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Original paper

PRODUCTION OF AN EFFECTIVE ANTI-*Bothrops*-TETANUS MIXED HYPERIMMUNE SERUM OF EQUINE ORIGIN

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ABSTRACT. The present investigation reveals the possibility of simultaneous immunization of horses with *Bothrops* or *Crotalus* snake venoms and Tetanus antigens for the production of anti-*Bothrops*-Tetanus or anti-*Crotalus*-Tetanus mixed serum, with high titers of the respective specific antibodies. *Bothrops* antivenoms with an average neutralizing titer of 4.16 mg venom/ml were obtained from plasma of horses with titers lower than 0.5 mg venom/ml when Tetanus antigens were not used. This suggests the existence of a synergism between *Bothrops* venoms and Tetanus antigens in the stimulation of the antibody response. The pooled plasma of the animals had a neutralizing titer of 21.0 mg/ml reference *Bothrops* venoms and 3,300 IU/ml to Tetanus antigens after purification by enzymatic digestion and ammonium sulphate precipitation. These experiments lead us to conclude that *Bothrops* envenomation therapy can be successfully performed using anti-*Bothrops*-Tetanus serum also serving as Tetanus prophylaxis. Anti-*Crotalus*-Tetanus serum can also be produced, although it is not of medical interest as *Crotalus* envenomation rarely results in local necrotizing lesions.

☑ **KEY WORDS:** antivenoms, immunization, *Bothrops*, Tetanus.

INTRODUCTION

In envenomation caused by *Bothrops* snakebites, lesions frequently occur in the venom inoculation area and the lesions may sometimes evolve to a necrosis phase. Bites usually occur on the hind limbs and when lesions evolve to necrosis, these lesions become an appropriate area for contamination by aerobe and anaerobe bacteria, such as *Clostridium tetani*. Due to this possible contamination, medical guidelines appeared in the" Manual de Diagnóstico e Tratamento de Acidentes Ofídicos"(**11**) indicating the need for Tetanus prophylaxis in characteristic cases and specifications. Considering the Tetanus incubation period (7 to 10 days), and in case of a not-previously vaccinated patient or one that is unaware of having been immunized, administration of the anti-Tetanus serum is imperative. In such a situation, the patient should receive a certain number of *Bothrops* antivenom vials added to the recommended dose of Anti-Tetanus serum.

It was thought that when Tetanus prophylaxis was needed, it could be applied with of a mixed anti-*Bothrops*-Tetanus serum. Thus, the volume of the heterologous serum and consequently the amount of protein administered would be lower turning both Tetanus treatment and prophylaxis easier, besides reducing treatment and serum production costs.

This study was aimed to demonstrate the possibility of immunizing horses with venoms from species of the *Bothrops* genus and antigens obtained from *Clostridium tetani* cultures(**4,8**). A purified hyperimmune serum with an adequate neutralizing potency to both offending agents under study is demonstrated.

MATERIALS AND METHODS

HORSES: Hyperimmune sera were obtained from horses of undefined breed, weighing about 380 kg, aged approximately eight years old, bought from Rio Grande do Sul breeders, Brazil. The animals were fed daily with six kilograms of a balanced feed containing 20% of protein, green foxtail grass and water "*ad libitum*". All the animals were already serum producers for at least one year and presented low serum neutralizing titers. Under these conditions**,** the horses were divided into three groups:

Group 1: five horses, previous anti-*Bothrops* hyperimmune plasma producers.

Group 2: three horses, previous Anti-Tetanus plasma producers.

Group 3: five horses, previous anti-*Bothrops-Crotalus* plasma producers.

MICE: Swiss mice weighing between 18 and 22g were provided by Butantan Institute Animal Facility.

SNAKE VENOMS: A pool of venoms from snakes of the *Bothrops* genus kept at the Butantan Institute Serpentarium, São Paulo, SP, Brazil, of the following species and in the respective proportions was used for immunizing the horses: *B. jararaca* 40%, *B. moojeni* 20%, *B. alternatus* 13.3%, *B. jararacussu* 13.3% and *B. neuwiedi* 13.3%. Each venom was collected from various specimens received from different regions of Brazil, mixed, lyophilized and kept at 4°C. The LD50 value for the *Bothrops* venom pool was 90.0µg per 18 to 22 g mice.

Crotalus venom was a pool of venoms collected from various specimens of *Crotalus durissus terrificus* from different regions of Brazil, lyophilized and kept at 4°C. *Crotalus* venom LD50 was 1.5 µg per 18 to 22 g mice.

