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Structure alteration and immunological properties of ^{60}Co-gamma-rays irradiated bothropstoxin-I

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Abstract In this work, the authors investigated the immunological behavior of bothropstoxin-I (BTHX-1), before and after irradiation process, and also the influence of scavengers substances on protein alterations induced by free radical production. Structural modifications were investigated by SDS-PAGE in reducing or non-reducing conditions. In vitro cytotoxicity assay was performed to test average toxic activities of BTHX-I. BALB/c Isogenic mice were immunized with irradiated or non-irradiated (native) forms of BTHX-I and antibody titers and isotypes were determined by ELISA method. Expression of murine cytokines was analyzed by using expression data obtained by quantitative real-time PCR (qPCR) assays. The results indicate that irradiation of proteins leads to significant structural modifications, and also changes the cytokines profile during immunization process, regarding a suitable approach to new immunogenic production.

Keywords Ionizing radiation · Immunological response · Cytokines

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Introduction

Ionizing radiation consists on electromagnetic waves that propagate with high velocity and energy. Irradiation of proteins in the dry state or in aqueous solution can induce a series of changes in protein structure, ranging from simple ionizations, to more drastic changes in its primary structure [1, 2]. Oxidation-induced changes may occur resulting from the interaction of the primary free radicals, produced upon radiolysis of water, with the protein molecule, changing its structure and may giving negative charging to these proteic structures [3]. However, these changes can be minimized by addition at the time of irradiation of the scavengers that combine readily with reactive specie-specific, preventing the action of the same in protein molecule [4].

About 5,000,000 ophidic accidents are registered worldwide (30,000 only in Brazil) [5] and serum therapy with equine antisera is the only efficient treatment available [6]. The venoms usually employed for immunizations are fairly toxic and some of them present low immunogenicity. Thus, a procedure that results in lower toxicity with preservation or even improvement of immunogenic properties would be useful.

Crude snake venoms or its isolated toxins present structural modifications if submitted to a gamma radiation effects in aqueous solution. This occurs due to reactions caused by the radiolysis products of water. Some scavenger substances, such as $NaNO_3$ and t-butanol, remove selectively the water radiolysis products [7].

Very promising results were obtained when crude animal venoms, as well as isolated toxins, were treated with gamma rays, yielding toxoids with good immunogenicity [8]. Ionizing radiation has proven to be a powerful tool in attenuating snake venoms toxicity without

affecting and even increasing their immunogenic properties.

The immune system is composed of cells and highly specialized 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. The innate immunity is mainly mediated by granulocytes and macrophages, while adaptive responses are mediated by lymphocytes, which provide a lasting immunity [9].

The adaptative response involves the proliferation antigen-specific T and B cells, which occurs through the interaction of receptors on the surface of these cells with antigens. There are two major types of effectors 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 cells and Th2 cells, which arise from a precursor Th0 and are differentiated according to the cytokines they produce, as are morphologically indistinguishable [10]. The Th1 cells produced interleukin 2, which induces the proliferation of T cells, and interferon-γ, which is responsible for the activation of macrophages which eliminate intracellular pathogens. There is a positive feedback regarding to the fact of interferon γ stimulate the Th0 cells to transform themselves into Th1 cells, while inhibiting the differentiation of Th2 cells.

The Th2 cells, in turn, produce the interleukins 4, 5, 6 and 10, which promote the production of antibodies. The interleukin 4 promotes a positive feedback that starts the induction of Th2 response and inhibits the differentiation of Th1 cells [11].

T-cells help B cells in antibody production and can also eliminate intracellular pathogens through the activation of macrophages and the infected cells annihilation. These processes are mediated by cytokines that stimulate the growth of B cells leading thus to their division and maturation in order to product antibodies [12]. Considering that numerous researches have shown the power of ionizing radiation to modify proteins, improving its immunological potential, which is very important in the search and improvement of immunogens or even vaccines, studies that can elucidate immune response generated against an irradiated protein is extremely necessary, especially in those cases where the substance is weakly immunogenic. Therefore, in the present work we have investigated the immunological behavior of bothropstoxin-I (BTHX-1), before and after irradiation process, in the presence of selective scavengers.

