Journal of Radioanalytical and Nuclear Chemistry, Vol. 269, No.3 (2006) 565–569

Immune response against antigens irradiated with ^{60}Co gamma-rays

J. A. Baptista,* P. J. Spencer, J. E. Oliveira, M. S. Casare, N. Nascimento

Centro de Biologia Molecular, Instituto de Pesquisas Energéticas e Nucleares
IPEN/CNEN, Avenida Prof. Lineu Prestes 2242, São Paulo, 05508-000, Brazil

(Received April 6, 2006)

In the present work, we investigated the immunological behavior of bothropstoxin-1, a K49 phospholipase from *Bothrops jararacussu*, and of ovalbumin before and after irradiation with ^{60}Co γ-rays. Isogenic mice were immunized with either native or irradiated proteins. The circulating antibodies were isotyped and titrated by ELISA. Results indicate that irradiated proteins were immunogenic and the antibodies elicited by them were able to recognize the native proteins in ELISA. Data also indicate that the irradiated protein induced higher titers of IgG2a and IgG2b, suggesting that Th1 cells were predominantly involved in the immune response. Structural modifications of the proteins were investigated by SDS-PAGE, mass spectrometry and size exclusion chromatography. According to our data, irradiation promoted structural modifications on both proteins, characterized by higher molecular weight forms (aggregates and oligomers). When analyzed by mass spectrometry, the irradiated bothropstoxin appeared in several oxidized forms. These 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 form.

Introduction

About 20,000 accidents involving snakebites are registered every year in Brazil. Serum therapy with equine antisera is the only efficient treatment.[1] The venoms employed for immunizations are fairly toxic and some venoms present low immunogenicity. Thus, it would be useful to get modified antigens with lower toxicity and preserved or improved immunogenicity.

Ionizing radiation has been successfully employed to modify the immunological properties of biomolecules.[2] Very promising results were obtained when crude animal venoms, as well as isolated toxins, were treated with γ-rays, yielding toxoids with good immunogenicity.[3] Obtaining of modified antigens with lower toxicity and preserved or improved immunogenicity would be useful. Ionizing radiation has proven to be a powerful tool to attenuate snake venoms toxicity without affecting and even increasing their immunogenic properties. However, not many are known about the modifications that irradiated molecules undergo and even less about the immunological response that such antigens elicit.

In the present work, we used bothropstoxin-1, a K49 phospholipase, and ovalbumin, typical egg-white proteins, as a model to 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.*[4] Our goal was to further characterize bothropstoxin-1 with focus on immunological aspects.

Ovalbumin, our control, is a thermally and chemically stable protein and is considered a classical allergen.[5]

Experimental

Reagents

All reagents were commercially obtained and had analytical grade. Bothropstoxin-1 was purified from *Bothrops jararacussu* crude venom (Instituto Butantan, São Paulo, Brazil).

Animals

B10.PL isogenic mice were obtained from the animal housing facility of IPEN/CNEN 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).

Protein irradiation

Bothropstoxin-1 and ovalbumin were dissolved in 0.15M NaCl to a final concentration of 2 mg/ml. This solution was irradiated with a 2000 Gy dose using γ-rays derived from a ^{60}Co source (GammaCell, Atomic Agency of Canada, Ltd.) at room temperature and in the presence of atmospheric O_2, with a 5170 Gy/h dose rate.

* E-mail: janabap@gmail.com

0236–5731/USD 20.00

Akadémiai Kiadó, Budapest
Springer, Dordrecht

SDS-PAGE

Bothropstoxin-1 (after purification) and ovalbumin, native or irradiated, were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[6] under reducing and not-reducing conditions.

HPSEC – high performance size exclusion chromatography

The assay determined the possible structural modifications of the molecules after being irradiated (^{60}Co γ-rays). The resin of size exclusion utilized was TSK – 2000 TOSOHAAS, equilibrated previously with sodium phosphate buffer (0.02M Na_2HPO_4) in pH 7.0, and 0.15M NaCl. The elution of ovalbumin, native or irradiated, was performed at a 1 ml/min flow rate.

