PERGAMON

Toxicon 37 (1999) 1131–1141

TOXICON

Toxicity and immunogenicity of *Crotalus durissus terrificus* venom treated with different doses of gamma rays

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Received 20 January 1998; accepted 14 November 1998

Abstract

Crotalus durissus terrificus venom (CDT venom) was irradiated with four different doses of gamma rays (2, 3, 5 and 10 kGy) from a ^{60}Co source and their structural, toxic and immunogenic properties were analysed. Venom irradiated with 2 and 3 kGy were, respectively, 2.7 and 13.5 times less toxic than the native one, whereas the 5 or 10 kGy irradiated venom were at least 100 times less toxic than nonirradiated venom. Irradiated venom with all doses were immunogenic and the antibodies elicited by them were able to recognise the native venom in ELISA. However the toxoid produced with 2 kGy irradiation dose had its immunogenicity improved. Antisera raised against this toxoid had a higher neutralising capacity than those produced against the native venom. Irradiation of venom with 2 kGy dose was the most effective to inactivate the CDT venom toxicity and improve its immunogenicity.

1. Introduction

The major treatment for snake bite victims is serum therapy. This consists of intravenous administration of antivenom serum obtained from immunised animals, usually horses. *Crotalus durissus terrificus* (CDT) venoms are considered

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0041-0101/99/$ - see front matter
PII: S0041-0101(98)00249-9

to be relatively poor immunogens (Scheaffer et al., 1988; Santos et al., 1988). One reason for the difficulty in obtaining a high humoral immune response against the crotalic venom is the presence of immunosuppressive components (Cardoso and Mota, 1997) and the injury suffered by the animals after venom injection. Many times in the past it has been suggested that detoxified venoms can be used to produce antiserum as an effort to protect the animals from the venom toxicity. It is therefore important to be sure that the detoxified venom does not lose its immunogenicity. Several techniques have been used to detoxify venoms, such as mixing the venom with carboxymethylcellulose (Moroz et al., 1963), irradiation by gamma rays (Baride et al., 1980), adding formaldehyde (Costa et al., 1985), controlled iodination (Daniel et al., 1987) and encapsulation of purified toxins in liposomes (Freitas and Frézard, 1997). Usually, the institutions responsible for producing immune sera do not use detoxified venoms due to the putative structural alterations of relevant epitopes that may impair the effectiveness of the antibodies produced against these toxins (Freitas and Frézard, 1997; Angulo et al., 1997). One method that has been shown to be effective for attenuating venom toxicity and maintaining immunogenicity is gamma radiation (Baride et al., 1980; Hati et al., 1990; Murata et al., 1990; Mandal et al., 1993; Rogero and Nascimento, 1995; Nascimento et al., 1996).

Proteins irradiated either in the dry state or in solution undergo chemical and physicochemical changes that can alter their primary, secondary and tertiary structures, while keeping many of their native immunological properties intact (Skalka and Antoni, 1970). Kume and Matsuda (1995), studying the radiation-induced changes in the structural and antigenic properties of egg albumin and bovine serum albumin, suggest that the main part of conformation-dependent antigenic structures (conformational epitopes) is easily lost by radiation, but some antigenicity, which is mostly due to the amino acid sequence-dependent antigenic structures (sequential epitopes) remains, even at high doses.

Preliminary experiments with crotalic venom (Murata et al., 1990) showed that diluted samples of CDT venom irradiated with 2 kGy doses become five times less toxic than the native venom. This irradiated venom had its chromatographic and electrophoretic profiles drastically changed, but preserved its antigenic and immunogenic properties. After irradiation it was observed that higher molecular weight products, probably in the form of aggregates, were formed. The amount of these aggregates increased according to the radiation dose.

When purified crotoxin, the most toxic component of CDT venom, was irradiated with a 2-kGy dose, protein aggregation and generation of lower molecular weight breakdown products were produced. These aggregated components were virtually nontoxic to mice and were used as antigens to produce antiserum. The antiserum obtained with these aggregates could cross-react and neutralise the toxic effects of the native crotoxin (Nascimento et al., 1996).

