DIFFERENTIAL UPTAKE OF IRRADIATED CROTOXIN BY CBA/J MICE PERITONEAL MACROPHAGES AND ITS INHIBITION BY PROBUCOL

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ABSTRACT

Ionizing radiation alters proteins and their biological activities, mainly by oxidation due to water radiolysis free radicals. After 2 kGy ^{60}Co gamma radiation, crotoxin, the main toxin of *Crotalus durissus terrificus* venom, was less toxic, with the same or even enhanced immunogenicity. Oxidized lipoproteins are preferentially recognized by scavenger receptors (ScvR) in macrophages. The immune response is started by phagocytosis of antigen by specialized cells, the antigen presenting cells(APC), mainly macrophages. To investigate the relationship between irradiated or native crotoxin and antigen-presenting cells(APC), we studied crotoxin endocytosis by CBA/J mouse peritoneal macrophages and the role of ScvR in this process, by a specific *in situ* ELISA, using Probucol as a ScvR blocker. The quantitative test was performed on cultured macrophages, with crotoxin specific antibody and revealed by immunoperoxidase systems. The results show the preferential endocytosis of irradiated crotoxin by macrophages, without affecting cell viability or function. Probucol induces a reduced uptake of irradiated crotoxin by macrophages, without affecting the uptake of native crotoxin, suggesting that the increased uptake of irradiated crotoxin is mediated by ScvR on macrophage surface. We argue that the better immunogenic properties of irradiated crotoxin could be ascribed to a preferential uptake by APC, using a specific receptor system by the ScvR pathway.

I. INTRODUCTION

Snakebites by South American rattlesnakes, *Crotalus durissus terrificus*, represent a health care problem, with the occurrence of more than 2000 accidents a year and with considerably high mortality rates, despite specific therapy with antiserum. Crotoxin, the main toxic component of this venom, has two non-covalently linked subunits and causes myotoxicity[1] after binding to the neuromuscular junction[2,3], with rhabdomiolysis[4]. The main obstacle in the production of antiserum, the only specific and effective therapy for venomous snakebites[5],is the toxicity of crotoxin. Due to its high toxicity, native venom or crotoxin may produce severe intoxication in serum-producing animals, frequently followed by death[6]. Several methods with chemical modifications of the toxin have been used to detoxify venoms, but most of them affect their antigenic properties[6], resulting in less efficient antiserum production. Irradiation with gamma rays of this purified toxin maintains or increases its immunogenic properties, with low toxicity[7]. The effect of gamma irradiation on this purified protein has been attributed to the action of water radiolysis free radicals, that, by inducing oxidation of aminoacid side chains[9], with intramolecular and

intermolecular new formed bridges in aggregation of protein molecules, as detected by SDS-PAGE[7], but the chemical nature of this process has not been clearly identified. Some results indicate that $OH^{\bullet}$ or aqueous e^{-}, main water radiolysis free reactive radicals, are involved in detoxification process[8]. The enhanced immunogenicity of the protein could not be explained solely by these radical effects on the protein, since other factors and interactions with immune cells must also be involved.

In another system, we have shown that ^{60}Co irradiation of recombinant 18kDa heat shock protein from *Mycobacterium leprae*, affects its immunogenicity, resulting in better cellular immune response, as shown in a hypersensitivity skin model, as compared to non-irradiated protein[10].

The antigen processing is a complex mechanism that involve the recognition and internalization of this antigen by specific cells. These cells possess a machinery to cooperate with the lymphocytes, the main cells in this process but not a phagocytic cell. Macrophages are the main antigen-presenting cells, aside other immune and inflammatory functions. These cells have scavenger receptors(ScvR), that mediate the recognition and uptake of a wide range of molecules, usually negatively charged, and are also implicated in atherogenesis, due its function in the pathologic deposition of cholesterol on arterial walls through endocytosis of oxidized LDL[11]. The recognition of these receptors enhances the uptake of proteins by cells, due its high frequency in the cell surface of macrophages and endothelial cells, by antigen presenting cells(APCs). This enhanced uptake will quantitatively favors this antigen presentation, as compared to non-recognized ScvR proteins.

