Life Sciences 124 (2015) 1–7

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Comparison of the neurotoxic and myotoxic effects of two Moroccan scorpion venoms and their neutralization by experimental polyclonal antivenom

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ARTICLE INFO

Article history:
Received 3 September 2014
Accepted 31 December 2014
Available online 24 January 2015

Keywords:
Androctonus mauretanicus
Buthus occitanus
Scorpion venom
Antivenom
Polyclonal
Scorpion toxin
ED50
Chick biventer cervicis method
Myotoxicity
Neurotoxicity
Lethality

ABSTRACT

Aims: Scorpion venoms contain complex mixtures of molecules, including peptides. These peptides specifically bind to various targets, in particular ion channels. Toxins modulating Na^+, K^+, Ca^{2+} and Cl^- currents were described from venoms. The *Androctonus* and *Buthus* geni of scorpions are widely distributed in Morocco. Their stings can cause pain, inflammation, necrosis, muscle paralysis and death. The myotoxicity is predominantly associated with neurotoxic effects and is a cause of mortality and morbidity. In this study, pharmacological effects of venoms were investigated in vitro on neuromuscular transmission.
Main methods: Effects of *Androctonus mauretanicus* (Am) and *Buthus occitanus* (Bo) venoms were investigated using the chick biventer cervicis nerve-muscle preparations. The protective activity of antivenom was also investigated. The antivenom was made from serum of horse that was hyperimmunized with Bo and *Androctonus australis hector* (Aah) venoms and one venom from Middle East species (Lq). The protective activity of the antivenom was assessed on the neuromuscular system by using stimulated chick nerve-muscle. The results were compared with lethal activity neutralization in mice.
Key findings: Am and Bo venoms contain myotoxins and postsynaptic neurotoxins. In agreement with lethal potencies of these venoms in mice, Am venom displays greater neurotoxicity and myotoxicity. The antivenom prevented lethality caused by Am, Bo and Aah venoms. The antivenom did not prevent toxic effects caused by Am venom whereas it neutralized Bo venom.
Significance: Am and Bo venoms contain distinct toxins that are responsible for myotoxicity and neurotoxicity. It would be appropriate to add Am venom to produce more efficient antivenom.

Introduction

Scorpionism is the main cause of animal envenomation and a serious medical concern in North African countries, such as Morocco. Due to a favorable hot climate, certain areas of Morocco are appropriate habitats of scorpions, thus increasing the probability of scorpionism. Treating envenomation in these areas is rendered difficult by the lack (or unavailability) of an efficient antivenom.

Scorpionism comprised 50 to 60% of animal envenomation cases that have been reported to the Poison Centre of Morocco (CAPM). The epidemiological data showed that 30,000 to 50,000 scorpionism cases are reported yearly, among which ca. 1000 cases were severe envenomations. About 10 to 100 deaths (mostly children under 10 years old) are reported each year from such envenomations. Stings occurred most often at home, during the night, and between July and August. This work showed that Am scorpion, age <15 and a post-sting delay >1 h were risk factors for death or complications as well as priapism, vomiting, hyper-sweating and fever [17].

A statistical analysis indicates that 83% of severe envenomation cases were caused by black scorpion whereas 14% of the cases were caused by yellow scorpion *Buthus occitanus* (Bo). Venoms from these scorpion species are highly potent and were reported to give rise to potential dysfunctioning of the gastrointestinal, neuromuscular, cardiovascular, respiratory and urinary systems [16].

The components of the venom are complex and 'specific' to each scorpion species; those of the Buthidae family being the most toxic to humans. The venom of this genus is very toxic, and associated symptoms of envenomation can include malignant hyperthermia, myocarditis and pulmonary edema [5,15].

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http://dx.doi.org/10.1016/j.lfs.2014.12.031
0024-3205/

The dysfunctionings of these body systems are likely due to targeted actions of the various venom components acting individually or synergistically. It is now well-established that neurotoxins bind to receptors/ion channels located on the surface of cell membranes causing stimulation of the peripheral nervous system and a release of cellular mediators and neurotransmitters. Besides, it is also admitted that mediators are the main cause of pathophysiological disorders observed after scorpion envenoming [15,19].

Toxic fractions of Am and Bo venoms have been reported to cause cytotoxicity, tissue necrosis, inflammation, and muscle paralysis in host. Indeed, myotoxicity is a cause of mortality and morbidity which is predominantly associated with neurotoxic effects, while neurotoxins are considered as the main compounds responsible for the pathophysiological disorders observed after scorpion envenomation [5,15].

Several studies indicate that immunotherapy would be the most important therapeutic measure to use after scorpion envenoming. The antivenom is considered as more efficient when given without delay after envenomation and in adequate administration (dose, injection route, etc.) [16].

Currently, the ability of antivenom to neutralize the venom-induced lethal effects is evaluated in in vivo assay using mice. However, little is known on the ability of the antivenom to actually neutralize the neuromuscular and myotoxic activities of scorpion venom [7–9,21].

In this study, our objectives were first to carry out a preliminary evaluation of the neurotoxic and myotoxic effects of two Moroccan scorpion venoms (i.e. Am and Bo), and second, to examine the efficacy of the commercial polyvalent antivenom regarding the neutralization of myotoxicity and neurotoxicity of Am and Bo venoms [18,19,21].

