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IN VITRO HEMOLYTIC ACTIVITY OF *Bothrops lanceolatus* (FER-DE-LANCE) VENOM

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ABSTRACT: *Bothrops lanceolatus* venom contains a variety of enzymatic and biological activities. The present work investigated the hemolytic activity of this venom and its phospholipase A_2 (PLA_2). *Bothrops lanceolatus* venom (6.7 µg/mL) caused indirect hemolysis of cow, horse, rat and sheep erythrocytes, with horse erythrocytes being the most sensitive; no direct hemolysis was observed. Hemolysis in sheep erythrocytes was concentration-dependent (5-11.7 µg/mL) and markedly attenuated by heating the venom for 30 minutes at ≥ 40°C and by the PLA_2 inhibitor *p*-bromophenacyl bromide. An acidic PLA_2 (5 µg/mL) purified from *B. lanceolatus* venom also caused hemolysis. This PLA_2 showed immunoprecipitin lines with antivenom against *B. lanceolatus*, which suggests that the enzymatic and hemolytic activities of this enzyme may be neutralized during antivenom therapy. These results indicate that *B. lanceolatus* venom and its PLA_2 can cause hemolysis *in vitro*.

KEY WORDS: *Bothrops lanceolatus*, hemolytic activity, phospholipase A_2, snake venom.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Bothrops lanceolatus is endemic to the Caribbean island of Martinique (1). Envenomation by this species produces local (edema, pain, hemorrhage, necrosis) and systemic (coagulopathy) effects, with coagulation disturbances (thrombosis) being the most important cause of clinical complications and mortality (2-4). Enzymes that may contribute to these effects include an esterase (5), a caseinolytic protease (6), a hemorrhagic metalloproteinase (7) and an acidic phospholipase (8). The hemolytic activity of *B. lanceolatus* venom and involvement of PLA_2 in this phenomenon have not been extensively studied, although Bogarín *et al.* (9) reported that the hemolytic activity of *B. lanceolatus* venom was neutralized by homologous antivenom. In this report, we describe some characteristics of the hemolysis caused by *B. lanceolatus* venom *in vitro*.

MATERIALS AND METHODS

Venom, PLA_2 and Antivenom

Bothrops lanceolatus venom was supplied by the Unité des Venins, Institut Pasteur (Paris, France) and was stored at –20°C until use. Acidic PLA_2 (fraction $F_3$2) was purified from *B. lanceolatus* venom by a combination of gel filtration and ion-exchange chromatography, as previously described (8). Antivenom (batch BO 278, Pasteur Institute, Paris) produced in horses immunized with *B. lanceolatus* venom was stored in lyophilized form.

Assay for Hemolytic Activity

Sheep blood was obtained from a commercial sheep breeder (Biotério Boa Vista, Fazenda São Sebastião, Patrocínio Paulista, SP, Brazil), cow and horse blood were obtained from a commercial slaughterhouse, and blood from Wistar rats was collected from animals provided by the university's Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP). Suspensions of washed erythrocytes were prepared in phosphate-buffered saline (PBS) using standard procedures whereas the indirect hemolytic activity was assayed in a mixture containing venom or PLA_2, hen egg-yolk lecithin and erythrocytes, essentially as described by Gutiérrez *et al.* (10). The relationship between venom concentration and hemolysis was investigated in sheep erythrocytes. Direct hemolytic activity was assessed in incubations without egg-yolk lecithin. Hemolytic activity was expressed as a

percentage of the total hemolysis (100%) obtained by lysing erythrocytes in distilled water.

Heat Stability of the Hemolytic Activity

The heat stability of the hemolytic activity was determined by incubating venom at 25, 40, 50, 70 and 90°C for 30 minutes followed by cooling and then assaying the residual hemolytic activity in sheep erythrocytes.

Role of PLA_2 in the Hemolytic Activity

The role of PLA_2 in the hemolytic activity of the venom was assessed by incubating venom with 2.88 mM *p*-bromophenacyl bromide (*p*BPB) (Sigma Chemical Co., USA) for 30 minutes at 37°C, after which the residual PLA_2 and hemolytic activities were assayed in 96-well plates, as described by Nishida *et al.* (11). Each well contained 200 µL of hen egg-yolk emulsion as substrate in PBS containing 2 mM $CaCl_2$ and 40 µL of venom. The increase in absorbance at 750 nm was recorded for up to 40 minutes in a SpectraMax 340® multiwell plate reader (Molecular Devices, USA) and the activity was expressed as the increase in absorbance at 750 nm/minute. Hemolytic activity was assayed as described above, using sheep erythrocytes. All assays were run in duplicate and corrected for appropriate blanks.