In the present study we investigated the uptake of native or ^{60}Co irradiated crotoxin by non-stimulated peritoneal macrophages, by a quantitative *in situ* ELISA using probucol as ScvR-blocking reagent.

II. MATERIALS AND METHODS

Materials

Purified *C.d.terrificus* crotoxin was assessed by SDS-PAGE, lyophilized and stored at -20°C[7,12].

Probucol [4,4'-isopropylidenedithio) *bis* (2,6-di-t -butyl phenol)] (Merrell Lepetit™) was used dissolved into a concentrated solution in DMSO.

CBA/J mice were obtained from the Instituto de Medicina Tropical colony and maintained in sterilized cages and absorbent media before use, tih food and water *ad libitum*.

Methods

Crotoxin irradiation schedule

Native crotoxin was purified from *C.d.terrificus* crude venom (Instituto Butantan, São Paulo, Brazil)[7,12] by molecular exclusion chromatography and isoelectric precipitation and subsequently submitted to 2kGy homogeneous irradiation in a ^{60}Co GammaCell 220 source (Atomic Energy of Canada Ltd.), in air and at room temperature. Molecular weight composition was detected by SDS-PAGE[13], with also recognition of samples by specific antibodies as determined by Western blot[14].

Experimental Design

Short-term macrophage cultures.

Macrophages from non-stimulated CBA/J mice were isolated as described[15] by peritoneal washings. Briefly, 3 ml of sterile PBS containing gentamycin (10μg/ml) were injected and recovered from the peritoneal cavity. The collected cells were washed with warmed serum-free RPMI 1640 medium (Sigma Chemical Co., St.Louis, Mo), and plated onto flat-bottom, 96-well microtiter plate (Corning Glass, Corning, NY) with RPMI 1640 medium containing 10% inactivated FCS at a concentration of $4x10^5$ cells. The plate was maintained at 37°C in a humidified 5.0% CO_2/air atmosphere incubator (Forma Scientific, Inc.) for 30 minutes to allow cell plate adherence. Adhesive cells were then washed with three changes of warmed serum-free RPMI 1640 medium and microplate was incubated for 24 h under the same conditions. The viability and quality of cells were also assessed by Trypan blue dye exclusion using inverted phase microscopy before the experiments. Only wells with an uniform and 95% viable monolayer were used.

In situ ELISA

Irradiated or native crotoxin (0.5 μg/well) was incubated with CBA/J mouse peritoneal macrophages (4×10^5/well) previously seeded on flat-bottom, 96-well microtiter plates (Corning Glass, Corning, NY) diluted in serum-free RPMI 1640 medium. Cells were assayed in quadruplicate

and at different times. After incubation, cells were fixed with 4% phosphate buffered paraformaldehyde, pH 7.2, for 10 minutes, permeabilized with methanol/acetone (1:1) for 30 seconds and endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide for 10 minutes. After washing with PBS, cells were incubated with anti-irradiated crotoxin rabbit serum for 60 minutes at room temperature in a humid chamber. After washing with PBST cells were incubated with anti-rabbit IgG peroxidase conjugate under the same conditions. Quantitative uptake was determined using a chromogenic solution of 1 mg/ml of o-phenylenediamine-HCl (Sigma Chemical Co., St.Louis, Mo) and 0.03% hydrogen-peroxide(Merck) in 0.05 M sodium citrate buffer, pH 5.6, for 10 minutes in the dark followed by spectrophotometry in a microplate reader at 492 nm. The cell concentration was monitored by staining the washed monolayers with 1% aqueous crystal violet and subsequent extraction of the dye with 33% glacial acetic acid. The OD at 575nm was determined in a microplate reader.

Probucol Assays

Mouse peritoneal macrophages were assayed with crotoxin samples (0.5 μg/well) and probucol (0-20 μg/well) in serum-free RPMI 1640 medium as described above for *in situ* ELISA, using a procedure similar to that described by Yamamoto and coworkers (1986)[16].

Statistical Analysis

Data are reported as means ± SD. After testing the homogeneity of variances' data were compared using the Student *t* test, and considered significant when the probability of equality was less than 0.05 ($p < 0.05$).

