It has been shown that Bothrops jararaca venom (BjV) induces a significant leukocyte accumulation, mainly neutrophils, at the local tissue damage. Therefore, the role of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1), LECAM-1, CD18, leukocyte function-associated antigen–1 (LFA-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) on the BjV-induced neutrophil accumulation and the correlation with release of LTB4, TXA2, tumor necrosis factor-α, interleukin (IL)-1 and IL-6 have been investigated. Anti-mouse LECAM-1, LFA-1, ICAM-1 and PECAM-1 monoclonal antibody injection resulted in a reduction of 42%, 80%, 66% and 67%, respectively, of neutrophil accumulation induced by BjV (250 μg/kg, intraperitoneal) injection in male mice compared with isotype-matched control injected animals. The anti-mouse CD18 monoclonal antibody had no significant effect on venom-induced neutrophil accumulation. Concentrations of LTB4, TXA2, IL-6 and TNF-α were significant increased in the peritoneal exudates of animals injected with venom, whereas no increment in IL-1 was detected. This results suggest that ICAM-1, LECAM-1, LFA-1 and PECAM-1, but not CD18, adhesion molecules are involved in the recruitment of neutrophils into the inflammatory site induced by BjV. This is the first in vivo evidence that snake venom is able to up-regulate the expression of adhesion molecules by both leukocytes and endothelial cells. This venom effect may be indirect, probably through the release of the inflammatory mediators evidenced in the present study.

**Key words:** Snake venom, Adhesion molecules, Cytokines, Eicosanoids, Leukocyte influx

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**Introduction**

The snake Bothrops jararaca is responsible for most snakebite accidents in Brazil. Its venom induces severe pathophysiological effects characterized by local tissue damage, with hemorrhage, pain, myonecrosis and inflammation.1–3 Several studies demonstrate that Bothrops venoms induce a significant leukocyte accumulation at the site of inflammation.4–6 However, the mechanisms involved in this event have not been elucidated. Leukocyte accumulation in inflamed tissue results from generation of chemoattractant factors as well as adhesive interactions between leukocytes and endothelial cells within the microcirculation.7

Leukocyte recruitment into inflammatory sites is known to be a multi-step process, which is characterized by an initial transient contact with the endothelium, or rolling, followed by firm adhesion and transmigration.8,9 The initial phase of the adhesion cascade, neutrophil rolling, is mediated by members of the selectin family (L-selectin on neutrophils, and P-selectin and E-selectin on endothelial cells). Next, intercellular adhesion molecule-1 (ICAM-1) (CD54), which belongs to the immunoglobulin superfamily, binds to leukocyte function-associated antigen–1 (LFA-1) (CD11/CD18) on leukocytes.10 This interaction results in arrest and firm adhesion of the neutrophil to the endothelium, and is required for transendothelial cell migration.11 Finally, platelet endothelial cell adhesion molecule-1 (PECAM-1) (CD31) is required for the activation of proteases needed for passage through endothelial junctions and/or the basement membrane.12,13 Each adhesion molecules is involved in a different phase of leukocyte emigration through the endothelium, and the synchronization of their expression and functions is crucial for recruitment of leukocytes from the bloodstream to the tissue.14 A range of inflammatory mediators regulates this cascade by a sequential release.15

In the present study we investigated the importance of some adhesion molecules (ICAM-1, LFA-1, PECAM-1, LECAM-1 and CD18 β2-integrin) in the...
migration of neutrophils into the peritoneum after injection of B. jararaca venom in mice and the release of some inflammatory cytokines and eicosanoids. Understanding the mechanisms of venom-induced cell influx will be helpful for the pharmacological modulation of inflammatory events associated with Bothrops venom-induced local injury.

Materials and methods

Chemicals and reagents

Heparin was obtained from Roche (Rio de Janeiro, Brazil). Murine capture antibody anti-IL-6 (clone MP5–20F3), recombinant IL-6 and detection antibody anti-IL-6 (clone MP5–32C11) were purchased from Pharmingen (CA, USA). Rat monoclonal antibodies directed against L-selectin (anti-mouse CD62L, clone MEL-14), ICAM-1 (anti-mouse CD54, clone 3E2), LFA-1α chain (anti-mouse CD11a, clone M17/4), PECAM-1 (anti-mouse CD31, clone MEC 13.3), β2 integrin β chain (anti-mouse CD18, clone GAME 46) were purchased from Pharmingen. Rat immunoglobulin (Ig)G anti-horse IgG (irrelevant antibody) derived from mouse myeloma (ascitic fluid) was kindly provided by Dr Irene Fernandes (Immunopathology Laboratory, Butantan Institute, S˜ao Paulo, Brazil). 2,2′-Azino-bis(3 ethylbenzthiazoline–6-sulfonic acid) (ABTS) was purchased from Southern Biotechnology Associates Inc. (AL, USA). All salts used were obtained from Merck (Darmstadt, Germany).