In this study we describe the effects of increasing different doses of gamma radiation on CDT whole venom in an effort to find out an optimal dose to produce a detoxified venom with large amounts of nontoxic, but still immunogenic aggregates.

2. Material and methods

Crude air dried venom from the South American rattlesnake, *C. durissus terrificus*, was purchased from the Institute Butantan (São Paulo, Brazil). Mice were obtained from the colony housed in the Department of Radiobiology at the IPEN/CNEN-SP (Brazil). The ^{60}Co source Gammacell 220 (Atomic Energy Agency of Canada) was available at the same institute.

2.1. Venom irradiation

Whole CDT venom was dissolved in saline solution (0.15 M NaCl), adjusted to pH 3.0 with concentrated HCl, and its protein concentration adjusted for 2 mg/ml as determined by the Bradford (1976) method. Samples were irradiated at a dose rate of 391 Gy/h with 2, 3, 5 and 10 kGy in the presence of oxygen, at 35°C.

2.2. Gel filtration

Irradiated samples were clarified by centrifugation (12,000*g*, 10 min at 4°C). The protein concentration was determined in the supernatants (soluble protein) according to Bradford (1976). The amount of precipitated protein was estimated by the difference between the initial (2 mg/ml) and final soluble protein concentration. After centrifugation, the soluble proteins from each irradiated sample were applied to a 1.6×90 cm Sephadex G-100 column previously equilibrated with 100 mM ammonium formate and 1.3 ml samples were collected with a flow rate of 11 ml/h. Elution was monitored by absorbance at 280 nm. The relative amounts of soluble aggregate and nonaggregate irradiated venom were calculated by the integration of absorbencies. Fractions were collected, pooled and concentrated in a Speed-Vac Sc-200. The exclusion volume of column (V_o) was calculated with Blue Dextran 2 mg/ml (Sigma Chemical Co, St Louis, MO, USA) and the elution was monitored by absorbance at 280 nm.

2.3. Lethality assays

Irradiated and nonirradiated venoms were dissolved in phosphate buffered saline (0.15 M, pH 7.8), and the LD_{50} was calculated. Injections were made i.p., in 25–35-g mice, according to methodology described by Aird and Kaiser (1985) using four mice per group. Survival was determined after 24 h and the data were analysed by the Spearman–Karber method (WHO, 1981).

2.4. Production of antibodies

Outbred female rabbits, weighing about 3.5 kg, were bled one day before immunisation to obtain preimmunised sera. Two animals were used for each analysed sample. Native and irradiated venom samples (2, 3, 5 and 10 kGy) were

dissolved in PBS (without being clarified by centrifugation) and emulsified with an equal volume of complete Freund's adjuvant to a final concentration of 1 mg/ml. Each animal received a total of 2.0 mg of antigen, which was injected i.d. at four dorsal sites (0.5 mg/site). After two weeks, an additional dose of antigen was injected i.d. using aluminium hydroxide as adjuvant. A third booster without adjuvant was given two weeks later, and six days later the animals were bled and the serum antibody concentration determined by enzyme linked immunossorbent assay (ELISA), performed as described by Nascimento et al. (1996). The antigen–antibody reaction was revealed with peroxidase-labelled anti-rabbit IgG (Sigma Chemical Co., St Louis, MO, USA), followed by orthophenylene diamine plus H_2O_2 as the enzyme substrate. Plates were read using an ELISA microplate reader (Dynatech MR 4000) and the titres are reported as the reciprocal of the highest dilution that causes an absorbance greater than 0.300 at 450 nm. Data are presented as the median of three independent experiments.

2.5. Protective ability of the antiserum

To determine the antiserum neutralisation titre, a constant amount of CDT venom (52 μg/ml) was mixed with different dilution of rabbit antiserum raised against the nonirradiated or irradiated venom. Following incubation at 37°C for 30 min, the mixtures were injected i.p. into mice at a dose of 10 μl/g of body weight. Antisera neutralising capacity (μg toxin/ml antiserum) was calculated as described by Kaiser et al. (1986).

2.6. SDS-PAGE

Native and irradiated venom were clarified by centrifugation and submitted to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using a concentration gradient between 3 and 20% of polyacrylamide. Another SDS-polyacrylamide gel (15%) using only native crude venom was performed for analysis by Western blotting.

