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Effect of *Bothrops bilineata* snake venom on neutrophil function

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ABSTRACT

The aim of the study was to evaluate the *in vitro* effects of *Bothrops bilineata* crude venom (BbV) on isolated human neutrophil function. We proved that BbV isn't toxic towards human neutrophils. During an incubation of human neutrophils with BbV hydrogen peroxide was produced. Moreover, BbV was able to stimulate neutrophil release of proinflammatory mediators such as IL-8 and IL-6 as well as PGE_2 and NETs liberation. There is no data in the literature showing the effect of BbV on the production of IL-6 and IL-8 or NETs liberation by isolated human neutrophils. Taken together our results testify that BbV triggers relevant proinflammatory events in human neutrophils.

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1. Introduction

Bothrops bilineata ((Wied-Neuwied, 1825) is an arboreal species which has a known distribution in the Amazon Forest, in some areas of the Atlantic Forests (Campbell and Lamar, 2004) and in the northeastern part of the state of Minas Gerais (Feio and Caramaschi, 2002; Bernarde et al., 2011). Recently, Carrasco et al. (2012) through morphology, phylogeny and taxonomy studies has suggested an arrangement of the *Bothrops* genus and also has recognized as sister clade synonymizing *Bothriopsis*, *Bothropoides* and *Rhinocerophis*.

It is important to note that there are few studies on the epidemiological and clinical aspects of envenomation by *B. bilineata* (Borges et al., 1999; Smalligan et al., 2004; Waldez and Vogt, 2009). And experimentally *B. bilineata* venom induces neuromuscular activity in nerve-muscle preparations isolated from vertebrates (Rodrigues-Simioni et al., 2011). In addition, *B. bilineata* venom induces a significant leukocyte accumulation at the site of tissue damage characterized by neutrophil migration (Porto et al., 2007). However, the activation state of these cells is still unclear.

Neutrophils, also named polymorphonuclear granulocytes (PMN), represent the majority of the leukocytes in peripheral blood. They have very short lifespans, spending only 8–12 h in circulation (Summers et al., 2010). However, various stimuli, such as cytokines and bacterial products were shown to prolong their survival (Colotta et al., 1992).

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They are considered the first line of defense in the organism due to their quick migration into infected tissue thus providing an acute inflammatory response (Nathan, 2006). At the inflammation site, neutrophils perform host defense functions such as phagocytosis, release of proteolytic enzymes, generation of reactive oxygen species (ROS), and synthesis of a number of inflammatory mediators including cytokines and lipid mediators (Cassatella, 1995, 1999; Nathan, 2006; Timár et al., 2013).

In addition to these well-known neutrophil functions, the literature documents the discovery of neutrophil extracellular traps (NETs) also capable of eliminating microorganisms in the extracellular space (Brinkmann et al., 2004). These extracellular vesicles represent a form of intercellular communication carried out by lipids, proteins, and nucleic acids (Timár et al., 2013).

So, the present study aimed to evaluate the effect of *B. bilineata* venom (BbV) on the functionality of human neutrophils such as cytokine production (IL-6 and IL-8) as well as that of PGE_2, hydrogen peroxide and release of NETs.

2. Material and methods

2.1. Chemicals and reagents

MTT, RPMI-1640, L-glutamine, gentamicin, phorbol myristate acetate (PMA), Histopaque 1077, DMSO, OPD (o-1,2-phenylenediamine dihydrochloride), horseradish peroxidase and nitroblue tetrazolium (NBT) were purchased from Sigma (MO, USA). FITC anti-human CD66b was purchased from BD Pharmingen (CA, USA). DuoSet Elisa human IL-6 and DuoSet Elisa human CXCL8/IL-8 were purchased from R&D Systems (Oxon, United Kingdom). PGE_2 enzyme immunoassay kit was purchased from Cayman Chemical (MI, USA). Quant-iT™ Picogreen dsDNA was obtained from Invitrogen (CA, USA). Fetal bovine serum was obtained from Cultilab (Brazil). All salts and reagents used were obtained from Merck (Darmstadt, Germany) with low endotoxin or endotoxin-free grades.

