Molecular Immunology 68 (2015) 412–420

Contents lists available at ScienceDirect

Molecular Immunology

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

Development of protective agent against *Hottentotta saulcyi* venom using camelid single-domain antibody

Maryam Darvish[a], Mahdi Behdani[a], Mohammad Ali Shokrgozar[b], Kamran Pooshang-Bagheri[a], Delavar Shahbazzadeh[a,*]

[a] *Biotechnology Research Center, Venom & Biotherapeutics Molecules Lab, Pasteur Institute of Iran, Tehran, Iran*
[b] *National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran*

ARTICLE INFO

Article history:
Received 9 August 2015
Received in revised form
25 September 2015
Accepted 4 October 2015
Available online 20 October 2015

Keywords:
Hottentotta saulcyi
Nanobody
Phage display
Heavy chain antibody (HC-Ab)
Antivenom

ABSTRACT

Hottentotta saulcyi, medically important scorpion species, causes some of harmful toxic exposure in Iran. Administrated, conventional antivenom-based immunotherapy is still limited and hardly meet ideal characteristic of effective treatment for scorpion envenomation. In this study we aimed to develop a neutralizing agent directed against scorpion venom based on VHH, variable domain of the *Camelidae* heavy chain antibody or Nanobody. This promising biomolecule is well-established as an advantageous tool for therapeutic purposes due to its small size, stability, monomeric performance and less immunogenicity. In this study, a large Nb library was constructed and phage displayed after successful camel immunization using *H. saulcyi* scorpion crude venom. After a series of biopanning rounds on Sephadex G50 purified venom fraction and screening by monoclonal phage ELISA, the best reactive Nb was retrieved and designated Nb12. The selected Nb was then expressed as soluble protein in *Escherichia coli*, purified and confirmed by SDS-PAGE analysis and western blotting. The lead candidate Nb12 bound scorpion venom with K_{aff} value of $5 \times 10^7\ M^{-1}$. Nb12 was shown to be capable of neutralizing 2 LD_{50} of whole venom of scorpion toxin when injected in the ratio of the Nb/toxin of 1.4:1 into C57BL/6 mice. In challenge experiment, Nb succeeded to rescue all i.p. lethal dose injected mice even when administrated i.v., 20 min after envenoming. These results with ease of production and superior neutralizing activity make Nb a suitable anti-toxin candidate for treatment of scorpion envenoming.

1. Introduction

Scorpion sting is a prevalent health problem in many parts of the world and particularly in Iran (Chippaux and Goyffon, 2008; Shahbazzadeh et al., 2009). Some scorpion stings are harmful to humans and cause a wide range of clinical manifestations, from just severe local pain without life-threatening symptoms to developing systemic symptoms resulting in morbidity and mortality especially among the children (Dehghani and Fathi, 2012; Ismail, 1995). The severity of scorpion venom depends on scorpion species, venom composition and the age of victims (Saucier, 2004). So far, more than 1500 scorpion species have been identified around the world of which 30 are venomous (Bawaskar and Bawaskar, 2012; Nejati et al., 2014). According to retrospective studies in Iran, recorded death of scorpion envenoming from 2001 to 2005 were 104 cases which mostly occurred in southwestern region of country (Dehghani and Valaie, 2005). Amongst dangerous Iranian scorpion fauna, seven species (Jalali and Rahim, 2014) are considered medically important including *Hottentotta saulcyi* which inflicts 6% of scorpion envenomation accidents (Dehghani and Fathi, 2012). The venom component of this member of *Butidae* family comprises heterogeneous structural and functional substances, mainly proteins and peptides responsible for neurotoxic effects and impairing voltage-gated channels and receptors (Possani et al., 1999; Quintero-Hernandez et al., 2013). Current available therapy for scorpionism is limited to antivenom derived from hyper immune horse sera, $F(ab')_2$ pepsin-cleaved fragments of whole antibodies which is a polyvalent antivenom (Brown and Landon, 2010). This serotherapy is the main resource of treatment measure and has been widely practiced in many countries which encounter poisonous animal stings. However, ineffectiveness of antivenoms in venom neutralization has been mostly due to rapid biodistribution of the small scorpion toxin molecules (4–7 kDa) (Jalali et al., 2005) and failure of large IgG fragments (100 kDa) to diffuse readily from vascular compartment to the peripheral tissues (Gutierrez et al., 1998). Therefore, high dose of antivenom administrated i.v. is

