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Development of a new approach of immunotherapy against scorpion envenoming: Avian IgYs an alternative to equine IgGs

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ABSTRACT

Antivenom treatment has been largely used against scorpion stings. Despite their efficacy, the use of mammalian antivenoms may cause adverse effects due to the immune system activation. IgYs from hyperimmunized laying hens against venoms could be a promising alternative to equine IgGs due to the various benefits that these antibodies can provide. Here we report the preparation of specific IgYs by immunizing laying hens with *Aah* (*Androctonus australis hector*) scorpion venom.

IgYs were isolated from egg yolks by water dilution and salt precipitation methods; they were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis, western blot and ELISA. The efficiency of these immunoglobulins on the pathophysiological effects induced by *Aah* venom was assessed by histological and metabolical analysis of the aorta and the heart. The inflammatory response was assessed by evaluating the granulocyte tissue infiltration and oxidative/nitrosative status.

Results revealed high IgYs titers against *Aah* venom by ELISA. Overall, these IgYs seem to protect efficiently mice against envenomation and neutralized the lethal effects of scorpion venom with a high efficacy; the median effective dose (ED50) was 221 µl/2 LD50; i.e. an amount of 79.23 mg of IgY scan neutralize 1 mg of *Aah* venom. IgY antibodies neutralize effectively the *Aah* venom lethality and could prevent severe pathological effects induced by scorpion venom and could be used as an effective alternative to equine IgGs against scorpion envenoming.

1. Introduction

Scorpion envenomation (SE) constitutes a real public health problem in Algeria with annually 30,000 to 50,000 cases of scorpion stings and an average of 60 deaths per year [1–3]. The most dangerous scorpion responsible for lethal cases of envenomation is *Androctonus australis hector* (*Aah*) [4]. The venom of this species is very toxic and the induced symptoms include myocardial inflammation and respiratory failure. Thus, a rapid and effective treatment is essential to reduce the symptomatology and the mortality from the victims [5].

Specific immunotherapy would be the most important therapeutic outcome after SE [6–8]. The current scorpion antivenoms used in Algeria for commercial purposes are $F(ab')_2$ immunoglobulin fragments purified from the blood of hyperimmunized horses with *Aah* venom [4]. The major disadvantages of this procedure are the suffering of the animals due to the hyperimmunization and the induced severe side reactions [9–11]. Mammalian antibodies are also known to activate the human complement cascade and to cause adverse effects [12].

To improve the antivenom production and to reduce the costs, many investigations are undertaken using new approaches [13–15]. Indeed, the employment of chickens to produce antivenom antibodies from egg yolks (IgY) represents a promising perspective on the future [16]. The use of IgYs on a large-scale has several positive aspects: i) IgY can be obtained noninvasively from the egg yolk, ii) IgY does not activate the complement system like IgG and does not interact with rheumatoid factors, iii) the amount of antibodies produced by a single hen is similar to that of a large mammal, and iv) maintenance costs may be more economical [17–20].

The efficacy of IgYs obtained from laying hens in neutralizing the lethality of snake venoms has already been demonstrated [9, 21–25]. However, only two studies have reported IgY antibodies production and their efficacy against scorpion venoms (*Leiurus quinquestriatus* and *Tityus caripitensis*) [16, 23].

Therefore, the aim of this study was to develop and to characterize

Abbreviations: Aah, *Androctonus australis hector*; ED50, median effective dose; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; Fab, Fragment antigen binding; IgY, yolk immunoglobulin; IgG, Immunoglobulin G; LD50, median lethal dose; NMRI, Naval Medical Research Institute; SE, scorpion envenomation; SOF, salting out fraction; WSF, water-soluble fraction

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chicken egg yolk antibodies (IgYs) against *Aah* scorpion venom and to evaluate in-vivo its neutralizing capacity on a murine model.

2. Material and methods

2.1. Venom

The antigen used is the lyophilized *Aah* scorpion venom provided from the LBCM (Laboratory of Cellular and Molecular Biology) of the Biological Sciences Faculty at USTHB (Algiers, Algeria).

2.2. Animals

Fifteen laying hens (*Babolnà tetra*), 18 weeks old, weighing 1.2 kg and obtained from the poultry group center *ORAC* SPA (Algiers, Algeria) were used in order to produce anti-*Aah* scorpion venom. Hens were housed in individual cages with standard food and water ad libitum and they were kept on a cycle of 12/12-h light/dark cycle at 20–23 °C.

Male NMRI mice (18–20 g) obtained from Pasteur Institute of Algeria were used for lethality neutralization test and in immunotherapy experiments. They were housed under standard laboratory environment (humidity, lighting and temperature) and fed the standard rodent diet and tap water.

Animal experiments were in compliance with the European Community Council Directive (86/609/EEC).

2.3. Reagents

Chemicals and reagents used were of high analytical quality and they were purchased from Sigma (St. Louis, MO), Merck (Mannheim, Germany) and Biochem (Montréal, Canada).

2.4. Methods

2.4.1. Laying hens immunization

Groups of hens were intramuscularly immunized (in the breast region) five times at 11-day intervals with *Aah* venom according to four different protocols (Fig. 1). For the initial immunization, venom sample was mixed with equivalent volumes of the Freund's complete adjuvant (FCA). On the 11^{th} and 22^{th} day after the first immunization, booster doses of antigen emulsified with Freund's incomplete adjuvant (FIA) (1:1, volume/volume) were administered. For the next immunizations, venom sample was diluted in normal saline solution (NaCl 0.9%). Blood samples were collected before each injection. Sera were separated through centrifugation (1398 ×*g* for 10 min). IgY antibodies isolated from egg yolks were collected from the individual chicken each day throughout the immunizations. Pre-immune serum and egg yolk samples collected from chickens were used as negative controls. They were stored in a freezer at −20 °C.

