EXPERIMENTAL AND NATURAL INFECTION OF NORTH AMERICAN BATS WITH WEST NILE VIRUS

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Abstract. Big brown (*Eptesicus fuscus*) and Mexican free-tailed (*Tadarida brasiliensis*) bats were inoculated with the New York 99 strain of West Nile virus to assess their potential to serve as amplifying hosts and determine the clinical effect of infection. Groups of three or four bats were bled at daily intervals between 1 and 6 days after inoculation to determine the pattern of viremia. Beginning 2 days after inoculation, virus was isolated each day from one or more *E. fuscus* bats, in titers ranging from 10 to 180 plaque-forming units per milliliter of serum. Virus was not isolated from any of the sera collected from *T. brasiliensis* bats. None of the bats from either species showed clinical signs associated with exposure to virus. Sera from an additional 149 bats collected in Louisiana in 2002 during an epizootic of West Nile fever were tested for antibodies to virus, and two were found to be positive. These data suggest that bats from these two widely distributed species are unlikely to serve as amplifying hosts for West Nile virus.

INTRODUCTION

Since West Nile virus (WNV) was first recognized in North America in 1999, it has spread widely across the United States, as well as into Canada and Mexico.[1–3] The major factor responsible for rapidity of spread appears to be movement of infected birds.[4] Despite the extraordinary susceptibility of corvids and certain other birds to WNV and their clear role as amplifying hosts, there has been considerable interest in defining other host species that might play a role in the enzootic transmission cycle of WNV. Bats are of particular interest as they have been found to experience natural infections with several flaviviruses, including St. Louis encephalitis (SLE), Japanese encephalitis (JE), Rio Bravo, Montana *Myotis* leukoencephalitis, and Tamana bat viruses.[5–9] Other studies have demonstrated viremia in bats experimentally infected with SLE and JE viruses.[5,9–11]

Information about bats and WNV is meager. Antibodies to WNV in Chiroptera were first identified in healthy *Rousettus aegyptiacus* fruit bats collected in Uganda and Israel.[12] WNV was isolated in India from the nearly indistinguishable *Rousettus leschenaulti*.[13] Subsequent interest in WNV as a Chiropterian pathogen appeared limited until its arrival into the New World. During the year 2000, 150 dead bats were submitted to the New York State Department of Health after initial testing for rabies, and two bats (one *Eptesicus fuscus* and one *Myotis lucifugus*) were found to be positive for WNV by immunofluorescence.[14] Subsequently, attempts were made to demonstrate WNV in an additional 306 bats submitted during 2000 to 2002 from the WNV-endemic zones of New York State. These animals represented several species, most of which were *E. fuscus*, and none were found to be positive by virus isolation and polymerase chain reaction testing (Baryl J, Bernard K, personal communication).

Like the numerous species of birds in the class of Aves, diversity within the order Chiroptera is extraordinary, with more than 1,100 species found throughout the world.[15] The current study focused on two species that are most numerous within the United States, *E. fuscus* (big brown bat) and *Tadarida brasiliensis* (Mexican free-tailed bat). Individuals from both of these species are likely to live within or near urban populations and frequently come into contact with humans.[16] *E. fuscus* bats are considered to be nonmigratory and hibernate during the winter months. Previous demonstrations of persistence of JE[17,18] and SLE[10] viruses through a period of experimental hibernation implied the possibility that hibernating *E. fuscus* might play a role in overwintering of WNV. The majority of *T. brasiliensis* are migratory, and millions of these animals fly between the southern United States and Latin America every year.[19] If *T. brasiliensis* were found to be significant hosts for WNV, this yearly event could promote the spread of WNV into countries currently free of the disease, as well as reintroduce the agent back into the southern United States. The purpose of this study was to elucidate the role these two species of bats might play as hosts in the WNV transmission cycle and to determine their clinical response to WNV infection. We also assessed the prevalence of WNV infection in *T. brasiliensis* bats living within a focus of endemic infection in Louisiana.

