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ABSTRACT: A captive colony of adult Big Brown Bats (*Eptesicus fuscus*) was experimentally infected with a rabies virus (RABV) variant isolated from the salivary glands of a naturally infected Big Brown Bat and passaged once through murine neuroblastoma cell culture. Bats were divided into 11 groups, which were composed of one to three noninfected and one to three infected individuals each. Twenty of 38 animals were infected intramuscularly into both left and right masseter muscles; they received a total of $10^{3.2}$ median mouse intracerebral lethal dose ($MICLD_{50}$) of Big Brown Bat RABV variant. Experimental outcome after viral exposure was followed in the bats for 140 days postinoculation (PI). Of 20 infected bats, 16 developed clinical rabies, and the mean incubation period was 24 days (range: 13–52 days). Three infected bats never seroconverted and succumbed early to infection (13 days). Four infected bats that survived until the end of the experiment without any signs of disease maintained detectable antibody titers until the third month PI, peaking between days 13 and 43, and consequent drop-off below the threshold for detection occurred by day 140. Limited excretion of virus in saliva of infected bats during the clinical course of disease was observed in two individuals on days 13 and 15 PI (<24 hr prior to onset of clinical illness). No bat-to-bat transmission of RABV to noninfected bats was detected.

Key words: Chiroptera, *Eptesicus fuscus*, infection, pathogenesis, rabies virus, transmission.

INTRODUCTION

Infection of humans with rabies virus (RABV) invariably results in death by acute encephalitis. In the United States (USA), the number of cases involving humans has dropped considerably in the past 50 yr, but surveillance has shown that rabies cases have increased in wildlife hosts (Smith, 1996; Blanton et al., 2007). An analysis of rabies cases in the USA from 1958 to 2006 reveals that >90% of cases of indigenously acquired rabies in humans were caused by RABV associated with insectivorous bats, although many of the patients had an undocumented bite history (Messenger et al., 2002; Blanton et al., 2007). Rabies has been documented in all species of insectivorous bats in the USA (Constantine, 1979), and monoclonal antibody and molecular typing has revealed that RABV is enzootic in most species (Smith, 1989; Smith, 1996). Many studies have shown that insectivorous bats in the USA are susceptible to different RABV variants (Burns et al., 1958; Constantine,

1966b, c; Constantine and Woodall, 1966; Baer and Bales, 1967; Constantine et al., 1968). However, the mechanisms of RABV transmission within wild bat populations and RABV pathogenesis in bats remain largely unknown.

Big Brown Bats (*Eptesicus fuscus*) range through most of North and Central America. In spring and summer, females form maternity colonies that typically consist of 25–75 bats (Davis et al., 1968; Mills et al., 1975). Adult males may roost with females or form male-only colonies (Davis et al., 1968). Maternity colonies in the eastern USA are often in man-made roosts, but western maternity colonies of *E. fuscus* can also be found in tree cavities (Brigham, 1991). A recent study has indicated that most *E. fuscus* individuals engage in short local migrations between maternity sites and winter hibernacula (Neubaum et al., 2006). Habitat loss and environmental alterations may explain the increasing trend of finding colonial species of bats in buildings, bridges, and other man-made roosts (Kunz and Reynolds,

2004). Alternatively, building roosts may provide superior habitat for Big Brown Bats and allow greater aggregation of individuals than is possible in natural roosts (Lausen and Barclay, 2006).