Experimental

Reagents

All reagents were commercially purchased with analytical grade. Bothropstoxin-1 was purified from *Bothrops jararacussu* crude venom maintained at the animal facility of Butantan Institute, São Paulo, Brazil.

Experimental groups

B10.PL and BALB/c isogenic mice, from animal housing facility of IPEN/CNEN/SP, have been maintained in sterilized isolators 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" (SBCAL/COBEA).

Concentration of scavengers

It was considered that the concentration of products formed in radiolysis of water and scavengers is equal to 1 (one), i.e. for each molecule of free radical formed, there is a molecule of scavenger working in the milieu.

- Concentration of sodium nitrate ($NaNO_3$) [scavenger for electron aqueous (e-aq.)]: 0.60 μM.
- Concentration of t-butanol (scavenger for hydroxyl radical): 0.70 μM.

Ion-exchange chromatography

Bothropstoxin-1 was purified using a single-step purification method. *B. jararacussu* venom was dissolved in 1 mL buffer (25 mM sodium phosphate, pH 7.8). After centrifugation, the supernatant was injected into a Resource-S cation exchange column connected to an FPLC system (Pharmacia®) and eluted with a linear salt gradient [13]. The samples resulting from the chromatography were collected, frozen in –80 °C and dried for later use. BTHX-1 purity assessed by SDS-PAGE.

Protein irradiation

Bothropstoxin-I, in the presence or not of scavengers, was dissolved in 0.15 M NaCl to a 2 mg/mL final concentration. 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

2.88 kGy/h dose rate. A control sample was kept outside of the irradiator during the process of irradiation.

SDS-PAGE

Native or Irradiated BTHX-1 purified samples were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples containing 40 μg of protein, as native or irradiated, were diluted in sample buffer, with or without β-mercaptoethanol, and heated to 95 °C for 5 min. After heating, samples were electrophoresed using SDS-PAGE protocol (Acrylamide: 15%; 90 V) [14], and analyzed after Coomassie Blue R-250 standard protocol staining.

Cytotoxicity assay

This assay determines the viable cell proportions in a monolayer culture upon exposure to the toxic agent (bothropstoxin-1). Bothropstoxin-1 (1 mg/mL), native or irradiated, was added in 96-well flat-bottomed microplates, in crescent dilutions, in a volume of 50 μL/well. Subsequently, CHO cells (3000 cells/well) were plated on described wells. The cell cultures was incubated at 37 °C in an atmosphere of 5% CO_2 for 72 h. After incubation, a mixture of MTS (dye) 0.2% and PMS 0.9% in PBS, was added in microplates. Which were kept at 37 °C in an atmosphere of 5% CO_2 for 2 h. The incorporation of the MTS was measured spectrophotometrically at 490 nm in a microplate reader.

Antibody production

Specific anti-native or anti-irradiated bothropstoxin-1 antibodies were obtained by immunizing B10.PL mice, inoculating the protein in its native or irradiated form, following an immunization protocol of three doses, one after 15 days and the other with an interval of 7 days [15]. 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 1 h after a 1/20000 or 1/40000 dilution in PBS. Peroxydase-labeled specific antibodies against total mouse IgG, and mouse IgG1 and IgG2b were then allowed to react individually with the bound antibodies. Finally, the reaction was developed by adding a chromogenic solution containing 0.5 mg/mL orto phenyl diamine in 50 mM citrate buffer pH 5.0 in the presence of 10 μL/mL hydrogen peroxide. After 20 min incubation, the reaction was interrupted by the addition of 50 mL of 2 M citric acid and the plates were analyzed on a microplate reader at 450 nm. The results were expressed in arbitrary index of reactivity (IR). The calculation was performed by the same ratio of the average values of OD (optical density) obtained for samples of the immunized animals and the average values of OD of samples from the control group (not immunized).

