MAJOR ARTICLE

Effects of Aerosolized Rabies Virus Exposure on Bats and Mice

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Between 1956 and 1977, 4 human cases of rabies virus infection were attributed to aerosolized rabies virus; however, little work has been done to address this topic since the late 1960s. Employing modern nebulization equipment coupled with serologic, cell culture, and molecular technology, we have continued the investigation into aerosolized rabies virus as a potential route of transmission. Laboratory mice and 2 species of bats were exposed, through aerosol, to 3 variants of rabies virus. All bats survived exposure to aerosolized rabies virus and produced rabies neutralizing antibody. Several mice died of rabies as a result of aerosol exposure. Antibody response was followed for 6 months before animals were given an intramuscular challenge of rabies virus. Poor protection from challenge was afforded in bats, despite the presence of neutralizing antibodies.

Rabies is a neurotropic disease that has the ability to affect all mammals. Humans are not the primary host of this viral disease, yet it is estimated that, worldwide, rabies virus kills in excess of 55,000 humans annually [1]. Dogs remain the most epidemiologically significant host of rabies virus, as they have been for the past 4000 years of recorded history [2]. In the United States, it was not until the 1950s that bats were recognized as competent vectors of rabies, and the presence of rabies in bat populations was observed to pose public health threats [3]. The natural biting behavior of hematophagous bats, the close associations with human and commensal bat species, and the recently described theory of cryptogenic rabies exemplify the concerns presented by bat rabies [4, 5].

Rabies virus is maintained in host populations and transmitted to other species, including humans, largely through bites by infected animals. Aerosol transmission has been postulated as an alternative route of exposure to rabies virus after a small number of both laboratory incidents/cases and apparently natural occurrences. Aerosol transmission has been implied in 4 reports of human rabies cases and documented in experimental work with animals [6–11]. In all instances when aerosol transmission was cited as the route of human infection, more plausible explanations may exist [4]. In the 2 cases involving spelunkers working in Frio Cave [10, 12], there were notations of direct contact between the spelunkers and the bats, although no documented bites were reported. In the 2 cases involving laboratory-acquired infections [7, 13], the scientists had other opportunities to be exposed to rabies virus in situations that did not include aerosol productions. Constantine [10] documented aerosol transmission of rabies virus to experimental animals in Frio Cave. This Texas cave, which is home to >10 million bats, was the location where 2 humans were believed to have contracted rabies via aerosol. Constantine was able to demonstrate transmission of rabies virus to animals housed in cages that excluded all but cave atmosphere. Rabies virus was isolated from samples collected via air condensation techniques that were used to monitor the atmosphere in this cave [14].

Additional work is warranted if we are to understand the role that aerosol exposures play in the epizootiology

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of rabies in mammals. The objectives of the present study were to investigate whether rabies virus, especially bat variants, can be transmitted to animals through aerosol exposures in the laboratory and to characterize the pathogenic and immunologic consequences of such exposures.

MATERIALS AND METHODS

Cell culture. Mouse C-1300 neuroblastoma (NA) cells were grown to confluence in T-25 flasks containing 7 mL of Eagle's growth medium (GM) consisting of minimum essential medium supplemented with 10% fetal bovine serum, 2.0 mmol/L glutamate, 100 IU of penicillin G, 50 μg of streptomycin, and 2.5 μg amphotericin B/mL. The numbers of cells containing rabies antigen were enumerated by immunofluorescence as described elsewhere [15].

