

Species and Regional Variations in the Effectiveness of Antivenom against the *in Vitro* Neurotoxicity of Death Adder (*Acanthophis*) Venoms

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Although viperlike in appearance and habit, death adders belong to the Elapidae family of snakes. Systemic envenomation represents a serious medical problem with antivenom, which is raised against *Acanthophis antarcticus* venom, representing the primary treatment. This study focused on the major *Acanthophis* variants from Australia and islands in the Indo-Pacific region. Venoms were profiled using liquid chromatography–mass spectrometry, and analyzed for *in vitro* neurotoxicity (0.3–10 µg/ml), as well as the effectiveness of antivenom (1–5 units/ml; 10 min prior to the addition of 10 µg/ml venom). The following death adder venoms were examined: *A. antarcticus* (from separate populations in New South Wales, Queensland, South Australia, and Western Australia), *A. hawkei*, *A. praelongus*, *A. pyrrhus*, *A. rugosus*, *A. wellsi*, and venom from an unnamed species from the Indonesian island of Seram. All venoms abolished indirect twitches of the chick isolated biventer cervicis nerve-muscle preparation in a dose-dependent manner. In addition, all venoms blocked responses to exogenous acetylcholine (1 mM) and carbachol (20 µM), but not KCl (40 mM), suggesting postsynaptic neurotoxicity. Death adder antivenom (1 unit/ml) prevented the neurotoxic effects of *A. pyrrhus*, *A. praelongus*, and *A. hawkei* venoms, although it was markedly less effective against venoms from *A. antarcticus* (NSW, SA, WA), *A. rugosus*, *A. wellsi*, and *A. sp.* Seram. However, at 5 units/ml, antivenom was effective against all venoms tested. Death adder venoms, including those from *A. antarcticus* geographic variants, differed not only in their venom composition but also in their neurotoxic activity and susceptibility to antivenom. For the first time toxicological aspects of *A. hawkei*, *A. wellsi*, *A. rugosus*, and *A. sp.* Seram venoms were studied.

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Death adders (genus *Acanthophis*) are unique among Australian snakes in both morphology and venom composition (Campbell, 1966; Fry, 1999). Although classified into the Elapidae family of snakes they are viperlike in appearance and habit (Campbell, 1966). They are characterized by a somewhat flattened, almost triangular head and a short, stout body terminating to a thin ratlike tail (Cogger, 1996). This makes them among the most specialized of all elapids and closely convergent in many respects with members of the family Viperidae.

Death adders are the widest ranging of the Australian elapids, being found not only in continental Australia, but north throughout the Torres Straight Islands, Papua New Guinea, Irian Jaya, and the Indonesian islands of Seram, Halmahera, Obi, and Tanimbar. Although there have been up to 12 species and 3 subspecies of death adders described thus far (Hoser, 1998), considerable debate remains about species identification (Wuster *et al.*, 1999). Of these, only the venoms of the common (*A. antarcticus*), northern (*A. praelongus*), and desert (*A. pyrrhus*) death adders have been studied.

In terms of biochemical and pharmacological properties, species variations between the venoms of *A. antarcticus*, *A. praelongus*, and *A. pyrrhus* have been reported. Many differences between venom profiles were found when each venom was applied to a cation-exchange Mono-S column (van der Weyden *et al.*, 2000). Another study showed significant differences in *in vitro* neurotoxicity between these three venoms (Wickramaratna and Hodgson, 2001).

In a comparative study of 25 snake venoms, Broad *et al.* (1979) ranked *A. antarcticus* venom as the ninth most lethal venom with a murine LD₅₀ of 0.338 mg/kg (sc, in 0.1% bovine serum albumin in saline). Kellaway *et al.* (1932) found the neurotoxic action of *A. antarcticus* venom to be attributable to a peripheral curare-like neuromuscular block. More recently, five postsynaptic neurotoxins have been isolated and sequenced from *A. antarcticus* venom (Sheumack *et al.*, 1979,

1990; Kim and Tamiya, 1981a,b; Tyler *et al.*, 1997). In the chick isolated stimulated biventer cervicis nerve-muscle preparation, *A. antarcticus* (Qld), *A. praelongus*, and *A. pyrrhus* venoms (3–10 $\mu\text{g/ml}$) caused time-dependent inhibition of twitches and blocked contractile responses to exogenous acetylcholine and carbachol, suggesting the presence of postsynaptic neurotoxins (Wickramaratna and Hodgson, 2001). In the same study the three venoms (3–10 $\mu\text{g/ml}$) were ranked according to their *in vitro* neurotoxicity as follows: *A. antarcticus* (Qld) \geq *A. pyrrhus* > *A. praelongus*.