Venom

Lyophilized crude venom of B. jararaca (BjV) was supplied by the Herpetology Laboratory of Butantan Institute. The venom was dissolved in 0.15 M NaCl solution and subsequently filtered through sterilizing membranes (0.22 μm pore size; Millipore Ind. Com. Ltd, Brazil) before use.

Animals

Male Swiss mice (18–20 g) were used. These animals were housed in temperature-controlled rooms and received water and food ad libitum until used. These studies were approved by the Experimental Animals Committee of Butantan Institute (protocol number 014/2001) in accordance with the procedures laid down by the Universities Federation for Animal Welfare.

Induction of inflammatory reaction

BjV (250 μg/kg), dissolved in 1 ml of sterile saline was injected by the intraperitoneal (i.p.) route. Control animals received 1 ml of sterile saline alone.

At selected time intervals, the animals were killed under a halothane atmosphere and the inflammatory exudate was withdrawn after washing the cavities with 2 ml of phosphate-buffered saline (PBS) (pH 7.2). Aliquots of the washes were used to determine total cell counts. The remaining volume was centrifuged at 500 × g for 6 min (at 4°C), and supernatants were stored at −70°C and later used for the determination of eicosanoids or cytokine concentration.

Leukocyte harvesting and counting

Leukocytes were harvested 6 h after i.p. injection of BjV or sterile saline by washing peritoneal cavities with 2 ml of PBS containing heparin (10 U/ml). Aliquots of the washes were used to determine total cell counts in a Neubauer chamber after dilution (1:20, v/v) in Turk solution (0.2% crystal violet dye in 30% acetic acid). For differential cells counts, cyto-spin preparations were stained with Hema3 stain (Biochemical Sciences Inc.). Differential cell counts were performed by counting at least 100 cells, which were classified as either polymorphonuclear or mononuclear cells, based on conventional morphological criteria.

Treatment with anti-adhesion molecules

Groups of mice were injected intravenously (i.v.), in the tail vein, with antibodies against adhesion molecules (1 mg/kg).16 Control animals received a similar injection of either saline solution or an equivalent dose of control rat IgG monoclonal antibody (mAb (LO-HoGa–3I), which recognizes horse IgG(T). After 30 min, mice were injected i.p. with BjV (250 μg/kg), and the neutrophil influx was assessed at 6 h, as already described.

Quantification of eicosanoid concentrations

Concentrations of LTB4 and TXB2 (stable metabolite of TXA2) were measured in the peritoneal washes at 30, 60 and 240 min after i.p. injections of BjV (250 μg/kg) or sterile saline, by a specific enzymatic immunoassay (ELISA) previously described by Pradelles et al.18 using a commercial kit (Cayman Chemicals, MI, USA), after extraction of eicosanoids on Sep Pak C18 columns eluted with ethanol. In brief, 100 μ1 aliquots of each extracted sample were incubated with the eicosanoids conjugated with acetycholines- terase and the specific rabbit antiserum, in 96-well microtitration plates coated with anti-rabbit IgG mouse monoclonal antibody. After addition of the substrate, the absorbances of the samples were recorded at 412 nm in a microplate reader, and the concentration of the eicosanoids was estimated from standard curves.

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Quantification of IL-6 or IL-1\(\alpha\) concentrations

Peritoneal washes were collected 30, 60, 180 and 360 min after i.p. injection of BjV (250 \(\mu\)g/kg) or saline. After centrifugation, the supernatants were used for determination of IL-6 and IL-1\(\alpha\) levels by a specific EIA, as described by Schumaker et al.\(^{19}\) Briefly, 96-well plates were coated with 50 \(\mu\)l of the first capture monoclonal antibody (anti-IL-6, 2.5 \(\mu\)g/ml; or anti IL-1\(\alpha\), 2 \(\mu\)g/ml) and incubated overnight at room temperature. Then 200 \(\mu\)l of blocking buffer, containing PBS/Tween-milk 5%, were added to the wells and the plates incubated for 2 h at room temperature. After washing, 50 \(\mu\)l of either samples or standards were dispensed into each well and the plates incubated for 2 h at room temperature. Wells were washed, and bound IL-6 or IL-1\(\alpha\) was detected by the addition of the biotinylated anti-cytokine monoclonal antibody (5 \(\mu\)g/ml, 50 \(\mu\)l/well). After incubation and washing, 50 \(\mu\)l of avidin-phosphatase or peroxidase-labeled streptavidin were added, followed by incubation and addition of the substrate (200 \(\mu\)l/ml of \(p\)-nitrophenylphosphate or 50 \(\mu\)l/ml of \(o\)-phenyldianizidine) for IL-6 or IL-1\(\alpha\) detection, respectively. Absorbances at 450nm were recorded and plotted against a standard curve prepared with recombinant IL-6 or IL-1\(\alpha\) in order to determine the concentration of this cytokine.