Quantitative real-time reverse transcription PCR (qRT-PCR)

Sample collection, RNA extraction and purification

All total RNA extraction procedures, amplification, detection o products and data analysis were carried out regarding the M.I.Q.E. guidelines [16]. Cell proliferation assays were carried out with splenocytes from BALB/c mice immunized with either the native or the irradiated protein, in the presence or not of scavengers substances, cultured in the presence of both antigens. After 48 h 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 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 frozen at −20 °C until performing qRT-PCR reactions. All primers were designed using Primer3 software (http://frodo.wi.mit.edu/) and the sequences were obtained in the database Nucleotide NCBI Databases (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore&itool=toolbar). Different primer sets were tested, and those that produced amplified products with efficiency between 90 and 100% were chosen. Preferred primer pairs are described in Table 1.

qRT-PCR reactions were performed in technical triplicates in SYBR Green-based tests on 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 setted to perform 95 °C for 10 min, 40 repetitions of 95 °C for 15 s and 60 °C for 1 min, followed by a step of specific-melt detection. The results were expressed in Cq values (quantification cycle) and calculation of the relative expression was determined by the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen [17], using β-actin expression levels as normalize parameter.

Table 1 Primers sequences for IFN-γ, IL-2, IL-4, IL-10 and β-actin and respective efficiency of standardization reaction of qRT-PCR

Primers	Sense	Antisense	Efficiency (%)
IFN-γ	5′-GTCCAGCGCCAAGCATTCAA-3′	5′-GTCCCCCACCCCCAGATACA-3′	90
IL-2	5′-TGGAGCAGCTGTTGATGGAC-3′	5′-GAGGGCTTGTTGAGATGATGCT-3′	93
IL-4	5′-ACCCCCAGCTAGTTGTCATC-3′	5′-CGAAAAGCCCGAAAGAGTC-3′	94
IL-10	5′-CTCAGTTCCCATTCTATTTATTCAC-3′	5′-GGATCTCCCTGGTTTCTCTTC-3′	92
β-Actin	5′-TGGAATCCTGTGGCATCCATGAAAC-3′	5′-TAAAACGCAGCACAGTAACAGTCCG-3′	91

Results

SDS-PAGE

Proteins SDS-PAGE profiles show that γ-radiation causes breakdown of polypeptide chains and also degraded high molecular weight molecules formation (Fig. 1).

Cytotoxicity assay

The results of cytotoxicity assay, performed with CHO cells, showed that the irradiated toxin was fivefold less toxic than its native counterpart (Fig. 2). 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.

Enzyme linked immunosorbent assay (ELISA)

Our results indicate that both forms of proteins induced detectable amounts of antibodies with employed dilutions (Fig. 3). We could also observe that animals' plasma immunized with native bothropstoxin-1 presented higher IgG1 titers when compared with the irradiated one. Also, our data indicate that the irradiated protein induces higher titers of IgG2b than the non-irradiated protein.

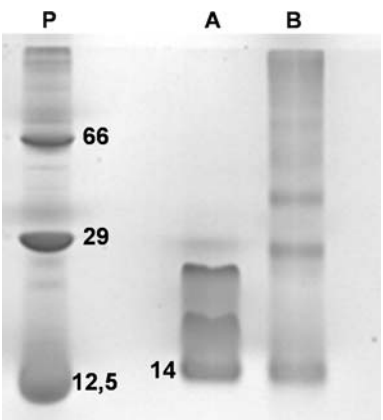

Fig. 1 SDS-PAGE profile of Bothropstoxin-1 (BTHX-1) Native (A) or Irradiated (B) in reduced conditions. (P) Molecular weight marker

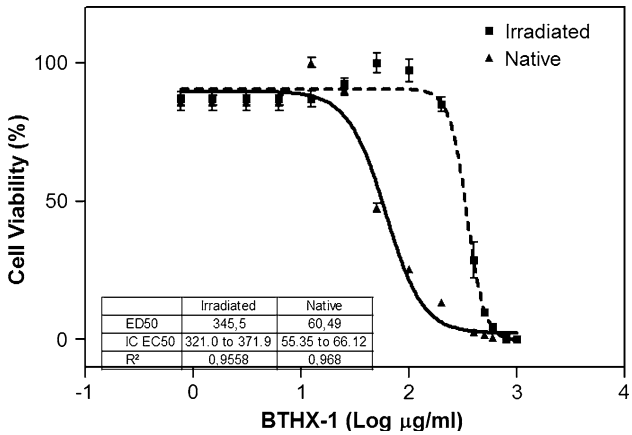

Fig. 2 Cytotoxicity assay of native and irradiated bothropstoxin-1, in CHO cells. ED 50% (effective dose 50%)