Death adder envenomations are a rare occurrence in Australia, although these are still a significant health problem in Papua New Guinea (Currie *et al.*, 1991; Sutherland, 1992; Laloo *et al.*, 1995). Clinical symptoms of envenomation by *Acanthophis* spp. include those relating to the paralysis of bulbar and ocular muscles, enlargement of regional lymph nodes, and death occurs through inhibition of respiration resulting from paralysis of the voluntary muscles (Campbell, 1966; Laloo *et al.*, 1996). CSL death adder antivenom, which has been raised against *A. antarcticus* venom, remains the principal therapy for death adder envenomation (White, 1998). This antivenom was found to be very effective in reversing the effects of death adder envenomation in Papua New Guinea (Campbell, 1966; Laloo *et al.*, 1996). A recent *in vitro* study showed that CSL death adder antivenom, while very effective against *A. praelongus* and *A. pyrrhus* venoms, was significantly less effective against the neurotoxicity of *A. antarcticus* (Qld) venom (Wickramaratna and Hodgson, 2001). Although death adder antivenom was raised against *A. antarcticus* venom it is possible that it may not have been raised against a pool of *A. antarcticus* venoms representative of all geographic variations. Thus, the antivenom may lack the ability to neutralize some neurotoxic components of venoms from subpopulations of *A. antarcticus* species (Schenberg, 1963; Wickramaratna and Hodgson, 2001). This explanation, however, remains to be investigated. With many new species of death adders described, from a clinical perspective, it is useful to know the effectiveness of CSL death adder antivenom against their venoms (Currie, 2000). However, no such study has been published.

To date, no pharmacological studies have been undertaken on whole venoms from the Barkly tableland (*A. hawkei*), black head (*A. wellsi*), or Irian Jayan (*A. rugosus*) death adders, nor from death adders from the Indonesian island of Seram (*A. sp. Seram*), considered by some herpetologists to be another possible species (Mark O'Shea, personal communication, 2000). Therefore, this study examined the *in vitro* neurotoxicity of these venoms and compared these to the previously studied *A. antarcticus*, *A. praelongus*, and *A. pyrrhus* venoms. In light of our recent findings, the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of these unstudied venoms was also examined. In addition, death adder venoms were profiled using on-line liquid chromatography–mass spectrometry (LC-MS) to determine the level of variations in venom

composition resulting from geographic location or species differences.

MATERIALS AND METHODS

Venom Collection

A. antarcticus venoms were obtained from populations in New South Wales (NSW), Queensland (Qld), South Australia (SA), and Western Australia (WA). *A. praelongus* venom was milked from populations in Cairns, Queensland; *A. pyrrhus* venom from Alice Springs, Northern Territory; *A. wellsi* venom from the Pilbarra region of Western Australia; *A. hawkei* venom from the Barkly tableland region of Northern Territory; *A. rugosus* venom from Irian Jaya (West Papua), and *A. sp. Seram* from the island of Seram, Indonesia. Venoms were either purchased from Venom Supplies Pty. Ltd., South Australia, or milked by the first author. For each geographic variant or species venoms were collected and pooled to minimize the effects of individual variations (Chippaux *et al.*, 1991).

Venom Preparation and Storage

Freeze-dried venoms and stock solutions of venoms, prepared in 0.1% bovine serum albumin in 0.9% saline, were stored at -20°C until required.

Liquid Chromatography–Mass Spectrometry (LC-MS)

Venoms were dissolved in 0.1% trifluoroacetic acid (TFA) to a concentration of 1 mg/ml. On-line LC-MS of venoms was performed on a Vydac C18 analytical column (2.1×250 mm, 5μ particle size, 300 \AA) with solvent A (0.05% TFA) and solvent B (90% acetonitrile in 0.045% TFA) at a flow rate of 130 $\mu\text{l/min}$. The solvent delivery and gradient formation of a 1% gradient from 0 to 60% acetonitrile/0.05% TFA over 60 min was achieved using an Applied Biosystems 140 B solvent delivery system. Electrospray mass spectra were acquired on a PE-SCIEX triple quadrupole mass spectrometer equipped with an ionspray atmospheric pressure ionization source. Samples (10 μl) were injected manually into the LC-MS system and analysed in positive ion mode. Full scan data were acquired at an orifice potential of 80 V over the mass range 400–2100 Da with a step size of 0.2 amu. Data processing was performed with the aid of the software package Biomultiview (PE-SCIEX, Canada).

Chick Isolated Biventer Cervicis Nerve-Muscle Preparation

Male White leg horn chicks aged between 4 and 8 days were killed with CO_2 and both biventer cervicis nerve-muscle preparations were removed. These were mounted under 1 g resting tension in organ baths containing Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; MgSO_4 , 1.2; KH_2PO_4 , 1.2; CaCl_2 , 2.5; NaHCO_3 , 25; and glucose, 11.1. The Krebs solution was bubbled with carbogen (95% O_2 and 5% CO_2) and maintained at 34°C . Twitches were evoked by stimulating the motor nerve every 10 s with pulses of 0.2 ms duration at a supramaximal voltage (Harvey *et al.*, 1994) using a Grass S88 stimulator. After a 30-min equilibration period, *d*-tubocurarine (10 μM) was added. Subsequent abolition of twitches confirmed selective stimulation of nerves. Twitches were then reestablished by thorough washing. Contractile responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s), and potassium chloride (KCl; 40 mM for 30 s) were obtained in the absence of stimulation (Harvey *et al.*, 1994). Electrical stimulation was then recommenced and the preparations allowed to equilibrate for a further 30-min period before commencement of the experiment. Venoms were left in contact with the preparations until complete twitch blockade occurred, or for a 4-h period. Contractile responses to ACh, CCh, and KCl were then obtained as previously described. Where indicated, death adder antivenom (1–5 units/ml) was added 10 min prior (Barfaraz and Harvey, 1994; Crachi *et al.*, 1999; Wickramaratna and Hodgson, 2001) to the addition of venoms (10 $\mu\text{g/ml}$).

