STUDY ON IMMUNE RESPONSE TO IRRADIATED COBRA VENOM IN RABBITS *

การศึกษาเกี่ยวกับการตอบสนองทางภูมิคุ้มกัน ในกระต่ายต่อพิษงูเห่าที่อาบรังสี

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ABSTRACT

The immune responsiveness of rabbits to irradiated Cobra venom was investigated. Partially detoxified venom could induce the production of antibody as demonstrated by immunodiffusion and immunoelectrophoresis methods as well as mouse neutralization test. Completely detoxified irradiated venom could induce the production of antibody which was effective for mouse protection against the homologous venom, although the antigenic property could not be detected by agar-gel diffusion methods. Electrophoretic separation of immunized rabbit serum also showed increased gamma globulin fraction

เรื่องย่อ

ได้ศึกษาถึงการตอบสนองของกระต่ายในการสร้างภูมิคุ้มกันต่อพิษงูเห่าที่อาบกัมมันตภาพรังสี พบว่าพิษงูที่ถูกอาบรังสีจนพิษอ่อนลงบ้างยังสามารถทำให้เกิด

* This study is the final part of the research project supported partly by grant from the International Atomic Energy Agency under Contract No. 661/RB

ภูมิคุ้มกันซึ่งตรวจพบได้โดยการทดลองด้วยวิธี Immunodiffusion และ Immunoelectrophoresis และด้วยวิธี neutralization ในหนูถีบจักร ส่วนพิษงูที่ถูกอาบรังสีจนหมดพิษแล้วก็ยังสามารถทำให้เกิดภูมิคุ้มกันซึ่งทำลายพิษงูชนิดเดียวกันได้ในเมื่อทดลองในหนูถีบจักร ถึงแม้ว่าการตรวจโดยวิธี agar-gel diffusion จะไม่ได้ผล จากการศึกษาด้วยวิธี electrophoresis พบว่าน้ำเหลืองกระต่ายที่มีภูมิคุ้มกันนี้ มี gamma globulin สูงขึ้น

INTRODUCTION

Snake venoms, the essential antigens in the production of antivenins, are highly toxic and complex substances. Freshly extracted venoms are generally contaminated with microorganisms. Besides, the enzymes of snake venoms may give rise to complications during immunization in horses and may influence the production of antisera. There is the requirement to obtain highly potent antisera to be fulfilled. Therefore, it is desirous to have sterile and less toxic snake venoms which retain much of antigenic character to produce antisera of optimal potency.

It has been found that following ionizing radiation snake venoms are less toxic but still retain their antigenicity[1].

Cobra and Russell's viper venoms collected from the Snake Farm of the Queen Saovabha Memorial Institute were subjected to study following radiation by cobalt-60 rays. The source of radiation was a Co-60 unit delivering a dose rate of approximately 5600 Mrads/minute at a temperature of about 30°C. The radiation processes were performed at the Office of Atomic Energy for Peace. All experimental samples were prepared from pooled lyophilized venoms. The aqueous samples were prepared by reconstituting the pooledlyophilized venoms with distilled water.

In a previous study[2] Cobra venom was studied following radiation at doses of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 Mrads in the aqueous form and up to 60 Mrads in the dry samples. The detoxification effect on these venoms was investigated in mice. It was found that in aqueous samples of 20 mg per ml of venom solution the toxicity was completely lost at a dose of 4 Mrads whereas in the dry samples the detoxification was not complete at doses up to 60 Mrads. The demonstrable antigenic properties were also studied by Ouchterlony immunodiffusion and immunoelectrophoretic techniques. The results of immunodiffusion and immunoelectrophoresis agreed with each other as well as with those of the detoxification test; that is no antigen-antibody reaction on the agar-gel diffusion can be seen at a dose of 4.0 Mrads.

Fresh Cobra venom was found to be free from contaminating organisms immediately after irradiation at a dose of 0.5 Mrad[2].

