

The neutralizing effect of a polyclonal antibody raised against the N-terminal eighteen-aminoacid residues of birtoxin towards the whole venom of *Parabuthus transvaalicus*

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Abstract

Scorpion venom is composed among other things of a large number of neurotoxic peptides affecting all major types of ion channels. The majority of the toxicity of the venom is attributed to the presence of these peptides. In our previous studies using a combination of HPLC and mass spectrometry, we showed that birtoxin like peptides are the major peptidic components of the venom of *Parabuthus transvaalicus*. These peptides are quite similar to each other differing by only few amino acid residues. In addition they all share a common N-terminus of eighteen amino acid residues. We hypothesize that neutralization of this domain will decrease the toxicity of the whole venom of *P. transvaalicus*. Polyclonal antibodies against the common N-terminal region of the peptides are generated. Here we show by bioassays that the polyclonal antibodies neutralize the venom of *P. transvaalicus* in a dose dependent manner and by mass spectrometry and western blotting that these peptides indeed react with the polyclonal antibodies. Previously antibodies generated against a single major toxic component of venom have proven to be an effective strategy for antivenin production. In the case of *P. transvaalicus* the generated antibody is against the majority of the peptidic fraction due to the presence of several highly similar and highly toxic components in this venom. We show that using the knowledge obtained through biochemical characterization studies it is possible to design very specific antibodies that will be useful for clinical applications against *Parabuthus* envenomation.

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1. Introduction

Scorpions use their venom to deter or kill predators and to immobilize prey. Scorpion venom consists of several main classes of peptide toxins. Among these most well studied groups are the SCNs (short chain neurotoxins) that

are 3000–4400 Da in molecular mass and act on potassium or chloride channels and LCNs (long chain neurotoxins) that are 6500–7800 Da in molecular mass and mostly act on sodium channels (Possani et al., 2000; Rodriguez and Possani, 2004; De la Vega and Possani, 2004; Possani et al., 1999). In the case of an envenomation the venom mixture is injected to the target (i.e. threat) and the peptide toxins that are present in this mixture act cooperatively for the ultimate goals of deterring, paralyzing or killing the targeted animal.

In this regard, *Parabuthus* species are medically the most important scorpions in South Africa. Bergman reports on

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the clinical features of human envenomation by this scorpion and the incidence rate of envenomation in Zimbabwe (Bergman, 1997). With the local production of specific and protective antiserum against the venom of *Parabuthus* species deaths due to their stings are minimized. South African Vaccine Producers Ltd Pty has locally pioneered this area and produces antiserum against *Parabuthus* species in the region. Antivenin production typically involves the direct injection of crude venom or the toxic peptidic fraction of venom into horses. The two major disadvantages associated with this method are potential toxicity to horses and inconsistency of the final product. Early death of horses is reported to disrupt the antivenin manufacturing process (Machado de Avila et al., 2004). Cross-linking of the toxic fraction to itself or to bovine serum albumin is shown to abolish toxicity while retaining antigenicity (Kharrat et al., 1997; Machado de Avila et al., 2004). At the molecular level effective and improved antivenin products have been developed by many research groups through utilizing the sequence information belonging to the most toxic components in venom, expressing recombinant fusion proteins consisting of toxin sequences and also by directing antibodies against specific epitopes of toxic peptides (Alvarenga et al., 2002; Legros et al., 2002; Calderon-Aranda et al., 1999). Often it is possible to reduce toxicity of venom by employing antibodies against a single most potent peptide (Zenouaki et al., 1997). This strategy although valid, has the disadvantage of leaving less toxic however highly bioactive components of venom not neutralized. Indeed Possani et al. (2000) cautions that selection of immunogens is critical for the final quality of antivenin. Devaux et al. (1996) describes a strategy for generating broadly cross-reactive antibodies through the analysis of a common neutralizing epitope in all scorpion toxins. They indicate that the N-terminal region of LCNs is both a neutralizing antigen and a conserved region. It is interesting to note that in spite of the existence of highly conserved regions in scorpion toxins in many cases antiserum raised against one toxin may not bind to other toxins of the same scorpion venom (DeLima et al., 1993; Devaux et al., 1996).