Quantification of TNF-\(\alpha\) concentrations

Peritoneal washes were collected 30 min, and 1, 3 and 6 h after i.p. injection of BjV (250 \(\mu\)g/kg) or saline. After centrifugation, the cells were used for determination of TNF-\(\alpha\) levels by a standard assay using the fibroblast continuous cell line L–929, as described by Ruff and Gifford.\(^{20}\) Monolayers of L–929 cells grown in RPMI–1640 medium were seeded at 3.5 \(\times\) \(10^4\) cells per well in microtiter plates and incubated in humidified air with 5% CO\(_2\) at 37°C for 18 h. Afterwards, 100 \(\mu\)l of the samples containing 2.5 \(\times\) \(10^4\) peritoneal cells were added serially and 0.2 \(\mu\)g/well of the anti-TNF-\(\alpha\) was added for the determination of the assay specificity. The plates were incubated during 4 h, and actinomycin D (2 \(\mu\)g/ml) was then added. After incubation for 18 h at 37°C, supernatants were removed and viable cells were assessed after fixation and staining with crystal violet (0.2% in 20% methanol). Cytotoxicity, expressed as a percentage, was calculated as follow (Abs\(_{\text{control}}\) – Abs\(_{\text{sample}}\) / Abs\(_{\text{control}}\)) \(\times\) 100. TNF-\(\alpha\) levels were then expressed as picograms per milliter, using a standard curve prepared with recombinant TNF-\(\alpha\).

Statistical analysis

Means and SEM of all data were obtained and compared by analysis of variance followed by the Tukey test, with significance probability levels of \(p < 0.05\).

Results

Effect of treatment with mAbs against adhesion molecules on neutrophil influx

To test whether adhesion molecules play a critical role on BjV-induced neutrophil in vivo, the effect of neutralizing mAbs against several adhesion molecules was examined. Since maximal neutrophil influx was observed 6 h after venom injection,\(^3\) we evaluated these mAbs on neutrophil accumulation at this time point. As shown in Fig. 1, i.v. injection of saline followed by i.p. injection of BjV (5 \(\mu\)g/mouse) caused accumulation of (1.3 \(\pm\) 0.1) \(\times\) \(10^6\) neutrophils at 6 h. Intravenous administration of mAbs anti-ICAM-1, anti-LECAM-1, anti-LFA-1 and anti-PECAM-1 (200 \(\mu\)g/mouse) 30 min before the i.p. injection of the BjV significantly reduced the number of recovered neutrophils to (0.44 \(\pm\) 0.11) \(\times\) \(10^6\), (0.75 \(\pm\) 0.15) \(\times\) \(10^6\), (0.26 \(\pm\) 0.09) \(\times\) \(10^6\) and (0.43 \(\pm\) 0.17) \(\times\) \(10^6\) neutrophils, respectively (\(p < 0.05\), compared with BjV), a response that was not significantly different from that seen after i.p. injection of saline [(0.6 \(\pm\) 0.1) \(\times\) \(10^6\) neutrophils]. In contrast, anti-CD18 mAbs did not inhibit neutrophils infiltration.
Changes in peritoneal exudate eicosanoid and cytokine release

To investigate the ability of BjV to release chemotactic mediators in the peritoneal cavity of mice, the concentrations of LTB$_4$ and TXA$_2$ in the peritoneal fluids of these animals were measured. BjV (250 μg/kg) induced a marked increase in peritoneal TXB$_2$ levels between 30 and 240 min. LTB$_4$ was significantly increased between 60 and 240 min (Fig. 2).

To further analyze the mechanisms of the inflammatory events induced by BjV, the concentrations of IL-6, IL-1$\alpha$ and TNF-$\alpha$ in the peritoneal fluid were measured. BjV induced a rapid elevation of IL-6 levels in the peritoneal exudate, peaking 3 h after the injection of the venom, and returning to normal values at 6 h (Fig. 3). The peak concentration was 14.5 ng/ml and control value was 5 ng/ml. No detectable levels of IL-1$\alpha$ were observed in the peritoneal exudates collected after the injection of BjV (data not shown).

