Journal of General Virology (2008), **89**, 1998–2010 **DOI** 10.1099/vir.0.83688-0

Susceptibility of North American big brown bats (*Eptesicus fuscus*) to infection with European bat lyssavirus type 1

R. Franka,[1] N. Johnson,[2] T. Müller,[3] A. Vos,[4] L. Neubert,[4] C. Freuling,[3] C. E. Rupprecht[1] and A. R. Fooks[2]

Correspondence
R. Franka
rpf5@cdc.gov

[1]Centers for Disease Control and Prevention, 1600 Clifton Road, Mail-Stop G33, Atlanta, GA 30333, USA

[2]Department of Virology, Rabies and Wildlife Zoonoses Group, WHO Collaborating Centre for the Characterization of Rabies and Rabies-related Viruses, Weybridge, Addlestone, Surrey KT15 3NB, UK

[3]Friedrich Loeffler Institute, Federal Research Institute of Animal Health, 16868 Wusterhausen, Germany

[4]IDT Biologika, Am Pharmapark, 06861 Dessau-Roßlau, Germany

The aim of this study was to determine the susceptibility of insectivorous bats (using the big brown bat as a model) to infection with European bat lyssavirus type 1a (EBLV-1a), to assess the dynamics of host immune responses and to evaluate the opportunity for horizontal viral transmission within colonies. Two isolates of EBLV-1a, originating from Slovakia (EBLV-1aSK) and Germany (EBLV-1aGE), were tested. Four different routes of inoculation were used with isolate EBLV-1aSK [$10^{4.8}$ mouse intracerebral median lethal dose ($MICLD_{50}$) in 50 μl]: intramuscular (i.m.) in the deltoid area or masseter region, *per os* (p.o.) and intradermal (i.d.) scratches. Isolate EBLV-1aGE ($10^{3.2}$ and $10^{2.2}$ $MICLD_{50}$ in 20 μl) was inoculated via the intranasal (i.n.), i.m. (low- and high-dose groups, into pectoral muscles); p.o. and intracerebral (i.c.) routes. None of the bats infected by the i.n., p.o. or i.d. route with either virus isolate developed disease during the experiments (91 or 120 days, respectively). Incubation periods were 9–12 days for i.c.-inoculated bats (66 % mortality), 12–33 days for bats inoculated i.m. with the higher dose (23–50 % mortality) and 21–58 days in bats inoculated i.m. with the lower dose of virus (57 % mortality). Virus or viral RNA in bat saliva was detected occasionally, as early as 37 days before death. All i.d.-inoculated and the majority of i.m.-inoculated bats seroconverted within 7–10 days of inoculation. These observations suggest that exposure of bats to varying doses of EBLV-1 from rabid conspecifics via natural (i.d.) routes could lead to an abortive infection and serve as a natural mode of immunization resulting in the presence of virus-neutralizing antibodies in free-ranging bats.

Received 26 December 2007
Accepted 6 March 2008

INTRODUCTION

Of the seven viral species belonging to the genus *Lyssavirus* and four putative newly isolated species, all except Mokola virus have reservoirs in bats. The European bat lyssaviruses are maintained by specific bat hosts with occasional spillover to other mammalian species. The importance of bats as rabies reservoirs and their public health significance have been reinforced by reports from the USA where rabies virus (RABV) variants (genotype 1) harboured by bat species, particularly *Lasionycteris noctivagans*, *Pipistrellus subflavus*, *Tadarida brasiliensis* and *Eptesicus fuscus*, have been responsible for most human rabies cases in the last 50 years (Messenger *et al.*, 2002, 2003).

In Europe, epizootiological studies have revealed that at least two distinct genotypes belonging to the genus *Lyssavirus* circulate in bat populations: European bat lyssavirus type 1 (EBLV-1, genotype 5) and European bat lyssavirus type 2 (EBLV-2, genotype 6). Both genotypes are further divided into 'a' and 'b' sublineages based on their phylogenetic relationships (Bourhy *et al.*, 1992; Amengual *et al.*, 1997).

According to EUROBATS, an organization dedicated to the conservation of European bat populations, 45 species of bat are geographically distributed throughout Europe (Eurobats, 2004). So far, EBLV-1 has been found predominantly in specific association with the serotine

bat (*Eptesicus serotinus*), although it has also been reported in *Nyctalus noctula*, *Vespertilio murinus*, *Myotis myotis*, *Myotis dasycneme*, *Myotis daubentonii*, *Pipistrellus pipistrellus*, *Pipistrellus nathusii*, *Myotis nattereri*, *Rhinolophus ferrumequinum* and *Miniopterus schreibersii* (Selimov *et al.*, 1991; Schneider & Cox, 1994; Serra-Cobo *et al.*, 2002; Van der Poel *et al.*, 2005; Kuzmin & Rupprecht, 2007). EBLV-2 has been reported less frequently, appears to be associated more commonly with *Myotis* species (*M. daubentonii and M. dasycneme*) and is isolated predominantly from bats in The Netherlands, the UK and Switzerland (Fooks *et al.*, 2003a; King *et al.*, 2004; Harris *et al.*, 2006).

The first report of bat rabies in Europe was in 1954 (Mohr, 1957). Fourteen cases of bat rabies were reported throughout Europe from 1954 to 1984 (Kappeler, 1989; Baer & Smith, 1991; Schneider & Cox, 1994; King *et al.*, 2004). The first documented human rabies case associated with European bats was reported in 1977 (not laboratory confirmed) and led to additional epizootiological investigations. Between 1977 and 2006, a total of 826 cases of bat rabies were detected in Europe and reported to the World Health Organization Collaborating Centre for Rabies Surveillance and Research at the Friedrich-Loeffler-Institute, Germany. This figure, however, is still a conservative estimate as not all European countries conduct surveillance for bat rabies and sampling bias is common (Harris *et al.*, 2006). By comparison, in the USA, 1408 rabid bats were reported in 2005 alone (Blanton *et al.*, 2006). In Europe, most rabid bats were reported from The Netherlands, Denmark, Germany and Poland, accounting for more than 90 % of all rabid bats recorded for this period (Müller *et al.*, 2007; Vos *et al.*, 2007). Bat rabies was also reported from France, Spain, Switzerland, Great Britain, the Czech Republic, Slovakia, Hungary, Ukraine and Russia (Ondrejkova *et al.*, 2004; Müller *et al.*, 2007).

Three laboratory-confirmed human rabies cases associated with EBLV-1 (Belgorod, Russia, 1985) or EBLV-2 (Helsinki, Finland, 1985; Dundee, Scotland, 2002) have been reported in Europe to date (Lumio *et al.*, 1986; Selimov *et al.*, 1986, 1989; Roine *et al.*, 1988; Fooks *et al.*, 2003a, b). Two additional human rabies cases, assumed to be associated with EBLV based on clinical symptoms and known bat exposure, were reported but not laboratory confirmed (Voroshilovgrad, currently Lugansk, Ukraine, 1977; Molodogvardeysk, Ukraine, 2002) (Scherbak, 1982, 1984; King *et al.*, 2004; Botvinkin *et al.*, 2005).

Only rare spillover events of EBLV to terrestrial mammals (sheep and stone marten) have been reported (Bourhy *et al.*, 1992; Stougaard & Ammendrup, 1998; Rønsholt, 2002; Müller *et al.*, 2004). Rarely, antibodies to EBLV-1 have been detected in domestic animals: two cats in Denmark (out of 152 tested) (Tjørnehøj *et al.*, 2004) and one sheep in Denmark (out of 2179 tested) (Tjørnehøj *et al.*, 2006). Conversely, a high seroprevalence of EBLV-neutralizing antibodies within bat populations (up to 60 % in *Eptesicus serotinus*, *Myotis myotis*, *Myotis nattereri*, *Miniopterus schreibersii*, *Tadarida teniotis* and *Rhinolophus ferrumequinum*) observed over many years suggests permanent circulation of EBLV (Grauballe *et al.*, 1987; Echevarría *et al.*, 2001; Serra-Cobo *et al.*, 2002; Amengual *et al.*, 2007).