Recently the venom of *Parabuthus transvaalicus* was studied in detail by two independent organizations and novel groups of peptide toxins that act on voltage gated sodium and potassium channels were isolated (Dyason et al., 2002; Inceoglu et al., 2003). This venom has several unique properties. Firstly, the majority of the peptide components are in the range of 6000–7000 Da in molecular mass. Secondly, the N-terminal sequences of at least six different peptides from this venom are identical. These peptides in total represent more than 80% of the peptidic content of the crude venom by UV absorbance and the majority of the toxicity of the venom of *P. transvaalicus* (Inceoglu et al., 2005). Thirdly, these peptides are members of a new group of LCNs with three disulfide bridges and slightly shorter in primary structure (Inceoglu et al., 2001). Indeed,

Martin-Eauclaire et al. (2005) recently described new members of birtoxin-like peptides family from the venom of another Buthid, *Androctonus australis*, showing the wider presence of six disulfide bonded LCNs in scorpion venom. The presence of many peptides with minor sequence differences justifies the idea of testing the protective effect of an antibody raised against a common epitope of those peptides. Here we report on the ability of a polyclonal antibody raised against a single conserved synthetic peptide to protect against the crude venom of *P. transvaalicus*.

2. Materials and methods

2.1. Animals, venom, antibodies

Venom of *P. transvaalicus* was milked by electrical stimulation, lyophilized at South Africa Vaccine Producers Ltd, mailed to UC Davis, and stored at -80°C . Venom of *P. leisoma* was from a laboratory maintained colony at UC Davis. Male Swiss-Webster mice are purchased from Charles River Inc. and maintained at the animal housing facility of UC Davis under standard conditions. Peptides are purified and sequenced according to Inceoglu et al. (2001). The 18 residue N-terminal portion of birtoxin-like peptides 'NH₂-ADVPGNYPLDKDGNTYKC' is commercially synthesized by Sigma and polyclonal antibodies against this peptide are raised by Sigma-Genosys. Briefly, the synthetic peptide is cross-linked to keyhole limpet hemocyanin and rabbits are immunized. The bleedings are done after the 4th, 5th and 6th booster doses and pooled. IgG molecules are purified using a Protein A antibody purification kit from Sigma following the manufacturer's instructions.

2.2. In-vitro neutralization studies

Crude venom (100 μg) is incubated with Protein A beads which are preloaded with peptide specific antibodies. The ratio of venom to antibodies reacted are 1:10, 1:25 and 1:50 wt/wt (venom:antibody). The flow through from this reaction is collected and passed through a centricon-30 filter to remove antibody molecules and bound toxins. This fraction now contains venom components that are smaller than 30 kDa and do not bind to the antibody molecules. The volumes are adjusted so that the same quantity of venom is injected in a constant volume.

2.3. Bioassays

Venom and antibody reacted venom are injected into the intracerebroventricle at a concentration of 2 times the LD₅₀ adjusted for the weight of mice using a 10 μl Hamilton syringe. The animals are observed for a period of 24 h although all the deaths occur within the first hour. Surviving animals show signs of recovery after three hours but are euthanized after the observation period.

2.4. Mass spectrometry and Western blotting

Components present in the venom: antibody reactions are identified by MS as described previously (Inceoglu et al., 2001). Briefly, the product from the neutralization reaction of 1:25 venom antibody mixture is processed as described in the above section and ran through a C18 RP column for desalting. All of the effluent is collected, pooled and injected to an Ultima Quattro ESI/MS instrument. Crude venom of *P. transvaalicus*, a closely related scorpion *P. leiosoma* (5ug total protein) and 250 ng of purified birtoxin are separated on an 18% SDS PAGE gel and electrophoretically transferred to a nitrocellulose membrane. The membrane is incubated in blocking buffer (5% nonfat dry milk in TBST [0.1% Tween 20, 150 mM NaCl, 10 mM Tris-Cl, pH 7.6]) overnight, and then incubated in the rabbit anti-birtoxin antibody, diluted 1:10,000. After extensive washing in TBST, the membrane is incubated for 30 min with the horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz), diluted 1:5000. The membrane is washed with TBST and the antigen is visualized using the Immun-Star HRP Chemiluminescent substrate (BioRad).

