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INFLUENCE OF IONIZING RADIATION ON CROTOXIN: BIOCHEMICAL AND IMMUNOLOGICAL ASPECTS

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N. D. Nascimento, C. S. Seebart, B. Francis, J. R. Rogero and I. I. Kaiser. Influence of ionizing radiation on crotoxin: biochemical and immunological aspects. *Toxicon* **34,** 123–131, 1996.—Irradiation of crotoxin and its subunits with 2000 Gy of γ-rays from ^{60}Co source leads to aggregation and generation of lower mol. wt breakdown products. Aggregates separated by gel filtration retain at least part of their higher-ordered structure, based on their reactivity with monoclonal antibodies known to react with conformational epitopes in native crotoxin. These same aggregates can serve as antigens to raise antisera that cross-react and neutralize crotoxin. Compared with native crotoxin, aggregates appear less myotoxic, are largely devoid of phospholipase activity, and are virtually non-toxic in mice. These results indicate that irradiation of toxic proteins can promote significant detoxification, but still retain many of the original antigenic and immunological properties of native crotoxin.

INTRODUCTION

Venoms in general are weakly immunogenic, yet fairly toxic (Magalhães, 1986). This causes problems because serotherapy is the treatment of choice in snakebite accidents, and horse antisera availability is dependent upon venom immunogenicity. To improve antisera production and extend the useful life of immunized horses much effort has been devoted to decreasing chronic venom toxicity. Several techniques have been employed to detoxify venoms, including ultraviolet rays (Tejasen and Ottolenghi, 1970), formaldehyde (Grasset, 1945), carboxymethylcellulose (Moroz *et al.*, 1963), X-rays (Flowers and Goucher, 1965), photooxidation (Kocholaty *et al.*, 1968), glutaraldehyde (Guidolin *et al.*, 1989), iodination (Daniel *et al.*, 1987; Heneine *et al.*, 1986) and gamma rays (Salafranca, 1973; Kankonkar *et al.*, 1975; Herrera *et al.*, 1986). Ionizing radiation appears promising as a venom detoxification tool. Proteins irradiated in either the dry state or in solution undergo poorly characterized chemical and physiochemical changes that can alter their primary, secondary or tertiary structures, without adding new immunological substances that may also act as antigens in the process and keeping the immunological properties intact. Murata *et al.*

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(1990) used crude venom from *Crotalus durissus terrificus* and showed that 2000 Gy was a good compromise in irradiation dosage for venom solutions, which promoted significant venom detoxification, yet maintained many of the venoms original immunological properties, as tested in mice, rabbits and horses. We have obtained results consistent with this using purified crotoxin, and have reported these findings in a preliminary report (Nascimento *et al.*, 1993).

Crotoxin is 23,000 mol. wt protein, and was used in these studies because it represents the most toxic component found in *C. d. terrificus* venom, and in different venom samples may represent up to 75% of the total dry weight. Intact crotoxin is composed of two non-covalently linked subunits and acts both presynaptically and postsynaptically at neuromuscular junctions (Hawgood and Smith, 1977; Chang and Lee, 1977; Bon *et al.*, 1979), although its primary mode of action is presynaptic (see review by Bon *et al.*, 1989). Crotoxin subunits are both less toxic than intact material, with the acidic subunit being non-toxic and the basic subunit showing only about one-tenth the toxicity of intact crotoxin (Horst *et al.*, 1972).

In this study we describe the effects of ionizing radiation on crotoxin and its subunits, with an emphasis on the subsequent biological properties and immunogenicity of the aggregates formed during irradiation.

MATERIALS AND METHODS

Crude air dried venom from the South American rattlesnake, *C. d. terrificus*, was purchased from the Instituto Butantan (São Paulo, Brazil). Monoclonal antibodies used were raised against the basic subunit of crotoxin (Kaiser and Middlebrook, 1988). Mice used in the experiments were obtained from the colony housed in the Department of Molecular Biology at the University of Wyoming.

