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Immunological properties of ^{60}Co gamma-rays irradiated bothropstoxin-I

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We investigated the immunological behavior of BTHX-1, before and after irradiation. SDS-PAGE showed that BTHX-1 irradiated in the presence of $NaNO_3$, had its structure preserved. Animals' plasma immunized with native BTHX-1 had high IgG1 titers. The irradiated protein induced high titers of IgG2b. When the toxin was irradiated with t-butanol, there was a slight decrease in the production of IgG2b. Real-time PCR showed that both the IL-2 as for IL4 was more expression from the cells of the animals immunized with BTHX-1 irradiated. These results indicate that irradiation of proteins leads to significant structural modifications.

Introduction

Ionizing radiation 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.[3,8]

Snake venoms and also isolated toxins present structural modifications when submitted to gamma-radiation in aqueous solution. This occurs due to reactions with the products of water radiolysis. Some scavenger substances, such as $NaNO_3$ and t-butanol, remove the water radiolysis products selectively.[6,11]

Very promising results have been described when crude animal venoms, as well as isolated toxins, were treated with gamma-rays, yielding toxoids with good immunogenicity.[4] Ionizing radiation has proven to be a powerful tool to attenuate snake venom toxicity without affecting and even increasing their immunogenic properties.

The immune system is composed of highly specialized cells and molecules with two types of fundamental immune response: the innate (or natural) and adaptive. The innate response occurs without requiring a prior exposure to the infectious agent, while adaptive response makes use of the production of antibodies against a particular agent and is acquired during the life of an individual as adaptive reaction to the presence of specific pathogens.[7]

The adaptative response involves the antigen-specific T and B cells proliferation, which occurs through the interaction between receptors, on the surface of these cells, and the antigens. There are two major types of effector T cells, the helper T lymphocytes (helper) (Th) and cytotoxic T lymphocytes (Tc), which carry on their surface molecules CD4 or CD8, respectively. The CD4+Th cells have two subtypes, Th1 and Th2 cells, which arise from a precursor Th0 and are differentiated according to the produced cytokines, since they are morphologically indistinguishable.[20]

The Th1 cells produce interleukin 2, which induces the proliferation of T cells. The Th2 cells, in turn, produce the interleukins 4, to promote the production of antibodies. The interleukin 4 promotes a positive feedback to promote the induction of Th2 response and inhibit the differentiation of Th1 cells.[9]

T cells help B cells in the production of antibodies. These processes are mediated by cytokines that stimulate the growth of B cells leading thus its division and maturation for the production of antibodies.[17]

About 30,000 ophidic accidents are registered every year in Brazil and serum therapy with equine antisera is the only efficient treatment.[19] But, the venoms employed for immunizations are fairly toxic and some venoms present low immunogenicity. Thus, to get modified antigens with lower toxicity and preserved or improved immunogenicity would be helpful.

Numerous researches have shown the power of radiation to modify proteins, improving its immunological potential. In this way, studies involving a better characterization of immune response generated against an irradiated protein would be very important, especially in those cases where the substance is weakly immunogenic. Therefore, in the present work we investigated the immunological behavior of bothropstoxin-I, before and after irradiation, in the presence of selective scavenger substances.

Experimental

Reagents

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

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Animals

B10.PL and BALB/c 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. No. 86–23, revised in 1985) and to the "Principles of Ethics in Animal Experimentation" (COBEA – Colégio Brasileiro de Experimentação Animal).

"Scavengers" dosage

It is considered that the concentration among the products formed during radiolysis of water and "scavengers" is equal to 1, that is, for every molecule of free radical formed, there will be a molecule acting as a "scavenger".

2 kGy irradiation: 1 mol – $6.02 \cdot 10^{23}$ molecules; X mol – $3.56 \cdot 10^{17}$ e^-_{aq}. Therefore, the concentration of "scavenger" for the electron aqueous is 0.60 μM.

X mol – $4.22 \cdot 10^{17}$ OH•. Therefore, the concentration of "scavenger" to hydroxyl radical is 0.70 μM.

Protein irradiation

Bothropstoxin-I was dissolved in 0.15M NaCl to a final concentration of 2 mg/mL. This solution was irradiated with 2 kGy dose using gamma-rays 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.