In order to study the toxoiding effect of radiation in small doses on snake venoms, Cobra venom was irradiated with cobalt-60 rays at doses ranging from 0.1 to 1.0 Mrad. The detoxification effect of radiation as well as antigenic properties of irradiated venoms were investigated. It was found that the toxicity of Cobra venom was proportionally destroyed by increasing dosage of radiation. Toxicity was reduced to two fifths at the highest exposure but there was no indication of significant effect on antigenic properties as demonstrated by immunodiffusion and immuno-electrophoresis methods.[3]

Russell's viper venom was also subjected to the same radiation at doses of 0.5, 1.0, 2.0, 3.0 and 4.0 Mrads in order to study the effects of radiation on venom of a family different from Elapid venom. It was found that the toxicity was reduced proportionally to the dosage of radiation and was completely destroyed at a dose of 4.0 Mrads as Cobra venom. The antigenicity loss as demonstrated by immunodiffusion and immunoelectrophoresis was proportional to lethality[4].

Due to the limitation of agar-gel diffusion methods in detecting the antigenic components of detoxified venoms, it is, therefore, necessary to study the antigenic properties of these venoms by direct immunization of animals in order to determine whether the venoms detoxified by radiation will be able to stimulate the production of antibodies which will neutralize the toxic effects of venoms. After failure of producing any protective antibody by immunizing sheep and mice with Cobra venom, rabbits were used as antibody-producing animals.

In a previous study[4] the antibody production was performed in rabbits using normal and irradiated Russell's viper venom and antibodies produced were studied by neutralization test in mice and by agar-gel diffusion methods. It was shown that sera from rabbits immunized with irradiated venom up to 4.0 Mrads possessed remarkable neutralizing capacity against Russell's viper venom, although the potency of these sera was not as high as that of serum from rabbits immunized with normal venom. These findings were supported by antigen-antibody reaction in immunodiffusion and immunoelectrophoresis methods.

Since Cobra venom is the essential antigen for the production of serum against the most common deadly poisonous snake found in Thailand, the present study deals with antibody production in rabbits by immunization with irradiated Cobra venom.

MATERIALS AND METHODS

Cobra venom, collected at the Queen Saovabha Memorial Institute was lyophilized and reconstituted with distilled water to make a 10 mg per ml solution and irradiated by Co-60 rays at a temperature of about 30° C. The radiation doses were 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 Mrads. A normal venom solution of 10 mg per ml was also prepared as control.

The process of immunization was the same as that in the study on Russell's viper venom[4], i.e., rabbits weighing about 2.5 kg were immunized with increasing doses of each venom solution (treated and untreated venoms) mixed with Freund's adjuvant (complete). Doses from 0.0125 mg/kg (about one tenth of M.L.D. in rabbit) to 1.5 mg/kg body weight were infected subcutaneously into the right and left thigh alternately at weekly intervals for a period of about three and a half months. (The schedule of immunization is shown in Table 1). Two rabbits were used for each venom solution. The animals were bled periodically for antibody testing purpose. The last bleeding was made 10 days after the final injection.

The antibody produced was studied by neutralization test in mice and by agar-gel diffusion methods against homologous venom.

The neutralization test was carried out in Swiss white mice by the same procedure as described in a previous study[4] and the potency of serum for protection was expressed as number of mouse LD_{50} values of venom neutralized by one ml of serum.

Antigen-antibody studies were also performed by Ouchterlony immunodiffusion and immunoelectrophoresis methods, the details of which were the same as described in the previous study on Rusell's viper venom[4] except in the last part the plates were stained with 0.5% Buffalo black to obtain better results for photographing.

Sera from rabbits immunized with normal and irradiated venoms were also subjected to polyacetate paper electrophoresis which was carried out at 300 V for 30 minutes using 0.025 M veronal buffer at pH 8.6.

RESULTS

Neutralization : The results of neutralization test are shown in Table II.
Immunodiffusion : Patterns of double diffusion in agar are shown in Figs. 1 and 2. The results can be summarized as folllows:

Antivenin from rabbits immunized with normal venom (2 in Fig. 2) gave at least 8 precipitation lines.