3. Results

The LD₉₉ value for this venom has previously been determined to be 4.8 µg/20 g mouse by administration into the intracerebroventricle (Inceoglu et al., 2001). Firstly this is confirmed. None of the mice ($n=5$) receiving the LD₉₉ dose survive. Administration of 1:10 venom:antibody (wt/wt) mixture ($n=12$) does not produce any protective effect. These mice show typical symptoms of envenomation by the venom of *P. transvaalicus*. The time to death ranges between 5 and 56 min. However, when the ratio of the antibody to venom is raised to 1:25 venom:antibody mixture ($n=10$) this results in significant increase in survival (Fig. 1). Further increasing the antibody quantity, administration of 1:50 venom:antibody mixture ($n=9$) does not show any more significant increase over the 1:25 venom:antibody mixture (Fig. 1). Mice are also administered the same quantity of venom incubated with preimmune serum. The survival rate in this group is 41%. This value is comparable to survival rate that is observed when 2 times the LD₅₀ is administered to mice in previous studies for other venoms (Alvarenga et al., 2002).

The generated primary antibodies strongly reacted with both crude venom of *P. transvaalicus*, a closely related species *P. leiosoma* and pure birtoxin (Fig. 2). This is shown both by western blotting and mass spectrometry. Western blotting showed the presence of multiple components that cross react with the generated antibody. In lane B of Fig. 2 pure birtoxin (250 ng) reacts with the antibody. Lanes A and C show that the antibodies react with multiple components

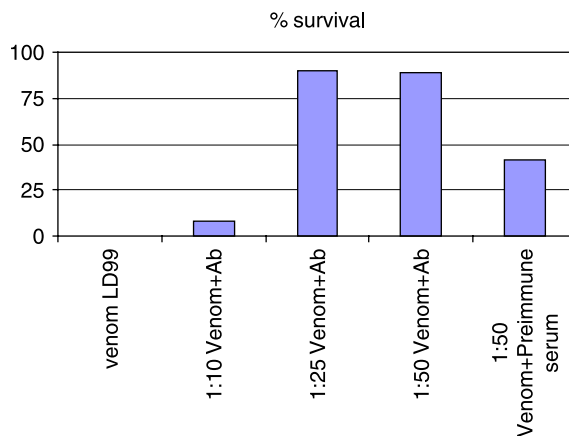


Fig. 1. Neutralizing ability of polyclonal antibodies. The antibodies were generated towards the common N-terminal 18 amino acid residues of birtoxin like peptides. The venom and purified antibodies are mixed in various ratios, the antibodies are captured with an affinity resin and the lethality of the unbound fraction is measured. These polyclonal antibodies neutralize the toxicity of the venom of *Parabuthus transvaalicus*.

in the crude venom of two species. This is expected because multiple peptides with identical N-terminal sequence are found in *P. transvaalicus* venom.

The components that bind to the antibodies are then investigated using mass spectrometry. Direct loop injection of this fraction does not produce reliable information due to the high content of salts that are used to elute this fraction.

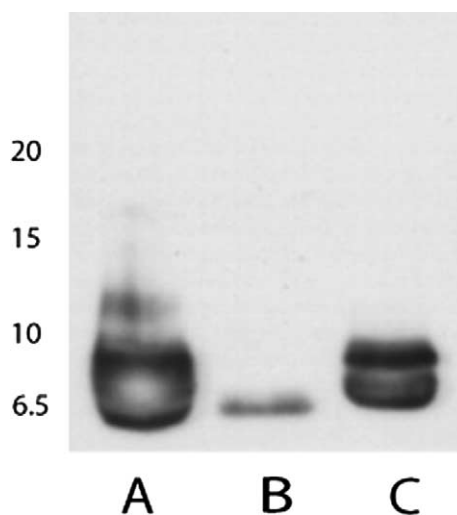


Fig. 2. Western blot using anti-birtoxin antibodies. Crude venom of *Parabuthus transvaalicus* (A), pure birtoxin (B) and venom of *P. leiosoma* (C) are separated on an SDS-PAGE gel and blotted to a nitrocellulose membrane. Bands are detected using the anti-birtoxin antibody. *Parabuthus transvaalicus* and *Parabuthus leiosoma* possess multiple reacting peptides.