Virus. Four rabies virus variants were used in this experiment: a variant isolated from *Tadarida brasiliensis mexicana* (Mexican free-tailed bat; *Tbv*), 2 variants isolated from *Eptesicus fuscus* (big brown bat; *Efv1* and *Efv2*), and a variant isolated from *Lasionycteris noctivagans* (silver-haired bat; *Lnv*). Virus variant identity was verified by the utilization of BLAST analysis (available at: http://www.ncbi.nlm.hih.gov/blast) based on the N gene sequence. The Colorado Department of Public Health and Environment provided carcasses of rabid *E. fuscus* and *L. noctivagans* bats, which were submitted for rabies diagnosis. Rabid *T. brasiliensis* were collected at a Texas field study site, where they were found grounded and moribund underneath a colony. Bat collection and handling followed protocols that were in compliance with established procedures of the Colorado State University Institutional Animal Care and Use Committee. Bats were dissected; brains and salivary glands were removed. Brain tissue from all bats was examined by the direct fluorescent antibody (DFA) procedure [16] to identify and verify rabid individuals. Homogenates (10% wt/vol) were made of salivary glands with a Ten-Broeck tissue grinder, using 1.0 mL of GM as diluent. To achieve consistent virus titers and adequate volumes for the aerosol and inoculation experiments, all virus isolates were passaged 3 times in NA cells. The resulting viral stocks contained infectivity titers for the *Efv1, Efv2,* and *Lnv* isolates of 1.0×10^5 $TCID_{50}$/mL and for the *Tbv* isolate of 1.0×10^6 $TCID_{50}$/mL. The virus employed in all intramuscular (im) challenges, *Efv2,* was obtained from a rabid *E. fuscus.* In a previously performed experiment, a challenge dose of 10^3 $TCID_{50}$/mL of this virus produced mortality levels of 67% (4/6) in *E. fuscus* and 20% (1/5) in mice (authors' unpublished data).

Viral genotyping. RNA extraction and reverse-transcription (RT) polymerase chain reaction (PCR) were performed on all inocula employed in this experiment, as well as on the tissue samples from killed experimental animals. For RNA isolation, 50 μL of the viral suspension were added to 1.0 mL of TRI-Reagent and processed using 1-bromo-3-chlorpropane as per the manufacturer's recommendations (Molecular Research Center). The resultant 50-μL volume containing isolated RNA was stored at −20°C for at least 1 h before the RT procedure. The RT procedure was performed with the SuperScript First-Strand Synthesis System according to manufacturer's instructions (Invitrogen), with minor modifications. Random hexamers from the SuperScript First-Strand Synthesis System were used in cDNA synthesis. The total volume of the RT reaction was 20 μL. The RT reaction conditions followed the manufacturer's recommendations. The cDNA was stored at −20° C until further processing.

Viral N gene cDNA was amplified using a Robocycler (Stratagene). The optimal reaction using the Turbo system (Stratagene) contained 5 μL of cDNA, 33 μL of water, 5 μL of 10× ($MgCl_2$) Turbo cloned plaque-forming units buffer, 1 μL of 50 mmol/L $MgCl_2$, 4 μL of dNTP, 1 μL of each primer (20 mmol/L), and 1 μL of 500 pfu Turbo DNA polymerase. To amplify a 320-bp PCR product, the optimal annealing temperature for the PCR primers 21 forward (5′-ATGTAACACCCCTACAATG-3′) and 304 reverse (5′-TTGACGAAGATCTTGCTCAT-3′) was 55°C. Two negative controls were run with each PCR, one substituting 5 μL of water for cDNA and a second substituting 5 μL of cDNA from the RT negative control. Positive controls were done with 5 μL of cDNA from CVS-11 rabies virus. PCR conditions were as follows: 1 cycle of 94°C for 1 min; 40 cycles of 94°C for 1 min, 55°C for 1 min, and 2 min at 72°C; followed by one 10-min cycle at 72°C. Amplicons were electrophoresed on a 1% SeaKem agarose gel (BMA Products). To verify the size of the amplicon, a 1-kb ladder was electrophoresed next to the negative control. The appropriate band was excised with a sterile razor blade and placed in a labeled sterile microcentrifuge tube. Electrophoresed cDNA was purified using the Qiagen Quick kit following manufacturer's recommendations (Qiagen). In separate microcentrifuge tubes, the purified PCR product and 8 μL of each primer (at 3 mmol/L) were sent to Davis Sequencing (University of California, Davis). Initial sequence analysis was done with CHROMAS software (version 1.45; School of Health and Science, Griffith University, Brisbane, Australia). Sequences were compared with other rabies sequences from the National Center for Biotechnology Information site (available at: http://www.ncbi.nlm.hih.gov/blast).

