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Efficacy of antivenom against the procoagulant effect of Australian brown snake (*Pseudonaja* sp.) venom: In vivo and in vitro studies

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Abstract

Snake venom induced consumption coagulopathy (VICC) is a common complication of snake bite due to prothrombin activators or thrombin-like enzymes in the venom. This study aimed to determine the efficacy and dose of antivenom for treating VICC in patients envenomed by brown snakes (*Pseudonaja* spp.), including in vitro coagulation studies. In serial blood samples from patients with brown snake envenoming, venom and antivenom concentrations were measured using enzyme immunoassays. In vitro mixtures of brown snake venom and antivenom were used to investigate antivenom binding, neutralisation of prothrombin activity, prevention of venom-mediated clotting and effect on thrombin generation parameters using a thrombinoscope. In 27 envenomed patients the median venom concentration was 20 ng/mL (Interquartile range[IQR]:12–44 ng/mL) prior to antivenom and was not detected after antivenom administration, including 9 patients given one vial. In vitro, 200 μg/mL of antivenom bound all free venom at venom concentrations seen in patients. In vitro prothrombinase activity of the venom (using a chromogenic substrate) was not neutralised by antivenom. However, for venom concentrations seen in humans, 100 μg/mL of antivenom prevented venom clotting activity in human plasma and 479 μg/mL neutralised procoagulant venom activity measured by triggering thrombin generation. One vial of antivenom appears to be sufficient to bind and neutralise all venom in patients with severe brown snake envenoming.

Keywords: *Pseudonaja* sp.; Snake envenoming; Antivenom; Procoagulant; Brown snake; Coagulopathy

1. Introduction

Worldwide, there are in the order of 2.5 million snake envenomings each year and about 100,000 deaths in healthy and productive individuals (White et al., 2003). This is a particular problem in the rural

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tropics and coagulopathy from snake bite occurs in many cases. Although often not recognised, snake venom induced consumption coagulopathy (VICC)(Isbister et al., 2002) is probably the commonest cause worldwide of acquired afibrinogenaemia due to prothrombin activators (Warrell and Arnett, 1976) or thrombin-like enzymes in the venom. (Milani et al., 1997). Although snakebite is an uncommon cause of death in Australia (1–4 per annum) (Sutherland and Leonard, 1995) it continues to be a significant treatment issue in regional and rural hospitals.

Brown snake (*Pseudonaja* sp.) envenoming is arguably the most important in Australia. Envenoming causes a rapid consumption of coagulation factors or VICC due to a potent prothrombin activator in the venom (Masci et al., 1988; Filippovich et al., 2005; Birrell et al., 2006). This results in a defibrination coagulopathy and the potential for life-threatening haemorrhage (Yeung et al., 2004; Currie, 2004b). Despite the presence of neurotoxins in the venom, neurotoxicity is rare (Currie, 2004b, 2000). Other effects including thrombotic microangiopathy and renal failure are also uncommon. Brown snake envenoming continues to cause deaths each year, most commonly from haemorrhage or multi-organ failure (Jelinek et al., 2005; White, 2000). The medically important toxin is a prothrombin activator complex that makes up a significant proportion of the venom (Masci et al., 1988; Birrell et al., 2006). The toxin and thus whole venom is procoagulant in vitro where it causes whole blood and plasma to rapidly clot, but in vivo it causes a coagulopathy due to consumption of essential clotting factors.

An antivenom has been available since 1956 for brown snake envenoming and has been used for the treatment of thousands of cases since that time. Over the last decade there has been increasing concern that larger doses of brown snake antivenom are required than the dose initially recommended by the manufacturer, CSL Ltd. (Yeung et al., 2004; Sprivulis et al., 1996; Tibballs and Sutherland, 1991). This has changed recommendations for the initial dose of antivenom and the manufacturer has doubled the antivenom content in each vial since its introduction. Most guidelines now recommend five vials (equivalent to 10 times the initial dose recommended when antivenom was introduced), (White, 2001; Isbister, 2004). Despite numerous anecdotes, case reports and animal studies, there are fundamental questions regarding the efficacy of this antivenom, the dose of antivenom and end-points for treatment.

Animal studies, (Tibballs and Sutherland, 1991) in vitro human studies (Sprivulis et al., 1996) and clinical studies (Yeung et al., 2004) have all suggested that larger doses are required to neutralise the procoagulant activity of brown snake venom. The usual explanation for this has been that polyclonal $F(ab')_2$ directed at the prothrombin activator has low binding affinity and so larger amounts are required to bind all of the prothrombin activator and neutralise its activity (Madaras et al., 2005; Sprivulis et al., 1996). However, no binding studies have been undertaken and studies investigating the effectiveness of the antivenom in neutralising the venom procoagulant activity provide contradictory results (Madaras et al., 2005; Tibballs and Sutherland, 1991; Sprivulis et al., 1996).

