Brazilian Journal of Medical and Biological Research (2002) 35: 1221-1228
ISSN 0100-879X

# Evaluation of antivenoms in the neutralization of hyperalgesia and edema induced by *Bothrops jararaca* and *Bothrops asper* snake venoms

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## Abstract

Neutralization of hyperalgesia induced by *Bothrops jararaca* and *B. asper* venoms was studied in rats using bothropic antivenom produced at Instituto Butantan (AVIB, 1 ml neutralizes 5 mg *B. jararaca* venom) and polyvalent antivenom produced at Instituto Clodomiro Picado (AVCP, 1 ml neutralizes 2.5 mg *B. aspar* venom). The intraplantar injection of *B. jararaca* and *B. asper* venoms caused hyperalgesia, which peaked 1 and 2 h after injection, respectively. Both venoms also induced edema with a similar time course. When neutralization assays involving the independent injection of venom and antivenom were performed, the hyperalgesia induced by *B. jararaca* venom was neutralized only when bothropic antivenom was administered *iv* 15 min before venom injection, whereas edema was neutralized when antivenom was injected 15 min or immediately before venom injection. On the other hand, polyvalent antivenom did not interfere with hyperalgesia or edema induced by *B. asper* venom, even when administered prior to envenomation. The lack of neutralization of hyperalgesia and edema induced by *B. asper* venom is not attributable to the absence of neutralizing antibodies in the antivenom, since neutralization was achieved in assays involving preincubation of venom and antivenom. Cross-neutralization of AVCP or AVIB against *B. jararaca* and *B. asper* venoms, respectively, was also evaluated. Only bothropic antivenom partially neutralized hyperalgesia induced by *B. asper* venom in preincubation experiments. The present data suggest that hyperalgesia and edema induced by *Bothrops* venoms are poorly neutralized by commercial antivenoms even when antibodies are administered immediately after envenomation.

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Research supported by FAPESP (No. 98/00257-0), Fundação Butantan and Vicerrectoría de Investigación, Universidad de Costa Rica (Project 741-A1-027).

Received November 26, 2001
Accepted July 12, 2002

**Key words**
- *Bothrops jararaca* venom
- *Bothrops asper* venom
- Hyperalgesia
- Edema
- Antivenom
- Neutralization

## Introduction

Antivenoms of equine or ovine origin constitute the only effective treatment of snakebite envenomation (1). The use of these products has greatly contributed to the reduction of mortality owing to their effectiveness in the neutralization of life-threatening, systemically acting toxins present in snake venoms (2-4). Randomized controlled clinical trials have demonstrated that antivenoms are highly effective in halting systemic bleeding, coagulopathies and other cardiovascular disturbances due to envenomations by snakes of the family Viperidae in Latin America (3,5-7).

In contrast, the effectiveness of antivenoms in neutralizing local effects induced by pit viper venoms is doubtful. Experimental studies have suggested that there is significant, albeit partial, neutralization of hemorrhage, edema, dermonecrosis and myonecrosis only when antivenom is administered rapidly after envenomation (8-10), even when using antibody fragments $F(ab')_2$ and Fab which have a larger volume of distribution and reach the interstitial fluid at a faster rate (11,12). Clinical observations also indicate that local tissue damage is only partially halted by antivenoms (2,3,13).

Most experimental studies concerning neutralization of local effects have dealt with myonecrosis, hemorrhage and edema (for a review, see Ref. 10), but little attention has been paid to the study of pain, which is a clinically relevant consequence of snakebites and occurs in the vast majority of patients bitten by *Bothrops* sp (3,13,14). Pharmacological studies carried out with *B. jararaca* and *B. asper* venoms have indicated that the hyperalgesic effect is based on the action of a number of inflammatory mediators such as leukotrienes, prostaglandins and platelet-activating factor, in the case of *B. jararaca* (15), and bradykinin and leukotrienes in the case of *B. asper* (16). However, there have been no investigations on the ability of antivenoms to neutralize this effect. The present study was designed to assess the neutralization of the hyperalgesic effect induced by *B. asper* and *B. jararaca* venoms by polyspecific antivenoms used clinically in Central and South America. For comparative purposes, the neutralizing ability of antivenoms against edema induced by both venoms was also investigated.