Rabies occurs in *E. fuscus* populations across the USA (Whitaker et al., 1969; Baer and Adams, 1970; Trimarchi and Debbie, 1977; Kurta, 1979), and three known RABV variants are specifically associated with Big Brown Bat populations (Nadin-Davis et al., 2001; Hughes et al., 2005; Shankar et al., 2005; Velasco-Villa et al., 2006). Passive surveillance in the USA indicates that Big Brown Bats account for the majority of rabid bat diagnoses in state health laboratories (Childs et al., 1994; Pape et al., 1999; Blanton et al., 2007); however, this species is also the most frequently submitted for testing. Despite frequent contact with humans, only one case of indigenously acquired rabies in humans from 1958 to 2006 was caused by a Big Brown Bat RABV variant (Messenger et al., 2002; Blanton et al., 2007). The relatively common prevalence of rabies in colonies of *E. fuscus*, their frequent contact with humans, and the low number of human rabies cases associated with this particular RABV variant all raise questions about the pathogenicity of Big Brown Bat RABV compared to RABV variants associated with other insectivorous bat host species, particularly *Lasionycteris noctivagans*, *Pipistrellus subflavus*, and *Tadarida brasiliensis*. Intramuscular (IM) inoculation of carnivores has indicated equivocal susceptibility of these animals to Big Brown Bat RABV (Constantine et al., 1968; Trimarchi et al., 1986) compared to other insectivorous bat RABV variants (Constantine, 1966a, b; Constantine and Woodall 1966; Constantine et al., 1968). However, basic susceptibility of *E. fuscus* to infection with Big Brown Bat RABV or RABV variants from other bat host species has not been tested.

The objectives of this study were to investigate the basic susceptibility of *E. fuscus* to a Big Brown Bat RABV variant, assessed through observations of incubation period, clinical signs, salivary excretion, serologic response, and potential for bat-to-bat transmission of RABV. These data will be used to develop models for studying RABV transmission from bats to humans, and bat-to-bat transmission among conspecifics and other colonial species.

MATERIALS AND METHODS

Animal collection and care

Experimental procedures and animal care at the Centers for Disease Control and Prevention (CDC) were performed in compliance with Institutional Animal Care and Use Committee guidelines. Thirty-eight adult Big Brown Bats were captured from building roosts in Atlanta, Georgia, between 4 May and 6 May 2005 (GA permit #29-WMB-01-129). Bats were held captive in quarantine for at least 1 mo. They were individually marked with ear tags. Samples of baseline sera and swabs indicated the naïve status of all experimental bats. Animals included 31 adult females (F/A), and seven adult males (M/A). All bats were aged on the basis of the degree of closure of the phalangeal epiphyses (Anthony, 1988). After quarantine, bats were randomly separated into 11 groups of three to six animals, allowing even distribution of males and females when possible. Each group was housed in a separate 813 mm × 305 mm × 254 mm stainless-steel cage, and all cages were housed collectively in a room at 75–80 F and 30% humidity.