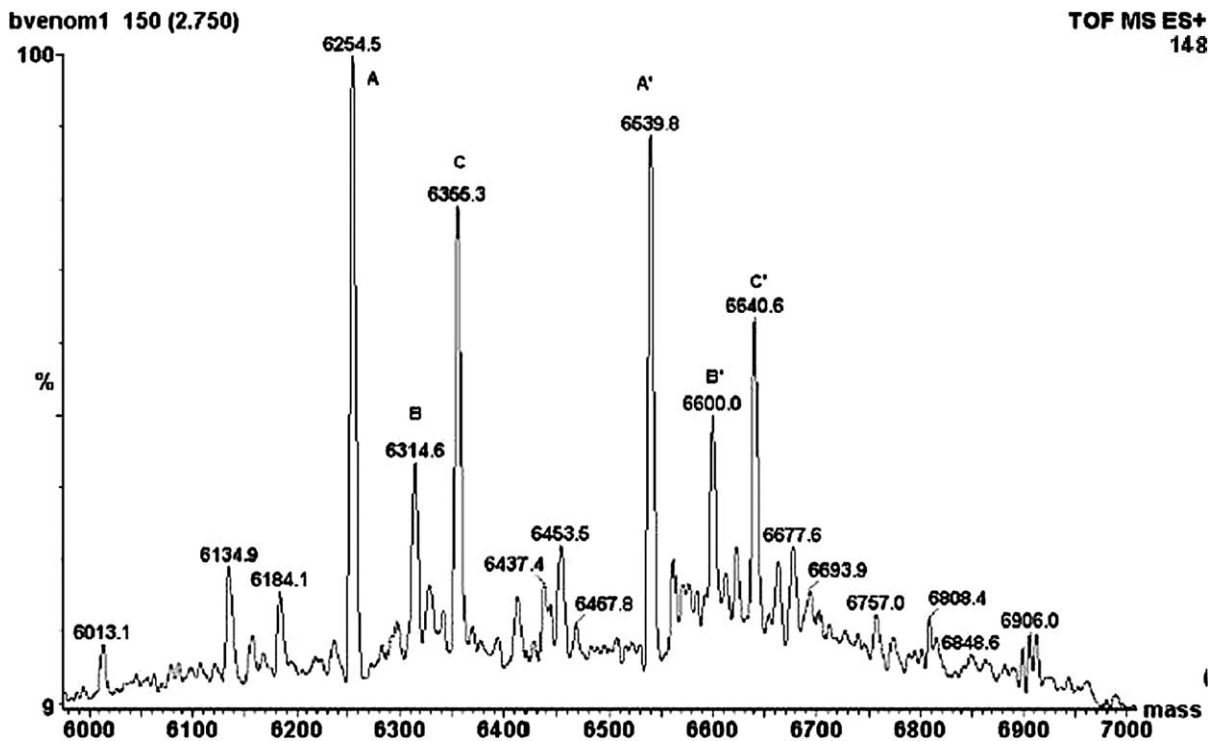


Fig. 3. Mass spectroscopic investigation of neutralized venom components. Venom components that are bound to the antibodies are detected and identified using ESI-TOF mass spectrometry. Polyclonal antibodies bind and recover birtoxin-like peptides from the venom. Peaks A', B', and C' are birtoxin, bestoxin and altitoxin respectively. Peaks A, B, and C are the proline cleavage products of birtoxin, bestoxin and altitoxin.

