Latent Infection of Rio Bravo Virus in Salivary Glands of Bats

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RIO BRAVO virus, also called bat salivary gland virus, has been isolated from submaxillary salivary glands of Mexican free-tailed bats, *Tadarida brasiliensis mexicana*, in California (*1*, *2*) and Texas (*3–5*, *6*), and identified with the group B arthropod-borne viruses (*1*, *3*, *5*, *7–9*). Although the role of the virus in nature is unknown, accidental infections of laboratory personnel are suspected to have occurred, and experimental infections in monkeys have been performed (*10*). Diagnoses of the cases in man were based on retrospective serologic evidence, association of the patient with free-tailed bats, and similarities in morbidity. Clinical disease was manifested by systemic or central nervous system illness with complicating orchitis or oophoritis. Infected monkeys developed an elevated temperature, drowsiness, transitory weakness of extremities, and nervousness.

The information reported here was developed incidental to research in bat rabies. Rio Bravo virus appeared as a lethal agent for mice used in inoculation tests conducted to isolate rabies virus from tissues of bats, and differentiation of the two viruses was necessary to verify rabies isolations. The result has been an accumulation of data relating to Rio Bravo virus infection rates in bats, persistence of the virus in captive bats, and a lack of obviously related host morbidity.

Materials and Methods

Rio Bravo virus was isolated after mice were inoculated in an attempt to isolate rabies virus. When serum-virus neutralization tests failed to identify isolates as rabies, the isolates were identified as Rio Bravo virus by challenge of immunized mice. In two instances (bats X–11 and X–74) the virus was identified by Dr. Robert E. Kissling, of the Communicable Disease Center, Public Health Service, in serum-virus neutralization tests with Rio Bravo virus-immune hamster serum, supplied by Dr. Harald N. Johnson of the Rockefeller Foundation.

Mouse inoculation tests and serum-virus neutralization tests were done by conventional methods (*11*, *12*). Albino Swiss mice, about 3 weeks old, were used. Trituration was done with mortar, pestle, and Alundum. In 1957 the diluent used was 10 percent horse serum in phosphate-buffered saline with a pH at 7.2 to 7.5; in 1958 the horse serum was replaced with 10 percent egg yolk; and in 1960, 0.75 percent solution of bovine albumin (Armour fraction V) was substituted for the egg yolk. Two milligrams of dihydrostreptomycin sulfate and 1,000 units of crystalline penicillin were added per milliliter of tissue suspension. Bat tissues comprised 10 percent by weight of suspension except salivary glands, which were 5 percent.

Rio Bravo virus was indentified by immunizing mice with suspect material, holding them a month, and then challenging the identification with Rio Bravo virus. In each test to identify Rio Bravo virus, six 21-day-old mice were immunized by injecting 0.03 ml. of a 10^{-1} or 10^{-2} dilution of the unknown virus into muscles

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of the thigh. Challenge was accomplished by intracerebral inoculation of at least 3 logs of Rio Bravo virus. Johnson provided the challenge virus and recommended the procedure.

Saliva specimens were collected from bats by swabbing the oral cavity with a small cotton swab, rinsed in ½ ml. of diluent. The diluent was tested by the mouse inoculation procedure. The glands examined were the large, superficial, submaxillary salivary glands.

A total of 1,175 bats, 1,075 of the Mexican free-tailed species and 100 cave myotis bats, *Myotis velifer*, were collected over a 5-year period (September 1957 to September 1962) during an investigation of rabies virus in bats. They were taken at several locations in the southwestern United States. Cave capture sites were Carlsbad Caverns, Eddy County, N. Mex., Frio Cave, Uvalde County, Tex., and Lava Cave, Socorro County, N. Mex. Other bats were taken at a roost between the beams of a railroad bridge in Eddy County, N. Mex., while foraging in Doña Ana County, N. Mex., and in a tunnel in Kendall County, Tex.