Crotoxin purification

Briefly, crude venom (150 mg) was dissolved in 4 ml of 0.1 M acetic acid (pH 3.0) at room temperature and clarified by centrifugation (10,000 × *g*, 10 min at 4°C). Solubilized protein was applied to a 2.5 × 80 cm column of Sephadex G-75 previously equilibrated with 0.1 M acetic acid, eluted, and the absorbance monitored at 280 nm. Appropriate fractions were collected, pooled and lyophilized. Crotoxin initially separated by Sephacryl S-200 at pH 4.0, followed by anion-exchange chromatography using the procedure of Aird and Kaiser (1985), gave material that had properties indistinguishable from the crotoxin used in this work.

Protein irradiation

Purified protein was dissolved in saline solution (0.15 M NaCl adjusted pH 3.0 with concentrated HCl) at a concentration of 2 mg/ml, as determined by the Bradford method (Bradford, 1976). This solution was irradiated with 2000 Gy using gamma rays derived from a ^{60}Co source Gammacell 220 (Atomic Energy Agency of Canada Ltd) in the presence of O_2 at room temperature, with dose rate of 554 Gy/hr. After irradiation, the protein was dialyzed (3500 mol. wt cut-off) against distilled water and lyophilized.

Isolation of aggregates

Crotoxin (15 mg) was irradiated, diluted with 2 ml saline solution, and passed over a column (3 × 98 cm) Sephacryl S-100 equilibrated and eluted with 0.1 M acetic acid (pH 3.0). Absorbance was determined at 280 nm and fractions corresponding to aggregates were pooled, dialyzed against water and lyophilized.

Electrophoresis of purified fractions

Purified fractions were submitted to analytical sodium dodecyl sulphate (SDS)–PAGE, using the procedure of Aird and Kaiser (1985). Samples, at 5 μg concentration, were treated (2–5 mM, SDS; 2.2 mM Na_2 EDTA; 0.03% Bromophenyl Blue), heated to 90°C for 5 min and applied to a 15% resolving gel with a 4.5% stacking gel. Electrophoresis was carried out at 35 mA for 3.5 hr. After fixing overnight in 5% acetic acid, the gel was silver stained using the procedure provided by the manufacturer (Pierce, U.S.A.).

Lethality assays

Lyophilized toxins were dissolved in phosphate buffered saline (PBS) (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 140 mM NaCl at pH 7.4), containing 1% of bovine serum albumin. Injections were made i.v. (Aird and Kaiser, 1985) in either of the dorsolateral caudal veins, in 20–40 g mice, placed in a 37°C incubator for 3–5 min prior to injection to induce vasodilation. Survival was determined after 24 hr and the data were analyzed by the Spearman–Karber Method (WHO, 1981).

Phospholipase assay

Phospholipase activity against egg yolk L-α-phosphatidyl choline was determined using a Radiometer pHM82 pH meter equipped with a TTT 80 titrator, an ABU 80 auto burette and a TTA 80 titration assembly. Fatty acids released by the reaction were titrated to pH 8.0 with 0.04 M NaOH under nitrogen at 37°C in an unbuffered reaction mixture.

Substrate was prepared using 90 mg of L-α-phosphatidyl choline, dried under nitrogen, and then suspended by sonication in 9 ml of 12.5 mM Triton X-100 (estimated mol. wt of 646), using a Virsonic Cell Disrupter, Model 16-850 (Virtis Company, Gardener, NY, U.S.A.). When the suspension was complete, 1.0 ml of 0.1 M $CaCl_2$ was added and the substrate–$CaCl_2$ mixture was then briefly resonicated. This yielded a roughly 2:1 molar ratio of Triton X-100:phospholipid. Phospholipase activity was assayed in 1.25 μg protein/2 ml of substrate/20 min. Activity was expressed in μmoles of fatty acids released/min/mg of protein.

