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Second generation snake antivenomics: Comparing immunoaffinity and immunodepletion protocols

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ABSTRACT

A second generation antivenomics protocol, based on affinity chromatography, was compared with a previously (first generation) immunodepletion protocol using as a proof of principle the pan-African EchiTAb-Plus-ICP® IgG antivenom and the venoms of *Echis ocellatus*, *Bitis arietans*, and African spitting cobras. The antivenom showed qualitatively similar immunoreactivity patterns using either antivenomic approach. Quantitative departures were noticed between both methods, which may be ascribed to differences in calculating the relative amounts of the non-recognized venom proteins. The smoother baseline in chromatograms of the affinity column allowed better resolution and more accurate quantification of the antivenomic outcome than the original immunodepletion protocol. Our results indicate that both methods can be used interchangeably to investigate the *in vitro* immunoreactivity of antivenoms. However, advantages of the second generation antivenomics are the possibility of analyzing $F(ab')_2$ antivenoms and the reusability of the affinity columns. These features contribute to the generalization, economy and reproducibility of the method.

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1. Introduction

The timely parenteral administration of antivenom remains, more than a century after the simultaneous development in 1894 of the first *serum antivenimeux* by Calmette (Calmette, 1894a, 1894b), and Phisalix and Bertrand (Phisalix and Bertrand, 1894a, 1894b) (Hawgood, 1999; Lalloo and Theakston, 2003), the only effective treatment of the scourge of the neglected tropical pathology of snakebite envenoming (Harrison et al., 2009, 2011; Williams et al., 2011; Gutiérrez et al., 2011a, 2011b). Antivenom manufacturing, mainly involving the hyperimmunization of horses, and to a minor extent also donkeys, sheep, and llamas, has remained relatively unchanged for the last decades (Gutiérrez et al., 2011a, 2011b). However, whereas first generation antivenoms comprised crude serum, current antivenoms consist of purified whole immunoglobulin (IgG) molecules or antibody divalent $F(ab')_2$ or monovalent Fab fragments, which have reduced the incidence and severity of adverse reactions associated with antivenom administration (WHO, 2010; Gutiérrez et al., 2011a, 2011b). Diverse biotechnological approaches, such as production of monoclonal antibodies, generation of recombinant scFV fragments, and affinity purification of neutralizing antibodies, provide technological alternatives for antivenom manufacture, although these developments have not gone beyond the experimental level (Gutiérrez et al., 2011a).

The use of transcriptome-based design of synthetic multiepitope DNA immunogens to design toxin-specific antivenoms with wide geographic and snake-species

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coverage has also been successfully explored at the experimental level (Wagstaff et al., 2006; Harrison et al., 2011). Another rational approach, being promoted by the Global Snakebite Initiative (GSI, http://www.snakebiteinitiative.org) for improving the availability and accessibility of antivenoms, is the organization of a multinational collaborative project to develop new regional polyvalent antivenoms in Asia and Africa using proteomic tools and quality control protocols for assessing cross-reactivity (Williams et al., 2011). Snake venomics, and its logical extension, antivenomics (reviewed in Calvete, 2011), offer the exciting possibility of improving both the design of immunogen mixtures and of providing a complementary protocol to *in vivo* neutralization assays for assessing the suitability of antivenoms against homologous and heterologous venoms (Gutiérrez et al., 2009, 2010; Calvete et al., 2009a). The central idea of this approach is to replace the traditional geographic- and phylogenetic-driven hypotheses for antivenom production strategies with a more rational approach based on a deep knowledge of the venom proteomes and their cross-immunoreactivity profiles (Calvete, 2010a, 2010b, 2011).

Using the original, first generation antivenomics protocol, antivenom immunoreactivity was inferred indirectly through the proteomic characterization of the toxin fraction that remains in solution after immunoprecipitation (Lomonte et al., 2008; Gutiérrez et al., 2008; Calvete et al., 2009b; Núñez et al., 2009). Immunoaffinity chromatography constitutes another possibility for assessing the ability of antivenoms to recognize venom components (Williams et al., 2011; Fahmi et al., 2012; Makran et al., 2012). The combination of immunoaffinity chromatography and proteomic analysis is the basis of a second generation antivenomics protocol, which provides information on both the set of toxins bearing antivenom-recognized epitopes and those showing poor immunoreactivity. As a proof of principle that the original (immunodepletion) and the new (affinity capture) antivenomic approaches yield compatible outcome, we have assessed the immunological profile of the pan-African EchiTAb-Plus-ICP® antivenom towards the venoms of a panel of African viperid snakes and spitting cobras, and compared these results with the previously assessed immunoreactivity of the same antivenom (Calvete et al., 2010; Petras et al., 2011).