All European bat species are protected by European Union regulations or by national legislation (EU Directives 92/43 and 97/62) (Harris *et al.*, 2006; Lina & Hutson, 2006). As a result, experimental infections of EBLV in natural indigenous hosts, such as *Eptesicus* or *Myotis* species, have not been studied rigorously. Kuzmin *et al.* (1994) studied the influence of hibernation on duration of the incubation period and virus distribution in *P. pipistrellus* infected with EBLV. Botvinkin *et al.* (1992) studied the susceptibility of *Myotis daubentoni* and *Myotis brandtii* to infection with RABV and EBLV. Limited experimental studies of EBLV pathogenesis have been conducted in non-reservoir species, such as Egyptian flying foxes (Van der Poel *et al.*, 2000), ferrets (Vos *et al.*, 2004a), red foxes (Baltazar *et al.*, 1988; Vos *et al.*, 2004b; Picard-Meyer *et al.*, 2008), dogs, mice and cats (Fekadu *et al.*, 1988) and sheep (Baltazar *et al.*, 1988; Tjørnehøj *et al.*, 2006; Brookes *et al.*, 2007).

The aim of this study was to investigate an insectivorous bat model for EBLV infection, to determine the susceptibility of the North American big brown bat (*E. fuscus*) to EBLV-1a via different routes of infection, to study the dynamics of host immune responses and virus excretion, and to evaluate the opportunity for intraspecific virus transmission. *E. fuscus* was selected as a model because of the close phylogenetic relationship and ecological similarities to *E. serotinus*, the natural reservoir of EBLV-1 in Europe.

METHODS

Virus. Two EBLV-1 isolates were selected for experimental infection of big brown bats. The first was isolated from the brain of a naturally infected serotine bat (*E. serotinus*), collected in the eastern part of Slovakia in Prešov in 1998 (EBLV-1aSK, GenBank accession nos AY863382, AY863323, AY863295 and AY863257; Ondrejkova *et al.*, 2004; Davis *et al.*, 2005). The second virus was isolated from a serotine bat in Osnabrück, Germany, in 1997 (EBLV-1aGE) (GenBank accession nos DQ522860 and DQ522892; Müller *et al.*, 2007). These isolates were selected because of their availability at relatively high titre after a limited number of passages in laboratories participating in this study and because of the availability of sequence information for both isolates. Each virus was used after the fourth or third intracerebral (i.c.) mouse brain passage in BALB/c and ORF1 mice, respectively. Mouse brain suspensions (20 %) were prepared in minimal essential medium (MEM-10; Gibco) supplemented with 10 % fetal calf serum and clarified by centrifugation at 3200 ***g*** for 10 min. Aliquots of the supernatant were frozen and used for bat inoculations and titration in mice.

Animals. Sixty-six adult big brown bats (*E. fuscus*) of both sexes were captured from two roosts in Georgia, USA. Animals were housed in autoclavable cages and fed *ad libitum* with mealworms and water. All animal-handling and experimental procedures were undertaken in compliance with Centers for Disease Control and Prevention (CDC) Institutional Animal Care and Use Committee (IACUC) guidelines

and German Animal Welfare guidelines with the recommendations of GV-SOLAS (Society for Laboratory Animal Science). Ethical approval was obtained for each study before experiments began (IACUC CDC, USA; Federal State of Saxony-Anhalt, Germany; Veterinary Laboratories Agency Local Ethical Committee; UK).

Bats were quarantined for at least 1 month before inoculation. The titre of virus-neutralizing antibody (VNA) was measured against EBLV-1 before the experiments began to assess pre-existing VNA titres and previous exposure to lyssavirus. Two bats that had measurable titres of VNA against EBLV-1 were used for the experiment with the EBLV-1aSK isolate. For identification, animals were individually marked with ear tags (Experiment 1) or a microchip (Experiment 2) inserted subcutaneously.

Two experimental studies were undertaken in parallel in the CDC laboratory (Atlanta, USA: Experiment 1) and in the IDT Biologika laboratory (Dessau-Roßlau, Germany; Experiment 2) to assess two different EBLV-1 isolates from geographically distinct locations in Europe.

Experiment 1. Four routes of inoculation were used with EBLV-1aSK. An inoculum of $10^{4.8}$ mouse i.c. median lethal dose ($MICLD_{50}$) in 50 µl was injected intramuscularly (i.m.) into the deltoid area or the masseter region, inoculated intradermally (i.d.)/subdermally (s.d.) by scratching with a 31-gauge needle on the leading edge of each wing or applied *per os* (p.o.) by direct oral administration using a syringe. Four animals in each group were inoculated and two or three others were left uninfected as controls for transmission in each cage.

Experiment 2. Four routes of inoculation were used with EBLV-1aGE. A dose of $10^{3.2}$ $MICLD_{50}$ in 20 µl was applied i.m. into the pectoral muscle or intranasally (i.n.) into one nostril or i.c. into the left side of the brain and p.o. by direct oral administration. For i.n. and i.c. inoculation, animals were sedated using inhalation of ether. Additionally, one group of bats (negative control) was infected i.m. with a placebo (10 % mouse brain homogenate in PBS) and another group i.m. with a lower diluted dose of $10^{2.2}$ $MICLD_{50}$ in 20 µl into the pectoral muscle. The negative-control group was inoculated first, with virus challenge groups inoculated sequentially to avoid cross-contamination. Each bat was identified by microchip and its number recorded before inoculation.

Observation. In both experiments, bats were observed daily and any signs of disease were recorded (loss of coordination, inability to fly, vocalization, seizures or aggression). Animals with severe disease signs and those surviving to the end of the study period were euthanized by injection of 50 µl Ketaset III (Fort Dodge Animal Health). A final blood sample was taken by cardiac puncture and tissue samples, including brain, submandibular and parotid salivary glands, brown fat, tongue, trachea, heart, lung, kidney, liver, spleen and bladder, as well as pectoral muscle and thyroid, were collected during necropsy and stored at −80 °C.

Sampling. Animals were observed for 120 days in Experiment 1 and 91 days in Experiment 2. Each bat was bled before inoculation (day 0) to determine baseline VNA titres and at 2-week (Experiment 1) or 3-week intervals (Experiment 2) until completion of the study. Blood (10–100 µl) was collected from the brachial artery or vein at the distal epiphysis of the humerus, the uropatagial veins or via intracardiac routes (terminal bleeding after euthanasia; Kunz & Nagy, 1988) with heparinized microcapillary tubes. An anti-bleeding product (Kwik-Stop; ARC Laboratories) was applied to the puncture site. Blood was centrifuged at 5000 ***g*** for 20 min and the serum was separated, heat-inactivated at 56 °C for 30 min and stored at −80 °C.

Oral swabs were taken weekly for the first 2 months post-inoculation (p.i.) and every 2 weeks for the remainder of the experiments. Swabs were collected daily when the animal started to show clinical signs of rabies. In Experiment 1, two swabs were collected at each time point using sterile cotton-tipped swabs, previously moistened in MEM-10. One swab was placed directly into 1 ml MEM-10 for virus isolation, the second swab was placed into a tube containing 1 ml TRIzol reagent (Gibco) for RNA extraction and both were then placed immediately on dry ice and stored at −80 °C. For Experiment 2, one swab was collected at each time point using a sterile dry cotton-tipped swab. Swabs were placed into 0.4 ml MEM-10 for virus isolation and RNA extraction; RNA was extracted from 0.2 ml MEM-10 using a commercially available RNA extraction kit (Invitrogen) and the remainder was used for virus isolation.

Laboratory tests.

Virus detection. Antigen was detected in brain samples using a direct fluorescent antibody (DFA) test following a published method (Dean *et al.*, 1996) using either a fluorescein isothiocyanate (FITC)-conjugated anti-RABV monoclonal antibody (Fujirebio Diagnostics) or an FITC-conjugated polyclonal antibody (SIFIN). Virus isolation was undertaken using a rabies tissue-culture infection test as described previously (Webster & Casey, 1996).

RT-PCR, hemi-nested RT-PCR and quantitative real-time PCR. In Experiment 1, RT-PCR and hemi-nested RT-PCR were performed as described previously (Kuzmin *et al.*, 2008) with the following modifications. Reverse transcription was carried out using forward primer N1001fw (5′-CAGAGTTGTGCACCCCATGAA-3′, nt 1061–1081, all primer positions are according to the Pasteur virus genome, GenBank accession number M13215) for 90 min at 42 °C. Additionally, reverse primer 304rv (3′-TTGACAAAGATCTTGC TCAT-5′, nt 1517–1536) for primary RT-PCR and forward primer 1066fw (5′-GAGAGAAGATTCTTCAGGGA-3′, nt 1136–1155) and reverse primer 304rv for hemi-nested RT-PCR were used.