## Material and Methods

### Animals

Male Wistar rats weighing 170 to 190 g were maintained under controlled temperatures (22-25ºC) and on a 12-h light/dark cycle, with free access to food and water.

### Venoms

Lyophilized crude venom of *B. jararaca* (BjV) was supplied by Laboratório de Herpetologia, Instituto Butantan, São Paulo, SP, Brazil, and lyophilized crude venom of *B. asper* (BaV) was supplied by Instituto Clodomiro Picado, Costa Rica. Both venoms were kept at -20ºC and dissolved in sterile 0.15 M NaCl (saline solution) at the time of use.

### Antivenoms

Bothropic antivenom of Instituto Butantan, batches 9710158 and 9805055 (AVIB - anti-*B. jararaca*, anti-*B. jararacussu*, anti-*B. moojeni*, anti-*B. neuwiedii* and anti-*B. alternatus*), and polyvalent antivenom of Instituto Clodomiro Picado, batch 2811096 (AVCP - anti-*B. asper*, anti-*C.d. durissus* and anti-*L.m. stenophrys*) were used in this study. Antivenoms were stored at 4ºC. The immunization protocols and adjuvants used have been previously described by Raw et al. (17) and Angulo et al. (18). Both antivenoms were liquid preparations used before their expiration date.

### Evaluation of hyperalgesia

The animals were injected with 5 µg of BjV or 15 µg of BaV dissolved in 100 µl sterile saline solution into the subplantar surface of one hind paw. Control animals were injected with the same volume of sterile saline. The contralateral paw was not injected. The pain threshold was measured at 1 and 4 h or 2 and 4 h after BjV and BaV injection, respectively, or after saline injection using an Ugo-Basile pressure apparatus, essentially as described by Randall and Selitto (19). Briefly, a force (in g) of increasing magnitude was applied to the paw. The force needed to induce the rat to withdraw its paw was recorded and represented the pain threshold.

### Evaluation of edema formation

Rats received an injection of 5 µg BjV or 15 µg BaV dissolved in 100 µl sterile saline solution (0.15 M NaCl) into the subplantar surface of one hind paw. An equal volume of saline was injected into the contralateral paw (control). The volume increase (edema) of the paws was measured plethysmographically at 1 and 4 h or 2 and 4 h after BjV and BaV injection, respectively, according to the method of Van Arman et al. (20). The difference between the values obtained for both paws expressed as percent increase in paw volume was used as a measure of edema.

### Neutralization studies

Two kinds of experiments were performed: 1) injection of antivenom at different times before or after venom administration and 2) *in vitro* incubation of venom and antivenom before injection.

*Assays with independent injection of venom and antivenom*. Antivenoms (0.5 ml) were administered *iv* 15 min or immediately before or 15 min after intraplantar injection of the venoms. Hyperalgesia and edema measurements were carried out as described above.

*Assays with preincubation of venom and antivenom*. A constant amount of venom was incubated with several dilutions of antivenom at the following antivenom/venom ratios: 1, 0.5, 0.25, 0.125 ml antivenom/mg venom. After incubation at 37ºC for 30 min, 100 µl of each mixture containing the appropriate amounts of venoms (5 µg BjV or 15 µg BaV) were injected by the intraplantar route, and hyperalgesia and edema were determined as described above.

*Cross-neutralization studies*. Cross-neutralization experiments were performed in order to determine if AVIB and AVCP antivenoms are able to neutralize the hyperalgesic and edematogenic effects induced by *B. asper* and *B. jararaca* venoms, respectively. The neutralization studies were carried out as described above for assays with independent injection of venom and antivenom or assays with preincubation of venom and antivenom. In preincubation assays, the antivenom/venom ratios used were 2 ml antivenom/mg venom for AVCP/BjV and 1 ml antivenom/mg venom for AVIB/BaV.

The antivenoms did not alter, *per se*, the pain threshold of the animals, nor did they induce an edematogenic response (data not shown).

### Statistical analysis

Data were analyzed statistically by analysis of variance and sequential differences among means were determined by Tukey contrast analysis, with the level of significance set at $P<0.05$ (21).