Irradiated toxins, alone or in the presence of $NaNO_3$ and t-butanol, were immunogenic and the antibodies elicited by them were able to recognize the native toxin (Fig. 4). It is also showed in Fig. 4, a slight reduction in the title of antibodies when the toxin was irradiated in the presence of t-butanol, compared to BTHX-1 irradiated in the presence of $NaNO_3$ and toxin irradiated without scavengers.

qRT-PCR

In Fig. 5 we found that spleen cells obtained from BALB/c mice immunized with the BTHX-1 native and stimulated in vitro with the irradiated form of the toxin, showed higher expression of IFN-γ and IL-2 (TH1 cells response). Spleen cells obtained from animals immunized with irradiated BTHX-1, and stimulated in vitro with the irradiated form of the toxin showed basal expression of INF-γ, IL-2 and IL-10.

In Fig. 6 we found that spleen cells of BALB/c mice showed higher expression of IL-2 and IL-10 when the cells were stimulated in vitro with BTHX-1 irradiated in the presence of t-butanol (B). While BTHX-1 irradiated in the presence of sodium nitrate (D), induced higher expression of INF-γ, compared to the irradiated toxin in the presence of t-butanol.

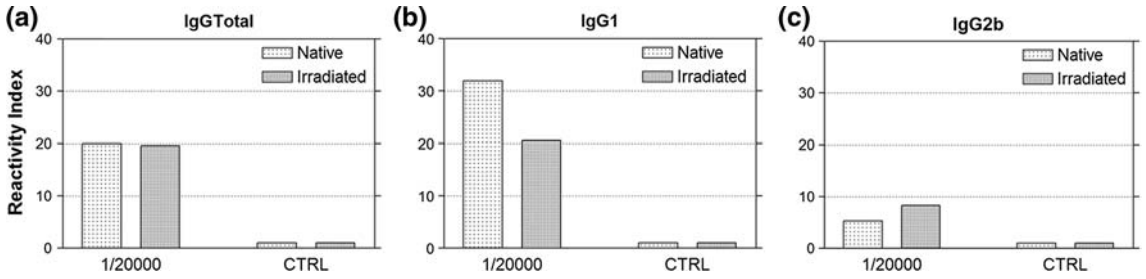

Fig. 3 Enzyme linked immunosorbent assay isotyping IgG Total (**a**), IgG1 (**b**) and IgG2b (**c**) of the antibodies rose against native and irradiated bothropstoxin-1 (BTHX-1) samples. *CTRL* control of non-immunized mice

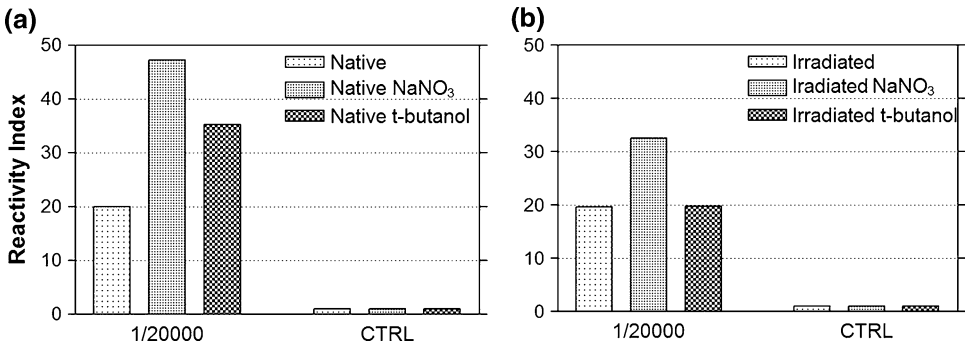

Fig. 4 Enzyme linked immunosorbent assay of the antibodies rose against native (**a**) and irradiated (**b**) BTHX-1 samples, with or without scavengers substances. *CTRL* control of non-immunized mice