Previous in vitro studies that were unable to demonstrate neutralisation of the procoagulant activity of the venom used very high venom concentrations (Madaras et al., 2005; Sprivulis et al., 1996). This has been based on the assumption that large amounts of venom enter the systemic circulation and are required to cause envenoming. We have recently shown in a small series that the concentration of brown snake venom in serum in envenomed cases ranges from 8 to 95 ng/mL (O'Leary et al., 2006) which is much lower than the concentrations of up to 50 μg/mL used in previous neutralisation studies (Madaras et al., 2005; Sprivulis et al., 1996).

We investigated the effect of antivenom on the venom, using four techniques: Enzyme immunoassay (EIA), prothrombinase activity, clotting times and thrombinoscopy. The aim of the study was to determine how much antivenom was required to bind and neutralise venom and to establish the dose of antivenom that is likely to neutralise the effects of brown snake venom in envenomed patients.

2. Methods

2.1. Materials

Brown snake (*Pseudonaja textilis*) venom was purchased from Venom Supplies, South Australia and Brown Snake Antivenom was produced by CSL Ltd. Hen anti-brown snake venom affinity-purified IgY was kindly donated by Frank Madaras (Madaras et al., 2005). Tetramethylbenzidine (TMB), horseradish peroxidase (HPO) and HPO-

conjugated anti-horse IgG were obtained from Sigma. HPO-conjugated IgY was prepared according to a standard procedure (Crowther, 1995). A 0.5% solution of BSA in PBS was used as blocking solution. Greiner Microlon 96-well high-binding plates were used for ELISA assays.

TRIS buffer is 0.05 M tris(hydroxymethyl)methylamine pH 8.0. S-2765 was supplied by Chromogenix. Phenomenex C18-U 100 mg/1 mL solid phase extraction (SPE) cartridges were conditioned with 1 mL methanol and 1 mL water before use. High-performance liquid chromatography (HPLC) was carried out on a Phenomenex Synergi 4u MAX-RP 80A 150 × 4.6 mm column, with mobile phase 50% MeOH at 0.5 mL/min. pNA was detected at 405 nm. Fresh, pooled citrated human plasma was used in the clotting and thrombinoscope experiments.

2.2. Samples from envenomed patients

Serum samples were obtained from patients with brown snake (*Pseudonaja* sp.) envenoming recruited from the Australian Snakebite Project (ASP) including a preceding prospective study of snake envenoming in Darwin (Currie, 2004b). ASP is a multicentre prospective observational study recruiting snake bite cases from over 40 tertiary and regional hospitals, and from referrals to three major poison information centres. Ethics approval has been obtained for all institutions involved in the study. Baseline demographic and clinical data, including features of envenoming and time of resolution of each feature are recorded on a standardised study datasheet, and all interventions are recorded on the study datasheet. A commercial snake venom detection kit (SVDK) is performed on the bite site swabs to identify the species group, and the snake is identified by an expert if brought in by the patient.

In this study samples were only included from patients where: (1) severe venom-induced consumptive coagulopathy occurred with complete defibrination defined as fibrinogen <0.5 g/L or PT >24 s with significantly raised XDP; (2) only monovalent brown snake antivenom was given; and (3) samples were available before and after antivenom administration. All samples were stored at $-80\,^{\circ}$C until assayed. Venom and antivenom concentrations were measured in all samples and prothrombinase activity was measured in cases where citrate-anticoagulated plasma was available.

2.3. Brown snake venom and antivenom immunoassays

Detection of free brown snake venom was carried out on an anti-brown snake IgY-coated plate, with HPO-conjugated labeled IgY as the detecting antibody. It has been previously shown by gel immunoelectrophoresis that the IgY has strong affinity for all the toxins in the venom (Madaras et al., 2005) and we have shown using our EIA method that the IgY binds to textilotoxin and the prothrombin activator (data not shown). Detection of brown snake antivenom (unbound) was carried out on a plate coated with brown snake venom, with labeled anti-horse IgG as the detecting antibody. Plates were read at 450 nm on a Bio-Rad Microplate Manager. The methods for the brown snake venom and antivenom enzyme immunoassays are described in detail elsewhere (O'Leary et al., 2006).

