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Lyssaviral Infection and Lead Poisoning in Black Flying Foxes from Queensland

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ABSTRACT: Pteropid lyssaviral infection, lead poisoning, and the difficulties in diagnosing pteropid lyssaviral infection using histopathological examination of tissues are described in wild black flying foxes (Pteropus alecto) from northern Queensland (Australia). An adult female P. alecto showed aggression before death in January 1995. Lead poisoning was diagnosed due to the presence of intranuclear lead inclusion bodies in renal proximal convoluted tubular epithelium and high concentrations of lead in renal and hepatic tissues, 370.03 ± 7.35 ppm and 16.76 ± 0.53 ppm, respectively. Renal inclusion bodies were composed of lead, calcium, phosphorus, and possibly sulphur; some inclusions had their granules arranged in concentric bands. This bat also had a moderate concentration (8.09 ± 0.18 ppm) of cadmium in renal tissue. An adult male P. alecto presented with ascending paralysis before it died in May 1996. Pteropid lyssaviral infection was diagnosed subsequently in both bats in September 1996 by immunofluorescent and immunoperoxidase antibody tests for rabies on brains and viral culture from brains. Neither bat had gross or microscopic lesions of the brain that suggested a lyssaviral infection, apart from occasional, subtle, eosinophilic cytoplasmic inclusions in the neurones of the brain stem of the female. These cases illustrate the need for a specific test to detect pteropid lyssavirus such as an immunofluorescent antibody test for lyssavirus rather than histopathological examination of tissues.

Key words: Bat, concurrent disease, flying fox, inclusion body, lead poisoning, pteropid lyssavirus, Pteropus alecto.

Lead poisoning has been reported in flying foxes (Zook et al., 1970; Sutton and Wilson, 1983; Sutton and Hariono, 1987). Zook et al. (1970) reported high concentrations of lead, more than 500 ppm, in the livers of captive grey-headed flying foxes (Pteropus poliocephalus) which had died after probably ingesting lead based paint. Sutton and Wilson (1983) and Sutton and Hariono (1987) reported cases of lead poisoning and high levels of lead in wild flying foxes. However, the above authors did not determine the composition nor the detailed structure of inclusion bodies in renal proximal convoluted tubules that occurred in lead poisoned flying foxes. Carroll et al. (1970) used an electron probe microanalyser to show that inclusion bodies in lead poisoned rats contained high levels of lead, calcium, and phosphorus. Therefore, transmission electron microscopy (TEM), scanning electron microscopy (SEM), and energy dispersive spectroscopy (EDS) were used to study the inclusions in the bats reported herein.

Five flying foxes infected with pteropid lyssavirus (PLV) have been reported in the literature (Fraser et al., 1996; Speare et al., 1997). Pteropid lyssavirus is most closely related to rabies and European bat lyssavirus and has caused the death of one human (Fraser et al., 1996; Allworth et al., 1996). This paper describes pteropid lyssaviral infection in two black flying foxes (P. alecto) from northern Queensland and the concurrent lead poisoning in one. The lyssaviral infection of these bats was first reported by Speare et al. (1997) but not described.

Bats were necropsied and samples from major organs were fixed in 10% buffered neutral formalin. Additional brain, liver and kidney samples were stored at −20°C. Fixed tissues were embedded in paraffin,
sectioned at 5 μm and stained routinely with hematoxylin and eosin (H&E) (Luna, 1968). Ziehl-Neelsen (ZN) stain was performed on kidney sections with inclusion bodies and phloxine tartrazine stain (Luna, 1968) was performed on brain sections from both bats. Gram staining (Luna, 1968) was done on tissues in which bacteria were seen histologically.

Deparaffinized kidney sections (20 μm) with inclusion bodies were carbon coated and then examined in a JEOL JXA 840A scanning electron microscope (JEOL, Sydney, Australia). Ultrathin sections of kidney, postfixed in osmium tetroxide and embedded in Spurr's resin, were examined unstained and stained with lead citrate and uranyl acetate in a JEOL JEM 2000FX transmission electron microscope (JEOL) operated at 80kv (Hayat, 1989). Tissue samples (0.250 g), which had been stored at −20 C, of brain, liver and kidney were prepared by routine microwave digestion for animal tissue (Jarvis et al., 1992) and analysed by inductively coupled plasma mass spectrometry (ICP-MS) (Robinson, 1996). Concentrations, expressed as an estimate followed by the standard deviation, of lead and cadmium were determined.