The RABV used for experiments was collected from the salivary glands of a naturally infected Big Brown Bat in Colorado in 2004 (CDC A04-0719). This virus was passaged once through murine neuroblastoma (MNA) cell culture. The virus was identified as a Big Brown Bat RABV variant by sequencing and phylogenetic analysis (Fig. 1). On day 0, one to three randomly selected bats from each cage were inoculated intramuscularly (IM) into both left and right masseter muscles. Each inoculated bat received a total of $10^{3.2}$ median mouse intracerebral lethal dose ($MICLD_{50}$) of Big Brown Bat RABV. All infected and noninfected bats were observed for 140 days postinoculation (PI). Blood samples were obtained from the brachial artery and vein at the distal epiphysis of the humerus or by intracardiac routes (Kunz and Nagy, 1988). Samples were collected with heparinized microcapillary tubes. Blood was centrifuged immediately after collection, and serum was separated and stored at −80 C. Blood samples were collected six
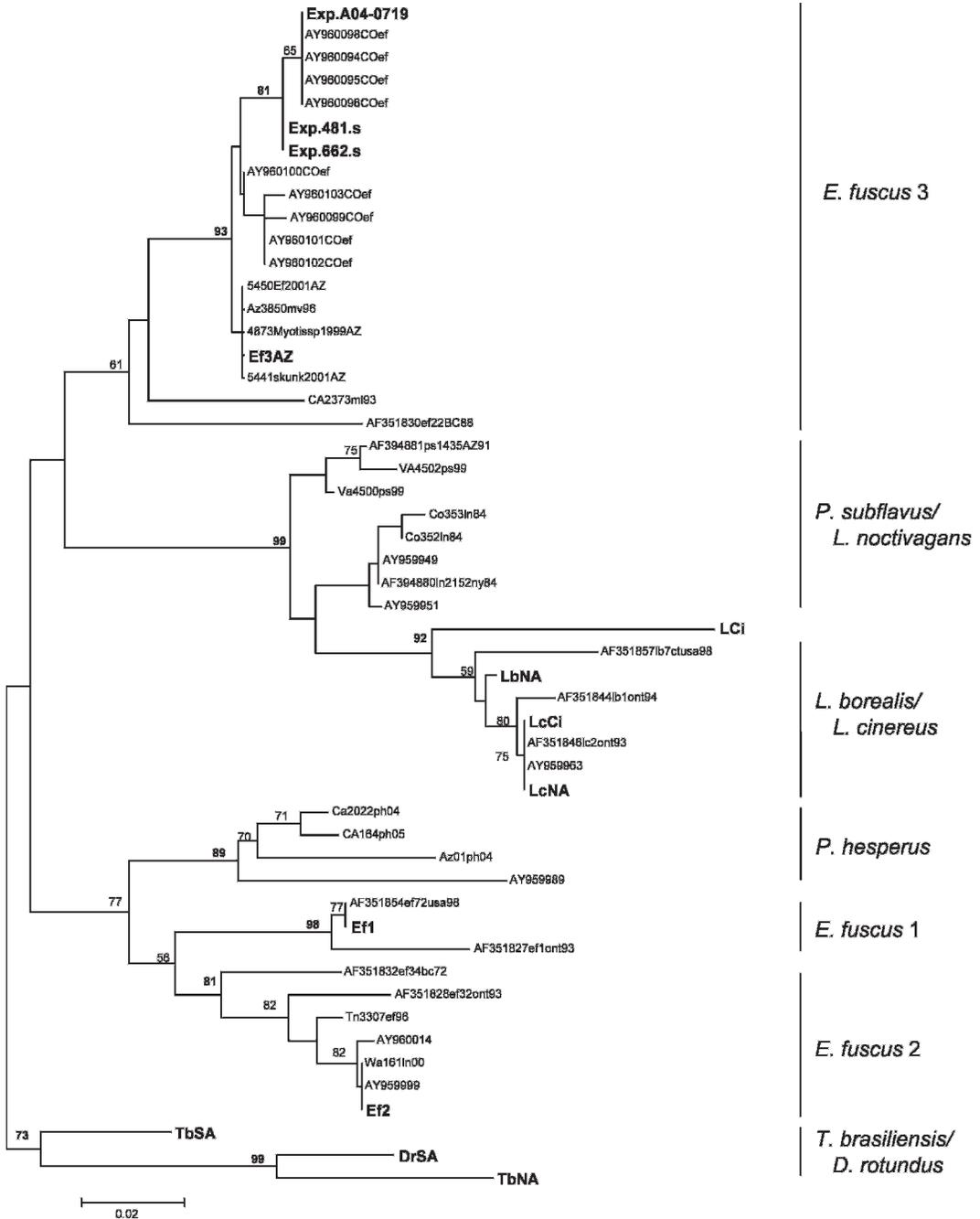
FIGURE 1. Phylogenetic tree of bat rabies virus (RABV) variants using neighbor-joining analysis (K2P model) on a portion of the 5′ end of nucleoprotein (N) gene-coding sequence (244 nts). Bootstrap proportions (of 10,000 replicates) are shown above nodes. Consensus sequences are included, as previously described in Velasco-Villa et al. (2006): EF1 and EF2=*Eptesicus fuscus* North America, EF3Az=*Eptesicus fuscus* Arizona, TbSA=*Tadarida brasiliensis* South America, TBNA=*Tadarida brasiliensis* North America, Dr=*Desmodus rotundus*, LCi=*Lasiurus* spp. Chile, LBNA=*Lasiurus borealis* North America, LCNA=*Lasiurus cinereus* North America, LCCi=*Lasiurus cinereus* Chile.

times between 15 August 2005 and 9 January 2006. Sera were assayed for the presence of rabies virus-neutralizing antibodies (VNA). The first measurement of VNA following inoculation was performed on samples collected on day 13 PI. In addition, duplicate oral swab samples were collected 11 times (on days 0, 6, 8, 15, 19, 23, 27, 30, 42, 51, and 91 PI) during the study from each animal, and samples were stored at -80 C in either minimal essential medium supplemented with 10% fetal bovine serum (MEM-10) (Invitrogen, Carlsbad, California, USA) or Trizol LS (Invitrogen).

