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Development of process to produce polyvalent IgY antibodies anti-African snake venom☆

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ABSTRACT

Polyvalent anti-*Bitis* and anti-*Naja* antivenom IgY antibodies were prepared using *B. arietans*, *B. nasicornis*, *B. rhinoceros*, *N. melanoleuca*, and *N. mossambica* venoms to immunize chickens. Blood and eggs were collected before and during the 10-month immunization period; the sera and yolk extracts were then prepared and assayed for the presence of antivenom antibodies by ELISA and Western blot methods. ELISA Antivenom antibody titers, referred to as U-ELISA/ml of serum or egg yolk extracts, absent in pre-immunization sera or yolk, increased sharply during the 4 weeks after immunization, reaching a plateau thereafter. Yolk extracts with high antivenom titers, as detected by ELISA were used to isolate and purify IgY. Purified IgY preparations recognized venom protein bands from 10 to 20 kDa to 60 and 70 kDa, as shown by Western blot. Recovery of antivenom antibodies from the whole yolk was over 80%. Final preparations exhibited high antivenom activity (>100,000 U-ELISA/ml) as well as efficacy in neutralizing venom lethality (1440 μg of IgY neutralize 62.2 LD_{50} of venom), and were free of toxic products, pyrogen or bacterial and fungal contaminations.

1. Introduction

The snake species usually found in Guiné, S. Tomé, Angola and Mozambique belong to the *Elapidae* and *Viperidae* families (Manaças, 1981–1982). *Elapidae* includes the genus *Aspidelaps*, *Elapsoidea*, *Hemachatus*, *Naja*, *Pseudohajc*, and *Dendroaspis*, comprising around 20 species. Their venoms are essentially neurotoxic. The local effects of bites from these snakes are usually minimal, restricted to small fang puncture wounds, exceptionally with tender local swelling, blistering and superficial necrosis (Reid, 1964; Viravan et al., 1986). Early systemic symptoms of envenoming are vomiting, "heaviness" of the eyelids, blurred vision, fasciculation, paraesthesiae around the mouth, hyperacusis, headache, dizziness, vertigo, hypersalivation, congested conjunctivae and "gooseflesh". Ptosis and external ophthalmoplegia appear soon after the bite, followed minutes or hours later by paralysis of the face, jaws, tongue, vocal cords, neck muscles and (bulbar) muscles of deglutition with pooling of secretions in the pharynx. The intercostals and diaphragm become paralyzed, leading to abdominal breathing, respiratory distress, with agitation, tachycardia, sweating, and central cyanosis (Warrell, 1996). *Viperidae*

☆ *Ethical statement:* The experiments with chickens were approved by the "Ethical Committee of Animal Welfare—UENF".

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includes the genus *Causus*, *Bitis*, *Vipera*, *Atheris*, and *Atractaspis*, with also around 20 species. Their venoms are rich in various enzymes, mainly proteinases. Swelling and bruising may develop at the site of the bite within a few minutes or hours. Swelling spreads rapidly and sometimes involves the whole of the bitten limb and trunk. Regional lymphatic vessel and lymph node involvement is common. Blistering and necrosis may develop at the site of the bite. Hemostatic abnormalities are clinically manifested by persistent bleeding from the fang puncture wounds and other partially healed wounds, suggesting coagulopathy. The blood clot alterations are sometimes systemically reflected as epistaxis, hematemesis, cutaneous ecchymoses, petechiae or discoid ecchymoses, hemoptisis, and subconjuntival, retroperitoneal, subarachnoid, intracranial hemorrhages (Warrell, 1996). These venom effects can be completely and acutely reversed by specific antivenoms, if the patients or bitten animals are properly treated. Snake venom poisoning is one disease process that has been successfully treated with specific polyclonal antibodies. This therapeutic tool has been used since the pioneering work of Sewall (1887) and Calmette (1894). Conventional antivenoms are prepared by immunizing large animals, usually horses, with individual venom or a range of different venoms obtained from several snakes to eliminate intraspecific variation (Theakston, 1996). In order to minimize the expected adverse reactions, upon injection into the bitten victims, the IgG immunoglobulins are partially purified, enzymatically cleaved by pepsin, and the resulting F(ab′)2 fragments are concentrated and tested for their antivenom neutralizing properties, and submitted to quality control, before release for use in human beings. As antivenoms prepared in horses are very expensive for use to treat domestic animals bitten by venomous snakes, alternative animals and methods are being tried. Previous studies in our laboratory have demonstrated that hens (*Gallus gallus*) immunized with *Bothrops* spp. and *Crotalus durissus terrificus* crude venoms, produce IgY antibody specific for these venoms, which can then be recovered from the egg yolks and purified (Almeida et al., 1998). The resulting IgY preparations were endowed with the ability to combine with venom components used to immunize the hens, and neutralize their toxic activities. The present study aimed to improve procedures to produce, on a large scale, IgY-specific antibodies to *Bitis* spp. and *Naja* spp. venoms to be used to treat domestic animals and eventually human patients bitten by African snake venoms. Before being released for use, these antivenoms will be submitted to pre-clinical trials. Based on the simplicity of the entire procedure, the low price of hens and, especially, the high capacity of the resulting antivenoms to neutralize the venoms' lethality, IgY antivenoms are indicated to treat animals bitten by snakes, especially in developing countries.