An indirect immunoperoxidase antibody test (IPAT) for rabies was performed on fixed brain sections from one bat (Fraser et al., 1996) and an immunofluorescent antibody test (IFAT) was conducted on smears of brain, which had been stored at −20 C, from both bats at the CSIRO Australian Animal Health Laboratory (Geelong, Australia) 20 and 4 mo after the two bats died respectively. Viral isolation was attempted from brains.

In January 1995 an adult, wild, female black flying fox (P. alecto) was behaving abnormally near a release cage for hand-raised juvenile flying foxes in the back yard of a house in Townsville (Queensland, Australia; 146°49’E, 19°15’S). The flying fox chased and bit other flying foxes including her own pup fracturing its maxilla and frontal bones and perforating its skull. The bat was captured and isolated in a cage. It refused water and died later that morning. At necropsy the right lung was consolidated and the meninges were congested. The pup died three days later. Histopathological examination of tissues from the adult revealed many eosinophilic intranuclear inclusion bodies in renal proximal convoluted tubular epithelium. The bodies varied in size and shape, although many appeared to fill the entire nucleus (Fig. 1). Some eosinophilic bodies were within the cytoplasm of proximal convoluted tubules and often were surrounded by a thin unstained halo. The proximal convoluted tubules were necrotic. Many nuclei were enlarged and had a vesicular appearance. Cell outlines were indistinct and the cytoplasm of cells occluded the lumen of tubules. The distal convoluted tubules were necrotic. Many cells were pyknotic (Fig. 1) and some had detached from the basement membrane. Ascending and descending tubules were necrotic with cytoplasm broken into numerous strands and some cells detaching from the basement membrane. Collecting tubules appeared normal apart from some loss of cytoplasm. A few of the renal inclusion bodies were acid fast with ZN staining. Many of the inclusions had a darker central disc with H&E staining (Fig. 1) which was more distinct with ZN staining.
Figure 2. Graph of energy dispersion analysis of an inclusion body in renal convoluted tubular epithelium of a black flying fox. Peaks correspond to the x-ray energy for a particular element. The silicon peak represents x-rays from the glass slide. The (l) indicates that the x-ray peak could be that of one or both elements. Ca = calcium; Fe = iron; K = potassium; P = phosphorus; Pb = lead; S = sulphur; Si = silicon; Zn = zinc.

Moderate congestion was seen in brain, liver and lung, and a few small focal perivascular hemorrhages were present in brain and lung. Mild interstitial inflammation was present in the lung, and one area contained bacterial colonies within airways. These were gram positive cocci and bacilli, and there was little to no inflammation near them. Eosinophilic material, which sometimes appeared refractile, was often next to or surrounded by these bacteria. It stained brown with gram staining and resembled plant material. A few focal areas of necrosis and mixed inflammatory cells were seen in the liver.

Energy dispersion analysis of five inclusion bodies in renal tissue, detected in backscatter electron mode, showed that they contained lead, calcium, phosphorus and possibly sulphur (Fig. 2). The lead was concentrated in 3 to 5 μm diameter circular areas which corresponded to inclusion bodies seen within nuclei in secondary electron mode (Figs. 3, 4). The backscattering of electrons from nuclei appeared uniform for the 100 to 500 μm depth that electrons were reflected (Fig. 4). Lead was not found in other areas of the kidney with the electron microprobe. Examination of inclusion bodies using TEM showed a dense granular core surrounded by a sparse fibrillar zone (Fig. 5). Unstained sections revealed that the core contained granules of varying sizes (<0.20 μm diameter). In some inclusion bodies there was a distinctive concentric arrangement of bands (Fig. 5). A very high concentration of lead and a moderate concentration of cadmium were measured in kidney tissue and a high concentration of lead was measured in liver tissue using ICP-MS (Table 1).

Paraffin embedded, formalin fixed brain and brain stored at −20°C stained strongly on IPAT and IFAT for lyssavirus, respectively, and PLV was isolated by viral culture. Re-examination of histological sec-

Figure 3. Secondary electron photomicrograph of inclusion bodies (arrows) in renal convoluted tubular epithelium of a black flying fox. Bar = 3 μm.

Figure 4. Backscattered electron photomicrograph of inclusion bodies (arrows) in renal convoluted tubular epithelium from a black flying fox. The bright areas correspond directly to lead with a high atomic weight. Bar = 3 μm.
tions of brain showed several eosinophilic cytoplasmic inclusions in the neurones of the brain stem (Fig. 6). Phloxine tartrazine staining of the cerebrum did not reveal any other cytoplasmic inclusion bodies. The brain of the pup was negative for lyssavirus on IFAT and viral isolation. The

pup had high concentrations of lead in renal and hepatic tissue (Table 1).

