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Presence of presynaptic neurotoxin complexes in the venoms of Australo-Papuan death adders (*Acanthophis* spp.)☆

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ABSTRACT

Australo-papuan death adders (*Acanthophis* spp.) are a cause of serious envenomations in Papua New Guinea and northern Australia often resulting in neurotoxic paralysis. Furthermore, victims occasionally present with delayed-onset neurotoxicity that sometimes responds poorly to antivenom or anticholinesterase treatment. This clinical outcome could be explained by the presence of potent snake presynaptic phospholipase A_2 neurotoxin (SPAN) complexes and monomers, in addition to long- and short-chain postsynaptic α-neurotoxins, that bind irreversibly, block neurotransmitter release and result in degeneration of the nerve terminal. The present study therefore aimed to determine within-genus variations in expression of high molecular mass SPAN complexes in the venoms of six major species of *Acanthophis*, four geographic variants of *Acanthophis antarcticus*. Venoms were separated by size-exclusion liquid chromatography under non-denaturing conditions and fractions corresponding to proteins in the range of 22 to >60 kDa were subjected to pharmacological characterization using the isolated chick biventer cervicis nerve-muscle (CBCNM) preparation. All venoms, except *Acanthophis wellsi* and *Acanthophis pyrrhus*, contained high mass fractions with phospholipase A_2 activity that inhibited twitch contractions of the CBCNM preparation. This inhibition was of slow onset, and responses to exogenous nicotinic agonists were not blocked, consistent with the presence of SPAN complexes. The results of the present study indicate that clinicians may need to be aware of possible prejunctional neurotoxicity following envenomations from *A. antarcticus* (all geographic variants except perhaps South Australia), *Acanthophis praelongus*, *Acanthophis rugosus* and *Acanthophis. laevis* species, and that early antivenom intervention is important in preventing further development of toxicity.

1. Introduction

The family Elapidae consists of a variety of highly venomous snake genera, including the death adders (*Acanthophis* spp.), which are considered elapids despite their viper-like appearance and behaviour. In addition, death adders have semi-mobile fangs which results in them being commonly regarded as the most evolved of all elapids and morphologically transitional between the Elapidae and Viperidae. Indeed, death adders are the widest ranging of the Australian elapids with a variety of species found throughout continental Australia, Irian Jaya, Papua New Guinea (PNG) and some eastern Indonesian islands including the Maluku islands of Seram (formerly Ceram), Halmahera, Obi and Tanimbar (Lalloo et al., 1996; Wüster et al., 2005). This wide range and ecological specialization of *Acanthophis* is consistent with its relatively early

☆ *Ethical statement*: The authors declare that all animal experiments described in the paper comply with Australian animal ethics regulations.

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divergence from its closest sister lineage around 9.1 Mya (Sanders et al., 2008).

Acanthophis species are the second most common cause of envenomation in the Central Province and National Capital District of PNG, second only to the Papuan taipan (*Oxyuranus scutellatus canni*). They have been reported to be responsible for *ca.* 11% of patients admitted for envenomation in these areas between January 1990 and June 1992 (Lalloo et al., 1994). The death adder has additionally been reported to be the cause of most serious envenomations in the Madang region of PNG (Hudson and Pomat, 1988) while in northern Australia death adder envenomation is also considered a serious problem (Currie, 2003). General symptoms of envenomation from *Acanthophis* species can include pain in regional lymph nodes, and non-specific systemic features such as abdominal pain, headache, drowsiness, nausea and vomiting. More problematic are neurological symptoms that typically involve ptosis, dysarthria, dysphagia, diplopia and flaccid muscle paralysis with death resulting from respiratory failure (Campbell, 1966; Lalloo et al., 1996). Myotoxicity is not usually associated with envenomation but there are clinical reports in PNG of rhabdomyolysis with *Acanthophis rugosus* (Lalloo et al., 1996). Significantly, delayed-onset neurotoxicity is a problem in death adder envenomations, occasionally with late presentation of neurotoxicity as the first feature of envenoming (Currie, 1989, 2003; Lalloo et al., 1996). This has resulted in a recommended hospital admission period of 24 h for patients in PNG and both central and northern Australia, and for children in any region (Currie, 2006).

