Experimental rabies infection in haematophagous bats *Desmodus rotundus*

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SUMMARY

In order to determine the susceptibility and serum neutralizing antibody response of *Desmodus rotundus* to rabies virus, bats were inoculated with a virus isolated from a naturally infected haematophagous bat. Bats were divided into four groups of 10 animals each. Dilutions of rabies virus containing 100, 1000, 10 000 and 100 000 $MICLD_{50}$ (lethal dose 50 % for mice inoculated by the intracerebral route) were administrated in the pectoral muscle. The presence of rabies virus was detected in brain and salivary glands by fluorescent antibody, mouse inoculation and RT–PCR. The observed mortality for each virus dose was 0, 20, 20 and 60 % respectively. Serum neutralizing antibodies were tested for by the rapid fluorescent focus inhibition test, and antibody titres greater than 0·5 IU/ml were found in 53 % of bats 30 days after virus inoculation. Resistance to infection was seen in bats that developed low or no detectable antibody response as well as in bats with high titres. Among the 10 bats that died of rabies, eight showed signs of paralytic rabies and two bats showed no clinical signs.

INTRODUCTION

While a combination of vaccination, surveillance and other control programmes have eliminated or decreased dog-related rabies in many parts of the world, the disease persists in a variety of wildlife hosts. Epidemiological data, together with antigenic typing using monoclonal antibodies and phylogenetic analyses of viruses from around the world have demonstrated the importance of the bats as reservoirs [1–4]. The haematophagous bat, *Desmodus rotundus*, has been shown to be the main wild reservoir of rabies in several countries in Latin American [5–7]. This bat was recognized as a rabies reservoir in 1911, when Carini [8] described an outbreak of rabies in cattle in Santa Catarina State, Brazil, and suggested that *D. rotundus* may have been the source of the infection, although isolation of the virus occurred only in 1916 [9]. Bat rabies continues to cause the loss of thousands of cattle every year in South America.

The first human death attributed to rabies acquired from vampire bats was reported on the island of Trinidad during the early 1930s [10], since then human rabies acquired from bats and human depredation by

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Table 1. *Experimental infection of* Desmodus rotundus *with rabies*

5 Dose*	Mortality (%)	Bat	Incubation period (days)	Clinical signs	Morbidity (h)
100	0	—	—	−	—
1000	20	A2	19	−	—
		B2	41	−	—
10 000	20	H7	29	+	24
		Y7	32	+	
100 000	60	Q12	5	+	48
		X12	5	+	
		Z12	5	+	
		K12	9	+	24
		R12	9	+	
		F12	14	+	18
Control	0	—	—	−	—

* LD_{50} in mice by the intracerebral route.

vampire bats has continued to be documented in Latin American countries [11–15].

In spite of the importance of this species as rabies reservoir, experimental studies of rabies in vampire bats have been limited. In this study, a rabies virus isolated from a naturally infected vampire bat, was used in an experimental infection to determine the susceptibility of *D. rotundus* to rabies virus, and the serum antibody response of bats to the infection.

Fifty *D. rotundus* adults bats (28 males, 22 females), were captured in caves between July 2001 and January 2002 from an area where wild rabies is not documented, dog and cat rabies had not been notified since 1986 and the last rabies case in cattle occurred in 1998 (Paraisópolis, southeast of the state of Minas Gerais). The bats were maintained in cages at temperatures between 19 and 23 °C, and were fed daily with defibrinated swine blood. They were quarantined for 30–45 days. Regular access to the animal room was permitted only to persons immunized against rabies and whose antibodies titres were monitored every 6 months.

The rabies virus variant used in this study (Brldr2918) was isolated in 1997 from the brain of a *D. rotundus* bat from Santa Branca City in São Paulo State. Sequencing and phylogenetic analysis performed at the Centres for Diseases Control and Prevention, Atlanta, GA, USA [16] showed the virus to segregate with high homology (96·11%) in one monophyletic group with other vampire bat, cattle and horse isolates. The titre of the virus stock (first passage in mouse) was $10^{5.99}/0.03$ ml $MICLD_{50}$ (lethal dose 50% for mice inoculated by the intracerebral route).

