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PREVALENCE OF RABIES SPECIFIC ANTIBODIES IN THE MEXICAN FREE-TAILED BAT (*TADARIDA BRASILIENSIS MEXICANA*) AT LAVA CAVE, NEW MEXICO

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ABSTRACT: Adult female and juvenile Mexican free-tailed bats (*Tadarida brasiliensis mexicana*) were collected bimonthly at Lava Cave, New Mexico from May through September. The purpose of this study was to examine the prevalence of active rabies infection as well as to determine individual immune status in these hosts. All bats were bled and examined for rabies antibody (total antibody versus IgM) utilizing a modified serum neutralization test. The brains were removed and examined by the fluorescent rabies antibody (FRA) test. No significant difference was observed in the number of adults with rabies neutralizing antibody (total) over the study period. Significant differences in rabies neutralizing antibody (total) were observed among the juveniles sampled during July and August. The number of adults with IgM specific antibody was low (15 of 750, 2%) and did not fluctuate significantly. However, the number of juveniles with IgM antibody did show increased levels in August and September. The number of adults positive by the FRA was low (4 of 750, <1%) and did not appear to fluctuate significantly over the study period. The number of juveniles positive by the FRA was three and one-half times higher than observed for the adults (14 of 600, 2%). These results indicate that the Mexican free-tailed bat appears to be exposed to rabies virus shortly after birth as evident by its immune status. The low prevalence (4 of 750, <1%) of active infection as determined by the FRA and mouse inoculation and the high prevalence (514 of 750, 69%) of IgG antibody in the adult females indicate that the Mexican free-tailed bat recovers from rabies virus infection.

Key words: Rabies virus, Mexican free-tailed bat, *Tadarida brasiliensis mexicana*, rabies microneutralization, prevalence, recovery, natural infections.

INTRODUCTION

The first documented evidence in humans of a viral paralytic disease caused by bats occurred in 1908 in Santa Catarina in southern Brazil although the etiology in domestic livestock was not diagnosed as rabies until 1911 and the bat was not proven to be the vector until 1921 (Carini, 1911; Haupt and Rehaag, 1921). For many years interest in bat rabies was confined to South America, Central America and Mexico where frugivorous and vampire bats implicated in the transmission of rabies are native.

Bat rabies was first suspected in the United States in 1951 when a 43-yr-old woman in Texas was bitten by a bat and subsequently died of rabies (Sulkin and Greve, 1954). Unfortunately, the bat was not recovered for testing or for determination of species. Bat rabies was first confirmed in the United States in 1953, when a rabid yellow bat (*Lasiurus intermedius floridans*) initiated an unprovoked attack on a 7-yr-old boy in Florida (Venters et al., 1954). As a result of these incidences, interest in bat rabies has increased. Since the early 1960's the percentage of rabies positive bats reported by State Health Laboratories has been consistent, ranging from 4 to 20% (Constantine, 1967a; Trimarchi and Debbie, 1977). However, the total number of bats tested and reported as rabid has increased, from eight in 1953 to 829 in 1985 (Centers for Disease Control, 1986). Rabies virus has been found in every species of bat in North America that has been adequately sampled. Rabies has been confirmed in 30 of 39 species of bats in the United States (Constantine, 1979).

Numerous field studies examining brain impressions from asymptomatic Mexican free-tailed bats (*Tadarida brasiliensis mexicana*) in the United States have in-

dicated a low prevalence (<1%) of infection (Burns et al., 1956a, b; Constantine et al., 1968b; Baer, 1975). In field studies of symptomatic Mexican free-tailed bats, infection rates varied from 25 to 50% (Constantine et al., 1968b; Baer, 1975). In serological studies involving populations of Mexican free-tailed bats, antibody levels ranging from 16 to 80% have been reported (Burns and Farinacci, 1955; Burns et al., 1956a; Constantine et al., 1968b; Baer, 1975). It is not known whether these clinically normal bats which have antibody are incubating the virus and may eventually succumb to its effect or have recovered from past infection and are possibly immune. The purpose of this report is to examine the prevalence of active infection as well as to determine immune status in one colony of Mexican free-tailed bats in the southwestern United States.

MATERIALS AND METHODS

Study site

The study site was Lava Cave, Engle, New Mexico (33°31'N, 106°52'W). The cave is a segment of intact lava tube approximately 70 m long, 15 to 18 m wide and 9 m high at its highest points. A constriction divides the cave roughly into two equal chambers, north and south (Constantine, 1967a). The maternity area is located at the back of the north chamber near the constriction. Adult bats in the north chamber were almost exclusively females. The population of the south chamber usually contained males and appeared to be a transient population which varied greatly in size. The study was conducted over a 5 mo period from 10 May 1986 through 13 September 1986.

