CROSS-REACTIVITY AND PHOSPHOLIPASE A_2 NEUTRALIZATION ABILITY OF ANTI-IRRADIATED *Bothrops jararaca* VENOM ANTIBODIES.

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ABSTRACT

Snake venom toxicity can in part be attributed to the enzymes included in it, so one of the goals of envenomation treatment is to neutralize these enzymes. This is usually achieved by serotherapy, the sera being produced in horses. However, the high toxicity of the venoms employed in the horses immunization schedule restricts the amount of the venom injected and about 10% of the animals die as a result of prime injection. Previous works of our group have shown gamma rays to attenuate snake venom toxicity without affecting its immunological properties. In the present work, we detoxified *Bothrops jararaca* venom, immunized rabbits with the toxoid obtained and investigated cross-reactivity of the antibodies obtained against autologous and heterologous venoms. We also investigated the ability of the IgGs, purified by affinity chromatography, from those sera to neutralize phospholipase A_2, an ubiquous enzyme in animal venoms. Results indicate that venom irradiation leads to an attenuation of toxicity of 84%. Cross-reactivity was investigated by ELISA and Western blot and all venoms were reactive to the antibodies. On what refers to phospholipase A_2 activity neutralization, the antibodies neutralized autologous venoms efficiently and, curiously, other venoms from the same genus were not neutralized, while *Lachesis muta* venom, a remote related specie, was neutralized by this serum. These data suggest that irradiation preserve important epitopes for induction of neutralizing antibodies and that these epitopes are not shared by all venoms assayed.

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, as a result of the immunogens high toxicity, 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]. 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 immunoglobulines proved to be useful as diagnostic and therapeutic agents.

In the present work, we detoxified *Bothrops jararaca* venom by mean of gamma radiation, immunized rabbits with the obtained toxoid and investigated cross-reactivity of the antisera raised against it. We also investigated the ability of the IgGs to neutralize phospholipase A_2 activity which, as suggested by other authors [5], is an indicator of antisera potency.

MATERIAL & METHODS

Venom Irradiation: *Bothrops jararaca* venom, purchased from Instituto Butantan, was dissolved in 150 mM NaCl to a 2 mg/ml final concentration and irradiated at room temperature and atmosphere with 2 kGy of ^{60}Co radiation, at a dose rate of 480 Gy/h in a Gamma cell device.

Toxicity: toxic activity was determined by Guarnieri [2] using lethal dose 50 % following the Spearman-Karber method as preconized by WHO [7].

Immunization: 3 month old male New Zealand rabbits were immunized as follows:

TABLE 1-Immunization Schedule

Time, in days	0	15	30
adjuvant toxoid amount inoculation route	complete Freund adjuvant 1 mg Intramuscular	incomplete Freund adjuvant 1 mg Subcutaneous	saline solution 1 mg Intraperitoneal

Enzyme Linked Immuno Sorbent Assay: 96 wells microplates were coated with 10 µg/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). Serial dilutions of the sera were assayed against those venoms. Reaction was developed by horseradish peroxidase coupled goat anti-rabbit IgG (Sigma) 1:2,000.

IgG purification: IgGs were purified from whole sera by protein-A affinity chromatography, according to a low salt schedule.

Western blot: The same venoms as above were submitted to SDS-PAGE (15% acrylamide) under non-reducing conditions and transferred to nitrocelluose membrane. After blocking with 1% BSA, the membrane was allowed to react with 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.

PLA_2 activity: This enzymatic activity was assayed by indirect hemolysis as described by Gutierrez *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 [7].

RESULTS

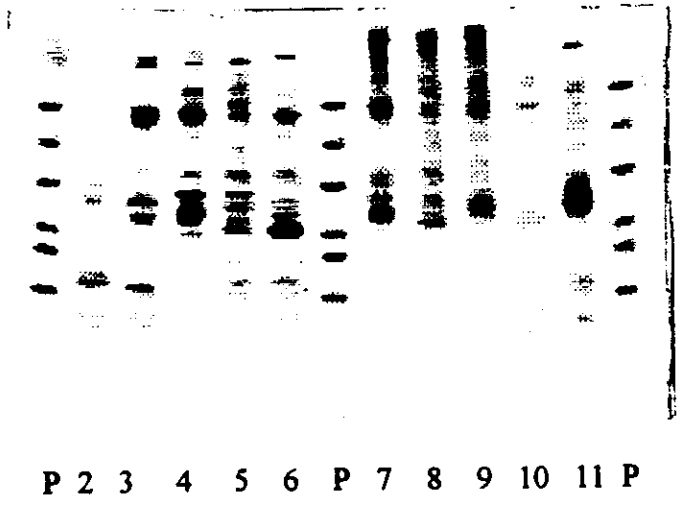
Irradiation resulted in a 6.5 folds toxicity attenuation (table 2).

