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Neutrophils regulate the expression of cytokines, chemokines and nitric oxide synthase/nitric oxide in mice injected with *Bothrops atrox* venom

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Abstract

Bothrops atrox crude venom injected intraperitoneal (i.p.) into BALB/c mice induced local afflux of inflammatory cells, one neutrophil-rich peak after 6 h and another macrophage-rich peak after 48 h. A similar pattern of local cell afflux plus edema, Delta lesions of some skeletal muscle cells, and hemorrhage were observed in mice intramuscular (i.m.) injected with the venom. Measurement of serum cytokines in neutrophil-depleted (by anti-mouse rat monoclonal antibody (mAb) RB6-8C5) and non-depleted BALB/c mice was performed by ELISA. With the exception of IL-1β (78 pg/ml), higher levels of IL-6 (1348 pg/ml), MIP-1β (437 pg/ml) and MIP-2 (904 pg/ml) were observed in neutrophil-depleted mice, in comparison to the values found in non-neutrophil depleted mice: IL-1β (437 pg/ml), IL-6 (750 pg/ml), MIP-1β (165 pg/ml) and MIP-2 (90 pg/ml). TNF-α was not detected. NO was detected (18 μM) 24 h after venom injection in neutrophil-depleted mice. RT-PCR using representative primers detected expression of mRNA in cells from BALB/c mice injected with *B. atrox* venom: (a) for IL-1β, IL-6, inducible nitric oxide synthase (iNOS), CXCR2, MIP-2 and RANTES in cells from mice that were neutrophil-depleted or not; (b) for CCR1, CCR5 and MIP-1β in cells from neutrophil-depleted mice; (c) for MIP-1α in cells from non-neutrophil-depleted mice; (d) TNF-α and TGF-β were not detected in either of the mice. These results indicate that neutrophils play a role in regulating the production of some cytokines and chemokines as well as locally expressed or liberated iNOS/NO in tissues injected with *B. atrox* crude venom.

Keywords: *Bothrops atrox*; Chemokines; Cytokines; Hemorrhage; Inflammation; Neutrophil; Venom

Introduction

Local pain, edema and hemorrhage are the immediate local consequences of *Bothrops* sp. snake bites, followed by the appearance of serous blood-filled bubbles,

Abbreviations: i.m., intramuscular; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; mAb, monoclonal antibody; PMN, polymorphonuclear

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myonecrosis and enlargement of regional lymph nodes. Systemic consequences such as haemostatic disturbances, hemorrhages and acute renal failure, although in variable proportions, usually occur (Siqueira-França and Santana Málaque, 2003). The early events of inflammation such as increases in vascular permeability and pain have been attributed to the local release of preformed molecules such as histamine and/or to newly generated peptides from plasma protein precursors such as bradykinin (Rocha e Silva et al., 1949; Ferreira and Silva, 1962) and anaphylatoxins (Dias da Silva and Lepow, 1967; Dias da Silva et al., 1967). Coagulopathy and spontaneous bleeding result from consumption and eventual depletion of clotting factors (Hutton and Warrel, 1993), while spontaneous bleeding is attributable to vascular endothelium-damaging hemorrhagins, some of which have been characterized as metalloproteinases (Paine et al., 1992).

More recently, some cytokines have also been implicated as putative mediators of some of these events (Moura da Silva et al., 1996; Barros et al., 1998). Cytokines act as soluble mediators of immune responses participating in the communication and regulation of inflammatory responses (Cerami, 1992). Some studies describing the involvement of cytokines in envenomation have been reported, including snake, scorpion and spider venom models (Petricevich et al., 2000; Meki and El-Dean, 1998; Tambourgi et al., 1998).

Two distinct classes of enzymes have been recently isolated from *B. atrox* venom and characterized with respect to their actions upon injection in mice. One of these classes is a type III metalloproteinase (Petretski et al., 2000, 2001), while the other includes two PLA_2s; a Lys-49 and an Asp-49 (Kanashiro et al., 2002). Local polymorphonuclear (PMN) cell accumulation in tissues injected with the type III metalloproteinase, but without hemorrhage, is a complement-dependent phenomenon (Rodrigues et al., 2004). PLA_2s exhibit strong myonecrotic, edematogenic, and mast cell histamine releasing activities (Kanashiro et al., 2002). These results suggest that although neutrophil accumulation in the *B. atrox* venom injection sites may not play a central role in the consequent rapid ensuing inflammation, it could regulate the subsequent slower events.

In the present study, we investigated the effects of neutrophil depletion on the expression of some cytokines, chemokines and inducible nitric oxide synthase (iNOS)/NO production induced by *B. atrox* crude venom to further understand the tissue lesions evoked by this snake venom. We report here using ELISA method and specific monoclonal antibodies (mAbs) that, with the exception of IL-1β (which was found in higher amounts in non-neutrophil depleted mice), the production of IL-6, MIP-1β, MIP-2 and NO was higher in neutrophil-depleted mice. In addition, we used RT-PCR and respective primers to detect the expression of mRNA in cells from BALB/c mice injected with *B. atrox* venom: (a) for IL-1β, IL-6, iNOS, CXCR2, MIP-2 and RANTES in cells from mice that were neutrophil depleted or not; (b) for CCR1, CCR5 and MIP-1β in cells from neutrophil-depleted mice; (c) for MIP-1α in cells from neutrophil non-depleted mice; (d) TNF-α and TGF-β were not detected. These results indicate that neutrophils play a role in regulating the production of some cytokines and chemokines as well as the up-regulation and liberation of iNOS/NO in tissues injected with *B. atrox* crude venom.

Materials and methods

Snake venom

Venom from adult specimens of *B. atrox* were provided by the Laboratório de Herpetologia, Instituto Butantan (SP, Brazil), in lyophilized form. The venoms were diluted in phosphate-saline buffer pH 7.2 (PBS), filtered through a 0.45 μm membrane and stored at −20 °C.

Animals

BALB/c, BALB/c nu^{++} mice (20 ± 2 g) and Wistar rats (250 ± 10 g) were provided, respectively, by Biotério de Camundongos Isogênicos and Biotério Geral, CBB, UENF, Campos dos Goytacazes, RJ. Animals were bred and reared under ethical conditions according to international animal welfare recommendations.

Cell cultures

RB6-8C5 was kindly supplied by Dr. Vera Calich (Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil). The fibroblast cell line, L929, was purchased from the American Type Cell Collection (ATCC, USA, CRL-2148). Cells were cultured in DMEM-F12 (Gibco BRL, USA) supplemented with 10% FCS and 20 μg/ml gentamicin, at 37 °C, 5% CO_2.

Production of anti-mouse neutrophil RB6-8C5 and non-specific rat IgG

To study the participation of neutrophils in inflammatory mediators production, RB6-8C5 hybridoma cell suspensions (2×10^6 cells/ml) in 300 μl of incomplete Freund's adjuvant (Difco, USA) were intraperitoneal (i.p.) injected into BALB/c nu^{++} mice, ascitic fluids were withdrawn 15 days later and stored at −20 °C. The presence of IgG light and heavy chains was monitored by SDS-PAGE analysis, whereas specific antibodies to cell surface neutrophil antigens were determined by