Drugs

The following drugs were used: acetylcholine chloride (Sigma, St. Louis, MO); bovine serum albumin (BSA; Sigma); carbamylcholine chloride (carbachol; Sigma); *d*-tubocurarine chloride (Sigma). Except where indicated, stock solutions were made up in distilled water. Death adder antivenom, which is raised against *A. antarcticus* venom in horses, was obtained from CSL Ltd. (Australia).

Analysis of Results and Statistics

For isolated tissue experiments, responses were measured via a Grass force displacement transducer (FT03) and recorded on a MacLab System. To compare the neurotoxicity of venoms, the time taken to cause 90% inhibition of nerve-mediated twitches (i.e., t_{90} values) was determined. t_{90} values were calculated for each experiment by determining the elapsed time after venom addition at 10% of the initial twitch height, and then the means and standard error of the means were calculated. A two-way ANOVA was performed for comparison of t_{90} values between venoms and concentrations. Where indicated, curves were compared by a two-way repeated measures ANOVA. Contractile responses to ACh, CCh, and KCl were expressed as a percentage of the respective initial response. These were analyzed using either Student's paired *t*-tests or, where stated, compared against the control response via a one-way ANOVA. All ANOVAs were followed by a Bonferroni post hoc test. Statistical significance was indicated when $p < 0.05$.

RESULTS

Liquid Chromatography–Mass Spectrometry Analysis of Venoms

Venoms were profiled using LC-MS to determine differences in venom composition. All venoms had essentially the same generalized elution profile: an early eluting component (~24% acetonitrile), a few percentage pause without any appreciable components eluting, and then the vast majority of components eluting between about 30 to 40% acetonitrile (Fig. 1). The first eluting component in all venoms had a mass similar to that of the short-chain neurotoxin Aa-c, previously isolated from *A. antarcticus* venom. A greater number of components were shared by *A. antarcticus* geographic variants (NSW, Qld, SA, WA) than between species (Table 1).

Neurotoxicity Studies

All *Acanthopis* venoms (10 $\mu\text{g/ml}$) caused time-dependent inhibition of nerve-mediated twitches, whereas vehicle (i.e., BSA) had no inhibitory effect on twitch height ($n = 4\text{--}8$; Figs. 2a and 2b). There was no significant difference in neurotoxicity between the *A. antarcticus* venoms at this concentration (two-way repeated measures ANOVA, $p < 0.05$). However, *A. hawkei*, *A. praelongus*, and *A. pyrrhus* venoms were significantly less neurotoxic than *A. sp. Seram* venom (10 $\mu\text{g/ml}$; two-way repeated measures ANOVA, $p < 0.05$). All venoms at 10 $\mu\text{g/ml}$ totally abolished contractile responses to exogenous ACh (1 mM) and CCh (20 μM), but not KCl (40 mM), whereas the vehicle had no significant inhibitory effect on the contractile responses to exogenous agonists ($n = 4\text{--}5$; Student's paired *t*-test, $p < 0.05$, data not shown).

All *Acanthopis* venoms (0.3–10 $\mu\text{g/ml}$; $n = 4\text{--}15$) caused

a concentration-dependent inhibition of nerve-mediated twitches (two-way ANOVA, $p < 0.05$; Figs. 3a and 3b). Because experiments were terminated after 4 h, t_{90} values for venoms (0.3 $\mu\text{g/ml}$) from *A. antarcticus* (SA), *A. praelongus*, *A. rugosus*, *A. wellsi*, and *A. hawkei* were not determined. Although not shown in Fig. 3b, the t_{90} values for *A. pyrrhus* and *A. sp. Seram* venoms (0.3 $\mu\text{g/ml}$; $n = 8$) were 180 ± 18 and 212 ± 32 min, respectively. *A. antarcticus* (SA) venom was significantly less neurotoxic than *A. antarcticus* (Qld) and *A. antarcticus* (NSW) venoms (two-way ANOVA, $p < 0.05$; Fig. 3a). *A. hawkei* venom was significantly less neurotoxic than *A. sp. Seram* venom (two-way ANOVA, $p < 0.05$; Fig. 3b). At lower concentrations (0.3–1.0 $\mu\text{g/ml}$) there was a greater degree of spread of neurotoxicity than at higher concentrations (3–10 $\mu\text{g/ml}$). In the case of *A. wellsi* and *A. pyrrhus* venoms, the rank order of venom neurotoxicity, based on t_{90} values, altered with the change in concentration (Fig. 3b).

Efficacy of Death Adder Antivenom

Prior incubation (10 min) of death adder antivenom (1 unit/ml) significantly delayed twitch inhibition produced by all *Acanthopis* venoms (10 $\mu\text{g/ml}$; $n = 4\text{--}8$; two-way repeated measures ANOVA, $p < 0.05$; Figs. 4a and 5a). Among the *A. antarcticus* geographic variants, antivenom markedly attenuated the neurotoxicity of *A. antarcticus* (Qld) venom, while having a significantly lesser effect on *A. antarcticus* (NSW) and *A. antarcticus* (WA) venoms ($n = 5\text{--}7$; two-way repeated measures ANOVA, $p < 0.05$; Fig. 4a). In addition, in the presence of antivenom (1 unit/ml) all four *A. antarcticus* venoms (10 $\mu\text{g/ml}$) continued to significantly inhibit contractile responses to exogenous ACh (1 mM) and CCh (20 μM) compared to the antivenom control (i.e., antivenom only; $n = 5\text{--}7$; one-way ANOVA, $p < 0.05$; Fig. 4b).