Upon the development of two or more definitive clinical signs (e.g., acute weight loss, ataxia, unusual vocalizations, paresis, or paralysis), bats were sedated (ketamine hydrochloride: 15 µl/25 g bat; 100 mg/kg), bled, and euthanized (pentobarbital sodium and phenytoin sodium: 10 µl/25 g bat; 390 mg/kg and 90 mg/kg, respectively). Brain tissue was collected and tested for the presence of RABV antigen by the direct fluorescent antibody (DFA) test, as described by Dean et al. (1996), using fluorescein isothiocyanate (FITC)–labeled monoclonal antibody (mAb) conjugate (Fujirebio Diagnostics, Inc., Malvern, Pennsylvania, USA).

Detection of RABV-neutralizing antibodies

The small quantity of sera collected (5–100 µl) required a modification of the rapid fluorescent focus inhibition test (RFFIT) to measure induced VNA (Smith et al., 1996). Briefly, the MRFFIT was performed as follows: approximately 10 µl of bat serum was heated at 56 C for 30 min to inactivate complement; 10 serial twofold dilutions in MEM-10 were performed in a 96-well plate (Fisher Scientific, Agawam, Massachusetts, USA), with the subsequent addition of a constant focus-forming dose (50 FFD_{50}) of RABV challenge virus standard (CVS-11) to each well. Negative and positive controls were used in each run of the assay (Smith et al., 1996). Plates were incubated for 90 min at 37 C and 0.5% CO_2 before the addition of 1.0×10^4 trypsinized MNA cells per well. The sera-virus-cell mixture was then transferred to a Terasaki plate (Fisher Scientific) for 18–20 hr incubation at 37 C and 0.5% CO_2. The presence of virus in MNA cells was visualized using FITC-conjugated RABV mAb (Fujirebio Diagnostics, Inc.). The MRFFIT results were expressed as endpoint titers using the Reed-Muench method (Reed and Muench, 1938). A positive VNA titer was considered as complete neutralization of the challenge dose by a serum dilution of at least 1:4. Titers were converted to IU/ml by comparison to a standard rabies immune globulin (SRIG) control containing 2 IU/ml (Smith et al., 1996). Titer results <0.05 IU/ml were considered to be negative.

Detection of viral RNA from saliva

Total RNA was extracted from oral swabs and analyzed for the presence of RABV genomic RNA using RT-PCR (Orciari et al. 2001). An aliquot (550 µl) of the oral swab sample (in Trizol LS) was used for RNA extraction with a Roche MagNA Pure LC (model JE 379, Roche Diagnostics, Indianapolis, Indiana, USA) system. Viral and reagent controls were included in the RNA extraction. A reaction including 2 µmol 1066F ('5-GAR-AGAAGATTCTTCAGRGA-3', position 1136–1155, Pasteur [PV] genome: M13215) and 5 µl of extracted RNA was denatured for 1 min at 94 C and then cooled on ice for 5 min. The RT MIX (1× buffer containing RNA-primer complex, 20 nM of deoxyribonucleotides, 16 U of RNase Inhibitor, and 8 U of avian myeloblastosis virus reverse transcriptase) (Roche Diagnostics) was added, and reverse transcription was achieved by incubation at 42 C for 90 min.