Collection of specimens

All collecting and subsequent work reported here was performed by personnel with laboratory confirmed rabies antibody titers. All laboratory procedures involving potentially infectious material were conducted in a biological safety cabinet (Baker Biogard, Class II; Baker Co., Inc., Sanford, Maine 04073, USA).

One hundred adult female and one hundred juvenile bats were collected bimonthly from 14 June 1986 through 13 September 1986. In addition, pregnant adult female bats were collected 10 May 1986 (n = 50) and 4 June 1986 (n = 100). Adult and juvenile bats were collected from the cavern roof in the maternity area using a fine mesh, long handled bat net and placed in small (30 cm^3) metal cages. All cages were placed into styrofoam ice chests immediately upon removal from the cave. The cages were cooled with refreezable ice packs for transportation to the laboratory. When bats arrived at the laboratory the cages were placed in perforated, humidified plastic bags and placed at 2 to 8 C until they were examined.

Specimen processing

After being removed from the refrigerator and allowed to "warm" in a 35 C incubator for 30 min, bats were lightly etherized and bled to extinction by collecting pooled heart blood. All blood was placed in Microtainer capillary blood serum separator tubes (Becton-Dickinson Vacutainer systems, Rutherford, New Jersey 07070, USA) and allowed to clot at room temperature for a minimum of 1 hr. The Microtainers were centrifuged in a Beckman J-6B (Beckman Instruments, Inc., Palo Alto, California 94304, USA) at 3,000 RPM for 20 min. After centrifugation, the serum was transferred to Flex-tubes (Eppendorf-Brinkmann Instruments, Westbury, New York 11590, USA) for storage at −70 C. The brains of all bats bled were removed for immunofluorescence testing and mouse inoculation.

Immunofluorescence

Bat brains were removed and multiple impression smears were prepared. The slides were fixed in cold acetone at −20 C for a minimum of 1 hr, allowed to air dry at room temperature, and stained by the fluorescent rabies antibody (FRA) test (Johnson, 1969) using fluorescein isothiocyanate conjugated Anti-Rabies Monoclonal Globulin (Centocor, Malvern, Pennsylvania 19355, USA) and examined with a Zeiss epi-fluorescent microscope (VWR Scientific, Denver, Colorado 80217, USA) at a magnification of 400×. The light source was a HBO 50 watt mercury bulb (Curtin Matheson, Dallas, Texas 75207, USA) with standard fluorescence filters.

Mouse inoculation

Bat brains were pooled (n = 10) for mouse inoculation unless they were determined to be positive by the FRA test. If a brain was positive for rabies virus that brain was processed separately. Brain suspensions were prepared as described previously (Velleca and Forrester, 1981). Suspensions of brain tissue (0.03 ml) were inoculated intracerebrally into four, 4- to 6-wk-old Swiss Webster albino laboratory mice (Charles River, Inc., Wilmington, Massachusetts 01887, USA) that were observed for 30 days. For mice dying after 3 days postinoculation the

brains were examined for rabies by direct immunofluorescence.

Virus

The ERA vaccine strain of rabies virus (obtained through the courtesy of J. Smith, Centers for Disease Control, Lawrenceville, Georgia 30245, USA) was propagated in BHK-21 cells.

Cell culture

Mouse Neuroblastoma Cells (MNA) (obtained through the courtesy of J. Smith, Centers for Disease Control, Lawrenceville, Georgia 30245, USA) were used for the microneutralization test. For cell subcultures and virus production, 75 cm^2 Corning tissue culture flasks (VWR Scientific, Denver, Colorado 80217, USA) were seeded with 20 ml of cell suspension containing approximately 100,000 cells/ml in Eagle's Minimum Essential Medium (Flow Laboratories, Inc., McLean, Virginia 22102, USA) supplemented with 10% fetal bovine serum, 2× vitamins, 2× amino acids and antibiotics (gentamicin 50 μg/ml and fungizone 2.5 μg/ml). The cells formed confluent monolayers in 2 to 3 days at 35 C and 95% air–5% CO_2. Confluent monolayers were subdivided twice weekly using 0.25% trypsin (Flow Laboratories, Inc., McLean, Virginia 22102, USA) in Ca^{++} and Mg^{++} free Hanks' Balanced Salt Solution (M. A. Bioproducts, Biggs Ford Road, Walkersville, Maryland 21793, USA).