Prior incubation of antivenom (1 unit/ml) prevented twitch inhibition by *A. hawkei*, *A. praelongus*, and *A. pyrrhus* venoms (10 $\mu\text{g/ml}$). However, in the presence of antivenom, *A. wellsi*, *A. rugosus*, and *A. sp. Seram* venoms (10 $\mu\text{g/ml}$) continued to significantly inhibit the twitch response compared to the antivenom control ($n = 4\text{--}8$; two-way repeated measures ANOVA, $p < 0.05$; Fig. 5a). Furthermore, in the presence of antivenom (1 unit/ml), *A. wellsi*, *A. rugosus*, and *A. sp. Seram* venoms continued to significantly inhibit contractile responses to exogenous ACh and CCh compared to the antivenom control ($n = 4\text{--}8$; one-way ANOVA, $p < 0.05$; Fig. 5b). However, antivenom prevented *A. hawkei*, *A. praelongus*, and *A. pyrrhus* venoms from inhibiting contractile responses to exogenous ACh and CCh ($n = 4\text{--}8$; Fig. 5b).

Prior incubation of antivenom (5 units/ml) prevented twitch inhibition by *A. antarcticus* (NSW, Qld, SA, WA), *A. rugosus*, *A. sp. Seram*, and *A. wellsi* venoms (10 $\mu\text{g/ml}$; $n = 3\text{--}4$; data not shown). Similarly, antivenom (5 units/ml) prevented these venoms from inhibiting contractile responses to exogenous ACh and CCh ($n = 3\text{--}4$; data not shown).

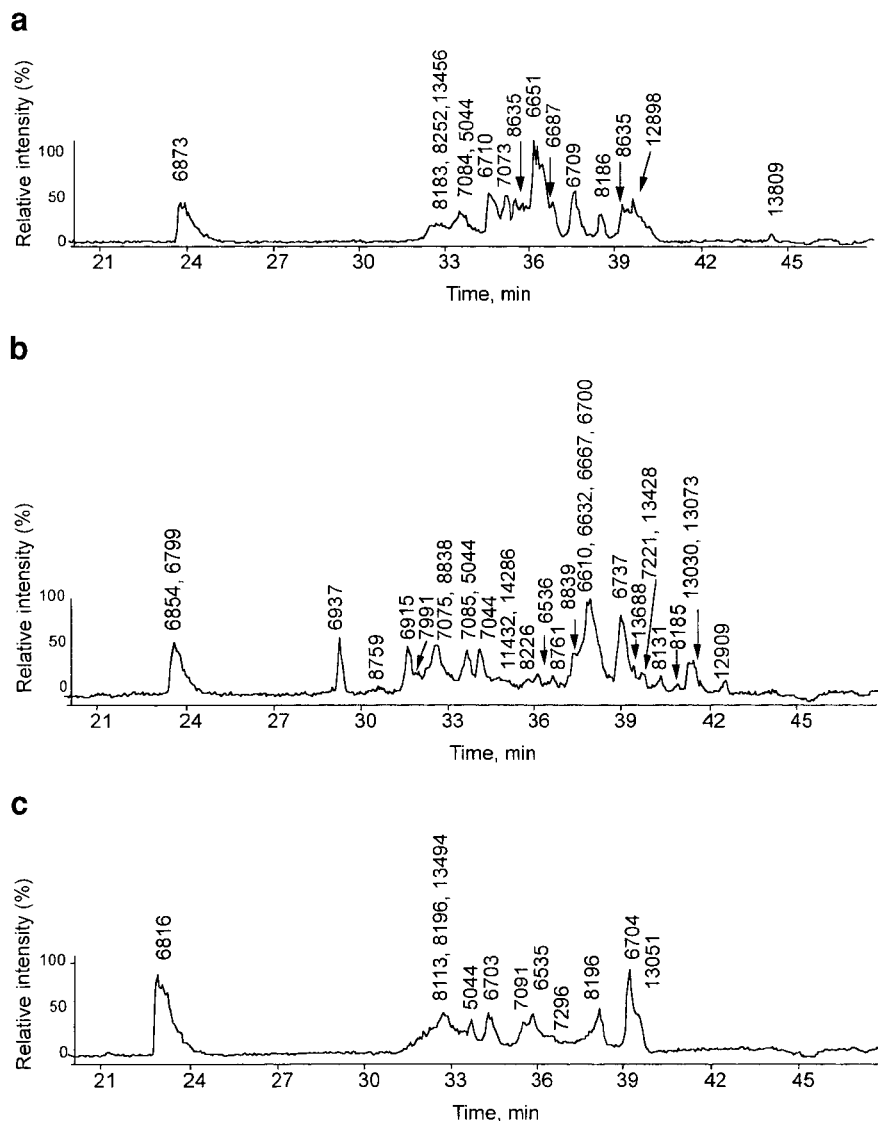


FIG. 1. Representative LC-MS profiles: comparison of the two main Australian *Acanthophis* species (a) *A. antarcticus* (NSW population) and (b) *A. praelongus* with (c) the venom of the species from the Indonesian island of Seram. LC-MS profiles for other venoms not shown.