## Results

### Neutralization of hyperalgesia and edema induced by *Bothrops jararaca* venom

The intraplantar injection of BjV (5 µg/paw) into the rat hind paw caused a signifi-

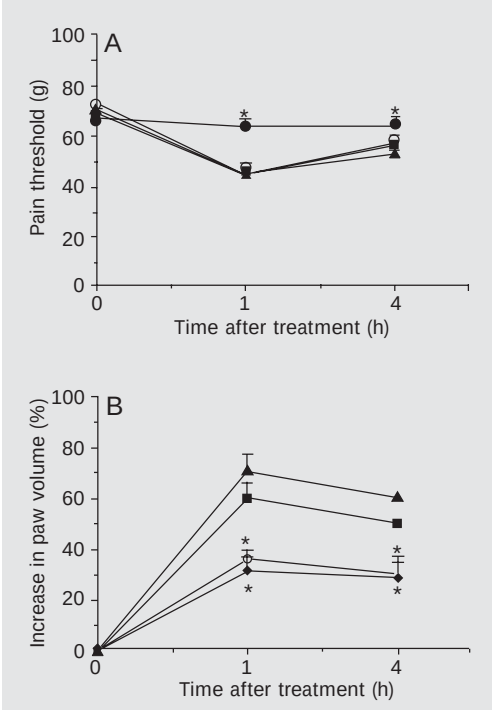


Figure 1. Neutralization of the hyperalgesia and edema activity of *Bothrops jararaca* venom by bothropic antivenom in assays in which venom and antivenom were injected separately. Antivenom (AV, 0.5 ml, 15 mg protein) or saline control (S, 0.5 ml) was injected *iv* 15 min before, immediately before or 15 min after intraplantar injection of the venom (V, 5 µg/paw). The decrease in threshold response (A) and increase in paw volume (B) were determined in rat hind paws before and 1 and 4 h after injection of the venom. Sensitivity to pain was measured as the threshold response to pressure and is reported in grams. Edema is reported as percent increase in relation to the initial volume of the paw. V + S (squares); V + AV 15 min before (filled circles); V + AV immediately before (open circles) or V + AV 15 min after (triangles). Each point indicates the mean ± SEM for 6 animals. *P<0.05 compared to the S + V group (ANOVA followed by Tukey test). Note that the abscissa scale is not linear.

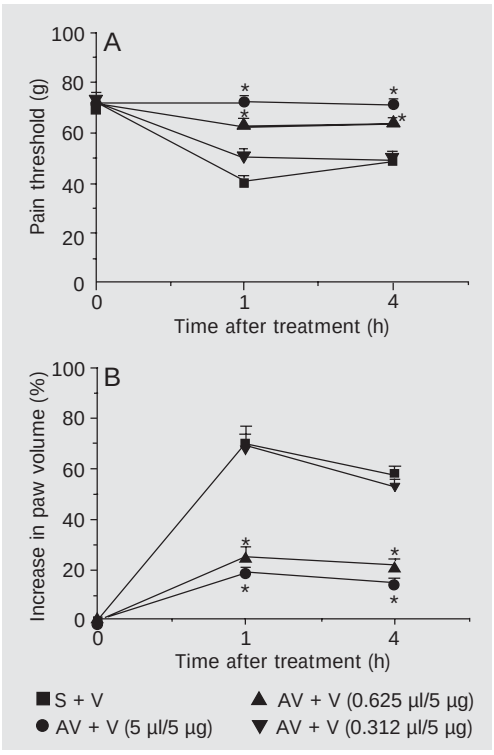


Figure 2. Neutralization of the hyperalgesia and edema induced by *Bothrops jararaca* venom by bothropic antivenom in assays in which venom and antivenom were incubated together before injection into the rat paw. The same amount of venom (V, 5 µg/paw), was incubated with various dilutions of antivenom (AV) or with saline (S, control) for 30 min at 37ºC. Then, 100 µl of the mixtures containing 5 µg of venom were injected by the intraplantar route. The decrease in threshold response (A) and increase in paw volume (B) were determined in rat hind paws before and 1 and 4 h after injection of the venom. Sensitivity to pain was measured as the threshold response to pressure and is reported in grams. Edema is reported as percent increase in relation to the initial volume of the paw. Each point indicates the mean ± SEM for 6 animals. *P<0.05 compared to the S + V group (ANOVA followed by Tukey test). Note that the abscissa scale is not linear.