Results

Infection rates. Table 1 summarizes data on infection rates. Except for one suckling bat, which was found to have Rio Bravo virus in its lungs, all 200 sucklings taken in July were negative for this virus. In contrast, the virus was found in salivary glands of as many as 3.4 percent of the adult bats tested at that time. In July, when the bat roosts included reasonably sedentary bat aggregations, primarily lactating females and young bats incapable of flying, no Rio Bravo virus was isolated from 100 adults occupying the well-ventilated spaces between the beams of a railroad bridge. However, the

Table 1. Data on Rio Bravo virus infection rates for specimens collected from asymptomatic bats

Date and place collected	Rio Bravo virus isolations from salivary glands				Percent infected	Tissues negative for Rio Bravo virus
	Adult males	Adult females	Immature males	Immature females		
Mexican free-tailed bat (*Tadarida brasiliensis mexicana*):						
June 1962, bridge roost	1/50	----------	----------	----------	2.0	49 salivary glands. 50 brains.
July 1959, bridge roost	----------	0/100	0/50	----------	0	150 brains. 150 salivary glands. 100 mammary glands.
July 1961, Frio Cave	2/50	4/125	0/50	0/50	2.2	275 brains. 269 salivary glands. 100 mammary glands. 275 lungs. 275 kidneys. 25 fetuses.
July 1961, Lava Cave	----------	2/100	[1] 1/25	0/25	2.0	150 brains. 150 salivary glands. 100 mammary glands. 149 lungs. 150 kidneys.
September 1957, Carlsbad Cavern.	1/50	1/50	0/50	0/50	1.0	200 brains. 198 salivary glands.
September 1960, bridge roost.	5/50	2/50	1/50	1/50	4.5	200 brains. 191 salivary glands.
October 1961, Frio Cave	[2] 2/32	[2] 1/18	----------	----------	6.0	50 brains. 47 salivary glands.
Cave myotis bat (*Myotis velifer*):						
July 1961, Frio Cave	0/25	0/25	0/25	0/25	0	100 brains. 100 salivary glands. 100 kidneys. 100 lungs.

[1] Lungs only. [2] Age unknown.

Table 2. Rio Bravo virus isolations from 5 asymptomatic captive Mexican free-tailed bats

Collection and identification data	Day after collection saliva tested [1]	Fate of bat	Virus in tissue at autopsy			
			Brain	Salivary gland	Lung	Kidney
Bridge roost:						
XS–20, June 19, 1958	191	Killed 219th day	Negative	Rio Bravo		
CB–66, immature male, Sept. 21, 1960.	[2] 6, 59, 556	Killed 681st day	do	Negative	Negative	Negative.
Foraging, Doña Ana County:						
CB–2, Adult male, May 27, 1959.	[3] 0, [2] 15, 55, 61–71, 97, 370, 373, 374	Inoculated with rabies, died 404th day.	Rabies	do	do	Do.
CB–10, adult male, Sept. 21, 1959.	[2] 1, [3] 282, 556	Killed 682d day	Negative	Rio Bravo	do	Do.
CB–15, adult male, Sept. 21, 1959.	24, 191	Killed 317th day	do	do	do	Do.

[1] No virus in saliva unless otherwise noted.
[2] Saliva killed mice; Rio Bravo virus identified by challenge.
[3] Saliva killed mice.

virus was isolated from 2 percent (2/100) of adults collected in July in somewhat ill-ventilated Lava Cave, which contained about 1 million clustered bats, and from 3.4 percent (6/175) of adults sampled in the same month at extremely ill-ventilated Frio Cave, which contained about 2 million clustered bats.

In the spring and fall, all roosts generally contain great proportions of migrants, and it would be far less meaningful to relate infection to a specific roost at these times. Thus in September the bridge roost contained adults with an infection rate of 7 percent (7/100) and immature bats with an infection rate of 2 percent (2/100). A 2 percent (1/50) infection rate was found in adult males taken at the same roost in June. All isolations were from Mexican free-tailed bats. One hundred cave myotis bats, *M. velifer*, were negative for this virus.

