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Production of monovalent anti-*Bothrops asper* antivenom: development of immune response in horses and neutralizing ability

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Abstract: A monovalent antivenom was produced by immunizing two horses with venom of the pit viper *Bothrops asper* (Ophidia: Viperidae). Although development of the immune response against four toxic and enzymatic activities of the venom was similar in both horses during the first two thirds of the immunization schedule, antibody response in one of the horses reached much higher levels in the last part of the immunization. Immunoelectrophoretic analysis indicates that there were precipitating antibodies in the sera of these horses during all the stages of immunization. However, immunoprecipitation did not correlate with the ability of sera to neutralize toxic activities of *B. asper* venom. Monovalent antivenom was more effective than the commercially available polyvalent antivenom in the neutralization of *Bothrops asper* venom. On the other hand, despite the fact that it neutralizes lethal and hemorrhagic activities of the venoms of *Lachesia muta* and *Crotalus durissus durissus*, it was less effective than polyvalent antivenom in these neutralizations. Moreover, it does not neutralize defibrinating activity induced by these two venoms, whereas it neutralizes this effect in the case of *B. asper* venom. It is proposed that monovalent antivenom may be highly effective in the case of envenomations induced by *Bothrops asper* venom; its use in treating accidents by *L. muta* and *C. durissus* would be indicated only if polyvalent antivenom is not available. Results also demonstrate that it is important to monitor antibody response individually in horses being immunized for antivenom production, due to the conspicuous variability in the response of different animals.

Bothrops asper is responsible for the large majority of snakebite cases in Central America (Bolaños 1982). Currently, the polyvalent antivenom produced at the Instituto Clodomiro Picado in Costa Rica is being used in several Central American countries to treat accidents induced by crotaline snakes. This antivenom is produced in horses by immunization with a mixture of equal parts of the venoms of *Bothrops asper, Lachesis muta* and *Crotalus durissus durissus* (Bolaños and Cerdas 1980). Previous studies have demonstrated that polyvalent antivenom is highly effective in the neutralization of toxic and enzymatic activities of Central American crotaline venoms (Gutiérrez *et al.* 1981, 1985, 1987a. Gené *et al.* 1985, Rojas *et al.* 1987). The only effect which is poorly neutralized is the edema-forming activity (Lomonte 1985; Gutiérrez *et al.* 1986).

In the past years there has been a significant increment in the production of polyvalent antivenom in Costa Rica. If this trend continues, there is a potential problem with venom supply, since although the reserves of *B. asper* venom are high, those of *L. muta* and *C. durissus* venoms have decreased recently due to the difficulty in collecting these snakes in the field. This fact, together with the observation that the majority of accidents are inflicted by *B. asper*, prompted us to produce a monovalent anti–*B. asper* antivenom in order to study its neutralizing capacity against homologous and heterologous crotaline venoms.

On the other hand, there is a need of more studies dealing with the development of immune response in horses used to produce antivenoms. In this work we followed the humoral immune response against toxic and enzymatic activities of *B. asper* venom in horses utilized to produce this monovalent antivenom.

MATERIAL AND METHODS

Venoms: The venoms of the snakes *Bothrops asper, Lachesis muta stenophrys* and *Crotalus durissus durissus* were obtained from specimens collected in different regions of Costa Rica and maintained at the Instituto Clodomiro Picado. Once obtained, venoms were frozen, lyophilized and kept at –20°C.

Immunization schedule and plasma fractionation: Two adult horses, which had not been previously injected with snake venoms, were immunized with venom obtained from adult *Bothrops asper* specimens collected in the Atlantic region of Costa Rica and kept at the Instituto Clodomiro Picado. The immunization schedule is shown in Table 1. At different times along immunization, blood samples were obtained from the jugular vein immediately before venom injection: sera were separated, distributed in aliquots and maintained at –20°C until used. At the end of immunization, horses were bled and plasma fractionated by ammonium sulfate precipitation. In some experiments, the polyvalent antivenom produced in Costa Rica (Bolaños and Cerdas 1980) was used for comparative purposes.

