Original Article

Antigenic Cross-Reactivity Anti-Birtoxin Antibody against *Androctonus crassicauda* Venom

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Abstract

Background: Antivenom is still widely used in the treatment of envenomation as there are no vaccines or other effective agents available against animal venoms. Recently, neurotoxins named birtoxin family have been described from *Parabuthus transvaalicus* and *Androctonus crassicauda*. The aim of the present study was to test the anti-birtoxin antibodies for their ability to neutralize the lethal effects of *A. crassicauda* scorpion venom.

Methods: SDS-PAGE and Western blotting used the presence of components from *A. crassicauda* and *P. transvaalicus* scorpion venoms and to determine the degree of cross-reactivity. The Minimum Lethal Dose (MLD) of venom was assessed by subcutaneously (sc) injections in mice.

Results: The MLD of the *A. crassicauda* venom was 35 µg/ 20g mouse by sc injection route. Western blotting showed the presence of components from *A. crassicauda* and *P. transvaalicus* scorpion venoms strongly cross react with the *A. crassicauda* antivenom. However, Western blotting of the *A. crassicauda* scorpion venom using the Refik Saydam Public Health Agency (RSPHA) generated antibody showed that not all the venom components cross reacted with the anti-birtoxin antibody. The antibodies only cross reacted with components falling under the 19 kDa protein size of *A. crassicauda* venom.

Conclusion: The bioassays and Western blotting of *A. crassicauda* venom with the anti-birtoxin antibodies produced against a synthetic peptide showed that these antibodies cross reacted but did not neutralize the venom of *A. crassicauda.*

Keywords: *Androctonus crassicauda*, Venom, anti-birtoxin antibody, Cross-reactivity

Introduction

Most of the medically important scorpion species belong to *Buthus*, *Parabuthus*, *Mesobuthus*, *Tityus*, *Leiurus*, *Androctonus* and *Centruroides* genera of the Buthidae family (Balozet 1971, Bücherl 1971, Efrati 1978). Scorpion venoms can be classified into two groups according to their molecular sizes, long-chain and short-chain neurotoxins. The short-chain neurotoxins are 3,000 to 4,400 Da and act on potassium or chloride channels. Long-chain neurotoxins are 6,500 to 7,800 Da and act mostly on sodium channels (Possani et al. 1999, 2000, Inceoglu et al. 2006, Ozkan et al. 2008). It has been estimated that 100.000 distinct peptides exist in scorpion venom but only limited number of these peptides have been described (Possani et al. 1999, 2000, Martin-Eauclaire et al. 2005, Inceoglu et al. 2006).

The unique specific treatment of scorpion envenomations is immunotherapy with antibodies from immunized horses (Ghalim et al. 2000). However, the venom is a complex mixture of antigens wherein not all components are equally important for the production of neutralizing antibodies. Thus, the identification of immunogenic protein(s) and/or their neutralizing epitopes may lead to the

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use of more clearly defined substances as immunogens to develop efficient antivenoms or to their use as antigens.

The venom of *P. transvaalicus* consists of recently described closely related neurotoxins named birtoxin family (Inceoglu et al. 2001, 2005). An antibody developed using a synthethic peptide composed of the first 18 amino acid residues of birtoxin displayed strong reactivity with the whole venom of *P. transvaalicus*, *P. leisoma* and pure birtoxin (Inceoglu et al. 2006). These antibodies also neutralized the venom of *P. transvaalicus* in mice. Recently, Calışkan et al. (2006) also reported the presence of peptides in *A. crassicauda* venom that belong to the birtoxin-like peptide family.

In this study, we tested the anti-birtoxin antibodies for their ability to neutralize the lethal effects of *A. crassicauda* scorpion venom.

Materials and Methods

Venoms

Venom was obtained from mature *A. crassicauda* scorpions (from Sanliurfa) by electrical stimulation of the telson. The venom was mixed with sterile double-distilled water and centrifuged at 15,000 rpm for 15 min at 4 ºC. The supernatant was immediately lyophilized at Refik Saydam Public Health Agency (RSPHA) and stored at -80 ^{o}C until use. Venom of commercially obtained *P. transvaalicus* scorpions were collected as described (Inceoglu et al. 2001, 2006) at University of California, Davis, CA.