SDS-PAGE

Native or irradiated purified bothropstoxin-1, in the presence of selective scavengers, were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and not-reducing conditions.[14]

Production of antibodies

Specific anti-native or anti-irradiated bothropstoxin-1 antibodies were obtained by immunizing B10.PL and BALB/c mice, with the protein in its native or irradiated form, either with or without scavengers, following an immunization protocol of three doses, one after 15 days and the other with an interval of 7 days.[12] Blood samples were collected and after centrifugation, the plasma was separated and frozen.

Enzyme linked immunosorbent assay (ELISA)

96 well microplates were coated with native bothropstoxin-1 (1.0 μg/well/100 μL) 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 1/4000 or 1/8000 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 by adding a chromogenic solution with 0.5 mg/mL orto phenyl diamine in 50 mM citrate buffer pH 5 in the presence of 1 μL/mL hydrogen peroxide. After 20 minutes incubation, the reaction was interrupted by the addition of 50 μL 2M citric acid and the plates were analyzed on a microplate reader at 450 nm.

Real time – PCR

Sample collection, RNA extraction: It was also performed a cell proliferation assay with splenocytes from BALB/c mice immunized with either native or irradiated protein, cultured in the presence of the both antigens. After 48 hours of culture, spleens cells were removed and immersed immediately in ten volumes of TriZOL™ (Invitrogen), and stored at –80 °C until the moment of total RNA extraction. The total RNA extraction from samples was performed according to the manufacturer's recommendations and their concentrations determined by the absorbance reading to 260, 280 and 320 nm in spectrophotometer Ultrospec300® (Pharmacia Biotech®). mRNA chains present in RNA samples supplied convenient templates to cDNA synthesis, in reactions catalyzed by M-MLV RT in presence of Oligo $d(T)_{12–18}$ primers (Invitrogen). cDNA samples were kept at –20 °C freezer until the performing of tests on real-time PCR. All primers were designed using Primer3 software (http://frodo.wi.mit.edu/) and the obtained sequences in the database Nucleotide NCBI Databases (http://www.ncbi.nlm.nih.gov). For use in real-time PCR, all primers were assayed and standardized reactions, showing efficiency equal to or greater than 90%, as can be seen in Table 1.

Real-time PCR tests were performed on the ABI PRISM ® 7300 Sequence Detection System (Applied Biosystems ®), using ® Power SYBR Green PCR Master Mix (Applied Biosystems ®) following the manufacturer's protocol. The thermocycler was scheduled to perform 95 °C for 10 minutes, 40 repetitions of 95 °C for 15 seconds and 60 °C for one minute, followed by a step of denaturation. The results were expressed in *Ct* values (cycle threshold) and for the calculation of the relative expression was used the method of $2^{-\Delta\Delta Ct}$, as described by LIVAK et al.[15]

Table 1.

Primers		Sequences	(bp)	Eficience, %
	Antisense	5'-GTCCCCCACCCCCAGATACA-3'		
IL-2	Sense	5'-TGGAGCAGCTGTTGATGGAC-3'	363	93
	Antisense	5'-GAGGGCTTGTTGAGATGATGCT-3'		
IL-4	Sense	5'-ACCCCCAGCTAGTTGTCATC-3'	307	94
	Antisense	5'-CGAAAAGCCCGAAAGAGTC-3'		
β-actina	Sense	5'-TGGAATCCTGTGGCATCCATGAAAC-3'	349	91
	Antisense	5'-TAAAACGCAGCACAGTAACAGTCCG-3'		

Results and discussion

SDS-PAGE

The polyacrylamide gel electrophoresis of BTHX-1 identified structural changes after irradiation (radiation γ ^{60}Co) process.

Proteins profiles show that the γ-irradiation causes breakdown of polypeptide chains and formation of degraded high molecular weight molecules (Fig. 1).

In lane C, we observed that the $NaNO_3$ did not interfere in the structure of native BTHX-1. When the protein was irradiated in the presence of $NaNO_3$ (lane A), 14 kDa band was preserved. The profile of native BTHX-1 (lane D) in the presence of t-butanol showed that this scavenger promoted changes at the gel bands. When irradiated in the presence of t-butanol (E lane), the protein has not significantly changed if compared with its native form. High molecular weight components production suggests that radiation induced peptide bond cleavage and produced fragments of proteins which suffer posterior aggregation.[11] This aggregation probably occurs due inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, and formation of disulfide bonds.[13,18]

Enzyme linked immunosorbent assay (ELISA)

Our results indicate that both forms of proteins have induced detectable amounts of antibodies (Fig. 2). The plasma of Native BTHX-1 immunized animals presented higher IgG1 titers than the irradiated one, indicating the predominance of a Th2 response. Also, our data indicate that the irradiated protein induced higher titers of IgG2b than the non-irradiated protein, suggesting that Th1 cells were predominantly involved in the immune response. This population of cells is involved in the up regulation of cellular response, specifically macrophage activation.[5]

Figure 3 showed that irradiated toxins, alone or in the presence of $NaNO_3$ and of t-butanol, in the presence of aqueous electron scavenger, were immunogenic and the elicited antibodies were able to recognize the native toxin. Similar fact was observed by CASARE[6] when working with crotamina (subunit of *Crotalus durissus terrificus* venom).

