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Active Immunization Of Rabbit With Gamma Irradiated Cobra *(Naja haje)* Venom Toxoid

Esmat A. Shaban
Department of Drug Radiation Research and Technology,
Atomic Energy Authority

Abstract

Cobra *(Naja haje)* venom detoxified by gamma radiation (15KGy or 25KGy) was used as toxoid for active immunization of rabbits following a short schedule (on day 0, 7,21) of immunization with complete Freund's adjuvant. Effective neutralization of venom toxin by immune sera of rabbits was observed. The presence of antibody in the immune sera was detected by immuno-diffusion test. The non-irradiated and the two dose levels gamma irradiated *Naja haje* venom, against the antivenin antibody produced with non-irradiated venom or against antivenin antibody produced with 15 KGy gamma irradiated venom all showed similar patterns. Also there was no change in the titer of antivenin solution obtained from the rabbit immunized with 15KGy irradiated venom , as compared with that antivenin solution obtained from rabbit immunized with non-irradiated venom. Sera of rabbits immunized with 15KGy irradiated venom toxoid (15KGy toxoid antiserum) neutralized venom lethality, 95% of protease activity and 50% of phospholipase A2.

Introduction

Cobra *(Naja haje)* is one of the major causes of snake bites death in Egypt. Detoxified venoms can be used to produce antiserum as an effort to protect the animals from the venom toxicity. Detoxification of antigen or toxoid production requires that venom loses its toxicity, but at the same time retains maximum immunogenicity (Khan *et al.,* 1977). Several techniques have been used to detoxify venom such as: treatment with formaldehyde (Khan et al. 1977), glutaraldehyde (Guidolin *et al.,* 1989), and iodine (Rocha *et al.,* 1992). Linking carboxymethyl-cellulose (Moroz *et al*.1963), functional group blockage (Chicheportiche *et al.,* 1972), and by encapsulation in lyosomes (Freitas and Frezard 1997). One method that has been shown to be effective for attenuating venom toxicity and maintaining immunogenicity is gamma radiation (Nascimento *et al.,* 1996). An effective toxoid against Russell's Viper venom was developed by gamma irradiation which yielded a potent antivenom (in rabbits without adjuvant) capable of neutralizing lethal, protease and phosphodiesterase activities of the crude venom (Hati *et al.,* 1990). Shaban (1990) also, developed a toxoid by gamma irradiation against Androctonus amoreuxi scorpion venom which produce a potent antivenom (in rabbits with complete Freund's adjuvant) which neutralize venom lethality as well as some biochemical and pharmacological venom toxicities. Preliminary experiments with *Naja haje* venom (Shaban *et al.,* 1996) showed that the lethality of *Naja haje* venom irradiated in the dry form was not affected up to a dose of 100 KGy. On the other hand, the venom irradiated in the aqueous solution form showed a decrease in lethality while the ability of the venom antigens to react with its corresponding antibodies was retained up to irradiation dose of 50KGy. Effective toxoids against (*Naja haje* and *Cerastes cerastes*) snake venom, were developed by Shaban (2003) using gamma irradiation (15 KGy). The toxoids obtained are devoided of toxicity while retaining their antigenicity.

In the present study immunization of rabbits with gamma irradiated *Naja haje* venom toxoid was carried out The neutralization capacities of the immune sera against lethality, phospholipase and protease activity of the Cobra *(Naja haje)* venom was studied.

Material and Methods

Animals:

Male albino Swiss mice weighing between 18-20g and male rabbits weighing about 3.5Kg were used in this study. Animals were maintained under standard conditions of boarding and given standard food *libitum.*

Venom:

Pooled venom of *Naja haje* (Egyptian Cobra) was obtained from serpentarium of the Medical Research Center, Faculty of Medicine, Ain Shams University extracted from healthy snake by milking, dried and lyophilized kept in desiccators at 4° till used.