2.4.2. Isolation and partial purification of IgYs antibodies

IgYs isolation from egg yolks of hyperimmunized hens, from the 1^{st}-76 day following the first immunization, was processed by collecting WSF (water-soluble fraction) following Akita and Nakai described procedure [26]. Briefly, the egg yolk was diluted 1:10 (volume/volume) with cold acidified distilled water (pH 5.2). The supernatant containing IgYs was collected by centrifugation (10,000 ×*g*, 20 min, 4 °C) and was precipitated with a 40% saturated ammonium sulfate solution. The salting-out fraction (SOF) containing the IgYs was collected by centrifugation (10,000 ×*g*, 20 min at 4 °C), dissolved in PBS buffer (pH 7.4, 0.1 M) and dialyzed against the same buffer and thereafter stored at −20 °C before use. Negative controls of the IgYs preparations were also purified from the egg yolks collected from non-immunized chickens.

Protein contents of egg yolks from hens immunized with protocol D were performed by using Bradford assay [27] with BSA as the comparative standard and analyzed by SDS-PAGE using an 8% acrylamide gel under native conditions [28].

2.4.3. Western blot analysis

Recognition of IgY and Fab' antibodies was carried out by Western blot according to the modified procedure of Towbin et al. [29]. After SDS-electrophoresis (8%), proteins were transferred onto nitrocellulose membrane in 48 mM Tris base (pH 8.3) containing 39 mM glycine, 0.0375% SDS and 20% methanol. After blocking the membrane for 1 h at room temperature with 5% nonfat dry milk in PBS buffer pH 7.4 containing 0.1% Tween 20, the membrane was incubated under stirring for 1 h with horseradish peroxidase-conjugated rabbit anti-whole chicken IgY (Sigma-Aldrich, USA) diluted 1:1000 in PBS-Tween 20. Finally, blots were incubated in peroxidase chromogenic substrate solution (0.1 M PBS pH 7.4 with 0.06% DAB and 0.03% H_2O_2) for 20 s. The immunoreactivity was stopped with distilled water.

2.4.4. Characterization of egg yolk antibodies by indirect antibody capture assay

The antibody titration in sera and egg yolks was assessed by ELISA following Theakston and coworkers procedure [11]. Polystyrene ELISA plates (Maxisorp, Germany) were coated with the *Aah* venom (5 µg/ml in 0.1 M carbonate bicarbonate buffer, pH 9.6) and kept overnight at 4 °C. After a washing step with PBS buffer containing 0.1% Tween-20, non-specific sites were blocked with 100 µl of 0.1 M PBS, pH 7.4 containing 5% skim milk for 1 h at 37 °C and washed five times with PBS-Tween 20. IgYs preparations diluted from 1:500 to 1: 2048000 in PBS buffer (0.1 M, pH 7.4) were added to each wells, followed by an incubation of 1 h at 37 °C. After washing, rabbit anti-chicken IgYs peroxidase-conjugated (whole molecule) diluted (1:2000) in PBS-Tween-20 0.1% were added. Wells were washed again and 50 µl of substrate buffer (phosphate citrate buffer 0.01 M and pH 5, 10 mg OPD, 4 mM H_2O_2) were added and the plates were incubated at room temperature for 10–15 min. Sulfuric acid (2 N, 50 µl/well) was added to stop the reaction. ELISA plate reader (Bio-TEK reader, USA) was used to record absorbance at 490 nm. IgYs extracted from eggs of pre-immunized chickens were considered as controls. Wells free of antigen were considered as blanks.

2.4.5. Antivenom neutralization assays (median effective dose = ED50)

The median effective dose value (ED50) of the IgY anti-Aah venom from egg yolk was measured following to the World Health Organization recommendations [30]. Five groups of five NMRI mice (18–20 g) were challenged with a mixture of varying volumes of IgYs antivenom (12.19 mg/ml) (150, 200, 250, 300 and 500 µl) containing two LD50 of *Aah* venom (2 × 0.85 mg/kg, 34 µg venom/20 g mouse) [31]. The antivenom/venom mixture pre-incubated for 30 min at 37 °C was intraperitoneally injected into mice for each dose. Negative control mice were injected with 2 LD50 of venom alone. ED50 was determined by probit analysis based on the recording of the number of death during 48 h of observation [32]. The neutralizing potency reflects the ratio of ml of antivenom/mg of venom or mg of antivenom/mg of venom.

2.4.6. Pepsin digestion of IgYs

Fab' fragments (45 Da) were obtained by the hydrolysis of the IgYs with pepsin prepared in sodium acetate buffer (0.05 M, pH 4.2, over night at 37 °C). The pH was adjusted to 8.0 to stop the digestion. Antibody titers of the obtained fraction were determined by ELISA as previously described [33].

2.4.7. Evaluation of venom neutralization efficacy of Fab' fragments on the pathophysiological effects induced by the venom

To evaluate the efficiency of the obtained IgYs on the pathophysiological and the inflammatory response effects induced by *Aah* venom, eight male mice NMRI (20 ± 2 g) received Fab' fragments (40 mg/kg; intraperitoneally), 30 min after the injection of the venom