

THE venom of the snake *Bothrops asper*, the most important poisonous snake in Central America, evokes an inflammatory response, the mechanisms of which are not well characterized. The objectives of this study were to investigate whether *B. asper* venom and its purified toxins – phospholipases and metalloproteinase – activate the complement system and the contribution of the effect on leucocyte recruitment. *In vitro* chemotaxis assays were performed using Boyden's chamber model to investigate the ability of serum incubated with venom and its purified toxins to induce neutrophil migration. The complement consumption by the venom was evaluated using an *in vitro* haemolytic assay. The importance of complement activation by the venom on neutrophil migration was investigated *in vivo* by injecting the venom into the peritoneal cavity of C5-deficient mice. Data obtained demonstrated that serum incubated with crude venom and its purified metalloproteinase BaP–1 are able to induce rat neutrophil chemotaxis, probably mediated by agent(s) derived from the complement system. This hypothesis was corroborated by the capacity of the venom to activate this system *in vitro*. The involvement of C5a in neutrophil chemotaxis induced by venom-activated serum was demonstrated by abolishing migration when neutrophils were pre-incubated with anti-rat C5a receptor antibody. The relevance of the complement system in *in vivo* leucocyte mobilization was further demonstrated by the drastic decrease of this response in C5-deficient mice. Pre-incubation of serum with the soluble human recombinant complement receptor type 1 (sCR 1) did not prevent the response induced by the venom, but abolished the migration evoked by metalloproteinase-activated serum. These data show the role of the complement system in bothropic envenomation and the participation of metalloproteinase in the effect. Also, they suggest that the venom may contain other component(s) which can cause direct activation of C5a.

Key words: *Bothrops asper* snake venom, Leucocyte infiltrate, Complement system, C5a, Metalloproteinase, Inflammatory reaction

***Bothrops asper* snake venom and its metalloproteinase BaP–1 activate the complement system. Role in leucocyte recruitment**

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Introduction

The acute inflammatory response is characterized by an increased passage of fluid and leucocytes, particularly neutrophils, out of the bloodstream into the damaged tissue.^{1,2} Neutrophils are one of the central inflammatory cells in the first line of host defence. Their adhesion to endothelial cells and the subsequent oriented transmigration are key events in their recruitment during inflammation. Such events are controlled by a dynamic interaction between adhesion molecules (i.e. selectins) expressed on leucocytes and the endothelial cells, as well as by a direct action of secreted chemotactic mediators

which bind to the expressing seven transmembrane G protein coupled receptors.^{2–5}

A local inflammatory reaction is characteristic of envenomations by snakes of the genus *Bothrops*, involving oedema formation and leucocyte mobilization.^{6–10} The mechanisms responsible for leucocyte recruitment in these envenomations are not well described. It has been shown that the accumulation of leucocytes in animals injected with some *Bothrops* sp. venoms is dependent on eicosanoid release^{7,9,10} and on chemotactic factors derived from the serum.¹⁰ The latter effect is probably due to complement activation as venoms from crotaline snakes are known to activate the complement system.¹¹ The pathophy-

biology of envenomation by *B. asper*, the most important poisonous snake in Central America,¹² includes systemic alterations such as blood coagulation disturbances,¹³ haemorrhage, cardiovascular shock and renal failure,¹⁴ besides prominent local tissue damage characterized by myonecrosis, haemorrhage and an acute inflammatory response.^{6,14-17} Although, it has been demonstrated that oedema evoked by the venom is multimediated,¹⁸ the mechanisms involved in the local leucocyte accumulation remain unknown.

The aims of the present study were to investigate whether *B. asper* venom (BaV) is involved in complement activation and to test whether this activation with the production of soluble complement anaphylatoxins leads to leucocyte recruitment. Our results show that the venom is able to activate classical and alternative complement system pathways, generating C5a component. Serum treated with BaV was a chemoattractant to neutrophils and we were able to confirm, as expected, that C5a mediated this effect. Indeed, an anti-rat C5a receptor (anti-ratC5aR) neutralized the chemotaxis activity. We propose that the metalloproteinase BaP-1 present in the venom is involved in complement activation. It remains to be tested whether complement activation can be attributed solely to this metalloproteinase or whether other components of the venom have synergistic actions.

Materials and methods

Chemicals and reagents

Crystalline bovine serum albumin (fraction V), eosin Y, lipopolysaccharide (LPS) from *Escherichia coli* (serotype 026:B6), oyster glycogen type II, Sepharose-polymyxin B columns were purchased from Sigma (St Louis, MO, USA). Ester cellulose filters (0.22 µm and 8.0 µm) were from Millipore (Brazil). Liquemine was acquired from Roche (Brazil).