Serologic analysis. The rapid fluorescent focus inhibition test (RFFIT) procedure, as described by Smith [17] and Shanker [18], was employed to determine the concentration of virus neutralizing antibody (VNA) .

Animals. Before laboratory exposure to virus, wild-caught *E. fuscus* and *T. brasiliensis* bats were quarantined for 8 and 6 months, respectively. For identification of individual bats, a passive integrated transponder tag was inserted subdermally into each *E. fuscus,* and an identification number was tattooed

onto the wing membrane of each *T. brasiliensis.* All bats were VNA negative before laboratory exposure to rabies virus. Four- to eight-week-old ICR mice were obtained from Taconic Farms and were identified by ear holes. Mealworms were fed ad libitum to *E. fuscus, T. brasiliensis* were fed a mixture of blended mealworms and beef baby food, and the mice were fed laboratory rodent pellets. All animals had continuous access to clean water. Bats were caged in $76 \times 61 \times 61$ cm enclosures lined with fly screen. Each cage housed 4 bats; species were not comingled. Mice were caged in $76 \times 31 \times 31$ cm enclosures with 3 mice/cage. The temperature was maintained between 29.4°C and 31.1°C, and the humidity was maintained at ~40%.

Clinically ill bats were given subcutaneous lactated Ringers solution and were hand-fed beef baby food during initial stages of clinical rabies, before the disease was differentiable from other illnesses that are occasionally encountered and successfully treated in captive bats. If clinical signs did not improve within 12 h, the bat was killed by intraperitoneal injection of pentobarbital (80 mg/kg). Mice were killed at the first sign of clinical illness by use of the same technique as described for bats. All animals were necropsied after killing.

After aerosol exposure to the virus, *E. fuscus* and mice were bled monthly. *T. brasiliensis* were bled bimonthly, because of fragility in captivity. Bats were bled from the uropatagial vein, and mice were bled from the tail vein, both by means of needle puncture with collection of blood in hematocrit tubes. All animals were bled 2 weeks before aerosol exposure to verify no detectable VNA and 2 weeks after im challenge (6-month bleeding). After exposure to the aerosolized virus, the animals were visually checked 3 times/day, and weights were recorded twice per week. Killed animals were necropsied, and brain smears were tested by DFA for the presence of rabies virus antigen.

Aerosol exposure procedure. Aerosols containing rabies virus were produced using an Inhalation Exposure System (Glas-Col). The apparatus was located in a biosafety level 3 suite, and a strict safety protocol was implemented to assure operator safety. A wire-mesh basket containing 5 compartments was used to house the animals during the aerosolization process. Bats and mice were exposed to virus simultaneously but were kept in separate compartments. *E. fuscus* and *T. brasiliensis* (4 of each species) and mice (3) were exposed to 1 of 3 bat variants of rabies virus (*Efv1, Tbv,* and *Lnv*) in 3 separate aerosolization experiments (1 virus variant/experiment), in an attempt to determine the immunological and pathological effects of an exposure to homologous and heterologous variants of rabies virus. For all experiments, a total of 10^6 $TCID_{50}$ of virus in 10 mL of GM was introduced as an aerosol into the chamber, over an 80-min time period. The infection chamber had a volume of 0.1416 m^3. Humidity and temperature were recorded during aerosol exposure; humidity was 45%, and temperature was 27.8°C–28.9°C.

Six-month challenge. Six months after exposure to aerosolized rabies virus, all surviving animals were challenged by im inoculation into the right deltoid muscle with 1.0×10^3 $TCID_{50}$ of *Efv2* challenge virus. Mice were anesthetized for challenge with 100 μL of ketamine/xylazine, and bats were injected without sedation.

To ascertain whether the animals that developed clinical rabies after im challenge were infected with the aerosolized rabies variant or with the challenge variant, sequence analysis of the N gene on viruses isolated from brains was performed, as described above. The sequence of the virus isolated from the killed animal was compared with the sequence of the aerosolized virus to which the animal had been exposed and to that of the im challenge virus.