In Experiment 2, three different RT-PCR tests were used for the detection of viral RNA: a single-round PCR assay (Vos *et al.*, 2004a), a nested PCR assay (Heaton *et al.*, 1997) and a quantitative real-time PCR (Wakeley *et al.*, 2005). Amplicons were sequenced using standard protocols to confirm virus identity.

Modified rapid fluorescent focus inhibition test. A modified rapid fluorescent focus inhibition test was used to measure VNA titre as described elsewhere (Smith *et al.*, 1973) using EBLV-1 as the challenge virus. For Experiment 1, a reduced volume of serum was used (1.3 µl) as described previously by Kuzmin *et al.* (2008) with fivefold serum dilutions from 1 : 25 to 1 : 3125. For Experiment 2, the test was undertaken using modifications as described by Cox & Schneider (1976). Samples were tested in duplicate with twofold serum dilutions starting at 1 : 10.

RESULTS

Experiment 1

All animals infected with EBLV-1aSK via the i.m. route into the deltoid area developed VNA (Table 1, Fig. 1). Two of the animals died of rabies at 18 and 21 days p.i. (M54, M56; 50 % mortality). Both bats from the group that had demonstrable pre-existing VNA to EBLV-1 on day 0 (M82 and F83) survived a lethal EBLV-1 challenge (Table 1). One (M82) died on day 110 p.i., but EBLV was not detected in the brain or other tissues and this observation was therefore considered a non-specific death.

Table 1. Experiment 1: routes of inoculation, dynamics of antibody response, detection of virus or viral RNA in saliva and mortality of bats after EBLV-1aSK infection

All bats were inoculated with EBLV1a-SK ($10^{4.8}$ $MICLD_{50}$ in 50 µl).

Route of inoculation	Mortality from inoculated animals	Sex/bat ID	Outcome/day of death or euthanasia p.i.	Titre of VNA at days p.i.:					Detection of virus or viral RNA in saliva
				0	7	14	28	120	
i.m. (deltoid area)	2/4 (50 %)	M54	Died day 21	<25	<25	**1800**	Dead	Dead	**Positive (14 and 21 days p.i.)***
		M56	Died day 18	<25	<25	**60**	Dead	Dead	**Positive (18 days p.i.)***
		M82	Died day 110†	**170**	**2200**	**3125**	**3125**	Dead	Negative
		F83	Survived	**19**	**33**	**70**	**60**	<25	Negative
		F90‡	Survived	<25	<25	<25	<25	<25	Negative
		F91‡	Survived	<25	<25	<25	<25	<25	Negative
i.m. (masseter region)	1/4 (25 %)	F59	Survived	<25	**360**	**1500**	**340**	**75**	Negative
		F65	Survived	<25	**125**	**1600**	**480**	**480**	Negative
		F60	Survived	<25	**1800**	**3125**	**2200**	**1800**	Negative
		F69	Died day 18	<25	**110**	**3125**	Dead	Dead	Negative
		F71‡	Survived	<25	<25	<25	<25	<25	Negative
		F79‡	Survived	<25	<25	<25	<25	<25	Negative
i.d./s.d. (scratch)	0/4 (0 %)	F84	Survived	<25	<25	**320**	**625**	<25	Negative
		F74	Survived	<25	<25	**800**	**95**	**50**	Negative
		F89	Survived	<25	<25	**1800**	**65**	**60**	Negative
		F61R	Survived	<25	<25	**3125**	**800**	**360**	Negative
		F69R‡	Survived	<25	<25	<25	<25	<25	Negative
		M62‡	Survived	<25	<25	<25	<25	<25	Negative
		F73‡	Survived	<25	<25	<25	<25	<25	Negative
p.o.	0/4 (0 %)	F77	Survived	<25	<25	<25	<25	<25	Negative
		M81	Survived	<25	<25	<25	<25	<25	Negative
		F96	Survived	<25	<25	<25	<25	<25	Negative
		M97	Survived	<25	<25	<25	<25	<25	Negative
		F98‡	Survived	<25	<25	<25	<25	<25	Negative
		F99‡	Survived	<25	<25	<25	<25	<25	Negative

*Detection of viral RNA by RT-PCR or real-time PCR.
†Non-specific death.
‡Uninfected controls.

All four bats inoculated with EBLV-1 into the masseter muscle developed VNA by day 7 p.i., with a gradual decrease in titre towards the end of the experiment (Table 1, Fig. 1b). One animal (F69) succumbed to the lethal EBLV challenge on day 18 p.i. despite a VNA titre of 1 : 3125. In all three animals that succumbed to disease (M54 and M56 from the i.m. deltoid area group and F69 from the i.m. masseter group), viral RNA was detected in several organs, including the salivary glands (see Table 3). Viral RNA was detected intermittently in saliva 1 or 2 days prior to observation of clinical signs. In one case (M54), viral RNA was detected in the saliva 8 days before clinical signs of illness were recorded. Virus was not isolated in cell culture from any of the samples tested (swabs and salivary glands; Table 3).

All bats administered virus p.o. survived infection and none developed detectable levels of VNA. Similarly, no viral RNA or viral antigen was detected in any of the collected organs or in saliva samples (Table 1).

All four bats inoculated via the i.d./s.d. route (via scratches on the leading edge of each wing) remained well and developed high titres of VNA at day 14 p.i., with a rapid decrease towards the end of the experiment, starting on day 28 p.i. (Table 1, Fig. 1c). No viral RNA or virus was detected in the saliva or organs of these animals.

Neither the transmission controls nor the p.o.-infected animals developed VNA (Table 1). No significant patterns of virus distribution in different organs were observed (Table 3). In those bats that developed disease, extensive virus distribution was observed in peripheral tissues. EBLV-1 RNA, but not live virus, was detected in the salivary glands of all three rabid animals infected by the i.m. route (Table 3).

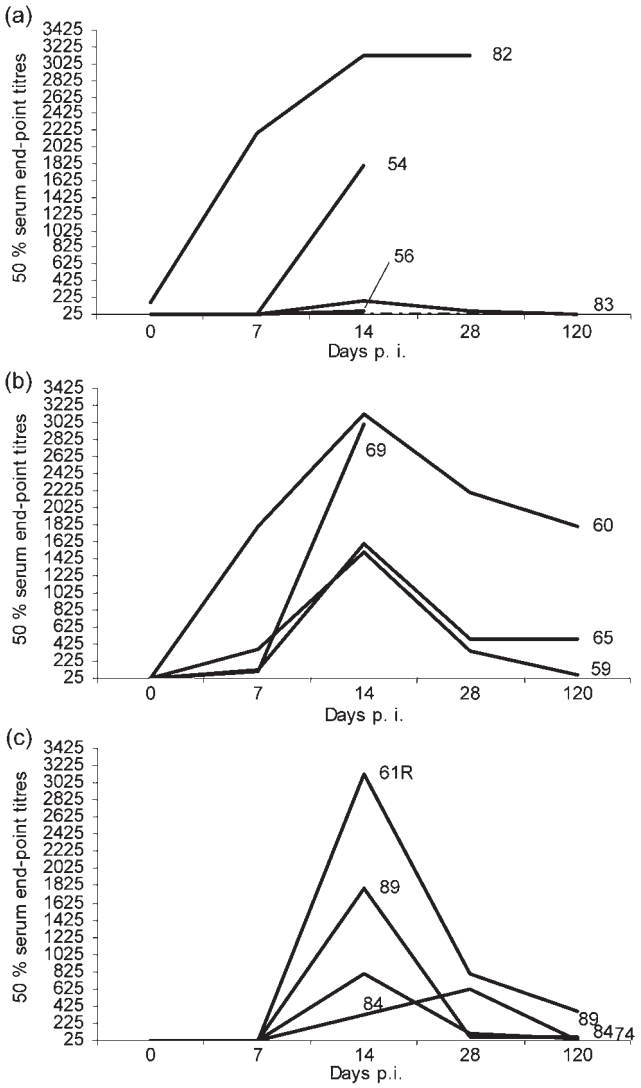

Fig. 1. VNA titres detected in bats injected i.m. with EBLV-1aSK into the deltoid area (a) or masseter region (b) or i.d./s.d. by scratches on the leading edge of each wing (c).

