Encephalitis Caused by a Lyssavirus in Fruit Bats in Australia

This report describes the first pathologic and immunohistochemical recognition in Australia of a rabies-like disease in a native mammal, a fruit bat, the black flying fox (*Pteropus alecto*). A virus with close serologic and genetic relationships to members of the *Lyssavirus* genus of the family *Rhabdoviridae* was isolated in mice from the tissue homogenates of a sick juvenile animal.

The *Lyssavirus* genus of the family *Rhabdoviridae* consists of five serotypes: classical rabies virus (serotype 1), Lagos bat virus (LBV) (serotype 2), Mokola virus (serotype 3), Duvenhage virus (DUVV) (serotype 4), and European bat virus (EBV) (serotype 5). The viruses within the genus share serologic relationships, but the serotypes and stable species-associated variants within serotypes can be distinguished by the reactivity profiles of monoclonal antibodies (Mab) directed against nucleoprotein and glycoprotein antigens. Analysis of the nucleotide sequence of the nucleoprotein gene has also shown genetic clusters along the same lines as serologic analysis, except that serotype 5, EBV, has been separated into two genotypes, EBV1 and EBV2 (1). Lyssaviruses have not been isolated in Australia before, although rhabdoviruses in the genus *Ephemerovirus* are present, and viruses with some serologic relationship to the *Lyssavirus* genus, for example Adelaide River virus (2), have been identified but not characterized. All members of the *Lyssavirus* genus can cause rabies or rabies-like diseases in infected animals.

Rabies-like disease has been recorded in bats on all continents except Australia. Classical rabies virus infections are common in insectivorous and hematophagous bats and less common in frugivorous bats in the Americas, while rabies-related viruses (EBV 1 and 2) are found in insectivorous bats in Europe. Two other rabies-related viruses, LBV and DUVV, are found in frugivorous bats and insectivorous bats, respectively, on the African continent. Rabies has been described in a flying fox (*Pteropus poliocephalus*) in India, although the virus causing the disease was not characterized (3). An outbreak of rabies involving several dogs occurred in the Australian island state of Tasmania in 1867 but was quickly eradicated (4). Two cases of rabies in children were reported in Australia (in 1987 and 1990). Both cases were caused by classical rabies virus and were contracted in endemic-disease countries (5). We report for the first time apparent endemic–lyssavirus-induced disease in Australia.

The four largest species of frugivorous bats in Australia are called flying foxes and belong to the genus *Pteropus* (Order Chiroptera, Suborder Megachiroptera, Family *Pteropodidae*). The Australian range of the flying foxes extends from temperate eastern and coastal Australia into the eastern tropics, around the tropical northern coastline, and down as far as the subtropical west coast. The gray-headed flying fox (*Pteropus poliocephalus*) range is the temperate and subtropical east coast, the black flying fox (*P. alecto*) inhabits primarily the subtropical and tropical range, and the little red flying fox (*P. scapulatus*) occupies the entire range except the coolest southern areas. The fourth species, the spectacled flying fox (*P. conspicillatus*) occupies a smaller range in tropical northeast Queensland. Large flying fox "camps," with possibly tens of thousands of foxes, often contain more than one species. Analysis of population genetic markers shows a considerable movement of both *P. alecto* and *P. poliocephalus* across their geographic ranges within Australia (6). The range of *P. alecto* extends to the north of Australia into Papua New Guinea and the eastern islands of Indonesia (7,8). Regular patterns of movement suggest that flying foxes move between northern Australia and Papua New Guinea (L. Hall, pers. comm.). It is, therefore, possible that the virus described in this paper also extends across

the range of these mammals outside Australia.

The flying foxes (*P. alecto*) described in this paper were wild native Australian animals collected near Ballina, in northern New South Wales, Australia. The first case, in 1996, was in a 5-month-old female black flying fox found under a fig tree, unable to fly. It was euthanized by intravenous sodium pentobarbitone injection. Fresh blood, lung, kidney, and spleen were submitted for equine morbillivirus (EMV) isolation; antibody to EMV has been detected in *P. alecto* (9), and it is conjectured that this species may be the reservoir for EMV. Paraffin-embedded formalin-fixed samples, processed by standard techniques, showed a severe nonsuppurative encephalitis. The second case, in 1995, was identified after a retrospective examination of archived paraffin-embedded tissues. The affected animal, a juvenile female of the same species, was reported to be more aggressive than usual, and was euthanized and necropsied in a similar manner to the first. Histologically, although encephalitis was very mild, many eosinophilic, cytoplasmic inclusion bodies were present in various parts of the brain. All tests for EMV were negative.