The bats were divided into five groups of 10 animals each. Each animal was inoculated intramuscularly (pectoral muscle) with 0·1 ml of the rabies virus suspension containing 100 (group 1); 1000 (group 2); 10 000 (group 3) and 100 000 $MICLD_{50}$ (group 4). The control group (group 5) was inoculated with phosphate-buffered solution. The animals were observed daily for 90 days for signs of rabies and changes in behaviour.

Serum samples from all of the animals were tested for rabies virus neutralizing antibody (VNA) by the rapid fluorescent focus inhibition test [17]. Blood samples were collected from a cephalic vein with a hematocrit tube [18] before virus inoculation, and on days 30, 60 and 91 of the experimental infection. The conventionally defined protective level of antibody in humans of 0·5 IU/ml was taken as the cut-off [17, 19].

Post mortem, the brain and salivary glands of all the bats were examined for the presence of rabies virus by the fluorescent antibody test (FAT) [20], the mouse inoculation test (MIT) [21] and RT–PCR. Viral RNA was extracted with Trizol™ (Gibco-BRL Inc., Carlsbad, CA, USA) and cDNA produced by reverse transcription and amplification by PCR with primers 21g and 304 as previously described [22, 23]. The primers annealed to the N gene, and the PCR amplified a 320-bp region of the nucleoprotein gene (bp1094–1413). Sequences were aligned and analysed with the Pile Up program and the Treeview program was used to obtain graphic output [24].

The results of the inoculation of rabies virus are shown in Table 1. The overall mortality rate after

Table 2. *Virus distribution after experimental infection of* Desmodus rotundus *with rabies*

| Virus dose* | Bat | FAT for antigen | | MIT† | | RT–PCR | |
		Brain	Salivary glands	Brain	Salivary glands	Brain	Salivary glands
1000	B2	+	+	5/0	5/1	+	+
	A2	+	+	5/0	5/0	+	+
10 000	H7	+	+	5/0	5/4	+	+
	Y7	+	−	5/0	—	+	−
100 000	F12	+	−	5/0	5/4	+	+
	K12	+	+	5/0	—	+	+
	R12	+	−	5/0	—	+	−
	Z12	+	−	5/0	—	+	−
	X12	+	−	5/0	—	+	−
	Q12	+	−	5/0	—	+	+
Survivors		−	−	5/5	5/5	−	−
Control		−	−	5/5	5/5	−	−

* LD_{50} in mice.
† Mouse inoculation test: number of mice inoculated/number of survivors.

intramuscular inoculation was 25%. In the groups that received 1000 or 10 000 $MICLD_{50}$ the mortality was 20%, and in the group that received 100 000 $MICLD_{50}$ mortality was 60%. No vampire bats given 100 $MICLD_{50}$ died.

The incubation period ranged from 5 to 41 days (mean 16·8 days) and the morbidity period lasted for 18–48 h. The mean incubation periods for groups 4, 3 and 2 were 7·7, 31·5 and 30 days respectively (Table 1). There was a negative correlation between the duration of the incubation period and the dose inoculated ($r = -0·89$). This inversely proportional correlation is similar to that seen in other animal species [25–27]. The incubation period and the morbidity period observed in this experiment are also similar to those reported by other authors [28, 29].

Rabies virus was detected by MIT, FAT and RT–PCR from the brain of the 10 bats infected, but it was detected from the salivary glands of 40% of infected bats by FAT and MIT and 60% of infected bats by RT–PCR. No virus was detected by any method in the bats in the control group and all survivors at the end of observation period (Table 2). The three techniques used had the same sensitivity for virus detection in bat brains (100%). However, the sensitivity of virus detection in bat salivary glands was higher by RT–PCR (60%) than FAT and MIT (40%). Others have also shown RT–PCR to be a rapid and sensitive diagnostic method [22, 30, 31] and to be sensitive in samples with a low viral load [32].