Experiment 2

None of the bats infected with EBLV-1aGE by either the i.n. or p.o. route succumbed to disease and none seroconverted during the course of the experiment (91 days). Of the seven bats infected i.c., one died immediately after i.c. inoculation (F61227) and four (4/6: 66.67 % mortality) died of EBLV-1 infection 9–11 days p.i., even though three of these animals (M63330, F62293 and F63955) developed VNA. Neither of the two surviving animals seroconverted (Table 2). For two animals (F62293 and F62793), virus antigen was detected in the brain and spinal cord but no other organ (data not shown). In two other animals from the i.c. group subjected to PCR, viral RNA was distributed widely in one case (M63330) in the brain, pectoral muscle, thyroid, lung, heart, spleen, kidney and bladder, whereas in a second case (F63955), viral RNA was limited to the brain and kidney (Table 3). Although no viral RNA was detected in the salivary gland of any animal inoculated by the i.c. route at the time of necropsy, virus was detected in the saliva of one animal (F62793), both by virus isolation and by RT-PCR, 2 days prior to death (Table 2).

For i.m. inoculation (in the pectoral muscle), two different doses of virus were used. In the group infected with a higher dose ($10^{3.2}$ $MICLD_{50}$ in 20 µl), three out of seven animals (43 % mortality) succumbed to EBLV-1 infection at day 12 (M62076 and F60803) and day 33 p.i. (F57599) (Table 2). None of the bats that died developed VNA. Of the four survivors, only one (F58563) developed a detectable VNA titre of 20 by day 35 p.i., which increased to 40 by the end of the experiment. Four out of seven bats (57 % mortality) inoculated i.m. with a lower dose of EBLV-1aGE ($10^{2.2}$ $MICLD_{50}$ in 20 µl) died at days 21, 32, 49 and 58 p.i. One bat seroconverted between days 11 and 35 p.i. but subsequently died at day 49 p.i. From three surviving bats, only one (M57612) seroconverted with a high titre of VNA and that bat maintained a high titre of VNA until the end of the experiment (Table 2).

One animal from the control group (F60161) inoculated with a placebo (10 % mouse brain homogenate in PBS) died 56 days after placebo inoculation. EBLV-1a was detected by DFA testing and PCR and was confirmed by sequencing to be identical to the inoculum (Table 3). None of the animals in the placebo group developed VNA.

Although 11 bats developed signs of rabies and subsequently died at 9–58 days p.i. (excluding the negative-control death), only four had virus detectable in saliva. One i.m. high-dose-inoculated bat (which died at 12 days p.i.) and two i.m. low-dose-inoculated bats (which died at 58 and 49 days p.i.) had detectable viral RNA in saliva only at a single sampling point during the experiment, on days 12, 21 and 35 p.i., respectively (Table 2). However, in only one of these cases was virus isolated in cell culture. One bat inoculated i.c., which died at 12 days p.i., had detectable viral RNA in saliva on days 10 and 11 p.i. Virus was only isolated in cell culture from the second sample (Table 2). Overall during the course of the experiment (91 days), from 34 inoculated bats (using various routes of inoculation), only two animals (i.c.-infected and i.m. low-dose-infected) had live virus isolated from a saliva sample at one sampling point during the infection. In one case, a bat (F67034) from the i.m. high-dose group that survived the experiment and did not display obvious clinical signs of rabies had detectable virus in saliva on day 28 p.i. (Table 2). No virus or viral RNA was detected in this bat after euthanasia at the end of the experiment.

No particular pattern of virus distribution in different organs was observed (Table 3). Besides the brain, viral RNA was most frequently detected in the tongue (not performed for EBLV-1aGE), pectoral muscle and lungs. Occasionally, viral RNA was found in the thyroid, heart, kidney, spleen,

Table 2. Experiment 2: routes of inoculation, dynamics of antibody response, detection of virus or viral RNA in saliva and mortality of bats after EBLV-1aGE infection

Virus (dose)	Route of infection	Mortality	Sex/bat ID	Outcome/day of death or euthanasia p.i.	Titre of VNA at days p.i.:					Detection of virus or viral RNA in saliva
					0	11	35	68	91	
EBLV-1aGE ($10^{3.2}$ $MICLD_{50}$ in 20 µl)	i.m. pectoral muscle high dose	3/7 (43 %)	M62076	Died day 12	<10	<10	Dead	Dead	Dead	Negative
			F61131	Survived	<10	ND	<10	<10	<10	Negative
			F67034	Survived	<10	ND	<10	<10	<10	**Positive (28 days p.i.)***
			F58563	Survived	<10	ND	**20**	**40**	**40**	Negative
			F57599	Died day 33	<10	ND	Dead	Dead	Dead	Negative
			F62124	Survived	<10	ND	<10	<10	<10	Negative
			F60803	Died day 12	<10	<10	Dead	Dead	Dead	**Positive (1 day p.i.)†**
	i.n.	0/7 (0 %)	F60947	Survived	<10	ND	<10	<10	<10	Negative
			F59501	Survived	<10	ND	<10	<10	<10	Negative
			F59699	Survived	<10	ND	<10	<10	<10	Negative
			M61399	Survived	<10	ND	<10	<10	<10	Negative
			F61808	Survived	<10	ND	<10	<10	<10	Negative
			M67087	Survived	<10	ND	<10	<10	<10	Negative
			F62057	Survived	<10	ND	<10	<10	<10	Negative
	i.c.	4/6 (66.6 %)	F61227	Died day 0‡	<10	Dead	Dead	Dead	Dead	ND
			M63330	Died day 9	<10	ND	**20**	Dead	Dead	Negative
			F62293	Died day 10	<10	**20**	Dead	Dead	Dead	Negative
			F63955	Died day 11	<10	ND	**40**	Dead	Dead	Negative
			F61400	Survived	<10	ND	<10	<10	<10	Negative
			F62793	Died day 12	<10	ND	Dead	Dead	Dead	**Positive (10† and 11* days p.i.)**
			F59027	Survived	<10	ND	<10	<10	<10	Negative
	p.o.	0/7 (0 %)	F66537	Survived	<10	ND	<10	<10	<10	Negative
			F58431	Survived	<10	ND	<10	<10	<10	Negative
			F61665	Survived	<10	ND	<10	<10	<10	Negative
			F66074	Survived	<10	ND	<10	<10	<10	Negative
			F65962	Survived	<10	ND	<10	<10	<10	Negative
			F57030	Survived	<10	ND	<10	<10	<10	Negative
			F60944	Survived	<10	ND	<10	<10	<10	Negative
EBLV-1aGE ($10^{2.2}$ $MICLD_{50}$ in 20 µl)	i.m. pectoral muscle, low dose	4/7 (57 %)	F60429	Survived	<10	ND	<10	<10	<10	Negative
			F63890	Survived	<10	ND	<10	<10	<10	Negative
			F61404	Died day 58	<10	ND	<10	Dead	Dead	**Positive (21 days p.i.)†**
			M57612	Survived	<10	ND	**160**	**640**	**640**	Negative
			F65219	Died day 49	<10	ND	**20**	Dead	Dead	**Positive (35 days p.i.)***

Table 2. cont.

Virus (dose)	Route of infection	Mortality	Sex/bat ID	Outcome/day of death or euthanasia p.i.	Titre of VNA at days p.i.:				Detection of virus or viral RNA in saliva
					0	11	35	68	91	
			F66582	Died day 32	<10	ND	Dead	Dead	Dead	Negative
			M66776	Died day 21	<10	ND	Dead	Dead	Dead	Negative
Placebo/negative	i.m. pectoral muscle	1/7	F66314	Survived	<10	ND	<10	<10	<10	Negative
			F66788	Survived	<10	ND	<10	<10	<10	Negative
			F62859	Survived	<10	ND	<10	<10	<10	Negative
			F60286	Survived	<10	ND	<10	<10	<10	Negative
			F60161	Died day 56	<10	ND	<10	Dead	Dead	Negative
			F61682	Survived	<10	ND	<10	<10	<10	Negative
			F66944	Survived	<10	ND	<10	<10	<10	Negative

*Isolation of virus in cell culture as well as PCR detection.
†Detection of viral RNA by RT-PCR or real-time PCR.
‡Died during anaesthesia immediately after i.c. inoculation.

bladder and liver. However, in the salivary glands, viral RNA was detected only in the group infected i.m. with the lower dose of virus. All four animals from this group had detectable levels of viral RNA.

Most notably, viral RNA was detected in the brain tissue and bladder in the bat that succumbed to rabies from the negative-control group. A typical pattern of behaviour, with some exceptions, was observed in animals developing signs of the disease in both experiments. Usually, the morbidity period lasted 1–2 days, but sudden death, without any other apparent signs, also occurred. Initially, sick animals either separated themselves or were excluded from the rest of the group. Paresis and paralysis later restricted the movement of sick bats and they remained at the bottom of the cage.