In May 1996 an adult, wild, male black flying fox with hindlimb paresis was found under a tree in Charters Towers (Queensland; 146°11′E, 19°53′S). Over the next 2 days it developed hindlimb paralysis and a clear discharge from its mouth and nose. The flying fox was found dead the next morning and its body was stored at −20°C until August 1996 when it was necropsied. A smear of frozen brain stained strongly on IFAT for lyssavirus and PLV was isolated by viral culture. Re-examination of the

![Figure 5](image-url)  
**Figure 5.** Ultrastructure of stained intranuclear inclusion body (small arrow) in renal convoluted tubular epithelium of a black flying fox. Nuclear membrane (large arrow). Note the granular core, the concentric bands of granules and the fibrillar coat of the inclusion body. Lead citrate and uranyl acetate. Bar = 1 μm.

![Figure 6](image-url)  
**Figure 6.** Histological section of cytoplasmic inclusion bodies (arrows) in a neuron of a black flying fox infected with pteropid lyssavirus. H&E. Bar = 10 μm.

<table>
<thead>
<tr>
<th>Bat</th>
<th>Lead</th>
<th>Cadmium</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Kidney</td>
<td>Liver</td>
</tr>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>370.03</td>
<td>16.76</td>
</tr>
<tr>
<td></td>
<td>(7.35)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(0.53)</td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>123.85</td>
<td>19.26</td>
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<td></td>
<td>(2.72)</td>
<td>(0.41)</td>
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<tr>
<td>3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NM&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.54</td>
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<td></td>
<td>(0.05)</td>
<td></td>
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<tr>
<td>4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.29</td>
<td>&lt;0.6</td>
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<td></td>
<td>(0.08)</td>
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<sup>a</sup> Townsville bat with PLV.
<sup>b</sup> Pup of Townsville bat.
<sup>c</sup> Charters Towers bat with PLV.
<sup>d</sup> Healthy bat which provides a control.
<sup>e</sup> Standard deviation in parentheses.
<sup>f</sup> NM, not measured.
<sup>⁺</sup> Indicates that the level of the element was below the minimum level which can be detected by ICP-MS.
brain and then examination after phloxine tartrazine staining did not reveal any lesions or cytoplasmic inclusions in neurons. The concentrations of lead and cadmium in hepatic tissue determined by ICP-MS analysis were not considered harmful (Table 1).

Both flying foxes had pteropid lyssaviral infection. However, the bat from Townsville also had lead poisoning based on the high concentrations of lead measured in its kidney and liver. Other flying foxes with lead poisoning had concentrations of lead in kidney and liver ranging from 20.5 to 44.6 ppm and 12.1 to 500 ppm respectively (Zook et al., 1970; Sutton and Wilson, 1983; Sutton and Hariono, 1987). The concentrations of lead in the Townsville case were very high when compared to concentrations found in other species and would be regarded in horses and cattle as confirmatory of lead poisoning (Jubb and Huxtable, 1993). Seawright (1989) states that lead concentrations of 25 ppm in kidney and 10 ppm in liver are of diagnostic significance. However, the concentrations of lead in tissues which are considered to be toxic varies and animals vary in their response to different amounts and durations of lead exposure (Seawright, 1985; Jubb and Huxtable, 1993). For example, horses are resistant to brief high doses and high blood levels must be sustained for several weeks to cause peripheral neuropathy, the characteristic feature of lead poisoning in this species. However, cattle develop clinical signs with high doses of lead, but if exposed to low amounts for long periods, they may accumulate high concentrations in tissues including brain with no ill effect. Zook et al. (1970) suggested that flying foxes may be relatively more resistant to lead than other animals.

The concentration of lead is usually much lower in nervous tissue than in viscera in most species with lead poisoning, despite clinical signs being almost exclusively neurologic (Jubb and Huxtable, 1993), as occurred in the bat from Townsville.

Seven flying foxes with lead poisoning have been reported in the literature; clinical signs ranged from an inability to fly to muscle fasciculation, uncoordinated movement, inappetance, excess saliva and diarrhea (Zook et al., 1970; Sutton and Wilson, 1983; Sutton and Hariono, 1987). Some pathologic features of lead poisoning have been reported in kidney, liver and brain of two grey-headed flying foxes (P poliocephalus) by Sutton and Wilson (1983) and similar features were seen in the bat from Townsville.