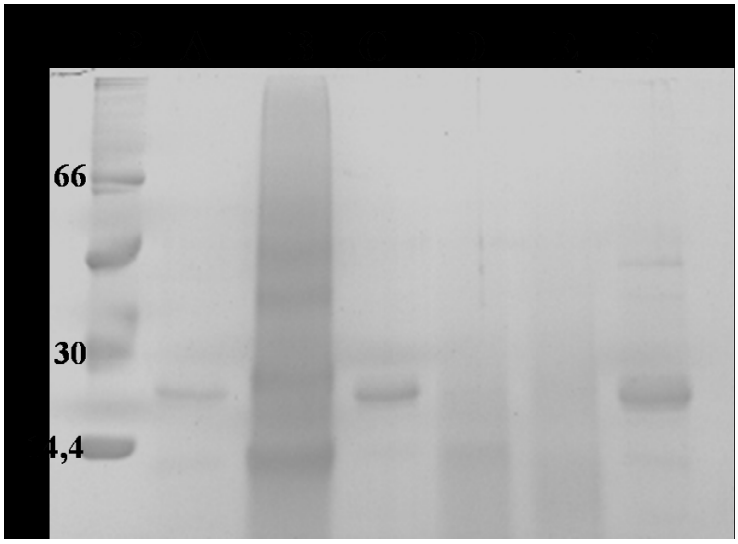

Fig. 1. SDS-PAGE profile of bothropstoxin-1 (BTHX-1); (A) Native BTHX-1; (B) irradiated BTHX-1; (C) native BTHX-1 with $NaNO_3$; (D) native BTHX-1 with t-butanol; (E) irradiated BTHX-1 with t-butanol; (F) irradiated BTHX-1 with $NaNO_3$. (P) Molecular weight marker

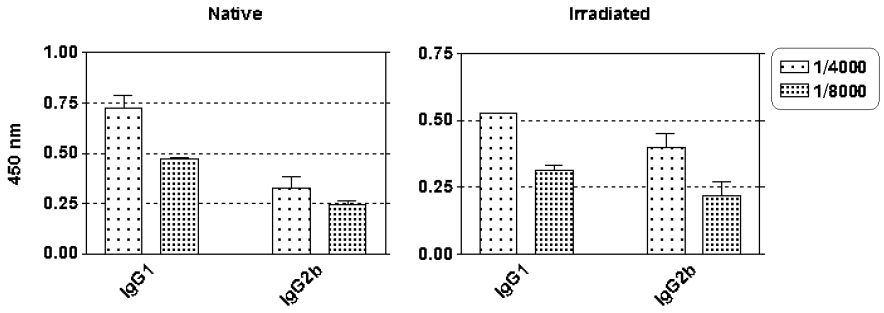

Fig. 2. Enzyme linked immunosorbent assay isotyping (IgG1 and IgG2b) of the antibodies rose against native and irradiated bothropstoxin-1 (BTHX-1) samples