DISCUSSION

Rabies in New World bats is characterized by a relatively low prevalence of virus (1–4 % in suspect bats) and a high prevalence of VNA in colonies [65–70 % in *T. brasiliensis mexicana* (Burns & Farinacci, 1955; Steece & Altenbach, 1989); 3–35 % in *E. fuscus*, 7 % in *Lasionycteris noctivagans* and 31 % in *Lasiurus cinereus* (O'Shea *et al.*, 2003)]. Similarly, a high prevalence of VNA against EBLV-1 was reported from colonies of insectivorous bats from Spain (Serra-Cobo *et al.*, 2002; Amengual *et al.*, 2007). Only low seroprevalence (0.7–5.1 %, but at one location up to 31.6 %) of VNA against EBLV-2 has been detected in *Myotis daubentonii* bats (Smith *et al.*, 2006). A relatively high seroprevalence of VNA against lyssaviruses is rather specific for the order *Chiroptera* (mostly for gregarious species with much less data available for solitary species), with other mammalian species having a significantly lower seroprevalence despite a relatively high incidence of rabies. Based on field observations, O'Shea *et al.* (2003) suggested that bats might acquire immunity through exposure to low doses of virus that do not result in a productive infection. Our experiments, mimicking the natural route of infection, showed that, after the i.d./s.d. route of infection (via scratches), all animals seroconverted and survived the infection (Table 1). Because of the superficial nature of inoculation, the virus dose was difficult to control and to keep constant, and was certainly lower than the dose delivered via the i.m. inoculation. Although this fact limits the potential for parallel comparisons with the other routes of inoculation such as the i.m. route where the virus dose could be controlled more precisely, it more closely imitates natural exposure.

The dynamics of VNA production following i.m. and i.d. inoculation was characterized by a decrease starting at 21–28 days p.i. (slow for the i.m. masseter route; relatively rapid for the i.d. route; Fig. 1). This observation corresponds with the findings of Hatten *et al.* (1968) that the immune response to bacteriophage ΦX174 in bats (*E. fuscus*) is characterized by a rapid increase and then an

Table 3. Distribution of EBLV-1 or viral RNA in different tissues as determined by nested RT-PCR, real-time PCR and rabies tissue-culture infection test

ND, Not done.

Virus	Route of infection	Bat ID	Brain	Salivary glands	Brown fat	Pectoral muscle	Tongue	Trachea	Thyroid	Lung	Heart	Liver	Spleen	Kidney	Blood	Bladder
EBLV-1a SK	i.m. (deltoid)	M54	+	+ RNA	+	ND	+	+	ND	+	+	+	+	+	−	ND
		M56	+	+ RNA	+	ND	+	+	ND	ND	ND	ND	ND	ND	−	ND
	i.m. (masseter)	F69	+	+ RNA	−	ND	−	−	ND	+	+	−	−	+	−	+
EBLV-1aGE	i.m. (pectoral, high dose)	M62076	+	−	ND	+	ND	ND	−	+	+	+	−	−	ND	−
		F57599	+	−	ND	+	ND	ND	−	+	−	−	−	−	ND	−
		F60803	+	−	ND	−	ND	ND	+	+	+	−	+	−	ND	−
	i.c.	M63330	+	−	ND	+	ND	ND	+	+	+	−	+	+	ND	+
		F62293*	+	−	ND	−	ND	ND	−	−	−	−	−	−	ND	−
		F63955	+	−	ND	−	ND	ND	−	−	−	−	−	+	ND	−
		F62793*	+	−	ND	−	ND	ND	−	−	−	−	−	−	ND	−
	i.m. (pectoral, low dose)	F61404	+	+	ND	+	ND	ND	+	−	+	−	+	−	ND	+
		F65219	+	+	ND	+	ND	ND	+	−	+	+	+	+	ND	−
		F66582	+	+	ND	+	ND	ND	+	+	−	−	−	+	ND	+
		M66776	+	+	ND	−	ND	ND	−	+	−	−	ND	+	ND	+
	Placebo/negative	F60161	+	−	ND	−	ND	ND	−	−	−	−	−	−	ND	+

*Only histological examination was performed (no PCR).

equally rapid decrease (of limited magnitude and duration) in VNA titres when compared with the response and VNA titre against the same test antigen in rabbit and guinea pig. Similarly, Sétien *et al.* (1998) observed short-duration circulation of VNA in *Desmodus rotundus* after oral vaccination with a recombinant vaccinia–rabies glycoprotein vaccine. Short-duration VNA production and circulation with persisting long-lasting memory B cells could explain the temporal fluctuation in VNA titres at the individual as well as the population level. In contrast, Kuzmin *et al.* (2008) observed that VNA in bats previously infected with Irkut virus persisted for 12 months after virus challenge. These findings indicate that the dynamics of VNA could depend on viral dose, route of exposure and the lyssaviruses species involved. Differences in serological results between Experiments 1 and 2 (Tables 1 and 2) could be attributed partly to different isolates and different doses of virus used for inoculation, as well as to slight differences in the methods used for VNA titration. Overall, our experimental observations suggest that exposure to varying doses of EBLV-1 from rabid conspecifics within a colony via natural (i.d. and also partly i.m.) routes could lead to an abortive infection and serve as a natural mode of immunization resulting in the presence of VNA in free-ranging bats.

Although all of the p.o.- and i.n.-infected animals survived EBLV infection, none developed VNA, in contrast to the bats infected parenterally. These observations suggest a possible role for innate immunity in peripheral clearance of lyssaviruses in bats. The absence of mortality, any signs of disease and immune responses after p.o. and i.n. infection with a concentrated experimental dose of virus significantly diminishes the importance of aerosol infection as a route of virus transmission within bat populations for this particular lyssavirus species.

Reagan *et al.* (1957) demonstrated bat-bite transmission of RABV (isolated from dogs) from experimentally infected bats to suckling hamsters. In contrast, transmission of bat RABV from *T. brasiliensis* to monkeys or guinea pigs was not demonstrated, even though the clinically sick bats bit them (Burns *et al.*, 1958). No transmission of EBLV-1aSK to non-infected conspecifics (assessed by the absence of seroconversion) was observed in our experiments (transmission of EBLV-1aGE was not tested). Similarly, Shankar *et al.* (2004) did not observe any transmission of RABV from rabid big brown bats to control bats housed in the same cage. Very limited shedding of virus in the saliva of infected bats together with the low number of bats per group (when compared with the size of natural populations of *E. fuscus*) are the most credible explanations. In the case of EBLV-1aSK, viral RNA could be detected in bat saliva only at a single sampling 8 days before the clinical signs of rabies were reported. In the case of EBLV-1aGE, two i.m. low-dose-inoculated bats (which died at 58 and 49 days p.i.) had detectable levels of viral RNA in saliva at a single sampling during the experiment, on days 21 and 35 p.i., respectively. In the first case (F61404), viral RNA was detected in saliva by PCR 38 days before the evident signs of disease appeared (although no virus was isolated in cell culture). In the second case (F65219), viral RNA was detected and virus was isolated 15 days before any clinical signs of rabies. A lack of suitable conditions mimicking the natural environment, including colonial gregarious behaviour with frequent interactions and bites, could explain the inability to demonstrate experimental virus transmission to conspecifics.

In studies on experimental RABV infection in various North American bat species, virus variants isolated from naturally infected bats of the same species were used and overt signs of CNS disease and/or death were observed (Constantine, 1966b; Constantine & Woodall, 1966). Infection was generally widespread, with virus being detected in brain, brown fat, kidney and lung tissue and in salivary glands and saliva. Bite transmission of RABV from bats showing clinical signs of irritability and aggressiveness to foxes, coyotes, mice and hamsters was demonstrated (Constantine, 1966a, c; Constantine *et al.*, 1968). No evidence of inapparent RABV infection in bats was obtained in these studies. In our experiments, a similar organ distribution of virus and viral RNA was detected in infected animals – a finding that suggests similarities in spread and organ distribution of different species of lyssaviruses in bats (Table 3). Animals that received a lower i.m. virus dose appeared to have a broader organ distribution than bats receiving the higher i.m. dose and bats inoculated i.c. Incubation periods also varied depending on dose and route of inoculation, with the i.c. route of infection having the shortest incubation period (9–11 days). The observed extent of organ distribution following EBLV-1 infection was positively correlated with the length of incubation period and negatively correlated with virus dose.

