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Feature Article

Development and Evaluation of a Snake Vaccine in Mice and Rhesus Monkeys

Kaushik Bharati†

Abstract
Snakebites kill more people in India than all the countries in the world put-together. India accounts for half of all snakebite deaths worldwide. The only treatment for venomous snakebite is the administration of antivenom. But it has limitations. A snake vaccine is an attractive alternative. This study focuses on the development of a prophylactic vaccine against Russell's viper using gamma radiation and its evaluation in mice and rhesus monkeys (*Macaca mulatta*).

Keywords: Russell's viper, gamma irradiation, rhesus monkeys, *Macaca mulatta*, immunization, toxoid.

Introduction

Snakebite is a major public health problem in India. The magnitude of the problem is exemplified by the fact that a staggering 50% of all global snakebite deaths occur in India alone, with the remaining 50% occurring in all the remaining countries put together [1]. The majority of deaths are caused by the "Big Four", namely, Biocellate cobra (*Naja naja*), Common krait (*Bungarus caeruleus*), Russell's viper (*Daboia russelii russelii*), and Saw-scaled viper (*Echis carinatus*) [2]. Since Russell's viper is a major contributor to snake mortality in India, effective preventive measures are imperative, given the fact that snake antivenom is scarce and expensive. In the present study, development of a Russell's viper venom (RVV) vaccine for immunization purposes was attempted.

Preparation of an effective snake venom vaccine involves detoxification of venom, but at the same time, retaining the immunogenicity of the product at its maximum. This process is termed as toxoidation and the resulting formulation is termed as a toxoid, which can be used as a vaccine. The toxoid has significantly reduced toxicity, yet sufficient immunogenicity to elicit a strong and robust immune response. Therefore, toxoid immunized animals have high circulating antibody titers, capable of neutralizing the venom, introduced post-immunization, such as by a snakebite or experimentally by injecting the crude venom [3].

Earlier attempts at toxoiding venoms have been made using various strategies. One of these approaches involve the treatment of venom with various chemicals, including formalin, glutaraldehyde, alcohol or 2, 5-dihydroxyterephthalic acid (DHTA). Other strategies involve irradiation with UV rays, X-rays, gamma rays, and photo-oxidation. However, these approaches have met with only partial success, as these are unable to combine

†Health Policy Consultant – UNESCO, New Delhi, **E-mail:** dr.kaushik.bharati@gmail.com
ORCID: Kaushik Bharati: https://orcid.org/0000-0003-3764-0186

a high level of detoxification with an efficient retention of immunogenicity [4-7].

There are however, not many reports of successful application of gamma radiation on RVV for toxoidation purposes. The basic purpose of the present study was to develop a gamma irradiated RVV toxoid for eventual use in humans. However, prior to clinical trials, the toxoid has to undergo rigorous testing in animal models. The present toxoid has shown promising results in Swiss albino mice [8] and New Zealand white rabbits [9]. The present work deals with the immunogenic potential of the toxoid in primate model, namely, rhesus monkeys (*Macaca mulatta*) – the last link between the pre-clinical trials in animals and the ultimate human clinical trials.

Aims and Objectives

The aim of the study was to develop and standardize a gamma irradiated RVV toxoid and test its safety and efficacy by immunizing rhesus monkeys. This was accompanied by venom neutralization, enzyme neutralization, and immunodiffusion studies to support the *in vivo* findings.

Methodology

The following methods were used in the study:

Snake Venom Lethality Assay

Venom-induced lethality was assayed in Swiss albino mice (n=6) [10]. The average weight of each mouse was determined to be 20 g. There were eight Experimental Groups and eight Control Groups with six mice in each group (n=6). The eight Experimental Groups were divided according to the dose of venom injected (5 µg, 10 µg, 15 µg, 20 µg, 25 µg, 30 µg, 35 µg, 40 µg), while the Control Group was injected with normal saline. Each experiment was repeated four times. So, the total sample size was 24 (n=24).

The unit of measurement of venom lethality is µg venom/mouse. The minimum lethal dose (MLD) is the least amount of venom (µg) that kills at least one mouse in the group, which in this case is 1/6 mice. The median lethal dose (LD_{50}) is the amount of venom (µg) that kills 50% of mice, which in this case is 3/6 mice. The absolute lethal dose (LD_{100}) is the least amount of venom (µg) that kills 100% of mice, which in this case is 6/6 mice. The minimum hemorrhagic dose (MHD) is the least amount of venom (µg) that causes a hemorrhagic lesion of 10 mm diameter 24 hours after intradermal (ID) injection in mice [11]. The minimum necrotic dose (MND) is the least amount of venom (µg) that causes a necrotic lesion of 5 mm diameter three days after ID injection in mice [11].

