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Enhanced immune sera and vaccine: Safe approach to treat scorpion envenoming

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ABSTRACT

Irradiation of *Androctonus australis hector* venom using a dose of 2 kGy has successfully abolished toxicity without reducing its antigenic or immunogenic properties. Toxicity of irradiated antigen was abolished until 20 times of LD_{50} of native venom. Analysis of physiopathological effects induced by native and irradiated venoms was assessed by the analysis of tissue damage, immunohistochemistry and metabolical analysis in the organs (heart, lungs and liver). Immunological response of Aah venom using native or irradiated venom showed high titers of IgG1 in the plasma of immunized animals with native venom suggesting that Th2 cells were predominantly involved in the immune response. In the other hand, irradiated venom induced high titers of IgG2, indicating a predominantly Th1 type response. A protective effect of immunized mice with irradiated venom was evaluated. Immunized mice were protected from the toxic effects of native venom doses at one, three and six months after immunization. Mice were protected against a challenge of 4 LD_{50} doses of native venom, one month after immunization. This protective effect was improved and effective at 3 and 6 months, all immunized mice were protected respectively against 6 and 10 LD_{50} of native venom. At the one-month time point, the protective effect of mice was associated with high levels of antibodies in the plasma of immunized mice. However, despite the persistence of higher protection levels, the antibody titers decreased in a time-dependent manner. These results suggest that additional factors other than circulating antibodies provided the long-term protective activity produced by immunization with irradiated venom.

1. Introduction

Annual accidental pathologies need a preventive therapy. Fatal accidents caused by scorpion envenoming are considered as a public health problem in subtropical and tropical countries, due to their lethal effects in human population, especially in children. In Maghreb region, and especially in Algeria, immunotherapy is the most commonly used approach to treat against lethality. This therapy is effective only when it is administered in the first hours after envenoming [1], its therapeutic value is limited because most of the patients arrive at the hospital too late after the accident. One approach to improve this therapy is to trap the toxic antigens as soon as they enter in the body, immediately after the scorpion sting.

Gamma irradiation has been described as a promising tool to detoxify scorpion venom or its toxic components [2–11]. Ionizing radiation can interact with biomolecules in two ways: directly, when the radiation interact on the substance, or indirectly when free radicals are generated by water radiolysis and interact with the target molecule [12]. This indirect effect of radiation is responsible for 99.9% of the protein structure alterations. Free radicals can modify the biological activities of peptides and proteins by reacting with some sites or groups of the molecules [13]. Considering these facts, irradiation of proteins in aqueous solution has been used, aiming to cause chemical and physicochemical changes in the secondary and tertiary structures of proteins but without adding any chemical group to the molecule. These changes are related with lower toxicity while keeping many of its immunological properties intact, a rather interesting combination for antisera and vaccine production.

Most lethal accidents caused by scorpions in Algeria result from envenomation by the most dangerous species: *Androctonus australis hector* (Aah). Aah venom contains low molecular-weight neurotoxins (~7 kDa). These basic toxins are cross-linked by four disulfide bridges and interact specifically with voltage-dependent sodium channels. They induce a complex pattern of clinical symptoms affecting the nervous, cardiovascular and respiratory systems, and produce a severe inflammatory response that can lead to the death [1,14–17].

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This study is undertaken to identify a detoxification strategy using different dose rate of 2 kGy-gamma radiation on Aah venom in order to find out optimal conditions for obtaining attenuated venom antigens while preserving or improving their immunogenic properties. We also evaluated the medium- and long-term immunoprotective properties of detoxified venom against the lethal effects of native Aah venom. Thus, this approach could be used to produce toxoids and vaccines.

2. Materials and methods

2.1. Venom and animals

Lyophilized *Androctonus australis hector* crude venom was obtained from the Pasteur Institute of Algeria. NMRI female mice (20 ± 2 g) and female rabbits (2–2.5 kg) were obtained from the animal breeding centre of Pasteur Institute of Algeria. The animals were housed in controlled temperature and humidity rooms, and received food and water *ad libitum* before being used for study. The experimental protocol was carried out according to the European community rules of the ethical committee for animals' welfare.

2.2. Venom detoxification

Venom was dissolved in saline solution (0.9% NaCl) at a concentration of 1 mg/ml. Samples were irradiated with a total dose of 2 kGy using various dose rates of 195 Gy/h, 765 Gy/h or 1788 Gy/h by a Cobalt-60 source in the presence of O_2 at room temperature. Irradiation was carried out at the Nuclear Research Centre of Algiers (CRNA) in the Department of Irradiation Techniques. After irradiation, samples were centrifuged at $3000 \times g$ for 10 min at 4 °C, and supernatants were stored in aliquots frozen at −20 °C.

2.3. Evaluation of residual toxicity of irradiated venom

Venom lethality was determined by injecting (i.p.) venom into various groups of mice and evaluating mortality after 48 h. A control group received saline solution (0.9% NaCl). A second group received a lethal dose (2 LD_{50}) of native venom. Three groups received a dose of either 10, 15 or 20 LD_{50} of irradiated venom with 2 kGy at different dose rates (195 Gy/h, 765 Gy/h or 1788 Gy/h). The LD_{50} of native Aah venom used throughout this study was 17 μg/20 g body mass given by i.p. injection [18].