Blood, sera, antibodies and other proteins

Normal sheep and rabbit blood, collected 1:1 in Alsever's old solution (pH 6.1, 114 mM citrate, 27 mM glucose, 72 mM NaCl), was obtained from the animal facilities of Institute Butantan and stored at 4°C. Normal human or rat blood was obtained in-house. Blood samples drawn to obtain sera were collected without anticoagulant and allowed to clot for 1 h at room temperature (RT). The normal human serum (NHS) or normal rat serum (NRS) were stored at -80°C. Anti-rat C5aR antiserum was produced in-house (P. Gasque) by injecting a rabbit with a multiple array C5a peptide (amino acids 12-35: TYDYS DSGTPNPDMPADGVYIPKME) corresponding to the N-terminal of the receptor involved in the C5a

binding. The IgG fraction was obtained by affinity purification on a protein A Sepharose column (Prosep A, Bioprocessing, Princeton, NJ, USA). The rabbit anti-C5aR stained specifically rat monocyte cell line NR83/83 and rat C5aR-transfected CHO cells (P. Gasque, unpublished observation). Soluble recombinant complement receptor type 1 (sCR 1) was obtained from Avant Immunotherapeutics (Needham, MA, USA).

Venom and purified toxins

Lyophilized crude BaV was a pool obtained from more than 50 adult specimens collected in the Atlantic region of Costa Rica and kept at the Serpentarium of Instituto Clodomiro Picado, Costa Rica. The venom was lyophilized and maintained at -20°C and dissolved at the moment of use. Prior to the experiments, the venom solutions were filtered through a cellulose ester membrane of 0.22 µm average pore size. Phospholipase A₂ myotoxins II and III and haemorrhagic metalloproteinase BaP-1 were purified from crude venom as previously described.¹⁹⁻²¹ In some experiments, BaV was loaded on to a Sepharose-polymyxin B column in order to remove any traces of LPS which could be present in the venom.

Animals

Male Wistar rats weighing 170-190 g were obtained from "Biotério Central do Instituto Butantan" and male B10/A (H-2^a) and A/J (H-2^a) isogenic mice strains weighing 17-20 g were obtained from "Biotério de Camundongos isogênicos do Instituto de Ciências Biomédicas da Universidade de São Paulo (São Paulo, SP, Brazil)". The animals were maintained with free access to standard rat food pellets and water *ad libitum*.

Haemolytic assay

In order to assess the capacity of BaV and related toxins to activate the complement system *in vitro*, samples of NHS were incubated with different concentrations of venom or toxin for 30 min at 37°C. Control samples were incubated with phosphate-buffered solution (PBS, pH 7.2, containing 10 mM Na phosphate, 150 mM NaCl) alone. The haemolytic assays were performed in 96-well microtitre plates. Sheep erythrocytes (E^s) were sensitized with rabbit antibodies against E^s (1:1000) for 30 min at 37°C. Aliquots (100 µl) of sensitized cells [2% suspension in veronal-buffered saline (VBS⁺⁺, pH 7.4, containing 10 mM Na barbitone, 0.15 mM CaCl₂ and 0.5 mM MgCl₂)] were incubated with dilutions of NHS (100 µl) as a source of complement for 60 min at 37°C. The plates were centrifuged to remove intact cells and the absorbance of the supernatant was

measured at 414 nm. E^s incubated without NHS were used as the standard for lysis and water-lysed E were used as the standard for 100% lysis. Alternative pathway complement activation was measured using serum diluted in VBS containing ethylene glycol tetraacetic acid (EGTA)-Mg⁺⁺ (VBS plus EGTA 0.1 M MgCl₂ and 0.1 M EGTA) and non-antibody sensitized rabbit E (E^r) as target cells.

Neutrophil preparations

Neutrophils were obtained from normal Wistar rats 4 h after the intraperitoneal injection of 20 ml of a 1% oyster glycogen solution in PBS. The animals were anaesthetized with ether and the cells collected by rinsing the abdominal cavity with Hanks' balanced solution (HBSS, containing 138 mM NaCl, 5.36 mM KCl, 1.25 mM Na₂HPO₄ · 12H₂O, 0.44 mM KH₂PO₄, 2.94 mM NaHCO₃, 0.13 mM CaCl₂, 0.1 mM MgSO₄, 5.5 mM glucose) plus 1 U/ml heparin. Cell viability was assessed by the eosin Y exclusion test. The final cell suspensions contained 1% crystalline bovine serum albumin in HBSS.

Neutrophil chemotaxis assay

The migration assay was performed as described by Boyden²² and Zigmond and Hirsch²³ using a multi-well chemotaxis chamber. In brief, aliquots of cell suspensions containing 1.5×10^6 neutrophils were added to the upper compartment of the chamber separated from the chemotactic agent(s) in the lower compartment by an ester cellulose filter (8 µm average pore size). The chemotactic agent was substituted for HBSS for assessment of random migration. The experiments were performed to evaluate: (1) the capacity of BaV to directly induce neutrophil chemotaxis, (2) the neutrophil chemotaxis in response to serum pre-treated with BaV (see below), (3) the capacity of myotoxins II or III and metalloproteinase BaP-1 to generate chemotactic factors derived from serum, (4) the participation of C5a/C5aR in the chemotaxis event. Activated serum was obtained by incubation with *E. coli* LPS (65 µg/ml) for 30 min at 37°C. Varying concentrations of LPS-activated serum were prepared using HBSS as the diluent.