cant decrease in pain threshold (hyperalgesia) and an increase in paw volume. The peak of the hyperalgesic and edematogenic responses occurred 1 h after venom injection. After this time both phenomena started to decrease (Figure 1) and completely disappeared at 24 h (data not shown). When venom and antivenom were injected separately, neutralization of BjV-induced hyperalgesia was observed only when AVIB was administered 15 min before venom injection (Figure 1A). Administration of AVIB 15 min or immediately before BjV induced a marked reduction of edema, although this effect was not completely neutralized (Figure 1B).

In experiments with incubation of venom and antivenom prior to injection, antivenom was effective in neutralizing the hyperalgesia and edema induced by BjV (Figure 2). Full protection against hyperalgesia was achieved at antivenom/venom ratios of 5 µl/5 µg (1 ml/mg) and 0.625 µl/5 µg (0.125 ml/mg) (Figure 2A), whereas edema was significantly reduced but not abolished at the same antivenom/venom ratios (Figure 2B).

### Neutralization of hyperalgesia and edema induced by *Bothrops asper* venom

The intraplantar injection of BaV (15 µg/paw) induced hyperalgesia and edema which peaked 2 h after venom injection, decreasing thereafter (Figure 3). AVCP administered 15 min or immediately before or 15 min after *B. asper* venom injection did not modify the hyperalgesia or edema induced by the venom (Figure 3). In experiments involving preincubation, AVCP neutralized hyperalgesia at the antivenom/venom ratio of 3.75 µl/15 µg (0.250 ml/mg), whereas a partial neutralization of edema was observed at the antivenom/venom ratio of 15 µl/15 µg (1 ml/mg) (Figure 4).

### Cross-neutralization studies

AVIB or AVCP injected 15 min before

BaV and BjV, respectively, did not modify the hyperalgesia or edema induced by both venoms (data not shown). In experiments involving incubation of venom and antivenom prior to injection, AVIB partially neutralized the hyperalgesia and edema induced by BaV (Figure 5A,B). On the other hand, AVCP did not modify either effect induced by *B. jararaca* venom. One hour after intraplantar injection of neutralized venom, the pain threshold was 47 ± 1.2 g and the increase in paw volume was 64 ± 8%. The data for the control (non-neutralized venom) group were 53 ± 2 g for pain threshold and 47 ± 8% for paw volume increase.

## Discussion

Bothropic and polyvalent antivenoms constitute the major therapeutic resource in snakebite envenomations in Brazil and Central America, respectively (3,22). Clinical investigations have demonstrated the efficacy of these products on the neutralization of life-threatening systemic effects associated with these envenomations (3,5,14). However, experimental and clinical evidence indicates that local effects in *Bothrops* sp envenomations are poorly neutralized by these and other antivenoms (3,8,10,13). The present results further demonstrate the complete ineffectiveness of these antivenoms in neutralizing the local edema and hyperalgesia induced by *B. asper* and *B. jararaca* venoms when antivenoms are administered to rats after envenomation.

The ability of antivenoms to neutralize hyperalgesia has not been previously addressed in experimental studies, despite the fact that pain is a common manifestation of *Bothrops* sp envenomations (3,13,23). We selected venom doses which caused an increase in the sensitivity to pain (hyperalgesia) and local edema, without inducing macroscopically evident hemorrhage or tissue damage. Such doses have been validated and used in previous pharmacological stud-

