STRUCTURE ALTERATION AND IMMUNOLOGICAL PROPERTIES OF ^{60}Co GAMMA RAYS IRRADIATED BOTHROPSTOXIN-I

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ABSTRACT

About 20000 ophidic accidents are registered every year in Brazil. Serum therapy with equine antisera is the only efficient treatment. The venoms employed for immunization are fairly toxic and some venoms present low immunogenicity. Thus, the obtention of modified antigens with lower toxicity and preserved or improved immunogenicity would be useful. These toxins, when submitted to gamma radiation, in aqueous solution, present structural modifications. This occurs due to reactions with the radiolysis products of water. Some scavenger substances, such as $NaNO_3$ and t-butanol, remove selectively the water radiolysis products. Ionizing radiation has proven to be a powerful tool to attenuate snake venoms toxicity without affecting and even increasing their immunogenic properties. However, the immune mechanisms involved in recognition, processing and presentation of irradiated antigens are yet unclear. In the present work, we investigated the immunological behavior of bothropstoxin-I (Bthx-1), before and after irradiation, in the presence of selective scavengers. Isogenic mice were immunized with either the native or the irradiated toxin, either with or without scavengers. After three immunizations, serum samples were collected and the antibody titers and isotypes were determined by Enzyme Linked Immuno Sorbent Assay. The antigenic characterization of native and irradiated bothropstoxin-I was performed by Western blot. The detection of expression of murine cytokines (IFN-γ e IL-10) was analyzed by RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction). According to our data, irradiation process has promoted structural modifications in the toxin, characterized by higher molecular weight forms of the protein (aggregates and oligomers). Our data also indicate that irradiated toxins, alone or in the presence of $NaNO_3$, an aqueous electron scavenger, were immunogenic and the antibodies elicited by them were able to recognize the native toxin. On the other hand, when the toxin was irradiated in presence of t-butanol, a discrete reduction in antibodies levels was observed, suggesting a role of hydroxil radicals in the modulation of immune response. Irradiated bothropstoxin-1 elicited antibodies responsive to both toxins forms, as demonstrated by Western blot. The cytokines profiles indicated that IFN-γ mRNA presence appeared to be higher for mices immunized with irradiated toxin, while IL-10 mRNA presence was predominant with the antigen in its native form. These results indicate that irradiation of proteins leads to significant structural modifications, and also to a modulation of the immunological response.

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1. INTRODUCTION

Ionizing radiation consists of electromagnetic waves resulting from nuclear transitions. It can interact with biomolecules in two ways: directly, when the radiation hits the molecule, or indirectly when free radicals are generated and react with the target molecule. With proteins, radiation promotes changes in their enzymatic, pharmacological and immunological properties; the two latter being more radioresistant [1,2,3].

The obtention of modified antigens with lower toxicity and preserved or improved immunogenicity would be useful. Snake venoms or its toxins, when submitted to gamma radiation, in aqueous solution, present structural modifications. This occurs due to reactions with the radiolysis products of water. Some scavenger substances, such as $NaNO_3$ and t-butanol, remove selectively the water radiolysis products [4,5].

However, the immune mechanisms involved in recognition, processing and presentation of irradiated antigens are yet unclear. In the present work, we investigated the immunological behavior of bothropstoxin-I (Bthx-1), before and after irradiation, in the presence of selective scavengers.

2. MATERIALS AND METHODS

2.1 Reagents

All reagents were commercially obtained and were of analytical grade. Bothropstoxin-1 was purified from *Bothrops jararacussu* crude venom (Butantan Institute).

2.2 Animals

B10.PL isogenic mice were obtained from the animal housing facility of IPEN/CNEN/SP and maintained in sterilized isolators and absorbent media, with food and water *ad libitum*. The manipulation of these animals before or during the experiments was according to the "Principles of Laboratory Animal Care" (NIH publ. N^o 86-23, revised in 1985) and to the "Principles of Ethics in Animal Experimentation" (COBEA – Colégio Brasileiro de Experimentação Animal).

2.3 Protein irradiation

Bothropstoxin-I was dissolved in 0,15 M NaCl to a final concentration of 2mg/mL. This solution was irradiated with a 2 kGy dose using gamma rays derived from a ^{60}Co source (Gamma Cell, Atomic Agency of Canada Ltd) at room temperature and in the presence of atmospheric O_2, with a 2,88 kGy/h dose rate.

2.4 SDS-PAGE

Purified bothropstoxin-1, native or irradiated one, were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE) [6] under reducing and not-reducing conditions.

2.5 Production of antibodies

Specific anti-native or anti-irradiated bothropstoxin-1 antibodies were obtained by immunizing B10.PL mice, with the protein in its native or irradiated form, following a classical immunization protocol [7]. Blood samples were collected and after centrifugation, the plasma was separated and frozen.