Neutralization of lethality: It was determined by incubating different proportions of venom and antivenom for 30 minutes at 37° C. Then 0.5 ml of the mixtures (containing 4 LD50 of venom) were injected intraperitoneally in groups of four mice (16–18 g body weight). Deaths were recorded during 48 hours and data were analyzed according to the Spearman–Karber method (W.H.O. 1981). Neutralization was expressed as effective dose 50% (ED50), defined as the antivenom/venom ratio which protects 50% of the population.

Neutralization of hemorrhage and proteolysis: The were determined by experiments with preincubation of venom and antivenom, as described by Gutiérrez *et al.* (1985). Neutralization was expressed as effective dose 50% (ED50), defined as the antivenom/venom ratio that reduces in a 50% the activity of venom alone.

Neutralization of indirect hemolysis: The technique described by Gutiérrez *et al.* (1988) was used. Venom and antivenom were mixed in different proportions and incubated for 30 minutes at 37°C. Then, samples of the mixtures were added to wells made on a gel containig 0.8% agarose with erythrocytes, egg yolk and CaC12. Plates were incubated at 37°C for 20 hours after which the diameter of hemolytic at 37°C for 20 hours after which the diameter of hemolytic halos were measured. Neutralization was expressed as effective dose 50%, defined as the antivenom/venom ratio that reduces in a 50% the diameter of the halo induced by venom alone.

Neutralization of edema: The technique of Yamakawa *et al.* (1976) modified by Gutiérrez *et al.* (1986) was used. Groups of four mice (20–22 g) were injected with various volumes of antivenom (100 µl, 200 µl and 400 µl) intravenously. Five minutes later, animals were injected subcutaneously in the right foot pad with a dose of venom equivalent to 6 minimum edema–forming doses dissolved in 50 µl of phosphate–buffered saline solution, pH 7.2. The left foot pad was injected with 50 µl of saline solution in the same conditions. Edema was evaluated 6 hours after injection as described elsewhere (Gutiérrez *et al.* 1986).

Neutralization of defibrinating activity: The procedure of Theakston and Reid (1978), modified by Gutiérrez *et al.* (1987), was followed. Several mixtures of venom and antivenom were incubated at 37°C for 30 minutes. Then, 0.2 ml (containing two minimum defibrinating doses of venom) were injected intravenously in the tail vein of groups of four mice (10–22 g). After one hour, mice were anesthetized with ether and bled by cardiac puncture. After incubation during one hour at room temperature, the formation of clots was observed. Neutralization was expressed as effective dose, defined as the

lowest antivenom/venom ratio which neutralized defibrinating activity in all mice tested.

Immunoelectrophoresis: Venoms were separated by electrophoresis on 1% agarose gels in 0.025 M barbital buffer, pH 8.2 (Ouchterlony and Nilsson 1978). After electrophoresis, two channels were made in the gel and filled with either polyvalent or monovalent ativenom. After 24 hours of diffusion at room temperature in a humid chamber, gels were washed several times with saline solution and stained with Amidoblack 10B. The number of precipitin arcs was recorded. For comparison, samples of polyvalent antivenom were tested simultaneously with each sample of monovalent antisera.

RESULTS

Development of immune response: Antibody production against *B. asper* venom components reached higher levels in horse No. 1 than in horse No. 2, although during the first two thirds of the immunization schedule both horses had a relatively similar response (Fig. 1). In agreement with previous publications (Gené *et al.* 1985, Gutiérrez *et al.* 1985) neutralization of hemorrhage and proteolysis was more effective than neutralization of lethality and indirect hemolysis. The development of antibody response against lethal, hemorrhagic, and indirect hemolytic effects showed a similar time-course. However, response against proteolytic activity presented a different pattern, since the striking difference observed between the two horses regarding neutralization of lethality, hemorrhage and hemolysis was not as pronounced in the case of of proteolysis (Fig. 1).

Analysis of immunoelectrophoretic results indicates the presence of precipitating antibodies against several venom components even at the earliest times of immunization (Fig. 2, Table 3). For instance, after the third injection (corresponding to 0.083 mg venom), sera from horses 1 and 2 formed seven and eight precipitin bands, respectively, when confronted against *B. asper* venom. At the end of the immunization, monovalent antisera formed 11 precipitin arcs when confronted with *B. asper* venom. In the same conditions, the commercially available polyvalent antivenom formed 11 bands against the same venom (Fig. 2).

