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EXPERIMENTAL RABIES INFECTION AND ORAL VACCINATION IN VAMPIRE BATS (*DESMODUS ROTUNDUS*)

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ABSTRACT

A rabies virus variant isolated from a vampire bat (*Desmodus rotundus*) and characterized by genome sequencing was used for the standardization of an experimental infection in this species. Theparenteral administration of 10^6 $MICLD_{50}$ of this variant was capable of inducing death from rabies in 89% of animals. The mean duration of post-challenge survival was 12 days. None of the experimental rabid vampire bats showed aggressive behaviour. A vaccinia-rabies glycoprotein recombinant virus vaccine was administered orally to vampire bats on days -120, -90, -30 or -18 pre-challenge, on the same day of challenge, or on day +5 post-challenge. A significant protection was noticed only in animals vaccinated on days -18 or -30 pre-challenge. A longer period of incubation was observed in animals vaccinated 5 days post- challenge.

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In tropical and subtropical latin american countries, the major sylvatic rabies vector is the vampire bat (Desmodus rotundus) [1,2]. This species is responsible for heavy losses in livestock and is increasingly involved in human rabies transmission [3]. Presently, the control of sylvatic rabies is attempted by reducing hematophagous bat populations by poisoning with anticoagulants, by pre-exposure immunization of cattle, and preventive vaccination and post-exposure treatments in humans [4].

Bat population reduction has been accomplished by systemic anticoagulant treatment in cattle (bats feeding on treated animals are poisoned) and anticoagulants administered on the back of captured bats (allogrooming behaviour results in poisoning of congenerics)[5-7]. During recent years, most of the research on the control of sylvatic rabies has concentrated on developing methods of oral vaccination of wild rabies vectors [8 9]. In Europe, three types of vaccine were developed and extensively used in the field [10]: SAD (Street Alabama Dufferin) attenuated strains of rabies virus (SAD B19, SAD bern and SAD P5-88), avirulent mutants of the SAD bern strain (SAG 1 and SAG2) [11] and a recombinant vaccinia virus which expresses the immunizing glycoprotein of rabies virus (V-RG) [12,13].

Between 1989 and 1995, about 8.5 million V-RG vaccine doses have been dispersed in western Europe for the vaccination of red foxes (*Vulpes vulpes*) and in the USA for the vaccination of raccoons (*Procyon lotor*) and coyotes (*Canis latrans*) [14].

Programmes of oral vaccination of wild vectors have led to the elimination of sylvatic rabies from large areas, which have consequently been freed from vaccination [10,14]. In a preliminary study, the authors have shown that the V-RG is innocuous to vampire bats whatever the route of administration (scarification, oral, parenteral and aerosol) (unpublished data). In this work, a rabies virus variant isolated from a vampire bat and characterized by genome sequencing [15] was used for the standardization of an experimental infection in this species. The authors have evaluated the efficacy of the V-RG vaccine, administered orally, to protect vampire bats against rabies.

Method

ANIMALS

Vampire bats were captured in an area where sylvatic rabies is not documented (state of San Luis Potosi, Mexico). Captures were performed with Japanese nets placed around cattle corrals.

Captive vampire bats were transported by airplane to the "Instituto Nacional de Investigaciones Forestales y Agropecuarias" (INIFA) (Mexico City) were they were housed in metallic cages (1m x 1m x 1m), maintained in a room (51 m^3) at a constant temperature of 23°C and a relative humidity of 70%.

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Vampire bats were fed daily with defibrinated cattle and/or sheep blood (20 ml per animal), with added vitamins and stored frozen in 1l non-toxic plastic bottles. The period of quarantine and conditioning to captivity varied from 60 to 90 days. All dead animals during this period were submitted to rabies diagnosis.

At the end of the quarantine period, all experimental vampire bats were marked with electronic microchips (Indexel®-Rhone Mérieux) injected subcutaneously in the dorsal region.

RABIES VIRUS

The rabies virus variant 'CASS-88', isolated in 1988 from a rabid vampire bat (INIFA, Mexico City), was used for establishing a standard rabies challenge in vampire bats.

This variant was characterized by the PCR amplification technique of nucleic acids followed by sequencing [16,17]. The sequencing was carried out in a region of 330 nucleotides (110 AA) covering the region 140-249 of the glycoprotein ectodomain [15]. This choice of region was relevant to its representation and because it covers the major antigenic site IIa of the rabies glycoprotein.

In a previous work, it was determined that the parenteral inoculation of $3.7x10^6$ mouse intracerebral lethal doses 50% ($MICLD_{50}$) ml^{-1} of the CASS-88 strain killed 100% of adult cattle [18]. The infectivity titer of this strain inoculated intracerebrally in 4-6 week-old BALB/C mice was $10^{8.03}$ LD_{50} ml^{-1}.