Virus production

The growth medium was removed from a confluent monolayer of MNA cells. The monolayer was washed twice with 10 ml of Hanks' Balanced Salt Solution (HBSS) and 2 ml of the ERA suspension was added. After 1 hr at 35 C, 18 ml of Virus Medium (0.1% lactalbumin hydrolysate instead of FBS) was added. The cells were incubated 3 to 4 days at 35 C and 95% air–5% CO_2. The virus was harvested by freeze/thawing once to disrupt the cells and release the virus, then centrifuged for 15 min at 2,000 RPM. The supernatant was aliquoted in 2 ml quantities and stored at −70 C. The optimal dilution for serum neutralization was determined by duplicate microtiter titrations. The dilution with a potency of 100 tissue culture infectious doses (100 $TCID_{50}$) per 0.025 ml was used for the microneutralization test.

Microneutralization

Test sera, positive and negative human serum controls (Biological Products Division, Centers for Disease Control, Atlanta, Georgia 30333, USA) were removed from the −70 C freezer and thawed in cold water. A 1:10 dilution was made by mixing 20 μl of serum with 180 μl of Dulbecco's Phosphate Buffered Saline (DPBS) and antibiotics. All sera were then heat inactivated at 56 C for 35 min. Briefly, equal volumes (25 μl) of serial dilutions (1:10 through 1:80) of serum and rabies virus (100 $TCID_{50}$) were incubated in Corning microtiter plates (VWR Scientific, Denver, Colorado 80217, USA) for 1 hr at 35 C and 95% air–5% CO_2. A cell suspension of 5×10^5 cell/ml in growth medium (100 μl) containing 10 μg/ml of DEAE-Dextran (Pharmacia LKB Biotechnology, Inc., Piscataway, New Jersey 08854, USA) was then added and the microtiter plate placed at 35 C, 95% air–5% CO_2 for 48 hr. After 48 hr the medium was removed and the cells were fixed in acetone (80%) and assayed for neutralization of virus by the FRA. A reduction of ≥50% was considered as the endpoint. All sera positive (≥10) for rabies antibodies were then absorbed with 0.5 ml of a packed 10% suspension of Omnisorb cells (Calbiochem Brand Biochemicals, San Diego, California 92112, USA) for 30 min at room temperature, then centrifuged at 3,000 RPM for 30 min. The supernatant was removed and 50 μl was set aside for serum neutralization. The remaining 100 μl was treated with 25 μl of 0.1 M 2-mercaptoethanol (Eastman Kodak Company, Rochester, New York 14650, USA) in HBSS for 30 min at 35 C. The two treatments were then assayed as previously described.

RESULTS

Rabies neutralizing antibodies

There were no significant differences (Chi-square analysis, $\chi^2 = 3.98$, df = 7, $P = 0.78$) in detectable rabies neutralizing antibody (total) observed among the adult female bats (Fig. 1). However, significant differences ($\chi^2 = 40.90$, df = 7, $P < 0.001$; $\chi^2 = 33.85$, df = 7, $P < 0.001$; and $\chi^2 = 27.47$, df = 7, $P < 0.001$, respectively) were observed in detectable rabies neutralizing antibody among the juveniles (Fig. 1) in the three samples collected from 19 July through 30 August 1986.

IgM neutralizing antibodies

The number of adult female bats with IgM class antibodies was low (15 of 750, 2%) and did not appear to fluctuate significantly during the study period (Fig. 2). The number of juvenile bats with IgM class antibodies was also low (57 of 600, 10%)

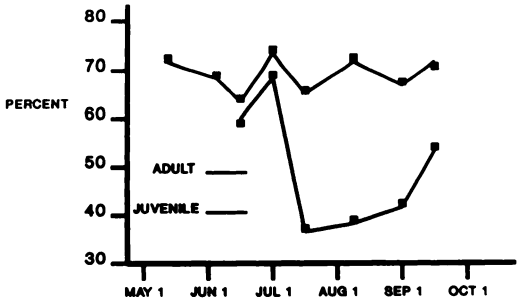

FIGURE 1. Percent (%) of Mexican free-tailed bats (*Tadarida brasiliensis mexicana*) with rabies neutralizing antibody.

but fluctuated more than the adults, with distinct increases in numbers observed during the 9 and 30 August and 13 September collection dates (Fig. 2).