2.4. Binding studies

To determine the amount of antivenom required to bind free venom in solution, the effect on EIA detection of free venom of adding increasing amounts of CSL brown snake antivenom to known amounts of venom was investigated. Three venom concentrations 12.5, 50 and 100 ng/mL in blocking solution, were mixed with increasing concentrations of antivenom ranging from 0 to 500 μg/mL. These venom concentrations were chosen because they were within the range of those found in patients. Venom–antivenom mixtures were allowed to stand for 2 h at room temperature in blocking solution before being applied to the IgY-coated plate. The percent free venom was then plotted against the antivenom concentration to determine the concentration when 5% or less venom remained unbound and when it was not detectable.

2.5. Chromogenic assay for prothrombin activity

Prothrombin enzymatic activity was assessed using the chromogenic substrate S-2765. This is a prothrombin analogue in the form of a derivatised tripeptide which releases a coloured product, paranitroaniline (pNA), when acted on by a prothrombinase, such as Factor Xa in plasma, or the prothrombin activator complex in venom. Antivenom and venom mixtures in blocking solution were allowed to stand for one hour before testing. To 100 μL of test solution in 1 mL of TRIS buffer in a

plastic tube was added 25 μL (43 nmol) of S-2765. After 10 min the reaction was stopped with 50 μL 50% acetic acid. The solution was then loaded onto a SPE cartridge slowly over 1 min. The cartridge was washed twice with 1 mL water and then eluted with 1 mL 50% methanol. The amount of pNA generated was then measured by HPLC. Prothrombin (chromogenic) activity was also measured in four patient samples prior to and after administration of antivenom to determine the effect of antivenom on neutralising prothrombin enzymatic activity in vivo.

2.6. Clotting times

Serial dilutions of AV (50 μL containing 0–4.22 mg;1 vial = 1000 U = 880 mg in 5.21 mL) and venom (50 μL containing 1000 ng, 500 ng, 100 ng, 50 ng, 20 ng and 10 ng) were made. Mixtures of 50 μL each of venom and AV were then incubated for 30 min at 37 °C. This was then added to 200 μL of pooled, citrated plasma kept at 37 °C to give final venom concentrations in plasma of 5000, 2500, 500, 250, 100, and 50 ng/mL. The time for cloudiness to appear was then observed, giving the clotting time. Clotting times were measured in seconds, and times greater than 1 h were regarded as having an infinite clotting time (i.e., no procoagulant effect detected).

2.7. Thrombinoscope

To further investigate the procoagulant effects of venom and the efficacy of antivenom in neutralising these effects, we made use of a Thrombinoscope™. This instrument is designed to measure thrombin generation in plasma. Full details of the method are available at www.thrombinoscope.com. In short, a fluorogenic substrate (an aminomethylcoumarin) is added to clotting plasma. The developing thrombin converts this into a fluorophore which is continuously measured by a fluorimeter. Calibration is performed by a modified thrombin which can convert the substrate to a fluorophore but which does not otherwise participate in the clotting cascade. Thrombinoscope software is used to analyse the fluorescence signals.

The standard thrombinoscopy method to initiate clotting of the plasma sample requires addition of Tissue Factor (thromboplastin). In our experiments we added venom instead of Tissue Factor to investigate the ability of venom to trigger the coagulation pathway. We were interested in the effect of increasing amounts of venom in pooled normal plasma on the thrombin generation parameters: lag-time, maximum concentration of thrombin [C_{max}], time to peak [T_{max}] and endogenous thrombin potential [ETP] calculated as the area under the curve. We expected venom to affect only the lag-time, T_{max} and C_{max} because venom would not change the intrinsic thrombin potential in normal plasma (i.e., the amount of prothrombin prior to activation). Once a venom concentration was determined that caused a maximal decrease on lag-time and T_{max} we investigated the effect of increasing antivenom concentration on lag-time and T_{max}.

Mixtures of venom and antivenom were allowed to stand in PBS buffer for 1 h at room temperature. 20 μL of the mixtures were placed in wells of an 8×12 microarray plate, each mixture being measured in triplicate. Sixty microliters of plasma was then added to each well, and the plate was placed in the instrument in accordance with the manufacturer's instructions. In the first experiment we tested the effect of a blank solution (no triggering agent), 25, 50, 75 and 100 ng/mL of venom and antivenom alone. In the second experiment we used a venom concentration of 100 ng/mL in all samples and varied the antivenom concentration from 13 up to 1667 μg/mL to determine the antivenom concentration that prevented venom activating the clotting cascade.