* Corresponding author. Fax: +98 21 66480780.
E-mail address: shahbazzadeh@yahoo.com (D. Shahbazzadeh).

http://dx.doi.org/10.1016/j.molimm.2015.10.002
0161-5890/

immediately needed after scorpion envenoming (Krifi et al., 2001). Moreover, polyclonal antivenoms obtained after complicated processes are potentially hazardous, due to early anaphylactic shock and late serum sickness associated with these treatments (Ismail, 2003; Krifi et al., 2001). As heterologous polyclonal antibodies are not robust enough, recent efforts focused on the development of monoclonal antibodies have raised many hopes for the production of innovative functional fragments, with the advantages in terms of specificity and homogeneity. There are a number of reports on research which has exploited recombinant toxin-neutralizing antibodies, such as single-chain variable fragment (Devaux et al., 2001; Mousli et al., 1999), which are highly tissue penetrable. However, their therapeutic value has been limited due to instability, in vivo immunogenicity and low neutralization capacity (Hmila et al., 2008; Juarez-Gonzalez et al., 2005; Zhang et al., 2012). Thus the current limitations prompted us to explore functional antitoxins capable of efficient in vivo neutralization of scorpion venom toxin. Typically apart from classical antibodies, heavy chain antibodies (HC-Abs) are unique natural immunoglobulins found in serum of *Camelidae* (camels and llamas) which lack light chain and CH1 domain (Hamers-Casterman et al., 1993). The antigen binding site of HC-Ab is confined to a single variable domain designated VHH or Nanobody (Nb) which is fully functional in term of N-terminal specific antigen binding. This highly stable minimal size Nbs (15 kDa) exhibit high affinity for recognition of inaccessible epitopes compared to conventional antibodies, due to their extended CDR3 loops (Muyldermans, 2001). The VHH sequence shares high degree of similarity with human VH (of family 3) resulting in less immunogenicity to humans (Kolkman and Law, 2010). However, The presence of a few signature amino acid substitutions in frame work 2 (FR2) renders VHH more soluble and well expressed in bacterial or yeast expression system (Rahbarizadeh et al., 2011). Considering the similar size of Nb to many of the toxic components of scorpion venom (15 kDa vs. 7 kDa), it is advantageous to have neutralizing antibodies that have similar volume of distribution to the toxins that they are targeting. With these remarkable features, many researchers have already been devoted to Nb development as therapeutic and diagnostic tools against a number of targets such as hapten peptides, enzymes and toxins (Bakherad et al., 2013; Muyldermans et al., 2009). Preliminarily reports demonstrated that after immunization with scorpion venom, *Camelus dromedarius* produced a high titer of antibodies (both HC-Ab and conventional antibody) capable of neutralizing venom induced toxicity in mice (Meddeb-Mouelhia et al., 2003) and might serve as a source to generate antigen-specific VHHs (Hmila et al., 2008). In general, specific VHH fragments could be selected from antibody repertoire by versatile well known phage display approach, based on ligand and target interaction. As *H. saulcyi* is responsible for some reported envenoming in Iran, we decided to explore potential VHH-based antitoxin against scorpion venom using phage display technology from an immunized camel. We demonstrated that isolated Nb had an outstanding neutralization activity to protect all envenomed mice from lethal effect of whole venom by intravenous injection. Furthermore, Nb successfully rescued mice even after delayed Nb therapy of envenomed mice.