Neutralizing capacity of antivenom: The final monovalent antivenom was tested for its ability to neutralize lethal, hemorrhagic, defibrinating and edema-forming activities of the venom of *Bothrops asper, Lachesis muta stenophrys* and *Crotalus durissus durissus.* Table 3 and fig. 2 show that monovalent antivenom was highly effective in neutralizing toxic activities of *B. asper* venom, with the exception of edema-forming effect which was only partially neutralized. On the other hand, monovalent antivenom was partially effective in neutralizing the venoms of *L. muta* and *C. durissus*, being more effective against the first one (Table 3, Fig. 3). Interestingly, monovalent antivenom neutralized the lethal effect of the three venoms tested. When compared with the polyvalent antivenom produced in Costa Rica at the Instituto Clodomiro Picado, the monovalent antivenom was more effective in the neutralization of *B. asper* venom, having a lower neutralizing ability against the venoms of *L. muta* and *C. durissus* (Table 3).

When confronted with *L. muta* and *C. durissus* venoms by immunoelectrophoresis, monovalent antivenom formed many precipitin arcs, corroborating the high degree of cross-reactivity between these venoms and that of *B. asper.* Nevertheless, there were bands resultant from the reaction between venoms and polyvalent antivenom that were absent when final monovalent antivenom was tested against heterologous venoms (Fig. 4).

DISCUSSION

Despite the fact that many centers in the world produce antivenoms (Chippaux and Goyffon 1983), there are very few studies related to the development of immune response in animals injected with snake venoms or toxins. In this work, antibody production against components responsible of four toxic and enzymatic activities of *B. asper* venom was monitored. Results indicate that there was a remarkably different response in the two horses studied, since although neutralizing ability was similar during the first stages of the immunization schedule, horse No. 1 developed a much higher antibody response at later stages. Interestingly, this difference was not associated