2.8. Analysis

Non-linear regression was used to fit antivenom and venom binding curves and lag time curves using Prism 4.0 and the most appropriate model. Prism 4.0 was also used to estimate the AV concentration required for binding or neutralisation effects of the antivenom.

3. Results

3.1. Serum venom and antivenom concentrations in envenomed patients

Samples collected before and after the first dose of antivenom had been given were available from 27 brown snake envenomed patients, including 9 cases where the snake was brought in and identified [*P. textilis* (2), *P. nuchalis* (5), *P. affinis* (1) and *Pseudonaja* sp. (1)]. The median patient age was 44

years (Interquartile range [IQR]: 32–52 yr) and 19 were male. All 27 patients had severe brown snake envenoming with VICC. No patient developed neurotoxicity, one patient developed a thrombotic microangiopathy with severe thrombocytopenia, haemolytic anaemia and renal failure and one had a mild rise in creatinine with normal urine output. There were no cases of intracranial bleeding or significant systemic bleeding.

The median brown snake venom concentration prior to the administration of antivenom was 20 ng/mL (IQR: 12–44 ng/mL; range: 4–95 ng/mL). Venom could not be detected in any of the samples (i.e., <4 ng/mL) taken a median of 1.3 h (IQR: 0.75–2.4 h) after the initial dose of antivenom. The range of initial doses of antivenom was 1–10 vials with a median dose of 3 (IQR: 1–5 vials) and in 9 patients only one vial was administered. The median brown snake antivenom concentration after administration of the initial dose was 770 μg/mL (IQR: 244–1930 μg/mL) and in those only administered one vial was 165 μg/mL (IQR: 154–236 μg/mL). Venom was not detected in any further samples collected from patients. Fig. 1 gives an example of serial venom and antivenom concentrations in one patient. In all patients clotting function returned to normal after antivenom administration. However, there was a lag in the time until clotting function was restored. The median time from the administration of the initial dose of antivenom until the prothrombin time was less than 24 s, was 11 h (IQR: 8–14 h).

3.2. Binding studies

The effect of increasing concentrations of antivenom on free venom detection by EIA (i.e., measurable free venom) is shown in Fig. 2. The graph demonstrates that for venom concentrations of 12.5–100 ng/mL, there is less than 5% free venom for antivenom concentrations greater than 100 μg/mL and free venom is undetectable (all bound) for antivenom concentrations over 200 μg/mL. This means that an antivenom concentration of 200 μg/mL is sufficient to bind all free venom for venom concentrations detected in patients (median 20 ng/mL [4–95 ng/mL]).

3.3. Prothrombinase activity

Chromogenic or prothrombinase activity was tested for a venom concentration of 100 ng/mL (maximum seen in human envenoming), with increasing antivenom concentrations. This is illustrated in Fig. 3, which shows that chromogenic activity increases rather than decreases with the

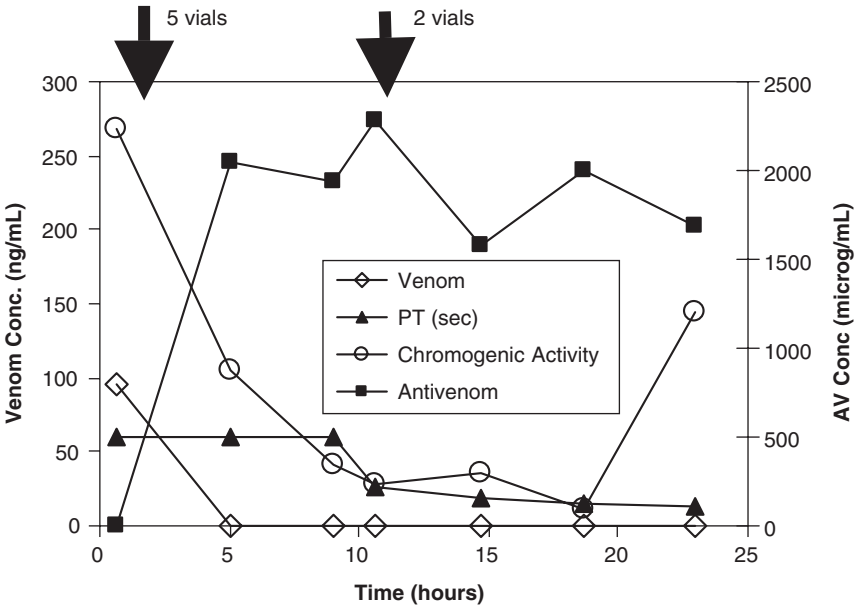

Fig. 1. Venom concentrations, antivenom concentrations, chromogenic activity and prothrombin time in a 9-year-old female with severe brown snake envenoming. The prothrombin time was more than 60 s and the activated partial thromboplastin time more than 120 s until 11 h after the bite, while fibrinogen remained undetectable (not shown).