Purification of the subunits from crotoxin

Crotoxin subunits were isolated by ion-exchange chromatography using FPLC, essentially as described (Aird *et al.*, 1990). For the basic subunit this consisted of dissolving crotoxin previously purified on Sephadex G-75 and lyophilized, in 50 mM Hepes buffer (pH 8.0) and applying to a 0.5 × 5 cm Mono Q column in about 10 mg aliquots. Bound basic subunit was eluted with a 0 to 1 M NaCl linear gradient in Hepes buffer (pH 8.0)–6 M urea.

Acidic subunit was isolated from crotoxin previously purified on Sephadex G-75 by dissolving the sample (about 10 mg per run) in 50 mM Tris–HCl (pH 7.2)–6 M urea and applying to a 0.5 × 5 cm Mono S column. Elution was carried out with a 0 to 0.5 M NaCl linear gradient in Tris–urea. Protein elution was detected by monitoring the absorbance at 280 nm.

Production of antibodies

Male BALB/c mice were bled 1 day before immunization to obtain pre-immunization sera. Lyophilized protein was dissolved in PBS and emulsified with an equal volume of complete Freund's adjuvant to yield 50 μg protein in 200 μl, which was injected intradermally at four dorsal sites in mice weighing about 30 g. After 1 week, the same amount of protein was injected, but alum was used as an adjuvant. A similar booster was performed 15 days later. Blood was collected at weekly intervals and antibody concentration determined by an enzyme linked immunoassay (ELISA). Six weeks after the first immunization, mice were challenged with either 15 or 46 LD_{50} of native crotoxin i.p., corresponding to 0.9 or 2.76 μg/g of the animal, respectively.

Enzyme-linked immunoassays (ELISA)

ELISAs were performed on Immulon II, microtiter plates (Dynatech). Wells were coated with either antigen or antibody at a concentration of 1 μg/ml in coating buffer (35 mM $NaHCO_3$, 15 mM Na_2Co_3 and 0.02% NaN_3 at pH 9.6) overnight at 4°C, washed with PBS containing 0.5% Tween-20 several times, and free sites were blocked by 1% gelatin in PBS for 1 hr at room temperature. On antigen-coated plates, mouse antisera obtained from the immunization schedule described above were initially diluted 600 times (as indicated), then serially diluted in 2-fold increments, and incubated at room temperature for 1 hr. After one wash, Sigma goat anti-mouse IgG peroxidase conjugate, diluted 1:1000 in wash buffer was added and incubated for 2 hr at room temperature. After a final wash, the chromogen 2,2′-azinobis (3-ethylbenzthiazoline sulfonic acid) at a concentration of 1 mg/ml in 50 mM citric acid (pH 4.0) with 0.03% H_2O_2 added immediately before use. Absorbances were read after 10 min at 415 nm in a Titertech Multiskan MC (Flow Labs; McLean, VA, U.S.A.).

On monoclonal antibody-coated plates, the coatings and washings were essentially as described above. The second sandwich component, the antigen, was initially at 2 μg/ml with subsequent sequential 5-fold dilutions. This was followed by rabbit antisera raised to the basic subunit of crotoxin diluted 1:1000 with PBS and finally Sigma goat anti-rabbit IgG peroxidase conjugate diluted 1:1000 in wash buffer. After washing, color was developed as described above.

Creatine kinase (CK) release assay

Groups of three animals were injected with 50 μg of either native crotoxin, irradiated crotoxin, native basic, irradiated basic, aggregates or non-aggregated irradiated material, in 100 μl saline (0.15 M NaCl). Toxins were injected into the soleus muscle and at different times following injection, the animals, under anesthesia effect,

were bled by orbital plexus. After 30 min at 37°C, the blood was centrifuged for 10 min at 12,000 rpm to separate the sera.

CK release assay was essentially as described by Sigma (Catalog no. CPK-1), except that volumes used were reduced to one-half the values given in the instruction sheet so that the final reaction volume was 5 ml. CK activity was determined from the standard calibration curve for creatine run in parallel with the sera samples. Sera samples were diluted from 1:10 to 1:50 with 0.15 M saline so that they fell within the calibration curve extremes. Absorbances were read at 520 nm in a Gilford 240 spectrophotometer.