The chemotaxis chambers were incubated in humidified air at 37°C for 60 min, followed by removal of the filters for fixation and staining of the cells. Neutrophil migration within the filter was determined under light microscopy by the "leading front" method,²³ in which the distance was measured from the top of the filter to the farthest plane of focus still containing two cells observed with the ×40 objective. Five fields were counted and averaged for each experiment, which was performed in duplicate.

Determination of the number and specific cell type of leucocytes of peritoneal exudate following BaV injection

To evaluate leucocyte recruitment induced by BaV, 10 µg of venom dissolved in 1 ml of sterile saline was injected into the peritoneal cavity of B10/A (H-2^a; C5-sufficient) and A/J (H-2^b; C5-deficient) isogenic mice strains under light ether anaesthesia. Match controls of each group of animals received 1 ml of sterile saline by the same route. After 4 h, the animals were re-anaesthetized with ether, the abdominal cavity was opened, and the exudate was withdrawn after washing the peritoneum with 3 ml of PBS containing 5 U/ml heparin. Total leucocyte counts were performed on Neubauer chambers and differential counts on smears stained with panchromatic dye.

Analysis of data

Means and standard error of the mean (SEM) of all data are presented and compared by Student's *t*-test or analysis of variance. When appropriate, the data were analysed by the Newman-Keuls test.²⁴

Results

Activation and consumption of the haemolytic activity of the complement system following treatment with BaV

The effect of BaV on the activity of the complement system was determined *in vitro*, using NHS as a

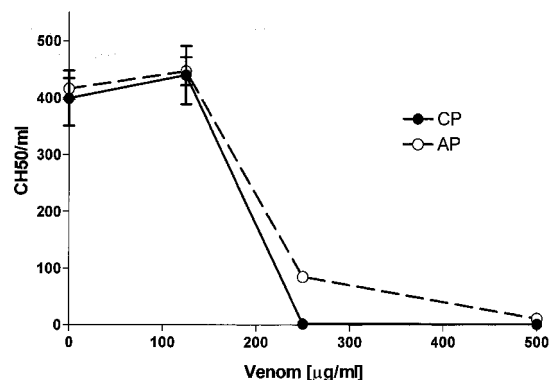


FIG. 1. Consumption of the haemolytic activity of the complement system following treatment with *Bothrops asper* venom (BaV). Samples of normal human serum (NHS) were incubated with different concentrations of venom for 30 min at 37°C. Control samples were incubated with phosphate-buffered solution (PBS) alone. Classical pathway (CP) activation of the complement system was determined by incubating aliquots of EA with dilutions of NHS in veronal-buffered saline (VBS) for 60 min at 37°C. The plates were centrifuged to remove intact cells and the absorbance of the supernatant was measured at 414 nm. Alternative pathway (AP) activation of the complement system was measured by using serum diluted in VBS containing EGTA-Mg⁺⁺ and rabbit E as target cells. Values are mean ± standard error (SE) of three experiments each performed in duplicate.