Antivenom (RSHC anti-Ac)

Antivenom of *A. crassicauda* was obtained as described (Ozkan et al. 2006a). Briefly, increasing venom doses, mixed half-and-half with adjuvants, were injected subcutaneously into horses on the 1st, 14th, 21st, 28th, 35th and 42nd days. On the 45th, 48th and 51st, days, blood samples were collected three times from the jugular vein of each animal and stored in containers with 10 % sodium citrate. After plasma separation, antivenom was obtained, from combined plasma, by the digestive method and kept in the dark at 4 ºC. One dose of RSHA anti-Ac was normalized to neutralize 2 MLD of *A. crassicauda* venom in rats when tested subcutaneously.

Anti-birtoxin antibody

The 18 residues N-terminal portion of birtoxin-like peptides 'NH_2-ADVPGNYPLD KDGNTYKC' was commercially synthesized by Sigma and polyclonal antibodies against this peptide were raised by Sigma-Genosys (Inceoglu et al. 2006). Briefly, the synthetic peptide was cross-linked to keyhole limpet hemocyanin and rabbits were immunized. The bleedings were done after the 4th, 5th and 6th booster doses and pooled. IgG molecules were purified using a Protein A antibody purification kit from Sigma following the manufacturer's instructions. Protein concentrations were determined using a BCA kit (Pierce, USA) with ovalbumin as the standard.

Determination of the Minimum Lethal Dose (MLD) in mice

All the experiments were performed according to the guidelines by the ethical committee of the Faculty of Veterinary Medicine in Ankara University. The Minimum Lethal Dose (MLD) of venom was assessed by subcutaneously (sc) injections in mice (20±2 g). The animals were kept in the experiment room under standard conditions throughout the experiment. Five mice per each dose-group were injected sc with doses of venom, diluted in 0.5 ml saline solution. An equivalent volume of 0.5 ml saline was injected into five mice as negative control group. The animals were observed for 48 h after venom injection in order to determine MLD.

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Serum-neutralization assays in mice

A solution of *A. crassicauda* venom (3 MLD for each mouse) diluted in physiologic saline solution (PSS) to a 2.5 ml volume, were prepared. The anti-birtoxin antibody was prepared at doses ranging from 0.5 ml to 1.5 ml. On the other hand *A. crassicauda* venom (1 MLD) also prepared for each mouse and mixed with 1.5 ml anti-birtoxin antibody. The final volume all dilutions were made up to 5 ml with PSS. The solutions were incubated for 60 min at room temperature. Then, 0.5 ml of each solution was subcutaneously injected into groups of eight mice previously injected with *A. crassicauda* venom. The control groups were only injected with 1 MLD of the venom diluted in PSS. The numbers of surviving mice were recorded up to 48 h. After administration, animals were monitored for 48 hours and the number of living animals was recorded. The anti-birtoxin doses that prevented 100 % deaths in the groups were considered the minimum effective doses (MED).

A solution of *A. crassicauda* venom (3 MLD for each mouse) diluted in physiologic saline solution (PSS) to a final volume of 2.5 ml. The anti-birtoxin antibody was prepared at doses ranging from 0.5 ml to 1.5 ml. Separately, *A. crassicauda* venom (1 MLD, 62 µl venom for each mouse) was prepared for each mouse and mixed with 1.5 ml anti-birtoxin antibody. All solutions were then diluted to a final volume of 5 ml using PSS. These solutions were incubated for 60 min at room temperature. Then, 0.5 ml of each solution was subcutaneously injected into groups of eight mice previously injected with *A. crassicauda* venom. The control groups were only injected with 1 MLD of the venom diluted in PSS using the same volumes. Following administrations the animals were monitored up to 48h and survival was noted. The anti-birtoxin doses that prevented 100 % deaths in the groups were considered the minimum effective doses (MED).

Gel electrophoresis of the venoms and Western Blotting

Venoms were analyzed by sodiumdodecylsulfate polyacrylamide gel electrophoretic (SDS-PAGE) analysis according to Laemmli (1970). Venom of *A. crassicauda* and *P. transvaalicus* scorpions were separated on precast NuPAGE 12 % Bis-Tris Gel are electrophoretically transferred to the nitrosellulose membrane (NCM) and divided to two sections. The membranes were incubated in blocking buffer (3% BSA in TBST [0.1 Tween 20, 150 mM NaCl, 10 mM Tris-Cl, pH 7.5]) for one hour. The membranes were then washed three times with TBST (Tris-Buffered Saline Tween-20, [0.1% Tween 20, 150 mM NaCl, 10 mM Tris-Cl, pH 7.4]) and strips of the membrane were exposed to pre-immune serum for each antivenom for 30 min followed by three washes and incubated with antivenom of *A. crassicauda* (1: 4000) and the anti-birtoxin Ab (1: 1000). Membranes were again washed 3 times with TBST, and then incubated with horseradish peroxidase-conjugated anti-horse antibody and HRP- conjugated anti-rabbit (1: 5000) for 60 min. The membranes were washed with TBST for 10 minutes and antigens were visualized using the Immun-Star HPR Chemiluminescent subtrate (BioRad). Membranes were exposed to X-ray film in a dark room and developed.