Detoxification:

Gamma irradiation of Cobra venom was carried out at the National Center for Research and Radiation Technology, Cairo, Egypt, using cobalt 60 gamma cell 220, manufactured by the atomic energy of Canada (AECL). The radiation dose rate was 1.4 rad per second.

Samples of Cobra venom in solution were subjected to 25 and 15KGy respectively. A non- irradiated sample of crude venom was used as control.

Lethality Assay:

Median lethal dose LD50 of the crude Cobra *(Naja haje)* venom and gamma irradiated venom (15KGy and 25KGy) were determined by i.p. injection into mice according to Spearnam Karber method (WHO1981).

Hyper immunization of rabbits:

Male rabbits weighing 3-3.5 kg were used . The rabbits were injected subcutaneously with venom each dose being emulsified in 0.5ml complete Freund's adjuvant (Defico. Laboratories, Detroit, Mich.) According to the following schedule : one injection of 200 µg in the first, seven and twenty one day respectively . A booster without adjuvant was given one week later. Six days the animals were bled. The same schedule was repeated using 15KGy gamma irradiated venom in two different doses 200µg and 1000 µg as well as 25KGy (200 µg) gamma irradiated venom . Pooled sera of two immunized rabbits for each set of immunization were used.

Immunodiffusion technique:

Immunodiffusion experiment was carried out according to the method described by Ouchterlony and Nelsson (1978) on glass slides (5x5cm) using 1.2% Noble agar (Defico. Laboratories, Detroit, Mich.) in 0.9% Nacl solution. Sodium azide in a concentration of 0.05% was added to retard bacterial growth. The wells were filled with 20 µl volumes of either the rabbit antiserum raised against non-irradiated or 15KGy irradiated venom. After developing of the precipitin bands (48 hrs at 25°c), the plates were washed for 24 hrs in saline dried and stained with amidoschwartz 10 B (0.5% in 5% acetic acid) for 7 min. washed with methanol acetic acid (9:1), dried in air and photographed.

Titer determination:

Using double diffusion experiments, the titer of the antibody from the animal, immunized with 15KGy irradiated venom, was compared to that of the animal immunized with non-irradiated venom.

A serial dilution of the antivenin, to be tested, was obtained using 0.9% Normal saline, as diluents. 20µl of *Naja haje* venom solution of 20mg/ml, was placed in the central well while in the six peripheral wells serial dilutions of the antivenin solution to be tested, were placed. After the incubation of the slides (20- 72 hrs), they were treated as Ouchterlony.

The titer of each sample is defined as: the reciprocal of the highest dilution giving a positive precipitin bands with *Naja haje* venom (20mg/ml) (Tizard 1984).

Venom neutralization test:

In this experiment *Naja haje* venom was incubated with the specific antivenin for one hour at 37°c in the proportion of 1ml serum to 10 LD50.

Protection against venom lethality in mice:

The venom antivenin mixture was shaken and dose equal the LD50 (5µg/20g mouse) was injected into each of 10 mice. Doses of the incubated mixture equal 2-5times the LD50 were injected each into a group of 10mice. All mice were observed for 24 hrs after injection.

Assay of venom enzymes:

Phospholipase A2 activity: it was assayed according to the method of Desnuelle *et al.,* (1995) and Nieuwenhuizen *et al.,* (1974). Lecithin was used as substrate, and was solubulised either with sodium cholate or triton X-100. the enzymes activity measured at A558mn. The phospholipase A2 was determined as corresponding to the decrease in O.D.

Proteolytic activity: The method by Labib et al., (1980 was followed for proteolytic activity using casein as substrate. The enzyme activity measured at A280.

Neutralization of Phospholipase and proteolytic activity:

Varying amount of antiserum were preincubated with 5 µl (25µg) crude venom in a volume of 0.1ml for one hour at 37 °C. Enzymes activities of the mixture were assayed as described earlier. Control experiments were carried out by using normal rabbit serum.

