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Intraspecific differences in the immunochemical reactivity and neutralization of venom from Argentinean *Bothrops* (*Rhinocerophis*) *alternatus* by specific experimental antivenoms

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ABSTRACT

The venoms of *Bothrops* (*Rhinocerophis*) *alternatus* (*B.a.*) from different regions of Argentina have shown biochemical, toxicological and immunological variations. Considering these variations, we produced nine experimental antisera (rabbit, IgG) against venoms from snakes of nine different regions and a pool of venom, comprised of equal amounts of venoms from each region. The immunologic studies (ELISA, Westernblot) showed significant cross reactivity among all regional antivenoms with all regional venoms, with no significant differences regarding the specificity of the immunogens used for the production of antivenom. Neutralization of hemorrhage was variable (although all the antivenoms neutralized this activity in all venoms) and the neutralization of coagulant and phospholipase activities were evident in all cases. Some antivenoms neutralized toxic activities that were absent or very low in the venoms used as immunogen, on other non-homologous venoms (e.g. thrombin like activity). Despite the different toxic potencies of regional venoms, antivenoms developed using venoms of snakes from a particular region showed high immunochemical reactivity and cross-neutralizing capacity on snake venoms from different and distant regions, in occasions over those of the homologous antivenoms. These findings could be used to improve the generation of pools of venoms for the production of antivenoms.

1. Introduction

Snake venoms may vary biochemically and toxicologically, not only at the genus and species levels, but also at the individual level (Chippaux et al., 1991). These variations may be related to environmental factors such as climate, geography and food availability (Chippaux et al.,

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1991; Daltry et al., 1996; Sasa, 1999; Gibbs and Rossiter, 2008).

Variation among venoms from a single species in different geographical locations has been widely described (Cavinato et al. 1998; Chanhome et al., 2009; Glenn et al. 1983; Saravia et al., 2002; Shashidharamurthy et al. 2002; Rocha and Furtado, 2005; Flight et al., 2006; Lanari et al., 2010; Costa de Oliveira et al., 2011). In a country with the size of Argentina (2,780,400 km^2) this recommendation is especially important in the case of *Bothrops [Rhinocerophis] alternatus* (hereafter *Bothrops alternatus*), which can be found from the province of Jujuy above the Tropic of Capricorn to about 38° of South latitude (more than 1800 km away) and from the Atlantic shore to pre-Andean region, nearly 1000 km apart (Giraudo et al., 2012; Ministerio de Salud, 2007).

In a previous work we demonstrated toxic and biochemical variation of *B. alternatus* venom from different geographical locations (Lanari et al., 2010). We also note a good variable neutralization of these venoms by polyvalent antibothropic antivenom (against *B. alternatus* and *Bothrops neuwiedi*), (de Roodt et al., 2011).

This variation in neutralization could be due to a differential toxicity of the venom of this species (Rocha and Furtado, 2005; Lanari et al., 2010) or to immunological differences between components of snake venoms from different regions. Barrio and Miranda (1966) using a polyvalent therapeutic antivenom observed immunochemical differences in the venoms of *B. alternatus* from different regions of Argentina. Nevertheless those studies were not done with a monospecific anti-*B. alternatus* venom,. At present, data are not available on possible intraspecific immunological differences of *B. alternatus* venoms from different geographic regions of Argentina. Therefore experimental antivenoms were produced by immunizing rabbits with snake venoms of nine regions of Argentina, and with a Pool formed by these regional venoms. These antivenoms were then immunologically tested against the different venoms and against their Pool.

2. Materials and methods

2.1. Venom

Adult specimens of *B. alternatus* were obtained from different regions of the country. Snakes from the province of Buenos Aires (5–9 snakes by region of the province) were provided by the Serpentarium of the National Institute for Production of Biologicals (hereafter INPB, specimens from Dock Sud, Baradero and San Nicolás) or by the Zoo "La Máxima" (city of Olavarría, donated by Dr. Sandra Botassi). Venoms of the Province of Entre Ríos came from the localities of Gualeguay and Concordia, from snakes housed in INPB (12–18 by region of the province). The venom of specimens of the province of Corrientes (20 snakes) was a donation from the Serpentarium "Palo Seco" in Monte Caseros in this province. The venom of species of the province of Misiones (24 snakes) was a gift from Dr. Alejandro Urs Vogt from Centro de Zootoxicología of the province of Misiones (Oberá, Misiones). The venom of animals in the province of Cordoba (30 snakes) was a gift from B. Sc. Gustavo Reati of the Center for Applied Zoology, National University of Cordoba. The animals from whom the venoms were collected, in all cases were housed in plastic boxes or terrariums with dark/light cycles of 12 h and were fed with albino mice or rats every 15 days and drinkable water *ad libitum*. For maintenance of the snakes, guidelines of the Institute for Laboratory Animal Research were followed (Pough, 1991).

2.2. Venom extraction and conservation

Venoms were obtained by manual milking and vacuum – dried immediately and stored at −20 °C until use. In all cases, previous to use they were dissolved in 0.15 M NaCl.

3. Animals

Antivenoms were obtained by immunizing New Zealand rabbits of 2.5–3 kg body weight. For the determination of hemorrhagic activity and its neutralization Wistar rats (200–250 g) were used. The animals were housed in plastic boxes under controlled environmental conditions, with dark–light cycles of 12 h, and fed with commercial specific food and drinkable water *ad libitum*. All animal experimentation was carried out in accordance with the suggestions of the Guide for the Care and Use of Laboratory Animals (National Research Council, 2002.)

4. Antivenoms production and purification

4.1. Immunization

Rabbits ($n = 3$ by Pool of venoms, Gualeguay, Dock Sud, Baradero and Olavarria and $n = 2$ per venom for the other regional antivenoms) were immunized with increasing doses of venom emulsified in complete Freund's adjuvant (100 μg, day 1), incomplete (200 μg, day 10), aluminum hydroxide 10% (500 ug, days 20 and 27) or only with 0.15 M NaCl (1000 μg, days 40 and 50). The antigen, in a final volume of 2 ml, was injected subcutaneously in the back of the animals in several points, in volumes of 0.2–0.5 ml per point. To assess the level of antibodies, rabbits were bled by marginal ear vein, and the immune response was evaluated by double immunodiffusion (Ouchterlony method) (Margni, 1990) or by counterimmunoelectrophoresis (Siles Villarroel et al., 1976/77).

4.2. Purification of antibodies

Rabbits were only bled when they reached 1/16 titer by Ouchterlony method, and the blood preserved with anticoagulant (1 volume sodium citrate 4% per 9 volumes of blood). The blood was centrifuged to separate the red blood cells, and the plasma obtained was treated with 6.7% caprylic acid under vigorous stirring for 60 min at room temperature. After that time, the plasma was filtered through filter paper. The filtrate was dialyzed at 4 °C against 0.15 M NaCl (twenty volumes with changes every 12 h) for ten days. The dialyzate was filtered through filters of 0.45 and 0.22 μm and stored in sterile containers at 4 °C. The IgG concentration was determined spectrophotometrically

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