Acta Tropica 149 (2015) 86–93

Contents lists available at ScienceDirect

Acta Tropica

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

Immunological cross-reactivity and neutralization of the principal toxins of *Naja sumatrana* and related cobra venoms by a Thai polyvalent antivenom (Neuro Polyvalent Snake Antivenom)

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ARTICLE INFO

Article history:
Received 3 March 2015
Received in revised form 23 April 2015
Accepted 25 May 2015
Available online 27 May 2015

Keywords:
Immunological activity
Cross neutralization
Polyvalent antivenom
Cobra venom toxins

ABSTRACT

The low potency of cobra antivenom has been an area of concern in immunotherapy for cobra envenomation. This study sought to investigate factors limiting the neutralizing potency of cobra antivenom, using a murine model. We examined the immunological reactivity and neutralizing potency of a Thai polyvalent antivenom against the principal toxins of *Naja sumatrana* (Equatorial spitting cobra) venom and two related Asiatic cobra venom α-neurotoxins. The antivenom possesses moderate neutralizing potency against phospholipases A_2 (*P*, potency of 0.98 mg/mL) and moderately weak neutralizing potency against long-chain α-neurotoxins (0.26–0.42 mg/mL) but was only weakly effective in neutralizing the short-chain α-neurotoxins and cardiotoxins (0.05–0.08 mg/mL). The poor neutralizing potency of the antivenom on the low molecular mass short-chain neurotoxins and cardiotoxins is presumably the main limiting factor of the efficacy of the cobra antivenom. Our results also showed that phospholipase A_2, which exhibited the highest ELISA reactivity and avidity, was most effectively neutralized, whereas *N. sumatrana* short-chain neurotoxin, which exhibited the lowest ELISA reactivity and avidity, was least effectively neutralized by the antivenom. These observations suggest that low immunoreactivity (low ELISA reactivity and avidity) is one of the reasons for poor neutralization of the cobra venom low molecular mass toxins. Nevertheless, the overall results show that there is a lack of congruence between the immunological reactivity of the toxins toward antivenom and the effectiveness of toxin neutralization by the antivenom, indicating that there are other factors that also contribute to the weak neutralization capacity of the antivenom. Several suggestions have been put forward to overcome the low efficacy of the cobra antivenom. The use of a 'proper-mix' formulation of cobra venoms as immunogen, whereby the immunogen mixture used for hyperimmunization contains a mix of various types of α-neurotoxins and cardiotoxins in sufficient amount, may also help to improve the efficacy and broaden the neutralization spectrum of the antivenom.

1. Introduction

In Asia, cobra (*Naja* sp.) bites constitute the major cause of high mortality and morbidity associated with snake envenomation (Warrell, 2010; World Health Organization, 2010). Progressive descending paralysis and local necrosis are the prominent neurological manifestation of cobra envenomation. The early signs of muscular paralysis usually begin with ptosis, diplopia, ophthalmoplegia, dysphasia and dysphagia, followed by limb weakness and areflexia, eventually progress to respiratory failure as a consequence of intercostal muscles and diaphragm paralysis (Ahmed et al., 2008; Alirol et al., 2010). Respiratory failure occurs in 10–50% of cases (Punde, 2005; Wongtongkam et al., 2005) and is the leading cause of death in cobra envenomation (Wongtongkam et al., 2005).

Antivenom therapy has hitherto been the only definitive treatment for snake envenomation. Unfortunately, numerous studies have reported that most of the existing commercially available Asiatic cobra antivenoms are generally low in potency (Balde et al., 2013; Kularatne et al., 2009). The low neutralizing potency of Asiatic cobra antivenom (typically <2 mg venom/mL antivenom) implies that a very large amount (>20 vials) of the antivenom may be required to treat severe cobra envenomation, as the amount of

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http://dx.doi.org/10.1016/j.actatropica.2015.05.020
0001-706X/

venom injected by a cobra in a bite may range from 100 to 600 mg (Mirtschin et al., 2006). Administration of a very large amount of antivenom is, however, undesirable as it not only results in a very high treatment cost but also greatly increases the risk of hypersensitivity.

The present study was undertaken as part of an effort to investigate factors limiting the neutralizing and cross-neutralizing potency of Asiatic cobra antivenom. Cross-neutralization of *Naja* venoms by heterologous antivenom is a well-known phenomemon (Cham et al., 2013; Kornhauser et al., 2013; Leong et al., 2012a). Neuro Polyvalent Snake Antivenom (NPAV) (produced against the venoms of the Thai monocled cobra, *Naja kaouthia*, and three other Thai elapid snakes) was chosen for this study as it is a commonly used polyvalent antivenom in Thailand and Malaysia, and extensive cross-neutralization data of this antivenom against venoms from various cobras are available for comparison purpose (Leong et al., 2012a). This study investigated the immunoreactivity and neutralization profiles of NPAV against various principal toxins of *Naja sumatrana* (Equatorial spitting cobra), *Naja kaouthia* (Monocled cobra) and *Naja sputatrix* (Javen spitting cobra) venoms. Findings from the current study may provide clues to formulation of a more potent and efficacious cobra antivenom. This is particularly relevant for the optimization of paraspecific antivenom use on species like *N. sumatrana* (a Category 1 medically important snake in the region), the bite from which unfortunately has no species-specific antivenom available as treatment. Besides, the findings will also reveal if there is a good correlation between immunoreactivity and neutralization potency of cobra antivenom, as earlier studies have demonstrated that immunoreactivity of an antivenom does not necessarily reflect the *in vivo* neutralizing potency of the antivenom (Casewell et al., 2010).