VACCINE

The vaccinia-rabies glycoprotein recombinant virus (V-RG) (Raboral®-Rhone Mérieux), propagated on cultured baby hamster kidney (BHK) cells, was used to establish a freeze-dried master stock from which all preparations were derived (<5 passages) (batch no. 5L24).

Lyophilized V-RG (titer: 10^8 $CCID_{50}$) was resuspended in 1 ml of defibrinated cattle blood, free of rabies antibodies.

After being left to fast for 48 h, vampire bats were administered orally with 1 ml of V-RG blood suspension by direct application into the mouth with a needleless syringe.

SEROLOGY

For rabies serological analysis, serum was obtained by centrifugation of 0,4 ml of blood collected from the marginal vein of the wing. All animals were bled before any treatment, and were demonstrated to be serologically negative for rabies virus antibodies.

Rabies virus neutralizing antibody (VNA) titers were determined by the rapid fluorescent foci inhibition test (RFFIT)[19] and expressed in International Units per ml (1.U. ml^{-1}) serum as determined by comparison with a standard serum. The conventionally defined level of 0.5 I.U. ml^{-1} in humans is considered indicative of successful rabies immunization.

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RABIES DIAGNOSIS

The presence of rabies virus in the brain of dead vampire bats was detected by the fluorescent antibody test (FAT) according to the recommendations of the World Health Organization [20].

EXPERIMENTAL PROTOCOLS

Experiment 1: rabies infection (Table 1). Thirty two vampire bats were divided into 4 separately housed groups, each containing 8 specimens (groups 1, 2, 3 and 4). On day 0, animals from groups 1, 2 and 3 were inoculated intramuscularly (dorsal muscle) with 10^6, $5x10^5$ and $5x10^4$ $MICLD_{50}$ respectively. Animals from group 4 were used as non-challenged controls and were injected intramuscularly with an equal volume of PBS (phosphate buffered saline, pH 7.4).

Table 1. *Experimental infection of vampire bats with the CASS-88 variant of rabies virus injected by the intramuscular route*

Bat no.	Challenge dose$_{50}$) (MICLD	Challenge results[a]	Clinical signs	Survival rate on day 90 post-challenge (%)	VNA on day 90 post-challenge (I.U. ml^{-1})
Group 1	10^6			12.5	
1		D (8)	+		
2		D (9)	+		
3		D (10)	+		
4		D (10)	+		
5		D (10)	–		
6		D (13)	–		
7		D (30)	–		
8		S			2
Group 2	5×10^5			37.5	
9		D (10)	+		
10		D (10)	+		
11		D (10)	+		
12		D (12)	+		
13		D (13)	–		
14		S			1.5
15		S			4.5
16		S			2.5
Group 3	5×10^4			50	
17		D (10)	+		
18		D (15)	+		
19		D (16)	–		
20		D (19)	–		
21		S			1
22		S			1
23		S			1
24		S			1.5
Group 4 25–32 (controls)	PBS	S		100	<0.5

[a]D: died (+ day of death)—FAT positive.
S: survived—euthanasia on day 90 post-challenge—FAT negative.

Experiment 2: oral vaccination (Table 2). In the first trial (trial 1), animals were challenged either 18 days after vaccination (10 bats), 5 days prior to vaccination (10 bats), or vaccinated and challenged simultaneously (9 bats). In the following two trials (trials 2 and 3), they were challenged either 30 (10 bats), 90 (9 bats) or 120 (8 bats) days after vaccination. In each trial, 10 unvaccinated challenged vampire bats were used as controls.

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Table 2. *Induction of virus-neutralizing antibodies and protection from rabies in vampire bats immunized orally with a vaccinia-rabies glycoprotein recombinant virus vaccine*

Group (n bats)	Vaccination time	Seroconversion rate on day −1 pre-challenge (%)	Fraction succumbing to challenge (+ mean duration of survival)	Fraction with clinical disease (+ duration)	Survival rate on day 90 post-challenge (%)
8 bats (trial 2)	day −120 pre-challenge	12.5	7/8 μ = 11.1 days	1/7 1 day	12.5
9 bats (trial 3)	day −90 pre-challenge	44	6/9 μ = 11.3 days	3/6 μ = 1.7 days	33
10 bats (trial 2)	day −30 pre-challenge	50	2/10 μ = 12.5 days	1/2 2 days	80[a]
10 bats (trial 1)	day −18 pre-challenge	0	4/10 μ = 15.2 days	4/4 μ = 2.7 days	60[a]
9 bats (trial 1)	day 0 (day of challenge)	0	9/9 μ = 11.9 days	2/9 μ = 1 day	0
10 bats (trial 1)	day +5 post-challenge	0	9/10 μ = 14.3 days[b]	3/9 μ = 1.3 days	10
Controls					
10 bats (trial 1)	non-vaccinated	0	9/10 μ = 11.5 days	3/9 μ = 1.7 days	10
10 bats (trial 2)	non-vaccinated	0	8/10 μ = 13.2 days	6/8 μ = 1.3 days	20
10 bats (trial 3)	non-vaccinated	0	10/10 μ = 10.7 days	3/10 μ = 1.3 days	0