Isolations from captive bats. Saliva specimens collected from captive Mexican free-tailed bats produced some information regarding occurrence of detectable virus in individual bats throughout extensive time periods (table 2). Bat XS–20, whose saliva specimen failed to kill mice 28 days before the bat was killed, had Rio Bravo virus in its salivary glands at death. Bat CB–2 had positive saliva the day it was captured and 15 and 55 days later, but virus was not recovered from saliva specimens taken between 61 and 374 days after capture. This bat died of experimental rabies on the 404th day, and its salivary glands were negative for both viruses. Bat CB–10 had Rio Bravo virus in its saliva the day after capture and 282 days after capture, but the virus was not recovered from saliva collected on day 556; however, Rio Bravo virus was identified in the salivary glands of this bat when it was killed 682 days following capture. Bat CB–15 had negative saliva 24 and 191 days after it was captured, but the virus was isolated from its salivary glands when it was killed 317 days after capture. Bat CB–66 had Rio Bravo virus in its saliva 6 days after being captured, but saliva samples were negative 59 and 556 days after capture. This bat's salivary glands were negative when the bat was killed on the 681st day after it was captured.

Rio Bravo virus infection and morbidity in bats. Table 3 lists field and laboratory data on 33 Mexican free-tailed bats infected naturally with Rio Bravo virus. The first 26 bats listed were asymptomatic; the last 7 were moribund or dead. Current investigations indicate that causes other than rabies account for occasional deaths during seasonal die-offs in this species of bat, but Rio Bravo virus has not been implicated as a cause. Rio Bravo virus has been isolated from groups of dead or moribund bats with no greater frequency than from asymptomatic bats of the same populations. Moreover, behavior of mice, inoculated by intracere-

Table 3. Test results of mice inoculated with salivary gland tissue positive for Rio Bravo virus from 33 naturally infected Mexican free-tailed bats

Bat identification and collection data	Original mouse test			Second mouse passage			Brain tissue used in challenge with immunized mice	Negative tissue
	Number died/ inoculated	Day of mouse death	Mouse symptom[1] and days duration	Number died/ inoculated	Day of mouse death	Mouse symptom[1] and days duration		
Asymptomatic bats								
Carlsbad Cavern, September 1957:								
X–11, adult female.[2]	2/4	7 10	------	------	------	------	1st passage mice.[3]	Brain.
X–74, adult male.[4]	4/4	8 10 10 10	0 Pa,1 Pa, 1 S, 1	------	------	------	---do[3]---	Do.
Bridge roost, September 1960:								
OV–528, adult male.	3/3	8 8 9	Pr, 1 S, 1 S, 1; Pr, 1	------	------	------	---do---	Do.
OV–532, adult male.	3/3	9 10 10	S, 1; Pr, 1 S, 1; Pr, 1 S, 1; Pr, 1	------	------	------	---do---	Do.
OV–605, adult male.	2/3	8 10	0 S, 2	2/2	7 8	S, 1 S, Pr, 2	2d passage mice.	Do.
OV–598, adult male.	3/3	8 9 10	S, 1 0 S, 1; Pr, 1	------	------	------	1st passage mice.	Do.
OV–590, adult male.	3/3	8 8 8	S, 1 S, 1 S, 1	------	------	------	---do---	Do.
OV–579, adult female.	2/3	8 10	S, 1 S, 2	2/2	8 8 7	S, 1; Pr, 1 S, 1; Pr, 1 S, 1	2d passage mice.	Do.
OV–682, adult female.	1/3	12	S, 1	3/3	7 7	S, 1 0	---do---	Do.
OV–696, immature male.	3/3	8 8 9	S, 1 S, 1 S, 1; Pr, 1	------	------	------	1st passage mice.	Do.
OV–551, immature female.	3/3	9 9 9	S, 1 S, 1 S, 1	------	------	------	---do---	Do.
Frio Cave, July 1961:								
IV–1551, adult male.	1/3	10	Pr, 1	3/3	6 6 6	0 0 Pr, 1	2d passage mice.	Brain, kidney, lung.
IV–1585, adult male.	1/3	9	Pr, 1	2/2	6 6	0 0	---do---	Do.
IV–1515, adult female.	1/3	9	Pr, 1	3/3	6 6 6	S, 1 S, Pr, <2 Pr, 1	---do---	Brain, kidney, lung, fetus.
IV–1642, adult female.	2/3	8 9	S, 1 Pr, 1	------	------	------	1st passage mice.	Brain, kidney, lung, mammary gland.
IV–1665, adult female.	1/3	11	S, 2	3/3	7 7 7	Pr, 1 Pr, 1 Pr, 1	2d passage mice.	Do.
IV–1673, adult female.	3/3	9 9 9	S, 1; Pr, 1 S, 1; Pr, 1 Pr, 2	------	------	------	1st passage mice.	Do.
Lava Cave, July 1961:								
IV–488, immature male.[5]	1/2	11	0	2/2	6 6	0 0	2d passage mice.	Brain, salivary gland, kidney.
IV–467, adult female.	3/4	9 9 9	S, 2 S, 1 S, 1	2/2	6 6	S, 1 S, 1; Pr, 1	1st passage mice.	Brain, lung, kidney, mammary gland.
IV–469, adult female.	2/4	9 19	0 Pa, 10	2/3	8 10	0 S, 2	2d passage mice.	Do.