The venom significantly induced a marked increment of TNF-$\alpha$ levels at all time intervals analyzed, except at 3 h. An early increase was observed 30 min after BjV injection, with maximum levels at 1 h (580% increment when compared with levels in control mice) (Fig. 4).

Discussion

Neutrophils migrate from vessels in response to a stimulus at the inflammatory site. The onset of
inflammation results in increased expression, or altered avidity of adhesion molecules, and thus increases in the adhesiveness of both the circulating cells and the endothelium.\textsuperscript{15}

The involvement of LECAM-1, ICAM-1, LFA-1, PECAM-1 and CD18 adhesion molecules in the leukocyte influx into the peritoneal cavity of mice after injection of BjV was investigated by pre-treating animals with selected anti-adhesion molecule mAbs. Previous results from our laboratory showed a significant accumulation of PMN cells into the peritoneal cavity of mice up to 24 h following BjV injection, with maximal counts of neutrophils between 3 and 12 h.\textsuperscript{3}

Present data showed that the anti-mouse LECAM-1 mAb significantly reduced the neutrophil migration. LECAM-1 is constitutively expressed on the neutrophil and is shed from the cell surface on cell activation, contributing to neutrophil rolling at the initial phase of the adhesion cascade.\textsuperscript{21} Thus, the obtained data suggest that BjV induces the expression of this leukocyte adhesion molecule from the selectin family that mediates rolling events. Previous reports have shown that L-selectin participates in leukocyte rolling after tissue trauma.\textsuperscript{22}

The expression of selectin adhesion molecules in leukocytes does not lead to firm adhesion and transmigration unless another set of adhesion molecules is engaged. For neutrophils, firm adhesion requires activation of the \( \beta_2 \) (CD18) adhesion molecule from the integrin family, resulting in binding to one of the intercellular adhesion molecules at the surfaces of endothelial cells.\textsuperscript{23} Results showed that anti-mouse LFA-1 (CD11a/CD18) mAb strongly inhibited the cell influx in our model. This further indicates the ability of the BjV to activate neutrophils to express adhesion molecules, in this case an integrin that is relevant for the firm adhesion of leukocytes. Conversely, no inhibitory effect of anti-CD18 mAb on neutrophil migration was observed. This result is unexpected because the literature shows that blocking mAbs directed against CD18 are effective inhibitors of neutrophil migration in various inflammatory models \textit{in vitro}.\textsuperscript{15} However, CD18-independent mechanisms of cell adhesion are also known to exist\textsuperscript{24,25} and it is well established that CD18-independent neutrophil migration occurs in pulmonary inflammation in numerous animal species.\textsuperscript{26,27} In addition, Winn and Harlan\textsuperscript{28} have described a CD18-independent neutrophil migration into the peritoneum of rabbits due to release of a factor by macrophage already present at the inflammatory site. However, we cannot discard the contribution of CD18 on BjV-induced neutrophil influx in other time intervals that were not evaluated in this study. It is possible that, at the period of time used to analyze the influx of neutrophils (6 h), the increased expression of other adhesion molecules could overcome the blockade of CD18, a hypothesis that needs to be further addressed.

In addition, we observed that venom-elicited peritoneal neutrophil influx was inhibited by both anti-ICAM-1 and anti-PECAM-1 mAbs. These results suggest that ICAM-1 and PECAM-1 may be required for the venom-induced trafficking of neutrophils and indicate the ability of BjV to activate the endothelium, since these molecules are expressed by endothelial cells.\textsuperscript{29} ICAMs are ligands for the integrin molecules LFA-1 and MAC-1 on leukocytes.\textsuperscript{10} ICAM-1 participates in leukocyte–leukocyte, leukocyte–endothelial, and leukocyte–epithelial cell interactions, transendothelial migration, and adhesion-dependent respiratory burst.\textsuperscript{30} Thus, in the present experimental conditions, ICAM-1 may be the ligand for LFA-1 and both molecules may contribute to neutrophil–endothelial interactions.

PECAM-1 is one of the most abundant proteins on the endothelial cell surface. It is located at the cell–cell borders of adjacent endothelial cells and plays an important role in transendothelial migration of neutrophils.\textsuperscript{51} PECAM-1 is important in the passage of neutrophils through endothelial junctions mainly in cytokine-activated transmigration; it seems to have little role in the chemotactic transmigration.\textsuperscript{13} Taking into account that large accumulation of neutrophils occurs at 6 h, we suggest that the venom may trigger a cytokine-activated transmigration process leading to the passage of neutrophils across endothelium and into the tissues. Our results, however, do not rule out the possibility that a chemotactic-dependent or a PECAM-1-independent mechanism may also operate in the passage of leukocyte through the endothelial cell junctions at the early stages of inflammatory response to the venom. To our knowledge this is the first report dealing with characterization of the adhesion molecules involved in the leukocyte influx induced by snake venoms.