[a]Survival rate significantly higher (as compared with in corresponding controls). Trial 1: $p = 0.0286$; trial 2: $p = 0.0115$ (unilateral Fischer test).
[b] Mortality delay significantly longer (as compared with corresponding controls). $p = 0.024$ (non-parametric Mann–Whitney test).

The rabies challenge was performed by intra- muscular inoculation of each bat with 10^6 $MICLD_{50}$. In both experiments, animals were monitored daily for a period of 90 days post-challenge and the presence of rabies virus in the brain of succumbing animals was detected by FAT. The day of death post-inoculation and the duration of clinical disease were recorded.

At the end of the observation period, the surviving bats were euthanized and examined by FAT for the presence of the rabies antigen.

Animals were bled for serological analysis either on day 90 post-challenge (survivors in experiment 1) or on day -1 before challenge (experiment 2).

Results

EXPERIMENT 1: RABIES INFECTION

As shown in Table 1, 87.5% (7/8 animals), 62.5% (5/8 animals) and 50% (4/8 animals) of vampire bats died from rabies when inoculated intramuscularly with 10^6, $5x10^5$ and $5x10^4$ $MICLD_{50}$ of the CASS-88 variant, respectively.

The mean duration of post-challenge survival was likely to vary according to the inoculum titer. In groups 1, 2 and 3, the mean number of days prior to death was 12.85, 11.8 and 15 respectively. The

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longest incubation period of the disease was 30 days while the shortest was 7 days; both these extreme periods were observed in animals inoculated with 10^6 $MICLD_{50}$.

Clinical signs (anxiety, altered reflex, tremor and paralysis) were observed in 10/16 (62%) rabid bats. The duration of clinical disease was less than 24 h before death. All specimens that died during the 90-day observation period were rabies-positive.

On day 90 post-challenge, all survivors had developed rabies antibody titers varying from 1 to 4.5 I.U. ml^{-1}.

EXPERIMENT 2: ORAL VACCINATION

Table 2 shows the seroconversion and survival rates in each vampire bat group challenged with 10^6 $MICLD_{50}$ of the CASS-88 rabies virus variant. For each group, the onset and mean duration of clinical disease and the mean duration of survival are also presented.

CONTROLS

Survival rates in non-vaccinated controls varied from 0 to 2/10. Among succumbing animals, 12/27 (44%) developed clinical signs of rabies virus infection and 56% died without prior neurological signs. The duration of post-challenge survival varied from 7 to 26 days (μ=12 days) and the duration of the clinical signs was 2 days maximum (t=1.4 days).

VACCINEES

Among vaccinees, the highest survival rate (8/10) was obtained in animals vaccinated 30 days before challenge. The survival rate was lower (6/10) for animals vaccinated 18 days before challenge but still statistically significantly higher than for the controls of the same trial (unilateral Fisher test: p=0.0286). When animals were vaccinated 90 days before challenge the authors observed a 33% protection which is not statistically significant. Vaccination 120 days before challenge or on the day of challenge or five days later did not show any protection at all.

In every trial, all animals that died during the observation period were shown to be positive for rabies diagnosis whereas all survivors were negative.

According to the non parametric Mann-Whitney test, vampires vaccinated on day +5 died later than controls (significant level: p=0.024). No statistical difference in mortality delay was observed between other vaccinees and the corresponding controls.

Only 9/31 (29%) vaccinated rabid animals belonging to groups showing no significant protection against challenge (group vaccinated either on day -120, -90, 0 or + 5) developed clinical signs of rabies infection, whereas 5/6 (83%) of vaccinated rabid animals belonging to groups showing protection (in group vaccinated either on day -18 or -30) developed clinical signs of rabies infection.

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Non-vaccinated controls, vaccinees on day -18 pre-challenge, on day 5 post-challenge or on the day of challenge were seronegative on day -1 pre-challenge.