A 398-nucleotide (nts) product of the 5′ end of the RABV N gene was amplified in a primary PCR reaction using the primers 304R ('5-TTGACGAAGATCTTGCTCAT-3', position 1514–1533, PV genome: M13215) and 1066F. The product from RT (20 µl) and 80 µl of PCR Mix (8 µl Tris buffer [pH 8.3], 1.25 µl primer 304R, 1.0 µl primer 1066F, 0.5 µl Taq) was initially denatured at 94 C for 1 min, and then cycled 40× (94 C/30 sec, 37 C/30 sec, 72 C/90 sec), before a final extension at 72 C for 7 min. Electrophoresis, using agarose gels incorporated with ethidium bromide, was used to visualize primary PCR products. If no product was apparent, hemi-nested PCR was used to amplify a 377-nts fragment from the 5′ end of the RABV nucleoprotein (N) gene using primers 304R and 1087F ('5-GAGAAR-GAACTTCARGAITA-3', position 1157–1176, PV genome: M13215). Reactions consisting of 90 µl of nested PCR MIX (7.7 µl Tris buffer [8.3 pH], 1.2 µl primer 304R, 1.2 µl primer 1087F, 0.48 µl Taq) and 10 µl of primary PCR product were cycled through the same conditions used in the primary PCR. Hemi-nested products were visualized on composite agarose gels (Bio-Rad, Hercules, California, USA; NuSieve GTG, BMA, Rockland, Maine, USA) incorporated with ethidium bromide. Amplicons were purified and sequenced using forward primer 1087F and reverse primer 304, as described previously. Sequencing reactions

were performed, using ABI PRISM® BigDye® Terminator v1.1 (Applied Biosystems, Foster City, California, USA), through 25 cycles (96 C/10 sec, 50 C/5 sec, and 60 C/4 min). Direct sequencing of the purified 377-nts amplicons was performed using an ABI 377 sequencer (Applied Biosystems).

Sequences were imported into BIOEDIT (Hall, 1999), and ambiguities were manually reconciled by eye. A nucleotide-nucleotide BLAST (blastn) search of the edited sequence (http://www.ncbi.nlm.nih.gov/BLAST) was used to find the closest match from sequences available in GenBank. To identify the RABV variant used for the experiment, a phylogenetic analysis was performed using >200 RABV sequences originating from different North American mammals available from GenBank. A neighbor-joining analysis with 10,000 bootstrap replicates, and using a Kimura-2 parameter (K2P) model of substitution (Kimura, 1980), was performed using MEGA v2.1 (Kumar et al., 2001) (Fig. 1). The RT-PCR protocol was also performed on brain tissue from all experimentally infected rabid bats for phylogenetic comparison to the viral inoculum used for infection.

Isolation of RABV from saliva

Swab samples that tested positive by RT-PCR were selected for attempted viral isolation. One milliliter of the swab sample (in MEM-10) was mixed with 0.5 ml of MEM-10 suspension containing 7.5×10^5 MNA cells in a 25 cm^2 (T25) cell culture flask. Six milliliters of MEM-10 were added to the flask and gently mixed. Subsequently, 2.4 ml of the mixture were removed and divided into an eight-well Labtek chamber slide (Nalge Nunc, Inc., Rochester, New York, USA) as a control of replication. Incubation was continued for 48–72 hr in a humidified incubator set to 0.5% CO$_2$ and 37 C. Following incubation, Labtek slides were rinsed with 0.01M phosphate-buffered saline (pH 7.5), fixed in acetone at −20 C for 30 min, and stained with FITC-conjugated RABV mAb to visualize RABV infection of cells. If no RABV antigen was detected on the slides, the T25 cultures were passaged again following the same procedure. The flasks were examined for two to three passages before being discarded.

RESULTS

Susceptibility

Of the 20 infected animals, 16 (80%) developed clinical rabies. Only four in-fected bats survived viral infection. Mean incubation period was 24 days (range: 13–52 days). Of the infected bats that developed clinical rabies, 12 (75%) had incubation periods between 13 and 17 days, whereas four (25%) had incubation periods of 52 days. Most clinically affected bats experienced acute weight loss, reclusive behavior from cage mates, ataxia, and paresis persisting for more than one day. In contrast, none of the noninfected bats presented signs of clinical rabies infection at any point in the study.

Antibody response to RABV infection

Prior to RABV infection, none of the bats had a positive RABV VNA titer. Following inoculation, 17 out of 20 bats (85%) seroconverted, and RABV VNA titers peaked between 13 and 43 days PI (Table 1). After 43 days, VNA titers declined at each time measured thereafter (Table 1). The four surviving bats (1888, 1889, 1882, and 1434) maintained RABV VNA titers for >3 mo PI, but none had detectable RABV VNA titers by the end of the experiment (day 139; Table 1). Of the four bats with the shortest incubation periods (13 days; Table 1), three (75%) did not develop detectable RABV VNA and were the only infected bats that failed to develop an antibody response. Bats that were not experimentally infected did not develop detectable RABV VNA titers at any time in the study.