Materials and methods

Venoms

The scorpion venom of Am and Bo were obtained by manual stimulation of wild animals in Captivity at the Experimental Centre of the Pasteur Institute of Morocco. These scorpions have been collected in a region of Morocco where the incidence of stings is the highest.

Crude venoms were obtained by electrical stimulation of telsons of appropriate scorpion species, and stored after freeze-drying at −50 °C, until use [13].

F(ab′)2 antivenom

Polyspecific anti-scorpion antivenom "Inoscorpi" was graciously given by Inosan Biopharma Institute from Mexico. This preparation was obtained from the serum of horses hyperimmunized with a mixture of venom from the most venomous scorpion species in north Africa and in the Middle East: *Leiurus quinquestriatus, Buthus occitanus* and *Androctonus australis hector*.

The immunoglobulin was then enzymatically digested to produce F(ab′)2 fragments, purified, lyophilized and packaged in individual dose vials. Protein content of the resulting product includes approximately 93% $F(ab')_2$ and 4% Fab fragments (the remaining 3% including low molecular weight compounds, dimers, soluble oligomers, and undigested IgG). The specifications for the reconstituted product are such as it contains no more than 30 mg of protein per ml, and has a neutralization potency of not less than 50 ED_{50}s per ml, with an intravenous challenge with 3 LD_{50}s.

Determination of the median lethal dose (LD_{50})

The lethal potency of venoms, as assessed by LD_{50} value determination (in micrograms of dried venom per mouse), was evaluated as recommended by the World Health Organization [20]. Groups of five CD-1 mice (18–20 g) were used per dose of venom. The venom doses were adjusted in 150 mM of NaCl, and injected in final volumes of 500 μl through intravenous (IV) and intraperitoneal (IP) routes.

Percent mortality was recorded 24 h after injection. The median lethal dose was determined by the method of Software package Prism 5 GraphPad Inc., according to the provided algorithm. Briefly, a non-linear curve fitting (variable slope) was generated using the four-parameter logistical equation; constraints were imposed on minimum (0% mortality) and maximum (100% mortality) values, and no weighing was used. The same package was used to calculate median doses. Plots were generated using KaleidaGraph 4.03 [4].

Determination of 50% effective doses (ED_{50})

A given amount (3 LD_{50}s) of venom was incubated with varying volumes of antivenom for 30 min at a temperature of 37 °C. Each mixture (0.5 ml) was injected through intraperitoneal and intravenous routes to five mice (CD-1 mice weighing 18–20 g) and deaths were recorded up to 48 h. As a negative control, mice received 3 LD_{50} of venom without antiserum. Results were analyzed by GraphPad Prism 5 software. The neutralization capacity of the antivenom was expressed as 50% effective dose (ED_{50}), defined as the volume (μl) of antivenom needed to prevent death in 50% of mice injected with 3 LD_{50} of venom.

Neurotoxicity and myotoxicity assays by chick biventer cervicis nerve-muscle preparation (CBCNM)

The pharmacological assays related to neurotoxicity and myotoxicity were performed using isolated chick biventer cervicis nerve-muscle [2,3,6,10]. Chick biventer cervicis muscles were dissected from 7 to 10 day-old chicks, humanely killed by diethyl oxide inhalation, as described in Ref. [6]. The tissues were mounted on double ringed electrodes, with a resting tension of approximately 1 g, in 5 ml organ bath containing Kreb physiological solution with the following composition: NaCl, 118.4; KH_2PO_4, 1.2; glucose, 11.1; $NaHCO_3$, 25; $CaCl_2$, 2.5; $MgSO_4$, 1.4 and KCl, 4.7. The physiological solution was constantly aerated and maintained at 37 °C. The tissues were equilibrated for 15–20 min before the addition of venom.

For neurotoxicity assessment, stimulation was achieved on the tendon, which triggers depolarization and releases endogenous acetylcholine from the presynaptic terminal into the synaptic cleft of the neuromuscular junction. The endogenous acetylcholine later binds to a (postsynaptic) receptor at the postsynaptic terminal of the neuromuscular junction. Then, it triggers the opening of specific ion channels, which leads to an increase in intracellular calcium concentration, eventually followed by muscle contraction. To detect any changes in postsynaptic sensitivity, responses to sub-maximal concentrations of exogenously applied acetylcholine (1–2 mM), carbachol (30–40 μM) and KCl (20–40 mM) were recorded in the absence of nerve stimulation, prior to the addition of venom and at the end of the experiment. The preparations were exposed to acetylcholine and KCl for 30 s, and carbachol for 1 min. After the wash-out stage of these drugs, the preparations were allowed to stabilize for 15–20 min before the addition of venom.

Table 1
Median lethal doses (LD_{50}) of scorpion venoms (Am, Bo and Aah species), using IV and IP routes of injection, with 95% confidence intervals and calculated by a non-linear regression.

Injection route	LD_{50}		
	Am venom	Bo venom	Aah venom
IV (μg/mouse)	4.7 (4.1–5.4)	15.2 (14.8–15.6)	5.2 (4.7–5.7)
IP (μg/mouse)	5.8 (5.3–6.4)	17.1 (16.7–17.5)	6.7 (6.4–7.1)