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Inflammatory mediators generated at the site of inoculation of *Loxosceles gaucho* spider venom

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ABSTRACT

Patients bitten by *Loxosceles* spiders generally manifest marked local inflammatory reaction and dermonecrosis. This report evaluated edema formation, leukocyte infiltration and release of inflammatory mediators at the injection site of *Loxosceles gaucho* venom. BALB/c mice were i.d. injected with venom and thereafter paws were disrupted and homogenized to obtain differential counts of migrated cells, as well to assay the levels of cytokines, chemokines and lipid mediators. Increased footpad thickness was detected as soon as 30 min after venom injection, and 24 h later was similar to that of the control group. *Loxosceles* venom mildly augmented the recruitment of leukocytes to the footpad in comparison with PBS-injected mice. Moreover, it stimulated the release of IL-6, MCP-1 and KC at 2 and 24 h after venom injection. In addition, higher levels of PGE_2 were detected 30 min after venom injection in comparison with control group. However, the venom failed to increase levels of IL-1β, TNF-α, TXB_2 and LTB_4. Our results demonstrate that *L. gaucho* venom evokes an early complex inflammatory reaction, stimulating the secretion of pro-inflammatory cytokines and lipid mediators (PGE_2), and recruiting leukocytes to the footpad which contribute to the local reaction induced by *L. gaucho* venom.

1. Introduction

Loxoscelism is a syndrome caused in humans by bites from the brown spider *Loxosceles* spp. In general, the clinical manifestations are characterized by inflammation and dermonecrosis at the site of the bite, although in some cases systemic hemolysis and coagulopathy are present, leading to acute renal failure (Hogan et al., 2004; Abdulkader et al., 2008). In Brazil, eight *Loxosceles* species are found, however, *Loxosceles gaucho*, *Loxosceles laeta* and *Loxosceles intermedia* are commonly implicated in accidents reported in humans (Barbaro and Cardoso, 2003), which occur mainly in the southern and southeastern regions of the country. In 2006, *Loxosceles* spiders were responsible for approximately 40% of 19,105 notified cases of spider envenomation reported to the Brazilian Ministry of Health (SINAN, 2006).

Loxosceles venoms are rich source of proteases, hydrolases, lipases, peptidases, collagenases, alkaline phosphatases, 5-ribonucleotidases, phosphohydrolases and other components. A family of phospholipases D has been mainly

Abbreviations: IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8; TNF-α, tumor necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; KC, keratinocyte chemoattractant; TXB_2, thromboxane B_2; LTB_4, leukotriene B_4; PGE_2, prostaglandin E_2; RANTES, regulated upon activation, normal T-cell expressed, and secreted; GM-CSF, granulocyte macrophage colony-stimulating factor; GRO-α, growth regulated oncogene-alpha.

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implicated in the genesis of dermonecrosis that occurs after the bite (Hogan et al., 2004; Silva et al., 2004; Barbaro et al., 2005; Kalapothakis et al., 2007). Venom components interact with the cellular membrane, degrade components of extracellular matrix, take part in complement system activation and recruit polymorphonuclear leukocytes and platelets, among others, thus contributing to the establishment of local injury (Futrell, 1992; Hogan et al., 2004; Silva et al., 2004). Such venom components may be important to induce an early inflammatory reaction, eliciting the release of many endogenous pro-inflammatory mediators that contribute to the development of the lesion (Hogan et al., 2004).

Loxosceles venoms have been demonstrated to stimulate cytokine production. Patel et al. (1994) showed that *Loxosceles reclusa* venom stimulated the production of IL-8 and the secretion of GM-CSF by endothelial cells, the migration of neutrophils to the injection site, and the release of granule content after neutrophil adhesion. Moreover, Málaque et al. (1999) observed that *L. gaucho* venom causes alterations in primary cultures of keratinocytes and stimulates TNF-α production. In addition, *Loxosceles deserta* has been shown to stimulate the expression of vascular endothelial growth factor (VEGF) in human keratinocyte culture (Desai et al., 2000). Induction of release of chemokines (IL-8, GRO-α, MCP-1, RANTES) by endothelial and epithelial cells has also been implicated in the pathogenesis of loxoscelism (Gomez et al., 1999). Recently, Souza et al. (2008) reported high levels of IL-6 and TNF-α in a patient bitten by *Loxosceles* spp. spider. Thus, based on these findings, a number of cytokines and chemokines seem to contribute to the pathogenesis of dermonecrotic arachnidism.