Therefore, a desalting step by running this fraction through a C18 RP column and pooling all the effluent except for the first five minutes of the run is performed. The bound fraction of 1:25 venom: antibody reaction contains three major peptidic components (Fig. 3). All three components are identified as birtoxin family of peptides previously characterized by our laboratory. These components are birtoxin (6540 Da), bestoxin (6600 Da) and dortoxin (6640 Da). Two other similar peptides known as ikitoxin and altitoxin are not observed in this study. However, several unidentified peptides of same molecular mass range can be seen in the transformed chromatogram. Peptides with molecular masses of 6254, 6314 and 6355 Da are identified as fragments of the three main toxin peaks, birtoxin (6540 Da), bestoxin (6600 Da) and dortoxin (6640 Da). This fragmentation is due to the ESI ionization process. The presence of proline residues is known to induce fragmentation of peptides under stringent ionization conditions (i.e. high voltage, high temperature). The mass of the 'ADV' fragment when subtracted from the three main toxin peaks gives the exact masses for the 6254, 6314 and 6355 Da species. This also confirms the identities of main toxic peaks as birtoxin, bestoxin and dortoxin. Other components that are immuno-purified are the subject of ongoing research in our laboratory.

4. Discussion

The quantity of venom we neutralized in this study is equivalent to 20 times of LD₉₉ through injection into the intracerebroventricle. Considering that 2.5 mg purified antibody fraction is able to neutralize 100 µg of venom and our antibody yield is about 8 mg/ml serum, 1 ml of serum is able to neutralize at least 60 times the LD₉₉ of the venom of *P. transvaalicus*. This value indicates the high potency of the antibody. However caution should be taken when extrapolating these numbers to scorpion stings. The venom of *P. transvaalicus* is at least ten fold less potent by subcutaneous injection. Although the existence of multiple peptides in *P. transvaalicus* venom that react with this antibody is well established we show that a closely related species *P. leisoma*'s venom also has multiple peptides that are cross-reacting. This further indicates the broader spectrum of the N-terminus of birtoxin like peptides and brings up the opportunity to use this antibody for isolating novel toxins from other species. Indeed the related scorpion *P. granulatus* is considered medically more important than *P. transvaalicus* (Müller, 1993). It remains to be seen however if this antibody will react with or neutralize the venom of this closely related species. Possibly the antibodies developed here or certainly a similar approach

may be valuable in addressing envenomization from the medically important species *P. granulatus*. The recent discovery by Martin-Eauclaire et al. (2005) of the existence of birtoxin-like peptides with identical N-term from another medically important species *A. australis*, although without activity on mammals, indicates the broader distribution of these peptides and thus wide-ranging utility of the antibodies against them.

The immuno-purified venom fraction was investigated by mass spectroscopy and the presence of the classical long chain neurotoxins (LCNs) was not observed. Therefore, we can exclude the possibility of this antibody reacting with LCNs based on our current detection limit by mass spectroscopy. Another piece of evidence that supports this view is seen in Fig. 1. As the amount of antibody is increased no improvement in neutralization capacity is observed meaning that all or most of the active toxin components are effectively neutralized. The 12% mortality that cannot be neutralized could be due to the presence of other LCNs and also the short chain neurotoxins, other small molecular mass components and/or their combination.

In this study several goals for making antivenin towards the venom of *Parabuthus* species are simultaneously achieved. Firstly, we show that a single synthetic peptide is sufficient to be used as an antigen. This is useful because the toxicity associated problems for antivenin-manufacturing process can be avoided through the use of synthetic peptides conjugated to carrier proteins or in the future by recombinant antibodies. Therefore, this approach can reduce the cost of the process both by utilizing a cheaper source of antigen and by prolonging the lives of horses used. Secondly, we show that the N-terminal domain of birtoxin-like peptides is a neutralizing domain. This provides a clue to the active surface of these toxins in addition to providing us a target site for directing antibodies. Thirdly, the synthetic peptide can recognize multiple very potent toxins, neutralizing them all. This increases the quality of the antivenin. One can argue that prevention of lethality although primary should be accompanied by reducing the cumulative synergistic effect of other toxic components in the venom. Clinically treating symptoms due to envenomation while targeting the venom by antivenin therapy does this. Neutralizing multiple toxic components simultaneously should have the added benefit of decreased pain, discomfort and faster recovery. As an extension of this thought using a combination of major classes of toxins as immunogens or antigens should be considered for future studies. Potential targets include the LCNs, SCNs and pore forming toxins and possibly new classes of toxins yet to be discovered. The antibodies developed in this study can be used to find other peptides in the venom related to the birtoxin group and to remove the birtoxin group from the venom to facilitate the search for novel toxins in the venom.

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