The primary treatment for death adder envenomation is CSL monovalent death adder antivenom, which has been raised against *Acanthophis antarcticus* (White, 1998, 2001). Death adder antivenom appears to be quite effective in preventing the progression of neurotoxicity from all species of death adder in Australia and PNG (Campbell, 1966; Lalloo et al., 1996), showing a rapid reversal of paralysis. However, conflicting reports with Australian death adder envenomation have suggested that the neurotoxic effects can be poorly reversed by antivenom or anticholinesterase (neostigmine) if the patient presents late after envenomation (Gunja et al., 2007). In support, an *in vitro* study involving *A. antarcticus*, *Acanthophis praelongus* and *Acanthophis pyrrhus* venoms found that all three venoms produced rapid postsynaptic neurotoxicity but antivenom displayed varying efficacy to reverse toxicity following neuromuscular blockade over a 4 h period. Indeed, there was only a 22% recovery of contractile responses following complete neuromuscular blockade with *A. antarcticus*, the venom to which the antivenom is raised (Wickramaratna and Hodgson, 2001).

It is commonly regarded that death adder neurotoxicity is mainly postsynaptic in origin, and therefore this suggests that the postsynaptic neurotoxins (so-called 'α-' or 'three-fingered' neurotoxins) present within death adder venoms may not be completely reversible or are 'pseudo-irreversible'. However, rapidly developing neurotoxicity from postsynaptic neurotoxins conceals the action of any underlying presynaptic neurotoxins or myotoxins that may be present in the venom, which typically have slower onsets of activity but bind irreversibly (Kelly and Brown, 1974; Wilson et al., 1995). In the case of patients with delayed-onset or slowly-developing neurotoxicity, presynaptic neurotoxins or myotoxins may play a significant role in the speed of recovery following antivenom therapy due to the irreversible nature of their actions, and may go some way to explain the above resistance to antivenom therapy. The presence of myotoxins in the venom of *A. praelongus*, *A. rugosus*, *Acanthophis* sp. Seram, and *Acanthophis wellsi* species has been previously determined (Hart et al., 2005; Wickramaratna et al., 2003a, 2003b) and rhabdomyolysis has been reported clinically with *A. rugosus* envenomation (Lalloo et al., 1996). However, this does not exclude the presence of presynaptic neurotoxins in these venoms, or in other major species or geographic variants of *A. antarcticus*.

It is therefore possible that *Acanthophis* venoms contain snake presynaptic phospholipase A_2 (PLA_2) neurotoxins (so-called 'β-neurotoxins' or SPANs) that bind irreversibly to motor nerve terminals (Rossetto and Montecucco, 2008). In support, highly toxic heteromultimeric SPANs have been identified and pharmacologically characterized from the venom of a number of other Australo-Papuan elapid snake venoms, including, paradoxin (inland taipan, *Oxyuranus microlepidotus*; Fohlman, 1979; Hodgson et al., 2007), taipoxin (coastal taipan, *O. scutellatus*; Fohlman et al., 1976), cannitoxin (Papuan taipan, *O. scutellatus canni*; Kuruppu et al., 2005b) and textilotoxin (common brown snake, *Pseudonaja textilis*; Su et al., 1983; Wilson et al., 1995) and the monomeric notexin (tiger snake, *Notechis s. scutatus*; Cull-Candy et al., 1976). These are potent presynaptically-active neurotoxins with an LD_{50} of 1–25 μg/kg (mouse i.v. or i.p.). Furthermore, a protein from *A. antarcticus* venom with significant N-terminal sequence homology to the γ-chain of the heterotrimeric SPAN taipoxin has been previously reported (Fry et al., 2002), suggestive of the presence of an SPAN in *Acanthophis* venom.

SPANs can cause irreversible neurotoxicity via the depletion of neurotransmitter, due to a modest increase in transmitter release and a block of vesicle recycling, as well as the degeneration of motor nerve terminals and intramuscular axons (Harris et al., 2000). Regeneration and functional reinnervation can take several days (Dixon and Harris, 1999; Harris et al., 2000) and in humans paralysis resulting from SPANs not promptly treated with antivenom can last many days. Some patients even require artificial ventilation and, in isolated cases, intensive care for prolonged periods (Connolly et al., 1995; Pearn, 1971; Trevett et al., 1995). While a number of monomeric PLA_2 proteins have been isolated from death adder venom (Chow et al., 1998; Sim, 1998; van der Weyden et al., 2000; van der Weyden et al., 1997), none of these have been characterized pharmacologically and there have be no previous reports of attempts at isolating multimeric SPAN complexes in the venom of *Acanthophis* spp.

Given the high potency of SPAN complexes from other Australian elapid venoms, the aim of this study was to determine whether the venoms of various species and geographic variants of Australian death adders also contain SPAN complexes. The presence of such components could explain reports of poorly reversible neurotoxicity in post-envenomated patients not treated promptly with antivenom.