Results

The MLD of the *A. crassicauda* venom was found to be 35 µg/20 g mouse (1.75 mg/kg) by sc injection route (Table 1). The potency of *A. crassicauda* antivenom (500 µl) has previously been determined to be neutralizing 2 MLD in 150g rats according to manufacturer's instructions.

Here this was confirmed to be the case. To assess the potency of the anti-birtoxin antibody, increasing doses of the antibody were used while the amount of *A. crassicauda*

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venom was kept constant (1 and 3 MLD). The Ac antivenom (0.8 mL) potently neutralized 3 MLD of the venom while all control mice died. However, 1.5 ml of anti-birtoxin antibody was not able to neutralize even 1 MLD of venom of *A. crassicauda* scorpion (Table 2). Despite the lack of the ability of anti-birtoxin antibodies to neutralize the venom of Ac western blots indicate that there is a certain level of cross reactivity between this Ab and the Ac venom (Fig. 1). Although the peptide toxins fall below 10 kDa molecular weight range often times on western blots a smear in the range of 5–15 kDa corresponds to these peptides and their heteromers due to the running conditions.

As shown by Western blotting, *A. crassicauda* antivenom strongly reacted with the components of both *P. transvaalicus* and *A. crassicauda* venoms (Fig. 1). Similarly, the anti-birtoxin Ab strongly reacted with both *P. transvaalicus* and *A. crassicauda* scorpion venoms as well. Fig. 1 shows that proteins that were detected using the anti-birtoxin Ab all fall under 19 kDa molecular mass.

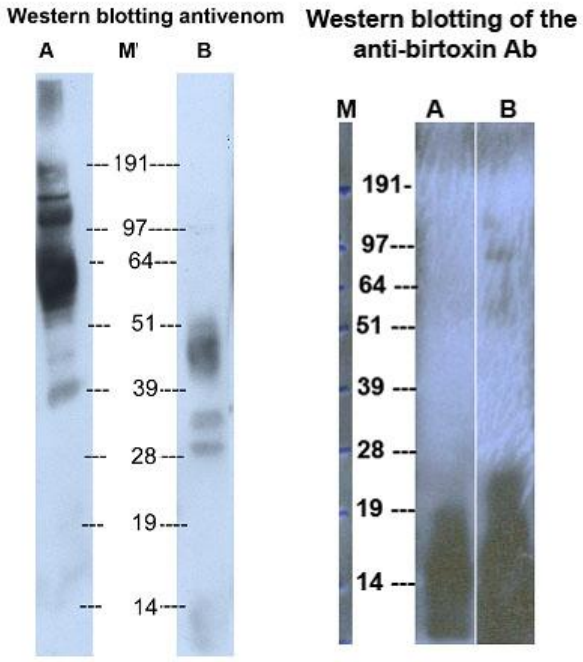

Fig. 1. The venoms from *Androctonus crassicauda* (**A**) and *Parabuthus transvaalicus* (**B**) were separated and transferred to membranes. Proteins were detected using the RSHC anti-Ac (1: 4,000) on the panel I and anti-birtoxin Ab (1: 1,000) on the panel II. Molecular weight (**M**) markers on the panel II are SeeBlue® Plus2 (Invitrogen Corporation, USA)

Table 1. Minimum Lethal Dose of *Androctonus crassicauda* venom

***Androctonus crassicauda* scorpion**		**Negative control**	
Venom (µg/mouse)	**Mice (Death/total)**	**PSS (µl/mouse)**	**Mice (Death/total)**
10	0/5		
15	1/5		
20	2/5	500	0/5
25	4/5		
35*	5/5*		
MLD: 35 µg/ 20g mouse			

Table 2. Neutralization capacity of the anti-birtoxin and antivenom was assayed for *Androctonus crassicauda* venom in mice