The reference venoms were provide by the National Institute of Health Control, Rio de Janeiro, RJ, Brazil.

TETANUS ANTIGENS: Tetanus toxin was concentrated by molecular ultrafiltration in 30,000 Nominal Molecular Weight Limit(**4**) and Tetanus anatoxin was purified by a combination of molecular ultrafiltration in 50,000 Nominal Molecular Weight Limit and gel filtration on Sephadex G-50(**10**).

ADJUVANT: According to the immunizing scheme indicated, the adjuvant was the Incomplete Multiple Emulsion (IME) prepared by the technique of Herbert(**7**), composed of Marcol 90%, Montanide 888 10% and Tween 80 2%. The proportion between antigens (aqueous phase) and the adjuvant IME was of 50% and the establishment of a stable emulsion was obtained through the use of a household mixer.

HORSE IMMUNIZATION SCHEMES:

Group 1: horses with anti-*Bothrops* basic immunization. These animals were subcutaneously inoculated according to **Table 1**.

Group 2: horses with Tetanus basic immunization. These animals were subcutaneously inoculated according to **Table 2**.

Group 3: horses with anti-*Bothrops* and *Crotalus* basic immunization. Animals from this group were subcutaneously inoculated according to **Table 3**.

In each group, inoculation of venoms was conducted around the spots (4 spots) of Tetanus antigen inoculation (Tetanus toxin or Tetanus anatoxin).

BLEEDING AND PLASMA SEPARATION:

Animals from all groups were bled five days after the last antigen dose.

Six liters of blood were taken from each animal by jugular punction, and kept in double-faced plastic bags and sterilized by cobalt-gamma radiation. The bags were interlinked, the first containing 400 ml of anticoagulant solution (Dextrose - 1.47%; Sodium citrate - 4.8%; Citric acid - 1.47%). After the bleedings**,** the bags were stored at 5°C ± 1.0**,** and 24 hours later**,** the plasma was transferred to a second bag.

PLASMA PURIFICATION: Plasmas were purified according to the industrial method described by Harms(**5**). Once pepsin has acted**,** the material was heated to 56°C for two hours in the presence of an adequate concentration of ammonium sulphate for the precipitation of denaturated non-neutralizing proteins.

ANTIVENOM AND ANTITOXIN NEUTRALIZING ASSAYS:

Lethality: Groups of six mice were injected intraperitoneally (i.p.) with several doses of venom dissolved in saline. Forty-eight hours later, deaths were recorded and results analyzed by probit test according to Finney(**3**). The mean lethal dose (LD50) is defined as the venom dose which killed 50% of the mice .

Determination of ED50: The proctetive effect of antivenom was estimated by determining the effective dose that protects 50% of the envenomed animals (ED50)(**1**). Reference venom (5 LD50) was mixed with different amounts of antivenoms dissolved in saline. The mixture was incubated at 37°C during 30 minutes and survival was recorded 48 hours after inoculation. Results were submitted to statistical analysis by the probit method according to Finney(**3**).

Flocculation Assay: According to Lying and Betzon(**9**) and the WHO(**12,13**) the recommended method was assayed with standard Tetanus Toxin containing 100 Lf/ml. Fixed volume mixtures of standard Tetanus toxin were prepared with different amounts of equine serum incubated in a water bath (45°C) and observed up to 120 minutes, for the presence of flocculation. The first tube presenting flocculation was considered for the titer estimation.

Serum-neutralizing Assay: The method described by the WHO(**12,13**) was used for this assay. This method establishes that the Tetanus toxin dose L+/10 (lethal limit /10) is the smallest amount of reference Tetanus toxin that combined with 0.10 IU of reference Tetanus antitoxin results in 100% mortality of Swiss mice (18 to 22g) in 96 hours. After the determination of the toxin dose L+/10, this was added to different serum concentrations or to the reference serum (control), kept at room temperature for 60 minutes and subcutaneously inoculated in mice. The animals were observed for 96 hours. Toxin-serum combinations which protected the animals against death presenting slight symptoms of Tetanus after 96 hours were considered as containing 1.0 IU/ml of antitoxin.