Mass spectrometry (ESI)

Bothropstoxin-1 samples (2 mg/ml), native or irradiated, were submitted to mass spectrometry analysis, 20 µl of each sample (2 mg/ml) was injected. The equipment utilized was a Q-Tof Ultima ES-MS Analysis.

Production of antibodies

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

Enzyme-linked immunosorbent assay (ELISA)

Ninety-six well microplates were coated with native bothropstoxin-1 or ovalbumin (1.0 µg/100 µl per well) 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 1/20,000 or 1/40,000 dilution in PBS. Peroxidase labeled antibodies specific against mouse IgG1, IgG2a or IgG2b were then allowed 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 50mM citrate buffer pH 5 in the presence of 1 µl/ml hydrogen peroxide. After 20-minute incubation, the reaction was interrupted by adding 50 µl of 2M citric acid and the plates were analyzed on a microplate reader at 450 nm.

Results and discussion

SDS-PAGE

SDS – PAGE profiles of the proteins show that γ-irradiation causes breakdown of polypeptide chains and, as a result, formation of degraded high molecular weight molecules (Fig. 1). The production of these high molecular weight components suggested that radiation induced peptide bond cleavage and produced fragments of proteins that suffer posterior aggregation.[5] This aggregation occurs through inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, and the formation of disulfide bonds.[8]

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.

HPSEC – high performance size exclusion chromatography

Ovalbumin, native (Fig. 2) or irradiated (Fig. 3), was submitted to size exclusion chromatography for better evaluating the probable structural modifications provoked by the irradiation. The chromatography process show that irradiation of ovalbumin (Fig. 3) resulted in the formation of high molecular weight products, due to aggregation caused by γ-radiation in the protein molecule, confirming the data observed in SDS-PAGE. The same fact was observed for crotoxin (major protein of *Crotalus durissus terrificus* venom).[9]

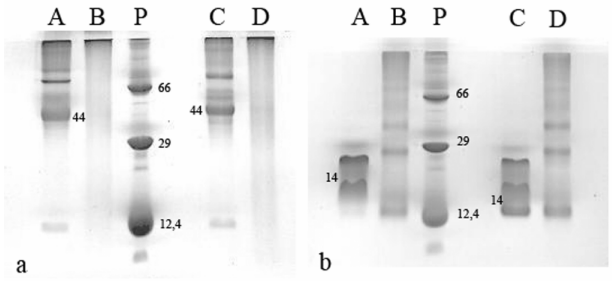

Fig. 1. SDS-PAGE profile of ovalbumin (a) and bothropstoxin-1 (b);
(A) ovalbumin or bothropstoxin-1 native and not-reduced;
(B) ovalbumin or bothropstoxin-1 irradiated and not-reduced;
(C) ovalbumin or bothropstoxin-1 native and reduced;
(D) ovalbumin or bothropstoxin-1 irradiated and reduced;
(P) molecular weight marker