Standardization of Gamma Radiation Dose for Preparation of Toxoid

Three samples of RVV in normal saline (0.9%) were irradiated by the application of gamma radiation from a Co-60 source (Gamma Chamber 900, Isotope Division, Bhabha Atomic Research Center, Trombay, Mumbai). Gamma radiation was delivered at a rate of 500 rad/minute at 17-19°C, relative humidity 45-50% and pH 6.5. The radiation was carried out at the Nuclear Chemistry Division, Saha Institute of Nuclear Physics, Kolkata. Three doses of gamma radiation were used: 100 Krad, 200 Krad, and 300 Krad, respectively. In order to check the extent of detoxification and suitability for immunization, three groups containing 10 mice each (n=10), were injected with each of the above irradiated venom samples. The immunization protocol was same as that for monkeys i.e., three consecutive doses on day 0, day 30, and day 60, followed by a lethal challenge with crude venom on day 90. The survival data helped to decide which dose of gamma radiation would be most suitable for adequately detoxifying the venom, yet retaining enough immunogenicity to elicit a robust immune response so that high titers of protective antibodies are generated.

Standardization of Optimal Concentration of RVV for Preparation of Toxoid

Graded concentrations of RVV were made in normal saline, starting from 0.5 mg/ml (through 1, 5, 10, 15, 20, 25, 30, 35, 40, 45) and ending at 50 mg/ml. These were irradiated with 100 Krad gamma radiation. The graded concentrations of toxoids were injected intramuscularly (IM) at a dose of 1 MLD in mice to determine the extent of detoxification. Taking safety limits from the murine model studies, three rhesus monkeys were injected with the toxoid in order to assess the safety limits in primate model.

Effect of Gamma Radiation on Lethal, Hemorrhagic and Necrotic Activities of Venom

The extent of detoxification of RVV by gamma radiation was determined by injecting several graded doses of gamma irradiated (100 Krad) venom (toxoid) IM into groups of six mice per dose, as described earlier in the 'lethality assay'. The survival of the mice and development of any pathological signs were observed for 48 hours. Mice treated similarly with crude (non-irradiated) venom served as controls. The effect of radiation on hemorrhagic and necrotic activity of RVV was studied by injecting the irradiated venom into mice as described earlier in the 'assay of MHD and MND'. Non-irradiated crude venom injected mice acted as controls. The assays were repeated four times.

Immunogenicity of Toxoid

The immunogenic potency of gamma irradiated RVV toxoid was determined by immunizing monkeys with the toxoid and observing the protection conferred to them against lethal challenge with crude venom.

The gamma irradiated RVV toxoid was used for immunizing six healthy monkeys (5-10 kg body weight) without any adjuvant, at a dose of 1 MLD (1 mg/ml). Intramuscular injections into the thigh were given on day 0, 30, and 60. Blood was drawn from the antecubital vein on day 0, 75, 100, 150, 260, and 300 for sera separation. On day 90, the monkeys were given a lethal challenge with crude venom at a dose of 1 MLD. The non-immunized control monkeys were similarly challenged with the same dose (1 MLD) of crude venom. The antibody response was observed, giving two intermediate booster doses on day 120 and 240, and ending on day 300. The antibody response in monkeys following basic schedule of immunization with 100 Krad of RVV toxoid was expressed in terms of the neutralizing activity of antisera towards the lethal effect of viper venom (LD_{50} neutralized/ml antiserum) [12].

Immunodiffusion Test: The immunization was confirmed by the immunodiffusion test performed using an agarose gel [13]. 1% (w/v) agarose in phosphate-buffered saline (PBS), containing 0.01% sodium azide (NaN_3) was heated to 60-80°C and the warm agarose was poured onto glass slides. It was kept as such for 15 minutes, after which wells were cut with a template (one central well and six peripheral wells, placed 7 mm from the center). Into the central well was added 20 µl (50 µg) of crude RVV solution. Into each of the peripheral wells was added the antiserum (20 µl) raised in monkeys at various stages of immunization against RVV toxoid. The slides were incubated at 37°C in a humidor for 30 minutes and then kept at 4°C for 48 hours until precipitin bands appeared.