RESULTS

Response of bats to aerosolized rabies virus. All 24 bats (12 *E. fuscus* and 12 *T. brasiliensis*) survived the 6-month observation period after exposure to aerosolized rabies virus and developed anti-rabies VNA within 3 months of aerosol exposure, with titers ranging from 0.2 to 37 IU/mL (figure 1). The VNA levels in *T. brasiliensis* exposed to *Efv1* aerosol peaked 3 months after aerosol exposure, and, in *E. fuscus* exposed to *Efv1,* the VNA level peaked at 4–5 months after aerosol exposure. Peak VNA level in all bats exposed to *Tbv* aerosol occurred 2–3 months after aerosol exposure. Among the bats exposed to *Lnv* aerosol, *E. fuscus* bats developed peak VNA levels within 2–4 months, and *T. brasiliensis* bats developed peak levels 5 months after exposure.

Response of mice to aerosolized rabies virus. One mouse exposed to *Efv1* and 1 mouse exposed to *Lnv* developed signs of rabies 17 and 8 days after aerosol exposure, respectively. Two mice exposed to *Tbv* developed signs of rabies, at 10 and 14 days after aerosol exposure. All 4 mice were killed and found to be rabies positive by the DFA . The 5 surviving mice developed anti-rabies VNA (figure 2). At ~5 months after aerosol exposure, the remaining mouse exposed to *Tbv* died of unknown causes.

Response of bats to im challenge. Of the 24 bats given an im challenge, 10 developed rabies (table 1). Within 2–4 weeks after im challenge, the clinically rabid bats developed respiratory distress and ataxia. These animals were killed, and all tested positive for rabies virus antigen by DFA test. Sequence analysis of the virus detected in the brain indicated that disease was due to the challenge virus, rather than to the virus originally delivered by aerosol. The bats that survived the im challenge had developed VNAs ranging from undetectable to 28.7 IU/mL before im challenge, whereas the titers of bats that did not survive the im challenge ranged from undetectable to 14.7 IU/mL before im challenge. An amenestic response to rabies virus challenge was noted in 21 bats after im challenge; however, 9