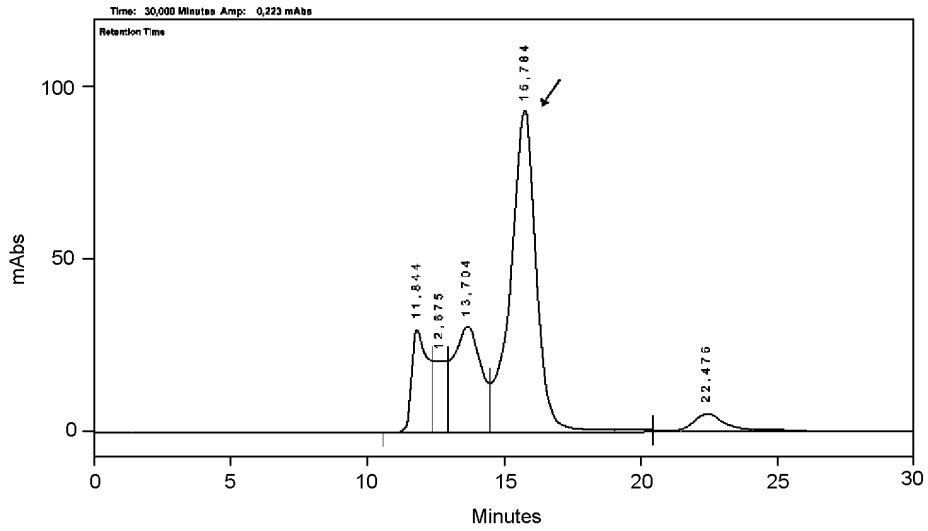

Fig. 2. Size exclusion chromatography of ovalbumin native (→) in column TSK 2000–TOSOHAAS (60 cm×7.5 mm internal diameter, pores of 125 Å and particles of 10 μm), previously equilibrated with sodium phosphate buffer (Na_2HPO_4) 0.02M, in pH 7.0, and 0.15M NaCl. Flow rate: 1 ml/min

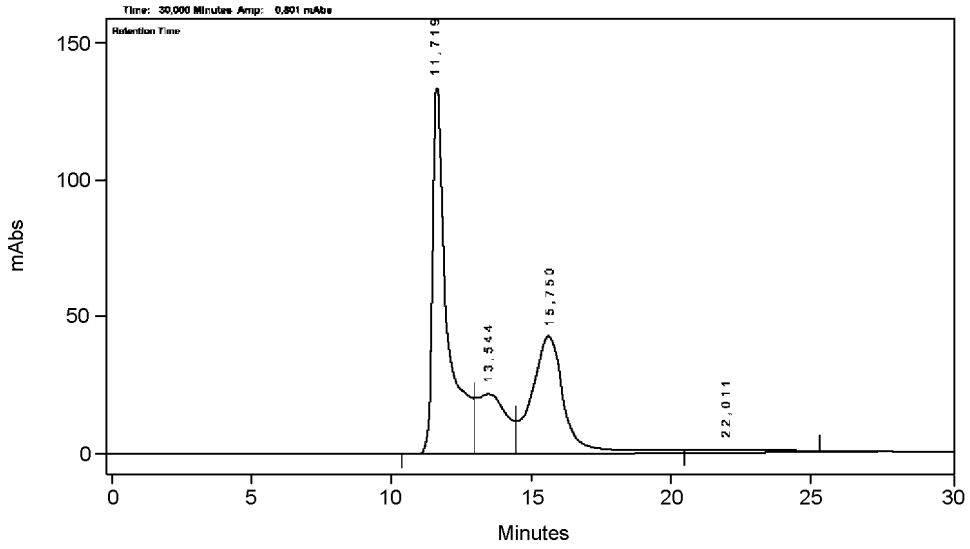

Fig. 3. Size exclusion chromatography of ovalbumin irradiated in column TSK 2000–TOSOHAAS (60 cm×7.5 mm internal diameter, pores of 125 Å and particles of 10 μm), previously equilibrated with sodium phosphate buffer (Na_2HPO_4) 0.02M, in pH 7.0, and 0.15M NaCl. Flow rate: 1 ml/min

Mass spectrometry (ESI)

The mass spectrometry of bothropstoxin-1 native (Fig. 4) or irradiated (Fig. 5) 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 irradiated crotoxin was better taken up by macrophages compared to uptake of the native form. This fact can be explained by the property that the radiation has to oxidate molecules.

Enzyme linked immunosorbent assay (ELISA)

The results indicate that both forms of the proteins induced detectable amounts of antibodies with the two dilutions used in the assay (Fig. 6). It could also be observed that the plasma of the animals immunized with native ovalbumin or bothropstoxin-1 had higher IgG1 titers, indicating the predominance of a Th2 type

response. This behavior was noticed in macrophage depleted animals.[11] 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, data indicate that the irradiated protein induced higher titers of IgG2a and IgG2b (Fig. 6), 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.