As reported previously by Sulkin *et al.* (1959) and Bell & Moore (1960) for RABV, we also detected EBLV-1aSK (EBLV-1aGE was not tested) in the brown fat of bats that succumbed to infection, but the quantity of viral RNA was lower than in the brain, as compared by a semi-quantitative gel-electrophoresis-based analysis. On the basis of our observations, we doubt that brown fat has any significant role in lyssavirus pathogenesis other than being a destination for virus spread through innervation. By contrast, Bourhy *et al.* (1992) did not detect virus in brown fat in bats naturally infected with EBLV-1. No viral RNA was detected in the brain, salivary gland and other organs or in the saliva of bats that survived the experiments.

Observed clinical signs associated with rabies after EBLV-1 infection included tremors, irritability, aggressiveness and paralysis, but sudden death without any apparent signs of disease also occurred in rare cases. However, intermittent observations may be insufficient to record subtle changes in behaviour.

Several authors have previously reported lyssavirus isolation from the brains and salivary glands of apparently

healthy, normal-behaving bats taken from the wild (Scatterday, 1954; Burns *et al.*, 1956). Sporadic reports suggest the existence of a 'carrier state' of rabies among asymptomatic free-ranging bats (Pawan, 1936a, b; Sulkin, 1962; Rønsholt *et al.*, 1998; Echevarría *et al.*, 2001; Serra-Cobo *et al.*, 2002; Wellenberg *et al.*, 2002; Aquilar-Sétien *et al.*, 2005). Conversely, however, a 'carrier state' was not demonstrated in experimentally infected insectivorous bats (Kuzmin & Rupprecht, 2007). In our experiments, shedding of virus or viral RNA in saliva was detected in two bats inoculated i.m. with a low dose of EBLV-1aGE 15 and 38 days before detectable signs of rabies (Table 2). However, in only one case was viral RNA as well as infectious virus detected in saliva. The bat did not develop obvious clinical signs of rabies, survived the experimental infection (i.m. high dose of EBLV-1aGE) and did not seroconvert, and no virus or viral RNA was detected in collected tissues after euthanasia. Based on the limited data gathered, we can only speculate about a possible mechanism behind this observation. A lick or bite from a rabid conspecific just before sampling cannot be excluded. We have no evidence and no intention to suggest that virus reached the CNS, spread to salivary glands and was cleared afterwards, as no VNA was detected in this particular animal. Because human error cannot be excluded and because this observation contradicts current knowledge and most published data about lyssavirus pathogenesis, further experiments with several similar outcomes need to be conducted before firm conclusions or public health recommendations can be made in this regard.

Two bats that were inoculated i.c. survived the infection without seroconversion. Variability in the virus dose used for inoculation (the virus dose used could have been less than bat LD_{100}; no previous or parallel titration of virus in bats was done) or differences among immune system responses in individual bats are possible suggestions to explain this phenomenon. Although unlikely, unsuccessful inoculation resulting from the small insulin needle (31-gauge) failing to penetrate the skull or the brain membranes of adult bats cannot be excluded.

One animal from the control group (F60161) inoculated with a placebo (10 % mouse brain homogenate in PBS) died at 56 days p.i. and EBLV-1a identical to the inoculum used was detected by DFA and PCR testing and confirmed by sequencing (Table 2). No VNA was detected in this animal. The negative controls were handled first to avoid contamination from the other virus-challenged animals. Each group of bats was sufficiently separated from each other to prevent viral transmission by direct contact (biting). In addition, each bat received a microchip implant at the beginning of the experiment and was allocated to a specific group. Under these circumstances, there was no opportunity to misplace the bats in an incorrect cage. The airborne route was excluded as a possible explanation as a positive airflow from the bottom to the top of the cages was maintained throughout the experiment, preventing aerosolized droplets from falling from the upper cage (i.c. challenge) on to the lower cage (controls). Additionally, our experiments showed that all i.n.- or p.o.-inoculated bats survived the infection without seroconversion, a finding that makes the airborne hypothesis less likely unless the affected animal was immunosuppressed. Similarly, Davis *et al.* (2007) showed that, although some mice infected with North American bat RABV variants via an aerosol succumbed to rabies, all bats infected by the same route survived and developed VNA. Likewise, under optimal experimental conditions, RABV but not EBLV-2 was transmitted by the airborne route in a dose-dependent manner between experimental mice (Johnson *et al.*, 2006). We speculate that the bat was infected via saliva droplets because the i.c.-inoculated group was housed above the control group. This would, however, contradict the observed inability to infect bats via the p.o. route. With the current level of knowledge about lyssavirus pathogenesis, no plausible explanation exists for this intriguing observation unless mislabelling of collection tubes is considered.

Our study represents the first experimental proof of the natural immunization hypothesis and offers an explanation for the observation of VNA in free-ranging bats. The exposure of bats to varying doses of virus from rabid conspecifics via i.d. routes (frequent biting resulting from colonial behaviour and grooming with small dermal scratches) could result in an abortive infection and would serve as a mode of natural immunization. Stress, malnutrition, an immature immune system and immunosuppression enable productive infection and circulation of virus within a population with certain levels of herd immunity.

ACKNOWLEDGEMENTS

The authors thank the staff at the Centers for Disease Control and Prevention (CDC), Veterinary Laboratories Agency (VLA), IDT Biologika, and Friedrich-Loeffler-Institute (FLI) for their outstanding technical expertise and contributions to this work. In particular, we thank Dr Ivan Kuzmin (CDC Atlanta), Drs Annette Denzinger and Ingrid Kaipf (University of Tübingen), Karen Mansfield (VLA) and Jeanette Kliemt (FLI) for their valuable comments, suggestions, help and advice. We would also like to thank Drs Stefan Svrcek and Anna Ondrejkova from the University of Veterinary Medicine in Kosice, Slovak Republic, for providing us with the EBLV-1aSK isolate from their collection. Richard Franka was funded by an American Society for Microbiology and National Centers for Infectious Disease post-doctoral fellowship. This work was supported in part by a grant from the UK Department for Environment, Food and Rural Affairs (DEFRA ROAME SE0524). Use of trade names and commercial sources are for identification only and do not imply endorsement by the US Department of Health and Human Services. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funding agency.

REFERENCES

Aguilar-Sétien, A., Loza-Rubio, E., Salas-Rojas, M., Brisseau, N., Cliquet, F., Pastoret, P. P., Rojas-Dotor, S., Tesoro, E. & Kretschmer,

R. (2005). Salivary excretion of rabies virus by healthy vampire bats. *Epidemiol Infect* **133**, 517–522.

Amengual, B., Whitby, J. E., King, A., Cobo, J. S. & Bourhy, H. (1997). Evolution of European bat lyssaviruses. *J Gen Virol* **78**, 2319–2328.

Amengual, B., Bourhy, H., Lopéz-Roig, M. & Serra-Cobo, J. (2007). Temporal dynamics of European bat lyssavirus type 1 and survival of *Myotis myotis* bats in natural colonies. *PLoS ONE* **2**, e566.

Baer, G. M. & Smith, J. S. (1991). Rabies in nonhematophagous bats. In *The Natural History of Rabies*, 2nd edn, pp. 341–366. Edited by G. M. Baer. Boca Raton, FL: CRC Press.

Baltazar, R. S., Blancou, J. & Artois, M. (1988). Study of rabies virus isolated from an European bat *Eptesicus serotinus* – virulence for sheep and fox. *Rev Med Vet (Toulouse)* **139**, 615–621.

Bell, J. F. & Moore, G. J. (1960). Rabies virus isolated from brown fat of naturally infected bats. *Proc Soc Exp Biol Med* **103**, 140–142.

Blanton, J. D., Krebs, J. W., Hanlon, C. A. & Rupprecht, C. E. (2006). Rabies surveillance in the United States during 2005. *J Am Vet Med Assoc* **229**, 1897–1911.

Botvinkin, A. D., Kuzmin, I. V. & Chernov, S. M. (1992). Experimental infection of bats with lyssaviruses serotype-1 and serotype-4. *Vopr Virusol* **37**, 215–218.

Botvinkin, A. D., Selnikova, O. P., Antonova, L. A., Moiseeva, A. B. & Nesterenko, E. Y. (2005). Human rabies case caused from a bat bite in Ukraine. *Rabies Bulletin Europe* **29**, 5–7.