2. Materials and methods

2.1. Animals

Albino mice (ICR strain, 20–25 g) were supplied by the Laboratory Animal Centre, Faculty of Medicine, University of Malaya. The animals were handled according to the guidelines given by CIOMS on animal experimentation (Howard-Jones, 1985). The experimental protocols for all animal studies were approved by the Animal Care and Use Committee (ACUC) of the Faculty of Medicine, University of Malaya (Ethics Reference no. 2013-06-07/MOL/R/FSY). The institutional ethics committee was informed about the possibility of animal death without euthanasia and has approved the protocol specific for such experiments, where lethality is considered the gold standard for the test, with 48 h as the standard and experimental endpoint. Animals were allowed to die without euthanasia, before the experimental endpoint because in venom study, there is no reliable standard humane endpoint besides the standard experimental endpoint. This is because the toxic effect of the venom/toxin can be reversible, making the judgment of a humane endpoint difficult before 48 h. Hence, to ensure the reliability of the quantitative data, the animals were allowed to die without euthanasia before the experimental endpoint. The total number of animals that died without euthanasia was about 40, and the cause of death was due to venom toxicity (neurotoxicity and cytotoxicity). The method of euthanasia used at the experimental endpoint was anaesthetic euthanasia with urethane. The health of the animals was monitored every 30 min in the first 12 h; and thereafter 12-hourly till end point. The venom/toxin injected intravenously was unlikely to cause much pain and hence there was no anesthesia given during venom/toxin administration, as anesthesia could actually affect the result due to interaction with the venom/toxin that may enhance the toxic effect of the venom/toxin in such experiment.

2.2. Venoms and antivenoms

Naja kaouthia (Thailand) and *Naja sputatrix* (Indonesia) venom was purchased from Latoxan (France) while *N. sumatrana* venom was a pooled sample obtained from several adult individuals captured in southern Peninsular Malaysia, supplied by Snake Valley (Seremban, Malaysia). After milking, the venom sample was instantly lyophilized and stored at −20 °C until use. Two antivenoms were used in this work: (a) Neuro Polyvalent Snake Antivenom (NPAV) (batch no. NP00109; expiry date May 10th, 2014), a lyophilized purified $F(ab')_2$ obtained from plasma of horses hyperimmunized against a mixture of four venoms: *Naja kaouthia* (Thai monocled cobra), *Ophiophagus hannah* (king cobra), *Bungarus candidus* (Malayan krait) and *Bungarus fasciatus* (banded krait), all of Thai origin; (b) *Calloselasma rhodostoma* monovalent antivenom (CRMAV) (Product name: Malayan pit viper antivenin; batch no. 0120406; expiry date November 2nd, 2014), a lyophilized purified $F(ab')_2$ obtained from plasma of horses hyperimmunized against the venom of Thai *C. rhodostoma* and served as control in this study. Both antivenoms are produced by Queen Saovabha Memorial Institute (QSMI), Thai Red Cross Society in Bangkok, Thailand. For neutralization studies, the antivenom was reconstituted according to the manufacturer's instruction: 10 mL normal saline was added to 1 vial of the lyophilized antivenom. According to the package insert, 1 mL NPAV is able to neutralize the following amount of snake venoms: 0.6 mg *N. kaouthia* venom, 0.8 mg *O. hannah* venom, 0.6 mg and 0.4 mg venoms of *B. fasciatus* and *B. candidus*, respectively.

2.3. Isolation and identification of principal toxins of N. sumatrana venom, long-chain neurotoxin of N. kaouthia venom and short-chain neurotoxin of N. sputatrix venom

Isolation and purification of the major *N. sumatrana* toxins were carried out as described by Yap et al. (2014) using Resource® S cation exchange-high performance liquid chromatography (HPLC) followed by reversed-phase HPLC. Isolation and purification of the long-chain neurotoxin of *N. kaouthia* venom and short-chain neurotoxin of *N. sputatrix* venom were carried out in a similar way as described below.

2.4. Cation exchange-high performance liquid chromatography (CE-HPLC)

Lyophilized venom (10 mg) was dissolved in 200 μL starting buffer (20 mM 2-(N-morpholino) ethane sulfonic acid (MES), pH 6.0) and subjected to a Resource® S cation exchange column (1 mL; GE Healthcare, Sweden) using a Shimadzu LC-20AD HPLC system (Shimadzu Corp., Japan). The column was pre-equilibrated with the starting buffer, and eluted with elution buffer (20 mM MES, pH 6.0 with 1.0 M NaCl) following a linear gradient flow system (0–30% elution buffer from 5 to 30 min, followed by 30–100% from 30 to 55 min) at the flow rate of 1.0 mL/min. Samples eluted at 280 nm absorbance peaks were collected manually. The fractions collected from CE-HPLC of the venoms were concentrated using Vivaspin®15R centrifugal concentrator (Vivascience, Germany) at 6000 × *g* for 35–40 min in Sorvall Biofuge Primo R refrigerated benchtop centrifuge (Thermo Scientific, USA).

2.5. Reversed-phase high performance liquid chromatography (RP-HPLC)

The concentrated fractions were further purified by a LiChrospher® WP 300 RP-18 (5 μm) column (Merck, USA) using the Shimadzu LC-20AD HPLC system. The column was pre-equilibrated with water containing 0.1% trifluroacetic acid (TFA), and elution