Table 3. Test results of mice inoculated with salivary gland tissue positive for Rio Bravo virus from 33 naturally infected Mexican free-tailed bats—Continued

Bat identification and collection data	Original mouse test			Second mouse passage			Brain tissue used in challenge with immunized mice	Negative tissue
	Number died/ inoculated	Day of mouse death	Mouse symptom[1] and days duration	Number died/ inoculated	Day of mouse death	Mouse symptom[1] and days duration		
Asymptomatic bats								
Frio Cave, October 1961:								
2V–236, male.	1/3	10	Pr, 2	4/4	6 6 7 7	0 0 Pr, 1 Pr, 1	1st passage mice.	Brain.
2V–1039, male.	1/4	9	S, 1	4/4	6 7 7 8	S, 1 S, 1; Pr, 1 S, 1; Pr, 1 S, 1; Pr, 2	--do---	Do.
2V–1202, female.	1/4	10	0	3/3	8 8 9	S, 1; Pr, 1 S, 1; Pr, 1 S, 1; Pr, 2	2d passage mice.	Do.
Bridge roost, June 1962:								
2V–319, adult male.	3/4	8 9 11	0 Pr, 1 S, 1; Pr, 2	------	------	------	1st passage mice.	Do.
Doña Ana County, September 1959–August 1961:[6]								
2V–136, adult male.	1/4	8	0	4/4	6 6 6 6	0 0 0 0	--do---	Brain, lung, kidney.
Doña Ana County, October 1959–August 1961:[6]								
2V–125, adult male.	1/4	8	0	4/4	7 7 8 8	S, 1 S, 1 S, 1; Pr, 1 S, 1; Pr, 1	--do---	Do.
Moribund or dead bats								
Doña Ana County, August 1961:								
IV–1009, adult male.	1/4	11	0	4/4	6 6 6 6	0 Pr, <1 Pr, <1 Pr, <1	2d passage mice.	Brain.
Lava Cave, September 1961:								
IV–1150, adult male.	3/4	8 10 11	S, 1 S, 1 S, 3	------	------	------	1st passage mice.	Do.
Frio Cave, October–December 1961:[6]								
2V–225, male.	1/4	9	0	4/4	7 7 7 8	S, 1 S, 1 S, 1 S, 1; Pr, 1	--do---	Do.
Tunnel, Kendall County, July 1962:								
2V–1085, adult male.	1/4	9	0	2/3	8 8	Pr, 1 S, 1	2d passage mice.	Do.
2V–1086, adult male.	1/4	8	Pr, 1	4/4	6 6 6 6	Pr, 1 Pr, 1 Pr, 1 Pr, 1	3d passage mice.	Do.