2.4. Immunogenicity of irradiated venoms

2.4.1. Rabbit immunization

Rabbits were immunized with irradiated or native venoms according to a schedule (Table 1). Before subcutaneous (s.c.) inoculation, an equal volume of adjuvant was added to the antigens. Freund's complete adjuvant (Sigma, USA) was used in the first inoculation and the incomplete adjuvant (Sigma, USA) was used in the second and third inoculations. The remaining inoculations were performed without adjuvant.

Six weeks after the first inoculation, the rabbits were bled and the serum antibody titers were evaluated by enzyme-linked immunosorbent assay (ELISA).

2.4.2. Evaluation of titer by ELISA

An ELISA was used to assess the efficiency of the produced antibodies raised against various prepared immunogens. Aah venom (5 μg/ml) in 0.1 M carbonate buffer pH 9.5 (100 μl) was adsorbed on 96-well plates overnight at 4 °C. After blocking with 100 μl of 0.1 M PBS, pH 7.4 containing 5% skim milk for 1 h at 37 °C and washing (five times with PBS-Tween 20), antisera raised against the native or irradiated venoms were successively diluted in PBS-5% skim milk of 1/500 to 1/2,048,000, added to the wells and incubated for 1 h at 37 °C. Following another washing step, the plates were incubated with anti-rabbit IgG conjugated with peroxidase (Sigma) (diluted at 1/500) for 1 h at 37 °C. The wells were then washed and 100 μl of OPD solution (10 mg/ml in phosphate buffer, pH 7.4) in presence of hydrogen peroxide (0.03%) was added. The reaction was stopped by adding 50 μl of 2 N H_2S0_4. Absorbance values were determined at 490 nm.

2.5. Cross-reactivity of rabbit anti-irradiated venom immune sera with Buthus occitanus tunetanus (Bot) venom

An ELISA was used to test the cross-reactivity between antibodies raised against Aah-irradiated venom at the rate of 765 Gy/h and native Bot venom. In this case, wells were coated with native Bot venom at a 5 μg/ml in 0.1 M carbonate buffer pH 9.5 (100 μl) overnight at 4 °C. The used procedure for this ELISA is the same as cited above.

2.6. Histological–immunohistochemical analysis and enzyme assays

Three groups of mice were used for this study. Animals were injected with 500 μl (i.p.) of the test solutions. The first group served as a control, received physiological saline solution (0.9% NaCl). The second group was injected with sublethal dose of Aah native venom (10 μg/20 g body weight). The third group was injected with irradiated venom at 2 kGy at a rate of 765 Gy/h (85 μg/20 g body weight).

Animals were anesthetized with ethyl ether and sacrificed 24 h after envenomation. Organs (heart, lungs and liver) were extracted and immersed in formalin fixative solution (4%) for three days at room temperature. They were then embedded in paraffin, sectioned (7 μm) and stained with hematoxylin and eosin for microscopic examination (Orthomat, Zeiss).

For immunohistochemical staining, sections (7 μm thickness) were processed as described by D'suze et al. [19] with slight modifications. Sections were deparaffinized, rehydrated and endogenous peroxidase activity was blocked in all tissues with 3% H_2O_2 in methanol for 30 min. All tissue sections were then treated with 0.5% SDS for 10 min at room temperature [20]. Nonspecific binding sites were blocked using 5% skim milk and 0.1% Triton X100 in PBS for 1 h at room temperature. Sections were then washed with PBS and incubated overnight at 4 °C with a primary antibody: 1/100 dilution of a horse $F(ab)'_2$ anti-Aah venom antibody produced in our laboratory. Afterwards, sections were washed three times for 5 min with PBS followed by incubation with a secondary antibody conjugate (anti-Horse IgG labeled with peroxidase) at a 1/100 dilution in PBS containing 2.5% skim milk and 0.1% Triton X100 for 1 h at room temperature. After washing three times for 5 min each with PBS, sections were incubated with a substrate solution containing 8% $NiCl_2$, 0.05% 3,3′ diaminobenzidine (DAB) and 0.02% H_2O_2 in 50 mM Tris–HCl buffer, pH 7.6. Finally, sections were washed with 50 mM Tris–HCl buffer, pH 7.6, counterstained with hematoxylin and mounted on slides and analyzed by microscopic examination (Motic Digital Microscope PAL System). This method results in a dark brown color of immunostained antigens. No positive reactions were observed in control tissue sections.

Mouse blood was collected 24 h after envenomation. The serum was prepared by centrifuging the whole blood at $10{,}000 \times g$ for 5 min and was stored at 4 °C. Soon after blood collection, heart and lungs were removed and homogenized in nine volumes of physiological saline solution. After that the homogenates were centrifuged at $10{,}000 \times g$ for 5 min, the supernatants were removed and used as tissue extracts. Creatine kinase (CPK), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities were assayed in the