA for sequencing. However, work by others suggests that this possibility is unlikely. Anderson and others[17] showed, by RT-PCR and direct sequencing, that WNV isolates before and after 1 to 3 passages in Vero cell culture were identical. In another similar study, Huang and others[18] found that WNV sequenced directly from a patient's cerebrospinal fluid was identical to virus from the same case recovered in cell culture. Thus we feel that *in vitro* cultural changes were probably not responsible for the observed nucleotide changes.

From a more pragmatic viewpoint, whether these genetic changes were the result of *de novo* mutations, or by selection of quasispecies, is not as important as whether the observed changes were associated with the increased renal tropism and loss of neurovirulence. Clinically, renal disease is not a manifestation of acute WNV infection in humans, although there

have been reports of the detection of WNV RNA in urine of a non-fatal case of encephalitis[19] and of WNV antigen in kidneys of fatal encephalitis cases.[20] In the hamster, chronic WNV renal infection appears to be asymptomatic and benign.[11] If the mutations observed in the current study form a molecular basis for new viral characteristics (i.e., reduced neurovirulence), then this information could potentially be useful in the design and development of new WNV vaccines. Further experiments are needed to verify this hypothesis and to pinpoint the specific mutations or combination of mutations responsible for these phenotypic changes.

Our findings are similar to earlier reports by Pogodina and others[21,22] of experiments done in rhesus monkeys with tick-borne encephalitis virus (TBEV) (*Flavivirus: Flaviridae)*. These authors reported that they were able to recover TBEV from organs (brain, liver, spleen, lymph nodes, and kidneys) of rhesus monkeys inoculated with the virus, for up to 2 years after infection. After several weeks, TBEV could be recovered from the tissues only by cocultivation, and not by direct organ culture, as observed in our studies of WNV persistent infection in hamsters.[11,12] Podogina and others[22] also observed that TBEV isolates recovered from tissues of persistently infected monkeys had lost pathogenicity for mice, and in some cases, the ability to produce CPE in vertebrate cell cultures. These experiments with TBEV were done more than 20 years ago, so the phenotypically different virus strains isolated from the persistently infected monkeys were not sequenced. However, we suspect these TBEV isolates may also have had comparable nucleotide changes associated with their loss of virulence for mice and their altered growth characteristics *in vitro*.[22]

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