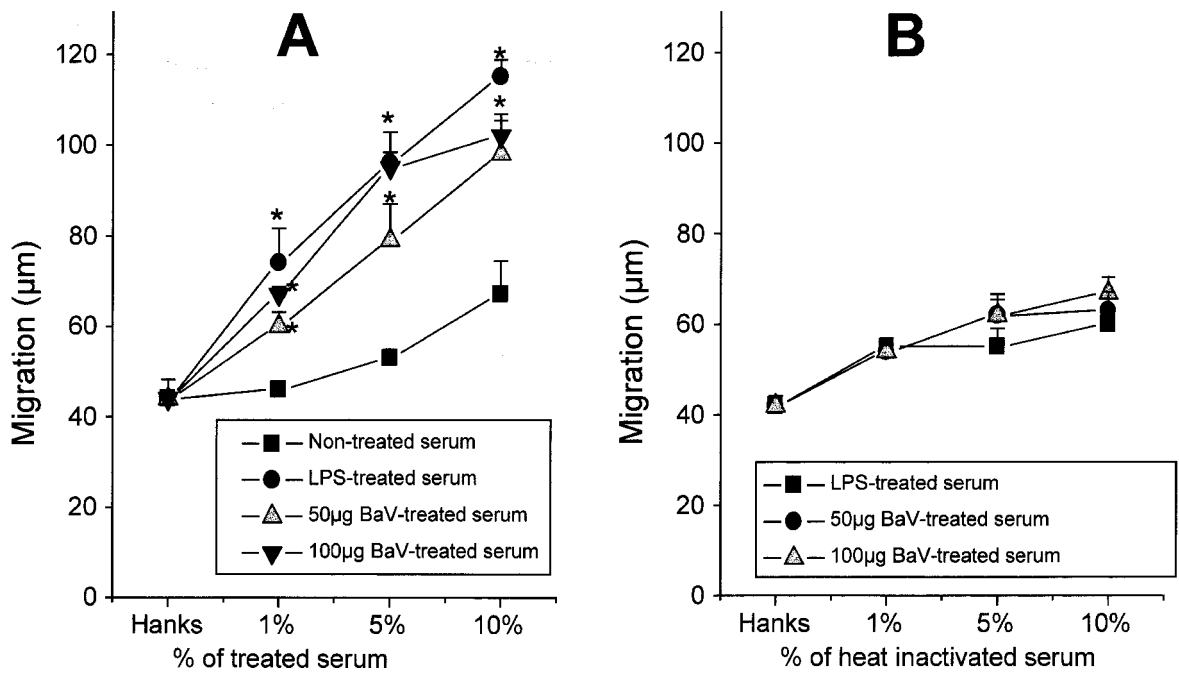


FIG. 2. Chemotactic responses of neutrophils to *Bothrops asper* venom (BaV) and to lipopolysaccharide (LPS)-treated serum. Neutrophils were placed in the top compartment of the chamber and allowed to migrate for 1 h. (A) BaV and LPS were incubated with serum for 30 min at 37°C, diluted in Hanks' balanced solution (HBSS) and used as chemotactic agents. (B) Heating the serum for 1 h at 56°C prior to the addition of BaV or LPS prevents its ability to induce neutrophil migration. Values are mean \pm standard error of the mean (SEM) of four experiments each performed in duplicate. * $p < 0.01$ versus values in the group using non-activated serum.

source of complement, with different doses of venom for 30 min at 37°C. The residual complement activity was determined by haemolytic assays performed in conditions for measuring classical or alternative pathway activation. Figure 1 shows that BaV induces a dose-dependent reduction in haemolytic activity as measured by both classical and alternative pathways.

Leucocyte chemotaxis assay

To evaluate the capacity of BaV *per se* to induce neutrophil migration, varying doses of venom (1, 10, 50 or 100 µg/ml) were dissolved in HBSS and placed in the bottom compartment of Boyden's chamber. The upper compartment was filled with neutrophils suspended in HBSS. The observed neutrophil migration was equivalent in all doses used, being similar to that observed in random migration (HBSS) (data not shown), indicating that BaV has no direct neutrophil transmigration activity.

In order to investigate the influence of BaV on neutrophil-oriented migration capacity, neutrophils were incubated with venom (1×10^7 cells suspended in 1, 10, 50 or 100 µg BaV/ml in HBSS) for 1 h at 37°C, centrifuged and immediately resuspended in HBSS for testing. These venom doses did not affect cell viability. The results indicated that pre-incubation with BaV does not alter neutrophil chemotactic response to LPS-activated serum. The migration obtained with

cells pre-incubated with different doses of BaV in response to HBSS, 1 or 10% of LPS-activated serum were similar and equivalent to that observed with cells pre-incubated with HBSS (data not shown).

To investigate the ability of BaV to generate complement chemotactic factors following complement activation, varying doses of venom were incubated with serum for 30 min at 37°C, diluted (v/v) in HBSS and tested in Boyden's chamber. The migration observed with serum treated with 1 or 10 µg BaV/ml was similar to that observed with non-treated serum (data not shown). However, higher doses of venom (50 or 100 µg/ml) were able to induce neutrophil migration equivalent to that observed with LPS-activated serum (Fig. 2A). This effect is not dependent on bacterial LPS components present in the venom, as equivalent migration was obtained with polymyxin-treated BaV (serum + BaV = 120 ± 9.22 µm, serum + polymyxin-treated BaV = 109 ± 8.65 µm). Heating the serum for 1 h at 56°C prior to the addition of venom completely abolished the ability to induce neutrophil chemotaxis (Fig. 2B).

