COMPARATIVE STUDIES OF ANTISERA AGAINST DIFFERENT TOXINS

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INTRODUCTION

Snake venom main components are proteins and peptides, many of them presenting enzymatic and/or toxic activities. Neutralization of these activities is one of the goals of envenomation treatment and this is usually achieved by mean of serotherapy. Snake venom antisera are produced in horses and, due to high toxicity of the immunogens, about 10% of the animals die after prime injection. Previous works of our group have shown gamma rays to attenuate snake venom toxicity without affecting its immunological properties, inducing an enhanced humoral and cellular immune response without the deleterious effects of native venom [1] [2] [3]. The venom attenuation induced by irradiation is a result of interaction of the venom components with the free radicals formed by water radiolysis, mainly hydroxyl radical and hidrated electron. These radicals act removing hidrogens, breaking S-S bonds or promoting deamination. As a result of the interaction of these radicals and others lesser known with the venom components, larger molecular weight aggregates are formed, with few if any toxic or enzymatic activity but presenting epitopes that induce a protective response when the irradiated venom is employed as immunogen [3]. These facts point toward venom irradiation as a solution to improve sera production. de Paula *et al.*[4], when immunizing rabbits with irradiated crotoxin, South American rattlesnake's main toxin, obtained polyclonal antibodies specific for the *Crotalus* genus and with high neutralizing capacity. These immunoglobulins proved to be useful as diagnostic and therapeutic agents. *C.d.terrificus* neurotoxic venom is composed by few protein fractions, mainly crotoxin, while the Brazilian most prevalent snake on what refers to accidents (85%), *Bothrops sp*, venom has proteolytic and coagulant activities, with several proteins and fractions involved, with phospholipase activity and minor neurotoxicity, serotherapy being the main treatment. These activities generate intense local signs in animals used in antisera production, with losses and low efficiency. In the present work, we detoxified *Bothrops jararaca* venom by mean of gamma radiation, immunized rabbits with the obtained toxoid and investigated the

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raised antisera efficiency against toxicity, cross-reactivity with other snake venoms from the Elapid and Crotalid group and neutralization of phospholipase A_2 activity which, as suggested by other authors [5], is an indicator of antisera potency.

MATERIAL AND METHODS

Venom Irradiation

Bothrops jararaca whole venom, purchased from Instituto Butantan, was dissolved in 150mM NaCl to a 2mg/ml final concentration and irradiated at room temperature and atmosphere with 2kGy of ^{60}Co radiation, at a dose rate of 480Gy/h in a Gammacell 220 (Atomic Energy of Canada Ltd, Canada) device.

Toxicity

Toxic activity was determined in mice by lethal dose 50% (LD50) and calculated by the Spearman-Karber method as preconized by WHO [6].

Immunization

Three month old male New Zealand rabbits were immunized with irradiated *B. jararaca* venom. Prime injection was of 1mg in complete Freund adjuvant. The second was of the same venom amount in incomplete adjuvant and the booster consisted of 1mg toxoid in saline solution.

Enzyme Linked Immuno Sorbent Assay (ELISA)

96 wells microplates were coated with 10μg/ml of either *B. jararaca, B. neuwiedi, B. cotiara, B. alternatus, B. moojeni, B. jararacussu, L. muta, M. frontalis, C. d. terrificus, C. d. cumanensis* and *C.d. terrificus* PLA_2 standard (Sigma Chemical Co, St. Louis, U.S.A.). Serial dilutions of the sera were assayed against those venoms. Reaction was developed by 1:2000 horse radish peroxidase coupled goat anti-rabbit IgG (Sigma), 0.02% OPD (orto-phenyldiamine) and 0.02% hidrogen peroxyde. The reaction was interrupted with 200mM citric acid. Optical densities were determined at 450nm in microplate reader (Dynatech MR5000). Negative controls consisted of rabbits serum collected before immunization. For comparative cross reaction between each venom, multiple sera dilution was performed, revealing that the best serum dilution to achieve 0.5 O.D. in ELISA was 1/2000, when reacting with autologous venom. The quantitative crossreactivity was expressed as percentage of autologous venom.