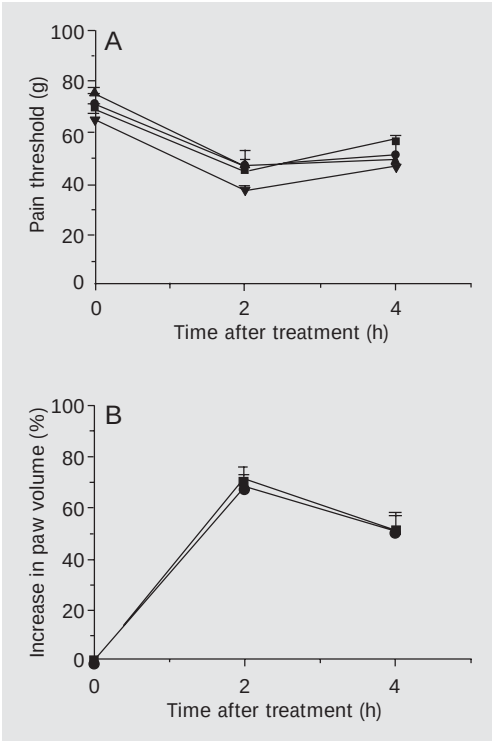


Figure 3. Neutralization of the hyperalgesia and edema activity of *Bothrops asper* venom by polyvalent antivenom in assays in which venom and antivenom were injected separately. Antivenom (AV, 0.5 ml, 22 mg protein) or saline (S, control group) was injected *iv* 15 min before, immediately before or 15 min after intraplantar injection of the venom (V, 15 µg/paw). The decrease in threshold response (A) and increase in paw volume (B) were determined in rat hind paws before and 2 and 4 h after injection of the venom. Sensitivity to pain was measured as the threshold response to pressure and is reported in grams. Edema is reported as percent increase in relation to the initial volume of the paw. V + S (squares); V + AV 15 min before (circles); V + AV immediately before (triangles) or V + AV 15 min after (inverted triangles). Each point indicates the mean ± SEM for 6 animals.

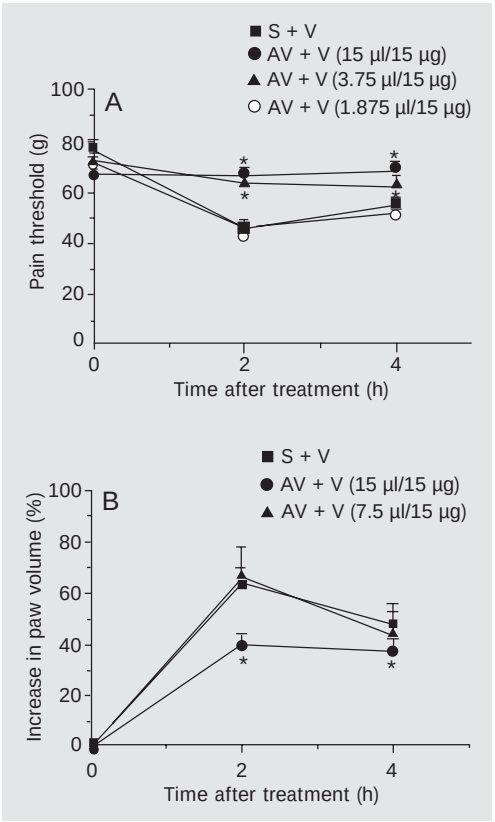


Figure 4. Neutralization of the hyperalgesia and edema induced by *Bothrops asper* venom by polyvalent antivenom in assays in which venom and antivenom were incubated together before injection into rats. A fixed amount of venom (V) was incubated with various dilutions of antivenom (AV) or with saline (S, control) for 30 min at 37ºC. Then, 100 µl of the mixtures containing 15 µg of venom were injected by the intraplantar route. The decrease in threshold response (A) and increase in paw volume (B) were determined in rat hind paws before and 2 and 4 h after injection of the venom. Sensitivity to pain was measured as the threshold response to pressure and is reported in grams. Edema is reported as percent increase in relation to the initial volume of the paw. Each point indicates the mean ± SEM for 6 animals. *P<0.05 compared to the S + V group (ANOVA followed by Tukey test).

