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Source: Journal of Wildlife Diseases, 25(3) : 329-334

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-25.3.329

Journal of Wildlife Diseases, 25(3), 1989, pp. 329–334

EVIDENCE FOR PRENATAL TRANSFER OF RABIES VIRUS IN THE MEXICAN FREE-TAILED BAT (*TADARIDA BRASILIENSIS MEXICANA*)

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ABSTRACT: Fetuses were collected from four Mexican free-tailed bats (*Tadarida brasiliensis mexicana*) and a fetal bat cell (FBC) line was established and tested for its ability to support the replication of the ERA vaccine strain of rabies virus. Cytopathic effects were detected in ERA virus-inoculated as well as uninoculated FBC's. Immunoflorescent antibody testing of uninoculated FBC's provided no evidence for the presence of rabies virus. However, mice inoculated intracranially with supernatant fluid from uninoculated FBC's died. Enzyme-linked immunosorbent assay and immunofluorescent antibody testing revealed rabies virus in the brains of these mice. Tests with a panel of monoclonal antibodies indicated that the isolate was the same as that isolated from Mexican free-tailed bats from the southwestern United States. We conclude that the fetuses from which the FBC line was derived had been infected in utero with rabies virus. We believe this may represent the first observation of prenatal transfer of rabies virus in naturally infected bats.

Key words: Rabies virus, Mexican free-tailed bat, *Tadarida brasiliensis mexicana*, in utero, prenatal infection, epidemiology, experimental study.

INTRODUCTION

Bat rabies was first detected in the United States in 1951 when a 43-yr-old person who was bitten by a bat subsequently died of rabies (Sulkin and Greve, 1954). Unfortunately, the bat was not available for laboratory confirmation of rabies virus infection or for determination of species. Bat rabies was first confirmed in the United States in 1953 when the virus was isolated from a yellow bat (*Lasiurus intermedius floridans*) that had attacked a 7-yr-old boy in Florida (Venters et al., 1954). Since then bat rabies has been reported from the 48 contiguous states (Centers for Disease Control, 1985). Detection of rabid bats in the United States has increased steadily, from eight in 1953 to 829 in 1985 (Centers for Disease Control, 1986). Of 39 species of bats considered residents of the United States, 30 have been reported infected with rabies virus (Constantine, 1979).

The mechanisms by which rabies virus infections are maintained in bat populations remain poorly understood. Possible routes of transmission of the virus from bat to bat include 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, 1967a; Constantine et al., 1968a). Airborne transmission may be of major importance in the dissemination of rabies virus in the colonial Mexican free-tailed bat (*Tadarida brasiliensis mexicana*) of the southwestern United States, one of the most well-studied bat species. This paper reports observations and laboratory studies indicating that prenatal infection with rabies virus also may occur naturally in the Mexican free-tailed bat.

MATERIALS AND METHODS

Cell culture preparation and vaccine strain of rabies virus

All work reported here was completed by rabies virus-immune field and laboratory personnel. Mexican free-tailed bats were collected at Lava Cave (Engle, New Mexico, USA; 33°10′N, 107°02′W) for a study of the prevalence of antibody to rabies virus in that population. Bats were collected by randomly netting clinically normal bats from the cave maternity area. Four bats not used in the seroprevalence study were used to establish a fetal bat cell (FBC) line. First

trimester (ca. 1.5 cm) fetuses of four Mexican free-tailed bats were aseptically removed and placed in a sterile Petri dish (100 × 15 mm); adult female bat carcasses were discarded without further testing. The four pooled fetuses were rinsed once in sterile calcium- and magnesium-free Hanks balanced salt solution (HBSS), placed in a sterile 10-ml syringe, and then were forced through the opening into a 250-ml Erlenmeyer flask containing 125 ml of 0.02% trypsin. The flask was stored at 37 C and shaken intermittently for 4 hr. The cell suspension was then filtered through sterile cheesecloth and was clarified by low speed centrifugation (1,000 rpm for 10 min) to pack the cells. The pellet was resuspended in Eagle's Minimum Essential Medium (Flow Laboratories, Inc., McLean, Virginia 22102, USA) supplemented with 2X vitamins, 2X L-glutamine and 10% fetal bovine serum; cells were counted in a hemocytometer. Plastic cell culture flasks (25 cm^2) were seeded with approximately 1×10^6 cells and placed in a 37 C incubator with 95% air–5% CO_2. After 24 hr the medium was decanted and the cells fed fresh growth medium. Monolayers were confluent about 3 days later. Fetal bat cells were subdivided (1:3 ratio) twice weekly. The ERA vaccine strain of rabies virus (obtained through the courtesy of J. Smith, Centers for Disease Control, Lawrenceville, Georgia 30245, USA) had been propagated in BHK-21 cells.