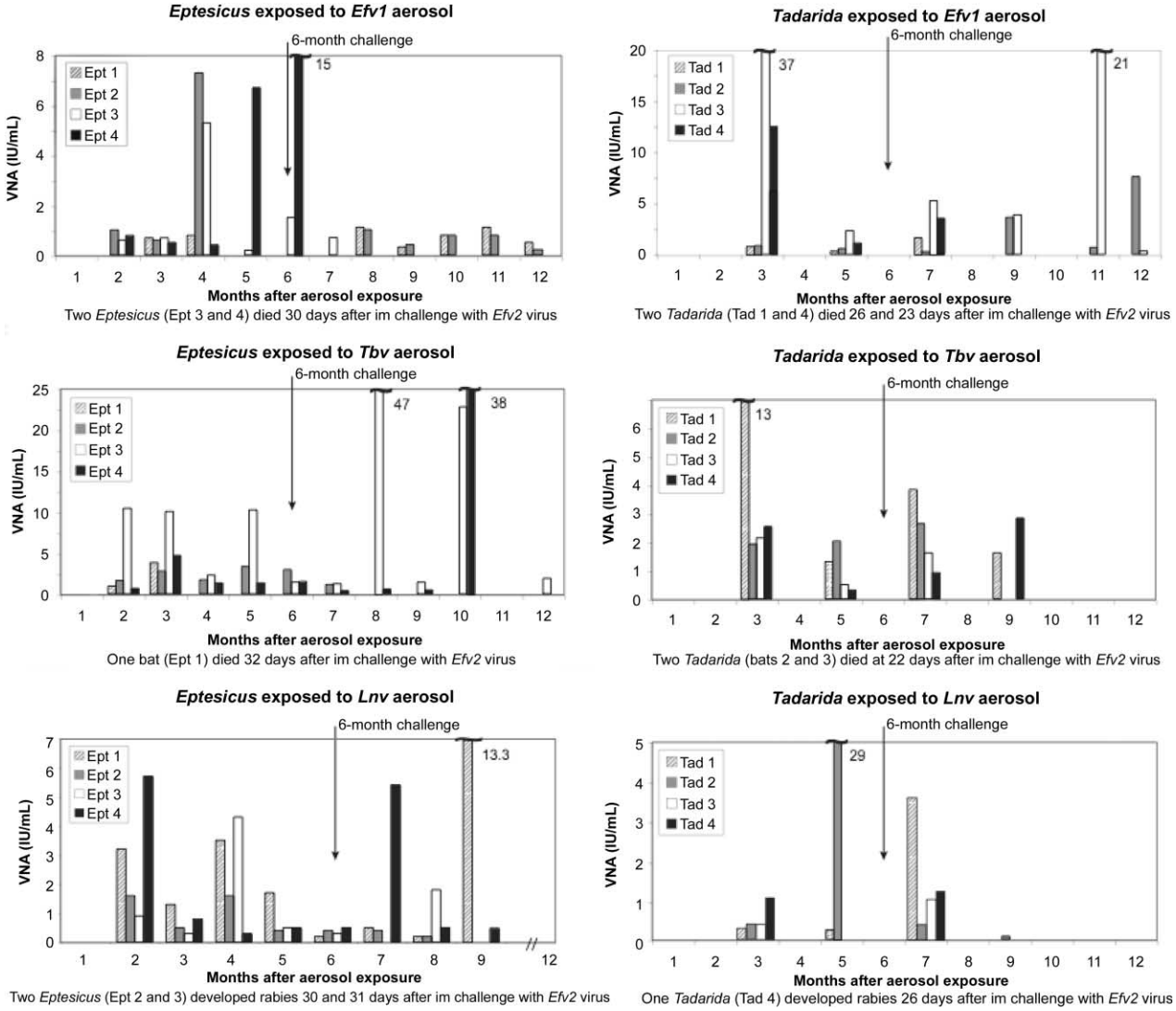

Figure 1. Anti-rabies neutralizing titers in 2 genera of bats (*Eptesicus* and *Tadarida*) after aerosol exposure to 1 of 3 variants of rabies virus and after an intramuscular (im) challenge 6 months after aerosol exposure. Bats were challenged im with 1.0×10^3 $TCID_{50}$ of *E. fuscus* variant 2 *(Efv2),* 6 months after aerosol exposure. The 6-month virus neutralizing antibody (VNA) results in *Eptesicus* represent serologic analysis from bleeding 2 weeks after im challenge. *Efv1, E. fuscus* variant 1; *Lnv, Lasionycteris noctivagans* variant; *Tbv, T. brasiliensis* variant.

of these bats subsequently developed rabies. The bats that survived the im challenge had a mean titer of 3.3 IU/mL in the serologic test before im challenge, whereas the bats that did not survive the im challenge had a mean titer of 1.5 IU/mL before the im challenge; the difference was significant ($P >$ .001, Student's t test). Although the Student's t test results indicate a significant difference among several of the groups, the results are not reported because they are considered unreliable because of the small sample size.

Response of mice to im challenge. With 1 exception, the 5 mice that survived for 6 months after aerosol exposure had detectable VNA levels before im challenge. Two weeks after the im challenge, the titer of the 1 mouse initially lacking antibody had increased to 1.1 IU/mL. On day 16 after im challenge, a mouse that had been exposed to *Lnv* aerosol appeared dehydrated and slightly ataxic. Before challenge, the titer of this mouse was 0.8 IU/mL. The mouse was killed, and rabies antigen was detected in the brain by the DFA. This was the only mouse to develop signs of rabies after im challenge. Sequence analysis of the infecting rabies strain indicated that it was consistent with the im challenge virus.