Results:

Lethality assay:

The i.p. LD50 for non irradiated, 15KGy and 25 KGy *Naja haje* venom were estimated to be 5µg/20g mouse, 16µg/20g mouse and 50µg/20g mouse respectively.

15KGY irradiated venom toxoid (200µg) and (1000µg) as well as 25 KGy irradiated venom toxoid produced antitoxin in rabbit sera after injection of three doses (on day 0, 7,21 and a booster dose) as revealed by neutralization test in mice. Incubation of *Naja haje* venom with the specific antivenin (obtained from non-irradiated venom toxoid or gamma irradiated venom toxoid) protected the mice against the lethal action of a dose of the venom equivalent to three fold the LD50. It also protects 80% of the mice against the lethal action of the dose of the venom equivalent to 4 times the LD50.

Injection of higher dose of 15KGy venom toxoid (1000µg) had little detrimental effect on the experimental animals yet did not result in higher neutralization power against venom lethality.

Determination of antivenin titer:

There was no change in the titer of antivenin solution obtained from the rabbits immunized with 15KGy irradiated venom toxoid, as compared with that of antivenin solution obtained from rabbits immunized with non- irradiated venom toxoid. (fig.1). Using the immunodiffusion experiment definite precipitin bands were obtained with a venom concentration of 20mg/ml, and serum dilution up to 1:32

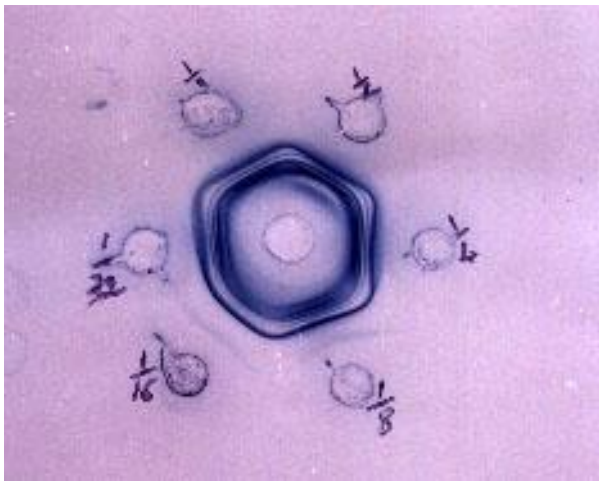

Fig.1 : Determination of titer of antibody using double diffusion reaction.
Ve: non-irradiated venom (in the central well)

In peripheral wells, rabbit serum antivenin raised against 15KGy irradiated venom, serially diluted up to 1:32

Double diffusion technique:

Immunodiffusion test (fig.2&3) demonstrated the presence of several antibodies in the immunized sera of rabbits. Multiple precipitin bands were observed in 15 KGY and 25KGy toxoid antisera. Inimmunodiffusion test antivenoms raised against non-irradiated, 15KGy and 25KGy gamma irradiated venom, showed similar results. The visible lines were identical and were joined smoothly at the corners, indicating that, they have the same antigenic determinant.

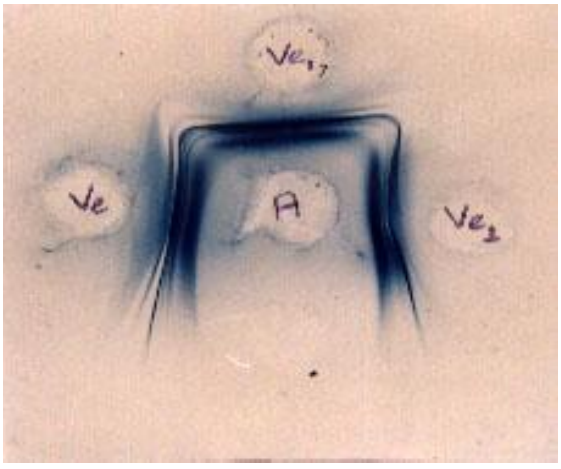

Fig.2 : Double diffusion reaction of rabbit serum antivenin from non-irradiated *Naja haje* venom (central well) with non-irradiated and gamma irradiated venom.
Ve: non-irradiated venom (20mg/ml).
Ve1: 15KGy gamma irradiated venom (20mg/ml).
Ve2: 25KGy gamma irradiated venom (20mg/ml).