2. Material and methods

2.1. Animals

Rhode Island Red female chickens (1.1–1.5 kg body mass), Swiss outbreed (18–20 g) mice, and rabbits (0.5–1.0 kg) were maintained in the animal facility of the Laboratório de Biologia do Reconhecer (LBR)/Centro de Biociências e Biotecnologia (CBB)/Universidade Estadual do Norte Fluminense—Darcy Ribeiro (UENF). Hens were used to produce IgY antivenoms, Swiss outbred mice were used to determine venom lethality potency and the neutralizing potency of antivenoms, and rabbits were used to produce anti-IgY antiserum. Animal care was provided by expert personnel, in compliance with the relevant laws and institutional guidelines.

2.2. Crude venoms from African snakes

The *Bitis arietans*, *Bitis nasicornis*, *Bitis rhinoceros*, *Naja melanoleuca*, and *Naja mossambica* snake venoms were purchased from Venom Supplies Pty Ltd (Tanunda, Australia).

2.3. Reagents and supplies

Freund's complete adjuvant (FCA), incomplete Freund's adjuvant (IFA), and rabbit anti- IgY antiserum were prepared in our Laboratory. The following reagents were purchased as indicated: Bovine serum albumin (BSA), goat anti-rabbit IgG peroxidase conjugate, 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma Aldrich Chemical Co., St Louis, MO, USA). All other reagents were of analytical grade as otherwise indicated. Polystyrene ELISA plates were utilized (Microlon Medium Binding, Greiner, Germany).

2.4. Determination of venom lethality

The lethal potencies of the venoms (LD_{50}) were determined by i.p. injection of Swiss outbreed mice (18–20 g) using eight mice per group. Mortalities were recorded after 48 h, and LD_{50} was calculated using the Bliss (1934) method.

2.5. Immunization schedule of chickens

Groups of eight chickens were immunized intramuscularly in the breast region at two or three sites with 20 μg of African snake venoms, alone or mixed as indicated in FCA: Group #1, *B. arietans*; Group #2, *B. nasicornis* plus *B. rhinoceros*; Group #3, *N. mossambica*; Group #4, *N. melanoleuca* plus *N. mossambica*; Group #5, non immunized. Three weeks later, the injections were repeated with the venoms in IFA. Three boosters were given with the venoms in 0.15 M NaCl by the same route, also at 3-week intervals. Blood samples and eggs were collected before immunization to be used as negative controls either in immunochemical assays or in immunoprotection tests. Eggs were collected every day from each immunized chick and refrigerated at 4 °C. Egg yolks were separated from the albumin and stored at −20 °C.

2.6. Antibody purification

2.6.1. Isolation and partial purification

Yolks from 30 eggs of the same immunized group of hens were diluted 10-fold with distilled water, pH 5.5.