Antivenin from rabbits immunized with:

0.5 Mrad irradiated venom (4 in Fig. 1) gave at least 3 precipitation lines.
1.0 Mrad (6 in Fig. 1) irradiated venom gave about 5 precipitation lines.
2.0 Mrads (7 in Fig. 1) irradiated venom gave about 4 precipitation lines.
3.0 Mrads (9 in Fig. 1) irradiated venom gave about 7 precipitation lines.
4.0 Mrads (12 in Fig. 2) irradiated venom gave 2 precipitation lines.
5.0 Mrads (14 in Fig. 2) irradiated venom gave 1 precipitation line.

Immunoelectrophoresis

Antigen-antibody reaction was further investigated by immunoelectrophoresis. Patterns are shown in Figs. 3 and 4. The results can be summarized as follows:

Serum from rabbits immunized with untreated venom (2 in Fig. 3) gave about 7 precipitation lines.

Serum from rabbit immunized with:

0.5 Mrad irradiated venom (4 in Fig. 3) gave about 6 precipitation lines.

1.0 Mrad irradiated venom (6 in Fig. 3) gave about 6 precipitation lines.

2.0 Mrads irradiated venom (7 in Fig. 3) gave about 5 precipitation lines.

3.0 Mrads irradiated venom (9 in Fig. 4) gave about 6 precipitation lines.

4.0 Mrads irradiated venom (12 in Fig. 4) gave 2 precipitation lines.

5.0 Mrads irradiated venom (14 in Fig. 4) gave 1 precipitation line.

For control neither normal rabbit serum now normal saline solution showed precipitation reaction in immunodiffusion as well as in immunoelectrophoretic techniques.

Results of electrophoretic separation of rabbit sera are shown in Table III. It was observed that gamma globulin fraction was found to be increased at the first trial bleeding and in three of the samples it was increased over 50% at the final determination.

DISCUSSION

In this study the Cobra venom was prepared in the same concentration as that of Russell's viper venom in order to compare the capacity of antibody formation between the two venoms.

It was observed that rabbits immunized with untreated venom as well as venom irradiated at lower doses developed ulcers at the sets of injection, in spite of the fact that the inactivated venom samples were proved to be free of microorganisms[2]. This might be due to the necrotizing effect of venom enzymes. This effect was also demonstrated in human victims bitten by cobra[5]. About 35 % of the animals died during the period of immunization.

It can be seen in Table II that one ml of serum from rabbits immunized with normal venom could neutralize 32 mouse LD_{50} values or 120 μg. Sera from rabbits immunized with irradiated venoms up to 3.0 Mrads also showed some protection against Cobra venom. The titers of these sera fluctuated and did not correlate with the dosage of radiation. This might be due to individual susceptibility of these animals to the antigens. The number of animals used was too small and not enough to give average results. However, the antibody titers of sera from rabbits immunized with irradiated venom decreased as the radiation doses increased. Neutralizing capacity of serum from rabbits immunized with venom at the highest exposure was only 8 LD_{50} or 30 μg which is not sufficient for protection.

It can be seen in Figs. 1 and 2 (immunodiffusion method) that serum from rabbits immunized with normal venom (2 in Fig. 2) showed the highest number of precipitation lines, then sera from rabbits immunized with irradiated venom at 3.0, 2.0 and 0.5 Mrad showed decreasing numbers of precipitation lines. Those from rabbits immunized with venoms irradiated at 4.0 and 5.0 Mrads showed only two and one precipitation line respectively. These results somewhat correspond to those of the neutralization test. However, upon further study of antigen-antibody reaction by immunoelectrophoresis (Figs. 3 and 4), it was found that sera from rabbits immunized with venoms irradiated at the lower four dose levels developed similar patterns of precipitation reaction with each other as well as with that of the control serum (rabbits immunized with normal venom). However, the control serum showed more precipitation lines. Sera from rabbits immunized with venom irradiated at 4.0 and 5.0 Mrads showed only two and one precipitation line respectively. These agreed with the results of immunodiffusion as well as those of the neutralization test in mice.

