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EASTERN EQUINE ENCEPHALOMYELITIS VIRUS IN EXPERIMENTALLY INFECTED BATS

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Abstract: Colonial bats *(Myotis* spp. and *Eptesicus* sp.) were infected with eastern equine encephalomyelitis virus by subcutaneous inoculation or by the bite of infected mosquitoes. Bats were maintained in an environment simulating conditions encountered in hibernacula or in summer maternal colonies. Virus was detected in the blood of hibernating bats at irregular intervals over a 42-day observation period; viremia perhaps was influenced by the amount of disturbance (arousal) involved in the blood sampling process. Target organs included brown fat, spleen, lung, kidneys, pancreas, and liver. Neutralizing antibody was not detected in sera collected from these bats between days 4 and 42 post-inoculation.

In nonhibernating bats, virus was recovered from mammary glands, brown fat, pancreas, lungs, kidneys, and liver, in addition to blood. Attempts to infect bats orally or to transmit virus to suckling mice by the bite of viremic bats were unsuccessful. Virus was transmitted from viremic chickens to *E. fuscus* by the bite of *Culiseta melanura* and *Aedes aegypti.*

INTRODUCTION

Although eastern equine encephalomyelitis (EEE) virus or antibody has been detected in naturally-infected colonial bats from Massachusetts,[7,10,14] Connecticut,[11] New Jersey,[8,9] and Georgia,[12] few laboratory studies designed to evaluate the significance of these findings or to elucidate the role of colonial bats as reservoir hosts of EEE virus have been published. LaMotte[13] reported, almost parenthetically in a laboratory study on Japanese B encephalitis (JBE) virus in hibernating bats, viremia in asymptomatic *Eptesicus fuscus* following injection with EEE virus. The present paper reports experimental EEE infections in colonial bats maintained in the laboratory under controlled temperature, relative humidity, and photoperiod simulating conditions encountered in hibernacula or in summer maternal colonies.

MATERIALS AND METHODS

Collecting, sampling, and testing procedures were reported previously.[14] Bats were held at least one week in the laboratory prior to any experimental work.

Maintenance Techniques: Hibernating bats were maintained in an environment controlled cabinet at 8 C and 80-90% RH. Drinking water, but not food, was available for the bats at all times. Nonhibernating bats were held at 35 C and 70-80% RH. These bats were fed mealworms or an homogenate of mealworms, cottage cheese, bananas, and vitamin supplements. Individual animals were force fed for the first few days in captivity and then allowed to feed at will. Only bats collected from hibernacula during the winter were maintained under conditions simulating hibernation; only summer bats were maintained in an active state. All bats

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were tested for circulating virus and antibody prior to experimental studies.

Inoculation techniques: Two strains of EEE virus were used in these experiments. In the first four experiments, the strain used originally was isolated from the brain of a horse from Massachusetts in 1966 and passed twice in chick-embryo tissue cultures (CETC). In the fifth experiment, an isolate from *Culiseta melanura* obtained from Connecticut in 1970 was used. This strain was passed twice as infected serum injected intramuscularly into 1-day old chicks prior to infection of the mosquitoes.

In experiments 1 and 2, bats were placed in hibernation following subcutaneous (sc) inoculation. In the first experiment, *Myotis lucifugus* were inoculated with 10^2, 10^4, 10^6, and 10^8 plaque-forming units (PFU) assayed in CETC (Table 1). In the second, *M. lucifugus, M. keenii, E. fuscus,* and *Pipistrellus subflavus* were inoculated with 10^6 PFU (Tables 2, 3).

In the next two experiments, *Myotis* bats were held at 35 C following sc inoculation with 10^6 PFU. Experiment 3 was designed to demonstrate tissue tropisms of EEE virus in nonhibernating bats at 48 and 72 h postinoculation

TABLE 1. Virus isolation attempts from hibernating *Myotis lucifugus* at various intervals following inoculation with one of four doses of eastern equine encephalomyelitis.