Immunological Analysis

Immunodiffusion of the venom and purified PLA_2 was done in PBS in 1% agar gels, as described by Ouchterlony (12), using a 10% (w/v) solution of *B. lanceolatus* antivenom. After incubation in a humidified chamber for 48 hours at room temperature, the slides were washed extensively in 0.9% (w/v) NaCl and the immunoprecipitin bands were detected by Coomassie blue staining.

Immunoelectrophoresis was done in 1% agar gels poured on glass slides. Samples of venom and purified PLA_2 were applied to the gels and run for one hour at 6 V/cm in 50 mM veronal buffer, pH 8.4 (13). Following electrophoresis, a 10% (w/v) solution of *B. lanceolatus* antivenom was placed in the central trough and the slides were incubated for 48 hours at room temperature in a humidified chamber. After extensive washing in 0.9% (w/v) NaCl, the gels were dried and stained with Coomassie blue.

Statistical Analysis

The results were expressed as the mean ± S.E.M., and statistical comparisons ($p < 0.05$) were done using Student´s *t*-test or analysis of variance (ANOVA) followed by the Tukey test.

RESULTS

Hemolytic Activity of *B. lanceolatus* Venom and Purified PLA_2

Figure 1A shows that *B. lanceolatus* venom caused indirect hemolysis of erythrocytes in all of the species examined, with horse erythrocytes being the most susceptible and sheep erythrocytes the least susceptible ($p < 0.05$); there was no significant difference between the degree of hemolysis of rat and cow erythrocytes ($p > 0.05$). No direct hemolysis was observed with any of these species (data not shown; n = 4). In subsequent experiments, sheep erythrocytes were used because these were readily obtained from an established laboratory.

Bothrops lanceolatus venom caused concentration-dependent hemolysis of sheep erythrocytes that was maximal at > 10 µg/mL (Figure 1B). This hemolytic activity was markedly attenuated by heating the venom for 30 minutes at ≥ 40°C, with complete inhibition of hemolytic activity occurring at ≥ 50°C (Figure 1C). Incubation with *p*BPB significantly ($p < 0.05$) inhibited the PLA_2 activity of the venom (from 74.0 A_{750nm}/minute to 3.7 A_{750nm}/minute) and completely abolished the hemolytic activity (n = 4). Purified PLA_2 (5 µg/mL) caused total hemolysis of sheep erythrocytes (a complete concentration-response curve of this hemolytic activity was not obtained because of the limited amount of pure PLA_2 available) (Figure 1D).