An indirect immunoperoxidase test for rabies was carried out on tissues from paraffin blocks (10) by using an antirabies Mab (HAM) (Clone 'HAM', c/o Drs. R. Zanoni and E . Peterhans, Institut für Veterinär-Virologie, Länggasstr. 122, CH-3012, Bern Switzerland) that gave good reactions without background staining when used at 1:100. The 1996 bat had positive results over wide areas of the brain, particularly in parts of the hippocampus, the mesenchymal cells of the trigeminal nucleus, and larger motor neurons of the medulla oblongata. The brain of the 1995 bat reacted strongly over all areas. The reactions were either granular, or characteristically, had ring formations in large neurons. In addition, similar reactions were seen in neuronal cytoplasms in nerve plexuses of the gastrointestinal tract from both bats. Electron microscopy examination of ultrathin sections of hippocampus from the 1996 bat showed aggregates of viral nucleocapsids within the cytoplasm of cell bodies. These inclusion bodies were specifically labeled with anti-rabies HAM Mab and gold-labeled rabbit–anti-mouse.

The only fresh samples available were blood, lung, kidney, and spleen from the 1996 bat. The blood was examined for neutralizing antibody to rabies virus (CVS-11) by the rapid fluorescent focus inhibition method (11). No neutralizing antibody was detected. Tissue homogenates (lung, kidney, and spleen) were injected into mouse neuroblastoma cells, individually injected intracerebrally into 3-week-old mice (five mice per sample), and, as a pool of the three tissues, injected into day-old suckling mice (two litters, 14 mice). No virus was isolated from cell culture after two serial passages of 4 days. One weanling mouse injected with kidney homogenate showed hind leg paraplegia 16 days postinoculation. All other mice remained normal until termination (suckling mice at 21 days and weanling mice at 28 days postinoculation). The affected mouse was euthanized, and acetone-fixed smears of brain material were positive for a lyssavirus when tested by the Centocor fluorescein-labeled Mab (Centocor Inc., 244 Great Valley Park, Malvern, PA 19355, USA). Formalin-fixed brain material showed nonsuppurative encephalitis and was positive to the indirect immunoperoxidase test for rabies virus by the HAM Mab.

Polymerase chain reaction (PCR) tests were done on nucleic acids extracted from the brain, lung, kidney and spleen of the 1996 bat and on paraffin-embedded formalin-fixed brain tissues from the 1995 and 1996 bats by using oligomers designed for the amplification of lyssavirus N protein (12) or for nested PCR amplification of the nucleocapsid protein (5). Results from these primers were consistently negative, presumably because of formalin-fixation and/or sequence heterogeneity. Therefore, another nested PCR system was devised for the amplification of N protein. Nucleic acids were extracted (5) and transcribed into cDNA by using a degenerate oligomer NP1087 (5' GAGAAAGAG[A/C]T[G/T]CAAGA[A/C/T]TA. Primary PCR was done with primers NP1087 and NP1279 (5' CAG AGACATATCT[G/C]C[G/T][G/T]ATGTG) with amplification conditions of 94°C for 1 min, 37°C for 2 min, and 72°C for 2 min for 35

Table 1. Amino acid sequences of the virus designated pteropid lyssavirus (PLV) and those of rabies and rabies-like viruses[a,b]

	PLV[c]	*PV*	*4FRA*	*POL*	*8FRA*	*AS*	*FIN*	*HOL*	*NGA*	*Genotype*
PLV	-									
PV	92	-								1
4FRA	93	99	-							1
POL	93	94	94	-						5
8FRA	93	94	94	100	-					5
AS	89	90	91	95	95	-				4
FIN	85	87	88	87	87	84	-			6
HOL	85	88	89	87	87	83	98	-		6
NGA	82	82	83	85	85	85	76	77	-	2
MOK	76	75	76	79	79	80	72	71	86	3

[a] Described in Ref. 13.

[b] Comparisons were made of cognate regions of the N protein (amino acids 298 to 426 inclusive). GenBank accession numbers are given in brackets. PV, PV rabies virus (X03673); 4FRA, fox rabies virus (U22844); POL, European bat virus (U22844, 8615POL, EBV1); 8FRA, European bat virus (U22845, 8918FRA, EBV1); AS, Duvenhage virus (U22848); FIN, European bat virus (U22846, 9007FIN, EBV2); HOL, European bat virus (U22847, 9018HOL, EBV2); NGA, Lagos bat virus (U22842), Mokola virus (U22843).