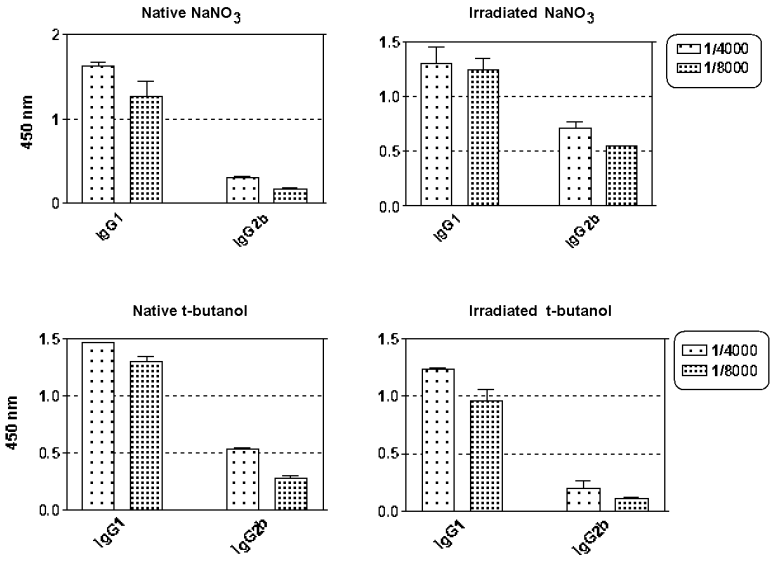

Fig. 3. Enzyme linked immunosorbent assay of the antibodies risen against native (A) and irradiated (B) BTHX-1 samples, with or without scavengers substances

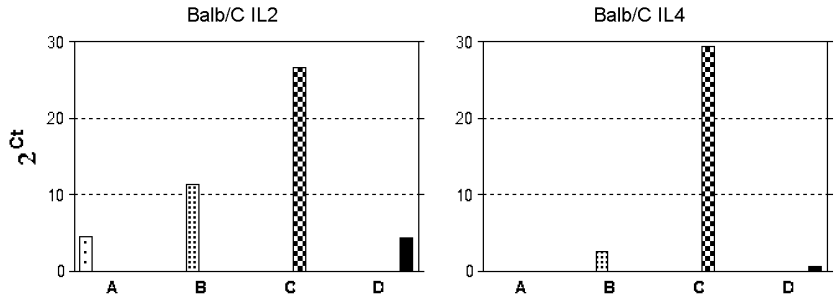

Fig. 4. Real time-PCR of INF-γ and IL10 from spleen cells, from the BALB/c mice, immunized with native or irradiated BTHX-1. Cells of mice immunized and in vitro stimulated with BTHX-1 native (A); Cells of mice immunized with BTHX-1 native and in vitro stimulated with irradiated toxin (B); Cells of mice immunized with BTHX-1 irradiated and in vitro stimulated with native toxin (C); Cells of mice immunized and in vitro stimulated with BTHX-1 irradiated (D)

The results of immunosorbent assay showed that the BTHX-1 irradiated with the $NaNO_3$, presented higher IgG2b titers than its native form, indicating the predominance of a response from Th1 cells. In contrast, when the toxin was irradiated with t-butanol, there was a slight decrease in the production of IgG2b antibodies, indicating a possible role modulator of the hydroxyl radical (OH•) in producing this antibodies and, to the

toxicity of protein. AKYON[1] studying the "scavenger" effect of astaxanthin, a carotenoid, on oxygen free radicals produced by cells of the immune system have observed similar results.

The results showed that, with the withdrawal OH•, by t-butanol, changes at the structure of the protein decrease, and there is a consequent decrease in the attenuation of toxicity. So, the immune response triggered by the protein irradiated in the presence of t-butanol, it would be very similar to the response from the native protein.

Real time – PCR

Figure 4 showed that cytokine profiles indicated that IL2 and IL4 mRNA presence appeared as much in the animals immunized with the native toxin as well as in those immunized with the irradiated toxin. However, we observed that both the IL2 as for IL4 there was greater expression by the cells of animals immunized with BTHX-1 irradiated and stimulated in vitro with the native toxin compared to the other groups tested.

The results obtained for IL2 and IL4 gene expression by real-time RT-PCR showed that both the IL2 as for IL4 was more expression from the cells of the animals immunized with BTHX-1 irradiated and stimulated in vitro with the native toxin. Similar fact was observed by HAN et al.[10] The authors examined the effects of γ-irradiation on Th1 and Th2 cytokines mRNA expression.

IL2 is an immunoregulatory cytokine, produced by Th1 cells. This cytokine stimulates the growth of T cells and hematopoietic cells. Therefore, the IL-4 produced by Th2 cells acts in the activation and growth of B cells and production of IgG1 antibodies. As well is able to inhibit the activation of macrophages.[2,16]

The immune response is the result of a series of interactions occurring between different immune cells and cytokines, therefore, examination of cytokine secretion profiles is an important step to understand the action of irradiated proteins on the immune system.

Our results indicate that irradiation of toxic proteins can promote significant modifications in their structures, but still retain many of the original immunological properties of native proteins. Ionizing radiation has proven to be a powerful tool in the production of better immunogens without affecting and even increasing their antigenic properties. These results indicate a potential use of irradiated proteins as antigens for immunization.

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