		Post-inoculation (days)							
Virus dose*	Tissues	2	3	4	5	6	7	8	9
10^8	Heart-Lung Pool		1/1**		1/1		1/1	1/1	
	Liver-Spleen Pool		0/1		0/1		0/1	1/1	
	Kidney		1/1		1/1		0/1	0/1	
10^6	Heart-Lung Pool		1/1					1/1	1/2
	Liver-Spleen Pool		0/1					0/1	0/2
	Kidney		1/1					0/1	0/2
	Blood								0/2***
10^4	Heart-Lung Pool	0/1			0/2		0/1		
	Liver-Spleen Pool	0/1			0/2		0/1		
	Kidney	0/1			0/2		0/1		
10^2	Heart-Lung Pool		0/1					0/1	0/2
	Liver-Spleen Pool		0/1					0/1	0/2
	Kidney		0/1					0/1	0/2
	Blood								0/2***
Control	Heart-Lung Pool		0/3						
	Liver-Spleen Pool		0/3						
	Kidney		0/3						

*PFU in CETC
**Number positive/number tested in CETC
***The four blood samples were also negative for EEE antibody by PRNT in CETC

PFU = Plaque forming units in CETC
CETC = Chick embryo tissue cultures
PRNT = Plaque reduction neutralization tests

TABLE 2. Isolation attempts from hibernating *Myotis* bats inoculated with 10^6 PFU* of eastern equine encephalomyelitis virus.

	Pre-inoculation**	Post-inoculation (week) 1	2	3	4	5
Blood***						
Myotis lucifugus						
Inoculated bats	0/38****	15/37	3/5	0/2	0/1	0/1
Control bats	0/16	0/16	0/2	0/2	0/2	
Myotis keenii						
Inoculated bats	0/20	5/20	2/4	1/5	0/2	0/1
Control bats	0/6	0/6			0/1	
Brown Fat		4/4	1/5	1/1	0/1 (control)	
Lung		4/4	2/5	1/1	0/1 (control)	
Kidney		3/4	2/5	0/1	0/1 (control)	
Heart		3/4	1/5	1/1	0/1 (control)	
Liver		3/4	0/3		0/1 (control)	
Pancreas		2/3	0/1		0/1 (control)	
Spleen		2/2	1/1		0/1 (control)	
Brain		1/4	1/5	0/1	0/1 (control)	
Salivary Gland		0/4	1/5	0/1	0/1 (control)	
Gall Bladder		0/1	0/1			
Urine		0/5	0/2		0/1 (control)	
Feces		0/4				

*See Table 1 for abbreviations.

**Pre-inoculation blood samples were negative for virus in CETC and antibody by PRNT.

***Blood samples taken from 2 to 5 weeks after inoculation were tested and found negative for EEE neutralizing antibody by PRNT.

****Number positive/number tested in CETC.

(pi). In experiment 4, attempts were made to transmit EEE virus orally by force-feeding bats on virus suspensions of approximately 10^6 PFU by injecting 0.03 ml into the buccal cavity with a syringe or on inoculated mealworms *(Tenebric molitor)* containing approximately 10^6 PFU/larvae. Attempts were made to transmit the virus to suckling mice by the bite of inoculated bats.

In experiment 5, each of six restrained nonhibernating *E. fuscus* were exposed overnight to eight mosquitoes 14 days after the mosquitoes had fed upon viremic chicks. Fed and unfed mosquitoes were frozen at -70 C the following day and later titrated in suckling mice. In all five experiments, uninfected bats were maintained with the infected bats as controls.

Mosquitoes: The mosquitoes used in these experiments were colonized *Cs. melanura* and *Aedes aegypti* maintained at 25 C and 80% RH. Both species have been maintained for at least five years in our insectary. Mosquitoes exposed to uninfected chicks and bats served as controls.

RESULTS

Experiment 1: EEE virus was not detected in the heart, lungs, liver, spleen, or kidneys of hibernating *M. lucifugus* inoculated with 10^2 or 10^4 PFU; virus was demonstrated in 3 of the 4 bats in-

TABLE 3. Isolation attempts on hibernating *Eptesicus fuscus* and *Pipistrellus subflavus* inoculated with 10^6 PFU* of eastern equine encephalomyelitis virus.

	Pre-inoculation**	Post-inoculation (weeks)									
		1	2	3	4	5	6	7	8	9	10
E. fuscus											
Inoculated	0/1***	0/1	0/1		1/1		1/1****				
Control	0/1	0/1			0/1			0/1			0/1
P. subflavus											
Inoculated	0/1	0/1*****									
Control	0/1	0/1									

*See Table 1 for abbreviations.

**Pre-inoculation samples were negative for virus in CETC and EEE antibody by PRNT.

***Number of blood samples positive/number of blood samples tested in CETC; blood samples taken from 2 to 10 weeks after inoculation were tested and found negative for EEE antibody by PRNT.