Rabies virus shedding and transmission

We detected RABV genomic RNA in the saliva of two (10%) of the infected bats. The positive swab samples both occurred immediately (<24 hr) prior to the onset of clinical illness (day 13 PI, bat 662; day 15 PI, bat 481). The two positive PCR results both occurred in male bats (of 3 males and 17 females infected). Phylogenetic analysis revealed 99.7% sequence similarity between the two salivary RNA amplicons (GenBank accession numbers: EU277849, EU277850) and the infecting virus (Fig. 1). Attempts to isolate the virus

TABLE 1. Twenty Big Brown Bats were infected intramuscularly (IM) with Big Brown Bat rabies virus (RABV). The gender and age of the bat, diagnostic (direct fluorescent antibody, DFA) result, incubation period before onset of clinical illness, and postinoculation rabies virus-neutralizing antibodies (VNA) titers are shown for all infected animals.

Cage	Bat no.	Sex/age[a]	DFA	Incubation (days)	Days postinfection[b]					
					0	13	43	75	105	139
					Rabies VNA titer (IU/ml)[c]					
A1	1858	F/A	+	14	0.02	**0.29**				
A2	1864	F/A	+	13	0.02	0.02				
A3	1874	F/A	+	17	<	**0.36**				
	199	F/A	+	17	<	**0.71**				
	1891	F/A	+	17	<	**4.77**				
A4	1894	F/A	+	17	0.02	**0.42**				
A5	1892	F/A	+	52	0.02	**1.19**	**1.00**			
A6	1882	F/A	−	−	0.02	**1.69**	**2.01**	**1.00**	**0.5**	0.02
	1434	M/A	−	−	0.02	**4.77**	**4.02**	**2.01**	**2.01**	0.02
B1	1885	F/A	+	52	0.02	**2.01**	**1.00**			
	1879	F/A	+	52	0.02	**6.78**	**5.76**			
	1886	F/A	+	52	0.02	**2.01**	0.03			
B2	1880	F/A	+	13	0.02	0.02				
	1889	F/A	−	−	0.02	**2.84**	**4.02**	**1.69**	**0.59**	0.02
B3	662	M/A	+	13	0.02	0.03				
	1888	F/A	−	−	0.02	**5.14**	**5.76**	**4.77**	**4.02**	0.02
B4	481	M/A	+	15	0.02	**4.77**				
	478	F/A	+	15	0.02	**8.04**				
	480	F/A	+	15	0.02	**8.04**				
B5	1863	F/A	+	13	0.02	**6.78**				

[a] F/A=female adult; M/A=male adult.

[b] Bats were challenged on 24 August 2005.

[c] Positive viral-neutralizing antibody (VNA) titers are listed in bold text.

from oral swabs (stored in MEM-10) collected from the bats showing PCR positives were unsuccessful. None of the four surviving infected bats showed evidence of viral shedding in their saliva throughout the duration of the study. Additionally, none of the noninfected bats showed evidence of viral shedding. Successful bat-to-bat transmission of RABV from infected bats to noninfected bats was not observed among cage mates.

Virus identification

The RABV used for the experiments joined clade EF3 (*E. fuscus* 3), which is formed by samples collected mostly from Big Brown Bats and, to a lesser extent, from *Myotis* sp. and striped skunks (*Mephitis mephitis*) (GenBank accession numbers: AY170404-16) in the western United States (primarily Arizona and Colorado) (Fig 1). Nucleotide sequence similarity between the Big Brown Bat RABV variant used for the experiment and other sequences belonging to the clade EF3 was 98.9%. Differences were based on four synonymous nucleotide transitions. All 377-nts fragments, from the brain tissue of experimentally infected bats, showed 100% identity with the infecting virus. According to present knowledge based on molecular typing and frequency in Big Brown Bats, this particular RABV variant is associated primarily with Big Brown Bats. Striped skunks (*M. mephitis*) and *Myotis* sp. likely represent spillover events (Shankar et al., 2005; Leslie et al., 2006).