addition of increasing amounts of antivenom. The amount of pNA generated by venom in the presence of AV is of the order of 2 to 3-fold that produced by venom alone. Increasing the AV concentration up to 4000 μg/mL caused no further suppression. The IgY antibody used for the EIA had a similar effect. CSL brown snake AV had no chromogenic activity itself, and it does not affect the chromogenic activity of FXa in plasma.

Chromogenic activity was measured in four patient samples before and after antivenom. Chromogenic activity was still detected in vivo in samples where free venom was not detectable although there did not appear to be an increase in activity. However, chromogenic activity did decrease over time, which is shown in Fig. 1 for one of the patients.

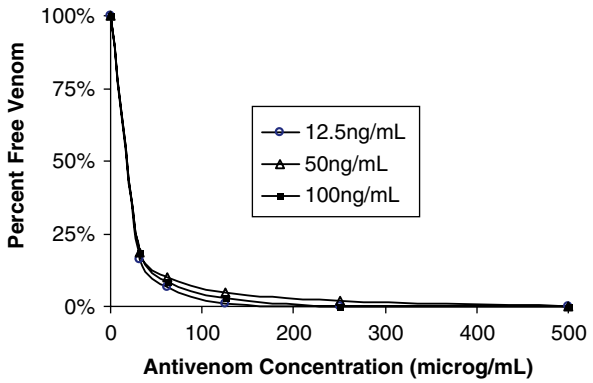

Fig. 2. Effect of the presence of antivenom on the amount of free brown snake venom detectable using the venom EIA. The venom EIA was performed on (A) 1.25 ng of venom in 100 μL of 0.5% BSA (12.5 ng/mL) [diamond], (B) 5 ng of venom in 100 μL of 0.5% BSA (50 ng/mL) [triangle] and (C) 10 ng of venom in 100 μL of 0.5% BSA (100 ng/mL) square] in the presence of increasing concentrations of antivenom. The data are plotted as percent free venom measured versus the antivenom concentration.

3.4. Clotting activity

Fig. 4 shows the effect of venom and antivenom on clotting times. Venom-induced clotting was neutralised for all concentrations of venom by increasing the antivenom concentration sufficiently. This was defined as a clotting time >1 h and the antivenom required to neutralise clotting activity for each venom concentration is reported in Table 1. High concentrations of venom (5000 ng/mL) caused rapid clotting that required very high concentrations of antivenom to neutralise. However, for venom concentrations in the upper range of those seen in humans (50 and 100 ng/mL), antivenom prevented the clotting activity at antivenom concentrations of 100 μg/mL or less.

3.5. Thrombinoscopy

In the first experiment the addition of all concentrations of venom decreased the lag time which is seen in Fig. 5(A) as a left shift of the curve. Antivenom alone had no effect. Venom also decreased the T_{max} but had no effect on the area under the curve, that is the ETP. In the second experiment the addition of increasing amounts of antivenom prevented the venom effect on lag time. Fig. 5(B) shows the relationship between antivenom concentration and lag time. This was fitted using non-linear regression with an exponential function rising from a plateau. Using this predicted model it was determined that an AV concentration of 479 μg/mL was required to reduce the venom effect on lag time by 90%.

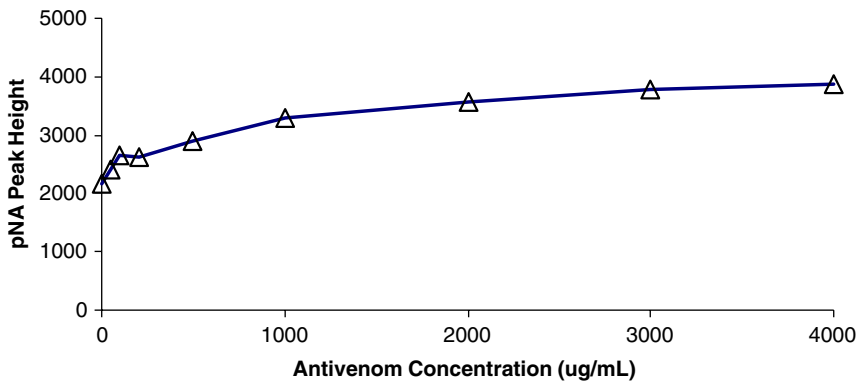

Fig. 3. Prothrombinase activity in samples containing 100 ng/mL venom and increasing concentrations of antivenom.