DISCUSSION

A. antarcticus crude venom was previously examined for lethality, neurotoxicity, myotoxicity, and its effects on blood coagulation, both experimentally and clinically (Fairley, 1929; Kellaway, 1929a,b; Campbell, 1966; Broad *et al.*, 1979; Sutherland, 1983). Recently, *A. praelongus* and *A. pyrrhus* venoms were studied for *in vitro* neurotoxicity, myotoxicity, and phospholipase A₂ activity (van der Weyden *et al.*, 2000; Wickramaratna and Hodgson, 2001). To date, no pharmacological studies have been carried out on whole venoms from *A. hawkei*, *A. wellsi*, *A. rugosus*, and *A. sp.* Seram. Therefore, this study examined the *in vitro* neurotoxicity of these crude venoms and compared these to the previously studied *A. antarcticus*, *A. praelongus*, and *A. pyrrhus* venoms. In addition, the

efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of these venoms was examined.

Venoms were profiled using on-line liquid chromatography–mass spectrometry to determine basic biochemical differences. As previously detailed, all venoms had essentially the same generalized elution profile. Given that these venoms are from snakes belonging to the same genus this is not surprising. However, close examination and comparison of each profile showed many differences in peak distribution and complexity between venoms from different species of death adder. Such species variations in chromatographic profiles were previously observed for *A. antarcticus*, *A. praelongus*, and *A. pyrrhus* venoms (van der Weyden *et al.*, 2000). In the present study, LC-MS profiles of venoms from *A. antarcticus* geographic

TABLE 1
Components Shared by Two or More *Acanthophis* Venoms

		Venom ^a (m/z)							
ant									
NSW	Qld	SA	WA	Ser	haw	pra	pyr	rug	wel
6873	6873	6873	6873						
						6914	6913		
8186	8184	8183	8183						
8252	8252	8252	8252						
5044	5044	5044	5044	5044	5044			5044	
6710	6710	6710	6711		6709				
7073	7070		7070				7075		
6651	6652	6652	6654		6653			6657	
6687	6686		6688						
	6676	6676	6679		6675		6676		6678
					13427	13428			
12898			12898						
13809	13810								

Note. Molecular weights of components are presented by order of elution.

^a ant = *A. antarcticus*; Ser = *A. sp. Seram*; haw = *A. hawkei*; pra = *A. praelongus*; pyr = *A. pyrhus*; rug = *A. rugosus*; wel = *A. wellsi*.

variants showed a lesser degree of variability. Furthermore, a greater number of components were shared by *A. antarcticus* geographic variants than between species. Previous reports suggest that variations in venom composition as a result of geographic location or difference in species are not unique to death adders (Jimenez-Porras, 1964; Williams *et al.*, 1988; Yang *et al.*, 1991; Assakura *et al.*, 1992; Daltry *et al.*, 1996). The large distances separating the *A. antarcticus* geographic variants may account for the variations in their venom composition. Daltry *et al.* (1996) showed that variations in venom composition as a result of geographic location reflect natural selection for feeding on local prey. LC-MS venom profiles may be used to suggest taxonomic relationships among death adder species, as was previously suggested for some spider venoms (Escoubas *et al.*, 1997).

Although it is beyond the scope of this study to fully characterize species variations in venom composition, preliminary observations can be made. The venom profile of *A. antarcticus* varies little over its vast range, with the majority of the venom components being conserved in this species but with minimal conservation among other species. Certain venoms, such as that of *A. praelongus*, appear to be much more complex than those of other species, such as *A. sp. Seram*. This may be the case but may also indicate that *A. praelongus*, in fact, is a species complex. Given that venoms used in the study were the result of pooling of venoms of *A. praelongus* across its range this has implications upon homology of samples. The apparent diversity in venom composition will be the focus of a follow-up study.

The first eluting components have masses corresponding to isoforms of the short-chain neurotoxin Aa-c (Kim and Tamiya,

1981b). *A. praelongus* is notable in being the only species with an intermediate eluting component. The characteristic masses for each species of this first peak allows for preliminary *m/z* fingerprinting and these components have been isolated for characterization. Molecular weights corresponding with PLA₂ toxins are also present in all the venoms, in greater quantities and molecular weight diversities than were expected. These components have also been isolated and are presently being characterized. A component of mass 5044 is present in the vast majority of venoms and does not correspond in molecular weight with other isolated components from elapids and thus may represent a new class of venom molecule.

Clinically, the most important symptoms of death adder envenomations are those relating to neurotoxicity, such as ptosis, generalized paralysis, and respiratory failure (Campbell, 1966; Laloo *et al.*, 1996). However, nothing is known about the neurotoxicity of *A. hawkei*, *A. wellsi*, *A. rugosus*, and *A. sp. Seram* venoms. Furthermore, we compared the venoms from *A. antarcticus* geographic variants to determine whether variations in venom composition, as a result of geographic location, are reflective of neurotoxic activity. Therefore, the neurotoxicity of death adder venoms was investigated using the chick biventer cervicis nerve-muscle preparation. In this preparation, all venoms caused time-dependent inhibition of indirect twitches. Furthermore, all venoms abolished contractile responses to acetylcholine and carbachol but not KCl, thus indicating the presence of postsynaptically acting neurotoxins in these death adder venoms. This is in agreement with previous studies showing that the neurotoxicity of *A. antarcticus* venom is mainly attributed to the presence of postsynaptic neurotoxins (Sheumack *et al.*, 1979, 1990; Kim and Tamiya,