****42 post-inoculation day organ samples - positive; brown fat, lung, heart, kidney; negative: brain, salivary glands.

*****3 post-inoculation day organ samples - positive; brown fat; negative: brain, salivary glands, lung, heart, liver, gall bladder, spleen, pancreas, kidney, feces, urine.

oculated with 10^6 PFU and in all four of the animals injected with 10^8 PFU (Table 1). All tissues were assayed in CETC.

Experiment 2: A transient viremia was detected in hibernating bats after only 24 h and as long as 42 days pi (Tables 2, 3). Target organs included the spleen, brown fat, lung, kidney, heart, pancreas, and liver. Virus seldom was recovered from the brain or salivary glands and never from the gall bladder, urine, or feces. Virus occasionally was isolated from the brown fat, spleen, and kidneys in the absence of a demonstrable viremia. Blood samples collected more than seven days after inoculation were tested and found negative for antibody by plaque reduction neutralization tests.

Experiment 3: Virus was detected in organs from 16 of 20 adult nonhibernating female *M. lucifugus* (Table 4). One of the four uninfected bats had natural EEE neutralizing (NT) antibody prior to inoculation; the remaining 19 bats did not have demonstrable antibody. Organs most frequently infected at 48 to 72 h pi included the mammary glands, brown fat, pancreas, lungs, kidneys and liver; virus occasionally was isolated from the brain, heart, salivary glands, and ovaries. No virus was detected in two fetuses or two suckling bats; however, virus was detected in only one gravid and one lactating female bat associated with these progeny. Virus was recovered from the blood of two additional *M. lucifugus* 48 h after inoculation.

Virus was isolated from the brown fat but not from the salivary glands, lungs, heart, kidneys, testes, pancreas, or brain of an inoculated nonhibernating *E. fuscus*.

Experiment 4: Virus was detected in the blood of 12 of 14 *M. lucifugus* injected sc with 10^6 PFU, but not in 12 *M. keenii* fed approximately the same amount of EEE virus (Table 5). Virus was not demonstrated in throat swabs from any of the 24 bats. Blood and saliva samples were taken 48 h after attempted infec-

TABLE 4. Virus isolation attempts on nonhibernating female *Myotis lucifugus* inoculated with 10^6PFU* of eastern equine encephalomyelitis virus.

	Post-inoculation (hours)	
	48** (15 bats)	72*** (5 bats)
Mammary glands	12/15****	2/4
Brown fat	10/15	2/5
Lung	9/15	2/5
Kidney	6/15	2/4
Brain	4/15	2/5
Pancreas	4/6	
Heart	3/15	2/5
Salivary glands	2/15	1/5
Liver	2/3	0/2
Ovary	0/2	1/2
Fetus	0/2	
Suckling bat	0/2	

*See Table 1 for abbreviations.
**Virus was not detected in any of the organs tested from two bats.
***Virus was not detected in any of the organs tested from two bats; one of the two uninfected bats had natural EEE neutralizing antibody prior to inoculation.
****Number of positive/number tested in CETC.

TABLE 5. Virus isolations from bats following attempted infection via the subcutaneous route and the oral route with eastern equine encephalomyelitis virus.

		Saliva	
	Blood	Throat Swab	Bite*
Subcutaneous Route *(Myotis lucifugus)*	12/14**	0/12	0/2
Oral Route *(Myotis keenii)*	0/12	0/12	
Controls (both species)	0/3	0/3	

*Each bat was forced to bite five suckling mice.
**Number positive/number tested.

tion. Virus was not detected in 10 suckling mice bitten by two additional viremic *M. lucifugus* 48 h pi.

Experiment 5: Five of 6 nonhibernating *E. fuscus* exposed to infected *A. aegypti* or *Cs. melanura* died after incubation periods ranging from 8 to 19 days (Table 6). These bats circulated between 10^1 and 10^2 suckling mouse LD_{50} of virus per 0.02 ml prior to death. Virus was recovered from all five bats post mortem (Table 7). No virus or antibody was detected in either control; one control was killed after 20 days and the other survived beyond the 30 day observation period. The sixth "infected" bat (No. 7), although fed upon by four infected *A. aegypti* did not circulate virus nor was complement-fixing (CF), hemagglutination-inhibiting (HI) or NT antibody detected in the sera up to day 20 when the animal was killed. Virus was not detected in any of the organ samples tested of this bat (Table 7).