RESULTS

Crotoxin purification

Our initial gel filtration run used 0.1 M acetic acid at pH 3.0 for solubility purposes; it is on the acidic side of the *p*Ka of both subunits (acidic subunit *p*Ka $\approx$ 3.5; basic subunit *p*Ka $\approx$ 9.5). Since both subunits should be positively charged under these conditions, they probably exist as dissociated subunits during the chromatography process. Their molecular dimensions are apparently close enough that no appreciable separation between the two is achieved. Redissolving the material in preparation for anion-exchange chromatography, following dialysis and lyophilization, would permit subunit reassociation. Chromatography on Mono Q gave two major peaks which both had associated crotoxin activity.

Basic and acidic subunits were isolated from purified crotoxin by cation and anion-exchange chromatography, respectively, in the presence of 6 M urea as described in Materials and Methods.

Irradiation of purified crotoxin and subunits

Intact crotoxin and its individual subunits were subjected to 2000 Gy of irradiation as described in Materials and Methods. In all cases, irradiated material appeared to retain solubility and subunits were used directly without additional fractionation. Irradiated crotoxin was subjected to gel filtration (Fig. 1) and the material eluting as a sharp peak was identified as aggregated crotoxin. Non-aggregated material was pooled and isolated from the indicated fractions in Fig. 1, and identified as non-aggregated irradiated crotoxin (NAIC).

Analytical SDS–PAGE

Electrophoretic analysis of various native and irradiated samples are shown in Fig. 2. It is clear that irradiation of intact crotoxin and each of its individual subunits leads to

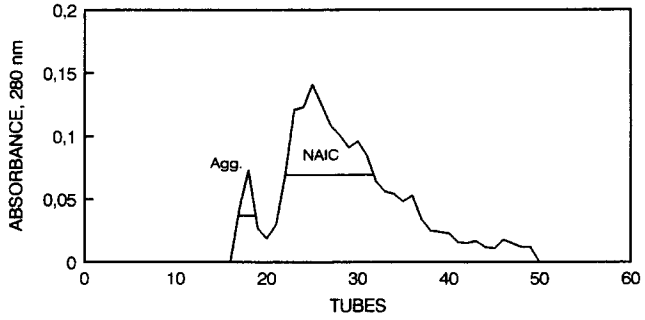

Fig. 1. Aggregated and non-aggregated irradiated crotoxin (NAIC) were separated by chromatography of irradiated crotoxin on Sephacryl S-100 in 0.1 M acetic acid. Fractions (11.4 ml) were collected and absorbancies were determined at 280 nm. Tubes were pooled for aggregates (17 and 18), and NAIC (22 to 31) fractions are indicated.