It is well known that LTB\textsubscript{4} and TXA\textsubscript{2} induce the expression of ICAM-1 and PECAM-1 adhesion molecules.\textsuperscript{32,33} To further analyze the inflammatory events that could be involved in the expression of adhesion molecules secondary to venom administration, the release of these eicosanoids into the peritoneal cavity was analyzed. Results showed that i.p. injection of BjV induced a significant increment in intraperitoneal concentrations of LTB\textsubscript{4} and TXA\textsubscript{2}. These results are in agreement with the previous observations showing the release of eicosanoids into the air pouch injected with BjV,\textsuperscript{6} as well as in the site of injection of venoms from other species of \textit{Bothrops} genus.\textsuperscript{5,34} Thus, our observations suggest that these mediators may contribute to the expression of ICAM-1 and PECAM-1 induced by BjV. Alternatively, a direct effect of this venom on the endothelium cannot be ruled out. On the other hand, Farsky \textit{et al.}\textsuperscript{6,35} showed that venoms of \textit{Bothrops asper} and \textit{Bothrops jararaca} do not alter the intrinsic mechanisms involved in leukocyte locomotion, in an experimental model \textit{in vitro}.
Cytokines have been identified as the key mediators responsible for up-regulation of the expression of adhesion molecules, and they participate in a variety of inflammatory conditions. Among them, TNF-α, IL-6 and IL-1α are highly relevant. Elevated concentrations of TNF-α in the peritoneal cavity were detected after i.p. injection of BjV. TNF-α induces the expression of ICAM-1, E-selectin and CD11b/CD18. As a mediator of inflammation, it triggers the release of practically all known mediators such as IL-1, IL-6 and all metabolites of arachidonic acid, thereby amplifying the inflammatory cascade. In our experimental model, TNF-α may be relevant for the expression of ICAM-1 thereby contributing for neutrophil influx. Moreover, release of TNF-α was a long-lasting effect following BjV injection, which suggests that this cytokine may be also important in the activation of leukocytes and production of hydrogen peroxide and nitric oxide observed after venom injection.

IL-6 is also likely to be involved in leukocyte infiltration induced by BjV. The i.p. injection of BjV induced a significant increment in the concentration of IL-6 in the peritoneal exudates. These results are in concordance with others showing an increase of this cytokine in the serum of mice after i.p. and subcutaneous injection of snake venoms from Bothrops sp. IL-6 is related to the systemic acute phase response associated with infection or injury, including the release of neutrophils into the circulation and the up-regulation of ICAM-1 expression. Thus, our results suggest that IL-6 may have a role in the expression of ICAM-1 following BjV injection, being relevant for firm adhesion of neutrophils. Moreover, since maximal production of IL-6 occurs at 3 h, a time when no release of TNF-α was observed, IL-6 may also act as a negative mediator of TNF-α production and/or secretion in this experimental model, in agreement with previous observations. On the other hand, levels of IL-1α were not detectable in our experimental model, in contrast with previous observations describing increments in IL-1 levels in serum of mice after i.p. injection of BjV. It is likely that non-peritoneal cells are responsible for the production of IL-1 after i.p. injection, resulting in the increments described in serum.

Many cell types produce and secrete inflammatory mediators involved in the leukocyte influx after stimulation. In this way, monocytes and macrophages are the central sources of the studied cytokines and eicosanoids. Mast cells represent another source of TNF-α and store this cytokine in specific granules. Taking into account that BjV is able to stimulate macrophages and neutrophils, we assume that these cells, mainly macrophages, are relevant sources of eicosanoids and TNF-α in our experimental model. However, the contribution of mast cells cannot be ruled out, since an earlier release of TNF-α was detected in the local of BjV injection. Phospholipase A2-homologues isolated from Bothrops jararacussu venom degranulate mast cells in rats.

In conclusion, we have shown that the neutrophil influx induced by i.p. injection of BjV venom in mice is related to the expression of LECAM-1, LFA-1, ICAM-1, and PECAM-1 adhesion molecules, responsible for the rolling, firm adhesion and transmigration events associated with neutrophil migration. This indicates that the venom is able to induce the expression of adhesion molecules from both leukocytes and endothelial cells. This effect may be indirect, via the release of eicosanoids LTβ4 and TXA2, as well as the cytokines TNF-α and IL-6, but not IL-1.

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