2. Materials and methods

2.1. Venoms and antivenoms

The venoms of *Bitis arietans arietans* (Ghana) and *Echis ocellatus* (Nigeria) were pools from adult specimens kept at the herpetarium of the Liverpool School of Tropical Medicine, and were kindly provided by Dr. Robert A. Harrison. The venoms of the spitting cobras *Naja nigricollis* (Togo), *Naja katiensis* (Burkina Faso), *Naja pallida* (Kenya), *Naja nubiae* (North Africa), and *Naja mossambica* (Tanzania) were purchased from Latoxan (Valence, France). All venoms were lyophilized and stored at -20 °C until used.

The polyspecific EchiTAb-Plus-ICP® antivenom comprised IgG molecules purified by caprylic acid fractionation of the plasma of four horses that had been immunized with a mixture (at a weight ratio of 1:1:1.33) of the venoms of *E. ocellatus*, *B. arietans* and *N. nigricollis* collected from Nigeria (Gutiérrez et al., 2005). The antivenom batch used (Batch 4260308PALQ, the same employed by Calvete et al., 2010 and Petras et al., 2011, in previous first generation antivenomics studies), had the following composition: protein concentration 69.6 g/L, sodium chloride 7.6 g/L, phenol 1.86 g/L, and pH 6.78. The antivenom batch passed all the quality control requirements at the Quality Control Laboratory of Instituto Clodomiro Picado.

2.2. Immunoaffinity capturing antivenomics protocol

To prepare immunoaffinity columns of EchiTAb-Plus-ICP® antivenom, 1 ml of NHS-activated Sepharose 4 Fast Flow (Ge Healthcare) was packed in a column, washed with 10–15 matrix volumes of cold 1 mM HCl, followed by two column volumes of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), and incubated for 4 h at room temperature with 35–50 mg of antivenom IgG molecules in coupling buffer. Non-reacting groups were blocked with 500 μL of 0.1 M Tris–HCl, pH 8.0 at 4 °C overnight using an orbital shaker. The affinity column was washed alternately at high and low pH, with three volumes of 0.1 M acetate buffer, 0.5 M NaCl, pH 4.0–5.0 and three volumes of 0.1 M Tris–HCl buffer, pH 8.5. This treatment was repeated 6 times and the column was equilibrated in binding buffer (PBS). Different amounts of venom (300 μg *B. arietans*; 400 μg *E. ocellatus*; 500 μg *N. mossambica*; 500 μg *N. nigricollis*; 300 μg *N. katiensis*; 500 μg *N. nubiae*; 500 μg *N. pallida*) in 500 μL of PBS were separately loaded and incubated with the affinity matrix in an orbital shaker overnight at room temperature (23 °C). The immobilized antivenom:venom ratio used corresponded to about 20 IgG molecules per "25 kDa of toxin molecule" (*E. ocellatus* and *B. arietans* venoms) or 8 IgG molecules per "10 kDa of Naja toxin". This molar ratio is the same employed by Petras et al. (2011) to immunoprecipitate Naja toxins, and 25× the antivenom:venom ratio used by Calvete et al. (2010) for immunodepleting toxins from Echis and Bitis venoms. After eluting the non-retained fraction, the column was thoroughly washed (5×) with PBS and the captured proteins were eluted with 5 column volumes of elution buffer (0.1 M glicine–HCl, pH 2.0). As specificity controls, 0.5 ml of Sepharose 4 Fast Flow matrix, without or with 30 mg of immobilized control (pre-immune) IgGs, were incubated with the same amount of venom and developed in parallel to the immunoaffinity columns.

Eluted fractions from the immunoaffinity columns were neutralized with 1 M Tris–HCl, pH 9.0, and the whole non-retained and retained fractions were analyzed by reverse-phase HPLC using a Teknokroma Europa C_{18} (0.4 cm × 25 cm, 5 mm particle size, 300 Å pore size) column and an Agilent LC 1100 High Pressure Gradient System equipped with DAD detector and micro-Auto-sampler. The flow-rate was set to 1 ml/min and the column was developed with a linear gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B), isocratically (5% B) for 10 min, followed by 5–25% B for 20 min, 25–45% B

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