III. RESULTS

Quantification of crotoxin uptake by macrophages by *In situ* ELISA

The *in situ* ELISA was used to detect quantitatively the uptake of toxins by macrophages. As shown in Figure 1, macrophages incubated with irradiated crotoxin presented two times higher uptake than macrophages incubated with native crotoxin. Time-dependent uptake was also observed, showing that this endocytosis, at these toxin concentrations, was not restricted to a few or groups of monolayer cells, showing excess ligand concentration. The protein toxicity to the cell layers was slight and had no effect on the cell contents as detected by crystal violet staining (Fig 1). Both results showed that the *in situ* ELISA was a good method yielding reproducible results.

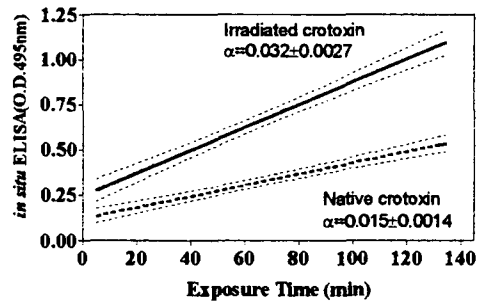

Fig 1- Determination of crotoxin uptake by peritoneal macrophages by *in situ* ELISA. Number express the rate of uptake/min($p<0.001$)

Addition of other proteins such as FBS affected the uptake of both proteins but were more intense in native crotoxin, suggesting that a nonspecific protein-dependent system may be partially responsible for the uptake of both toxins but is the main pathway in the native toxin. (Fig.2).

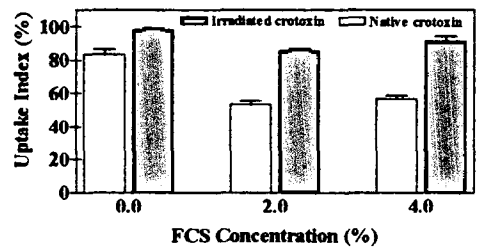

Fig 2. Effect of fetal calf serum concentration in the uptake of irradiated and native toxins by peritoneal macrophages.

Blocking Assays

When macrophages were assayed with toxins and increasing concentrations of probucol, the enhanced irradiated crotoxin uptake was abolished without affecting native crotoxin internalization, in a concentration-dependent manner and with a tendency to decreased ScvR-dependent uptake. Probucol showed no significant changes in native crotoxin uptake, suggesting that this form of toxin is not recognized or internalized by ScvR (Fig 3).

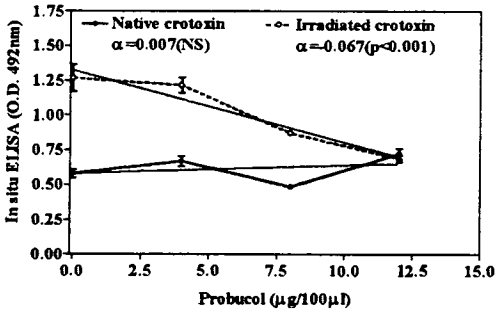

Fig 3- *In situ* ELISA of macrophages assayed with irradiated crotoxin, using probucol as ScvR-specific blocker.

IV. DISCUSSION

Our results indicate that ^{60}Co irradiation of crotoxin induces a time-dependent increased uptake compared to native crotoxin, with few or no changes in the morphology of challenged cells, as observed by immunohistochemistry. When a similar quantitative assay was used, this increased uptake was approximately twice higher and was found to depend on ScvR on the macrophage surface, as suggested by blocking experiments with probucol.. These experiments were adequately controlled and highly reproducible, with small dispersion around the average resulting in greater significance in statistical analysis.

We have studied the biodistribution of irradiated crotoxin in several organs by immunohistochemistry, showing that irradiated crotoxin was present mainly in organs rich in phagocytic mononuclear cells (Cardi, Nascimento & Andrade Jr., manuscript in preparation), as confirmed by the present study.