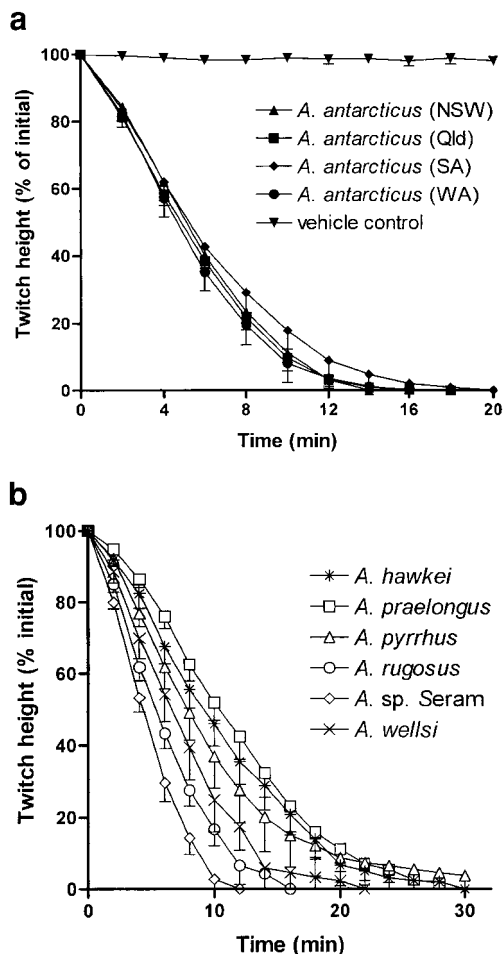


FIG. 2. (a) Effect of venoms ($10 \mu\text{g/ml}$; $n = 4-5$) from *A. antarcticus* geographic variants or vehicle ($n = 8$) on nerve-mediated twitches in the chick isolated biventer cervicis nerve-muscle preparation. (b) Effect of venoms ($10 \mu\text{g/ml}$; $n = 4-5$) from other *Acanthophis* spp. on nerve-mediated twitches in the chick isolated biventer cervicis nerve-muscle preparation.

1981a,b; Tyler *et al.*, 1997; Wickramaratna and Hodgson, 2001).

Because of the complex regulatory requirements involved in gaining approval for murine LD₅₀ studies in many countries, including Australia, they have been largely superseded by *in vitro* studies. One method of measuring the neurotoxicity of venoms is by determining t_{90} values in isolated skeletal muscle preparations (Harvey *et al.*, 1994). Comparison of t_{90} values, at $10 \mu\text{g/ml}$, indicated the following rank order of neurotoxicity: *A. sp. Seram* \geq *A. antarcticus* (Qld) \geq *A. antarcticus* (WA) \geq *A. antarcticus* (NSW) \geq *A. rugosus* \geq *A. antarcticus* (SA) \geq *A. wellsii* \geq *A. hawkei* \geq *A. pyrrhus* \geq *A. praelongus*. However, for some venoms the rank order of neurotoxicity altered with a change in venom concentration. This was especially the case with regard to the potency of *A. wellsii* and *A. pyrrhus* venoms. This possibly results from the fact that death adder venoms contain a number of neurotoxins with various quantities of each (Sheumack *et al.*, 1979, 1990; Kim and Tamiya,

1981a,b; Tyler *et al.*, 1997). Thus, the neurotoxicity of the whole venom is dependent not only on the toxicity of each neurotoxin but also on the quantity of each neurotoxin within the venom. Therefore, particularly at lower concentrations of whole venom, the quantity of each neurotoxin within the venom becomes important. Although there were no significant differences in neurotoxicity between venoms from *A. antarcticus* geographic variants at a concentration of $10 \mu\text{g/ml}$, there were significant differences when other concentrations were taken into account. *A. antarcticus* (SA) venom was significantly less neurotoxic than *A. antarcticus* (Qld) and *A. antarcticus* (NSW) venoms. Therefore, venoms from *A. antarcticus* geographic variants differ not only in their venom composition but also in their neurotoxic activity.

Whether the rank order of neurotoxicity is representative of the rank order of venom LD₅₀ values for this genus remains to be elucidated. However, it is tempting to speculate that this could be the case because lethality of death adder venoms is

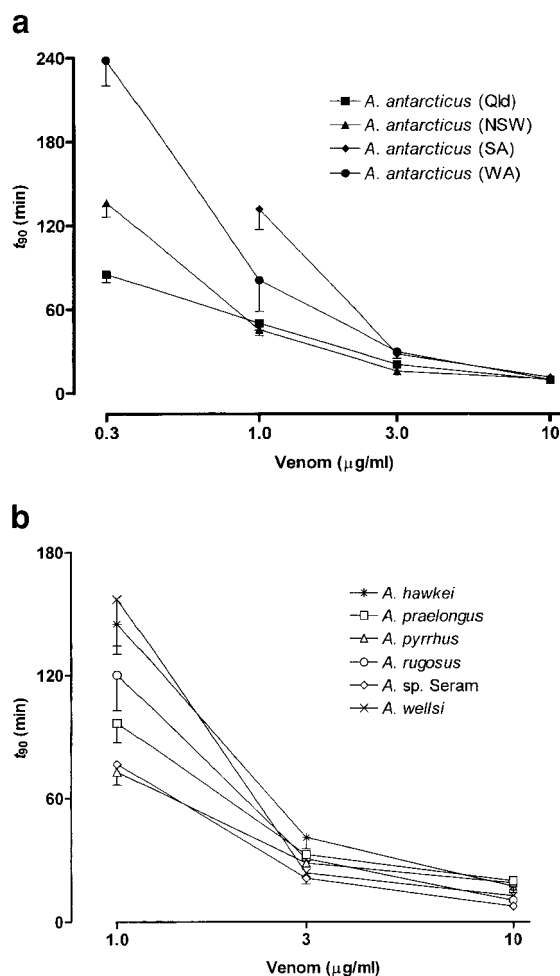


FIG. 3. (a) Graph showing t_{90} values (min) against venom concentrations ($n = 4-5$) for *A. antarcticus* geographic variants. (b) Graph showing t_{90} values (min) against venom concentrations ($n = 4-15$) for other *Acanthophis* species.