MATERIALS AND METHODS

Experimental infections. *E. fuscus* bats were captured from roosts in Fort Collins, Colorado, in summer 2002 prior to any recognition of WNV infections in Colorado. *Tadarida brasiliensis* (*T. brasiliensis*) bats were collected in Austin, Texas, in September 2002. In both cases, appropriate state and federal wildlife permits were in place. The bats were held in quarantine within a biosafety level 3 facility in groups of 3 to 6 for 4.5 (*E. fuscus*) or 1.5 (*T. brasiliensis*) months prior to inoculation with WNV. They had free access to water and were fed mealworms, supplemented occasionally with beef baby food. Prior to inoculation with WNV, blood was collected from the uropatagial vein of the *T. brasiliensis* bats, and those sera were screened for antibodies to WNV and other flaviviruses using a blocking enzyme immunoassay with antigen consisting of lysates of WNV-infected C6/36 mosquito cells, as previously described.[20] This screening test was used because of the small quantities of serum available. Sera were tested in this assay using monoclonal antibodies (MAb) 6B6C-1, which has reac-

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tivity against WN, SLE, Murray Valley encephalitis, yellow fever (YF), JE, and all four serotypes of dengue viruses, and MAb 3.1112G, which is specific for WNV/Kunjin.[21] Seventeen of the 34 *T. brasiliensis* bats tested had serum antibodies that blocked binding of MAb 6B6C-1 to WNV antigen (>30% inhibition; range 40–84%), but none of the sera blocked binding of MAb 3.1112G.

Bats of both species were inoculated subcutaneously with 0.1 mL containing between 6,000 and 7,000 plaque-forming units (PFU) of New York 99 strain of WNV (4132), isolated initially from an infected crow and passaged once in Vero cells, once in C6/36 mosquito cells, and once in BHK-21 cells. All bats were examined for clinical signs of disease twice daily for the duration of the project by rousing for feeding and observing their movement in their cages. Three *E. fuscus* bats were euthanized via pentobarbital overdose daily on Days 1 through 6 and on Days 14 and 40 after inoculation. Four *T. brasiliensis* bats were euthanized daily from Days 1 to 6 after inoculation; within each group of four bats, two were among those that had antibodies that reacted in the flavivirus group-specific immunoassay, and two were serologically negative in that assay. At the time of euthanasia, blood was collected by heart puncture, and a cotton swab wet with BA-1 (medium M199-Hanks' salts, 0.05 M Tris pH 7.5, 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 50 mg gentamicin/L, 2.5 mg amphotericin B/L) was rotated within the oral cavity, then swirled in 0.5 mL of BA-1. Duplicate 0.1 mL samples of undiluted sera and oral swab samples were assayed for WNV using a double overlay plaque assay on Vero cells, with plaques counted on Days 3, 4, and 5. For *E. fuscus* bats, samples of brain, heart, lung, liver, kidney, and spleen collected at necropsy were homogenized in BA-1 to yield 10% suspensions, and those were assayed for virus by plaque assay on Vero cells. Sera from the six *E. fuscus* bats euthanized 14 and 40 days after virus inoculation were tested for neutralizing antibody using a plaque-reduction neutralization test (PRNT) as previously described.[22] Maintenance and care of all bats complied with the National Institutes of Health guidelines for the humane use of laboratory animals.

Field studies. As part of a larger field study in St. Tammany Parish, Louisiana, in late summer 2002, 149 *T. brasiliensis* bats were bled by cardiac puncture under isoflurane anesthesia. An 0.2-mL sample of blood was added to 0.9 mL BA-1, allowed to clot, and centrifuged to recover the equivalent of a 10^{-1} dilution of serum. These sera were tested for neutralizing antibodies to WNV by PRNT.

RESULTS

None of the *E. fuscus* or *T. brasiliensis* bats inoculated with WNV showed clinical signs of infection, with the exception of one *E. fuscus* bat that became abnormally vocal and aggressive 10 days into the study. This bat was euthanized on Day 14 and found to be rabid by direct immunofluorescence testing of brain. This animal had been in quarantine for approximately 20 weeks prior to showing signs of rabies.