In the Townsville case, lead was shown to occur predominantly in inclusion bodies within renal epithelium. Lead was probably evenly distributed within inclusions because backscattering of electrons appeared uniform for the 100 to 500 μm depth that electrons were reflected under SEM (Watt, 1985). Goyer et al. (1970) found that 80 to 90% of renal lead was concentrated within nuclei and that at least 50% of this was recovered from inclusion bodies in lead acetate poisoned rats. They also found that the ratio of lead to protein in inclusion bodies was at least double that in the remainder of the nucleus. The renal inclusion bodies from the Townsville case were found to contain high levels of calcium, phosphorus and possibly sulphur as well as lead. Carroll et al. (1970) also found high levels of lead, calcium and phosphorus in inclusion bodies in rats dosed with lead acetate, which they stated, supported the finding of Goyer et al. (1970, 1971) that lead inclusion bodies are composed of lead-protein complexes.

The lead inclusion bodies in the Townsville bat seen under TEM are similar to other lead inclusion bodies reported by Richter et al. (1968) in that they have a dense granular core and are surrounded by a fibrillar coat. The inclusions with concentric bands of granules, which also were seen under TEM (Fig. 5), are similar to the concentric laminar inclusions in lead poisoned mice reported by Vicente-Ortega et al. (1996). The concentric bands may
have formed due to intermittent development of the inclusion body.

Sutton and Hariono (1987) found that lead levels were much higher in flying foxes from urban environments. They suggested that the atmosphere was the most likely source of lead. Therefore, it is not surprising that the black flying fox from the urban environment of Townsville had high lead tissue levels. The mechanism of lead poisoning in flying foxes has been partially explained by Hariono (1991) who found experimentally that flying foxes absorbed between 55% and 75% of ingested inorganic lead; much higher than rats (16%) and most species of animals (5–10%) (Hariono, 1991).

The pup of the female bat from Townsville also had high concentrations of lead in kidney and liver tissue and low concentrations in the brain. Lead may have been transferred from mother to pup. Hariono (1991) reported similar findings in a small number of female flying foxes and their pups. Lead has been shown to cross the placenta in animals and humans, and is secreted in small amounts in the milk of cows with clinical symptoms of lead poisoning (Hariono, 1991).

The concentration of cadmium in renal tissue of the bat from Townsville was possibly harmful (Table 1) since cadmium is toxic at lower concentrations than lead to Escherichia coli (Mariscal et al., 1995). Hence, cadmium also should be considered in cases of poisoning in flying foxes.

Neither of the adult black flying foxes had encephalitis although brain smears from both reacted strongly to the IFAT for lyssavirus antigen. In contrast, both black flying foxes with PLV from Ballina in the index report had non-suppurative encephalitis (Fraser et al., 1996). The Townsville case had occasional, subtle, cytoplasmic inclusions, of variable shape and size, in the neurons of the brain stem (Fig. 6), similar to Negri bodies found in rabies infection. However, a central basophilic granule which occurs in Negri bodies was not seen (Perl, 1975). One of the flying foxes in the index report had neuronal cytoplasmic inclusions (Fraser et al., 1996).

Neurological signs were present in both flying foxes from northern Queensland infected with PLV. The aggression shown by case 1 was abnormal. Flying foxes will exhibit aggressive behaviour in specific types of social interactions, but the aggression shown by this bat was considered to be excessive. As excessive aggression has not been seen in lead poisoned flying foxes (Zook et al., 1970; Sutton and Wilson, 1983; Sutton and Hariono, 1987) and one of the bats with PLV reported by Fraser et al. (1996) was more aggressive than usual, the aggression may have been solely due to PLV. The clinical signs of paralysis and aggression seen in these bats with PLV infection are similar to those seen in classical rabies infection in other animals (Geering et al., 1995).

These cases show that lyssaviral infection can be easily overlooked. The reasons for not diagnosing lyssaviral infection at first were (1) that lyssavirus was not known to occur in Australia at the time and therefore not considered, (2) there was evidence of another disease, lead poisoning, which could explain the neurological signs and death of one of the bats, and (3) there was no obvious histopathology to suggest lyssaviral infection. These cases also show that to detect PLV a specific test for lyssavirus such as an immunofluorescent antibody test is needed because histopathological examination of tissues is inadequate.

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