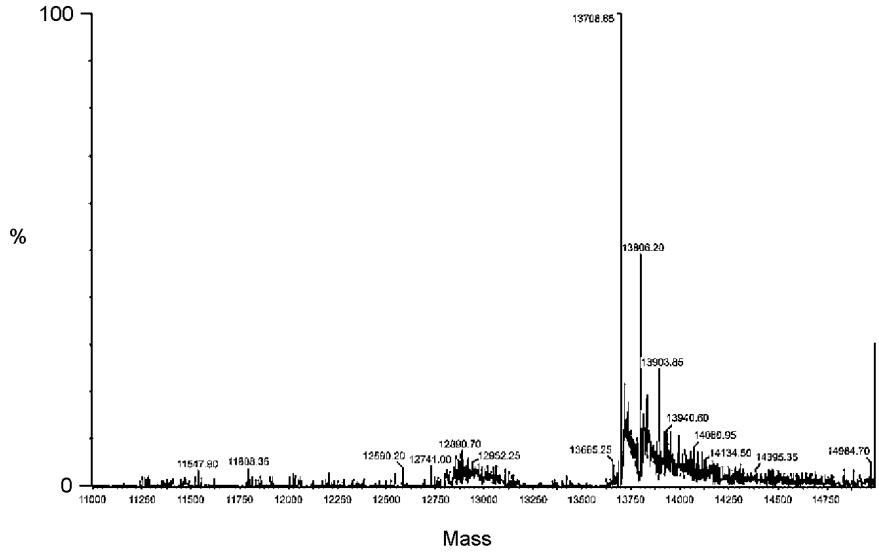

Fig. 4. Mass spectrometry of bothropstoxin-1 native in Q-Tof Ultima ES-MS analysis

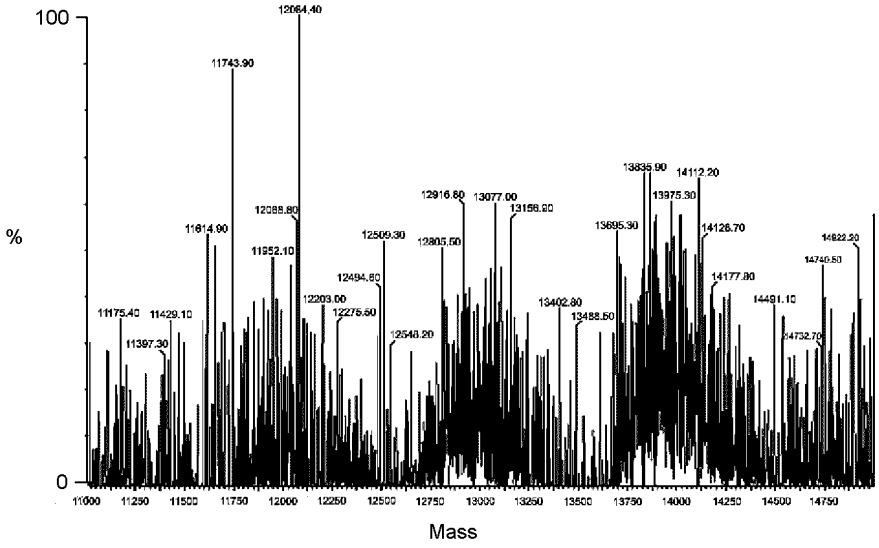

Fig. 5. Mass spectrometry of bothropstoxin-1 irradiated in Q-Tof Ultima ES-MS analysis