Bourhy, H., Kissi, B., Lafon, M., Sacramento, D. & Tordo, N. (1992). Antigenic and molecular characterization of bat rabies virus in Europe. *J Clin Microbiol* **30**, 2419–2426.

Brookes, S. M., Klopfleisch, R., Müller, T., Healy, D. M., Teifke, J. P., Lange, E., Kliemt, J., Johnson, N., Johnson, L. & other authors (2007). Susceptibility of sheep to European bat lyssavirus type-1 and -2 infection: a clinical pathogenesis study. *Vet Microbiol* **125**, 210–223.

Burns, K. F. & Farinacci, C. J. (1955). Rabies in nonsanguivorous bats of Texas. *J Infect Dis* **97**, 211–218.

Burns, K. F., Farinacci, C. J. & Murnane, T. G. (1956). Rabies in insectivorous bats of Texas. *J Am Vet Med Assoc* **128**, 27–31.

Burns, K. F., Shelton, D. F. & Grogan, E. W. (1958). Bat rabies: experimental host transmission studies. *Ann N Y Acad Sci* **70**, 452–466.

Constantine, D. G. (1966a). Transmission experiments with bat rabies isolates: responses of certain Carnivora to rabies virus isolated from animals infected by nonbite route. *Am J Vet Res* **27**, 13–15.

Constantine, D. G. (1966b). Transmission experiments with bat rabies isolates: reaction of certain Carnivora, opossum, and bats to intramuscular inoculations of rabies virus isolated from free-tailed bats. *Am J Vet Res* **27**, 16–19.

Constantine, D. G. (1966c). Transmission experiments with bat rabies isolates: bite transmission of rabies to foxes and coyote by free-tailed bats. *Am J Vet Res* **27**, 20–23.

Constantine, D. G. & Woodall, D. F. (1966). Transmission experiments with bat rabies isolates: reactions of certain Carnivora, opossum, rodents, and bats to rabies virus of red bat origin when exposed by bat bite or by intrasmuscular inoculation. *Am J Vet Res* **27**, 24–32.

Constantine, D. G., Solomon, G. C. & Woodall, D. F. (1968). Transmission experiments with bat rabies isolates: responses of certain carnivores and rodents to rabies viruses from four species of bats. *Am J Vet Res* **29**, 181–190.

Cox, J. H. & Schneider, L. G. (1976). Prophylactic immunization of humans against rabies by intradermal inoculation of human diploid cell culture vaccine. *J Clin Microbiol* **3**, 96–101.

Davis, P. L., Holmes, E. C., Larrous, F., Van der Poel, W. H., Tjornehoj, K., Alonso, W. J. & Bourhy, H. (2005). Phylogeography, population dynamics, and molecular evolution of European bat lyssaviruses. *J Virol* **79**, 10487–10497.

Davis, A. D., Rudd, R. J. & Bowen, R. A. (2007). Effects of aerosolized rabies virus exposure on bats and mice. *J Infect Dis* **195**, 1144–1150.

Dean, D. J., Abelseth, M. K. & Athanasiu, P. (1996). The fluorescence antibody test. In *Laboratory Techniques in Rabies*, 4th edn, pp. 88–93, Edited by F. X. Meslin, M. M. Kaplan & H. Koprowski. Geneva: World Health Organization.

Echevarría, J. E., Avellón, A., Juste, J., Vera, M. & Ibáñez, C. (2001). Screening of active lyssavirus infection in wild bat populations by viral RNA detection on oropharyngeal swabs. *J Clin Microbiol* **39**, 3678–3683.

Eurobats (2004). Agreement on the Conservation of Populations of European Bats, EUROBATS. http://www.eurobats.org/documents/agreement_text.htm

Fekadu, M., Shaddock, J. H., Chandler, F. W. & Sanderlin, D. W. (1988). Pathogenesis of rabies virus from a Danish bat (*Eptesicus serotinus*): neuronal changes suggestive of spongiosis. *Arch Virol* **99**, 187–203.

Fooks, A. R., Brookes, S. M., Johnson, N., McElhinney, L. M. & Hutson, A. M. (2003a). European bat lyssaviruses: an emerging zoonosis. *Epidemiol Infect* **131**, 1029–1039.

Fooks, A. R., McElhinney, L. M., Pounder, D. J., Finnegan, C. J., Mansfield, K., Johnson, N., Brookes, S. M., Parsons, G., White, K. & other authors (2003b). Case report: isolation of a European bat lyssavirus type 2a from a fatal human case of rabies encephalitis. *J Med Virol* **71**, 281–289.

Grauballe, P. C., Baagøe, H. J., Fekadu, M., Westergaard, J. M. & Zoffmann, H. (1987). Bat rabies in Denmark. *Lancet* **1**, 379–380.

Harris, S. L., Brookes, S. M., Jones, G., Hutson, A. M., Racey, P. A., Aegerter, J., Smith, G. C., McElhinney, L. M. & Fooks, A. R. (2006). European bat lyssaviruses: distribution, prevalence and implications for conservation. *Biol Conserv* **131**, 193–210.

Hatten, B. A., Allen, R. & Sulkin, S. E. (1968). Immune response in chiroptera to bacteriophage ΦX 174. *J Immunol* **101**, 141–151.

Heaton, P. R., Johnstone, P., McElhinney, L. M., Cowley, R., O'Sullivan, E. & Whitby, J. E. (1997). Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses. *J Clin Microbiol* **35**, 2762–2766.

Johnson, N., Phillpotts, R. & Fooks, A. R. (2006). Airborne transmission of lyssaviruses. *J Med Microbiol* **55**, 785–790.

Kappeler, A. (1989). Bat rabies surveillance in Europe. *Rabies Bulletin Europe* **13**, 12–13.

King, A. A., Haagsma, J. & Kappeler, A. (2004). Lyssavirus infections in European bats. In *Historical Perspective of Rabies in Europe and the Mediterranean Basin*, pp. 221–241. Edited by A. A. King, A. R. Fooks, M. Aubert & A. I. Wandeler. Paris: World Organization for Animal Health (OIE).

Kunz, T. H. & Nagy, K. A. (1988). Energy budget analysis. In *Ecological and Behavioural Methods for the Study of Bats*, pp. 283–285. Edited by T. H. Kunz. Washington, DC: Smithsonian Institution Press.

Kuzmin, I. V. & Rupprecht, C. E. (2007). Bat rabies. In *Rabies*, 2nd edn, pp. 259–307. Edited by A. C. Jackson & W. H. Wunner. San Diego: Academic Press.

Kuzmin, I. V., Botvinkin, A. D. & Shaimardanov, R. T. (1994). Experimental rabies and rabies-related infections in bats. *Vopr Virusol* **1**, 17–21.

Kuzmin, I. V., Franka, R. & Rupprecht, C. E. (2008). Experimental infection of big brown bats (*Eptesicus fuscus*) with West Caucasian bat virus (WCBV). *Dev Biol (Basel)* **131**, 327–337.

Lina, P. H. & Hutson, A. M. (2006). Bat rabies in Europe: a review. *Dev Biol (Basel)* **125**, 245–254.

Lumio, J., Hillbom, M., Roine, R., Ketonen, L., Haltia, M., Valle, M., Neuvonen, E. & Lähdevirta, J. (1986). Human rabies of bat origin in Europe. *Lancet* **1**, 378.

Messenger, S. L., Smith, J. S. & Rupprecht, C. E. (2002). Emerging epidemiology of bat-associated cryptic cases of rabies in humans in the United States. *Clin Infect Dis* **35**, 738–747.

Messenger, S. L., Smith, J. S., Orciari, L. A., Yager, P. A. & Rupprecht, C. E. (2003). Emerging pattern of rabies deaths and increased viral infectivity. *Emerg Infect Dis* **9**, 151–154.

Mohr, W. (1957). Die Tollwut. *Med Klin* **52**, 1057–1060 (in German).

Müller, T., Cox, J., Peter, W., Schafer, R., Johnson, N., McElhinney, L. M., Geue, J. L., Tjornehoj, K. & Fooks, A. R. (2004). Spill-over of European bat lyssavirus type 1 into a stone marten (*Martes foina*) in Germany. *J Vet Med B Infect Dis Vet Public Health* **51**, 49–54.

Müller, T., Johnson, N., Freuling, C. M., Fooks, A. R., Selhorst, T. & Vos, A. (2007). Epidemiology of bat rabies in Germany. *Arch Virol* **152**, 273–288.