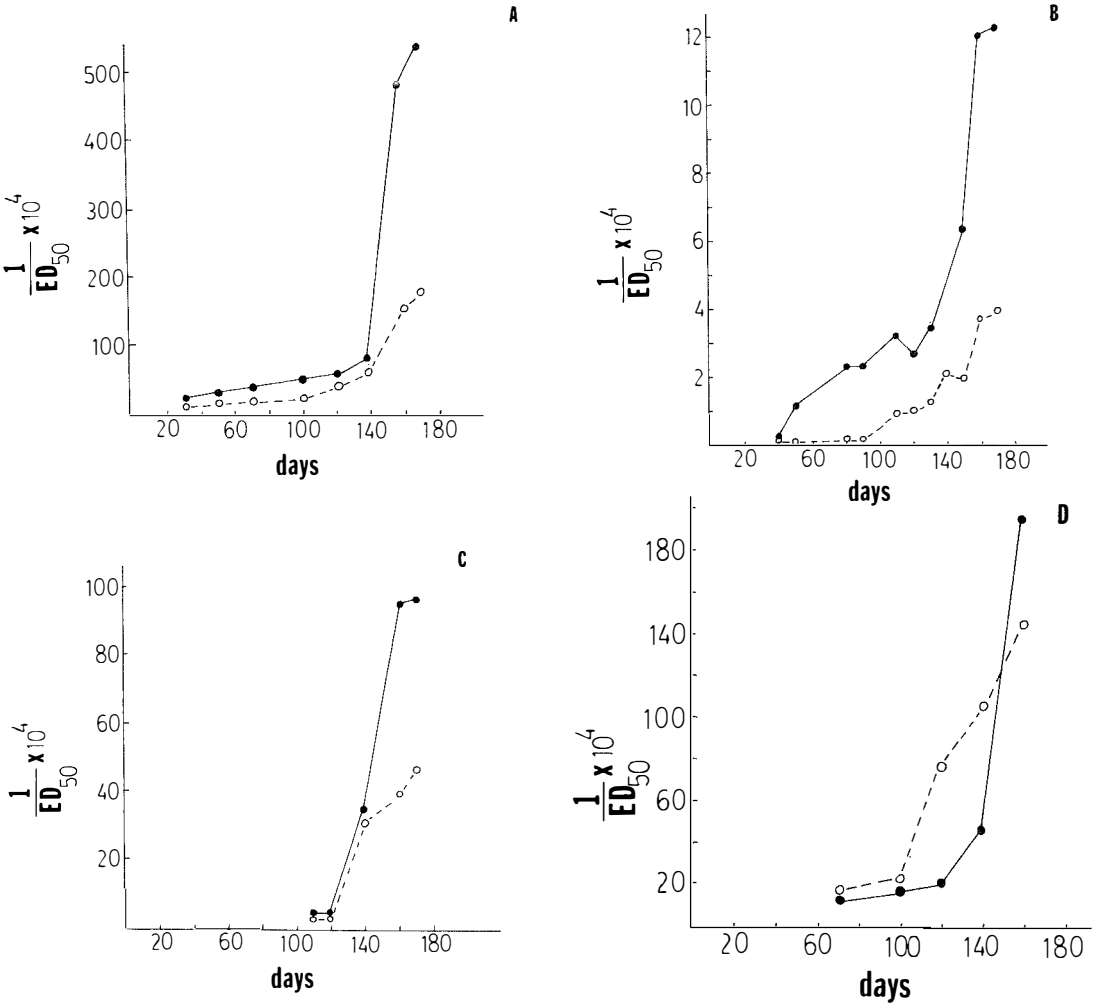

Fig. 1. Time-course of the development of antibody response in two horses immunized with *Bothrops asper* venom against four toxic and enzymatic activities of the venom. Neutralizing ability is expressed as (1/ED50) x 10^4, where ED50 is defined as the antivenom/venom ratio which neutralizes venom activity in a 50°/₀₀. The immunization schedule is shown in Table 1. (A) Development of antibody response against hemorrhagic activity; (B) Development of antibody response against indirect hemolytic activity; (C) Development of antibody response against lethal activity; (D) Development of antibody response against proteolytic activity. (●——●) horse No. 1, (○——○) horse No. 2.

with a different clinical response of both horses to venom injections. Since individual horses respond so differently to the immunization, it is important to monitor antibody response in each animal during immunization. This would help in the selection of horses with a good antibody titer, in order to bleed them and obtain a pool of plasma with high neutralizing ability. Such individual monitoring could be performed by *in vitro* techniques such as the one described by Gutiérrez *et al.* (1988) who showed that, in the case of the polyvalent antivenom produced in Costa Rica, neutralization of indirect hemolysis *in vitro* correlates significantly with neutralization of lethality. Since this is a simple and inexpensive assay, antibody response in individual horses could be easily evaluated by studying the neutralization of indirect hemolysis, thereby avoiding the large scale use of mice.

Antibody response (expressed as ED50) raised earlier and reached higher levels against hemorrhagic toxins and proteases than against phospholipases A2 and lethal toxins. This finding parallels previous results in that polyvalent antivenom is more efective in the neutralization

TABLE 1

*Immunization schedule used in the production of Monovalent anti–*Bothrops asper *antivenom*

Day No.	*Venom injected (mg)**	*Adjuvant*
1	0.083	Incomplete Freund
10	0.083	Sodium alginate
20	0.083	Sodium alginate
30	0.12	Sodium alginate
40	0.23	Sodium alginate
50	0.35	Sodium alginate
60	0.50	Sodium alginate
70	0.70	Sodium alginate
80	1.0	Sodium alginate
90	1.5	Sodium alginate
100	3.0	Sodium alginate
110	5.0	Sodium alginate
120	10.0	Sodium alginate
130	15.0	Sodium alginate
140	30.0	Complete Freund
150	30.0	Sodium alginate
160	50.0	Sodium alginate

* Injections were made subcutaneously.

of proteolysis and hemorrhage than in the neutralization of indirect hemolysis and lethality (Bolaños and Cerdas 1980, Gutiérrez *et al.* 1981, 1985, 1987a, Gené *et al.* 1985). Thus hemorrhagic and proteolytic components are more immunogenic, due perhaps to their high molecular weight which contrast with the relatively low molecular weight of phospholipases A2 (Tu 1977).