Seven of the 24 *E. fuscus* bats were viremic at the time of euthanasia (Table 1). Viremia was detected between 2 and 6 days after inoculation, with titers ranging from 10 (limit of detection) to 180 PFU/mL. In contrast, WNV was not isolated from any of the sera collected between Days 1 and 6 from the

TABLE 1
Viremia in *Eptesicus fuscus* bats after inoculation with West Nile virus

	Plaque-forming units per milliliter serum on						
Bat	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 14
A	< 10	180	< 10	130	0	< 10	< 10
B	< 10	< 10	10	10	0	< 10	< 10
C	< 10	< 10	< 10	50	50	60	< 10

* Three bats were euthanized each day; Bats A, B, and C are different bats on each day.

24 *T. brasiliensis* bats. Virus was not isolated from any of the oral swabs collected from either species of bat, nor from any of the tissues collected at necropsy from *E. fuscus* bats. None of the sera collected from the *E. fuscus* bats euthanized 14 and 40 days after inoculation with WNV had neutralizing antibody to WNV when tested at a dilution of 1:10 (90% reduction).

Of the 149 *T. brasiliensis* sera collected in Louisiana, two were positive for neutralizing antibody to WNV, with reciprocal PRNT (90% reduction) titers of 20 and 40, and <10 to SLE virus.

DISCUSSION

The primary goal of this study was to assess the potential role of common insectivorous bats as hosts for WNV. Both of the species investigated are present in large numbers over broad regions of North, Central, and South America, and both species are commonly found in close association with humans.

Seven of 24 serum samples collected from *E. fuscus* bats over the first 6 days after inoculation with WNV contained virus. However, the level of viremia observed was low, the highest titer being 180 PFU/mL, and unlikely to serve as a source of virus for feeding mosquitoes.[22–24] In the case of *T. brasiliensis* bats, we were unable to isolate WNV from any of the sera collected during the 6 days after inoculation. Prior to challenge, half of these bats did have antibodies that cross-reacted with WNV antigen. However, these antibodies recognized a flavivirus group-specific epitope, did not react with a WNV-specific epitope, and may thus have represented antibody to SLE or Rio Bravo viruses.

It was surprising that we failed to detect a neutralizing antibody response on Days 14 or 40 after inoculation of WNV into *E. fuscus* bats. This lack of immune responsiveness may be due to poor viral replication in these animals. Lack of neutralizing antibody was also observed in *E. fuscus* bats sampled 17 days after inoculation with SLE virus.[11]

Insectivorous bats have been experimentally infected with several flaviviruses. Needle inoculation of SLE virus resulted in development of viremia in some *E. fuscus* bats, but the magnitude of viremia was very low and not considered infectious to mosquitoes, and clinical signs of disease were not observed.[11] Inoculation of *E. fuscus* and *Pipistrellus subflavus* bats with JE virus also resulted in development of viremia, but the titers achieved were not reported.[25] In another study, it was found that bats experimentally infected with JE virus were capable of producing a viremia sufficient to infect mosquitoes.[17]

Insectivorous bats feed on numerous types of insects, including occasional mosquitoes, and may possibly be infected

with arboviruses through oral exposure. Indeed, it has been demonstrated that bats could become infected with YF virus through feeding on a single infected mosquito,[26] and that feeding of three JE virus-infected mosquitoes to a *P. subflavus* led to development of viremia.[17]

We found 2 of 149 Mexican free-tailed bats from an endemic focus in Louisiana to have neutralizing antibody to WNV. Free-ranging birds sampled from this region during the same period of time had an overall WNV seroprevalence rate of 28% (Komar and others, submitted).

Our objective was to assess the potential of bats to serve as amplifying hosts and thereby contribute to WNV transmission in the Americas. Although it is possible that some bat species may react to WNV infection in a substantively different manner than the two species we investigated, *E. fuscus* and *T. brasiliensis* bats together probably represent the most prevalent bats in North America. The major finding of this study is that neither species of bat investigated developed more than a low-titer viremia after inoculation with a low-passage isolate of WNV. It was also found that the prevalence of antibodies to WNV in bats collected from an endemic focus was very low. Collectively, these observations suggest that *E. fuscus* and *T. brasiliensis* bats are unlikely to serve as amplifying hosts for WNV. Additional surveillance to characterize seroprevalence and presence of virus in dead bats is indicated to more fully understand possible interactions of WNV and bats.

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