2.6 Enzyme linked immunosorbent assay (ELISA)

96 wells microplates were coated with native bothropstoxin-1 (1,0µg/well/100µl) overnight. The plates were then blocked with 5% skim milk in phosphate buffered saline (PBS). The plasma samples were then incubated for one hour after a 1/20000 or 1/40000 dilution in PBS. Peroxydase labeled antibodies specific against mouse IgG1, IgG2a or IgG2b were then allow to react individually with the bound antibodies. Finally, the reaction was developed adding a chromogenic solution containing 0.5 mg/ml orto phenyl diamine in 50 mM citrate buffer pH 5 in the presence of 1µl/ml hydrogen peroxide. After 20 minutes incubation, the reaction was interrupted by the addition of 50 µl 2 M citric acid and the plates were analyzed on a microplate reader at 450 nm.

2.7 Western Blot

SDS-PAGE samples were transferred electrophoretically to a nitrocellulose membrane, followed by blockade of membrane free binding sites with 5,0% milk in phosphate buffer (PBS), pH 7,2, over nigth. Specific anti-BTHX-1, native or irradiated, mouse IgG was added over nigth, at 4°C with constant shaking. After washing, peroxidase anti-mouse IgG conjugate was applied. The reaction was developed by the addition of a chromogenic solution (3,3´-diamino-benzidine; H_2O_2; pH 7,2) and then stopped by repeated washing with distilled water.

2.8 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Sample collection, RNA extraction and RT-PCR: Reverse transcription, amplification and detection of products

Spleens cells of immunized and control mice (BALB/c and B10.PL) were aseptically removed and immersed immediately in ten volumes of TriZOL™ (Invitrogen), and stored at -80ºC until the moment of total RNA extraction. The total RNA extraction from samples was performed according to the manufacturer recommendations. mRNA chains present in RNA samples supplied convenient templates to cDNA synthesis, in reactions catalyzed by M-MLV Reverse Transcriptase (Invitrogen) in presence of Oligo $d(T)_{12-18}$ primers (Invitrogen). The libraries obtained by this method were tested against presence or absence of IL-10 expression using specific primers in PCR reactions (Sense: CTCAGTTCCCATTCTATTTATTCAC; Antisense: GGATCTCCCTGGTTTCTCTTC; T_a= 62,1ºC; 336 bp) and IFN-γ expression (Sense: GTCCAGCGCCAAGCATTCAA; Antisense: GTCCCCCACCCCCAGATACA; T_a= 71,6ºC; 391 bp). For control of the reaction we used a 349 bp fragment corresponding to β-actin expression (Sense: TGGAATCCTGTGGCATCCATGAAAC; Antisense:

TAAAACGCAGCACAGTAACAGTCCG, T_a=55,6ºC). Amplified sequences were resolved in silver-stained polyacrilamide gels (6% / 60V).

3. RESULTS AND DISCUSSION

Figure 1 showed that irradiated toxins, alone or in the presence of $NaNO_3$ and t-butanol, an aqueous electron scavenger, was immunogenic and the antibodies elicited by them were able to recognize the native toxin. Similar fact was observed by CASARE (2003) when worked with the crotamina (subunit of *Crotalus durissus terrificus* venon).

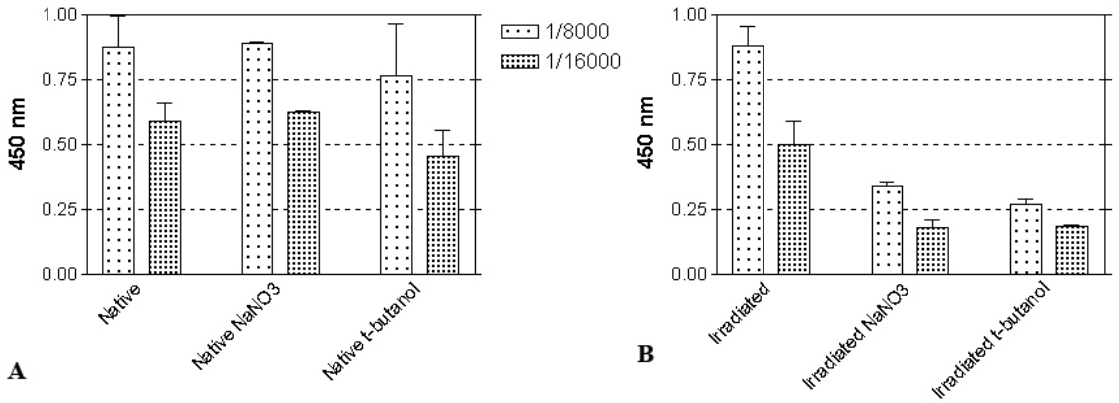

Figure 1 - Enzyme Linked Immunosorbent Assay of the antibodies rose against native (A) and irradiated (B) BTHX-1 samples, with or without *scavengers* substances.

In Western blot (Figure 2) was demonstrated that irradiated bothropstoxin-1 elicited antibodies responsive to both toxins forms. CARD and cols. (1998) observed the same fact when worked with crotoxin (toxin of *Crotalus durissus terrificus* venon).