Neutralization of Lethal, Hemorrhagic and Necrotic Activities

Neutralization of lethal, hemorrhagic, and necrotic activities were carried out by the conventional venom neutralization test [10, 14]. Neutralization of lethality was studied by injecting 0.2 ml incubated venom-immune sera mixture (several LD_{50}'s of crude RVV in 0.1 ml normal saline with 0.1 ml of undiluted immune sera obtained from immunized monkeys) IM in mice and recording the mortality up to 48 hours. The number of LD_{50} neutralized per ml of antiserum was taken as a reflection of the antibody titer.

Neutralization of hemorrhagic activity was studied by injecting 0.1 ml incubated venom-immune sera mixture (several MHDs in 0.05 ml saline with 0.05 ml undiluted immune sera) ID in mice and observing for any hemorrhagic spot, 24 hours later.

Neutralization of necrotic activity was studied similarly, except that the skin was removed 3 days after ID injection to observe for any necrotic lesions.

In all the neutralization experiments, venom-immune sera mixtures were incubated for 1 hour at 37°C, centrifuged for 2 minutes and the clear supernatants used. Non-immune serum, treated similarly was used as control. In all the experiments, 6 mice/venom-immune sera incubate were used and the experiments repeated at least twice (n=12).

Neutralization of Enzyme Activities by Antiserum Raised in Monkeys

The enzymes present in snake venom can act as potent toxins that cause degradation of tissue at the site of envenomation. In the context of RVV, the prominent toxic enzymes are phosphodiesterase, phospholipase A, hyaluronidase and protease.

For the enzyme neutralization studies, varying amounts of antisera were pre-incubated with 5 µl (25 µg) crude venom in a total volume of 0.1 ml for 30 minutes at 37°C. All the enzyme assays, described below, were performed in triplicate. While all the experimental samples contained crude venom, the control samples did not contain any venom and were assayed using non-immunized monkey sera. Spectrophotometric readings were taken in a SICO-SPEC 200 GL UV-VIS spectrophotometer.

Assay of Phosphodiesterase: Phosphodiesterase activity was determined by measuring the liberated p-nitrophenol spectrophotometrically, using calcium bis- (p-nitrophenyl) phosphate as substrate. The absorbency was measured at 400 nm against water blank that contained all reagents except the enzyme [15].

Assay of Phospholipase A: Phospholipase A (PLA) activity was determined by turbidimetry using egg yolk as substrate. Enzyme activity was estimated from the change in absorbance between 5 and 15 minutes, using a spectrophotometer at 740 nm. A decrease in absorbance of 0.01 was defined as one unit of activity [16, 17].

Assay of Hyaluronidase: Hyaluronidase activity was measured by turbidimetry. The decrease in turbidity was measured at 600 nm against water blank [18].

Assay of Protease: Protease activity was assayed using azocasein as substrate. The absorbance of the released dye was taken at 366 nm [19].

Assessment of Reversion of Toxicity of the Toxoid

A batch of the gamma irradiated RVV toxoid was stored at 4°C in the dark for the entire period of the study and tested for reversion of toxicity in Swiss albino mice of either sex. The parameters tested included LD_{50}, MHD, and MND. These parameters were determined on day 0, day 100, day 200, and day 300. The generated data provided an index of reversion of toxicity of the toxoid upon long-term storage [10].

Results

Snake Venom Lethality Assay

The MLD in Swiss albino mice was found to be 10 µg/mouse, LD_{50} was 16.59 µg/mouse (~20 µg/mouse), and LD_{100} was 35 µg/mouse (Table 1). The MHD in mice was found to be 12 µg/mouse (Figure 1) and MND was 5.2 µg/mouse (Figure 2).