Participation of C5a component on the neutrophil chemotaxis induced by the venom

In order to evaluate the possible involvement of the complement-derived chemotactic fragment C5a in the neutrophil chemotaxis induced by BaV, rat neutrophils were pre-incubated for 15 min with

Cells incubated with:

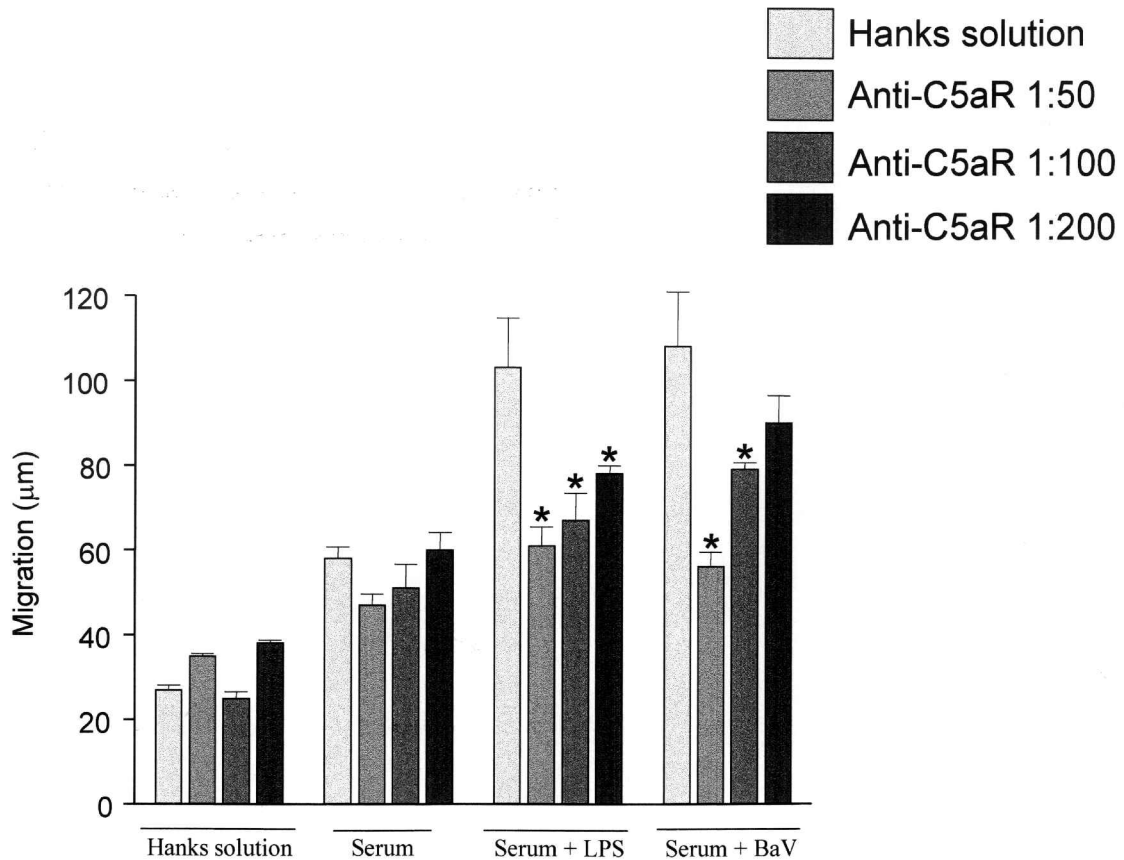


FIG. 3. Chemotactic responses of neutrophils incubated with different concentrations of anti-rat C5a receptor antibody before testing with *Bothrops asper* venom (BaV)-treated and lipopolysaccharide (LPS)-treated serum. Neutrophils were incubated with the antibody at 37°C for 15 min, placed in the top compartment of the chamber and allowed to migrate for 1 h. BaV and LPS were incubated with serum for 30 min at 37°C, diluted in Hanks' balanced solution (HBSS) and used as chemotactic agents. Values are mean \pm standard error of the mean (SEM) of three experiments each performed in duplicate. * $p < 0.01$ versus values obtained with incubated serum with HBSS in each group.

different dilutions of anti-rat C5a receptor serum and tested in chemotaxis assays. Figure 3 shows that the migration of neutrophils was inhibited by anti-rat C5aR antibody, in a dose-dependent manner. The anti-C5aR antibody was similar in blocking neutrophil chemotaxis induced by LPS-activated serum.

Effect of venom phospholipases and metalloproteinase on the generation of chemotactic factors from serum

Chemotaxis assays were performed with serum pre-incubated with different concentrations of phospholipases A₂ myotoxin II or III and metalloproteinase BaP-1 at 37°C for 30 min. Data obtained showed that only metalloproteinase-activated serum was able to induce neutrophil chemotaxis. The magnitude of the response induced by 5 or 10 $\mu\text{g/ml}$ was equivalent to that induced by LPS-activated serum (Fig. 4). Pre-heating the serum at 56°C for 1 h abolished the ability of the metalloproteinase to induce neutrophil chemotaxis (data not shown). Serum incubated with

myotoxin II or III (doses of 1, 10, 50 $\mu\text{g/ml}$) did not induce *in vitro* neutrophil locomotion (data not shown).