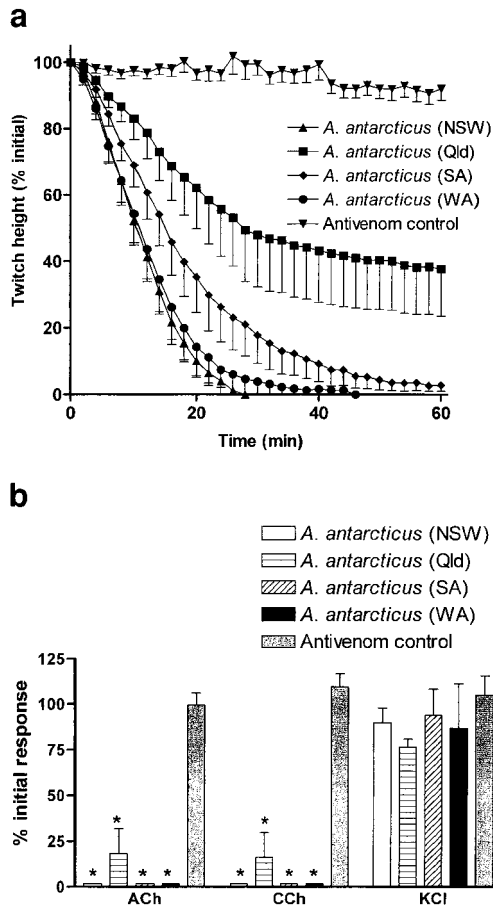


FIG. 4. (a) Effect of venoms ($10 \mu\text{g/ml}$; $n = 5-7$) from *A. antarcticus* geographic variants, in the presence of antivenom (1 unit/ml), or antivenom control ($n = 8$) on nerve-mediated twitches in the chick isolated biventer cervicis nerve-muscle preparation. (b) Effect of venoms ($10 \mu\text{g/ml}$; $n = 5-7$) from *A. antarcticus* geographic variants, in the presence of antivenom (1 unit/ml), or antivenom control ($n = 8$) on contractile responses to exogenous ACh, CCh, and KCl in the chick isolated biventer cervicis nerve-muscle preparation. * $p < 0.05$, significantly different from antivenom control response, one-way ANOVA.

largely the result of respiratory failure (Kellaway, 1929b, 1932; Campbell, 1966). Although it should be noted that murine LD50 determinations are based on “quantity” (i.e., what concentration of venom kills 50% of mice usually over a 24- to 48-h period), whereas t_{90} values are based on how “quick” a venom acts. Therefore, it is possible to have an extremely “lethal” venom (based on LD50 values), which takes a long time to produce its effects. Therefore, knowledge of both parameters is desirable. In the present study, it was not possible to determine the presence of presynaptic neurotoxins in death adder crude venoms. Presynaptic neurotoxins have a slow onset of action, with a latency period of up to 60 min resulting from internalization and disruption of the presynaptic processes (Chang and Su, 1982; Chang, 1985). Therefore, it is possible that the action of any presynaptic neurotoxins was masked by the faster acting postsynaptic neurotoxins in the

death adder crude venoms (Lee, 1979). However, fractionation of these venoms will allow the identification of any presynaptic neurotoxins.

In Australia, CSL death adder antivenom is indicated for use in envenomation by any death adder species (AMH, 1998). A previous *in vitro* study by us showed that CSL death adder antivenom (1 unit/ml), although very effective against *A. praelongus* and *A. pyrrhus* venoms, was significantly less effective against the neurotoxicity of *A. antarcticus* (Qld) venom (Wickramaratna and Hodgson, 2001). However, no studies have examined the efficacy of antivenom against venoms from *A. hawkei*, *A. wellsi*, *A. rugosus*, and *A. sp. Seram*. Furthermore, it was of interest to determine the efficacy of antivenom against venoms from other *A. antarcticus* geographic variants. Therefore, the efficacy of CSL death adder antivenom was determined according to the procedure described by Barfaraz and Harvey (1994). Prior incubation of antivenom (1 unit/ml) significantly attenuated twitch inhibition produced by all venoms. Antivenom (1 unit/ml) totally pre-