[c]PLV, the virus now reported, provisionally designated pteropid lyssavirus; PV, PV rabies virus (X03673); 4FRA, fox rabies virus (U22844); POL, European bat virus (U22844, 8615POL, ELB1); 8FRA, European bat virus (U22845, 8918FRA, EBV1); AS, Duvenhage virus (U22848); Fin, European bat virus (U22846, 9007FIN, EBV2); HOL, European bat virus (U22847, 9018HOL, EBV2); NGA, Lagos bat virus (U22842), Mokola virus (U22843).

cycles. Nested PCR was done by using primers NP1087 and NP1227 (5' CTTCA [C/T]C[G/T]ACC[A/T][C/T][C/T]GTTC ATCAT) as above except that the number of cycles was reduced to 25. PCR products were excised and sequenced. Positive PCR amplification signals were derived from the tissue culture virus and paraffin-embedded formalin-fixed brain tissues by using primers NP1087 and NP1227. Sequence analysis of these products showed that they were identifical. Sequence comparisons were done by using the nucleocapsid proteins of known lyssaviruses and the virus reported in this paper, designated pteropid lyssavirus (PLV) (Table 1). Nucleotide sequence comparisons showed that PLV had a 75% homology with LBV, 75% homology with EBV-2, and 79% with Pasteur vaccine rabies virus; at the amino acid level, the virus was 85% homologous with both EBV-2 and LBV (but 92% homologous with the rabies virus), 89% with DUVV, and 93% homologous with EBV-1 viruses. Phylogenetic analysis of both the nucleotide and amino acid sequences (not shown) showed that the virus is closely related to the EBV as well as the classic street rabies strains (12).

Brain material from the affected mouse was repassaged by intracerebral inoculation into 3-week-old mice, in which neurologic signs developed 8 to 11 days postinoculation. Examination of brain homogenate from these mice by negative-contrast electron microscopy showed classical bullet-shaped rhabdoviruses. The isolate was also passaged to mouse neuroblastoma cells, which were acetone-fixed and tested by indirect immunofluorescence using a panel of Mabs against various rabies and rabies-like viruses. The CVS-11 strain of rabies was also tested for comparison. The results (Table 2) confirm that the isolate is a lyssavirus but is different from previously described isolates. Additional nucleocapsid Mab reaction patterns (results not shown) indicated a unique profile that shared the greatest number of positive reactions with serotype 1 rabies (CVS-11) compared with published profile data on other viruses (15). Preliminary testing of the isolate in a modified (incubated 3 days) rapid fluorescent focus inhibition neutralization assay indicated that the virus was neutralized by antisera to rabies virus (mouse anti-Evelyn-Rokitnicki-Abelseth [ERA] virus). The titer of the immune mouse serum against CVS rabies virus was 1,194, and against the bat virus, 1,640.

This is the first evidence of an endemic lyssavirus in Australia. The isolate described has been provisionally called pteropid lyssavirus. The natural history of this virus in bats in Australia needs to be investigated. Further genetic and antigenic analyses are

Table 2. Reactivity patterns of nucleocapsid monoclonal antibodies (Mab) with rabies (CVS-11) and pteropid lyssavirus (PLV)

		IFAT[a]	
MAb[b]	Specificity[a]	CVS-11	PLV
W502-2[c]	lyssavirus	+	+
HAM[d]	lyssavirus	+	+
C15-2[e]	rabies	+	-
62-143-1[f]	rabies	+	+
62-3-1[f]	rabies +, EBV +	+	-
62-146-3[f]	rabies +, DUVV -	+	+
W422[c]	Mokola +, LBV +	-	-

[a]+ indicates a positive reaction; - indicates a negative reaction
[b]Monoclonal antibody were specificities indicated by the following sources:
[c] (14,15); [d] (10); [e] (J Smith, pers. comm.); [f] (15,16)

also needed to fully determine the relationship of the virus to existing *Lyssavirus* serogroups and genogroups and to confirm its separate identity from other as yet uncharacterized rhabdoviruses isolated in Australia. The virus has been submitted to the Rabies Laboratory at the Centers for Disease Control and Prevention, Atlanta, for further Mab profile analysis and crossprotection studies with classical rabies vaccines. Findings will result in a better understanding of the public health implications of this newly emerged lyssavirus.

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Addendum

Since this report was submitted in September, 1996, the host and geographic range of the virus have been extended. The virus has been recognized by immunohistochemical techniques in five bats in three different virus isolations. Some of these bats were from another species, (the little red flying fox [*P. scapulatus*]), and from locations as far apart as 1,700 km along the Australian east coast.