The poor efficiency of current strategies (serotherapy and corticoid administration) to treat loxoscelism in Brazil is attributed to at least two causes. One of them is the rapid tissue injury that occurs after *Loxosceles* envenomation (Barbaro and Cardoso, 2003; Hogan et al., 2004; Silva et al., 2004). The other is the delayed medical assistance, since patients seek medical care usually 12–24 h after the spider bite (Barbaro et al., 1992b; Málaque et al., 2002). Thus, the effectiveness of loxoscelism treatment should take into consideration both the intrinsic venom toxic activity and the local inflammatory reaction that develops after envenomation.

Loxosceles venom induces dermonecrosis in rabbits, guinea pigs and humans but not in mice (Futrell, 1992; Barbaro et al., 1996a,b; Domingos et al., 2003; Hogan et al., 2004). However, *Loxosceles* venom causes an important inflammatory reaction at the injection site and, depending on the dose, it can be lethal to animals (Barbaro et al., 1994). Domingos et al. (2003) demonstrated that the size and availability of local sphingomyelin may be important in determining the outcome of *Loxosceles* envenomation in different mammalian species. Since we are interested to verify the inflammatory reaction in the absence of dermonecrosis, in the present work we investigated the effect of *L. gaucho* venom in evoking an acute inflammatory response by means of the evaluation of edema formation and the release of inflammatory mediators (cytokines, chemokines and lipid mediators) after injection of *L. gaucho* venom in mouse paws.

2. Materials and methods

2.1. Animals and venom

BALB/c mice (male, 18–20 g) were provided by the Butantan Institute animal house. The procedures involving animals were conducted according to national laws and policies controlled by Butantan Institute Animal Investigation Ethical Committee (protocol n° 316/06). Specimens of adult *L. gaucho* spiders were collected in São Paulo State, Brazil. The spiders were kept in quarantine for 1 week without food before venom extraction. Venoms were obtained as previously described (Barbaro et al., 1992a). Protein concentrations were determined by the bicinchoninic acid (BCA) assay (Smith et al., 1985). Standard curves were constructed using bovine serum albumin (Sigma Chemicals, St Louis, MO, USA) diluted in duplicate.

2.2. Experimental model

Mice ($n = 4$–8) were injected (30 μL) in the right hind paw with *L. gaucho* venom (3, 5, or 10 μg), PBS (negative control), or lipopolysaccharide (LPS) from *Escherichia coli* (1 μg, Sigma Chemicals, St Louis, MO, USA, positive control). Edema-forming activity was studied at three time intervals (0.5, 2 and 24 h), and thereafter the right paws were removed at the level of the tibiotarsal joint, disrupted and homogenized. Following centrifugation (400 *g*) at 4 °C for 10 min, supernatants were recovered to assay the release of cytokines, chemokines and lipid mediators by enzymatic immunoassay. Cell pellets were recovered to perform cell counts.

2.3. Edema measurement after footpad injection

Mice were injected (30 μL) in the footpad with different doses of *L. gaucho* venom or PBS. Edema formation was measured using a plethysmographer (Ugo Basile, IT). Results expressed the difference in paw volume (μL) prior to (control) and after (experimental) injection. Edema was measured at different time points (0.5, 2 and 24 h) after injection.

2.4. Leukocyte recruitment to footpads

Total leukocyte counts from footpad homogenates were performed by Trypan blue exclusion (Sigma–Aldrich, USA) using a hemocytometer; differential counts were accomplished using cytocentrifuge slides stained with HEMA-3 (Fischer Scientific Company, MI, USA). For differential cell counts, 100 leukocytes were enumerated and identified as macrophages, lymphocytes or neutrophils, based on staining and morphologic features.

2.5. Assays for cytokines, chemokines and lipid mediators

Levels of cytokines (IL-1β, IL-6 and TNF-α), chemokines (KC and MCP-1) and lipid mediators (LTB_4, PGE_2 and TXB_2) were measured in supernatants of paw tissue extracts (as described in section 2.2) or in supernatants of murine