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High-density peptide microarray exploration of the antibody response in a rabbit immunized with a neurotoxic venom fraction

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ABSTRACT

Polyvalent snakebite antivenoms derive their therapeutic success from the ability of their antibodies to neutralize venom toxins across multiple snake species. This ability results from a production process involving immunization of large mammals with a broad suite of toxins present in venoms. As a result of immunization with this wide range of toxins, many polyvalent antivenoms have a high degree of cross-reactivity to similar toxins in other snake venoms – a cross-reactivity which cannot easily be deconvoluted. As a proof of concept, we aimed at exploring the opposite scenario by performing a high-throughput evaluation of the extent of cross-reactivity of a polyclonal mixture of antibodies that was raised against only a single snake venom fraction. For this purpose, a venom fraction containing short neurotoxin 1 (SN-1; Uniprot accession number P01416, three-finger toxin (3FTx) family), which is the medically most important toxin from the notorious black mamba (*Dendroaspis polylepis*), was employed. Following immunization of a rabbit, a specific polyclonal antibody response was confirmed by ELISA and immunodiffusion. Subsequently, these antibodies were investigated by high-density peptide microarray to reveal linear elements of recognized epitopes across 742 3FTxs and 10 dendrotoxins. This exploratory study demonstrates in a single immunized animal that cross-reactivity between toxins of high similarity may be difficult to obtain when immunizing with a single 3FTx containing venom fraction. Additionally, this study explored the influence of employing different lengths of peptides in high-density peptide microarray experiments for identification of toxin epitopes. Using 8-mer, 12-mer, and 15-mer peptides, a single linear epitope element was identified in SN-1 with high precision.

1. Introduction

Each year worldwide, snakebite envenoming causes mortality and morbidity to an overwhelming number of victims, particularly in rural tropical parts of the world (Gutiérrez et al., 2010; Harrison et al., 2011; Harrison and Gutiérrez, 2016). Despite recent biotechnological and medicinal chemistry developments within snakebite envenoming therapy, antivenom derived from the serum of immunized animals is still the only effective treatment option used clinically (Gutiérrez et al., 2011; Laustsen et al., 2016). Consisting of antibodies generated by the animal immune system, antivenoms are capable of neutralizing the toxic effects of the venoms used in the immunization procedure. In many cases, however, antivenom can also cross-neutralize venom(s) from other species than those included in the immunization mixture (Williams et al., 2011). This desirable quality, referred to as cross-reactivity or para-specificity, must be investigated for each antivenom in a preclinical setting and cannot necessarily be assumed *a priori*. Traditionally, preclinical efficacy and cross-reactivity have been studied using ELISA-based methods and *in vivo* models. More recently, the antivenomics protocol based on immuno-affinity chromatography has successfully been introduced as a tool to assess cross-reactivity and to predict efficacy of antivenoms prior to rodent testing (Pla et al., 2012; Gutiérrez et al., 2013, 2017). Although the antivenomics method has proven useful in describing antivenom cross-reactivity on the protein family level, it lacks the capacity to reveal details on the amino acid residue level and to

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explain, in molecular detail, why some similar toxins are recognized equally well, while others are not. Due to this missing information, rational improvement of antivenoms can only be achieved through trial and error.

Aiming at obtaining further in-depth understanding of cross-reactivity on an amino acid residue-level, mapping of antibody binding sites (epitopes) can be performed. Ideally, such studies should entail structural elucidation of antibody antigen complexes (Van Regenmortel, 2009). However, less extensive identification of linear elements of epitopes has previously been found to be helpful in understanding the interaction(s) between antibodies and single snake toxins (Ferreira et al., 2006; Lomonte, 2012; De-Simone et al., 2013; Castro et al., 2015; Ramos et al., 2016; Schneider et al., 2016). Recently, we introduced high-density peptide microarray technology for high-throughput epitope mapping of toxins into the field of antivenom research, making it possible to study antivenom cross-reactivity on a larger scale (Engmark et al., 2017, 2016). So far, almost all epitope mapping studies of snake toxins have studied complex mixtures of antibodies, derived from pooling of sera of multiple animals immunized with mixtures of several whole venoms. In studies of such complex systems, it is challenging to understand how the immune systems of the individual animals recognize single venom components and to elucidate how cross-reactivity is derived. One unanswered question remains regarding whether a polyvalent antivenom derives its broad cross-reactivity mainly from the injection of venom mixtures in each animal in the immunization process or from the pooling of sera from different immunized animals. Also, it is unclear to which extent the non-toxic venom components in the immunization mixture affect the immune response against the critical toxins. Hypothetically, a medically important toxin could go unnoticed by the animal immune system if the toxin has low immunogenicity, whereas less important, but immunogenic, venom components may give rise to a strong antibody response (Laustsen et al., 2017). Therefore, both the “polyvalent” recognition (based on pooled sera from animals individually immunized with complex mixtures of toxins) and the recognition originating from single animals against single toxins may provide important complementary information to understanding the complex phenomena underlying the production and performance of cross-reactive antivenoms. Potentially, such knowledge of Nature's choice of linear epitope elements can also be exploited in the development of antivenoms by more targeted approaches. As an example, such approaches could involve the use of mixtures of monoclonal antibodies, where each antibody must be carefully engineered and selected to ensure desired cross-reactivity of the entire antivenom antibody cocktail. Investigation of immune responses of both pooled and single sera may therefore unlock new knowledge and create a better foundation for understanding the immunological interaction between venom and antivenom.