DISCUSSION

EEE virus was demonstrated in about one-third of the blood samples from inoculated hibernating bats. Viremia was detected at irregular intervals over the 42-day observation period. In at least two bats, virus was recovered from organs but not from blood taken at the same time. Other workers have reported that bats inoculated with JBE, St. Louis encephalitis (SLE), or Venezuelan equine encephalitis virus and maintained at hibernating temperatures either failed to develop demonstrable viremia or circulated virus in low titers.[6,13,19,21,23,24]

The importance of the brown fat in bats as a site of virus replication and its role as a possible "reservoiring mechanism" for certain arboviruses (JBE and SLE) and rabies virus has been stressed by Sulkin and his co-workers.[17-20,25,26] Several viruses, including rabies,[3-5,22] Rio Bravo,[2] and SLE[27] have been isolated from the brown fat of naturally-infected bats. In the present study, EEE virus was recovered from this organ in 23 of 32 experimentally-infected bats. Virus was isolated from this tissue in the absence of viremia in at least two bats. It was the only tissue infected in three bats; however, virus was not recovered from the brown fat of nine infected bats. Virus was demonstrated in the adipose tissue in hibernating bats at intervals ranging from 24 h to 42 days after inoculation.

The mammary glands were frequently infected in inoculated lactating or gravid *M. lucifugus*. Glandular and fatty tissue in 14 of 16 infected bats contained EEE virus. In three bats, the mammary glands were the only tissue found to be

TABLE 6. Nonhibernating *Eptesicus fuscus* experimentally infected with eastern equine encephalomyelitis virus by mosquito bite.

Bat No.	No. of mosquitoes feeding*	Virus Titer of mosquitoes**	Days Post-exposure										
			1	2	3	4	5	6	7	8	9	10 . . . 19	20 . . . 30
1	1	4.5		.***						died****			
2	2	4.7			-	-					+(1.0)	died	
3	3	4.4	+(1.0)					+(1.5)				-died	
4	4	4.9					+(1.5)					+(1.4)	sacrificed; moribund
5	3	5.1			+(1.0)					+(2.0)		died	
6	control	-		-									-sacrificed
7	4	4.5		-					-				-sacrificed
8	control	-	-										-

**Culiseta melanura* fed on bats 1 and 6; all other bats exposed to *Aedes aegypti.*

**log of the suckling mouse (ic) LD_{50}

***Virus isolation attempts from blood; log of the suckling mouse (ic) LD_{50} given in parentheses; all pre- and post-exposure blood samples were negative for complement-fixing, hemagglutination-inhibiting, and neutralizing antibodies (assayed in suckling mice by ip inoculation) against eastern equine encephalomyelitis virus.

****See Table 7 for details of necropsies.

TABLE 7. Virus isolation attempts* on *Eptesicus fuscus* experimentally-infected by mosquito bite.

Bat Number:	1	2	3	4	5	6	7
Days post-infection:	8	9	10	10	19	20	30
Tissue							
Blood		1.0**	-	1.4		-	-
Brown Fat	-	-	-	1.7	tr	-	-
Lung	1.0	-	2.0	1.7	1.7	-	-
Kidney	-	-	-	2.4	-	-	-
Heart	-	-	3.8	3.7	4.4	-	-
Liver	1.4	-	-	1.5	-	-	-
Pancreas	tr	-	-	2.0	-	-	-
Spleen	-	-	-	tr	-	-	-
Brain	-	-	-	1.5	-	-	-
Salivary Glands	-	-	-	1.5	-	-	-
Gall Bladder	-	-	-	-	-	-	-
Urinary Bladder	-	-	-	-	-	-	-
Bone Marrow	-	-	-	-	-	-	-
Skeletal muscle	-	-	-	-	-	-	-
Skin	-	-	-	-	-	-	-

*Virus isolation attempts in suckling mice (ic); All pre- and post- infection blood samples were negative for complement-fixation, hemagglutination-inhibition, and neutralizing antibody (assayed in suckling mice by ip inoculation) against eastern equine encephalomyelitis virus.

**Log of LD_{50} in suckling mice (ic); - = $\leq$0.5; tr = trace.

infected and in one bat, the mammary glands and the brown fat were the only positive tissues.