Immunoelectrophoresis was more sensitive than neutralization assays in detecting antibody response since even at the earliest venom inoculations there were several precipitin bands when venom was confronted with antisera. However, immunoprecipitation was not useful in predicting the neutralizing ability of antisera as the number of precipitin bands was similar in sera from both horses at all times tested, in contrast to neutralizing ability which was higher in horse No. 1.

Monovalent anti-*B. asper* antivenom has a higher neutralizing capacity when tested against *B. asper* venom than polyvalent antivenom (Table 3). Moreover, it cross reacts significantly with the venoms of *L. muta* and *C. durissus,* as revealed by immunoelectrophoresis and neutralization assays. This type of neutralization against heterologous venoms has been also observed with other monovalent anti-*Bothrops* antivenoms (Siles *et*

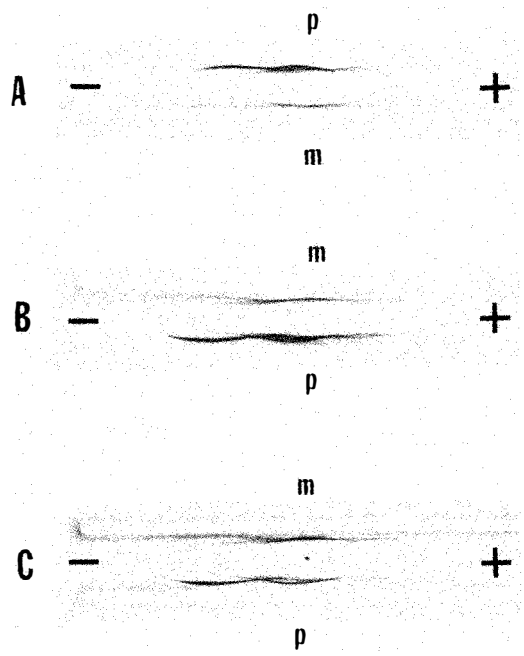

Fig. 2. Immunoelectrophoresis of sera obtained from horses immunized with *B. asper* venom at different stages in the immunization.For comparative purposes, commercial polyvalent antivenom was also confronted with *B. asper* venom. (A) Serum from horse No. 1 collected at day 20 of immunization; (B) Serum from horse No. 1 collected at day 60 of inmunization; and (C) Serum from horse N°1 collected at day 90 of immunization. Commercial polyvalent antivenom was placed in channels (P) whereas samples of monovalent antisera were placed in channels (M). Notice the presence of many precipitin arcs even at a very early stage in the immunization process.

TABLE 2

Immunoelectrophoretic analysis of the antibody response in horses immunized with Bothrops asper *venom*

Dose of venom injected (mg) 10 days before	Number of precipitin arcs in immunoelectrophoresis*	
	Horse No. 1	Horse No. 2
0.083	7	8
0.12	8	7
0.23	7	7
0.35	8	8
1.0	8	8
1.5	7	8
3.0	8	9
10.0	12	11
30.0	12	11
50.0	11	11

* For comparison, commercially–available polyvalent antivenom formed 11 precipitin arcs when confronted with *B. asper* venom in identical experimental conditions.

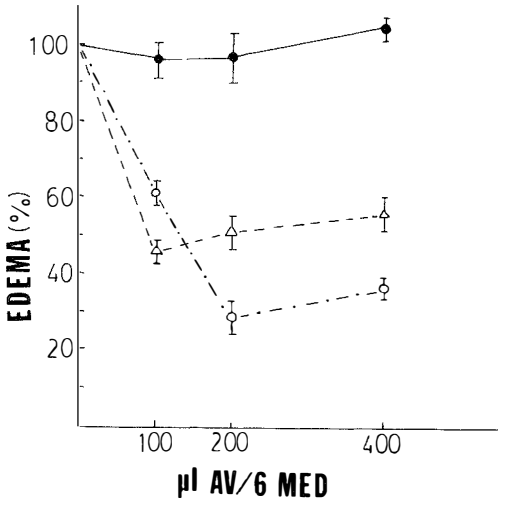

Fig. 3. Neutralization of edema-forming activity of three snake venoms by monovalent anti *B. asper* antivenom. In order to express neutralization of edema on percentage basis, swelling induced by venoms alone was considered as 100% edema. (●——— ●) Neutralization of *C. durissus* venom; (○- - - - ○) Neutralization of *L. muta* venom; (△——— △) Neutralization of *B. asper* venom. In the cases of *L. muta* and *B. asper,* antivenom reduced edema-forming activity significantly ($P < 0.05$) at all doses tested.