DISCUSSION

In the present study, 24 bats (12 *E. fuscus* and 12 *T. brasiliensis*) and 9 mice were exposed to an aerosol of 1 of 3 strains of bat variant rabies virus. Survivorship and antibody levels were tracked after animals were initially exposed to the aerosolized

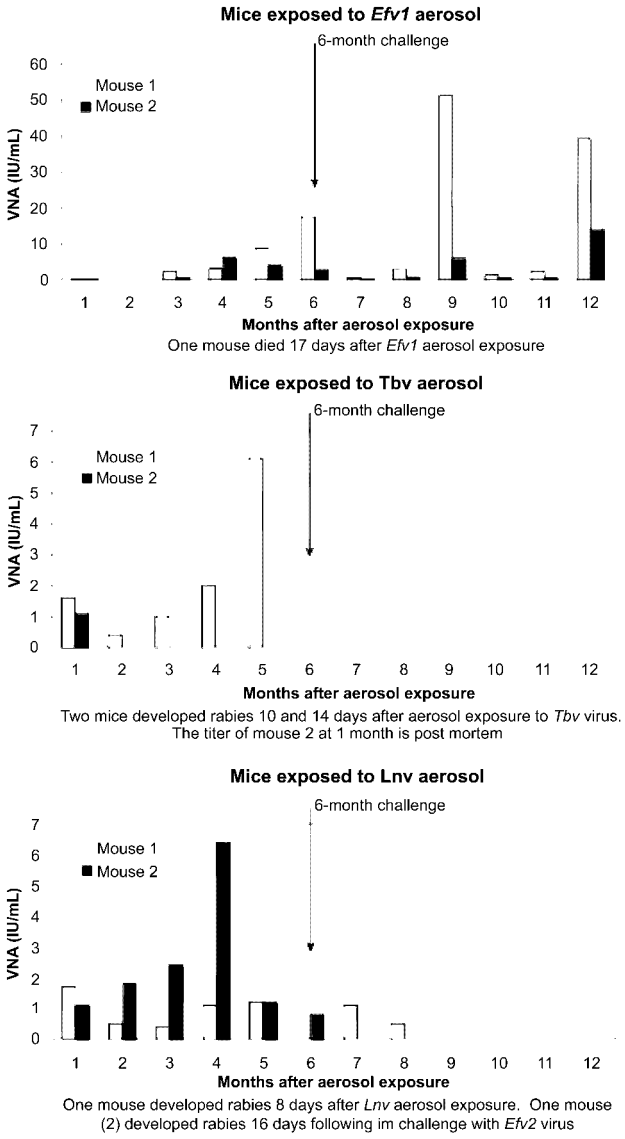

Figure 2. Anti-rabies neutralizing titers in mice after aerosol exposure to 1 of 3 variants of rabies virus and after an intramuscular (im) challenge 6 months after aerosol exposure. Mice were challenged im with 1.0×10^3 $TCID_{50}$ of *Eptesicus fuscus* variant 2 *(Efv2),* 6 months after aerosol exposure. The 6-month virus neutralizing antibody (VNA) results represent serologic analysis from bleeding 2 weeks after im challenge. *Efv1, E. fuscus* variant 1; *Lnv, Lasionycteris noctivagans* variant; *Tbv, Tadarida brasiliensis* variant.

rabies virus and then after an im challenge 6 months later. All bats were seronegative for rabies neutralizing antibody before the experiments, but developed demonstrable neutralizing antibody after aerosol exposure and survived for 6 months, until the im challenge. Four of the 9 mice died of rabies as a result of the aerosol exposure, and the remaining 5 mice developed demonstrable neutralizing antibody. One mouse died of rabies after the 6-month im challenge.

The bats used in this study were wild-caught, had possibly been previously exposed, and thus possibly were sensitized to rabies virus. Such priming could account for the immune response to aerosolized rabies virus that was seen in all bats and for the failure of the aerosol exposure to produce clinical rabies virus infection in any bat. Previous experiments employing aerosolized rabies virus have examined the lethality and the viscerotropic nature of aerosol exposure in laboratory settings [8, 9, 19, 20]. Under field conditions, Constantine demonstrated that certain species of animals can develop rabies when exposed to aerosolized rabies virus in a cave environment [10, 11]. Susceptibility ranking among animals exposed to rabies [21] can only be verified by inoculation experiments [22]. The present study, utilizing aerosol inoculation under laboratory conditions, suggests that bats are less susceptible than laboratory mice to 3 variants of aerosolized bat virus.