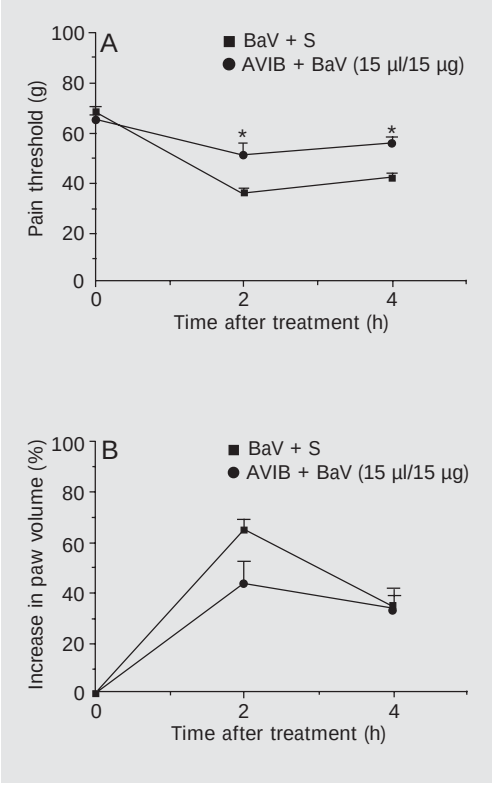


Figure 5. Neutralizing activity of bothropic antivenom (AVIB) against *Bothrops asper* venom in assays involving incubation of the venom and the antivenom together before injection. A fixed amount of venom was incubated with dilutions of antivenom or with saline (S, control) for 30 min at 37ºC. Then, 100 µl of the mixture containing 15 µg of *B. asper* venom (BaV) was injected by the intraplantar route. The decrease in threshold response (A) and increase in paw volume (B) were measured in rat hind paws before and 2 and 4 h after the injection of BaV. Sensitivity to pain was measured as the threshold response to pressure and is reported in grams. Edema is reported as percent increase in relation to the initial volume of the paw. Each point indicates the mean ± SEM for 6 animals. *P<0.05 compared to the BaV + S group (ANOVA followed by Tukey test).

ies (15,16).

Bothropic antivenom produced against *B. jararaca* at Instituto Butantan was ineffective in neutralizing edema or hyperalgesia when administered after envenomation. Only when antivenom was injected before venom was neutralization achieved. In the case of *B. asper* venom, polyvalent antivenom produced in Costa Rica was ineffective in neutralizing edema or hyperalgesia when administered either before or after envenomation, in agreement with prior observations in mice (8,10). Moreover, Rucavado and Lomonte (24) demonstrated that the presence of antibodies in the circulation before injection of *B. asper* venom in mice reduces, but does not abolish, local tissue damage.

Such poor neutralizing efficacy cannot be explained on the basis of lack of neutralizing antibodies in these antivenoms, since both hyperalgesic and edematogenic responses were abolished if venom and antivenoms were incubated prior to injection. A similar situation occurs regarding the neutralization of hemorrhage and myonecrosis induced by *B. asper* venom (8-10). It has been suggested that the observed poor neutralization is based on the fact that hyperalgesia and edema develop at an extremely rapid rate after venom injection, associated with the release of endogenous mediators involved in these pharmacological effects (15,16,25,26). Thus, by the time antivenom antibodies reach the affected tissues, it is likely that the cascade of inflammatory events associated with hyperalgesia and edema has already started. Moreover, since antivenoms are administered intravenously, the amount of antibodies diffusing to the affected tissues is probably insufficient and delayed. Thus, an evident mismatch occurs between the pharmacokinetics and pharmacodynamics of venoms and antivenoms in this experimental model and, very likely, in the clinical situation as well.

The present study also addressed the issue of cross-neutralization of these effects by antivenoms. Previous investigations have documented cross-neutralization between bothropic and polyvalent antivenoms when assayed against a variety of venoms from Central and South America (27-30). The present results demonstrate that bothropic antivenom partially neutralizes *B. asper* venom, whereas polyvalent antivenom is largely ineffective in the neutralization of edema and hyperalgesia induced by *B. jararaca* venom. It is likely that components immunologically related to hyperalgesic and edematogenic toxins in *B. asper* venom are present in one or more of the venoms included in the antigenic mixture used to produce bothropic antivenom in Brazil.

The present study reports the first experimental evidence showing that hyperalgesia induced by *Bothrops* sp venoms is not neutralized when antivenoms are administered after envenomation. In view of the clinical relevance of pain in *Bothrops* sp envenomations, it is necessary to gain further under-

standing of the pharmacological mechanisms involved in this effect, as well as of the characteristics of the hyperalgesic components present in these venoms in order to develop therapeutic strategies aimed at controlling pain in these envenomations.

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