Analysis of the mechanism of C5a generation in rat serum induced by BaV and metalloproteinase BaP-1

In order to investigate the mechanism of complement activation induced by the venom and metalloproteinase BaP-1, normal serum was incubated with different concentrations of sCR1 at 37°C for 5 min before venom or metalloproteinase addition. The same experimental procedure was performed on LPS-activated serum. The solutions were incubated at 37°C for 30 min and used in chemotaxis assays. The results obtained showed that sCR1 did not inhibit the generation of chemotactic factors in venom-treated serum (Fig. 5). In contrast, sCR1 was able to reduce the neutrophil chemotaxis serum activity induced by both metalloproteinase and LPS (Fig. 5).

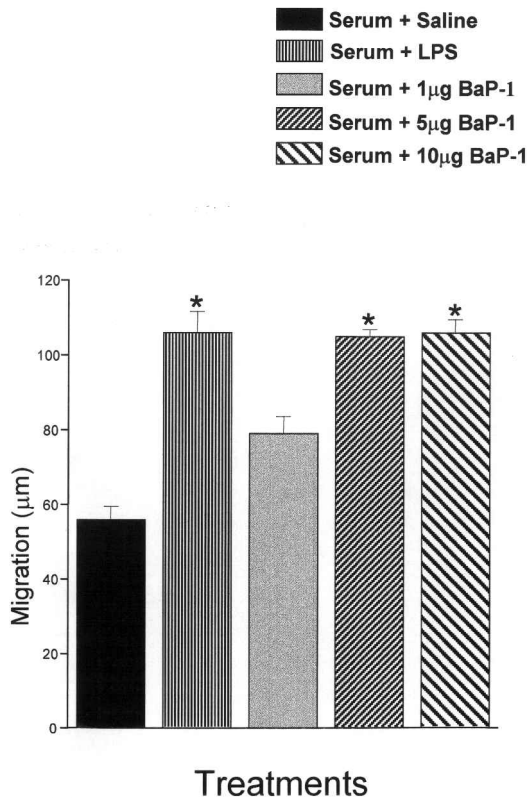


FIG. 4. Chemotactic responses of neutrophils to serum treated with *Bothrops asper* metalloproteinase BaP-1 or with lipopolysaccharide (LPS). Neutrophils were placed in the top compartment of the chamber and allowed to migrate for 1 h. BaP-1 or LPS were incubated with serum for 30 min at 37°C, diluted in Hanks' balanced solution (HBSS) and used as chemotactic agents. Values are mean \pm standard error of the mean (SEM) of four experiments each performed in duplicate. * $p < 0.01$ versus values obtained in the group using non-activated serum.

Local leucocyte mobilization induced by venom in C5-deficient mice

To evaluate the participation of C5a component on local leucocyte recruitment induced by BaV, 10 µg of venom was injected into the peritoneal cavity of B10/A (H-2^a, C5-sufficient) and A/J (H-2^b, C5-deficient) isogenic mouse strains and local leucocyte mobilization was determined 4 h after envenomation. The leucocyte mobilization into the peritoneal cavity of the C5-deficient animals was significantly reduced (50%) in comparison with that obtained in the C5-sufficient animals (Fig. 6A). Differential counts showed no significant migration of polymorphonuclear cells in C5-deficient mouse strain (Fig. 6B).

Discussion

BaV induces pronounced local effects, such as myonecrosis, haemorrhage and inflammatory reaction, characterized by long-lasting oedema and leucocyte infiltration.^{6,18,25,26} The mediators involved in the

development of oedema by this venom have been partially studied¹⁸ and the mechanisms involved in leucocyte recruitment remain unknown. Our results indicate that BaV activates the complement system leading to the genesis of C5a, which is notably involved in the chemotaxis of neutrophils during envenomation. Metalloproteinases, such as BaP-1, present in the venom may participate in the effect, but other component(s) may be involved.

In vitro chemotaxis studies suggest that BaV neither activates neutrophil-specific membrane receptors nor alters the intrinsic mechanisms involved in leucocyte locomotion. These conclusions are supported by the observations that venom *per se* is unable to evoke an oriented cell locomotion and does not modify the intrinsic ability of neutrophils to migrate in response to a chemotactic factor (LPS-activated serum). However, BaV was able to generate chemotactic factors when incubated with serum. Elimination of this activity by heating the serum at 56°C suggests that the serum factor(s) inducing cell migration may be complement activation products. Our observations of *in vitro* reduction of serum haemolytic activity by the venom corroborate this hypothesis.

It is well known that fragments generated during complement activation, such as C5a and C5a des arg, cause neutrophil activation, up-regulation of adhesion molecules, emigration and chemotaxis.²⁷ C5a is also a chemotactic factor to monocytes, macrophages, eosinophils and basophils.²⁸ Our results clearly demonstrate that C5a is involved in both *in vitro* and *in vivo* leucocyte locomotion induced by BaV. This was clearly evidenced by the use of a rabbit antibody generated against the N-terminal domain of the C5aR involved in binding activity. These observations were positively correlated with the experiments using C5-deficient mice. In both cases, the chemotaxis response was significantly reduced.