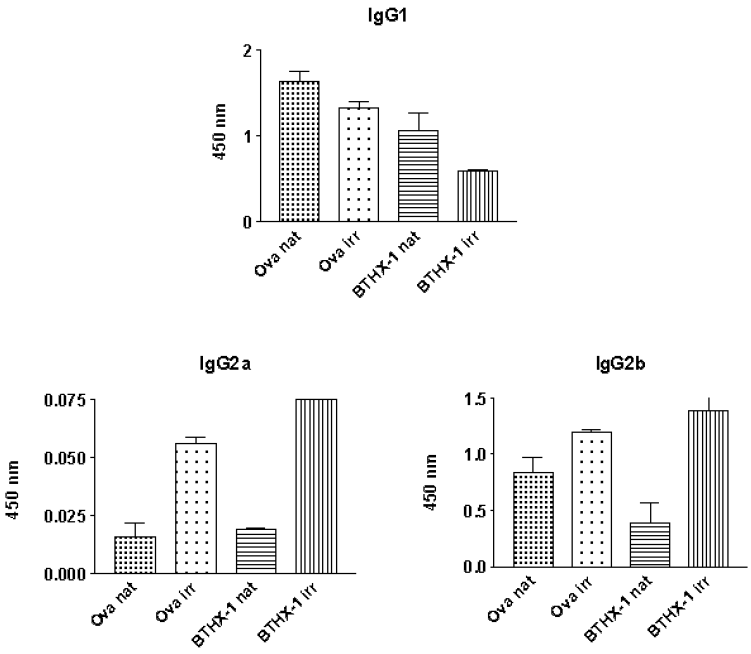

Fig. 6. Enzyme-linked immunosorbent assay isotyping (IgG1, IgG2a and IgG2b) of the antibodies rose against native and irradiated ovalbumin or bothropstoxin-1 (BTHX-1) samples. Ova nat: ovalbumin native; Ova irr: ovalbumin irradiated; BTHX-1 nat: bothropstoxin-1 native; BTHX-1 irr: bothropstoxin-1 irradiated

Conclusions

A dose of 2000 Gy ^{60}Co γ-rays causes modifications in the molecules of ovalbumin and bothropstoxin-1.

The mass spectrometry of irradiated bothropstoxin-1 shows that the γ-radiation promoted the oxidation of the toxin.

The preferential activation of Th1 and Th2 cells depends on whether the antigen is in its native or irradiated form, since Th1 response is observed preferentially when irradiated antigens are employed.

The native and irradiated proteins were able to stimulate the immune system and the resulting antibodies were able to react with the respective native form.

*

The authors wish to thank CAPES from Brazil.

References

1. B. SOERENSEN, Animais Peçonhentos, Livraria Atheneu Editora, Rio de Janeiro, Brazil, 1990.
2. N. NASCIMENTO, C. S. SEEBART, B. FRANCIS, J. R. ROGERO, I. I. KAISER, Toxicon, 34 (1996) 123.
3. B. A. CARDI, N. NASCIMENTO, H. ANDRADE Jr., Intern. J. Radiat. Biol., 73 (1998) 557.
4. P. J. SPENCER, S. D. AIRD, M. BONI-MITAKI, N. NASCIMENTO, J. R. ROGERO, Braz. J. Med. Biol. Res., 31 (1998) 1125.
5. T. KUME, T. MATSUDA, Radiat. Phys. Chem., 46 (1995) 225.
6. U. K. LAEMMLI, Nature, 227 (1970) 680.
7. E. HARLOW, D. LANE, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, USA, 1988.
8. S. MOON, K. B. SONG, Food Chem., 74 (2001) 479.
9. N. NASCIMENTO, Ph.D. Thesis, IPEN/CNEN, São Paulo, Brazil, 1995.
10. B. A. CARDI, Ph.D. Thesis, IPEN/CNEN, São Paulo, Brazil, 1999.
11. J. M. BREWER, J. RICHMOND, J. ALEXANDER, Clin. Exp. Immunol., 97 (1994) 164.
12. J. SPRENT, C. D. SURH, Annu. Rev. Immunol., 20 (2002) 551.
13. P. J. DELVES, I. M. ROITT, Adv. Immunol., 343 (2000) 37.
14. T. L. CHANG, C. M. SHEA, S. URIOSTE, R. C. THOMPSON, W. H. BOOM, A. K. ABBAS, J. Immunol., 145 (1990) 2803.