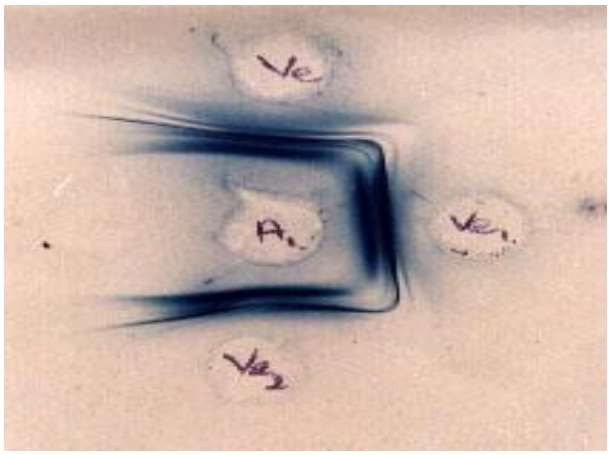

Fig. 3: Double diffusion reaction of rabbit serum antivenin from 15KGy irradiated venom (central well) with non-irradiated venom and gamma irradiated venom.
Ve: non-irradiated venom (20mg/ml).
Ve1: 15KGy gamma irradiated venom (20mg/ml).
Ve2: 25KGy gamma irradiated venom (20mg/ml).

Neutralization of phospholipase and proteolytic activities:

A significant inhibition of both phospholipase A2 and protease activities was obtained when neutralized with 15KGy irradiated venom toxoid. (Fig.4).

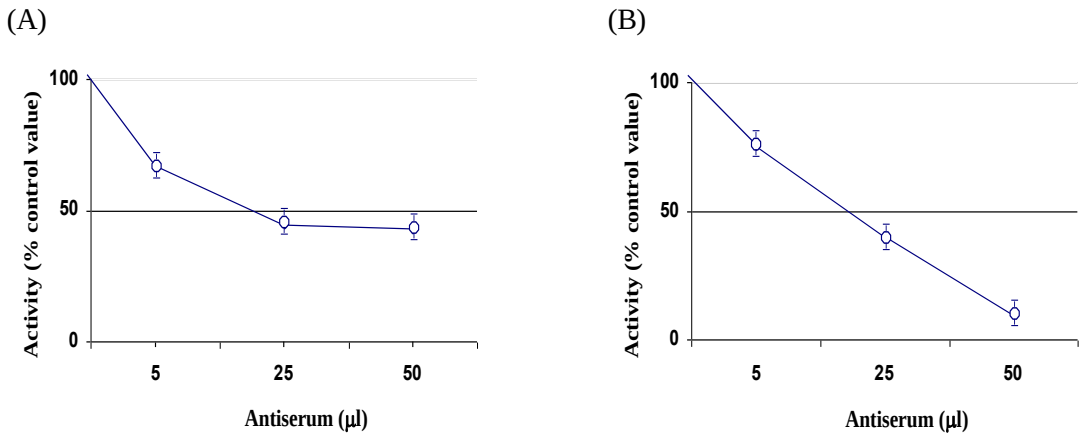

Fig 4 : Neutralization of phospholipase A2 and protease activities of crude venom by immune sera .