The presence of serum rabies neutralizing antibody alone does not define protection to clinical rabies virus infection [23–25]. Antibody levels of 1:20 to 1:39, as measured by the RFFIT (IU data were not provided), protected 98 of 98 dogs and 67 of 68 cats when they were challenged im with a 100% lethal dose of rabies virus [26]. In the present study, however, the presence of aerosol-induced anti-rabies VNAs was not associated with protection from an im challenge.

In the present study, the isotype of the VNA induced in bats and mice by aerosol exposure to rabies virus was not determined. The immune response resulting from the presentation

Table 1. Results of an intramuscular (im) challenge in bats previously exposed to aerosolized rabies virus.

Virus variant, genus of bat (incubation time, days)	Virus neutralizing antibody titer, IU/mL	
	Before evidence of clinical signs	At time of killing
Efv1		
Tadarida (23)	1.7	15
Tadarida (26)	3.5	4.9
Eptesicus (30)	0.7	ND
Eptesicus (30)	17	ND
Tbv		
Tadarida (22)	3.8	1.6
Tadarida (22)	2.6	0.5
Eptesicus (32)	1.1	1.1
Lnv		
Tadarida (26)	1.3	0.2
Eptesicus (30)	0.4	0.2
Eptesicus (31)	15	1.8

NOTE. Twenty-four bats (12 *T. brasiliensis* and 12 *E. fuscus*) were challenged im with 1.0×10^3 $TCID_{50}$ of rabies virus *E. fuscus* variant 2 *(Efv2)* 6 months after aerosol exposure to 1 of 3 variants of rabies virus (4 bats/species/virus variant). At necropsy, the *Efv2* variant was isolated from the brains of all symptomatic bats. *Efv1, E. fuscus* variant 1; *Lnv, Lasionycteris noctivagans* variant; ND, not done; *Tbv, Tadarida brasiliensis* variant.

of antigens to the mucosal immune system is principally of the isotype S-IgA [27]. S-IgA, produced after antigens were presented to mucosal inductive sites of the upper respiratory tract, would, by mucosal homing, be directed to mucosal effector sites [28]. Serum levels of IgA do not reflect the mucosal level of IgA, nor are serum IgA and mucosal IgA produced in the same compartments [29]. Monoclonal IgA has previously failed to protect when administered im in mice 24 h before im challenge with rabies virus; the same IgA monoclonal was protective against rabies virus in vitro [30]. Nasal immunization has been shown to protect against certain infections when the infectious agent's normal portal of infection is respiratory [27]. In the majority of cases, rabies virus has a transcutaneous portal of entry. This brings into question the protective effect of aerosol-induced IgA antibody in resistance to a parenteral challenge of rabies virus. Perhaps, in the present study, an aerosol challenge with rabies virus would have resulted in a different outcome.

The prevalence of neutralizing anti-rabies antibody in bat populations has been reported, but the source of the immune-system stimulation that elicits this antibody has not been identified [31–35]. Colonial bat species live in very dense clusters, suggesting a scenario for repeated aerosol exposures to rabies. Constant vocalization and frequent echolocation in densely packed colonies are potential sources of aerosol from the oral and nasal mucosa of bats that are infected with rabies virus. Clinically ill rabid bats have been observed to emit incessant, abnormal vocalization (B. Schmidt-French, R. Rudd, and C. Trimarchi, unpublished data). Published reports [36, 37] and unpublished data (A. Davis) demonstrate the presence of infected tissue in the nasal and oral mucosa of colonial bat species. The present study offers evidence that aerosolized rabies virus can induce the production of serum neutralizing antibody in exposed bats and mice. However, what role, if any, is played by aerosolized rabies virus in naturally arising rabies immunity is not understood. The lack of morbidity and mortality in bats after exposure to aerosolized rabies virus suggests that an aerosol route of exposure does not play a major role in bat-to-bat transmission of rabies, although it could be responsible for the naturally occurring antibody found in bat populations.

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