Study of serum proteins by paper electrophoresis also showed increase of gamma globulin fraction in the sera from all rabbits especially that from rabbits immunized with normal venom. However, sera from rabbits immunized with venom irradiated at 4.0 and 5.0 Mrads showed only slight increase of gamma globulin fraction. This finding corresponds to the results of the neutralization test as well as those of the agar-gel diffusion methods.

In our previous studies it was found that the toxicity of Cobra venom irradiated at doses of 0.5 and 1.0 Mrad was reduced to about one half and two fifths of that of normal venom respectively[3] and was completely destroyed at a dose of 2.0 Mrads for 10 mg per ml venom solution[2]. According to the present study, it can be seen in Table II that venom irradidted at 3.0 Mrads, which was proved to be non-toxic, still could stimulate antibody formation in rabbits giving rise to about 65% protection against the homologous venom as compared to that stimulated by normal venom. The antibody formation was supported by immunodiffusion and immunoelectrophoresis tests. The results agreed well with those of irradiated Russell's viper venom in the previous study[4]. although the antibody titers were not as high as those of Russell's viper venom. This may be due to the low molecular weight of the toxic principle of Cobra venom[6] which hardly stimulates antibody formation. These experiments demonstrate that venoms detoxified by radiation still retain antigens which are capable of inducing effective antibody formation despite the fact that the antigenic properties could not be detected by agar-gel diffusion methods.

For practical use Cobra venom irradiated at 3.0 Mrads should be applicable for antibody production.

CONCLUSION

The antigenic properties of irradiated Cobra venom were studied by using rabbits as antibody-producing animal. It was found that sera from rabbits immunized with irradiated venoms up to 3.0 Mrads could neutralize considerable amounts of Cobra venom as tested in mice, though the titers were not as high as that of serum from rabbits immunized with normal venom. The results were supported by antigen-antibody reaction in immunodiffusion and immunoelectrophoresis methods.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Svasti Srisukh, director of the Office of the Atomic Energy Agency for Peace, for the use of radiation facilities, to Dr. Sriprapai Phong-Aksara, Director of the Queen Saovabha Memorial Institute, for permitting the publication of this report and for her critical review of the manuscript and to Mr. Suthinai Jiamsavatpun and Mrs. Charoenjai Jeamcharoen for their technical assistance.

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Table I Immunization Schedule for Production of Antivenin in Rabbits

Day	Venom/kg $_{mg}$ body weight
0	0.0125
7	0.025
14	0.050
21	0.075
28	0.100
35	0.150
42	0.225
49	0.300
56	0.400
63	0.500
70	0.625
77	0.750
84	0.875
91	1.000
98	1.250
105	1.500
115	bleeding

Table II Neutralization Capacity of Sera from Rabbits Immunized with Irradiated and Unirradiated Cobra Venom

Sera from rabbits immunized with venom irradiated at Mrads	Cobra venom neutralized (expressed in multiples of LD_{50} values)
0	32
0.5	12
1.0	18
2.0	18
3.0	21
4.0	12
5.0	8