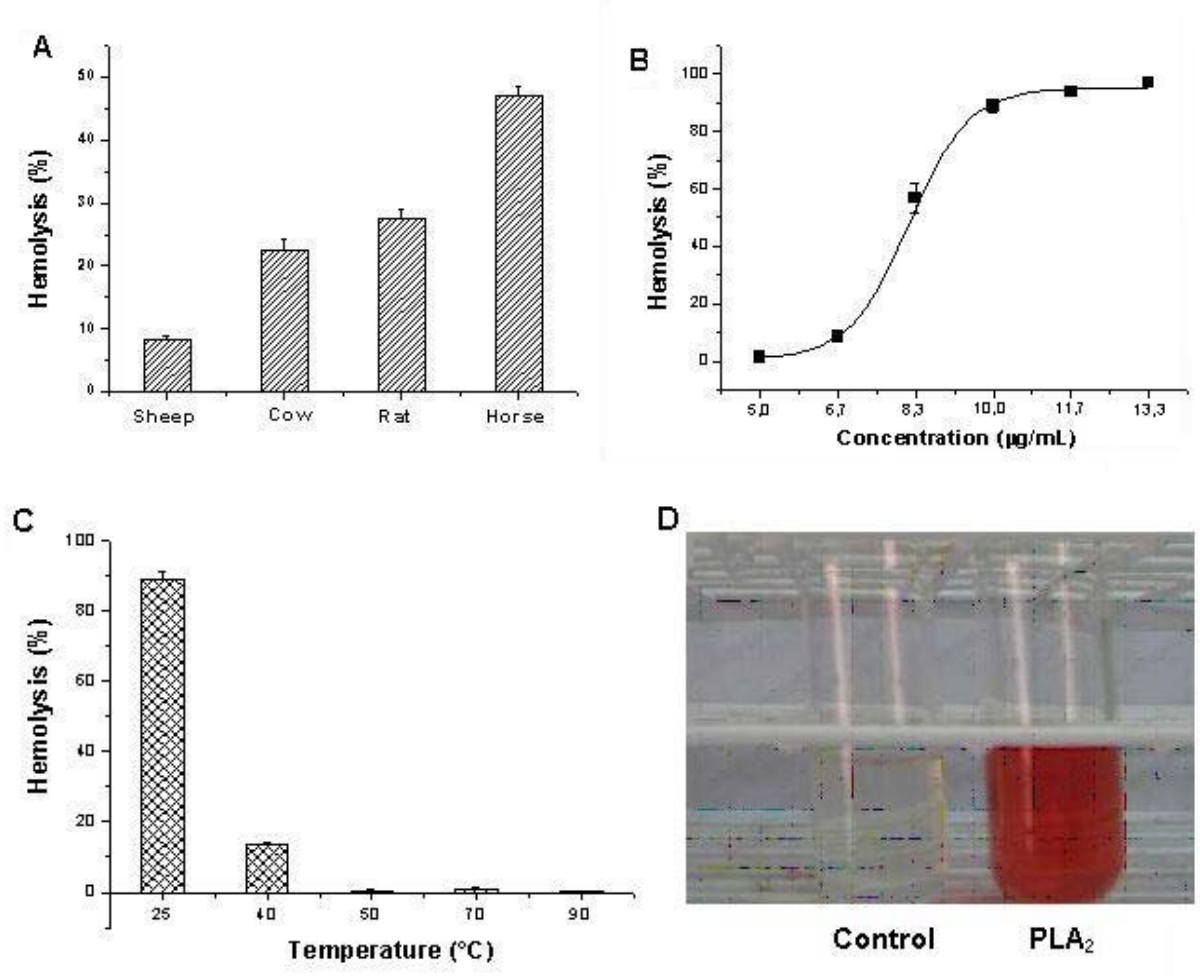

Figure 1. Hemolytic activity of *B. lanceolatus* venom (6.7 µg/mL) and its purified PLA_2. (**A**) Indirect hemolysis of erythrocytes from various species. (**B**) Concentration-dependent hemolysis of sheep erythrocytes by *B. lanceolatus* venom. (**C**) Thermolability of the hemolytic activity of *B. lanceolatus* venom (10 µg/mL) in sheep erythrocytes. (**D**) Hemolysis of sheep erythrocytes by purified PLA_2 (5 µg/mL). The control tube on the left shows no hemolysis. In panels **A** to **C**, hemolysis was expressed as a percentage of the total hemolysis (100%) obtained by lysing erythrocytes in distilled water (mean ± SEM, n = 4 independent experiments).

Immunological Studies

Figure 2 shows the immunoprecipitin lines obtained with *B. lanceolatus* venom and purified PLA_2 after incubation with antivenom. Multiple lines were obtained with the venom but only one major line was seen with PLA_2 in immunodiffusion and immunoelectrophoresis.