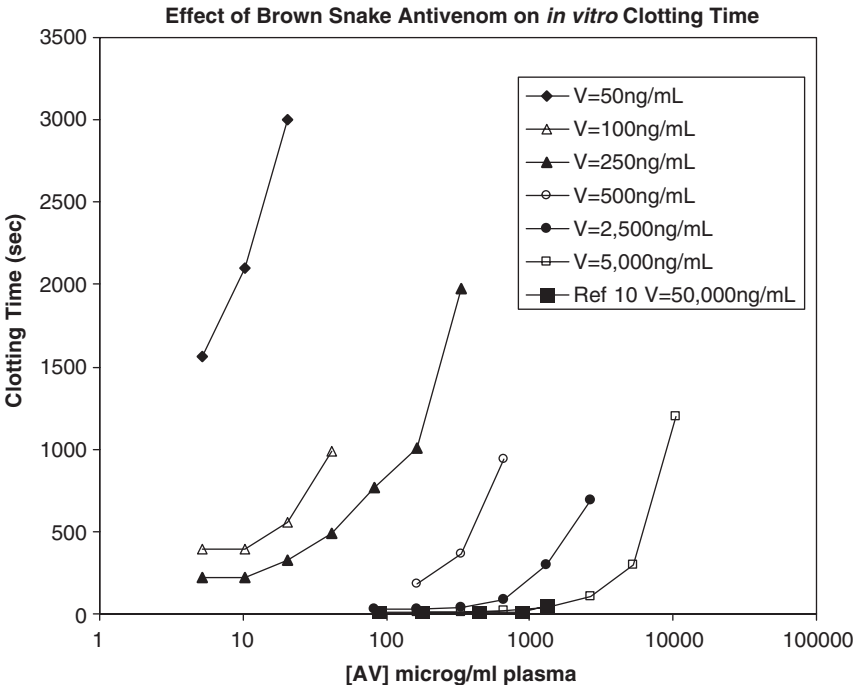

Fig. 4. Clotting times of normal citrated plasma after the addition of mixtures of venom (constant) and antivenom with increasing antivenom in each series of experiments. Each connected series of points is for a constant venom concentration with increasing antivenom concentration (logarithmic axis). The solid black squares are from a previous study for comparison (Sprivulis et al., 1996).

Table 1
Antivenom concentration required to prevent venom-induced clotting observed as a clotting time >1 h

Venom concentration (ng/mL)	Antivenom concentration (μg/mL) when clotting time >1 h
50	41
100	83
250	660
500	1320
2500	5280
5000	21,110

4. Discussion

This study has shown that one vial (1000 Units or 880 mg) of antivenom is sufficient to bind all free venom in severely envenomed patients and that an antivenom concentration of 200 μg/mL will bind all free venom in vitro, for venom concentrations observed in envenomed patients. Although antivenom does not neutralise the enzymatic/chromogenic activity of the venom it does neutralise the ability of the venom to clot blood in vitro for similar antivenom concentrations and block the activation of thrombin. It therefore suggests that only one vial and two at the most are sufficient to treat severe brown snake envenoming and VICC, contrary to previous animal and clinical studies.

There are clear limitations in extrapolating in vitro data to the clinical setting, however in this study we have examined in vivo effects in envenomed human patients to confirm the in vitro findings as well as make sure the in vitro experiments were done at the appropriate venom concentrations that are seen in humans. The major difference in our in vitro studies compared to previous work (Sprivulis et al., 1996; Madaras et al., 2005) was that we used much lower venom concentrations to more accurately reflect the range of venom concentrations found in patient sera. Venom concentrations found with severe brown snake envenoming were low and near the limit of quantification in a few cases. Similar low concentrations were found in earlier studies (Pearn et al., 1981; Coulter et al., 1980).

