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29. ANTIVENIN PRODUCTION

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Introduction

The production of antivenins in Iran was beset with many difficulties. Since the date of preparation of this serum at Razi Institute, continued efforts have been made to improve the therapeutic properties of the product. From 1960 till now a great number of poisonous snakes from various localities of Iran have been made to improve the therapeutic properties of the product. From month for venom. The venoms are lyophilized and kept for hyperimmunization and titration. Razi Institute produces the following antivenins:

a) Monovalent antivenins against the venom of:

Naja naja, Vipera lebetina, Vipera xanthina, Vipera persica persica, Echis carinatus, Agkistrodon halys.

b) Polyvalent antivenin against the venom of:

Naja naja, Vipera lebetina, Vipera xanthina, Vipera persica persica, Echis carinatus, Vipera ammodytes, Agkistrodon halys.

c) Polyvalent antivenin against the venom of:

Naja naja, Bungarus fasciatus (Krait), *Echis carinatus, Vipera russelli.*

All antivenins are produced as pepsin digested, ammonium sulphate concentrated solution containing 10-18 gr per cent protein.

Materials and Methods

We produce our antivenins according to methods used in Butantan Institute, São Paulo, Brazil. Production begun as early as 1960 and serum was issued for therapeutic use in 1961. The snakes are obtained by purchase from people who catch them and bring them to the laboratory. The snakes are milked once a month for venoms. For obtaining the venom from the poisonous glands in *Naja*, the fangs are inserted on the rubber of the dishes and in VIPERIDAE the fangs are pressed directly inside the dishes down to the glass. The venom is refrigerated at $-18^{\circ}C$. for 24 hours and dried in dessicator under vaccum over P_2O_5. The dried venom is kept away from light and humidity. For immunization as well as serum titration we always employ a pool of dried venom from several batches for preparing our stock. The dried venom is weighted

very carefully in an analytical balance. A ten per cent solution is prepared for hyperimmunization using 50% sterile physiological salt and glycerin with a piece of camphor, as stock solution. To determine the CLD, LD_{50} and serum potency titration, one per cent solution is prepared using sterile physiological salt as the initial dilution; determinations were made in white mice weighing 16-18 gr through the intravenous route.

For the production of the antivenin, healthy horses more than five years old (usually 7-8) are selected. Observations indicate that very young and old horses do not respond very well to immunization procedures. The general scheme followed in the immunization of horses is to gradually build up their antivenin titre by the regular subcutaneous injection of increasing doses of the unmodified venoms employed for hyperimmunization which is prepared by diluting the stock solution as already mentioned. According to our observations, it is better to start the injections of the horses with a small amount of venom as indicated in Table I, to avoid producing toxic symptoms and also to get a higher titre in polyvalent antivenins. The range of the mixture of venoms is shown in Table II; the initial dose contained the equivalent of 0,1 mg in 10 ml of volume, the injections were given twice a week up to the amount of 10 mg, then were followed weekly in volume of 20 ml intervals, the highest amount of injected venom is 120 mg; Re-immunization began with 2 mg of unmodified venom. During the period of immunization three injections of one ml sterile oil, as adjuvant is sufficient. The oil is prepared for adjuvant using 75 gr arabic white gum, 300 ml olive oil, by distilled water up to 600 ml total volume, issued and autoclaved.

TABLE I — PROTOCOL OF IMMUNIZATION SCHEDULE OF HORSES

Period of injection	Antigen		Administration
	Venom mg.	Total volume with saline	
1	0,1	10 ml	Subcutaneous
2	0,2	10 ml	Subcutaneous
3	0,4	10 ml	Subcutaneous
4	0,7	10 ml	Subcutaneous
5	1	10 ml	Subcutaneous
6	2	10 ml	Subcutaneous
7	4	10 ml	Subcutaneous
8	7	10 ml	Subcutaneous
9	10	+ 1 ml oil = 20 ml	Subcutaneous
10	20	20 ml	Subcutaneous
11	30	+ 1 ml oil = 20 ml	Subcutaneous
12	40	20 ml	Subcutaneous
13	60	+ 1 ml oil = 20 ml	Subcutaneous
14	80		Subcutaneous
15	100	20 ml	Subcutaneous
bleeding			
bleeding			
bleeding	6 to 8 lit. for each horse		

TABLE II — RANGE OF POLYVALENT MIXTURE OF VENOMS EACH 1/12 EQUIVALENT TO 200 MG DRY VENOM IN 2 ML SOLUTION

Venoms	P_1	P_2
Naja naja	3/12	4/12
Vipera lebetina	3/12	1/12
Echis carinatus	2/12	3/12
Vipera xanthina	1/12	—
Vipera persica persica	1/12	—
Vipera ammodytes	1/12	—
Agkistrodon halys	1/12	—
Vipera russelli	—	2/12
Bungarus fasciatus (Krait)	—	2/12

Purification and concentration of antivenin

Methods devised for the purification and concentration of antitoxins have been applicable to antivenins also. The technique adapted in this laboratory is that originated by Pope and developed by Grasset and Christensen (1947), and Delsal and Mirchamsy (1953). The routine procedures followed in Razi Institute are outlined below.