2. Materials and methods

2.1. Venom preparation and purification

The crude venom of *H. saulcyi* scorpions was milked after electrical stimulation of the telson, freeze dried and preserved until needed. The distilled water solution of venom was homogenized, centrifuged at 10000 × *g* for 10 min at 4 °C to discard mucus and other particles and supernatant solution was stored at −20 °C until further use. Water-extracted venom (40 mg) was purified over size-exclusion chromatography (SEC) on Sephadex G50 (1.6 × 100 cm; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) in ammonium acetate buffer (0.2 M, pH 6.8) on an AKTA purifier system (GE Healthcare Bio-Sciences). Fractions of 2 ml per tube were collected at a flow rate of 0.4 ml/min and monitored at 280 nm. The toxicity of major peak was confirmed in an in vivo experiment. This neurotoxic peptide collected from fractions (tube 45–79) were pooled and used as an appropriate antigen for the biopanning process. Both crude venom and G50 fraction were analyzed by Tricin-SDS-PAGE (Schägger, 2006), widely used for scorpion peptide analysis.

2.2. Camel immunization

To develop an immune library, a one-year-old camel (*Camelus dromedarius*) was used for immunization. The dromedary was subcutaneously injected with 100, 200, 400, 800, 1600 and 3200 μg of crude venom at weekly intervals. Antigen was mixed with the equal volume of complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for subsequent injection. Before the first injection and after each injection, serum samples were collected for evaluation of serum response using ELISA and an in vivo experiment.

2.2.1. ELISA

The camel antibody activity was detected by indirect ELISA. Briefly, each well of Maxisorp plate (Nunc) was coated by *H. saulcyi* venom (5 μg per well; bicarbonate buffer 50 mM, pH 9.6) at 4 °C overnight. Control wells were coated only with bicarbonate buffer. After washing with phosphate buffered saline (PBS), wells were blocked with blocking buffer [2% (w/v) BSA in PBS, pH 7.2] for 1 h at room temperature. Then, 100 μl of serially diluted immune and non-immune sera (1:4000–1:32000) were added to the wells and incubated for 1 h at room temperature (RT). The wells were washed five times with PBS-T [PBS plus 0.1% (v/v) Tween-20] and incubated with anti-camel rabbit antibody (1:5000) for 1 h at RT. After incubation and washing steps, mouse anti-rabbit antibody conjugated to HRP (horseradish peroxidase) (Sigma) was used at a final concentration of 1:3000. Finally, the reaction was developed using tetra-methylbenzidine (TMB) after 15 min incubation and stopped with 100 μl/well 2 N H2SO4 and optical density (OD) was read at 450 nm.

2.2.2. In vivo experiments

In order to determine whether antiserum was able to neutralize toxic effects of crude venom, first LD_{50} was identified: groups of 5 mice (C57BL/6, 16–18 g) were injected intravenously (i.v.) with different doses of crude venom dissolved in 150 μl of PBS, while the negative control group was injected with the equal volume of PBS. The median lethal dose of crude venom known as LD_{50}, administered dose at which 50% of mice survived the treatment, was calculated after 24 h according to probit analysis (Finney, 1971). For antivenom neutralization activity, various concentrations of venom (1–15 LD_{50}) were mixed with a constant amount of antivenom (150 μl). The mixtures were incubated for 30 min at 37 °C and injected intravenously into mice, four mice per dose. Control group receive 2 LD_{50} of venom alone. The numbers of surviving mice were recorded after 24 h and the median effective dose was expressed as the amount of venom neutralized per ml of antivenom.

2.3. Strains and vectors

The pHEN4 vector (Ghahroudi et al., 1997) was used for VHH library construction containing PelB leader signal to secrete the VHH in the periplasmic space, HA-tag for VHH-detection and the M13 bacteriophage PIII gene downstream of an Amber stop codon.