Table 1: Determination of MLD, LD_{50} and LD_{100} in mice

Group	Dose of Venom (µg/mouse)	Mortality	Mortality (%)
1	5	0/6	0
2	10	1/6	16.66
3	15	2/6	33.33
4	20	3/6	50
5	25	4/6	66.66
6	30	5/6	83.33
7	35	6/6	100
8	40	6/6	100

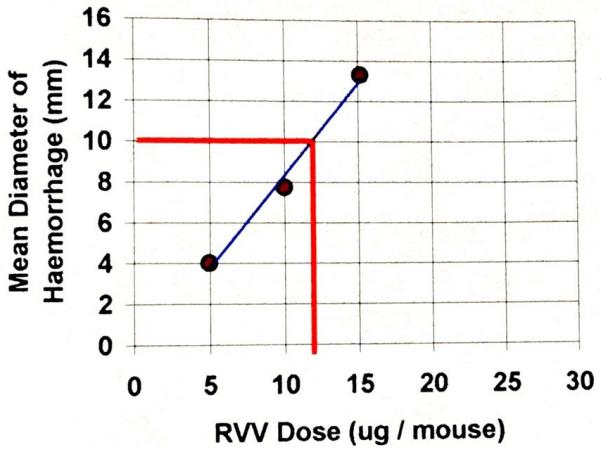

Figure 1: Determination of Minimum Hemorrhagic Dose (MHD)

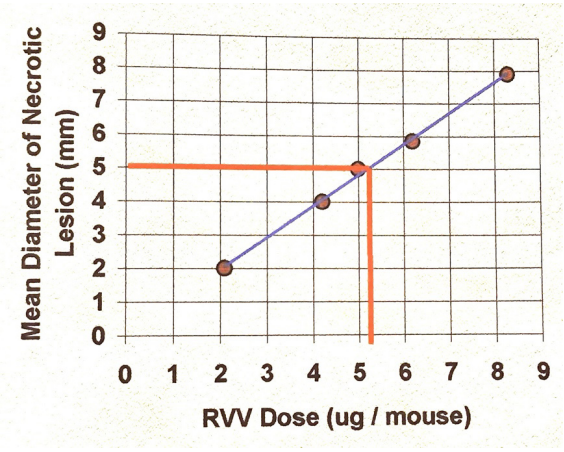

Figure 2: Determination of Minimum Necrotic Dose (MND)

Standardization of Gamma Radiation Dose for Preparation of Toxoid

Three groups of mice (n=10) were immunized with three doses of gamma irradiated RVV (100 Krad, 200 Krad, 300 Krad). It was noted that after challenge with crude venom, 100% survival was observed only in the 100 Krad group (Table 2).

Table 2: Correlation of gamma irradiation dose with survival of mice

Group	Dose of Gamma Radiation	Survival of Mice
1	100 Krad	100%
2	200 Krad	50%
3	300 Krad	20%

Although the higher doses of gamma radiation (200 Krad and 300 Krad) are better capable of inactivating the snake venom toxins, they also simultaneously reduce the immunogenic potential of the toxoid. Hence, in order to strike a balance between toxicity and immunogenicity, 100 Krad was chosen for further immunization studies in monkeys [20].

***Standardization of Optimal Concentration of RVV for Preparation of Toxoid** [21]*

Standardization of the optimal concentration of RVV for preparing the gamma irradiated toxoid was conducted in mice. To check the extent of detoxification, graded concentrations of the toxoid (0.5 mg/ml through 50 mg/ml) were used. 1 MLD of each of the toxoids was injected into groups of 10 mice (n=10) and the mortality was recorded (Table 3).

Based on data from Table 3, a graph was plotted, taking graded toxoid concentrations (mg/ml) along the X-axis and mortality (%) along the Y-axis (Figure 3).

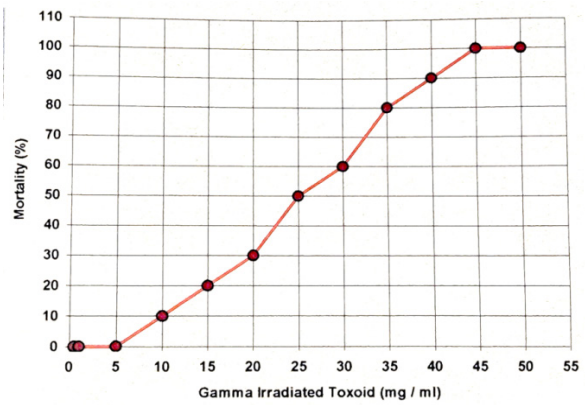

Figure 3: Variation of mortality with toxoid concentration

It is evident from Table 3 and Figure 3 that only three toxoids (0.5 mg/ml, 1 mg/ml, 5 mg/ml) could be used for immunization purposes, because they were non-toxic and didn't show any mortality. Of these three, the toxoid having a concentration of 5 mg/ml would be most suitable as it had the highest antigenic composition with zero toxicity.