Samples containing 30 μg protein were dissolved in HCl/Tris 500 mM, pH 6.8, containing 10% glycerol, 10% SDS, 0.05% Bromophenol Blue, boiled for 5 min and applied to the gel. Electrophoresis was carried out at 20 mA for 8 h. The gel was fixed for 1 h with 12.5% acetic acid and stained with 0.25% Commassie Brilliant Blue R-250. The molecular weight markers were lysozyme 14.3 kDa, trypsinogen 24 kDa, carbonic anhydrase 29 kDa, egg albumin 45 kDa and bovine serum albumin 66 kDa (Sigma Chemical Co., St Louis, MO, USA).

2.7. Western blotting

C. durissus terrificus whole venom submitted to 15% SDS-PAGE was transferred onto a nitro-cellulose paper in buffer, as described by Towbin et al. (1979). The nitro-cellulose paper sheet was incubated with increasing dilution of

rabbit antiserum raised against the native and irradiated (2 kGy) venom at 37°C for 2 h with gentle agitation. The immuno-reactive proteins were detected using peroxidase labelled anti-rabbit IgG and revealed with 0.05% 4-chloro-1-naphtol in 15 vol% methanol, in the presence of 0.03 vol% H_2O_2. Normal rabbit serum was used as control.

3. Results

3.1. Gel filtration

The nonirradiated control venom and the venom submitted to different levels of gamma radiation were subjected to gel filtration (Fig. 1). The irradiated material eluted in the positions corresponding to the main components of the nonirradiated control venom (Fig. 1a: tube 83–123) was pooled and is identified as nonaggregate irradiated venom (NAIV), while the material eluted between the V_o and the NAIV was identified as aggregates. The dose of 5 kGy gamma rays produced more aggregates and destroyed practically all principal components of the venom (Fig. 1d). The dose of 2 kGy maintained part of these toxins intact and produced fewer aggregates (Fig. 1b). Table 1 illustrates the percentage of aggregated and nonaggregated material produced after irradiation of the crotalic venom. The gamma rays dose responsible by the formation of higher percentage of soluble aggregates was 5 kGy, while 10 kGy produced practically total precipitation of soluble proteins.

3.2. SDS-PAGE

Electrophoretic analysis of native venom and venom irradiated with 2, 3 and 5 kGy are shown in Fig. 2. The higher molecular weight components of venom were

Table 1
Precipitate quantification and relative areas (%) of aggregated and nonaggregated fractions obtained by centrifugation and gel filtration (Fig. 1b–e)

Irradiation dose (kGy)	Soluble fraction (mg/ml)	Percentage precipitated (±S.D.)	Percentage of soluble aggregates (±S.D.)	Percentage of NAIV[a] (±S.D.)
0	2.00	0	–	–
2	1.78 ± 0.05	11 ± 2.6	51 (±3.1)	49 (±3.05)
3	1.66 ± 0.19	17 ± 9.5	61 (±1.9)	39 (±1.94)
5	1.37 ± 0.05	31 ± 2.8	88 (±0.38)	12 (±0.36)
10	0.09 ± 0.02	95 ± 1.0	67 (±0.38)	33 (±0.36)

[a] Nonaggregated irradiated venom.