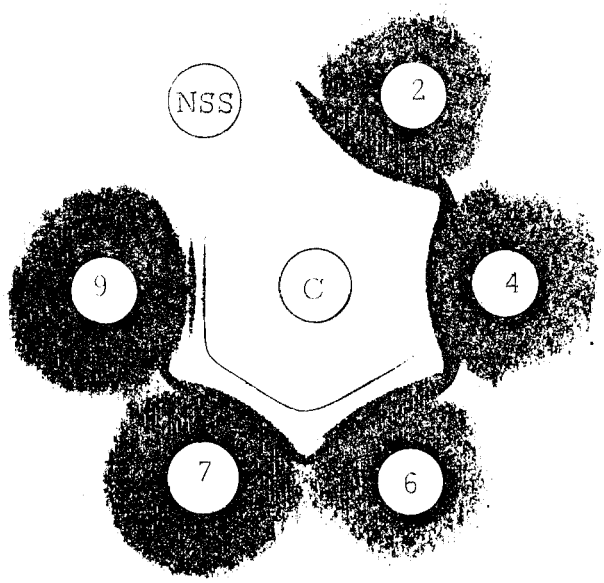

Fig. 1

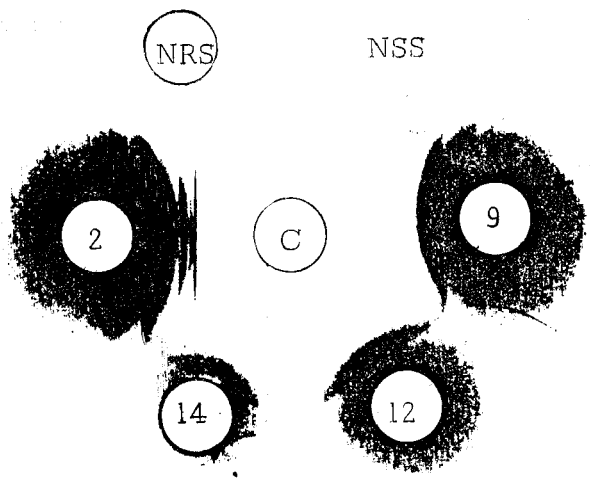

Fig. 2

Figures 1 and 2 Ouchterlony reaction of Cobra venom (C) against serum from rabbits immunized with normal venom (2), venom irradiated at 0.5 MR (4), 1.0 MR (6), 2.0 MR (7), 3.0 MR (9), 4.0 MR (12) and 5.0 MR (14). NSS and normal rabbit serum (NRS) were used as controls.

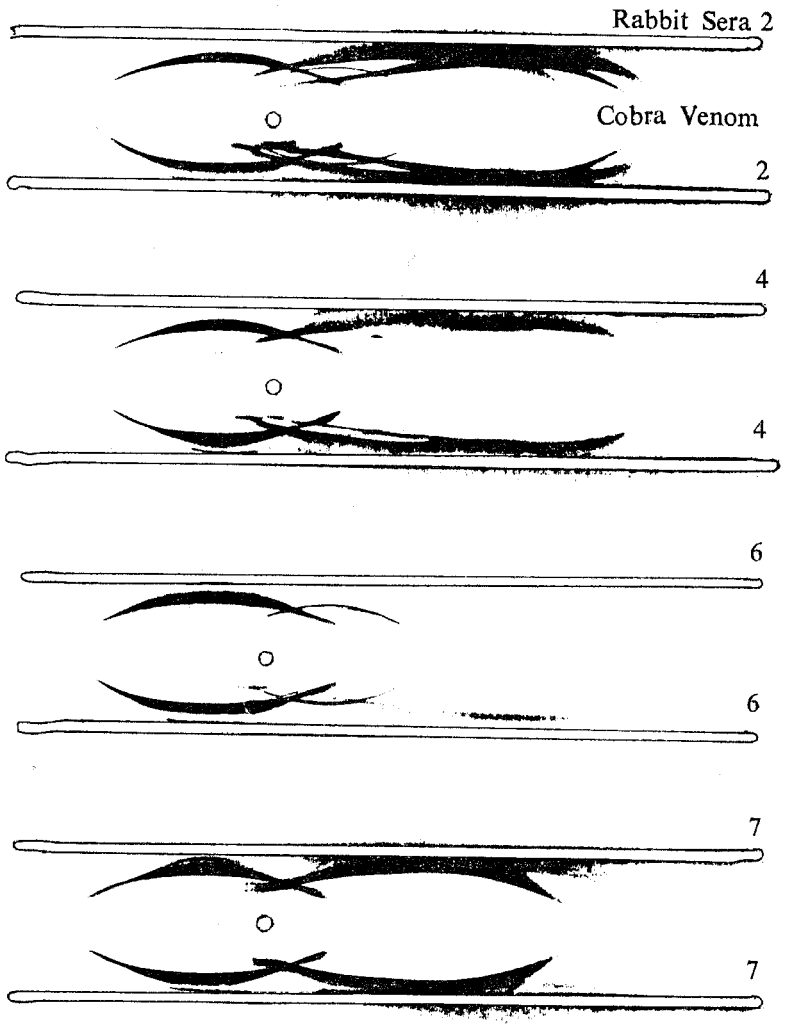

Figure 3 Immunoelectrophoretic patterns of Cobra venom (in the wells) against serum from rabbits immunized with normal venom (2), venom irradiated at 0.5 MR (4), 1.0 MR (6) and 2.0 MR (7).

