Epidemiol. Infect. (2005), **133**, 517–522. © 2005 Cambridge University Press
doi:10.1017/S0950268805003705 Printed in the United Kingdom

Salivary excretion of rabies virus by healthy vampire bats

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(*Accepted 22 July 2004*)

SUMMARY

Salivary excretion of rabies virus was evaluated in 14 adult vampire bats (*Desmodus rotundus*) intramuscularly injected with a large dose (10^6 $MICLD_{50}$) of vampire rabies virus variant CASS88. Saliva samples were obtained from surviving bats every other day for 30 days, then weekly for 2 months, and finally 1 and 2 years later. Rabies virus was isolated in murine neuroblastoma cells and in randomly selected cases by PCR. Rabies virus was not detected in the saliva of any of the 11 animals that succumbed (somewhat early) to rabies challenge, nor in the control bats. In contrast, virus was detected early, and only once (days 6, 6 and 21) in each of the three animals that survived rabies challenge and remained healthy for at least 2 years after challenge. At that time even vigorous dexamethasone and cyclosporine administration failed to provoke further viral excretion.

INTRODUCTION

The common vampire bat (*Desmodus rotundus*) is a major wildlife reservoir of rabies in tropical and subtropical areas of the American continent. The introduction of cattle to this part of the world by Spanish settlers brought a sudden increase in food resources (i.e. cattle blood) and probably allowed vampire bats to reproduce vigorously. This in turn led to a rise in the prevalence of rabies in these areas [1]. More than 500 cases of human rabies caused by *D. rotundus* have been reported in Latin America since 1975. This bat is also responsible for about 100 000 cases of fatal bovine rabies each year [1, 2]. Moreover, a bat (*Lasionycteris noctivagans*) rabies virus variant has caused human rabies in the United States and Canada [3–5], heralding perhaps an emerging role of bats in the transmission of rabies in these countries.

The role of bats in rabies transmission was first established in 1911 [6]. In 1936, when virology was a new branch of science and was still using very crude tools, Pawan speculated that such animals would not

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only excrete the virus during the course of the infection, but, unlike other rabies vectors, could sometimes recover and excrete virus in saliva for months [7]. Recently, Lyssavirus (EBV1) infection was detected in 13 oropharyngeal swabs, but only in five brains of 34 wild-captured bats (*Eptesicus serotinus*) from which simultaneous brain and oropharyngeal material had been obtained [8]. These results, and the sizable number of healthy, seropositive bats (EBV1 and rabies virus) [9–11] led Echevarría [8] to suggest that the clinical expression of EBV1 – and also rabies virus – infection in bats is usually a mild, not necessarily fatal, extra-neurological disease. Nevertheless, exact data on salivary excretion of rabies virus from bats remains limited [1]. In 1980 Moreno & Baer [12] found salivary excretion of rabies virus just before the onset of fatal disease in vampire bats following the parenteral administration of $0{\cdot}562 \times 10^3$ intramuscular and $5{\cdot}6 \times 10^3$ subcutaneous $MICLD_{50}$. Surviving bats, however, did not excrete virus in the saliva. They concluded that a carrier state does not occur in bats.

In this study, we attempted to measure the salivary excretion of rabies virus in vampire bats receiving a much larger ($\sim$2–3 log) viral challenge (i.e. 10^6 $MICLD_{50}$) than that employed by Moreno & Baer [12].

METHODS

Animals

Twenty-three common vampire bats (weight 25–45 g) were captured with 'mist' nets complying with Mexican regulations (Norma Oficial Mexicana NOM-011-SSA2-1996, Para la Prevención y Control de la Rabia). Animals were captured in an area where, up to then, bovine rabies had not been documented (San Luis Potosi 1999, municipio el Naranjo, Mexico, 22·30° N, 96·38° W). They were confined in individual cages in a biosafety level II security room, at constant temperature ($23 \pm 2{\cdot}5$ °C) and 70% relative humidity for a 30-day adaptation period, receiving defibrinated blood (free of rabies antibodies) from healthy bovine sources as described [13, 14]. All these bats lacked detectable anti-rabies antibodies at capture, and at the end of the adaptation period.