2.2. Venom

The venom from the *B. bilineata* (BbV) snake was acquired from CEBIO-UNIR,RO. The licenses for scientific purposes are from: Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis – IBAMA and Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio. Numbers: 11094-2, 11094-1, 10394-1 e 15484-1.

2.3. Neutrophil isolation

Peripheral blood neutrophils were obtained from buffy coats of self-reportedly healthy donors (18–40 years), and approval for use in this study was given during the blood draw. A prior agreement from all involved was made in order to be included in the study, and the Center of Tropical Medicine Research (Rondonia, Brazil) Research Ethics Committees (number 108/2010) approved this study. Briefly after, local asepsis blood was collected in vacuum tubes containing heparin and diluted in phosphate buffered saline (PBS, 14 mM NaCl, 2 mM $NaH_2PO_4H_2O$, 7 mM $Na_2HPO_412H_2O$), pH 7.4. In order to separate the leukocytes Histopaque 1077 was added to the tubes and then the diluted blood was added carefully to the reagent. After centrifugation at 400× *g* for 30 min, the neutrophils were collected from the bottom of the tube, along with erythrocytes and transferred to another tube. Lysis of erythrocytes was performed using lysis buffer (9.98 mM $KHCO_3$, 0.1 mM Na_2EDTA). Then the solution was homogenized, incubated at −8 °C for 5 min, and centrifuged. Neutrophils were washed with PBS and an aliquot of isolated neutrophils was used for determining the total number of neutrophils in a Neubauer's chamber after cell staining (1:20, v/v) with Turk solution (violet crystal 0.2% in acetic acid 30%). The purity of the isolated cell population was determined by Panotic staining of cytospin preparations and by flow cytometry analysis with CD-66b as a granulocyte marker (FACscan). The mean purity achieved by our isolation technique was 98.5% neutrophils.

2.4. Cytotoxic assay

Neutrophils (2×10^6 cells/mL) were suspended in an RPMI culture medium, supplemented with gentamicin (100 µg/mL), L-glutamine (2 mM) and 10% fetal bovine serum. Then the cells were incubated in duplicate in 96-well plates with BbV at concentrations of 1.5, 3, 6, 12.5, 25, 50 e 100 µg/mL or RPMI (control) for 2 and 15 h, at 37 °C in a humid atmosphere (5% CO_2). Next, 10 µL of MTT (5 mg/mL) was added and incubated for 2 h. After centrifugation at 400× *g* for 5 min, the supernatant was removed and 100 µL of DMSO was added to dissolve the crystals that formed. Subsequently, the plates were kept for 18 h at room temperature. The crystals of formazam formed were evaluated in a spectrophotometer at 540 nm. The results were expressed in terms of optical density compared to the control.

2.5. Determination of hydrogen peroxide (H_2O_2) production by human neutrophils

Shortly, neutrophils (2×10^5/50 µL) were resuspended in 1.0 mL of phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 0.56 mM phenol red) containing 0.05 mg/mL of horseradish peroxidase. Then the cells were incubated with BbV at 1.5, 3, 6, 12.5, 25, 50 and 100 µg/mL (experimental group), PMA (positive control group) and RPMI (negative control group) for 90 min at 37 °C in a humid atmosphere (5% CO_2). After this, the reaction was stopped by the addition of 1 M sodium hydroxide (10 µL). The absorbance was measured spectrophotometrically at 620 nm against a blank of phenol red medium. The data generated were compared to a standard curve conducted for each test. The results were expressed as µM of H_2O_2 produced.

2.6. Determination of prostaglandin E_2 (PGE_2) production by human neutrophils

PGE_2 concentration was measured in the supernatant of neutrophils (2×10^5 cells/mL) suspended in RPMI culture medium, supplemented with gentamicin (100 µg/mL), L-

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