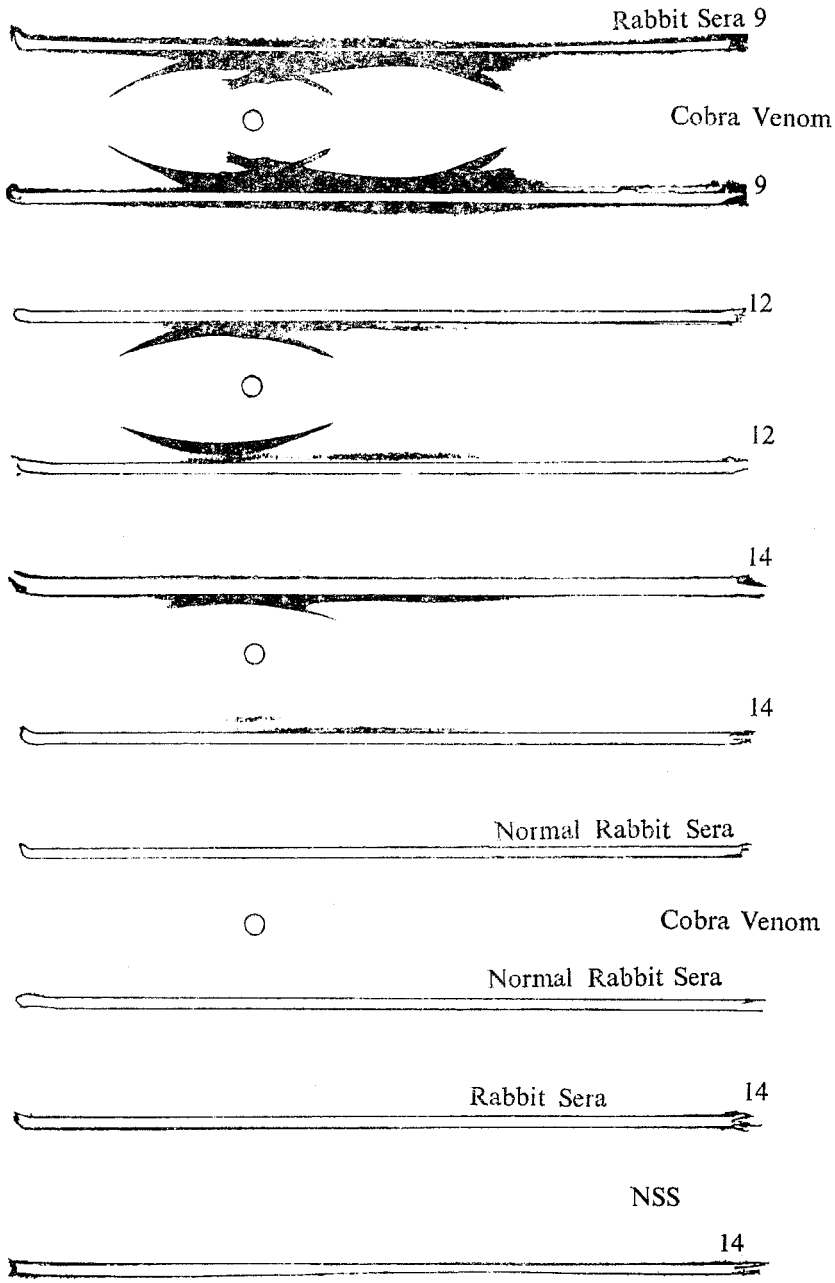

Figure 4 Immunoelectrophoretic patterns of Cobra venom (in the wells) against serum from rabbits immunized with irradiated venom at 3.0 MR (9), 4.0 MR (12) and 5.0 MR (14). Normal rabbit serum against Cobra venom and NSS against immunized rabbit serum (14) were also used as controls.