Percent of control values of enzyme activities in presence of varying amounts of immune sera were calculated from the value (100%) obtained by incubating venom with corresponding amount of normal sera. Control (100%) activities for phospholpase and protease were 1.8 O.D. at 558nm and 0.10 O.D. at 280nm respectively. Control values present the mean value of three sets of experiment; 25µg of protein was used in the assay of each enzyme. (a) Phospholipase A2 ; (b) protease.-o- 15KGy toxoid antiserum. Data are mean of + S.E.M. of three separate determination.

Discussion

Our study describes the active immunization of rabbits by gamma irradiated Cobra *Naja haje* venom toxoid. Effect of ionizing radiation on the proteins and peptides in aqueous solution is mediated either directly or indirectly (Alexender, 1960). In a direct effect several reactive species such as O_{-2}, e_{-aq}, H and oH formed from the radiolysis of water react rapidly with a large variety of biological molecule, although the reaction may be specific to a certain site or group in the molecules. The production of different antibodies in the immune sera of rabbits (Fig. 2) demonstrated that antigenicity of some components of venom was retained in the toxoid used in the study.

Experimental evidence suggests that neutralization of lethal effect of venom by antisera may not correlate with the neutralization of pathopharmacological effect *(Gene et.al, 1985)*. Standardization of antivenoms based on the neutralization of specific pathopharmacological effects has been recommended by the World Health Organization (WHO, 1981). Several enzymes in the venom may be responsible for some of its pathopharmacological effects *(Warrell, 1987; Jimenez-porras, 1968)*.

The LD_{50} values for natural venom and the two dose levels irradiated venom were 5 μg / mouce,16 μg / mouce, and 50 μg / mouse, respectively. The LD_{50} for 15 KGy and 25 KGy irradiated venom were 3.2 times and 10 times less toxic than natural venom, respectively. Similar results were reported by other authors, *Murata et al. (1990) and Clissa et al (1999)*, who suggested that toxicity of irradiated venom would be reduced by the formation of aggregate, enabling the formation of a new complex.

Sera of rabbits immunized with 15 KGy irradiated venom (200 μg) toxoid neutralized venom lethality (three fold LD50). Injection of higher dose of irradiated venom (1000μg) would have had little detrimental effect on the experimental animal yet did not resulted in higher titer. or neutralizing power. In the present study, hyperimmunizing rabbits against non-irradiated, 15KGy and 25KGy gamma irradiated *Naja haje* venom, over a period of one month, yielded specific antivenin of high potency.

Using the immunodifussion experiments, non-irradiated as well as the two dose levels irradiated *Naja hage* venom (15 and 25 KGy), all showed the same pattern (Fig2,3) when tested against control antivenin, produced with non-irradiated venom and against antivenin produced with 15KGy irradiated venom. The four distinct visible lines, obtained in the immunodiffusion test, were identical, and joined smoothly at the corners indicating that there was no change in the antigenic determinants. The results obtained in the present work are in the agreement with those of other investigators. *Sundaram et. al (1970)* reported that inspite of the break down of normal human serum components in solution form after exposure to doses up to 25 M rads (25KGy), it was able to form precipitin lines with its specific antiserum as demonstrated by polyacrlaminde gel, electrophoresis and immunodiffusioin testes

Kankonkar et al. (1975) reported that cobra venom irradiated in dry (lyophilized) form, had no changes in toxicity and antigenicity, as shown by immunodiffusion and immunoelectrophoresis with doses up to 4 M rads (40KGy) whil for aqueous samples, the antigenicity was destroyed proportionally with the destruction of toxicity. Biloaicol .

There was no change in the titer of antivenin solution obtained from the rabbits immunized with the irradiated venom, as compared with that of antivenin solution obtained from rabbits immunized with non-irradiated *Naja haje* venom, using the immunodiffusion venom concentration of 20 mg/ml, and serum dilution up to 1:32. Both groups were immunogenic and induced specific antibody formation.

