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# Immune Response Against Bothropstoxin-I Irradiated With $^{60}$Co Gamma Rays

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## ABSTRACT

Ionizing radiation has been successfully employed to modify the immunological properties of biomolecules. Very promising results were obtained when crude animal venoms, as well as isolated toxins, were treated with gamma rays, yielding toxoids with good immunogenicity. Ionizing radiation has proven to be a powerful tool to attenuate snake venoms toxicity without affecting and even increasing their immunogenic properties. However, little is known about the modifications that irradiated molecules undergo and even less about the immunological response that such antigens elicit. In the present work, we investigated the immunological behavior of bothropstoxin-I, a K49 phospholipase, before and after irradiation. Structural modifications of the toxin were investigated by SDS-PAGE and mass spectrometry. Aiming to compare the toxicity between native and irradiated forms of the toxin, an *in vitro* cytotoxicity assay, using CHO cells, was performed. Isogenic mice were immunized with either the native or the irradiated toxin. The circulating antibodies were isotyped and titrated by ELISA. According to our data, irradiation promoted structural modifications in the toxin, characterized by higher molecular weight forms of the protein (aggregates and oligomers). When analyzed by mass spectrometry, the irradiated bothropstoxin appeared in several oxidized forms. The citotoxicity assay showed that the modified toxin was 5 folds less toxic than its native counterpart. Irradiated toxins were immunogenic and the antibodies elicited by them were able to recognize the native toxin in ELISA. These results indicate that irradiation of toxic proteins can promote significant modifications in their structures, but still retain many of the original immunological properties.

## 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]. Radiation has been successfully employed to modify biomolecules, reducing or abolishing their biological activity without affecting their immunogenic properties [4]. This methodology could be used to produce toxoids and vaccines. The production of modified antigens with lower toxicity and preserved or improved immunogenicity would be useful. However, in order to develop such

methodology, a good comprehension on the immunological behavior of irradiated antigens would be necessary. In the present work, we used bothropstoxin-1, a K49 phospholipase, as a model for further characterize the immune response against irradiated proteins.
*Bothrops* venoms are complex mixtures of components with a wide range of biological activities. Among these substances, myotoxins have been investigated by several groups. Bothropstoxin-1 (Bthtx-1) is a phospholipase A2-like basic myotoxin from *Bothrops jararacussu* [5]. Our goal was to further characterize bothropstoxin-1 from the immunological point of view.

## 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-1 was dissolved in 0,15 M NaCl to a final concentration of 2mg/mL. This solution was irradiated with a 2000 Gy 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 5170Gy/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 Mass Spectrometry (ESI)

Bothropstoxin-1 samples (2mg/mL), native or irradiated one, was submitted to analyze for mass spectrometry. It was injected 20 μL of each sample (2 mg/mL). It was employed a Q-Tof Ultima ES-MS Analysis equipment.

### 2.6 Cytotoxicity assay

This assay determines the survival of viable cells in a monolayer cell culture upon exposure to the toxic agent (bothropstoxin-1). Bothropstoxin-1 (1mg/mL), native or irradiated one, was dispensed in 96-well flat-bottomed microplates, in crescent dilutions, in a volume of 50 μL/well. Subsequently the CHO cells (3000 cells/well) were added in the microplates containing the toxins. The microplates were incubated at 37°C in an atmosphere of 5% $CO_2$ for 72 hr. After incubation, a mixture of 0.2% MTS and 0.9% PMS in PBS, was added in the

wells and incubated at 37°C in an atmosphere of 5% $CO_2$ for 2 hr. The incorporation of the MTS was measured in a microplates reader at 490 nm.

### 2.7 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.

### 2.8 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.

## 3. RESULTS AND DISCUSSION

### 3.1 SDS-PAGE

Proteins SDS – PAGE profiles show that γ-irradiation causes breakdown of polypeptide chains and formation of degraded high molecular weight molecules (Figure 1). The production of these high molecular weight components suggested that radiation induced peptide bond cleavage and produced fragments of proteins which suffer posterior aggregation [5]. This aggregation occurs through inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, and the formation of disulfide bonds [8,9].
Bothropstoxin-1 in presence of the reducing agent did not present dissociation of the subunits, suggesting that the irradiation induced the formation of resistant covalent bonds.

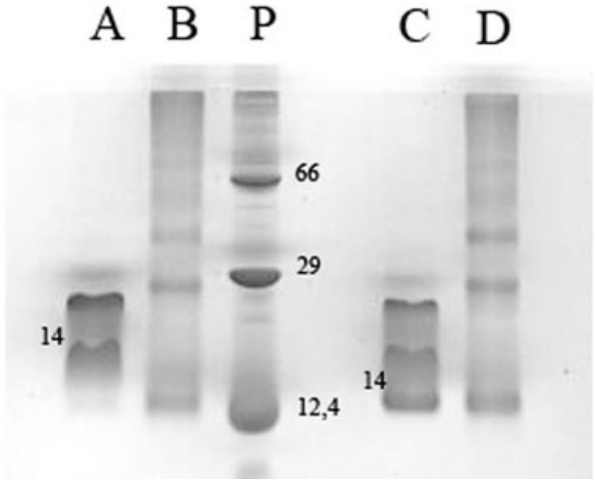

Figure 1 – SDS-PAGE profile of bothropstoxin-1. (A) Bothropstoxin-1 native and not-reduced; (B) Bothropstoxin-1 irradiated and not-reduced; (C) Bothropstoxin-1 native and reduced; (D) Bothropstoxin-1 irradiated and reduced. (P) Molecular weight marker.