Aiming at exploiting the potential of the high-throughput microarray platform in characterizing the cross-reactivity of a polyclonal antibody response in a single animal towards a single toxin, a rabbit was immunized with a venom fraction predominantly containing the medically most important toxin from the notorious black mamba (*Dendroaspis polylepis*), short neurotoxin 1 (SN-1; Uniprot accession number P01416) (Laustsen et al., 2015b; Petras et al., 2016). Specific recognition of SN-1 by the polyclonal rabbit antibodies was confirmed by ELISA and immunodiffusion. Subsequently, this response was investigated by high-density peptide microarray to reveal linear epitope elements and cross-reactivity to other three-finger toxins (3FTxs) to evaluate the extent of cross-recognition obtained in the immunization process. Furthermore, the high-density peptide microarray setup employed here allowed investigation of the influence of the length of peptides on a microarray on the signal output.

This study is the first to explore the immune response from a single animal immunized with a single snake venom toxin by high-density peptide microarray technology and therefore exploratory in nature and limited in its scope and conclusions.

2. Methods

2.1. Preparation of rabbit anti-SN-1 antiserum

Dendroaspis polylepis venom was obtained from Latoxan SAS, Valence, France. The venom is a pool obtained from several specimens collected in Kenya. Fractions of short neurotoxin 1 (SN-1) were isolated as described (Laustsen et al., 2015b) and pooled. Purity of SN-1 was determined using MALDI-TOF. In brief, the sample was mixed with an equal volume of a saturated solution of α-cyanohydroxycinnamic acid in 50% acetonitrile/water containing 0.1% trifluoroacetic acid and 1 μL was spotted onto an Opti-TOF plate, and analyzed by MALDI-TOF on an Applied Biosystems 4800 Plus instrument in positive linear mode. Data acquisition was performed by accumulation of 500 laser shots with a laser intensity of 3800. The peaks were integrated and the relative purity of SN-1 was estimated to 85 mol%, on the basis of ion peaks intensity.

A white New Zealand rabbit (approx. 2.5 kg) was immunized by injection of SN-1 emulsified in Freund's adjuvant, or adsorbed to aluminum hydroxide, according to the scheme in Table 1, following protocols approved by the Institutional Committee for the Use and Care of Animals (CICUA), University of Costa Rica. The immunization scheme was designed to mimic the immunization protocols used for antivenom production (Gutiérrez et al., 2005). The use of Freund's complete adjuvant was limited to one injection, followed by one injection of Freund's incomplete adjuvant, aiming to minimize the adverse effects of the adjuvant in the animal. The rabbit was bled 14 days after the last immunization. Serum was separated by centrifugation and stored at −20 °C.

2.2. ELISA protocols

Two ELISA experiments with either venom fractions or whole venoms were performed in two different labs containing different equipment, why the two assays were performed following two slightly different protocols.

For investigating the response of anti-SN-1 antiserum against venom fractions, the wells in MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight with 0.6 μg of each HPLC venom fraction dissolved in 100 μL PBS. Wells were blocked by adding 100 μL of PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma) and mixed at room temperature for 1 h. Plates were washed five times with PBS. 100 μL of a 1:2000 dilution of anti-SN-1 serum in PBS containing 2% BSA was added to each well in triplicates and incubated for 2 h followed by five additional washings with PBS. Then, 100 μL of a 1:2000 dilution of conjugated antibody (Sigma A6063, rabbit anti-horse IgG (whole molecule)-alkaline

Table 1
Immunization protocol for the rabbit employed in this study.

Day	Dose SN-1 (μg)	Adjuvant	Route
0	20	Freund's complete	s.c.
15	30	Freund's incomplete	i.m.
27	60	$Al(OH)_3$	s.c.
47	120	$Al(OH)_3$	i.m.
67	120	$Al(OH)_3$	s.c.
90	60	$Al(OH)_3$	i.m.
106	120	$Al(OH)_3$	i.m.