Detection of neutralizing antibodies

Blood was drawn from the marginal vein of the forearm membrane at capture and at the end of the adaptation period (before challenge), as well as 90 and 710 days after viral challenge in surviving bats. Anti-rabies virus-neutralizing antibodies were measured by the rapid fluorescent focus inhibition test (RFFIT) [15] (sensitivity $\geqslant 0{\cdot}25$ IU/ml) and were expressed as international units per ml (IU/ml) compared with a standard reference serum.

Virus challenge

Fourteen adult vampire bats (six males and eight females, 35–45 g) (nos. 1–14, Fig.) were selected (criteria: healthy, vigorous, alert, non-pregnant) from the original group and were inoculated with a single 10^6 $MICLD_{50}$ dose of vampire bat rabies virus variant CASS88 [16], injected in the dorsal muscle at its insertion site over the scapular cartilage 4 mm deep [13, 14]. Such large intramuscular challenges kill 80–90% of adult vampire bats [13, 14].

Four healthy, control adult vampire bats (two males and two non-pregnant females, 35–40 g) (nos. 5–18, Fig.) received phosphate-buffered saline (PBS) instead, under identical conditions, and were kept individually caged in the same animal room.

Animals were observed daily and clinical signs of rabies (depression, lack of coordination, tremor and diminished blood consumption, flaccidity) were recorded for 90 days after viral challenge, and then weekly for an additional 690 days.

Salivary samples

Salivary samples were obtained by rinsing the oral cavity with 0·4 ml of PBS approximately every other day for 30 days (Fig.), and then weekly for the next 2 months. Additional samples were taken from the surviving and control bats at 360 and 690 days, at which time bats were given massive doses of dexamethasone (Opticortenol®, Ciba, Mexico) (five daily 0·1-mg intramuscular doses), followed by cyclosporine (Sandimmun®, Sandoz, Mexico) (four daily 1-mg oral doses). Saliva samples were obtained every other day for 20 additional days in these animals.

Diagnosis of rabies

The diagnosis of rabies was established in brain smears from succumbing bats using fluorescent antibody testing (FAT) as recommended by the World Health Organization [17].

https://doi.org/10.1017/S0950268805003705 Published online by Cambridge University Press