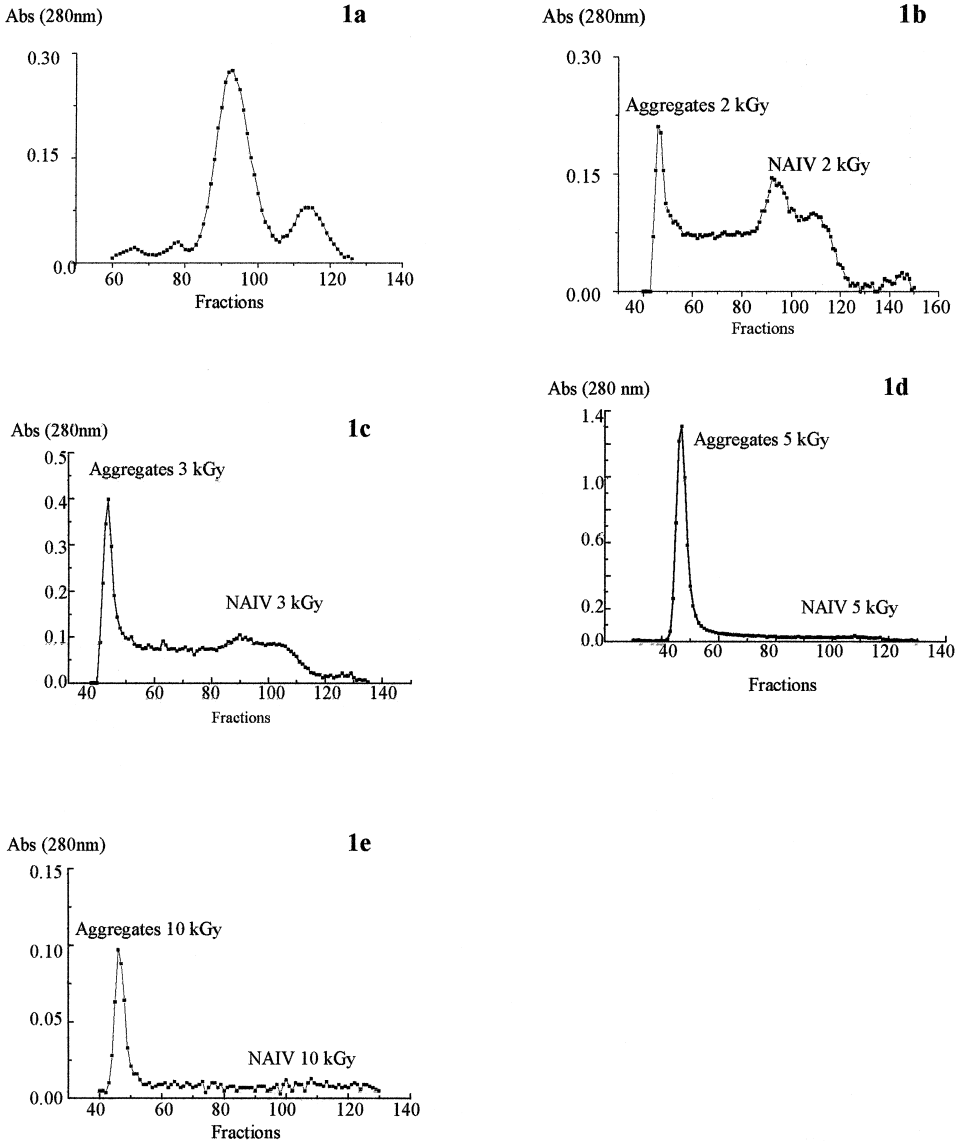

Fig. 1. Gel filtration chromatography of native and irradiated venoms: samples corresponding to native or soluble irradiated venom were applied on Sephadex G-100 in 0.1 M ammonium formate. Fractions (1.3 ml) were collected and absorbance was determined at 280 nm. (a) 4.0 mg of native venom and (b) 6.3 mg of 2 kGy-irradiated venom. Aggregated (fractions 45–85) and nonaggregated irradiated venom (NAIV) components (fractions 86–123). (c) 5.6 mg of 3 kGy-irradiated venom. Aggregated (fractions 41–81) and NAIV components (fractions 82–120). (d) 4.9 mg of 5 kGy-irradiated venom. Aggregated (fractions 43–82) and NAIV components (fractions 83–123). (e) 0.4 mg of 10 kGy-irradiated *crotalic* venom. Aggregated (fractions 43–83) and NAIV components (fractions 84–123).