Detection of virus in chronically infected FBC's

After the seventh passage the medium was removed from three flasks containing confluent monolayers of FBC's. Cell monolayers were washed with HBSS, and cells in two of the flasks were inoculated with the ERA strain of rabies virus. After adsorption for 1 hr at 37 C, all flasks were refed serum-free Eagle's Minimum Essential Medium and were reincubated at 37 C. The cells were examined by light microscopy 48 hr later.

Four 4- to 6-wk-old Swiss Webster albino laboratory mice (Charles River, Inc., Wilmington, Massachusetts 01887, USA) were inoculated intracranially with 0.03 ml of supernatant fluid from uninoculated FBC cultures in which 50 to 75% of the cells were detached, refractile, rounded or otherwise showing signs of cytopathic effects (CPE). The mice were observed daily for 30 days and were discarded at that time if asymptomatic. Brains of mice found dead or with signs of illness were removed surgically, homogenized in sterile HBSS using a mortar and pestle, and frozen at −70 C; a biological safety cabinet (Baker Biogard, Class II; Baker Co., Inc., Sanford, Maine 04073, USA) was used for all manipulations of cell cultures and tissues. The resulting approximately 10% suspension was clarified by centrifuging it at low speed, and the supernatant fluid was inoculated intracranially into four other weaned mice. If these mice died their brains were removed aseptically, and a 10% mouse brain suspension was prepared, clarified and stored at −70 C for additional studies (FBC-M). Vero (African green monkey, *Cercopithicus aethiops*) cells (American Type Culture Collection, Rockville, Maryland 20852, USA) also were inoculated with supernatant fluid from FBC's with CPE. The Vero cells were observed daily for signs of CPE and were frozen at −70 C when CPE were observed (FBC-V).

Immunofluorescent antibody testing

For examination by direct immunofluorescence, monolayer cultures of FBC's were scraped into the medium using a sterile cotton swab. The suspended cells were placed in a 15-ml conical centrifuge tube and were clarified at low speed. All but 1 ml of the supernatant fluid was removed, the tubes were shaken and the cells were resuspended in the remaining supernatant fluid. About 20 μl of the suspension was dropped on each of four spots on Teflon-coated microscope slides (Cell-Line Associates, New Field, New Jersey 08344, USA) used for fluorescent microscopy, the suspension was allowed to air dry at room temperature, and the slides were fixed in cold acetone at −20 C for a minimum of 1 hr. Brains of mice that died or showed signs of illness were removed and impression slides were prepared from them (Velleca and Forrester, 1981), allowed to air dry at room temperature, and fixed in cold acetone at −20 C for a minimum of 1 hr. Cells were stained by the fluorescent rabies antibody (FRA) test (Johnson, 1969) using fluorescein isothiocyanate conjugated Anti-Rabies Monoclonal Globulin (Centocor, Malvern, Pennsylvania 19355, USA) and were examined with a Zeiss epi-fluorescent microscope (VWR Scientific, Denver, Colorado 80217, USA) at a magnification of ×400. The light source was an HBO 50-watt mercury bulb (Curtin Matheson, Dallas, Texas 75207, USA) with standard fluorescence filters. Rabies virus typing was done by indirect immunofluorescence at the Centers for Disease Control (Lawrenceville, Georgia 30245, USA) by methods described previously using monoclonal antibodies to rabies virus (Smith et al., 1986).