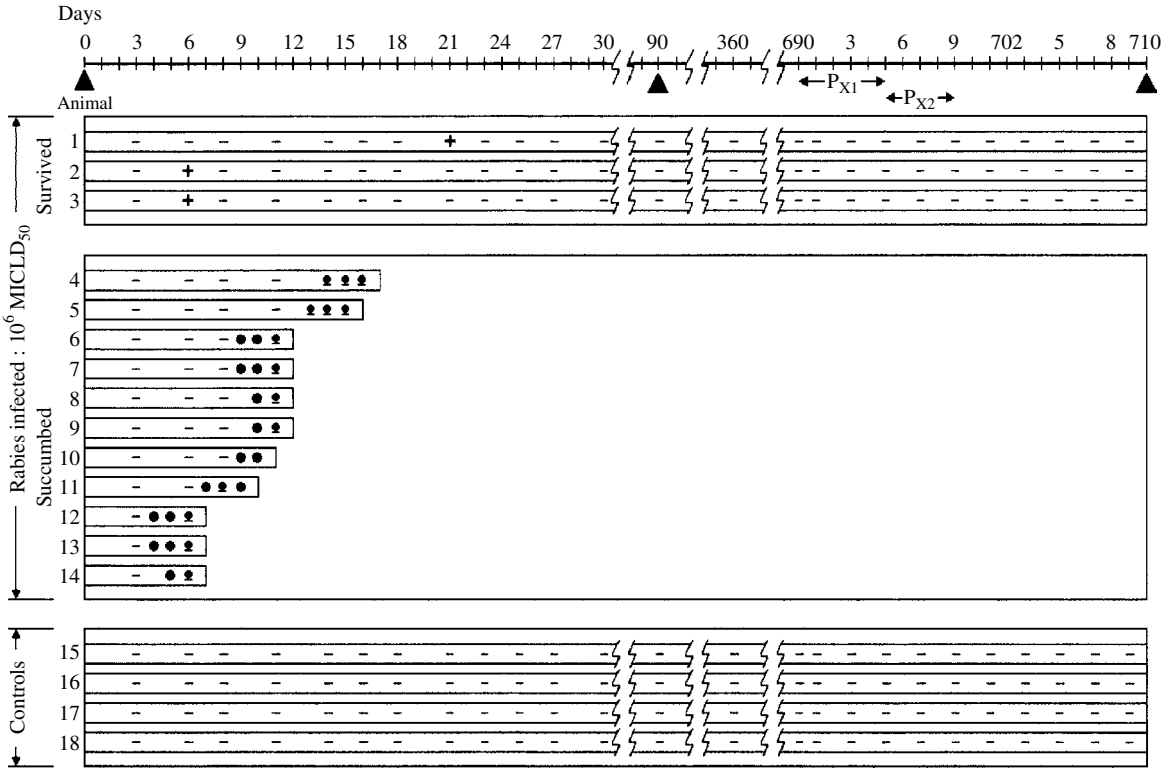

Fig. Survival and salivary virus in rabies virus-challenged bats. Negative (–), no virus isolated; positive (+), virus isolated from saliva; clinical signs of rabies (●), diminished blood consumption, depression, lack of coordination, tremor; dexamethasone (P_{X1}), five daily 0·1-mg intramuscular doses; cyclosporine (P_{X2}), four daily 1-mg oral doses; serological samples (▲).

Rabies virus isolation

Rabies virus isolation was attempted from saliva using CCL131 neuroblastoma cells and identified with rabies anti-nucleocapsid fluorescent conjugate [18]. PCR, a more sensitive viral detection test that employs rabies virus primer pairs specific for the N gene that amplify a 648-bp product [19], was applied to all saliva samples in four randomly selected infected bats (bat nos. 2, 11–13) and in the four control bats (bat nos. 15–18) (Fig.) (negative PCR controls). In total, 166 saliva samples were tested by PCR (40 from infected animals and 126 from controls). According to Shankar et al. [19], the smallest amount of virus-specific RNA that was detected by PCR amplification corresponded to 9 p.f.u. of rabies virus.

Virus isolated in CCL131 neuroblastoma cells was subsequently inoculated intracerebrally in BALB/c mice (21 g) for viral pathogenicity confirmation and rabies diagnosed by FAT.

Virus identification

The inoculated and re-isolated viruses were identified using a PCR/RFLP test with primers flanking the non-coding ψ pseudogene of the rabies virus genome, and the restriction enzymes *Bsa*WI, *Bsr*GI, *Bam*HI and *Stu*I, as described by Loza-Rubio et al. [20].

RESULTS

Eleven (79%) of the 14 infected bats, succumbed to rabies, always preceded by a short period of depression, hypoactivity and anorexia, but no aggressive behaviour (Fig.). The first sign noticed in animals dying of rabies was a decrease in blood consumption (4 days before death), leading to dehydration. Some remained quietly in the corner of the cage, withdrawn, without any aggressive behaviour (72 h before death). Neurological signs such as paralysis of the wings (unable to maintain an upright position) and in the hind-legs (hanging from one leg only) and tremors were evident in three animals and were detected only 48 h before death.

The three bats alive at 90 days after challenge (bat nos. 1–3; Fig.), had anti-rabies antibodies at this time (2·5, >10 and 4 IU/ml respectively) and remained clinically healthy, with sustained blood consumption (15–20 ml/day) for the next 690 days, and had

https://doi.org/10.1017/S0950268805003705 Published online by Cambridge University Press

anti-rabies antibody titres $\leqslant 0{\cdot}25$ IU on day 710. The control bats also remained healthy, seronegative and with a comparable sustained blood consumption during the 710 days of this study (Fig.). Thus, a total of 267 salivary samples were assayed in this study, 141 from rabies-infected animals (42 from 11 bats that eventually died of rabies, 99 from the three surviving bats) and 126 from the four non-infected controls.