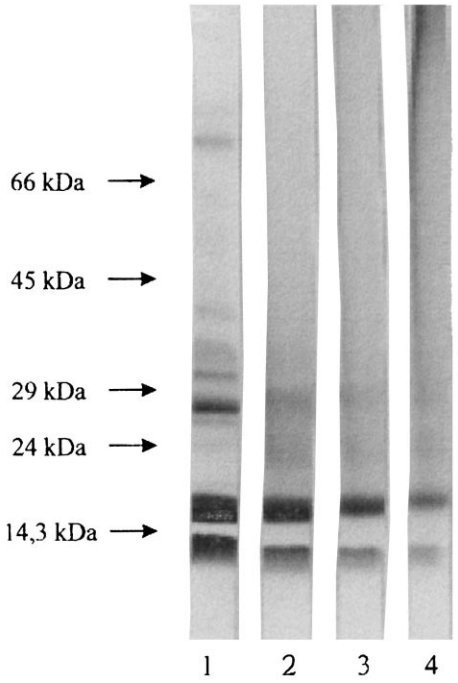

Fig. 2. SDS-PAGE of native and irradiated venoms: Samples of 30 μg of *C. d. terrificus* of nontreated native venom (lane 1), irradiated venom with 2 kGy (lane 2), irradiated venom with 3 kGy (lane 3), irradiated venom with 5 kGy (lane 4) were submitted to SDS-PAGE (3–20%) and bands revealed by Coomassie Brilliant Blue G-250. Numbers at the left correspond to migration of mol. wt. markers.

destroyed after irradiation, but some intact low molecular weight proteins still remained even after irradiation with the highest dose. Venom irradiated with 10 kGy was not submitted to SDS-PAGE due to its practically total precipitation (Table 1).

3.3. Lethality assays

Toxicity assays for the venoms subjected to different doses of radiation are shown in Table 2. The venom irradiated with 2 and 3 kGy were, respectively, 2.7 and 13.5 times less toxic than the native venom, while venom irradiated with either 5 or 10 kGy showed virtually no toxicity.

3.4. Production of neutralising antibodies and Western blotting

All antigens, native and irradiated venom, were immunogenic and the antibodies elicited by them were able to recognise the native venom in ELISA (Table 3). The antiserum against the venom irradiated with 2 kGy and the native venom had higher antibody titres, while the antiserum against venom irradiated

Table 2
Toxic activity of nonirradiated and irradiated *crotalic* venom

Samples	LD_{50} (μg/g)	Relative toxicity (%)
Nonirradiated, control venom	0.148 (0.258–0.085)[a]	100
Irradiated venom (2 kGy)	0.40 (0.76–0.21)	37
Irradiated venom (3 kGy)	2.00 (3.8–1.5)	7.4
Irradiated venom (5 kGy)	> 14.8	< 1
Irradiated venom (10 kGy)	> 14.8	< 1

[a] Figures in parentheses are 95% confidence limits.

with 3, 5 and 10 kGy had lower antibody titres. Only the anti-2 kGy irradiated venom and the anti-native venom sera were effective in neutralising the lethality induced by the crude venom at the tested dose (300 μl of antiserum). However, we observed that 1.0 ml of the antiserum prepared with 2 kGy irradiated venom could neutralise 297 μg of native venom, whereas 1.0 ml of the antiserum prepared with native venom could neutralise only 165 μg. It is important to note that by Western blotting analysis, the sera raised against the native venom and the 2 kGy-irradiated venom recognised equally all the bands present on the native crotalic venom, but the ELISA titre of recognition for each toxin was higher for the serum anti-2 kGy irradiated venom than for the serum anti-native venom (Fig. 3).

4. Discussion

Studying the influence of ionising radiation on purified crotoxin, Nascimento et al. (1996) showed that a 2 kGy dose of gamma-rays kept the material totally

Table 3
Ability of antiserum raised with native or irradiated venom to neutralise the toxic activity of *crotalic* venom and ELISA titres of rabbit anticrotalic venom serum

Antisera obtained with	ASNC[a]	V_{50} [b]	Antibody titres
Native venom	165.0	225	1/48,000
Irradiated venom (2 kGy)	297.6	125	1/98,000
Irradiated venom (3 kGy)	ND	> 300	1/12,000
Irradiated venom (5 kGy)	ND	> 300	1/24,000
Irradiated venom (10 kGy)	ND	> 300	1/12,000

[a] Antisera neutralising capacity (μg of venom/ml of antiserum) calculated according to Kaiser et al., 1986.
[b] Volume of antiserum (μl) which reduces the lethality of a 1 ml venom injection solution (52 μg/ml) by 50%.
ND means not determined.