Immunofluorescence and mouse inoculation

The number of adult female bats positive for rabies was low (4 of 750, <1%) and did not appear to fluctuate significantly over the study period (Fig. 3). The total number of juvenile bats rabies positive by FRA was three and one-half times higher than those observed for the adult females, but was also low (14 of 600, 2%). Rabies positive juvenile bats were observed only from the middle to the end of the study period (14 July to 13 September).

In this study we pooled brains (n = 10) of FRA negative bats and performed mouse inoculation. There were no discrepancies between the FRA and mouse inoculation.

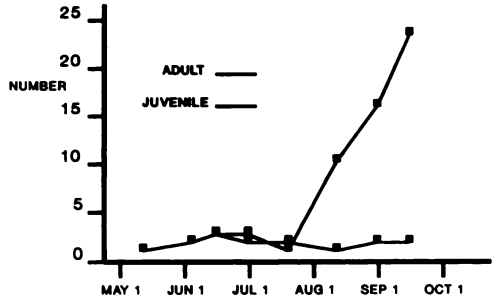

FIGURE 2. Number of Mexican free-tailed bats (*Tadarida brasiliensis mexicana*) with IgM class rabies neutralizing antibody.

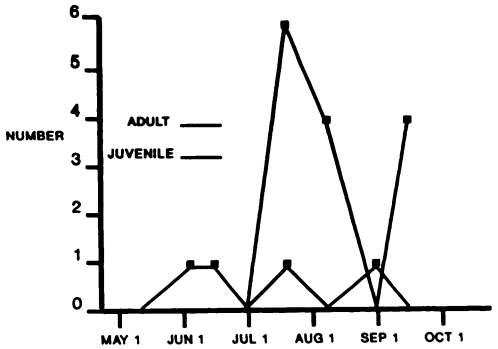

FIGURE 3. Number of Mexican free-tailed bats (*Tadarida brasiliensis mexicana*) positive by the fluorescent rabies antibody test.

DISCUSSION

A population density of at least 300 bats per square foot has been reported for Lava Cave (Constantine, 1967a). Considering this population density, possible rabies infection may occur early in life by direct contact (bite), aerosol transmission, ingestion of virus-infected milk from a virus-infected mother, and transplacental infection (Sims et al., 1963; Constantine, 1966a, b, 1967b; Constantine et al., 1968a; Steece and Calisher, 1989). Bats frequently survive experimental exposure to rabies virus; some never have clinical evidence of disease and others survive after recovering from clinical rabies (Baer and Bales, 1967; Constantine, 1967a). In a natural setting bats appear to survive exposure to rabies virus since a high proportion have serum neutralizing antibodies and the virus is not present in their tissue (Constantine, 1967a). However, it is not known whether these clinically normal bats which have antibody are incubating the virus and may eventually succumb to its effect or have recovered from past infection and are possibly immune.

In a typical primary mammalian immune response IgM class antibodies are first produced when stimulated by an antigen followed by the production of IgG class antibodies (Roitt et al., 1985). Antibodies of IgM class are generally short-

lived (<6 mo) and their presence indicates current or recent infection. The absence of IgM and the presence of IgG indicates recovery from an infection and probable immunity.

In this study the high prevalence of IgG class antibody (514 of 750, 69%) and low prevalence of IgM class antibody (15 of 750, 2%) along with a low prevalence of positive rabies virus by the FRA (4 of 750, <1%) and mouse inoculation in the adult female bats at Lava Cave suggest that these bats indeed survive exposure to rabies virus.

Serum IgM and IgG have been separated by different methods: ultracentrifugation (Vesikari and Vaheri, 1968), Staphylococcal Protein A (SPA) binding of IgG (Ankerst et al., 1974), ion exchange (Gupta et al., 1971; Sugg et al., 1979), and more recently by Streptococcal Protein G binding of IgG (Kawano et al., 1986). It was discovered that certain *Staphylococcus aureus* strains express type I FC receptors (Protein A) on their cell surface or as a secreted protein (Forsgren and Sjoquist, 1966). A number of studies have examined the binding capabilities of this protein receptor with regard to immunoglobulins of different classes and sub-classes from many mammalian species (Miele and Krakowka, 1981). Staphylococcal Protein A has strong affinity for immunoglobulins (IgG) from humans, rabbits and guinea pigs, but weak affinity for immunoglobulins from sheep, rats and goats. Recently the discovery of a similar protein (Protein G) from *Streptococcus* sp. strains has been shown to react with IgG from a wide variety of mammalian species with a much higher efficiency than Protein A (Bjorck and Kronvall, 1984). The use of Omnisorb Cells (Streptococcal Protein G) provided excellent adsorption of immunoglobulin from bat sera when analyzed by serum electrophoresis.