The abolishment of local neutrophil recruitment observed in C5-deficient mice implicates that the complement system and particularly C5a act as an important pathogenic component of the inflammatory response following envenomation. Other inflammatory mediators, as yet uncharacterized, certainly play additional roles in the leucocyte recruitment induced by BaV injection. A role for lipoxygenase pathway-derived products in leucocyte recruitment after injections of other *Bothrops* sp. venoms has already been described.^{7,9,10} Furthermore, our results indicate that the complement system has a central role in this phenomenon.

The presence of complement activators/inactivators has been demonstrated in a variety of animal venoms.^{11,29-31} Venoms of species classified in the families Elapidae, Crotalidae and Viperidae contain components with a broad range of action on the complement system. Some act by cleaving directly a

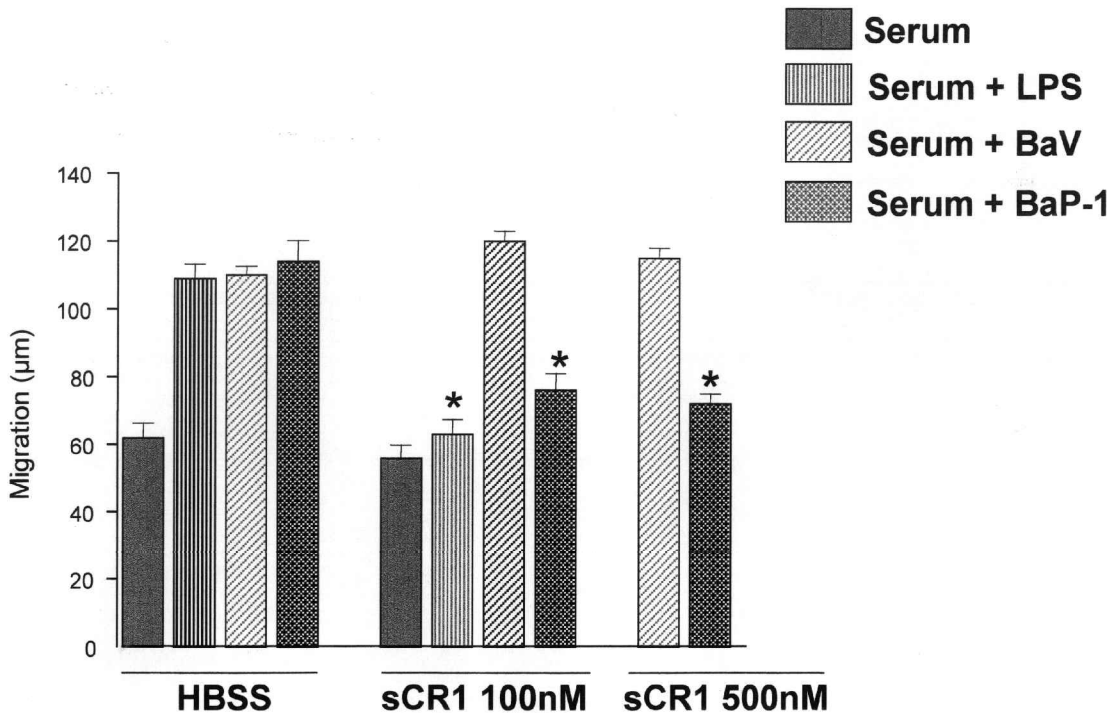


FIG. 5. Effect of soluble recombinant complement receptor type 1 (sCR1) on neutrophil chemotaxis induced by *Bothrops asper* venom (BaV), metalloproteinase BaP-1 or lipopolysaccharide (LPS)-treated serum. sCR1 was added to serum 5 min prior to the addition of BaV, BaP-1 or LPS. The solutions were incubated at 37°C for 30 min, diluted in Hanks' balanced solution (HBSS) and used as chemotactic agents. Neutrophils were placed in the top compartment of the chamber. Migration was determined 1 h after incubation of the chamber at 37°C. Values are mean ± standard error of the mean (SEM) of three experiments each performed in duplicate. * $p < 0.001$ versus values in the corresponding samples of serum without sCR1.