Based on the results from the murine model studies, three monkeys were injected with ½ MLD toxoid (5 mg/ml). However, within 24 hours, all three monkeys expired. The experiment was repeated in two monkeys, using the toxoid having

Table 3: Toxoid concentration and corresponding mortality

Group	Toxoid Concentration (mg/ml)	Toxoid Dose (IM Injection)	Mortality	Mortality (%)
1	0.5	1 MLD	0/10	0
2	1	1 MLD	0/10	0
3	5	1 MLD	0/10	0
4	10	1 MLD	1/10	10
5	15	1 MLD	2/10	20
6	20	1 MLD	3/10	30
7	25	1 MLD	5/10	50
8	30	1 MLD	6/10	60
9	35	1 MLD	8/10	80
10	40	1 MLD	9/10	90
11	45	1 MLD	10/10	100
12	50	1 MLD	10/10	100

a concentration of 1 mg/ml at a dose of 1 MLD. Both monkeys survived without exhibiting any untoward effects. Based on these findings, it was decided to use the toxoid having a concentration 1 mg/ml for safe immunization of monkeys.

Effect of Gamma Radiation on Lethal, Hemorrhagic and Necrotic Activities of Venom

Gamma irradiation caused detoxification or abolishment of lethal, hemorrhagic, and necrotic properties of RVV. A quantitative evaluation of the effect of gamma radiation (100 Krad) on these biological activities of RVV showed that the lethality (LD_{50}) was reduced 3-fold, while MHD and MND were reduced 1.5 and 4-folds, respectively (Table 4) [22].

Immunogenicity of Toxoid

The experimental monkeys after being challenged with crude venom (1 MLD) on day 90, survived without exhibiting any untoward effects. Two control monkeys expired within seven days observation period. After completion of the basic immunization schedule, the immune response was observed up to 300 days. The immunodiffusion banding pattern for the various stages of the immunization process is presented in Figure 4 [23].

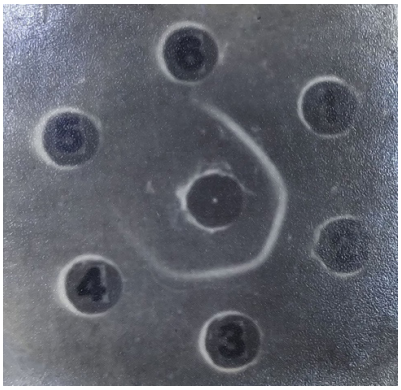

Figure 4: Immunodiffusion banding pattern for the various stages of immunization

Central well: 20 µl (50 µg) crude RVV; peripheral wells: antiserum (20 µl) raised in monkeys against RVV toxoid; well 1: day 100; well 2: day 150; well 3: day 260; well 4: day 300; well 5: day 0; well 6: day 75

The monkeys bled on day 100 after the challenge (day 90) showed an antiserum titer of 60-75 LD_{50}/ml. After a booster dose (1 MLD) administered IM on day 120, the antiserum titer shot up to 75-90 LD_{50}/ml, but gradually declined to around 25-50 LD_{50}/ml on day 200. Next booster dose on day 240 again boosted up the antiserum titer to 60-90 LD_{50}/ml, which again declined to an all-time low titer of 25-40 LD_{50}/ml on day 300 (Figure 5) [24].

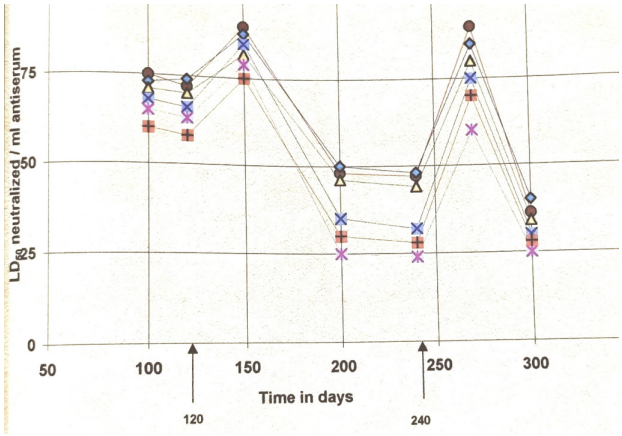

Figure 5: Antibody response in monkeys following basic immunization (day 0, 30, and 60) with RVV toxoid