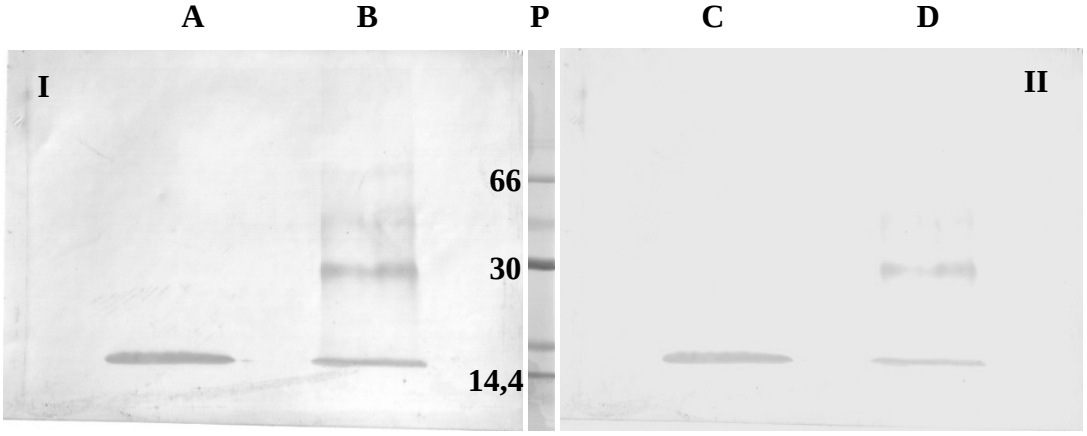

Figure 2 - Western blot assay, using BTHX-1 Native (A,C) e Irradiated (B,D), in reduction condictions. Mouse anti-BTHX-1 Irradiated (I) or anti-BTHX-1 Native (II) was employed as primary antibody.

Figure 3 showed that cytokines profiles indicated that IFN-γ and IL10 mRNA presence appeared as much in the animals immunized with the native toxin as well as in those immunized with the irradiated toxin. These results were obtained with the cells of the immunized animals. Our results indicate that irradiation of proteins leads to significant structural modifications but the irradiated toxin, alone or in the presence of scavengers substances, were able to stimulate the immune system and the resulting antibodies were able to react with the native form of them.

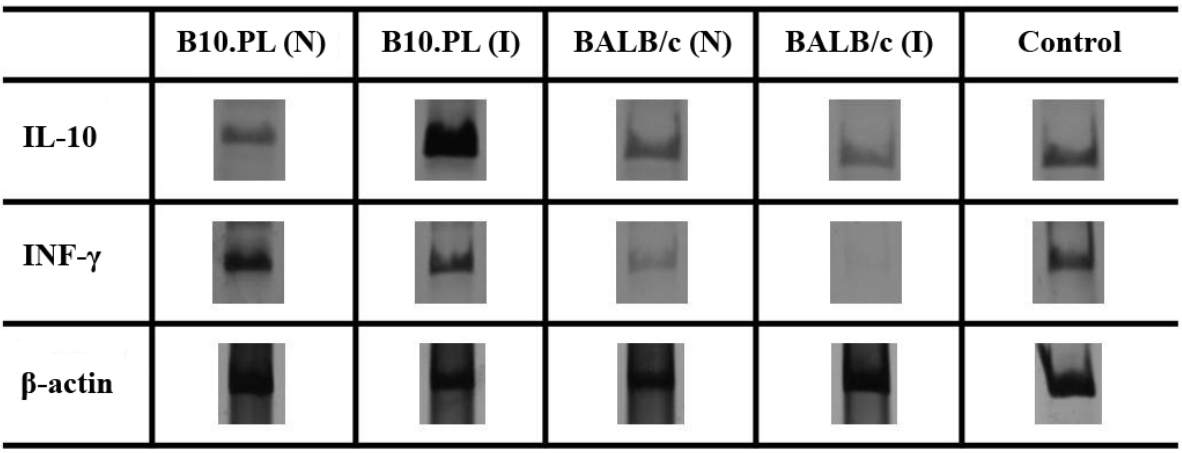

Figure 3 – Digital imaging records of IL10, INF-γ and β-actin RT-PCR from spleen cells, from the B10.PL and BALB/c mice, immunized with native protein (N) and irradiated protein (I). The control was a non immunized animal.

4. CONCLUSIONS

BTHX-1 irradiated, alone or in the presence of scavengers substances, an aqueous electron scavenger, was immunogenic and the antibodies elicited by them were able to recognize the native toxin.
The Western Blotting assay has indicated that the antibodies formed against the irradiated BTHX-1 present highly response for both forms of toxin.
Irradiation of proteins promotes both significant structural modifications and an immunological response modulation.
Altough RT-PCR data showed differences between IFN-γ expression in studied groups, further evaluation of expression of another cytokines and experiments using *in vitro* antigen stimulated spleen cells may resolve remaining questions about immune response polarization.

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