One study has shown that *P. textilis* can deliver up to 20 mg of venom when milked, (Williams et al., 1994) although the average yield is less than 10 mg. However, it is clear from our study that even if the

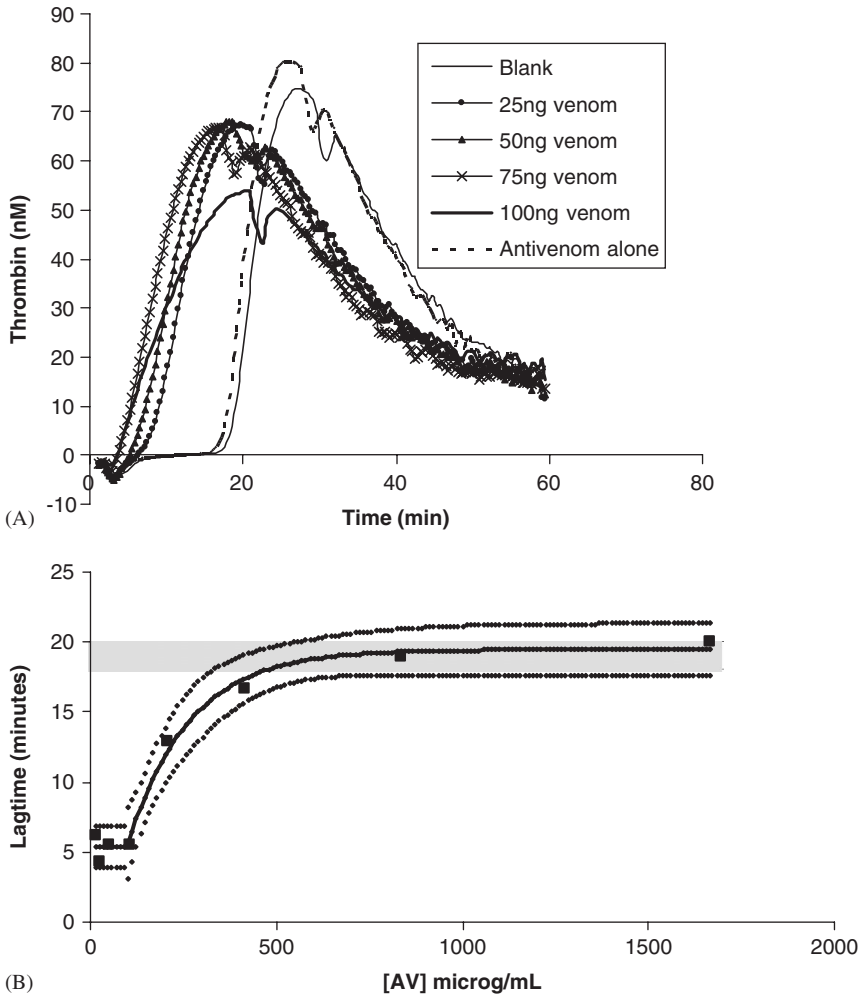

Fig. 5. Thrombin generation: (A) thrombin generation curves for increasing amounts of venom without antivenom; (B) lag time for thrombin generation with increasing amounts of antivenom with 100 ng/mL venom. The shaded area enclosed by the dotted line shows the range of normal lag times based on both experiments.

snake delivers this amount when biting, only a small proportion of this reaches the systemic compartment. If as suggested previously (Sprivulis et al., 1996) 10 mg of venom is distributed in 3 L of plasma we would expect a concentration of 3.3 μg/mL in envenomed patients. However we found that the median concentration was 20 ng/mL (4–95 ng/mL), suggesting that only about 1% reaches the systemic circulation. This is not unexpected, given that a significant amount of venom is left on the skin, as easily detected from bite swabs using the CSL snake venom detection kit (Currie, 2004a).

The dose of antivenom required and given has generally been seen to be dependent on the amount of venom delivered by the snake. This has been especially noted for vipers and snakes delivering large amounts of venom (Ho et al., 1990; Dart and McNally, 2001). However, our study suggests that for brown snakes there is not necessarily such a relationship between venom delivered and antivenom required. Severe envenoming occurred even with initial venom concentrations less than 10 ng/mL and envenoming appeared clinically no worse with much higher venom concentrations. Antivenom concentrations were 3–4 orders of magnitude greater than venom concentrations even after the administration of one vial which gives a median plasma concentration of 165 μg/L. On a molar basis the ratio of AV to venom is even higher because the prothrombin activator complex is approximately 250 kDa (Birrell et al., 2006) whereas $F(ab')_2$ molecules are approximately 150 kDa. The

antivenom concentrations found after one vial were also consistent with those expected for initial distribution into the vascular space.

These results also suggest that the specific antibodies ($F(ab')_2$ fragments) in the AV directed at the prothrombin activator complex are not directed toward the active site of this enzymatic toxin (structurally similar to human factor X). The antibodies must attach themselves to the prothrombinase in such a way that they do not completely cover the active site. Furthermore, our results suggest the antibodies somehow increase the exposure of the active site to the small tripeptide substrate S2765 used in the chromogenic assay. However, the binding of the $F(ab')_2$ fragments does prevent interaction with its intended physiological substrate prothrombin, as evident in the clotting activity and thrombinoscopy studies. It is likely that several $F(ab')_2$ antibody fragments become attached to each venom prothrombinase complex and that these prevent the molecule coming into close association with prothrombin.