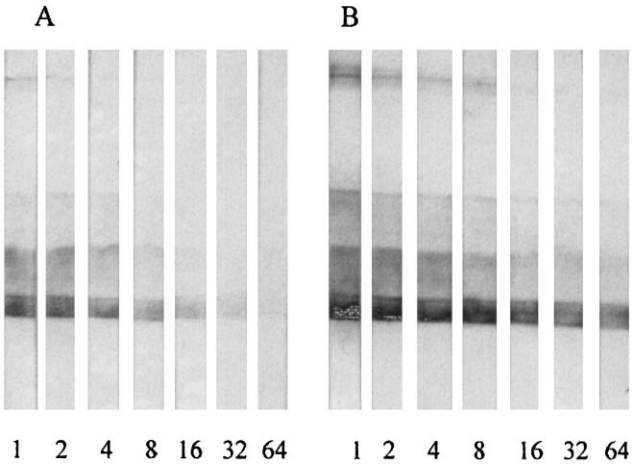

Fig. 3. Titration by Western blotting of antiserum raised against native and irradiated venom: samples of 30 μg of native venom were submitted to Western blotting and revealed by crescent dilutions of rabbit anti-native venom (A) or anti-irradiated venom with 2 kGy (B). The numbers at bottom correspond to the reciprocal of serum dilution ($\times 10^3$).

soluble and susceptible to separation by gel filtration. This procedure resulted in two distinct peaks, denominated respectively as 'aggregated' and 'irradiated crotoxin without aggregates — (ICWA)'. Comparison of native crotoxin with ICWA, showed that the largest aggregates although having several of their biological activities reduced (CK release, phospholipase activity and lethality), were still immunogenic. These results suggested that the aggregates of crotoxin could be an ideal immunogen to be used to obtain snake antivenom with a minimum distress to the animals.

In order to eliminate the step of crotoxin purification and increase the amount of atoxic but still immunogenic aggregates, we used whole *C. durissus terrificus* venom irradiated with crescent doses of gamma rays. After irradiation, we observed the generation of insoluble, soluble aggregated and nonaggregated irradiated venom components (NAIV), in opposite of only soluble aggregated and ICWA, observed when purified crotoxin was irradiated (Nascimento et al., 1996). The 5 kGy dose of gamma rays produced the largest amount of aggregates. Interestingly, the serum raised against 2 kGy irradiated venom had a higher titre of antibodies against the crotalic venom than the serum raised against the native venom. Both antiserum recognised crotoxin when submitted to protein blotting, but the anti-2 kGy serum had a higher neutralising ability than the antiserum raised against native venom. This suggested that some important immunogenic epitopes are intact following low level irradiation. In order to ensure that the improvement in immunogenicity of 2 kGy-irradiated venom was due to the presence of aggregates and not to minor amounts of intact proteins left after irradiation, we produced a antiserum against native venom following an immunisation protocol in which the amount of intact protein injected was similar

to the amount of intact protein present in the 2 kGy irradiated venom. The antiserum obtained in this way had a low ELISA titre and low neutralising ability (data not shown).

Irradiation of proteins in solution is more advantageous than in the dry state, because the dose needed to cause structural modifications in the proteins is lower because of the highly reactive free radicals produced by water radiolyse (Butter et al., 1984). The irradiation of the whole crotalic venom with 2 kGy (under a dose rate of 900 Gy/h) reduced its lethality five times (Murata et al., 1990). In our study, irradiation of the same venom with the same dose, under a dose rate of 391 Gy/h, reduced its toxicity 2.7 times. This discrepancy is probably due to differences in the dose rate of irradiation.

Higher doses of radiation (5 and 10 kGy) destroyed practically all the venom toxicity though keeping its immunogenicity. However, the 2 kGy dose showed to be the best radiation dose to promote venom detoxification with maintenance of its immunogenicity. Thus, our present results suggest that the treatment with gamma radiation is a suitable way to detoxify the crotalic venom without affecting its immunogenicity provided that a proper dose is used.

Acknowledgements

The authors would like to thank Dr. Ivan Kaiser and Dr. Ivan Mota for critically reading the manuscript and Dr. Wilson Ap. Calvo and his assistants for their technical support in the irradiation experiments. This work was supported by a grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

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