No rabies virus was isolated from the saliva of animals that succumbed to the disease, nor from the control animals. Saliva samples from dying bats were collected when they were closest to death. In all bats except in bat nos. 10 and 11, saliva samples were taken the same day they died; in bat no. 10 saliva was collected 48 h before death and in bat no. 11, 24 h before death (Fig.). All these samples were negative for virus isolation (Fig.). In contrast, virus was isolated in cell cultures from the saliva of the three surviving infected animals (bat nos. 1–3) although only on days 6, 6 and 21 respectively after virus inoculation (Fig.), and not later, even after high doses of dexamethasone and cyclosporine (Fig.). In bat nos. 1 and 3, salivary virus titrated to $1{\cdot}8 \times 10^3$ and $0{\cdot}9 \times 10^3$ respectively for rabies-specific fluorescent foci/ml 4 days after incubation in CCL131 neuroblastoma cell monolayers. In bat no. 2 (in which viral RNA was originally detected by PCR; Fig.) infectious virus was isolated only after three passages in CCL131 neuroblastoma cells ($0{\cdot}5 \times 10^3$ rabies-specific fluorescent foci/ml titre).

When RT–PCR/RFLP was applied to the virus isolated from the saliva of the three infected surviving bats, migration patterns of digested amplicons fully corresponded with the pattern obtained with the vampire bat rabies virus variant CASS88 employed for challenge. Mice inoculated intracerebrally with the rabies virus isolated from the saliva of the three surviving bats, succumbed to rabies by 10 ± 4 days.

DISCUSSION

Salivary viral excretion was found early and transiently in the three survivors (the number expected to survive the challenge dose used in this study) of 14 rabies-challenged bats. The events that follow infection with bat-derived Lyssavirus are not completely understood [1] but it is assumed that such bats do not become asymptomatic carriers [12, 21]. In other words, an infected and infectious bat is usually regarded as a bat doomed to succumb to rabies. Our results, however, show that vampire bats that survive a massive parenteral challenge with rabies virus (10^6 $MICLD_{50}$) for 2 years occasionally and transiently excreted virus in their saliva, without ever showing clinical signs of CNS rabies.

The viral genome moves centrifugally through peripheral nerves from the CNS to the salivary glands, where infectious virions bud from the apical (luminal) surface of mucosal cells and are released at high concentrations into the saliva [22]. Hence, by the time viral replication in the CNS leads to aggressive behavior and indiscriminate biting, the saliva is already highly infective as in the classical transmitting species such as dogs, foxes, cats, raccoons and skunks [22]. Clearly then, in bats the subtle timing of viral dissemination must differ from the classical carnivore story.

The main difference between our study and that of Moreno & Baer [12] was the $\sim$200-fold intramuscular and $\sim$2000-fold subcutaneous larger virus challenge employed by us. Most bats died after a relatively brief incubation period (Fig.), confirming the efficacy of the model employed, but at the same time raising the question of whether such a short period may be sufficient to allow the virus to reach the saliva before death. The presence of virus in the salivary gland tissue itself was not explored.

Rabies virus has not been isolated from saliva samples obtained from rabid bats just prior to death. Moreno & Baer [12] reported that only two out of five experimentally infected (562 LD_{50}, intramuscularly) bats presented virus in salivary glands at death. In *Tadarida brasiliensis mexicana*, virus was detected in the saliva in one of the five intramuscularly infected animals [21] and Sulkin & Allen [23] reported the frequency of isolation of rabies virus from brain, brown fat, and salivary glands to be 92, 30 and 17% respectively in experimentally infected *Myotis* bats. It is, therefore, not rare in experimentally infected bats to find little or no infectious virus in the saliva [1]. It is possible, however, that although the saliva contained some rabies virions they could have been in a non-infectious state because elements of the innate immune system and/or specific antibodies neutralized the virus.