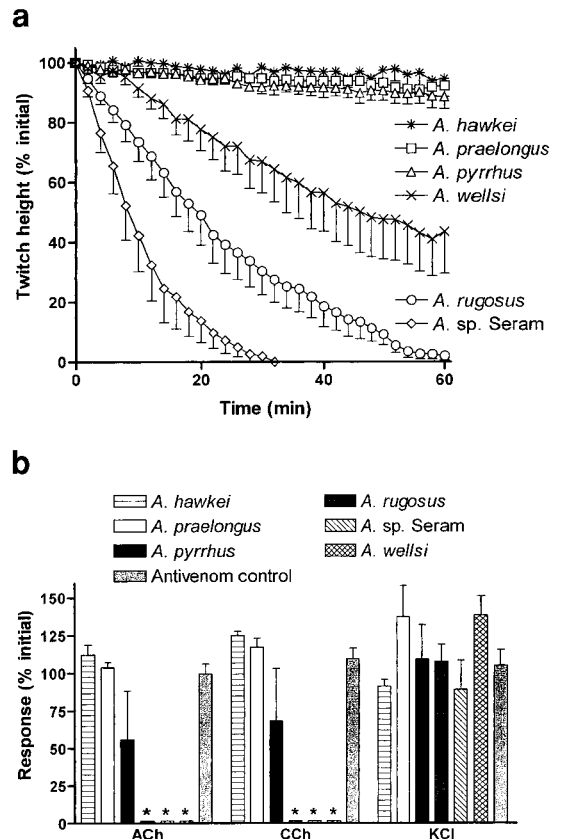


FIG. 5. (a) Effect of venoms ($10 \mu\text{g/ml}$; $n = 4-8$) from other *Acanthopis* spp., in the presence of antivenom (1 unit/ml) on nerve-mediated twitches in the chick isolated biventer cervicis nerve-muscle preparation. (b) Effect of venoms ($10 \mu\text{g/ml}$; $n = 4-8$) from other *Acanthopis* spp., in the presence of antivenom (1 unit/ml), or antivenom control ($n = 8$) on contractile responses to exogenous ACh, CCh, and KCl in the chick isolated biventer cervicis nerve-muscle preparation. * $p < 0.05$, significantly different from antivenom control response, one-way ANOVA.

vented twitch inhibition by *A. hawkei*, *A. praelongus*, and *A. pyrrhus* venoms. In addition, antivenom prevented *A. hawkei*, *A. praelongus*, and *A. pyrrhus* venoms from inhibiting contractile responses to exogenous ACh and CCh. Thus, antivenom raised against *A. antarcticus* venom is very effective against the neurotoxicity of *A. hawkei*, *A. praelongus*, and *A. pyrrhus* venoms. However, antivenom (1 unit/ml) was markedly less effective against venoms from *A. antarcticus* (Qld), *A. antarcticus* (SA), *A. rugosus*, and *A. wellsi*. Interestingly, antivenom (1 unit/ml) only delayed the neurotoxicity of venoms from *A. sp. Seram*, *A. antarcticus* (NSW), and *A. antarcticus* (WA). Furthermore, in the presence of antivenom (1 unit/ml), *A. antarcticus* (Qld, SA, NSW, WA), *A. rugosus*, *A. wellsi*, and *A. sp. Seram* venoms continued to significantly inhibit contractile responses to exogenous ACh and CCh.

Given that *A. hawkei*, *A. praelongus*, and *A. pyrrhus* venoms (10 µg/ml) were the least neurotoxic, it makes sense that these venoms were the most affected by the antivenom. However, it is surprising that *A. antarcticus* (Qld) venom was the most affected by antivenom compared to the other *A. antarcticus* geographic variants. In this case, *A. antarcticus* (Qld) venom was the most neurotoxic, whereas *A. antarcticus* (SA) venom was the least neurotoxic. Thus, it may have been expected that antivenom would have a greater effect on *A. antarcticus* (SA) venom than on *A. antarcticus* (Qld) venom. Furthermore, it is surprising that antivenom raised against *A. antarcticus* venom was markedly less effective against the neurotoxicity of all venoms from *A. antarcticus* geographical variants compared to venoms from other death adder species. It is possible that death adder antivenom may neutralize some neurotoxins within the venoms better than others. Thus, the effectiveness of antivenom may be dependent on its ability to neutralize the different neurotoxins within the whole venoms.

To further study the efficacy of CSL death adder antivenom the concentration of antivenom was increased. Prior incubation of antivenom (5 units/ml) prevented twitch inhibition by *A. antarcticus* (NSW, Qld, SA, WA), *A. rugosus*, *A. sp. Seram*, and *A. wellsi* venoms. Similarly, antivenom (5 units/ml) prevented these venoms from inhibiting contractile responses to exogenous ACh and CCh. Thus, at higher concentrations antivenom is capable of completely neutralizing all death adder venoms. Usually, in clinical situations antivenom is administered until symptoms of neurotoxicity (e.g., ptosis) are reversed (AMH, 1998; Currie, 2000). Clinical studies have shown that CSL death adder antivenom is very effective in reversing the effects of death adder envenomation in Papua New Guinea (Campbell, 1966; Lalloo *et al.*, 1996). This is not surprising if, as suggested by O'Shea (1996) and Lalloo *et al.* (1996), *A. praelongus* or a variant of this species is responsible for envenomations in some parts of Papua New Guinea. A detailed taxonomic study of the Indonesian/New Guinean death adders may be required before the taxonomy of this widespread and complicated genus can be fully understood.

In conclusion, all death adder venoms are predominately

postsynaptically neurotoxic. Some venoms were significantly more neurotoxic than other death adder venoms. CSL death adder antivenom (1 units/ml) was found to be very effective against the neurotoxicity of *A. hawkei*, *A. praelongus*, and *A. pyrrhus* venoms, while markedly less effective against *A. antarcticus* (NSW, SA, WA), *A. rugosus*, *A. wellsi*, and *A. sp. Seram* venoms. However, a higher concentration of antivenom was effective against all death adder venoms. Death adder venoms, including those from *A. antarcticus* geographic variants, differed not only in their venom composition but also in their neurotoxic activity. Although this study was based on an *in vitro* preparation, it is anticipated that these findings will have a clinical significance in the event of death adder envenomation.

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