Enzyme-linked immunosorbent assays (ELISA's)

Suckling 2- to 4-day-old mice were inoculated intracranially with FBC-M or FBC-V and were examined daily for signs of illness. Brains from

mice dead or dying after such inoculations were used, as 10% clarified suspensions, to infect Vero cells grown to monolayers in sterile Immulon 2 (Dynatech Laboratories, Inc., Alexandria, Virginia 22021, USA) 96-well flat-bottomed polystyrene plates. One day before CPE were expected, as determined by previous studies, supernatant fluid was decanted from the wells. This was treated as infectious material and was disposed of appropriately. To each of the 96 wells 20 μl of 3% formalin in phosphate-buffered saline (PBS), pH 7.2, was added, and the plates were covered and stored overnight at ambient temperature (ca. 22 C). Formalin was then decanted from the plates, flushed with PBS containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Missouri 63178, USA) and washed 15 times with PBS containing 0.5% Tween 20, using a mechanical plate-washing device (Titertek Microplate washer; Flow Laboratories, Inc., McLean, Virginia 22102, USA). Phosphate-buffered saline containing heat-inactivated fetal bovine serum (200 μl) was added to each well and the plates stored at 4 C overnight. The wells were again washed 15 times. To each well 50 μl of 1:400 dilutions of various antibody preparations was added. These antibodies were produced in mice hyperimmunized with individual viruses or with multiple related or unrelated viruses (Tikasingh et al., 1966); antibodies to rabies virus, to more than 250 arboviruses, and to certain other viruses were used. Plates were again incubated (1 hr at 37 C), wells were washed 10 times, and 50 μl of goat anti-mouse IgG conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories, Inc., Avondale, Pennsylvania 19311, USA) was added to each well. After further incubation for 1 hr at 37 C and washing, 75 μl of substrate (ABTS; Kirkegaard and Perry Laboratories, Gaithersburg, Maryland 20879, USA) was added to each well, plates were stored at ambient temperature for 3 to 5 min, and the optical density (OD) of each well was measured at 409 nm in an automatic device (Titertek Multiskan, Flow Laboratories, McLean, Virginia 22102, USA) and recorded by the same instrument.Controls included uninfected Vero cells and wells with no cells. The test was considered positive when the ratio of the OD of infected Vero cells to the OD of uninfected Vero cells was >2.0.

RESULTS

Forty-eight hr after inoculation 50 to 75% of all FBC's, infected or uninfected with the ERA vaccine strain of rabies virus, showed CPE. Although repeatedly harvested separately and stained with rabies virus-specific fluorescein-conjugated antibody, no fluorescence was observed in the uninfected cells and little (<five infected cells/field) specific fluorescence was observed in the ERA vaccine virus-infected cells. Twenty days after inoculation with supernatant fluid from FBC's that had spontaneously undergone degenerative changes, one weaned mouse became ill. All four mice inoculated with a subpassage of the virus from the brain of this mouse showed signs of illness 8 days after inoculation. The sick mice were not initially examined for rabies by the FRA test because of the negative results obtained with uninoculated FBC's. However, when brain impressions were examined by FRA they were positive. Of 16 suckling mice inoculated with a second weaned mouse passage of FBC-M, 10 were dead and six moribund on the third day after inoculation. Vero cells infected with FBC-M or FBC-V showed CPE on the sixth day after infection.

The only positive ELISA result was that obtained with a polyvalent ascitic fluid prepared in mice immunized with rabies, lymphocytic choriomeningitis, vaccinia, herpes type 1 and Newcastle disease viruses. However, confirmation of the identification of the isolate as a strain of rabies virus was accomplished by FRA tests on infected mouse brain (courtesy of J. Smith, Centers for Disease Control). Its reactivity with a panel of monoclonal antibodies prepared with rabies virus was identical to that of rabies virus isolates from Mexican free-tailed bats from the southwestern United States (Table 1).

DISCUSSION

Although rabies in bats was first confirmed in 1953 in the United States, it is probably not a recent phenomenon (Steece et al., 1982); the number of infected bats has increased since 1953, but the percentage of rabies-positive bats has not changed significantly (Constantine, 1967a). In-

TABLE 1. Results of immunofluorescent antibody tests with three isolates using monoclonal antibodies against the ERA vaccine strain of rabies virus.

	Reaction by immunofluorescence with hybridoma[b]															
Isolate[a]	143	146	97-3	97-1	3	62	52-1	71	22	52-2	141	24-10	61	8	24-1	41
FBC-M	−[c]	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
TBM	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
ERA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] FBC-M, isolate from fetal *Tadarida brasiliensis mexicana*; TBM, prototype rabies virus from *Tadarida brasiliensis mexicana*; ERA, vaccine strain of rabies virus.
[b] For details regarding these antibody preparations see Smith et al. (1986).
[c] −, no immunofluorescence; +, fluorescence of cells infected with isolate FBC-M, TBM or ERA vaccine strain.

creasing numbers of rabies-positive bats may reflect increased public awareness of this potential public health problem and concomitantly increased surveillance for the virus.