O'Shea, T. J., Shankar, V., Bowen, R. A., Rupprecht, C. E. & Wimsatt, J. H. (2003). Do bats acquire immunity to rabies? Evidence from the field. *Bat Research News* **44**, 161.

Ondrejkova, A., Franka, R., Ondrejka, R., Svrcek, S., Suli, J., Benisek, Z., Zubricky, P., Bajova, V., Bugarsky, A. & other authors (2004). Identification of a lyssavirus isolate from a bat (*Eptesicus serotinus*). *Bulletin of the Veterinary Institute in Pulawy* **48**, 11–13.

Pawan, J. L. (1936a). The transmission of paralytic rabies in Trinidad by vampire bat (*Desmodus rotundus murinus* Wagner, 1840). *Ann Trop Med Parasitol* **30**, 101–130.

Pawan, J. L. (1936b). Rabies in the vampire bat of Trinidad with special reference to the clinical course and the latency of the infection. *Ann Trop Med Parasitol* **30**, 401–422.

Picard-Meyer, E., Brookes, S. M., Barrat, J., Litaize, E., Patron, C., Biarnais, M., Healy, D. M., Johnson, L., Fooks, A. R. & other authors (2008). Experimental infection of foxes with European bat lyssaviruses type-1 and -2. *Dev Biol (Basel)* **131**, 339–346.

Reagan, R. L., Yancey, F. S. & Brueckner, A. L. (1957). Transmission of rabies from artificially infected bats to Syrian hamsters. *AMA Arch Pathol* **63**, 278–280.

Roine, R. O., Hillbom, M., Valle, M., Haltia, M., Ketonen, L., Neuvonen, E., Lumio, J. & Lähdevirta, J. (1988). Fatal encephalitis caused by a bat-borne rabies-related virus. Clinical findings. *Brain* **111**, 1505–1516.

Rønsholt, L. (2002). A new case of European bat lyssavirus (EBL) infection on Danish sheep. *Rabies Bulletin Europe* **26**, 15.

Rønsholt, L, Sørensen, K. J., Bruschke, C. J., Wellenberg, G. J., van Oirschot, J. T., Johnstone, P., Whitby, J. E. & Bourhy, H. (1998). Clinically silent rabies infection in (zoo) bats. *Vet Rec* **142**, 519–520.

Scatterday, J. E. (1954). Bat rabies in Florida. *J Am Vet Med Assoc* **124**, 125.

Scherbak, Y. N. (1982). *Epidemiology of natural rabies.* DSc thesis, Kiev, Ukraine, USSR (in Russian).

Scherbak, Y. N. (1984). Virologic studies of rabies in the Ukrainian SSR. In *Viruses and Viral Diseases*, vol. 12, pp. 11–16. Kiev: Zdorovye (in Russian).

Schneider, L. G. & Cox, J. H. (1994). Bat lyssaviruses in Europe. *Curr Top Microbiol Immunol* **187**, 207–218.

Selimov, M. A., Tatarov, A. G., Antonova, L. A., Shcherbak, Y. N., Shablovskaya, E. A., Smekhov, A. M. & Mogilevsky, B. Y. (1986). To the issue of chiropteric rabic infection. *Rabies Information Exchange* **14**, 9–12.

Selimov, M. A., Tatarov, A. G., Botvinkin, A. D., Klueva, E. V., Kulikova, L. G. & Khismatullina, N. A. (1989). Rabies-related Yuli virus: identification with a panel of monoclonal antibodies. *Acta Virol* **33**, 542–546.

Selimov, M. A., Smekhov, A. M., Antonova, L. A., Shablovskaya, E. A., King, A. A. & Kulikova, L. G. (1991). New strains of rabies-related viruses isolated from bats in the Ukraine. *Acta Virol* **35**, 226–231.

Serra-Cobo, J., Amengual, B., Abellán, C. & Bourhy, H. (2002). European bat lyssavirus infection in Spanish bat populations. *Emerg Infect Dis* **8**, 413–420.

Sétien, A. A., Brochier, B., Tordo, N., De Paz, O., Desmettre, P., Péharpré, D. & Pastoret, P. P. (1998). Experimental rabies infection and oral vaccination in vampire bats (*Desmodus rotundus*). *Vaccine* **16**, 1122–1126.

Shankar, V., Bowen, R. A., Davis, A. D., Rupprecht, C. E. & O'Shea, T. J. (2004). Rabies in a captive colony of big brown bats (*Eptesicus fuscus*). *J Wildl Dis* **40**, 403–413.

Smith, J. S., Yager, P. A. & Baer, G. M. (1973). A rapid tissue culture test for determining rabies neutralizing antibody. *Monogr Ser World Health Organ* **23**, 354–357.

Smith, G. C., Brookes, S. M., Harris, S. L., Aegerter, J. N., Jones, G. & Fooks, A. R. (2006). EBLV-2 prevalence in the United Kingdom as determined by surveillance testing. *Dev Biol (Basel)* **125**, 265–271.

Steece, R. & Altenbach, J. (1989). Prevalence of rabies specific antibodies in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*) at Lava Cave, New Mexico. *J Wildl Dis* **25**, 490–496.

Stougaard, E. & Ammendrup, E. (1998). Rabies in individual countries: Denmark. *Rabies Bulletin Europe* **22**, 6.

Sulkin, S. E. (1962). Bat rabies: experimental demonstration of the "reservoiring mechanism". *Am J Public Health Nations Health* **52**, 489–498.

Sulkin, S. E., Krutzsch, P. H., Rae Allen, R. & Wallis, C. (1959). Studies on the pathogenesis of rabies in insectivorous bats: I. Role of brown adipose tissue. *J Exp Med* **110**, 369–388.

Tjørnehøj, K., Rønsholt, L. & Fooks, A. R. (2004). Antibodies to EBLV-1 in a domestic cat in Denmark. *Vet Rec* **155**, 571–572.

Tjørnehøj, K., Fooks, A. R., Agerholm, J. S. & Rønsholt, L. (2006). Natural and experimental infection of sheep with European bat lyssavirus type-1 of Danish bat origin. *J Comp Pathol* **134**, 190–201.

Van der Poel, W. H. M., Van der Heide, R., Van Amerongen, G., Van Keulen, L. J. M., Wellenberg, G. J., Bourhy, H., Schaftenaar, W., Groen, J. & Osterhaus, A. D. M. E. (2000). Characterization of a recently isolated lyssavirus in frugivorous zoo bats. *Arch Virol* **145**, 1919–1931.

Van der Poel, W. H., Van der Heide, R., Verstraten, E. R., Takumi, K., Lina, P. H. & Kramps, J. A. (2005). European bat lyssaviruses, The Netherlands. *Emerg Infect Dis* **11**, 1854–1859.

Vos, A., Müller, T., Cox, J., Neubert, L. & Fooks, A. R. (2004a). Susceptibility of ferrets (*Mustela putorius furo*) to experimentally induced rabies with European bat lyssaviruses (EBLV). *J Vet Med B Infect Dis Vet Public Health* **51**, 55–60.

Vos, A., Müller, T., Neubert, L., Zurbriggen, A., Botteron, C., Pöhle, D., Schoon, H., Haas, L. & Jackson, A. C. (2004b). Rabies in red foxes (*Vulpes vulpes*) experimentally infected with European bat lyssavirus type 1. *J Vet Med B Infect Dis Vet Public Health* **51**, 327–332.

Vos, A., Kaipf, I., Denzinger, A., Fooks, A. R., Johnson, N. & Mueller, T. (2007). European bat lyssaviruses – an ecological enigma. *Acta Chiropt* **9**, 283–296.

Wakeley, P. R., Johnson, N., McElhinney, L. M., Marston, D., Sawyer, J. & Fooks, A. R. (2005). Development of a real-time, TaqMan reverse transcription-PCR assay for detection and differentiation of lyssavirus genotypes 1, 5, and 6. *J Clin Microbiol* **43**, 2786–2792.

Webster, W. A. & Casey, G. A. (1996). Virus isolation in neuroblastoma cell culture. In *Laboratory Techniques in Rabies*, 4th edn, pp. 96–104, Edited by F. X. Meslin, M. M. Kaplan & H. Koprowski. Geneva: World Health Organization.

Wellenberg, G. J., Audry, L., Rønsholt, L., van der Poel, W. H., Bruschke, C. J. M. & Bourhy, H. (2002). Presence of European bat lyssavirus RNAs in apparently healthy *Rousettus aegyptiacus* bats. *Arch Virol* **147**, 349–361.