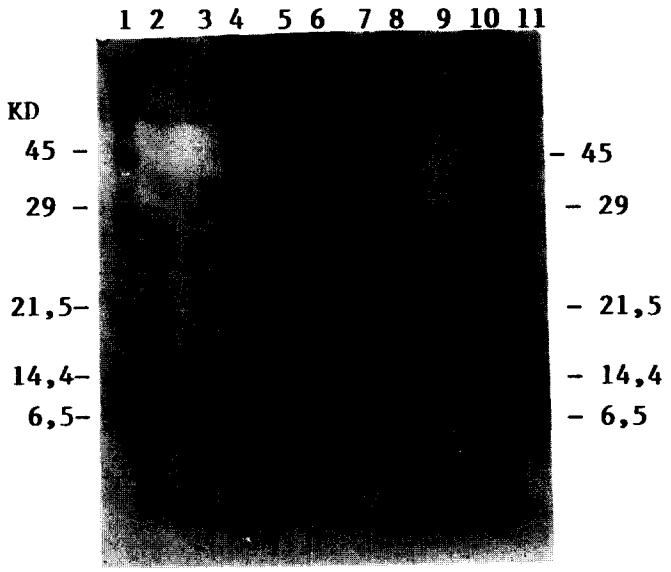

Fig. 2. Silver-stained SDS–polyacrylamide gel (15%) of *C. d. terrificus* crude venom (lane 2), intact crotoxin (lane 3), irradiated crotoxin (lanes 4 and 10), NAIC (5), aggregates (lane 6), native basic (lane 7), irradiated basic (lane 8), and native acidic (lane 9). Prior to electrophoresis, all samples (5 μg) were dissolved in protein-solubilizing solution without DTT. Protein standards (lanes 1, 11) were run with the mol. wts shown in kilodaltons in the figure, and correspond to the following: aprotinin, 6.5; lysozyme, 14.4; trypsin inhibitor, 21.5; carbonic anhydrase, 29; and egg albumin, 45.

aggregation. Highly aggregated material can be partially removed from irradiated samples by gel filtration, although this silver-stained gel indicates that the separation is not complete.

Lethality assays

Toxicity assays for various irradiated and non-irradiated crotoxin samples are shown in Table 1. Unfractionated, irradiated crotoxin shows about one-half the toxicity of native crotoxin. Material identified as NAIC shows lethality similar to native crotoxin, even though there is evidence of aggregation by SDS–PAGE (Fig. 2). Highly aggregated material recovered from Sephacryl S-100 (Fig. 1) shows only a fraction of this toxicity based on LD_{50} values in mice. Reconstitution studies using different subunit combinations suggest that modifications induced in the basic subunit determine toxicity loss.

Phospholipase assays

Irradiated crotoxin exhibits about one-half the phospholipase activity of native crotoxin, when assayed against phosphatidyl choline in Triton X-100. Separation of phospholipase-inactive aggregated crotoxin from NAIC material increases NAIC phospholipase activity to normal values. Irradiation of the basic subunit by itself reduces its phospholipase activity by greater than 90%. Reassociation of the irradiated basic subunit with either native or irradiated acidic subunit reduces its phospholipase activity to about one-half that found in reconstituted crotoxin formed by the combination of native subunits. Reassociation of native basic subunit with either native or irradiated acidic subunit, decreases its

phospholipase activity to about the same extent, indicating that acidic subunit irradiation did not alter its ability to inhibit the phospholipase activity associated with the basic subunit (Table 1).

Creatine kinase release

Creatine kinase release has been used by Gutiérrez *et al.* (1984) as a measure of myotoxicity. When various native and irradiated samples were injected into the soleus muscle of mice and their sera analyzed for creatine kinase activity at 1, 2.5, and 4.5 hr following injection, the values shown in Table 1 were obtained. There is a slight decrease in creatine kinase release in both unfractionated, irradiated basic subunit and irradiated intact crotoxin, relative to their native controls. When non-aggregated irradiated material is removed from the irradiated intact crotoxin sample, its creatine kinase generating activity is reduced even further. These results indicate that the most highly aggregated sample recovered from irradiated crotoxin, is also the weakest myotoxin as measured by creatine kinase release.

Antibody production

Samples of aggregated and non aggregated irradiated material were used to immunize mice. Both antigens generated antisera which recognized native crotoxin in ELISAs to about the same extent. Antisera raised to aggregated material still gave a 50% response in ELISAs at a serum dilution of $1:10^3$ against native crotoxin-coated plates. Under similar conditions, non-aggregated material gave a 50% response at the same dilution as above. By comparison, antisera raised to normal crotoxin in rabbits (Kaiser *et al.*, 1986) gave a 50% response against crotoxin-coated plates at a antiserum dilution of about $1:10^5$ (data not shown). When mice that were immunized and boosted two times over a 4 week period and then were challenged after 6 weeks with (0.9 μg/g) 15 LD_{50} values of native crotoxin

Table 1. Creatine kinase activity, LD_{50} values, and phospholipase activities of crotoxin and its subunits before and after irradiation