Antibody response was measured in terms of neutralization of lethality in mice. Arrow marks indicate the day on which booster doses of toxoid were injected. Venom neutralization test for each monkey at each observation point (day 100, day 120, day 150 etc.) was done four times (n=4)

Neutralization of Lethal, Hemorrhagic and Necrotic Activities [25]

The antiserum was highly effective in neutralizing the lethal, hemorrhagic, and necrotic activities of RVV. Neutralization of lethality was expressed by

Table 4: Comparison of LD_{50}, MHD and MND in crude venom and toxoid

LD_{50} (µg/mouse)			**MHD (µg/mouse)**			**MND (µg/mouse)**		
Crude Venom	Toxoid	Fold Decrease	Crude Venom	Toxoid	Fold Decrease	Crude Venom	Toxoid	Fold Decrease
16.59	51.41	3.09	12	18.96	1.58	5.2	21.1	4.05

the number of LD_{50}'s neutralized/ml of antiserum. The lethality was significantly reduced (data not shown).

Neutralization of hemorrhagic activity was highly effective by antiserum following immunization with 100 Krad toxoid. The antihemorrhagic titer on day 30 was around 78 MHD/ml, which decreased to 50 MHD/ml on day 50. A booster injection on day 60 resulted in a significant ($p<0.01$) increase in antihemorrhagic titer (140 MHD/ml) on day 100, as compared to the initial titer on day 30 (Figure 6).

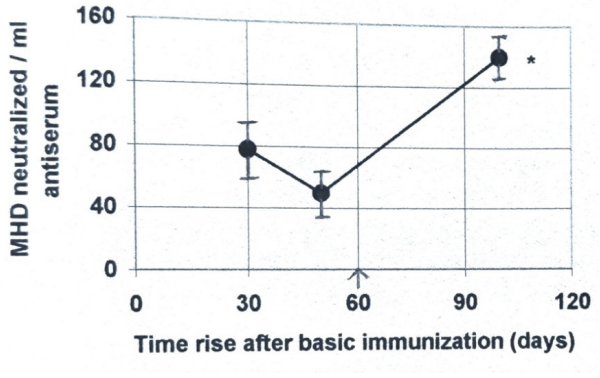

Figure 6: Neutralization of hemorrhagic effects by antiserum raised in monkeys against RVV toxoid

* Significant ($p<0.01$) increase in antihemorrhagic titer as compared to that on day 30. Arrow mark indicates the day on which booster dose of toxoid was given. Values are mean ± SE (n=12)

Regarding neutralization of necrotizing effect by the same antiserum, it could be observed that on day 30, it had an anti-necrotic titer of 140 MND/ml, which declined to 80 MND/ml on day 50. After boosting, the titer increased (150 MND/ml on day 100), but insignificant when compared to the initial titer on day 30 (Figure 7).

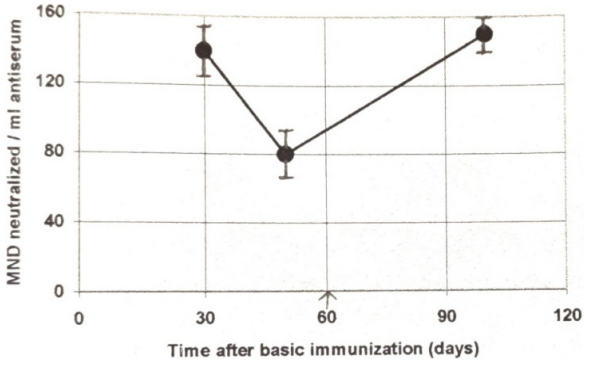

Figure 7: Neutralization of necrotic effects by antiserum raised in monkeys against RVV toxoid

Arrow mark indicates the day on which booster dose of toxoid was given. Values are mean ± SE (n=12)

Neutralization of Enzyme Activities by Antiserum Raised in Monkeys

A significant inhibition (98%) of protease activity was observed when neutralized by the toxoid antiserum (0.05 ml). Next in line came hyaluronidase (80% inhibition with 0.1 ml antiserum), followed by phosphodiesterase (65% inhibition with 0.1 ml antiserum). PLA activity was inhibited least (55%) even with 0.1 ml antiserum (Figure 8) [26].