TABLE 2: Toxicity of the Native and Irradiated Samples.

Samples	LD_{50} (mg/kg)	Relative toxicity
Native venom	1.94 (1.52-2.48)	1.0
Irradiated venom	12.55 (9.82-16.04)	6.5

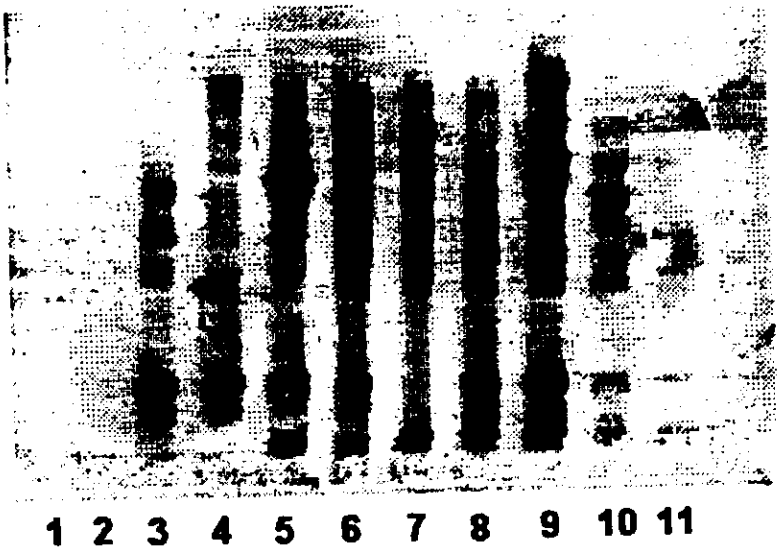
On what refers to immunization, antibodies titers were similar to those obtained when immunizing the animals with native venom (data not shown). However, local symptoms ,when present, were very discrete, suggesting once again attenuation of venom activity. Immunoenzymatic assay indicates similar immunoreactivity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

Western blot data (Figs. 1 & 2) indicate a similar immunoreactivity of all bothropic venoms, excepting *B. jararacussu* which presented no detectable reactivity in the low molecular weight region. 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.



P-Molecular weight standards
2-C. d. terrificus
3-C. d. cumanensis
4-B. jararaca
5-B. neuwiedi
6-B. cotiara
7-B. alternatus
8-B. moojeni
9-B. jararacussu
10-L. muta muta
11-M. frontalis

FIGURE 1- SDS-Page of the Assayed Venoms.



1-PLA_2
2-M. frontalis
3-L. muta muta
4-B. jararacussu
5-B. moojeni
6-B. alternatus
7-B. cotiara
8-B. neuwiedi
9-B. jararaca
10-C. d. cumanensis
11-C. d. terrificus$_2$

FIGURE 2-Western Blot of the Venoms Separated Above.

When assaying PLA_2 activity neutralization, 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, lachetic venom was almost totally neutralized (table 3).

TABLE 3: Neutralization of The Phospholipase A_2 Activity of the Assayed venoms.

Venoms	Hemolysis halo (cm)		
	Pure antibodies	1:10 diluted antibodies	PBS
B. jararaca	0.00	0.80.	1.00
B. neuwiedi	0.00	0.00	0.00
B. cotiara	0.00	0.00	0.00
B. alternatus	0.00	0.00	0.00
B. moojeni	0.65	0.75	0.80
B. jararacussu	1.10	1.20	1.20
L. muta	0.40	0.60	1.00
M. frontalis	2.00	2.00	2.00
C. d. terrificus	1.10	1.20	1.30
C. d. cumanensis	0.00	0.00	0.00
PLA_2 standard	1.10	1.20	1.20

DISCUSSION

ELISA and Western Blot indicate a high level of cross-reactivity of all the venoms assayed suggesting shared antigenic determinants in the cross-reactive proteins, and since the antibodies induced by irradiated venom were able to recognize proteins in the molecular weight range of these enzymes in almost all venoms, we were expecting PLA_2 to be neutralized. However, this did not occur, suggesting that these highly conserved epitopes are not important for the enzyme activity. On the other hand, irradiation preserved neutralizing antibodies inducing epitopes that may be shared by lachetic PLA_2 .However, as suggested by other authors [8] the toxic and the enzymatic site of toxic phospholipases A_2 may be located on different sites of the enzyme molecule. Considering this hypothesis, we can not speculate wether the antibodies neutralize or not the enzyme toxicity. Further experiments should be realized with purified enzymes in order to confirm these findings.

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