Sample	Creatine kinase units*			i.v. LD_{50}†	PLA_2 activity‡
	1	2.5	4.5		
Native crotoxin	300	390	550	0.06	84
Irradiated crotoxin	190	280	360	0.11	39
Non-aggregated irradiated crotoxin	405	550	700	0.06	90
Aggregated crotoxin	190	185	110	>2.5	0
Native basic subunit	190	380	660	0.6	330
Irradiated basic subunit	325	350	480	>2.5	17
Native acidic subunit	ND	ND	ND	>2.5	0
Acidic + Basic	ND	ND	ND	0.08	49
Acidic§ + Basic	ND	ND	ND	0.11	49
Acidic + Basic§	ND	ND	ND	0.22	20
Acidic§ + Basic§	ND	ND	ND	0.22	21
Saline	40	75	35	ND	ND

* Creatine kinase units/ml are reported for 1, 2.5, and 4.5 hr post-injection. One creatine kinase unit is equal to one Sigma unit, which will transform 1 nmole of substrate per min at 25°C. Data represent the average value from three mice per assay and are representative of results from eight independent experiments.
† Reported as μg/g in mice.
‡ μMoles fatty acid released/min/mg protein.
§ Irradiated subunit.
ND, Not determined.

i.p., only one of four animals immunized with the aggregated antigen died. None of the four challenged with 15 LD_{50} of crotoxin that were immunized with non-aggregated irradiated material died. At 46 LD_{50} (2.76 μg/g), four out of four animals immunized with aggregates died; whereas two of four animals immunized with non-aggregated irradiated material died.

Monoclonal antibodies assay

Four monoclonal antibodies raised to the basic subunit of crotoxin (Kaiser and Middlebrook, 1988) were used to compare reactivity of native and irradiated crotoxin. Earlier work indicated that these monoclonal antibodies largely recognized conformational epitopes, since reduction of the basic subunit prior to SDS–PAGE destroyed reactivity of all monoclonal antibodies on Western blots. When native and irradiated crotoxin and its basic subunit were coated on microtiter plates and screened for reactivity against all four monoclonal antibodies, no consistent differences in reactivity were observed (data not shown).

DISCUSSION

Murata *et al.* (1991) showed that irradiation with 2000 Gy of crude venom of *C. d. terrificus* was the ideal irradiation dose, promoting venom detoxification with maintenance of its immunogenicity. From these data, isolated crotoxin, the main toxin from *C. d. terrificus* venom, was tested because the elimination of the other venom proteins reduces the complexity of the irradiated protein mixture.

Irradiation of crotoxin by γ-rays from ^{60}Co (2000 Gy) results in aggregation and the generation of lower mol. wt breakdown products. These products can be largely separated from each other by gel filtration and further characterization of these lower mol. wt breakdown products have not been carried out. The largest aggregates are greatly reduced in their ability to promote CK release when injected into muscle tissue, are largely devoid of phospholipase activity and are virtually non-toxic to mice, relative to native crotoxin. These same aggregates show about the same level of reactivity toward four different monoclonal antibodies raised against the native basic subunit of crotoxin as does native crotoxin. That is, when solutions of protein-A purified monoclonal antibodies were prepared at the same concentrations, and serially diluted in ELISA plates coated with either native crotoxin or aggregated crotoxin, the ELISA titration curves (absorbance at 414 nm vs monoclonal antibody dilution) were virtually identical. This suggests that considerable secondary and tertiary structure is maintained, since the monoclonal antibodies are unable to recognize native basic subunit on Western blots following disulfide bond reduction. Aggregated crotoxin appears highly antigenic and can be used to raise antibodies in mice which cross-react with native crotoxin. Two out of four mice immunized with aggregated immunogen survived a challenge of 15 LD_{50}s of native crotoxin. Initial comparative amino acid analyses and peptide mapping of irradiated and native crotoxin subunits were not able to provide insight into reactive amino acids. These results indicate that irradiated toxic molecules with γ-rays can reduce toxicity and other degradative activities while maintaining enough structure to mimic immunologically the native toxin molecules.

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