Table 3 continued on next page.

See footnotes at end of table.

Table 3. Test results of mice inoculated with salivary gland tissue positive for Rio Bravo virus from 33 naturally infected Mexican free-tailed bats—Continued

Bat identification and collection data	Original mouse test			Second mouse passage			Brain tissue used in challenge with immunized mice	Negative tissue
	Number died/ inoculated	Day of mouse death	Mouse symptom[1] and days duration	Number died/ inoculated	Day of mouse death	Mouse symptom[1] and days duration		
Moribund or dead bats								
Carlsbad Cavern, August 1962:								
2V–788, adult male.	3/4	10	Pr, 1	-------	------	----------	1st passage mice.	Brain.
		10	Pr, 1					
		13	S, 1; Pr, 3					
Doña Ana County, September 1962:								
2V–886, male.	4/4	8	Pr, 1	-------	------	----------	---do---------	Do.
		8	S, 1					
		9	S, 1; Pr, 1					
		10	S, 2; Pr, 1					

[1] S=early signs of malaise, Pa=paralysis, Pr=prostration, 0=no syndrome seen before death.
[2] Montgomery laboratory No. 851.
[3] Serum-virus neutralization test used.
[4] Montgomery laboratory No. 852.
[5] Infected in lungs only.
[6] Collected during this time period, exact date unknown.

bral route with the virus in routine mouse inoculation tests is similar whether the virus originates in asymptomatic bats or in bats found dead or moribund.

The clinical syndrome in mice is characterized by a "humping" of the spine and lessened activity, followed or concurrent with a roughness in the texture of the hair coat. This condition degenerates to apparent dehydration and progressive paralysis until the animal is completely prostrate. The syndrome develops rapidly, as indicated in table 3. It will be noted that the number of days from inoculation until death is shortened markedly in second-passage mice.

Discussion

Association of higher Rio Bravo virus infection rates in Mexican free-tailed bats living in congested, ill-ventilated, bat-cave roosts during a period when the bat aggregations are sedentary, that is, returning to the same roost, seems consistent with the suggestion by Sulkin (*13*) that this virus may be transmitted by air. Similar conditions are associated with transmission of rabies by air (*14*). Negative results of efforts to isolate this virus must be observed with reservations, as low mouse mortality ratios occurred in many cases where positive results developed. Thus, it seems probable that many isolates were missed, a development which may have been avoided had we been in a position to use suckling mice as regularly as Johnson used them (*1*).

On the basis of results at hand (table 2), it would appear that free-tailed bats may carry this virus for periods approaching 2 years, while other bats may rid their salivary glands of the virus. Rio Bravo virus was conspicuously absent from brains, mammary glands, kidneys, lungs, fetuses, and suckling bats except for an isolation from lungs only of a suckling bat (IV–488, table 3). By fall a 2 percent infection rate was found in young bats, so it would appear that infection occurs or manifests itself in salivary glands sometime between the ages of 2 weeks and 2½ months.

Summary

Rio Bravo virus infection rates in 1,075 adult Mexican free-tailed bats and 100 cave myotis bats examined in this study varied from none, where the bats regularly inhabited ventilated roosts, to 3.4 percent, where they regularly inhabited extremely ill-ventilated roosts. Mixed groups of adult migrants had infection rates

as high as 7 percent. The virus was absent in bats 2 weeks of age except for one bat, which was positive in lungs only. Otherwise, the virus was not recovered from brains, mammary glands, lungs, kidneys, or fetuses. Young bats were found to be infected when they were about 2½ months old.

A captive bat that appeared normal was infected throughout a period of 682 days, after which it was killed. Other infected captives, held for prolonged periods, failed to evidence symptoms. Two bats had the virus in salivary glands when captured, but their saliva eventually became negative, and virus was not isolated from salivary glands when the bats were killed 404 and 681 days after capture.

Although Rio Bravo virus was isolated from symptomatic bats in nature, the infected bats comprised a proportion of symptomatic bats no greater than their proportion in asymptomatic bats.

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