DISCUSSION

Our study showed that Big Brown Bats are susceptible, with a relatively short incubation period, to IM infection with high doses of Big Brown Bat RABV variant. Incubation periods in RABV-infected insectivorous bats generally range from 14 to 175 days (Brass, 1994; but see Moore and Raymond, 1970) and do not appear to be influenced by the species of the infected individual or the RABV variant responsible for infection. In captive experiments studying susceptibility of *T. brasiliensis* to rabies virus collected from naturally infected *T. brasiliensis*, a low RABV dose led to longer incubation periods and higher viral titers in the brain and salivary glands, occasionally higher than the infective dose (Baer and Bales 1967). In our study, which used a high viral dose that resulted in 80% mortality, we observed short incubation periods (13–17 days) in the majority of infected bats relative to the general range of incubation periods observed in RABV-infected bats. If higher brain and salivary viral titers are associated with longer incubation periods, then the probability of RABV transmission to conspecifics should also increase when infected roost mates exhibit longer incubation periods. Infection of *E. fuscus* with a lower dose of Big Brown Bat RABV variant may lead to longer incubation periods, increased detection of RABV shedding in the saliva of bats, and a higher probability of observing transmission from infected bats to noninfected bats.

Presence of VNA against RABV can also significantly influence shedding of virus by means of viral clearance. In our experiment, 85% of infected animals developed a detectable RABV VNA response. No viral RNA was detected in saliva of most of these animals, except two bats, which had detectable viral RNA in the saliva ≤24 hr before the onset of clinical illness (day 13 PI, bat 662; day 15 PI, bat 481). Of those two animals, bat 481 developed a relatively

high level of VNA, but bat 662 did not seroconvert. Given these results, the association between RABV VNA titer immediately preceding or upon death and salivary RABV excretion is unclear.

RABV transmission from infected animals to noninfected cage mates was not detected. Bite transmission of RABV between roost mates resulting in productive clinical infection may be a comparatively rare event. Short incubation periods and acute presentation of infected bats in this study may have lessened the likelihood of successful bat-to-bat transmission. Recent studies have suggested that frequent contact to sublethal doses of RABV in wild colonies of Big Brown Bats may result in the establishment of immunity, protecting individuals from subsequent infections (O'Shea et al., 2003; Shankar et al., 2004). The clinical presentation of free-ranging vs. captive bats may differ. In addition, the dose, duration, and types of exposure may vary. Other experiments with Big Brown Bats have documented possible bat-to-bat viral transmission, as suggested by the development of a serologic RABV antibody response in two bats that were exposed to naturally infected conspecifics in captivity (Shankar et al., 2004). Clearly, additional studies that more closely mimic the ecologic conditions of RABV exposure and infection in wild bat colonies would improve our understanding of natural infection dynamics.

Our results suggest that the immune response of *E. fuscus* can prevent a productive RABV infection. Initial blood samples following experimental infection were taken 13 days PI, and therefore we cannot exclude the possibility that the RABV VNA response was a secondary response of memory cells primed by natural abortive infection that occurred prior to our capture of the animals in wild. The possibility of this scenario is exemplified by our observation of four bats that developed VNA titers, survived infection, but had no detectable VNA titer by

140 days PI. In our study, levels of rabies VNA in bats following viral exposure declined over time; similar results have been reported for other mammals. It is unclear whether abortive infections in bats lead to long-lasting immunity. Further experiments focused on challenge of previously infected survivors without detectable levels of rabies VNA should be conducted to evaluate protection conferred in bats by abortive infections.

Our study showed that *E. fuscus* individuals are susceptible to high doses of Big Brown Bat RABV variant. Most bats seroconverted, only two experimental bats shed virus in their saliva shortly before displaying clinical signs of rabies, and four animals survived a high viral dose and showed a decline in VNA levels below the threshold of detection by the end of the experiment. No bat-to-bat transmission of RABV from infected to uninfected cage mates was detected. Further pathogenesis studies with lower viral doses, different routes of inoculation, other RABV variants, and alterations of the experimental colony size are necessary to model the role of dose and route of infection in natural transmission of RABV among susceptible bats.

ACKNOWLEDGMENTS

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