Post-vaccinal seroconversion (day -1 pre-challenge) was observed in 5/10 vaccinees 30 days pre-challenge (VNA titers from 0.7 to 4.2 I.U. ml^{-1}) (5 survivors), in 4/9 vaccinees 90 days Rre-challenge (VNA titers from 0.66 to 1.12 I.U. ml^{-1})(2 survivors-2 non-survivors) and in 1/10 vaccinees 120 days pre-challenge (VNA titer: 0.7 U.I. ml^{-1}) (1 survivor). Among the 18 bats that were vaccinated before challenge and resisted challenge, 8 had seroconverted to rabies (in group vaccinated either 120, 90 or 30 days pre-challenge). Low titers (0.66 I.U. ml^{-1}) of seric antirabies antibodies were also detected in two succumbing animals that were vaccinated 90 days before challenge. Ten animals exhibiting undetectable levels of VNA also resisted challenge (in group vaccinated either 18, 30 or 90 days pre-challenge).

DISCUSSION

When adding data from experiments 1 and 2, a total of 34/38 (89%) unvaccinated vampire bats succumbed to the intramuscular inoculation of 10^6 $MICLD_{50}$ of the CASS-88 rabies virus variant (Tables 1 and 2). Succumbing animals either developed clinical signs of rabies virus infection (47%) or died without prior neurological signs (53%). The duration of the clinical disease was 2 days maxim um (average: 1.3 days) and the duration of post-challenge survival was between 30 and 7 days (average 12 days). These results attest to the efficacy of the experimental rabies infection. Vampire bats seem to be less sensitive to their homologous virus variant than foxes are. It was demonstrated that 0.33 $MICLD_{50}$ of the 'vulpine' homologous rabies virus variant is capable of killing 50% of red foxes [21] whereas $5x10^4$ $MICLD_{50}$ of the CASS-88 'vampire bat' homologous variant is required to kill 50% of vampire bats.

No aggressive behaviour against their congenerics was detected in experimentally infected bats. This observation suggests that aerosols, allogrooming behaviour or food sharing could play a role in the intraspecific transmission of the disease.

Our results demonstrate that V-RG, administered by orally to vampire bats, is capable of eliciting the production of rabies antibodies and confering protection against severe rabies challenge. Up to 60% of vaccinated vampires were protected against a challenge administered 30-18 days later, and this killed 80-90% non-vaccinees respectively. With longer or shorter delays between vaccination and challenge, non-significant protection was observed. This partial and short-lived immunity contrasts with the one observed in some terrestrial wild vectors similarly vaccinated with the VR-G. The duration of protection reached a minimum of 18 months in 100% red foxes (*Vulpes vulpes*)[22] and 6 months in 80% raccoons (*Procyon lotort*)[23].

The absence of seroconversion and partial lethality observed in animals vaccinated 18 days before challenge could be due to the fact that the rabies challenge was performed too early after vaccination. The longer mean duration of clinical disease (2.7 days rather than 1.7 days in the

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control group) observed in succumbing bats from this group could be indicative of an immunization outset.

Resistance to challenge was also observed in seronegative bats vaccinated on day -30 or -90. Such a protection, despite the absence of detectable antirabies antibodies, also reported in the red fox [13], suggest the relevance of cell-mediated immunity in defence against rabies.

A longer mean duration of post-challenge survival (14.3 days) also occurred in animals vaccinated 5 days after challenge. A similar 'late death' phenomenon was observed in foxes [24] as well as in mice [25] vaccinated after challenge a few days before the onset of clinical disease in non-vaccinated controls.

In previous studies, it was also demonstrated that an 'early death' phenomenon may occur in foxes and mice vaccinated and challenged simultaneously or vaccinated early after challenge (before the production of antirabies antibodies)[24,26]. This 'early death' phenomenon was suggested to be an immunopathological consequence (involving B lymphocytes or antirabies antibodies) of interactions between vaccination and infection [27]. In the present work, this phenomenon was not observed in vampire bats vaccinated and challenged on the same day.

If the control of vampire bat population is difficult using traditional measures that were furthermore shown to be ineffective for some time, more attention should be focused on the possibility of forming immune barriers by means of vaccination.

Regarding the present results, further research is required to obtain a vaccination procedure capable of confering a more durable protection to vampire bats. Furthermore, studies on the ecology and behaviour of this species are still needed to determine the most applicable route of vaccine administration in field conditions and consequently for planning a strategy of vaccine distribution. Even if the oral route of administration (directly in defibrinated blood or administered on the back of captured bats like anticoagulants) seems to be the most appropriate, other routes (scarification, aerosol) should be tested. Further studies are also needed to describe the excreting patterns of rabies virus in the vampire bat and the possibility of vaccinated bats excreting and transmitting VR-G.

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