The uptake of irradiated crotoxin has been proposed to be dependent on the protein aggregation effect that occurs after this treatment, usually resulting in 50 to 60% of aggregates[7] with low toxicity but without losing their immunogenicity[17]. We used the same amount of protein of native or irradiated toxin in our experiments, to minimize this problem, contrary to other studies with detoxification of crotoxin, when large amounts of detoxified protein were used, resulting in greater antigen amounts as compared to native protein[7,8,12].

Furthermore, the same mass of aggregates or native protein presented the same amount of epitopes useful for antigen presentation by specialized cells. In our previous studies, the same amount of irradiated protein was found to be a better or at least a similar immunogen when compared to native protein. Working with an 18 kDa recombinant protein of *Mycobacterium leprae*, native or 2000Gy ^{60}Co irradiated, in an *in vivo* immunization model, we showed that the irradiated protein presented greater immunogenicity in spite of the absence of aggregates[10]. The only explanation is those irradiation results in a higher recognition of antigen by the immunological system and probably the antigen-presenting cells were the target of this action, as shown here.

We have also shown that the rapid uptake of irradiated crotoxin by macrophages is probably due to the presence of ScvR that recognize negatively charged or oxidized macromolecules[18] and were blocked by probucol and dextran sulfate. Gamma radiation could produce oxidation of macromolecules[19] and affects both carbon and sulfur atoms present in radicals such as cysteine, oxidized by irradiation products[20]. The free radicals react to disulphide, $O_2^{\bullet}$ but not $HO_2^{\bullet}$, and free radicals produced by radiolysis of water are capable of oxidizing proteins, as in BSA, where some of the crosslinking is due to formation of disulphide bridges[21]. Upon reduction of the intramolecular disulphide links[22] thiol radicals are formed which partly combine intermolecularly thus forming higher molecular weight material[23]. Aromatic radicals, as two oxidized tyrosyl units combined could lead to the 2,2'-didhydroxibipherin derivatives that are an important pathway in the crosslinking of protein[19]. Other radicals formed involved the oxidation of carbon molecules resulting in negatively charged carbonyl groups. All of these radical groups with negative charges, present in irradiated molecules, could be recognized by the ScvR pathway, facilitating the antigen uptake by these cells. After this facilitated uptake, the metabolism and presentation of the irradiated antigen could be the same, and, as shown here, more molecules are internalized and presented, resulting in better immunogenicity. Abraham and coworkers[24] showed modulation of immunogenicity and antigenicity of proteins after maleylation. These authors also showed that ScvR could recognize changed proteins and suggested this model for vaccine approaches. This fact could represent a selective way for the APC to recognize external antigens, because polymorphonuclear cells and other phagocytic cells produce free radicals in the defense process that

could result in antigens similar to those induced by radiation. This would result in an alternative and saving pathway for antigen presentation, maximizing the response to foreign antigens processed by defense cells, in contrast to the native proteins that are internalized by regular pathways of lower efficiency.

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REFERENCES

[1]. Azevedo-Marques, M.M.,P. Cupo, and S.E. Hering. 1986. Detecção precoce da mioglobina em acidente crotálico humano. *Rev.Soc.bras.Med.Trop. 19S: abs 010.*

[2]. Vital-Brazil, O. 1966. Pharmacology of crystalline crotoxin -II- Neuromuscular blocking action. *Mem.Inst.Butantan. 33: 991.*

[3]. Cardi, B.A., N. Nascimento, J.R. Rogero, and H.F. Andrade Jr. 1992. Immunochemical detection of purified crotoxin from *Crotalus durissus terrificus* venom in motor end plate of striated muscle in CBA/J mice. *Braz.J.Med.Biol.Res. 25: 905.*

[4]. Azevedo-Marques, M.M., S.E. Hering, P. Cupo. 1987. Evidence that *Crotalus durissus terrificus* (South American Rattlesnake) envenomation in humans causes myolysis rather than hemolysis. *Toxicon. 11:1165-1168.*

[5]. Barraviera, B. & M.T.S. Peraçoli. 1994. Soroterapia Heteróloga, In *Venenos Animais: Uma visão Integrada.* Barraviera, B., ed. Ed. Publ.Científicas, São Paulo, pp361-372.