Among the 10 bats that died of rabies, eight exhibited signs of paralytic rabies: weakness, muscular tremors, muscular spasms, inability to stand on their feet and thumbs, lack of coordination of posterior limbs, irritability to light, wind and sounds, paralysis and prostration. Two of them had purulent conjunctivitis and one developed urinary incontinence. The development of the disease was rapid. Initially, when the animal was in its group, the signs were almost unnoticeable. Only when the animal was separated from the group and forced to move around, were the first signs (difficulty standing on their feet and thumbs, lack of coordination of posterior limbs) clearly observable. No signs associated with furious rabies were observed, unlike in previous studies where abnormal aggressiveness, an increased tendency for biting or viciously attacking other animals in the cage, dashing against the wire mesh or darting violently from one side of the cage to the other, have been reported [33].

Loss of body weight during the 2 days before death ranged from 11·5% to 22·6%. Three bats were very thin and no brown fat was found post mortem. In contrast, among the survivors, at the end of the observation period, 53·3% had gained weight.

In two bats inoculated with 1000 $MICLD_{50}$, which were found dead on days 19 and 41, no clinical signs were observed. These bats had only slight loss of weight (1·2% and 1·1% of body weight respectively), and showed no signs of decreasing blood consumption. They were examined the day before they died,

when they were apparently well and even descended to feed. Several authors have also previously observed lack of clinical signs of rabies in *D. rotundus* bats [29, 33–36].

None of the bats was seropositive to rabies before the inoculation. VNA titres above 0·5 IU/ml were found in 52·7% of bats on day 30, 43·7% on day 60 and 34·5% on day 90 after inoculation. Only one animal (inoculated with 100 MICLD$_{50}$) show no increase in level of antibodies on day 30, and maintained a low antibody titre until the end of the observation period. In all the groups some animals showed only a small increase of the titre on day 30 (0·2 or 0·3 IU/ml), which decreased on day 60 or day 90.

VNAs have a critical role in immunoprotection, and the presence of VNA in the blood is often considered an important index of protection against infection. However, the degree of resistance indicated by the presence of VNA in serum has been questioned and antibody titres do not necessarily correlate with immunity. Survival is associated with a T-cell response, the production of antibodies, a high expression of major histocompatibility complex and the presence of many apoptotic cells [37].

In this experiment, resistance to infection in seronegative animals or in those with low titres of antibodies was observed in all groups, regardless of the dose inoculated. Setien et al. [29] also reported that animals exhibiting undetectable amounts of VNA resisted challenge with high doses of virus. However, animals that received the highest inoculum developed the highest antibodies titre. All four surviving bats from the group that received 100 000 MICLD$_{50}$ showed high titres ($>2·2$ IU/ml) on day 91. In contrast, only three of the 25 bats surviving in other groups developed such antibody titres. This difference in the amounts of VNA produced between animals that received 100 000 MICLD$_{50}$ and animals of the other groups was significant [$P = 0·0002$, χ^2 uncorrected test in the Epi-Info 6.0 (CDC, Atlanta, GA, USA)].

Compared to other wild animals inoculated experimentally with homologous rabies strains [25–27, 38], *D. rotundus* bats were extremely resistant to their homologous virus variant. Another experimental study showed 5×10^4 MICLD$_{50}$ to produce disease in 50% of the animals [29], a result comparable to this study in which 10^5 MICLD$_{50}$ produced disease in 60% of the animals.

Our results demonstrate that *D. rotundus* is indeed very resistant to rabies disease and can produce antibody titres that would be considered protective in humans. This resistance may explain the role of these bats in rabies transmission in nature.

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