The clotting tests showed that 100 μg/mL of antivenom was sufficient to block the clotting effect of the whole venom for venom concentrations seen in human envenoming. Previous experiments by (Sprivulis et al. 1996) and (Madaras et al., 2005) appear to have used venom concentrations that are 1000 times greater than those seen in human envenoming. This may well explain why they were unable to reverse the effect with antivenom. However we have shown that with sufficiently large concentrations of antivenom, the effects of even these excessively high venom levels can be reversed (Fig. 4). Importantly, much lower antivenom concentrations reflecting levels that can be produced with only one vial of antivenom were able to reverse the clotting effect for the venom concentrations seen in human envenoming.

In the first group of thrombinoscopy experiments (Fig. 5(A)) the effect on lag time, T_{max} and C_{max}, but not the AUC is consistent with the venom accelerating the generation of thrombin but not increasing the endogenous thrombin potential, which is an intrinsic property of the plasma sample and related to total prothrombin present. By not adding any trigger substance to the sample the lag time in our studies was increased by 3 to 4 times compared to 5.5 min which is reported for normal controls (www.thrombinoscope.com). The addition of venom reduced the lag time to about twice the normal value. This is consistent with the expected effect of the venom activating prothrombin to thrombin, which is then measured fluoroscopically.

In the second group of thrombinoscope experiments (Fig. 5(B)) we showed that the addition of increasing amounts of antivenom returned the lag time back to that seen in normal samples without any trigger agent added. The amount of antivenom concentration required was more than for the clotting experiments but still less than the concentrations expected following 2 vials of antivenom in envenomed patients. This proved a useful way to assess the neutralising effect of the antivenom on procoagulant effect of the venom in an in vitro system similar to that which occurs in vivo. That antivenom neutralised thrombin generation caused by the venom, but did not neutralise the prothrombinase enzyme activity in the chromogenic assay, demonstrates the importance of assessing the effect of venom (and antivenom) on the whole system. These results are further supported by the fact that AV also neutralised the clotting activity in simple clotting experiments.

A literature review did not identify any previous use of thrombin generation measurement in the assessment of procoagulant snake venoms. We believe the modified use of the thrombinoscope provides a valid alternate method for detecting the procoagulant effect of venom on triggering thrombin generation. There was a discrepancy between the antivenom concentration required to completely neutralise the effect on thrombinoscopy and that required to neutralise the in vitro clotting effects. This may simply reflect the sensitivity of thrombinoscopy to small amounts of procoagulant substances which would not cause measurable clotting. The amount of antivenom required in the thrombinoscopy experiments is likely to be the maximum required, which was 2 vials in the case of brown snake venom. Thrombinoscopy provides a straightforward method for assessing and comparing the neutralising ability of antivenom on procoagulant venoms and should be included in assessments of the efficacy of antivenoms in the future. Such an approach would be equally applicable for other venoms with prothrombin activators including other Australasian elapids and the internationally important *Echis* genus which is responsible for a huge burden of snake bites worldwide and VICC (Reid, 1977). Further modifications would be required for venoms with procoagulant toxins that have thrombin-like activity (Marsh, 1994).

Although this study provides an estimate of circulating antivenom concentrations in humans the assumption that antivenom only distributes to the vascular compartment is unlikely to be correct and the pharmacokinetics of brown snake antivenom needs to be analysed. Previous pharmacokinetic analysis of $F(ab')_2$ antivenoms have used a 2 compartment model to describe the concentration time relationship of antivenom (Ho et al., 1990; Gutierrez et al., 2003). However, the redistribution of antivenom to the peripheral compartment appears to occur over hours, so immediately after intravenous administration all antivenom will be able to bind venom.

This study suggests that one vial of antivenom is sufficient to bind and neutralise all brown snake venom in patients with severe *Pseudonaja* sp. envenoming and that a clinical trial of low dose (1 or 2 vials) versus high dose (10 vials—as currently recommended by some authorities) antivenom is warranted. The study also provides a useful and more comprehensive approach to assessing the efficacy of antivenoms for VICC and establishing estimates of effective doses that can then be used in clinical trials. Such methods can be applied to snakes worldwide including regions where snakebite is a significant public health problem.

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