Rabies is endemic in vampire bats throughout their range. Thus, it is possible that the three surviving bats in our study had a low, undetected level of pre-existing acquired immunity from a previous exposure to rabies virus, and were now responding anamnestically to the viral challenge, eventually reaching high antibody titres (2·5, 4 and >10 IU/ml). Vampire

bat no. 2 (titre >10 IU/ml), in particular, may have responded in this way. The large viral inoculum employed in this study probably elicited some antibody response in all of the bats, irrespective of whether or not they survived the challenge. Unfortunately, however, the next sera in this study were obtained at 90 days, by which time all the dying bats had succumbed.

Resistance to rabies virus correlates with the immune response. Experiments in mice have shown that animals that survive a lethal challenge with rabies virus produced early and high-titre serum anti-rabies neutralizing antibody and could, thus, clear virus from the CNS [24]. Future studies of bat survival after lethal challenge with rabies virus will have to consider how soon anti-rabies antibodies appear and what titre they reach.

On the other hand the development of clinical CNS disease is associated with the restriction of virus replication within the CNS. In mouse studies [24], resistant, asymptomatic mice had low viral replication in the spinal cord, and no viral replication in the brain. In contrast in resistant-recovered mice, rabies virus infected the spinal cord and then the brain before ceasing replication in the CNS. In these animals, clinical disease was frustrated, but paralysis was irreversible. These results suggest that in the surviving bats, rabies virus may never have actually reached the brain. It is, therefore, possible that in the three surviving bats, virions arrived in the spinal cord and, without causing significant damage, proceeded centrifugally to the salivary glands. The observation of Echevarría et al. [8] using Lyssavirus EBV1 supports this concept.

Most patients dying from *Lasionycteris noctivagans* rabies virus in the United States had no history of bites, scratches or other contact with this or other species of bat [3]. The pattern of excretion of the CASS88 virus used in our study may thus represent an atypical and hitherto undescribed feature of this viral variant, at least in some of the challenged animals (i.e. those with pre-existent but undetectable immunity).

Bats exposed to sublethal doses of rabies virus in a colony cohabitating with infected vampire bats, may develop low titre, sometimes undetectable, anti-rabies antibodies [13, 14], and this give rise to an anamnestic response on re-exposure to the virus, as may have been seen in bat nos. 1–3. Sublethal doses of rabies virus can be disseminated through routes such as aerosol [21], mutual grooming, or reciprocal food-sharing of regurgitated ingested blood [25]. In a recent report, the emergence of latent rabies virus infection after transportation stress was suggested in otherwise healthy zoo bats [26]. Mechanisms for rabies virus latency have been proposed: some authors reported that the virus can stay latent in brown fat and reactivates when the animal wakes up from a hibernation period. As already discussed, Sulkin & Allen [23] reported that bats of the genus *Myotis*, experimentally infected with rabies virus, had the virus more frequently in brown fat (30%) than in salivary glands (17%). However, we think this an unlikely hypothesis for vampire bats, as they do not hibernate, and after a period of adaptation to captivity, they completely lose their scarce brown fat. Ray et al. [27] proposed that rabies virus can remain latent in macrophages. These authors reported that rabies virus was isolated from bone marrow macrophages in mice that survived a lethal challenge for more than 69 days. Future studies on the presence of rabies virus in bat macrophages should be undertaken.

The existence, albeit rare, of asymptomatic bats potentially transmitting rabies may help explain such epidemiological phenomena as intra/interspecies persistence and dissemination, as well as the high incidence of paralytic bovine rabies outbreaks recorded in Latin America.

ACKNOWLEDGEMENTS

This work was partially supported by a grants from the European Union (contract no. CI1*-CT92-0068) and from the CONACYT (Mexico) (contract no. Z00-6-B).

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