Antivenom raised against 15KGy irradiated *Naja haje* venom was also found to neutralized venom lethality with the same degree (three fold LD_{50}), as well as the antivenom raised against non-irradiated venom (crude venom). These results showed that antibodies generated against

the 15KGy irradiated venom, were reactive towards the non-irradiated venom and vice-versa showing no change in the immunogenecity.

This agree with flowers (1970) who showed that crotalied snake venom exposed to X-rays, revealed a decrease in its local reactivity (50-70%) and an increase in the LD50, in mice (six folds). This inactivation was accompanied with little change in its antigenic character. At the same time, the antivenin produced when using X-irradiated venom, neutralized both the local and the lethal effects of the venom. This effect was more or less, equal to that obtained with control antivenin produced when using non-irradiated venom.

However *Netto et al. (2002)* showed that the use of irradiated cortalus durissus terrificus venom in sheep immunization induces a powerful and lasting humoral immune response shown by both in vitro neutralization and potency tests and by indirect Elisa antibody level detection technique. Sera from the irradiated group were five times more potent than the sera from the natural group.

Protease and PLA enzymes are known to contribute to the venom toxicity or pathophysiological process(*Harrivis et a l., 2000, Fuly et al. 2003),* . Toxoid antivenom showed less neutralization capacity towards PLA as compared to protease activity. Gamma irradiation might have impaired the antigenic site (s) of the enzymes (PLA) molecule. *Tejasen and Ottolenghi (1970),* also reported that antive-nom against U.V. irradiated Agkistrodon bisciveorus venom was devoid of antienzymes activity (proteinase or PLA).

Bharati and Hati (2000), immunized Rhesus monkeys (Macaca mulatto) with irradiated Russell's viper venom toxoid, without any adjuvant. The antiserum ther-eby raised producing approximately half-antilethal, half-antihaemorrhagic and half antinecrotic titres as compared by the commertial hyperimmunized horse antise-rum. Adsorption of toxoid with suitable adjuvant may be more effective imm-unizing agent.

Toxoiding by gamma irradiation required much less time as compared to that of a slow and stepwise formal treatment. *Salofranco (1971),* reported that venom toxoid irradiated (from 60 co) Cobra *Naja naje* philippinesis, was more immunogenic as compared to untreated or formal (1%) heated venom.

Further investigation must be done to compare antibody (raised against gamm airradiated venom toxoid) titer and commercial antivenin on the basis of its ability to neutralize lethality and other pathopharmcaologial effects of *Naja haje* venom

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تحصين الأرانب شبه سم الكوبرا (نجا هاج) المشعع جاميا

د. عصمت عبد السلام شعبان
أستاذ مساعد بقسم البحوث الدوائية الإشعاعية
المركز القومي لبحوث وتكنولوجيا الإشعاع – هيئة الطاقة الذرية

تم تحضير مصل مضاد لسم ثعبان الكوبرا في الأرانب بعد حقنها يشبه السم المشعع (25 ، 15 كيلو جراي). ولقد أجريت عليه بعض التجارب المناعية مثل تجارب الانتشار المزدوج واختبار معادلة المصل لتأثيرات سم الثعبان ، وقد وجد أن العيار الحجمي للأجسام المضادة المكونة في الأرانب ضد السم غير المشعع أو المشعع ب15 كيلو جراي لم تتغير .

وفي تجارب الانتشار المزدوج كون السم (غير المشعع والمشعع ب 25 ، 15 كيلو جراي) مع المصل المضاد المكون ضد السم الغير مشعع خمس حزم في الترسيب على الأقل، وكانت هناك أيضا تفاعلات مماثلة مع المصل المضاد المكون ضد السم المشعع ب15 كيلو جراي . ثبت أيضا فاعلية المصل المضاد في حماية الفئران من التأثيرات القاتلة للسم ومعادلة النشاط الإنزيمي للسم ، فوجد أن كل من انزيم فوسفوليبيز A_2 والبروتييز يتم معادلة نشاطهم بنسب مدتلفة (50% و 95%) على التوالي.