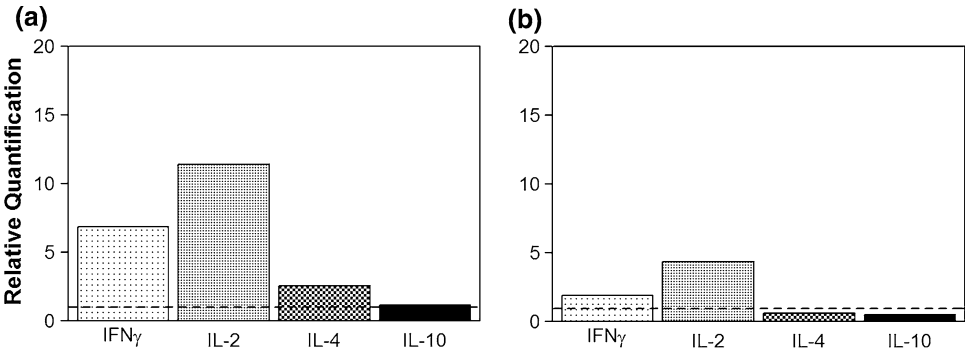

Fig. 5 Relative quantification of INF-γ, IL-2, IL-4 and IL-10 from spleen cells, from the BALB/c mice, immunized with native or irradiated BTHX-1. **a** Spleen cells of mice immunized with native BTHX-1 and in vitro stimulated with irradiated BTHX-1; **b** Spleen cells of mice immunized with irradiated BTHX-1 an in vitro stimulated with irradiated BTHX-1. *Dashed line*: Control of β-actin

Discussion

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

The production of high molecular weight components suggested that ionizing radiation have induced peptide bond cleavage and also produced fragments of proteins which suffer posterior aggregation [7]. This aggregation occurs through inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, and the formation of disulfide bonds [18, 19].

BTHX-1 in the presence of the reducing agent did not present dissociation of the subunits, suggesting that the irradiation have induced a resistant covalent bonds formation.

The cell cytotoxicity assay of BTHX-1 (Fig. 2), native or irradiated, performed in CHO cells, showed that was necessary a very higher amount of irradiated toxin to reach the capable concentration to eliminate 50% of the cellular population exposed to the BTHX-1. Cell cytotoxicity assays developed with medically important South American snake venoms suggest the existence of a correlation between ED50% (effective dose 50%) and biological activities of these venoms [20].

Concerning to immunological aspects, we could also observe that animals' plasma immunized with native bothropstoxin-1 had higher IgG1 titers (Fig. 3), indicating the predominance of a Th2 type response. This behavior was observed in macrophage depleted animals [21]. The authors observed that after depletion, the animals presented an increased IgG1 level, which is under control of Th2

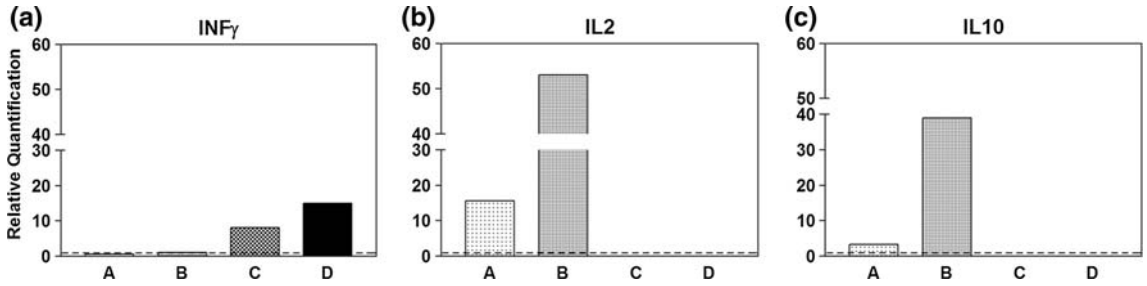

Fig. 6 Relative quantification of INF-γ (**a**), IL-2 (**b**) and IL-10 (**c**) from spleen cells, from the BALB/c mice, immunized with native or irradiated BTHX-1, in presence of $NaNO_3$ or t-butanol. A: native BTHX-1 with t-butanol; B: irradiated BTHX-1 with t-butanol; C: native BTHX-1 with sodium nitrate; D: BTHX-1 with irradiated sodium nitrate. *Dashed line*: Control of β-actin

cells, a cell type involved in the humoral immune response, modulating the production of antibodies by B lymphocytes [22].