### 3.3 Mass Spectrometry (ESI)

The mass spectrometry of irradiated bothropstoxin-1 (Figure 3) shows that $^{60}$Co γ-rays induced oxidative changes in toxin. In the case of crotoxin it was evaluated the uptake of native or irradiated toxin by non-stimulated peritoneal macrophages, analyzing the biochemical and immunological characteristics of irradiated crotoxin [10]. The author observed that the irradiated crotoxin, when compared with the native form, presented higher uptake by macrophages. This fact can be explained due the oxidation property of radiation on the molecules

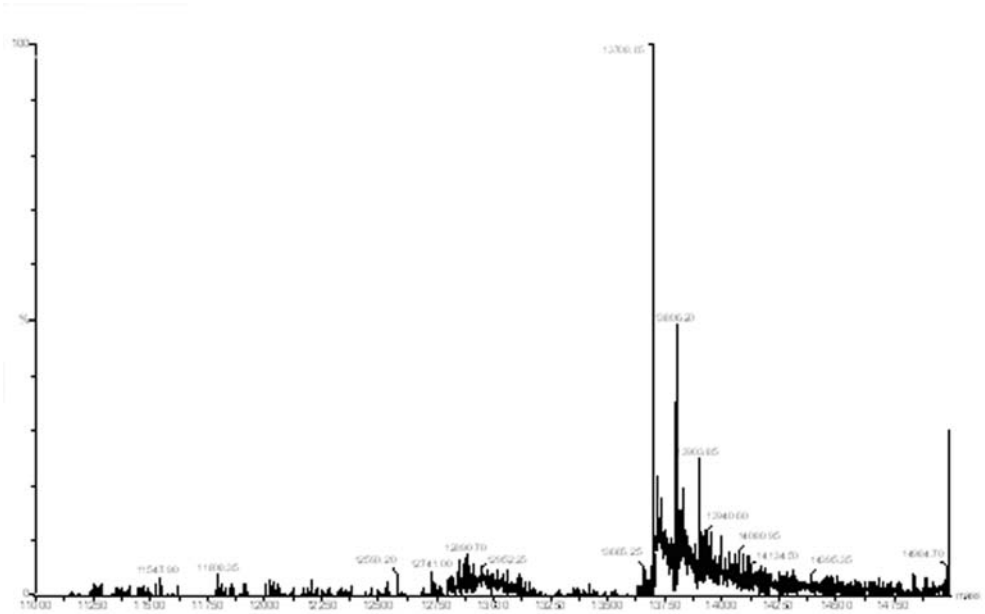

**Figure 2 – Mass spectrometry of bothropstoxin-1 native in Q-Tof Ultima ES-MS Analysis.**

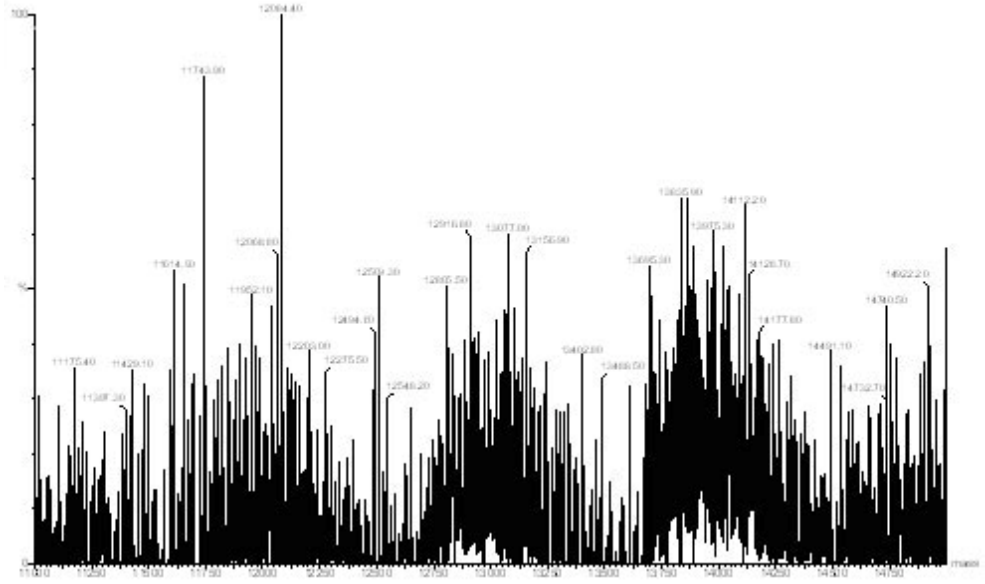

**Figure 3 - Mass spectrometry of bothropstoxin-1 irradiated in Q-Tof Ultima ES-MS Analysis.**

### 3.4 Cytotoxicity assay

The results of citotoxicity assay showed that the modified toxin was 5 folds less toxic than its native counterpart (Figure 4). It means that it was necessary a very higher amount of Bthx-1 to reach the capable concentration to eliminate 50% of the cellular population exposed to the toxin. Cell citotoxicity assays developed with medically important South American snake venoms suggest the existence of a correlation between EC50% and biological activities [11] of these venoms.