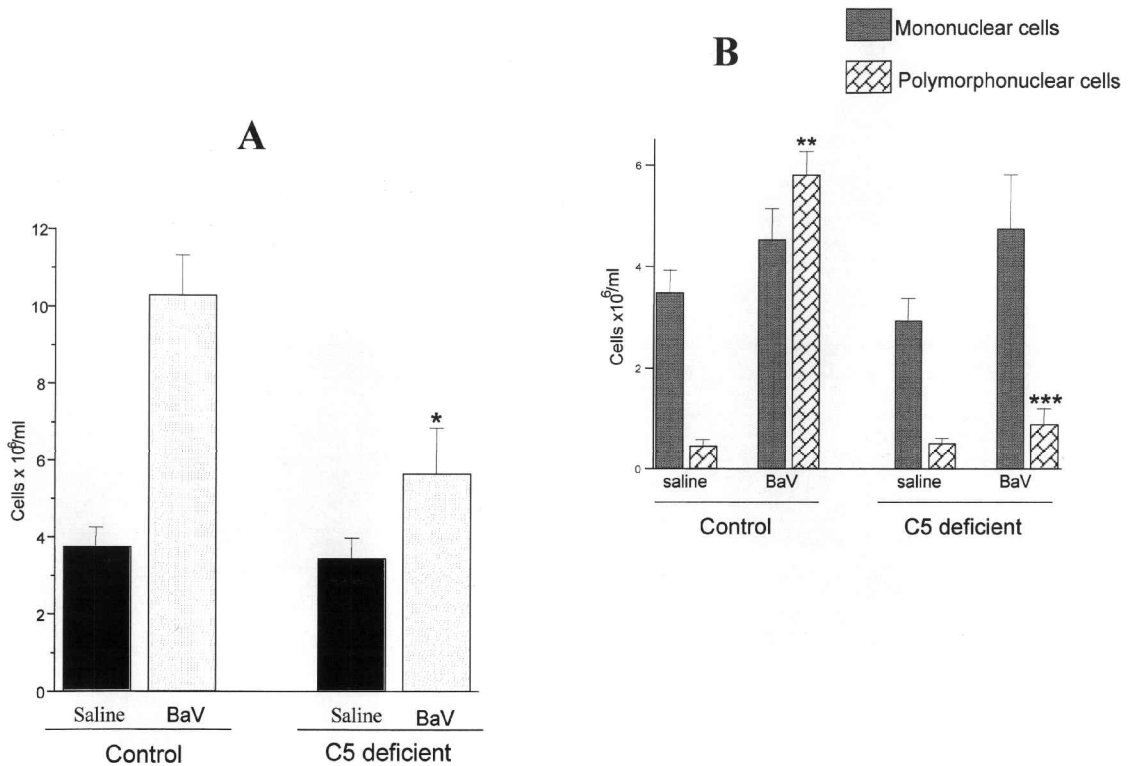


FIG. 6. Number of total (A) and differential (B) leucocytes migrated into the peritoneal cavity of male B10A (H-2^a) or A/J (H-2^b) mice 4 h after intraperitoneal injection of 10 µg of *Bothrops asper* venom (BaV) dissolved in 1 ml of sterile saline. Both groups of animals received venom or the equivalent volume of sterile saline. Values are mean ± standard error of the mean (SEM) of five animals in each group. * $p < 0.01$ versus values obtained in control animals treated with BaV, ** $p < 0.001$ versus values obtained in control animals treated with saline and *** $p < 0.001$ versus values obtained in control animals treated with BaV.

particular component, while others interact with complement components with the resulting complex being able to activate part of the complement cascade.

The complement activation induced by BaV was not attributed to the action of bacterial LPS. All venom solutions were sterilized by filtration and the eventual contamination by LPS was removed following polymyxin B column purification. Interestingly, venom-induced complement activation was not affected by incubation of the serum with sCR 1, whereas this soluble complement inhibitor abrogated neutrophil chemotaxis of LPS-activated serum.

Various tissue-damaging toxins have been isolated from BaV. There are at least four myotoxic phospholipases A₂ which induce acute muscle damage by directly affecting the integrity of the muscle cell plasma membrane.¹⁷ These myotoxins also induce oedema.^{17,32} In addition, five haemorrhagic metalloproteinases have been purified from this venom.^{21,33,34} Besides affecting the integrity of capillary blood vessels,^{35,36} metalloproteinases also induce myonecrosis,^{36,37} skin damage³⁸ and oedema.³⁷

Our results indicate that metalloproteinase BaP-1 is able to activate the complement system, as shown by the decrease in the haemolytic activity of the serum with the toxin (data not shown). In addition, neutrophil chemotaxis was evidenced when exposed to serum treated with BaP-1. In contrast, the two myotoxic phospholipases A₂ did not activate the complement system nor induce complement-dependent chemotaxis. Interestingly, the mechanism of complement activation by metalloproteinase seems to be dependent on C3 convertase assembly, as the addition of sCR 1 to BaP-1-treated serum prevented the generation of chemotactic factors. In contrast, complement activation by venom does not seem to be dependent on C3 convertase formation. Hence, sCR1 was unable to inhibit the neutrophil chemotaxis in response to venom-treated serum, suggesting that specific venom component(s) may act on the complement system by cleaving directly C5 component to generate a functional C5a anaphylatoxin fragment.

The precise mechanism by which the venom induces complement activation and the identification of other venom component(s) involved in the generation of C5a are the subjects of further study.

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