[6]. Guidolin, R., W. Dias da Silva, H.G. Higashi, C.P. Caricati, M.L.S.R. Lima, J.F. Morais, J.R. Pinto, and J.R. Marcelino. 1989. Hiperimunização de cavalos soroprodutores com venenos botrópicos e crotálicos tratados com glutaraldeído. *Mem.Inst.Butantan 51: 85.*

[7]. Nascimento, N., C.S. Seebart, B. Francis, J.R. Rogero & I.I. Kaiser. 1996. Influence of ionizing radiation on crotoxin: biochemical and immunological aspects. *Toxicon 34(1):123.*

[8]. Andriani, E.P. 1994, *Irradiação da crotoxina em solução aquosa: Influência das proncipais espécies reativas nas alterações estruturais, biológicas e imunológicas.* (Master Dissertation - IPEN/CNEN/SP - Brazil).

[9]. Wales, A. and J.R. Kusel. 1992. Biochemistry of irradiated parasite vaccines: Suggested models for their mode of action. *Parasitology Today 8: 358.*

[10]. Pinho, J.R.R., B.A. Cardi, H.F. Andrade Jr, P.J. Barr, I.C. Bathurst, E.J. Vicente, A.C. Schenberg. 1995. Immunogenic properties of the *M. leprae* recombinant 18-Kda antigen purified from *Saccharomyces cerevisiae*; Enhancement of delayed-type hypersensitivity after gamma-irradiation. *Int.J.Leprosy 63(3):381-390.*

[11]. Henriksen, T.; E.M. Mahoney; D. Steiberg. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins. *Proc.Natl.Acad.Sci.USA 78(10):6499-6503.*

[12] Nakazone, A.K., J.R.Rogero and J.J.Gonçalves. 1984. Crotoxin -I- Immunology and interaction of the subunits. *Braz.J.Med.Biol.Res. 17:119.*

[13]. Laemmli, U.K.. 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227:680-685.*

[14]. Towbin, H. and J. Gordon. 1984. Immunoblotting and dot immunobinding: current status and outlook. *J.Immunol.Meth. 72:313.*

[15]. Adams, D.O. 1979. Macrophages, In *Cell Culture.* W.B. Jakoby, and I.H. Pastan eds. *Meth.Enzymol. 58: 494.*

[16]. Yamamoto, A., S. Takaichi, H. Hara, O. Nishikawa, S. Yokoyama, T. Yamamura and T. Yamaguchi. 1986. Probucol prevents lipid storage in macrophages. *Atherosclerosis 62:209.*

[17]. Baride, R.M., S.D. Jain and B.B. Gaitonde. 1980. Biochemical studies on the toxoids of venoms of poisonous Indian snakes. *Indian J.Med.Res. 72: 571.*

[18]. Goldstein, J.L., Y.K. Ho, S.K. Basu and M.S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc.Natl.Acad.Sci.USA 76(1):333.*

[19] Bugota, G and A.M. Dancewicz. 1983. Radiolytic and enzymatic dimerization of tyrosine residues in insulin, ribonuclease, papain and collagen. *Int.J.Radiat.Biol. 43:249.*

[20]. Al-Thannon, A.A., J.F. Barton, J.E. Packer, R.J. Sims, C.N. Trumbore and R.V. Winchester. 1974. The radiolysis of aqueous solutions of cystein in the presence of oxygen. *Int.J.Radiat.Phys.Chem. 6:233.*

[21]. Schessler, H. and H. Shilling. 1984. Oxygen effect in the radiolysis of proteins. Part 2. Bovine serum albuminates *Int.J.Radiat.Biol. 45:267.*

[22]. Schessler, H. and J.V. Davies. 1983. Radiation-induced reductions with bovine serum albuminates. *Int.J.Radiat.Biol. 43:291.*

[23]. Schessler, H. and K. Freundl. 1983. Reactions of formate and ethanol radicals with bovine serum albumin studied by electrophoresis. *Int.J.Radiat.Biol. 44:1729.*

[24]. Abraham, R., N. Singh, A. Mukhopadhyay, S.K. Basu, V. Bal, S. Rath. 1995. Modulation of Immunogenicity and Antigenicity of Proteins by Maleylation to Target Scavenger Receptors on Macrophages. *J. Immunol.* 154:1-8.