TABLE 3

Comparison of the ability of monovalent and polyvalent antivenoms to neutralize lethality, hemorrhage and difibrination induced by the venoms of Bothrops asper, Lachesis muta *and* Crotalus durissus durissus.

Venom	Toxic activity	Neutralizing ability (ED50) * Monovalent antivenom	Polyvalent antivenom
Bothrops asper	Lethality	216	333**
	Hemorrhage	70	135***
	Defibrination	400	600****
Lachesis muta	Lethality	333	400**
	Hemorrhage	250	85***
	Defibrination	>4000	600****
Crotalus durissus durissus	Lethality	1390	666**
	Hemorrhage	840	150***
	Defibrination	>4000	400****

* Expressed as effective dose 50% (ratio of µl antivenom/mg venom in which the effect is neutralized 50%).

** According to Bolaños and Cerdas (1980).

*** According to Gutiérrez *et al.* (1985).

**** Gené, J.A. (unpublished data).

al. 1978/79). However, despite the fact that monovalent anti–*B asper* antivenom was effective in neutralizing lethality and hemorrhage of *L. muta* and *C. d. durissus* venoms, it was ineffective against defibrinating activity (Table 3). Regarding edema, monovalent and polyvalent antivenoms have a similar neutralizing ability (Gutiérrez *et al.* 1986). Surprisingly, neither antivenom was capable to reduce edema

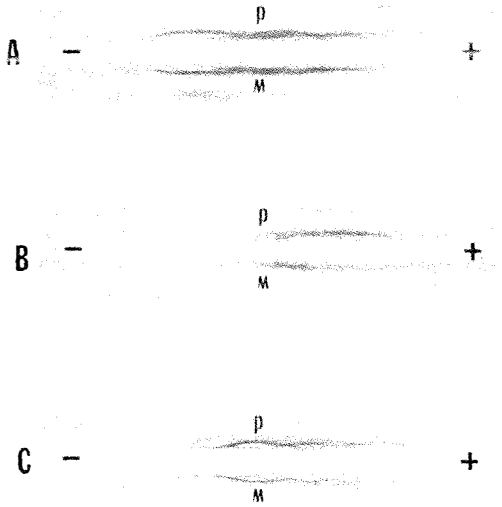

Fig. 4. Immunoelectrophoresis of polyvalent and monovalent antivenoms against the venoms of *Bothrops asper* (A), *Crotalus durissus durissús* (B) and *Lachesis muta stenophrys* (C). Channels labelled as (P) correspond to polyvalent antivenom and those labelled as (M) correspond to monovalent antivenom.

induced by *C. d. durissus* at any of the doses tested (Lomonte 1985, Rojas *et al.* 1987). In conclusion, it is proposed that although monovalent anti *B. asper* antivenom is highly effective in the neutralization of *B. asper* venom, its use in treating accidents by *L. muta* and *C. d. durissus* seems to be indicated only if polyvalent antivenom is not available.

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RESUMEN

En el Instituto Clodomiro Picado se produjo un suero monovalente contra el veneno de

la serpiente *Bothrops asper* (terciopelo). Durante las primeras etapas del proceso los caballos inmunizados mostraron un título similar en lo que respecta a la neutralización del veneno; sin embargo, uno de ellos mostró un título muy superior en la última fase de la inmunización. El suero monovalente fue más efectivo que el suero polivalente comercial para neutralizar el veneno de *B. asper*. Por otra parte, el suero monovalente fue menos efectivo que el polivalente para neutralizar las actividades letal y hemorrágica de los venenos de *Lachesis muta* (cascabel muda) y *Crotalus durissus* (cascabel). Más aún, el monovalente no neutralizó la actividad desfibrinante de estos dos venenos. Se propone que el suero monovalente anti–*B asper* es altamente efectivo en la neutralización del veneno homólogo, en tanto que su empleo en el tratamiento de envenenamientos por *L. muta* y *C. durissus* se justifica sólo cuando el suero polivalente no esté disponible.

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