The epidemiology of rabies in bats from North America is poorly understood. The Mexican free-tailed bat, a colonial species, appears to have two main pathways of migration in the southwest; bats from the western range (Arizona, New Mexico, and Colorado) winter primarily in western Mexico, whereas those from the eastern range (Texas, Oklahoma, and Kansas) appear to winter in eastern Mexico. Bats at or near the centers of these regions migrate either east or west (Villa and Cockrum, 1962; Glass, 1982). Such migration routes place Mexican free-tailed bats in sympatric situations with vampire bats (*Desmodus rotundus*), providing a possible source of exposure of Mexican free-tailed bats to rabies virus (Constantine et al., 1968b; Hill and Smith, 1984). It has been suggested that the reservoir of rabies virus infection in insectivorous bats may be infected vampire bats in Mexico or Central America (Martin, 1959; Moreno and Baer, 1980) and that infection of these insectivores may take place during such interspecies interactions. However, the distinctness of the isolates from Mexican free-tailed bats examined thus far by monoclonal antibody analysis suggests that if the initial exposure was from vampire bats, the virus has evolved uniquely in the Mexican free-tailed bat population.

There are several possible explanations of the mechanisms by which rabies virus is maintained in bat populations and the significance of bats as reservoirs for rabies virus in terrestrial mammals. It has been shown that rabies virus can be aerosol-transmitted from bats to other mammals and also from bats to other bats, particularly in colonial species, such as the Mexican free-tailed bat (Baer and Bales, 1967; Constantine, 1967b; Constantine et al., 1972). However, although transplacental transfer of rabies virus has been demonstrated in bats under laboratory conditions (Sims et al., 1963), it has been suggested that prenatal infection of bats with rabies virus does not occur under natural conditions. This was based on an examination of 22 pregnant wild-caught bats, 15 of which were Mexican free-tailed bats, and their fetuses (Constantine, 1986). We demonstrated that prenatal rabies virus infection does occur in Mexican free-tailed bats, but we used an even smaller number of fetuses. Further studies involving more specimens are needed to determine the prevalence and incidence of prenatal transfer of rabies virus in nature. Detection of prenatal rabies virus infection in Mexican free-tailed bats suggests that this route also may be a source of rabies virus infection in other bat species.

When the FBC's were tested by FRA, no typical fluorescence was seen in the uninoculated cells and only weak fluorescence was detected in cells inoculated with the ERA vaccine strain of rabies virus.

However, when the FBC-M virus was amplified by inoculation into mice, the mice died and their brain cells were positive by FRA, with characteristic staining patterns. It appears that the FBC line may have only limited susceptibility to rabies virus and limited capacity to support the replication of this virus. It is possible also that preexisting infection of the cells with bat rabies virus interfered with superinfection by the ERA strain. Alternatively, because none of the ERA-uninfected cells but some of the ERA-infected cells contained typically fluorescing rabies virus inclusions, the bat rabies virus strain may have established a persistent infection in these cells, with only a few fetal cells expressing little detectable antigen. It has been shown that brown fat cells of bats chronically infected with rabies virus can be passed many times (Sulkin and Allen, 1974). Fetal bat cells also have been shown to sustain persistent infection with rabies virus (Sims, 1969). It is unlikely that our isolate is a laboratory contaminant because the only rabies virus strain with which we worked during this period was the ERA vaccine strain, which is readily identifiable by monoclonal antibody typing.

In summary, prenatal infections with rabies virus occur in Mexican free-tailed bats and may conceivably occur in solitary long-ranging migratory species such as the hoary bat (*Lasiurus cinereus*), which could provide a mechanism for both maintenance of rabies virus in resident bats and spread of virus to northern colonial species. It is now important to determine both the prevalence of prenatal infections in bat populations and the significance of this mode of vertical transfer in maintaining rabies virus in bats.

ACKNOWLEDGMENTS

We thank Jean S. Smith, Centers for Disease Control, Lawrenceville, Georgia, for testing these viruses with a battery of monoclonal antibodies to rabies virus. Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

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Received for publication 9 June 1988.