In addition, our data indicate that the irradiated protein induced higher titers of IgG2b if compared with other subclasses (Fig. 3), suggesting that Th1 cells are predominantly involved in the immune response. This population is involved with the up regulation of cellular response, specifically macrophage activation [9]. Pavanelli [23], working with BALB/c mice, found high levels of IgG2a after immunization with bradykinin, indicating a response of Th1 cells.

The differential activation of T cells (Th1 or Th2) could be explained due 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 [21]. It has been shown that the uptake of proteins by macrophages is enhanced when they are in its irradiated form, increasing the association with macrophages, through scavenger receptors, involved with oxidized biomolecules processing [8]. Thus, irradiated proteins would be preferentially processed by macrophages, explaining the switch towards Th1 response in the animals immunized with the irradiated sample.

Quantification of cytokine gene expression and protein secretion is essential for the assessment of specific immune responses [24].

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) is becoming a widely used method to quantify cytokines from cells. This method allows direct detection of PCR product during the exponential phase of the reaction, combining amplification and detection in a single step [25].

The results obtained for gene expression by qRT-PCR showed that BALB/c mice (Fig. 5), which have a tendency to Th2 cells response [26], there was greater expression of IFN-γ and IL-2, in comparison for IL-4 and IL-10, for the cells of animals immunized with both forms of the toxin, native or irradiated, and stimulated in vitro with irradiated BTHX-1. IFN-γ and IL-2 was a key immunoregulatory cytokines, produced by Th1 cells, crucial for a variety of distinct cellular programs. They can activate macrophages and regulate cell-mediated immune responses. The IL-2, in turn, stimulates the growth of T cells and hematopoietic cells [27]. Han et al. [28] examined the effects of γ-irradiation on Th1 and Th2 cytokines mRNA expression. The authors observed that mRNA level of interferon IFN-γ, was reduced after 3 h post-irradiation, whereas the expression of IL-10 was increased.

The cells obtained from BALB/c mice (Fig. 6) immunized with BTHX-1 irradiated in the presence of $NaNO_3$, showed higher expression of INF-γ in comparison to cells of animals immunized with the native toxin plus $NaNO_3$. The sodium nitrate is the scavenger that has affinity for the aqueous electron (e-aq.), one of the products formed in the radiolysis of water. The default of immune response induced in animals immunized with BTHX-1 irradiated in the presence of this scavenger was predominantly Th1, which leads us to believe that the damage caused by e-aq. in irradiated toxin does not exert a role very important in the pattern of immune response induced by it. This is because, with the removal of e-aq. by $NaNO_3$, the pattern of immune response induced by irradiated BTHX-1 was not changed. The cells obtained from animals immunized with BTHX-1 irradiated in the presence of t-butanol, showed higher expression of IL-2 and IL-10 as compared to cells from animals immunized with the native toxin plus t-butanol. The IL-10 produced by Th2 cells down-regulate the effective function of Th1 responses, and also mediate immunosuppressive effects [24, 28].

These facts reinforce the involvement of $OH^{\bullet}$ in the modulation of immune response against the irradiated toxin. That is, with the removal of $OH^{\bullet}$ by t-butanol, the changes in the structure of the protein decrease, so there is a consequent decrease in the attenuation of protein toxicity. Therefore, the immune response triggered by the irradiated protein in the presence of t-butanol, is quite similar to the

response obtained with the native protein. Anraku et al. [29] observed that structural changes in human albumin caused by free radicals of oxygen were reduced by the action scavenger of chitosan.

The immune response is the result of a series of complex interactions occurred 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.

Conclusions

Irradiation process has promoted structural modifications on BTHX-I. The Cytotoxicity Assay showed that the modified toxin was 5 folds less toxic than its native counterpart, but still immunogenic. Irradiated BTHX-1 elicited antibodies were responsive to native BTHX-I form. In the presence of t-butanol, irradiated BTHX-I presented a discrete reduction in isotype-specific antibodies production. The cytokines expression profiles indicated that IFN-γ mRNA presence appeared to be higher in irradiated toxin immunized mice, while IL-10 mRNA expression was predominant to native antigen. Our results indicate that irradiation of toxic proteins can promote significant modifications in their structures, but still retain many of the original antigenic and immunological properties of native proteins. These results indicate a potential use of detoxified proteins as antigens for immunization.

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