IgG Purification

IgGs were purified from whole antisera by affinity chromatography in Protein-A Sepharose medium (Pharmacia, Uppsala, Sweden), according to a low salt schedule [7].

Western Blot

30μg of the same venoms as above were submitted to SDS-PAGE (15% acrylamide) under non-reducing conditions and transferred to nitrocellulose membrane (0.45μm pore; Sigma). After blocking with 1% BSA, the membrane was allowed to react with 20μg of the

purified IgG for 2 hours at room temperature and incubated with peroxydase conjugated anti-rabbit IgG. The reacting bands were revealed with incubation with diaminobenzidine 1mg/ml and H_2O_2 0.01% in citrate buffer. Reaction was intensified using cobalt chloride as enhancer. The proteins remaining in the overloaded separating gel were stained with Coomassie Brilliant Blue 250-R (Sigma).

Phospholipase A_2 Activity

This enzymatic activity was assayed by indirect hemolysis as described by Guttierrez *et al* [5] with a slight modification: instead of sheep erythrocytes, mice red blood cells were employed. Previous experiments have shown that this little change had no effect on the assay sensitivity [8]. 0.3µg of each venom were incubated with either phosphate buffered saline, pure or 1:10 diluted antiserum and applied to 2mm wells punched on a glass plate covered with a mixture of 1.2% fresh washed mice erythrocytes, 1.2% of 1:4 diluted egg yolk and 10mM calcium chloride in 0.8% agarose medium. Phosphate buffered saline was used as negative control. The diameter of the hemolysis haloes were measured after 24 hours incubation at 37°C.

RESULTS

Irradiation resulted in a 6.5 folds toxicity attenuation (table 1).

On what refers to immunization, antibodies titers, assayed by ELISA were similar to those obtained when immunizing the animals with native venom (data not shown). However, local signs following injection,when present, were discrete, suggesting once again attenuation of venom activity. Immunoenzymatic assay indicates similar immunoreactivity for all venoms assayed excepting *Micrurus* venom which presented lower reactivity.

Table 1. Toxicity of native and irradiated samples as determined by Guarnieri [2]

Samples	LD50 (mg/kg)	Relative toxicity
Native venom	1.94 (1.52-2.48)	1
Irradiated venom	12.55 (9.82-16.04)	6.5

Table 2. Quantitative crossreaction between B.jararaca irradiated venom antiserum and other snake venoms, as determined in ELISA

Venoms	% of Autologous venom
B. jararaca	100
B. neuwiedi	98
B. cotiara	97
B. alternatus	99
B. moojeni	96
B. jararacussu	96
L. muta	94
M. frontalis	30
C. d. terrificus	70
C. d. cumanensis	97