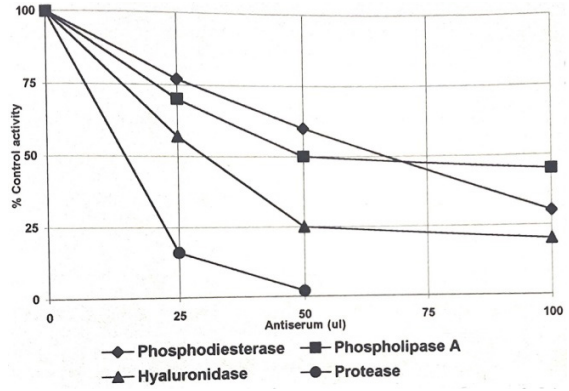

Figure 8: Neutralization of enzymatic activities of RVV by toxoid antiserum

Assessment of Reversion of Toxicity of the Toxoid

The LD_{50}, MHD, and MND values for the gamma irradiated toxoid, as determined initially on day 0 and subsequently on day 100, day 200, and day 300 showed that there was no significant change in the above parameters, indicating no appreciable reversion in the toxicity of the gamma irradiated toxoid due to long-term storage (Table 5) [27].

Table 5: Data on reversion of toxicity of RVV toxoid upon long-term storage

Timepoint	LD_{50}	MHD	MND
Day 0	51.41 ± 0.25	18.96 ± 0.16	21.1 ± 0.17
Day 100	51.23 ± 0.14	18.45 ± 0.24	20.96 ± 0.23
Day 200	50.94 ± 0.32	17.86 ± 0.18	20.42 ± 0.12
Day 300	50.37 ± 0.15	17.22 ± 0.21	20.23 ± 0.19

Discussion

This study is an attempt to produce a vaccine suitable for human use against Russell's viper envenomation [28]. Gamma irradiation resulted in a significant detoxification of lethal and local tissue-damaging effects of venom [29].

This was accompanied by a pronounced loss of activities of the enzymes studied (PLA, protease, phosphodiesterase, and hyaluronidase) [26]. Similar observation was made on the effect of UV light on snake venom toxicity and enzymatic activity [30].

Snake venoms are complex mixtures of toxins and enzymes that are responsible for different clinical conditions, as a result of envenomation [31,32]. When crude venom is exposed to gamma radiation, all these components of the venom become susceptible to radiation damage, although radiation susceptibility varies from one protein to another [33]. Reduction of proteolytic activity is significant because of its possible association with the hemorrhagic, hemolytic [34] and toxic manifestations of the venom [31].

A significant radiation effect was evidenced on the basis of altered biological activities as demonstrated by detoxification of lethal, hemorrhagic and necrotic effects, as well as inactivation of some venom enzymes. This suggests that gamma radiation might have caused inactivation of some groups or amino acids that are responsible for toxicity and enzyme activity, without affecting the primary structure of proteins, as because the antigenicity remained unhindered.

The mode of action of antivenom in neutralizing venom toxins is not known and it may differ from one toxin to another [35]. Antivenom (against gamma irradiated viper venom) neutralized the different toxic effects of viper venom varyingly, which only reflects the essence of the above observation by Theakston (1989) [35].

Immunogenicity of the toxoid could be improved further by incorporating well-chosen purified toxin or polypeptide fraction of a toxin into the toxoid preparation. And such a preparation, when linked to a suitable carrier/adjuvant, may be used for preventive immunization of individuals at high risk of venomous snakebite [3,35]. However, application of this approach was beyond the scope of the present study.

The major implication of the results obtained in this primate model of immunization is that gamma irradiated (optimum dose: 100 Krad) RVV is a safe and effective immunogen, capable of eliciting quick immune responses in monkeys and generating antibodies that neutralize the lethal and several other pathophysiological manifestations of viper venom poisoning.

Conclusion

The present investigation is one of several preparatory steps to develop a snake vaccine for use in humans to negate the toxic effects of Russell's viper envenomation. The developed toxoid could also be used for quick and safe immunization of horses for commercial antivenom production, cutting down the cost.

The study demonstrated that development of a snake venom toxoid using gamma radiation is possible, which can be used for immunization of small, as well as large animals, such as mice, rabbits, and monkeys. Furthermore, the study also demonstrated that the gamma irradiated toxoid was safe and effective in raising protective antibodies in primate model.

The study can be considered as a stepping stone for further development of the vaccine and conducting clinical trials in human populations.

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