The use of 2-mercaptoethanol (2-ME) in serologic assays to indicate that the residual antibodies tested were indeed IgM has been widely used (Handsher and Fogel, 1977; Kawano et al., 1986).

The presence of rabies antibody in the adult bats did not vary significantly from month to month (63 to 73%, $\bar{x} = 69\%$; $\chi^2 = 3.98$, df = 7, $P < 0.78$) which is generally consistent with other published reports demonstrating antibody levels of 16 to 80% (Burns and Farinacci, 1955; Burns et al., 1956a; Constantine et al., 1968b; Baer, 1975). The presence of rabies antibody in the juvenile bats did vary significantly ($\chi^2 = 40.90$, df = 7, $P < 0.001$; $\chi^2 = 33.85$, df = 7, $P < 0.001$; $\chi^2 = 27.47$, df = 7, $P < 0.001$ for the three samples, respectively) over the study period (36 to 68%, $\bar{x} = 49\%$). The percentage of juvenile bats with rabies antibodies at the beginning of the study period approximates the percentage found in the adult female population and may indicate prenatal transfer of antibody. This has also been observed previously (Constantine, 1967b). The decrease in the percentage of juvenile bats with rabies antibodies early in the study period and subsequent rise as the summer progresses may indicate that the young bats are exposed to rabies virus at a very young age. The levels of IgM class rabies antibodies of juvenile bats in August and September are different from those of the adult bat population, providing additional evidence that the juvenile bats are exposed early in life.

The presence of IgM class antibody in suckling bats in June may provide some evidence for the support of prenatal infection. In a typical mammalian immune response IgM class antibodies do not pass the placenta; therefore, the presence of IgM in very young bats may indicate prenatally acquired infections as well as infections acquired shortly after birth (Roitt et al., 1985).

The FRA test has been reported to correctly identify 98% of rabies infected brains. However, mouse inoculation has been shown to be slightly more sensitive than the FRA. The FRA combined with

mouse inoculation reportedly approaches 100% sensitivity (Kissling, 1975). In our study, we observed no discrepancies between the FRA and mouse inoculation. The prevalence of active infection in the adult female bats by the FRA and mouse inoculation was <1%. The prevalence of active infection in juvenile bats was 2% or three and one-half times the number for adult females. The combined prevalence of active infection for all bats examined was 1%. This does not vary significantly from the numerous field studies examining brain impressions from asymptomatic Mexican free-tailed bats in the United States which have generally indicated a low prevalence (<1%) of active infection (Burns et al., 1956a, b; Constantine et al., 1968b; Baer, 1975). Rabies positive adult females were observed throughout the study period. Rabies positive juvenile bats were observed only from the middle of the study period (14 July) through the end (13 September). The increased presence of juvenile bats rabies virus positive in July to September may result from the disappearance of maternal antibody and the loss of "protection" resulting in a large number of susceptible (non-immune) members in the population.

In an ideal host (bat) versus parasite (rabies virus) relationship it is advantageous for the parasite not to cause death in its host. Isolates from Mexican free-tailed bats examined by monoclonal antibody analysis suggest that rabies virus has evolved uniquely in this population (Smith et al., 1986). Additionally, it has been suggested that airborne transmission plays a major role in the dissemination and maintenance of virus in this population (Baer and Bales, 1967; Constantine, 1967b; Constantine et al., 1972). In this study we have observed a low prevalence of active infection as determined by the FRA and mouse inoculation with a high prevalence of IgG antibody in the adult Mexican free-tailed bats. This high level of "immunological recovery" in the absence of observable clinical symptoms suggests that rabies infections in this population may be primarily subclinical (asymptomatic).

In summary, in the Mexican free-tailed population it appears that the earliest exposure to rabies virus occurs shortly after birth and, in some cases, prenatally as indicated by immune status. The low prevalence of active infection as determined by the FRA and mouse inoculation and the high prevalence of IgG antibody in the adult females indicate that the Mexican free-tailed bat recovers from rabies virus infections. It is now important to determine the prevalence of clinical (symptomatic) versus subclinical (asymptomatic) infections and the role this may play in the dissemination and maintenance of rabies virus in the Mexican free-tailed bat.

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