The immune horses are bled into Sodium citrate. Six to eight liters of blood are removed from each horse on each of the three occasions in three days. The plasma is separated from the red cells by siphon, and sufficient phenolether mixture is added to give a phenol concentration of 0.5 per cent. 100 litres of plasma are mixed with 200 litres of Saline and the mixture is adjusted by pH 3.2, pepsin (1/10000 titre) is added to 0.5 gr per cent of plasma and the reaction is brought to pH 3.2. After 30 minutes, digestion at 30°C. 0.2 per cent of trisodium phosphate, 0.1 per cent of toluine, 14 per cent of ammonium sulphate are added to the mixture, while being stirred mechanically. The reaction is brought to pH 5.2, the mixture is steam-heated rapidly to 55°C and maintained at this temperature for 60 minutes. At the end of the heating period the mixture is water-cooled and the denatured protein is removed by filtration through filter cloth under slight positive pressure. The filtrate is added 0.2 per cent of trisodium phosphate and adjusted to pH 7.4, with 17 per cent of ammonium sulphate. Antibody-carrying protein precipitate is removed by filtration through filter cloth, pressed, weighted, dialized for 48 hours in running water. The volume of the dialized solution is determined and 40 per cent of aluminium gel is added (Aluminium Gel prepared at Willstater technique). Then it is heated at 50°C. for 60 minutes; the gel is removed by filtration or centrifugation and the requisite amounts of salt and phenol are added. pH 6.8-7.2, phenol concentration 0.35 per cent, protein 10-18 per cent, albumine negative. The solution of refined globulins is sterilized by filtration, checked for pH, protein, electrophoreses, phenol, sterility, innocuity, pyrogenicity and potency and ampouled for issue. The final results of polyvalent antivenin are a recovery 37-65 (52%), purification indices 1.1-2.39 (main 1.76), concentration indices 1.30-2.7 (main 1.85). The titration is made by different methods, but the

routine procedure is outlined below. Different amounts of pooled standard venom are added to a series of haemolytic tubes containing 0.1 ml of serum, total volume 0.2 ml is injected intravenously to white mice weighting 16-18 gr. Each dilution is injected to five mice and neutralization is calculated as mg weight of dry venom neutralized one ml of serum. For example the potency test of polyvalent antivenin for two batches as indicated in Tables III and IV.

TABLE III — TITRE OF PLASMA AND SERUM AGAINST VARIOUS SNAKE VENOMS OBTAINED IN IRAN. THE TITRE ARE GIVEN IN MG. VENOM NEUTRALIZED BY 1 ML OF PLASMA OR CONCENTRATED SERUM. THE M.L.D. OF VENOMS IN MG

Antivenin issued for Iran	*Naja naja*	*Vipera lebetina*	*Vipera xanthina*	*Vipera persica*	*Echis carinatus*	*Vipera ammodytes*	*Agkist. halys*
Polyvalent P_1							
Plasma	0.2	0.8	0.2	0.4	0.5	0.3	0.3
Refined Serum	0.6	2.2	1	1.4	1.7	1.2	4.4
M.L.D.	0.009	0.010	0.007	0.028	0.004	0.010	0.014
Monovalent							
Plasma	0.6	1.5	0.4	0.6	0.6	0.8	0.6
Refined Serum	0.8	3	1.6	2.4	1	1.2	0.8

TABLE IV — TITRE OF PLASMA AND SERUM AGAINST THE VENOMS OF FOREIGN ORIGIN

Antivenin issued for neighboring countries	*Naja naja*	*Bung. fasc.* (Krait)	*Echis carinatus*	*Vipera russelli*	*Vipera lebetina*
Polyvalent P_2					
Plasma	0.2	0.8	1.2	0.5	0.4
Refined Serum	0.6	1	1.6	1.2	1.6
M.L.D.	0.009	0.020	0.015	0.020	0.010

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References

1. CHRISTENSEN, P. A., *South-African Snake Venoms and Antivenoms*, 1955.

2. DELSAL, J. L., and MIRCHAMSY, H., *Rev. Immunol.*, **17**, 110, 1953.

3. GRASSET, E., and CHRISTENSEN, P. A., *Royal Soc. trop. Med. Hyg.*, **41**, 2, 1947.

4. GRASSET, E., *Trans. royal Soc. Med. Hyg.*, **26**, 1932.

5. GRASSET, E., *Bull. Wld. Hlth. Org.*, **16** (7), 122, 1957.

6. GRASSET, E., *Quart. Bull. Hlth. Org. of the League of Nations*, 1936.

7. HAZRA, A. K., *Bull. Wld. Hlth. Org.*, **12**, 15-46, 1945.

8. LEON, W., and SALAFRANCA, E., *Phillipine J. Sci.*, **85**, 1956.

9. POPE, C. G., *Brit. J. exp. Path.*, **20**, 1939.

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BIOQUÍMICA

BIOCHEMISTRY