RESULTS AND DISCUSSION

Table 4 shows the comparison of neutralizing titers both before and after the immunization with the combined antigens. Titers were significantly increased after immunization with associated antigens when compared with titers obtained from horses immunized with either venom or Tetanus antigens. Our results showed the possibility of inducing protective antibodies in relatively high titers, against venoms and Tetanus toxin when animals were immunized simultaneously with both antigens. The animals with low titers of *Bothrops* and *Crotalus* venoms present satisfactory titers after the association of venoms with Tetanus antigens. These findings suggest a synergism of these two types of antigens to elicit an immune response in horses, according to the results observed with **Group 3**. Opposite result seems to occur for Anti-Tetanus titers if a reduced scheme of immunization was used, as showed in **Group 1**. *Bothrops* venom probably acts as an adjuvant to Tetanus antigen due to its content in lectins in which the lectins induce T and B lymphocyte non-specific proliferation(**6**). It has also been described in the literature(**2**) the possibility of *Bothrops* and *Crotalus* venoms to induce an increase in TNF-, IL-1, IL-6 and IL-8 which promotes an increase in the macrophage functional activity and also augments the number of mononuclear and polymorphonuclear cells.

Results of **Group 2** also suggest that previously immunized horses with Tetanus antigens fail to respond satisfactorily to *Bothrops* antigen.

The pool of plasmas of **Group 1** was purified by the enzymatic digestion method. The product obtained was capable of neutralizing 21.0 mg/ml of *B. jararaca* reference venom, having a titer of 3,300 IU/ml to the Tetanus component. Under these conditions, the serum concentrate allowed a final dilution of 1:3.5, and was still able to neutralize 6 mg/ml of the bothropic component, and had 680 IU/ml to the Tetanus component. Yields and degrees of purification in this batch were as follows:

***Bothrops* component:** 40% and 201.7 mg/g of proteins

Tetanus component: 53.6% and 23,404 IU/g of proteins

The protein concentration obtained in this preparation was similar to that found in anti-*Bothrops* serum alone. Based upon our calculations, it is possible to conclude that a mean Tetanus titer of 300 IU/ml in the plasma of immunized animals would be sufficient to assure adequate treatment to patients bitten by *Bothrops* snakes and also to provide the respective Tetanus prophylaxis.

TABLE 1. Horse immunization scheme for **Group 1**.

DAY	ANTIGENS (Doses)		
	Bothrops venoms mg	Tetanus Anatoxin Lf	Tetanus Toxin Lf
1	2.5	100*	-
4	-	200	-
7	-	300	-
10	-	400	-
13	-	800	-
15	5.0	1,200	-
18	-	1,500*	-
21	5.0	2,000	-
24	5.0	3,000	-
28	-	-	4,000
31	5.0	-	5,000

TABLE 2. Horse immunization scheme for **Group 2**.

DAY	ANTIGENS (Doses)	
	Bothrops venoms mg	Tetanus Toxin Lf
1	2.5*	-
21	5.0	1,000
28	2.5	1,000
31	2.5	3,000
33	2.5	-
38	5.0*	3,000
45	5.0	4,000
52	10.0	5,000

TABLE 3. Horse immunization scheme for **Group 3**.

DAY	ANTIGENS (Doses)			
	Bothrops venoms mg	*Crotalus* venoms mg	Tetanus Anatoxin Lf	Tetanus Toxin Lf
1	2.5	2.5	1,000*	-
3	-	-	200	-
6	5.0	-	300	-
9	-	5.0	400	-
15	5.0	5.0	1,200	-
18	2.5	2.5	750*	-
25	5.0	5.0	-	500

TABLE 4. *Bothrops* and *Crotalus* antivenoms titers expressed as mg of reference venoms neutralized by 1ml of serum and of Tetanus antitoxin in International Units by 1ml of serum in horses before and after mixed immunizations.

Group	Horse N°	BEFORE ANTIGEN ASSOCIATION			AFTER ANTIGEN ASSOCIATION		
		Bothrops Antivenoms	*Crotalus* Antivenoms	Tetanus Antitoxin	*Bothrops* Antivenoms	*Crotalus* Antivenoms	Tetanus Antitoxin
	34	< 0.50	FT	< 1.0	0.59	FT	> 2,000
	216	< 0.50	FT	< 1.0	3.64	FT	500
1	540	< 0.50	FT	< 1.0	0.89	FT	1,800
	830	< 0.50	FT	< 1.0	8.20	FT	1,000
	832	< 0.50	FT	< 1.0	7.50	FT	800
	208	FT	FT	500	1.35	FT	640
2	538	FT	FT	500	2.39	FT	1,200
	651	FT	FT	300	1.40	FT	640
	496	< 3.00	0.3	FT	3.77	0.45	640
	637	3.00	0.8	FT	4.18	0.62	350
3	741	< 3.00	0.5	FT	4.26	0.87	350
	754	< 3.00	0.3	FT	6.73	0.45	350
	777	< 3.00	0.4	FT	8.50	0.83	640

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