EEE virus does not appear to be particularly neurotropic in bats; isolations were made from the brains of only two inoculated hibernating bats and seven experimentally infected nonhibernating bats. The lack of central nervous system involvement in the majority of infected bats may account for the apparent lack of encephalitic symptoms. Virus was recovered from the livers in 7 of 15 infected bats.

The clustering behavior of these gregarious mammals, both in the summer maternal colonies and in the winter hibernacula, is conducive to the spread of various pathogenic agents via arthropod vectors, by bite, or by contact transmission. In the present study, EEE virus was transmitted to bats by the bite of infected *A. aegypti* and *Cs. melanura*, but none of the control bats maintained with inoculated bats became infected, suggesting that oral or contact transmission does not occur.

Although bats are known to eat mosquitoes,[11] these insects are not a major source of food for insectivorous bats.[1,15,16] However, the opportunity for bats to feed upon infected mosquitoes while foraging is always present. The possibility of transmission by the ingestion of EEE virus was examined in this study. Virus was not detected in the blood or throat swab samples from 12 bats 48 h after feeding on EEE virus. Therefore, it is suggested that oral transmission through the ingestion of infected arthropods is not likely to be an effective method of perpetuating the virus among bats.

The recovery of virus from the mammary glands in many of the gravid or lactating *M. lucifugus* inoculated with

EEE virus suggests the possibility of infection through the mothers' milk. Only one suckling bat from an infected female was tested; virus was not demonstrated in the young bat although it was found in the mammary glands and other tissues of the mother. Oral infection could not be demonstrated although no attempts were made to infect suckling bats.

EEE virus was isolated from the lungs from 23 of 32 experimentally-infected bats; thus, direct contact via inhalation of virus-laden droplets expelled by sneezing and coughing is a possible mode of transmission. However, the lack of infection in 12 bats force-fed EEE virus indicates that an oral portal of entry is unlikely.

Infected droplets of urine are another possible method of transmission among colonial bats. EEE virus was not found in eight urine samples from infected bats; however the kidneys from six hibernating and nine nonhibernating bats were infected.

Transmission through fecal contamination could not be demonstrated; fecal samples from five infected hibernating bats were negative for virus.

The frequent recovery of rabies and other viruses from the saliva and/or salivary glands of naturally infected bats suggests the possibility of transmission by bite. EEE virus was recovered from the salivary glands of only one hibernating and four nonhibernating bats inoculated during the present study. Attempts to isolate the virus in throat swabs taken from 12 additional inoculated *M. lucifugus* (10 viremic) failed, as did two attempts to infect suckling mice by the bites of two viremic bats. Transmission by bite, therefore, seems unlikely.

Transplacental transmission may be another source of infection, but only one fetus from an infected *M. lucifugus* was tested for EEE virus in the present study. The entire fetus was negative although virus was present in the brown fat and mammary glands of the mother.

Conclusions: Natural infections of EEE virus occur in colonial bats of the genera *Myotis* and *Eptesicus* in southern New England.[14] The prevalence of acquired immunity in adult nonhibernating, colonial bats was 2.3% during the period studied (1966-1976).

Colonial bats are susceptible to infection with EEE virus by subcutaneous inoculation with a low passage strain of equine origin and by the bite of mosquitoes infected with a strain originally isolated from *Cs. melanura.*

Prolonged infections over a 42-day observation period were observed in inoculated bats maintained in simulated hibernation. Circulating virus was detected at irregular intervals in hibernating bats, but viremia may be influenced by the amount of disturbance (arousal) involved in the bleeding process. Virus also was demonstrated in various organs, especially the brown fat, lungs, spleen, kidneys, and heart, occasionally in the absence of viremia. The brain and salivary glands seldom were infected.

A lipotropism of EEE virus was demonstrated in experimentally infected lactating or gravid *M. lucifugus;* virus was frequently recovered from the mammary glands and the brown fat.

A statistical analysis of data from surveyed bats indicates that the incidence of EEE antibody is greater among nonhibernating than among hibernating bats.[14] Experimental evidence suggests that hibernating bats fail to develop specific neutralizing antibody.

Transmission via the bite of infected bats, the ingestion of virus, and contamination by infected urine or feces could not be demonstrated.

Population dynamics, plus the prolonged infections possible under hibernating conditions, indicate that colonial bats can be suitable reservoir hosts for the overwintering of EEE virus in southern New England.

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