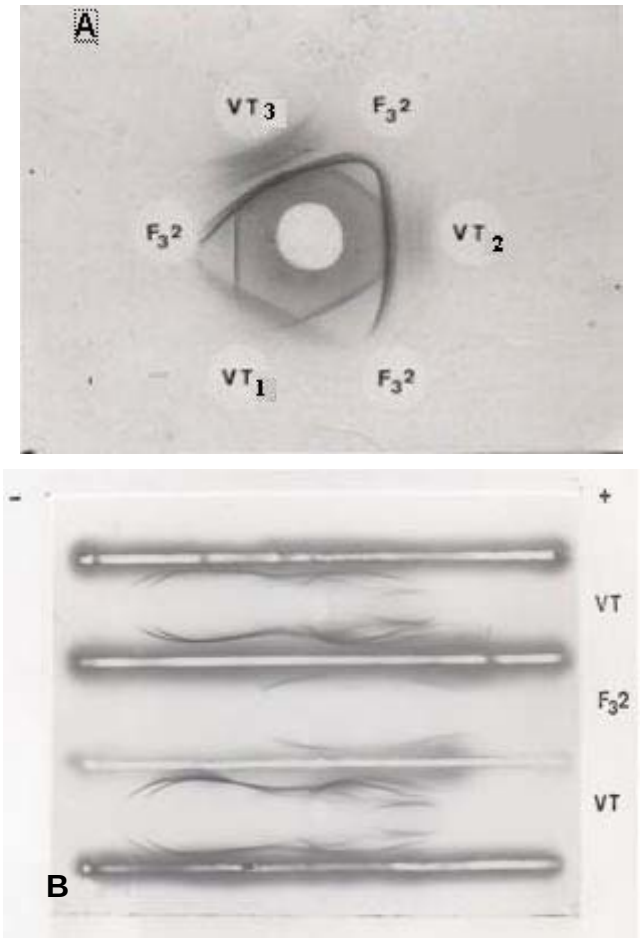

Figure 2. Immunoreactivity of *B. lanceolatus* venom (VT) and purified PLA_2 (F_32) with *B. lanceolatus* antivenom in (**A**) immunodiffusion and (**B**) immunoelectrophoresis. (**A**) The antigen concentrations were 0.1, 0.5 and 1.0 mg/mL (VT_1, VT_2 and VT_3). (**B**) The antigen concentration was 2 mg/mL.

(–) and (+): negative and positive poles, respectively.

DISCUSSION

The results of this study indicate that *B. lanceolatus* venom can cause indirect hemolysis of erythrocytes from a variety of mammalian species. This action on erythrocytes was indirect since there was no hemolysis in the absence of egg-yolk lecithin. The hemolytic activity was concentration-dependent and thermolabile since heating the venom at ≥ 40°C resulted in little or no hemolysis. These findings generally agree with reports on the hemolytic activity of other *Bothrops* venoms (14-16). Variation in species sensitivity to hemolysis as seen herein (sheep < cow/rat < horse) has also been observed with other *Bothrops* venoms, e.g., *B. asper* venom

causes direct hemolysis of mouse erythrocytes but has no effect on goat, horse, rabbit, sheep and toad erythrocytes; in contrast, lysis of human erythrocytes requires bovine serum albumin and Ca^{2+} (17). These variations may reflect differences in the phospholipid composition of the erythrocyte plasma membrane of these species (18-20), as well as the accessibility of the cellular membrane targets such as negative phospholipids to PLA_2 action (17, 18). Incubation of the venom at different temperatures indicated that the venom component responsible for hemolysis is thermolabile. Other biological activities of *B. lanceolatus*, such as rat paw edema, leukocyte migration and hemorrhagic activity, are also thermolabile (7, 21, 22).

A role for venom PLA_2 in the hemolytic activity was suggested by the finding that the loss of hemolysis paralleled the inhibition of PLA_2 activity by *p*BPB. In addition, purified PLA_2 also caused hemolysis. In agreement with this, various studies have shown that PLA_2s are the principal component of *Bothrops* venoms responsible for hemolysis (23-26). In addition to the hemolytic activity described herein, the PLA_2 of *B. lanceolatus* venom also contributes to neutrophil migration during inflammation caused by this venom (21) and may be involved in the anticoagulant action of the venom (27).

The immunoprecipitin lines observed with purified PLA_2 and *B. lanceolatus* antivenom in immunodiffusion and immunoelectrophoresis indicated that this protein was antigenic and was recognized by the antivenom. This finding supports the results of Bogarín *et al.* (9), who showed that this antivenom neutralized the indirect hemolytic activity of *B. lanceolatus* venom. Other studies have also shown that bothropic antivenoms can neutralize the PLA_2 and hemolytic activities of homologous and heterologous *Bothrops* venoms (16, 28-32).

Although intravascular hemolysis has been observed following envenomation by *Bothrops* species (33), this phenomenon has not been documented after bites by *B. lanceolatus*, the main effects of which are edema, pain and coagulation disturbances (2-4). Hence, the clinical relevance of the findings described herein remains to be determined.

In conclusion, *B. lanceolatus* venom causes concentration-dependent hemolysis *in vitro*, with the order of susceptibility among erythrocytes from different species being sheep < cow/rat < horse. This activity is thermolabile and is apparently mediated by the PLA_2 of the venom. The cross-reactivity of the venom and PLA_2 with antivenom suggests that the hemolytic activity may be neutralized by antivenom.

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