MED of *Androctonus crassicauda* antivenom			**MED of anti-birtoxin antibody**		
Venom (µg/mouse)	**Antivenom (µl)**	**Mice (Surviving /total)**	**Venom (µg/mouse)**	**Antibody (µl)**	**Mice (Surviving /total)**
105	400	5/8	105	500	0/8
105	800	8/8	105	1000	0/8
105	1000	8/8	105	1500	0/8
35 (Control)	-	0/8	35	1500	0/8*
MED: 800 µl			MED: No determined		

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Discussion

Venom effect on the autonomic nervous system

The antivenom therapy plays an important role in the treatment of scorpionism cases. As several studies stated that upon poisoning caused by scorpion stings, it is often recommended that the patients treated with species-specific antivenom and this must be administered early upon envenomation (Alexander 1984, El-Amin 1992, Ismail 1993, Sofer et al. 1994).

The potentially dangerous and medically important scorpion species venom effect on the autonomic nervous system (Bawaskar 2005). *Androctonus crassicauda* is considered as the most significant species of scorpions in Turkey and neighbouring countries Iran, Iraq and Syria causing a large number of envenomations every year (Radmanesh 1990, Ozkan et al. 2006b, Chippaux and Goyffon 2008, Dehghani and Khamechian 2008, Antopolsky et al. 2009, Bosnak et al. 2009, Dehghani et al. 2009, Shahbazzadeh et al. 2009, Dehghani and Fathi 2012). *Parabuthus* species are medically the most important scorpions in South Africa thus Bergman (1997) reports on the clinical manifestations of human envenomation by *P. transvaalicus* and the incidence rate of envenomation in Zimbabwe.

Krifi et al. (1998) reported the difficulties in standardization the venom quality and LD_{50} determination which are partly related to geographical origin, the age of the venomous species, the season and venom extraction procedures, the number of specimens milked, the breeding conditions, and also the species' strain, of the test animal body weight and administration route. These parameters must be accepted as important factors for standardization of the venom toxicity and the antivenom efficacy. Thus, potency of antivenom is estimated by national or regional control authority and is described as a toxin neutralization unit according their standards (Theakston et al. 2003, Ozkan et al, 2007). Therefore in the present study, MLD and MED were determined instead of LD_{50} and ED_{50}. In our study, the MED of the antivenom against 3 MLD *A. crassicauda* venom was 0.8ml while 1.5ml of anti-birtoxin antibody was not able to neutralize even 1 MLD of the venom of *A. crassicauda* scorpion. Therefore the MED of the anti-birtoxin was not determined.

Inceoglu et al. (2001) determined that native birtoxin from *P. transvaalicus* also has the average molecular mass of 6543.6 Da. Besides, Martin-Eauclaire et al. (2005) described new members of birtoxin-like peptides familiy from the venom of *A. austrailis*. Moreover they notified that this new family might probably exist in other "Old-World" Buthidae venoms (Martin-Eauclaire et al. 2005). Recently, this species from Turkey has been studied and five toxins described by Calıskan et al. (2006) two of which (Acra 1, 6496.8 Da and Acra 2 7848.6 Da), were lethal to mice.

In this study, Western blotting showed the presence of components from *A. crassicauda* and *P. transvaalicus* scorpion venoms strongly cross react with the *A. crassicauda* antivenom. However, Western blotting of the *A. crassicauda* scorpion venom using the RHSC generated antibody showed that not all the venom components cross reacted with the anti-birtoxin antibody. The antibodies only cross reacted with components falling under the 19 kDa protein size of *A. crassicauda* venom. This is not unexpected since most of the neurotoxic peptide components fall under this range. However these findings indicate that in contrast to the *P. transvaalicus* venom, in *A. crassicauda* venom the birtoxin like peptides contribute minimally to the neurotoxicity. This reiterates the fact that species differ-

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ences and geographical variations which result in a diverse number of neurotoxic peptides in the venom needs to be considered as an important factor in developing antivenoms that are effective. The finding that *P. transvaalicus* venom can be neutralized with a polyclonal antibody raised against the first 18 amino acid residues of birtoxin seems to be an exception (Inceoglu et al. 2006) though it remains to be seen if horse derived antivenom against *A. crassicauda* will neutralize the venom of *P. transvaalicus.* Similarly, it remains to be seen if the anti-birtoxin antibodies will neutralize the venom of closely related species including *P. granulatus.*

Conclusion

The bioassays and Western blotting of *A. crassicauda* venom with the anti-birtoxin antibodies produced against a synthetic peptide showed that these antibodies cross reacted but did not neutralize the venom of *A. crassicauda.*

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