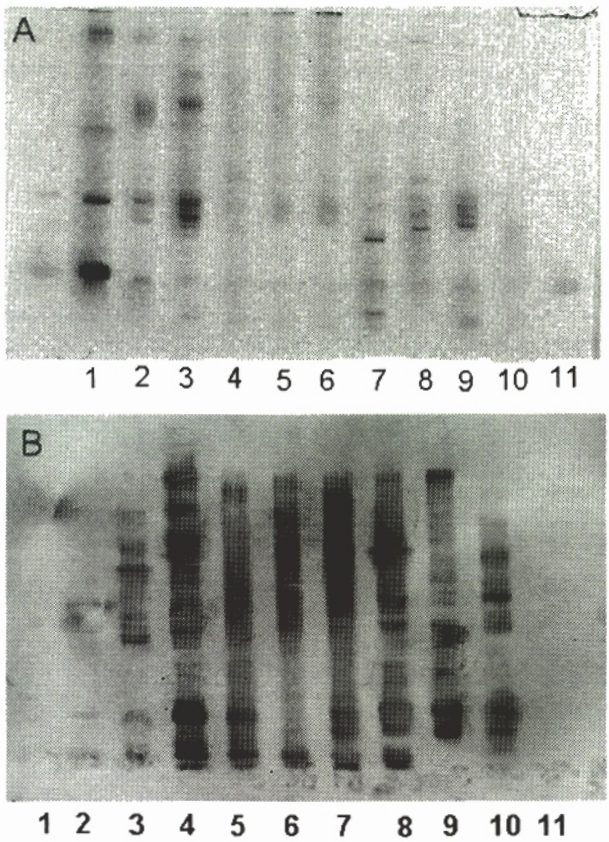

Figure 1. A. SDS-Page of the Assayed Venoms. B. Western Blot of the Assayed Venoms. Lane 1= C.d.terrificus, 2- C.d.cumanensis, 3- B.jararaca, 4- B.neuwiedi, 5- B.cotiara, 6-B.alternatus, 7-B.moojeni, 8-B.jararacussu, 9-L.muta, 10-M.frontalis, 11-C.d.terrificus Phospholipase A_2.

Table 3. PLA_2 Activity Neutralization

	Hemolysis halo (cm)		
Venoms	Pure Ab	1:10 diluted Ab	PBS
B. jararaca	0	0.80	1
B. neuwiedi	0	0	0
B. cotiara	0	0	0
B. alternatus	0	0	0
B. moojeni	0.65	0.75	0.80
B. jararacussu	1.10	1.20	1.20
L. muta	0.40	0.60	1
M. frontalis	2	2	2
C. d. terrificus	1.10	1.20	1.30
C. d. cumanensis	0	0	0
PLA_2 (standard)	1.10	1.20	1.20

Western blot data (Figs. 1a & 1b) indicate a similar immunoreactivity of all bothropic venoms, excepting *B. jararacussu* which presented lower detectable reactivity in the low molecular weight region at the end of the membrane. This might be due to species-specific myotoxins which may not cross-react since *B. jararaca* venom does not contain such compounds, although these myotoxins are structurally close to phospholipase A_2 with low molecular weight [9].

When assaying PLA_2 activity neutralization (table 3), the antisera neutralized efficiently the autologous venom, while venoms from other species of the same genus were only partially neutralized. Other bothropic venoms (*B. neuwiedi, B. cotiara and B. alternatus*) presented no detectable PLA_2 activity although previous standardization assays demonstrated this assay to be quite sensitive *C.d. terrificus* venom was not neutralized by the antibodies tested, neither did standard phospholipase or elapidic venom. Curiously, *L. muta* venom was almost neutralized.

DISCUSSION

The detoxification induced by gamma rays in Bothrops venom was less efficient as than the one described for *C.d.terrificus* venom. This fact could be ascribed to the diversity of fractions present in the bothropic venom, associated with a less specific activities of some of the components. Other explanation is the presence of small peptides and proteins that could protect and compete with the main components for the free radical generated by radiation. The toxoid, despite its relatively high toxicity, was able to induce an antisera in rabbits that presents a good protective activity against the original venom. ELISA and Western Blot analysis indicate quantitative and qualitative high level of cross-reactivity of all the venoms assayed against this antibody, suggesting shared antigenic determinants in most venoms, as expected by the close relationship of the tested snakes. Phylogenetically more distant snake venoms presented less cross reactivity but almost all presented antigens recognized in the molecular weight range of PLA_2 enzymes. When the direct inhibitory action of this antisera was tested against this enzymatic activity of each venom, it efficiency was not equal or proportional, suggesting that these conserved cross reacting epitopes are not important for the enzyme activity. Irradiation preserves epitopes that could induce neutralizing antibodies that may be shared by *L.muta* PLA_2, which was almost completely neutralized by the antisera, but others venoms presented the original activity. As suggested by other authors [10] the toxic and the enzymatic site of toxic phospholipases A_2 may be located on different sites of the enzyme molecule and we can not speculate whether the bound antibodies neutralize or not the enzyme toxicity. Further experiments should be done with purified enzymes in order to confirm these findings. As a whole, the irradiation of bothropic venom is an attenuation method that could induce better antisera, that could help to solve the chronic problems of antiophidic sera production.

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