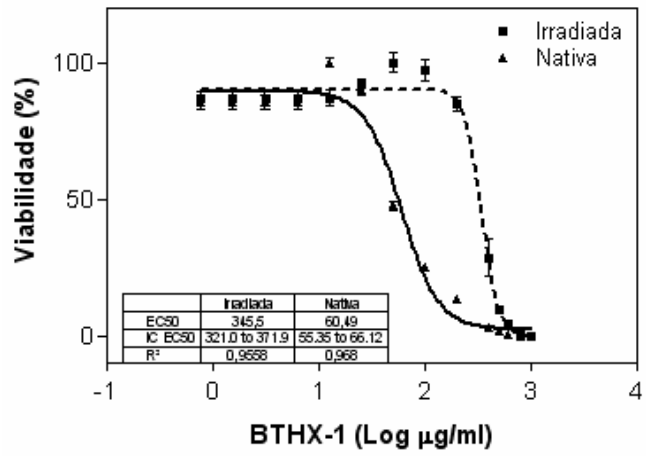


**Figure 4 – Cytotoxicity assay of native and irradiated bothropstoxin-1, in CHO cells. EC50% (toxic concentration 50%).**

### 3.5 Enzyme linked immunosorbent assay (ELISA)

Our results indicate that both forms of proteins induced detectable amounts of antibodies with both dilutions tested (Figure 5). We could also observe that animals' plasma immunized with

native bothropstoxin-1 had higher IgG1 titers, indicating the predominance of a Th2 type response. This behavior was observed in macrophage depleted animals [11]. The authors observed that after depletion, the animals presented an increased IgG1 level, which is under control of Th2 cells, a cell type involved in the humoral immune response, modulating the production of antibodies by B lymphocytes [12].
Also, our data indicate that the irradiated protein induced higher titers of IgG2a and IgG2b (Figure 5), suggesting that Th1 cells were predominantly involved in the immune response. This population is involved in the up regulation of cellular response, specifically macrophage activation [13].
The differential activation of T cells (Th1 or Th2) could be explained by the fact that these subpopulations respond selectively to antigens presented by different antigen-presenting cells (APC) : Th2 cells proliferate intensely when stimulated by antigens presented by B cells, while Th1 cells respond to antigens presented by macrophages [14]. It has been shown that the uptake of proteins by macrophages is enhanced when they are in the irradiated form, increasing their association with macrophages, through scavenger receptors, involved with oxidized biomolecules processing [3]. Thus, irradiated proteins would be preferentially presented by macrophages, explaining the switch towards Th1 response in the animals immunized with the irradiated sample.

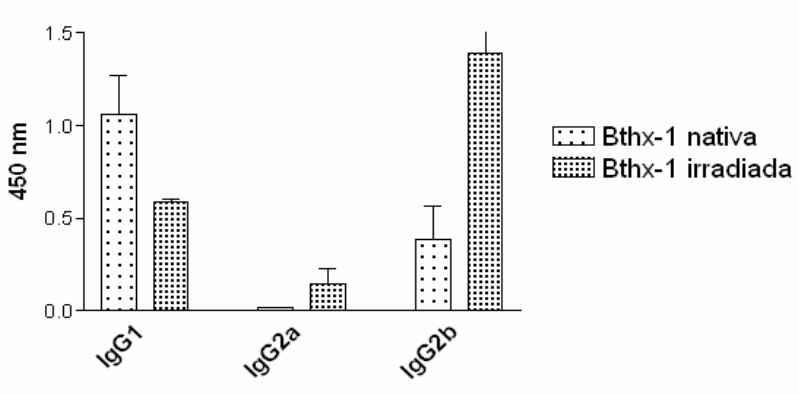


**Figure 5 - Enzyme Linked Immunosorbent assay isotyping (IgG1, IgG2a and IgG2b) of the antibodies rose against native and irradiated bothropstoxin-1 (BTHX-1) samples.**

## 4. CONCLUSIONS

The $^{60}$Co γ-rays, at 2000 Gy dose, cause modifications in the molecules of bothropstoxin-1.
The mass spectrometry of irradiated bothropstoxin-1 shows that the γ-radiation promotes the oxidation of toxin.
The preferential activation of Th1 and Th2 cells depends on, amongst other things, whether the antigen is in its native or irradiated form, since Th1 response is observed, preferentially, when irradiated antigens are employed.
The irradiated proteins were able to stimulate the immune system and the resulting antibodies were able to react with the native form of them.

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