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Effects of *Bothrops atrox* venom and two isolated toxins on the human complement system: Modulation of pathways and generation of anaphylatoxins

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ABSTRACT

The complement system plays important biological roles, including the activation of inflammatory processes in response to the generation of proteolytic fragments of its components. Here we evaluated the effects of *Bothrops atrox* venom and two of its toxins (the P-I metalloprotease Batroxase and the acidic phospholipase A_2 BatroxPLA$_2$) on the human complement system, evaluating their effects on the classical (CP), lectin (LP) and alternative (AP) pathways, as well as on different complement components associated to the generation of anaphylatoxins. Primarily, the venom and both toxins modulated the hemolytic activity of the complement CP, with the venom and Batroxase reducing this activity and BatroxPLA$_2$ increasing it. ELISA deposition assays indicated that *B. atrox* venom and Batroxase were also capable of modulating all three activation pathways (CP, LP and AP), reducing their activity after incubation with normal human serum (NHS), while BatroxPLA$_2$ apparently only interfered with AP. Additionally, the venom and Batroxase, but not BatroxPLA$_2$, promoted significant degradation of the components C3, C4, Factor B and C1-Inhibitor, as shown by Western blot and SDS-PAGE analyses, also generating anaphylatoxins C3a, C4a and C5a. Therefore, *B. atrox* venom and Batroxase were able to activate the complement system by direct proteolytic action on several components, generating anaphylatoxins and affecting the activation pathways, while BatroxPLA$_2$ only interfered with the hemolysis induced by CP and the C3 deposition related to AP. Our results indicate that Batroxase and possibly other metalloproteases should be the main toxins in *B. atrox* venom to induce pronounced effects on the complement system.

1. Introduction

Bothrops snakes are the major responsible for snakebite accidents in Brazil, causing envenomations that predominantly induce local effects in the victims, such as edema, pain, hemorrhage and necrosis, but can also lead to systemic effects that include disseminated coagulation and hemorrhage, hypotension and kidney failure (Farsky et al., 1997; Gutiérrez et al., 2005). These snake venoms are composed of complex mixtures of bioactive proteins and peptides, including phospholipases A_2 and metalloproteases, which possess different functions in the characteristic processes of local and systemic injury promoted after envenomations (Chippaux and Goyffon, 1998). Various biological activities of these venoms and toxins have been investigated, including some related to inflammatory responses (Teixeira et al., 2003, 2005). One of the immune response systems that could play important roles after envenomations is the complement system. Its activation by different snake venoms can mediate many different phenomena including hemolysis, cell lysis, chemotaxis and modulation of immune responses with subsequent tissue damage (Pidde-Queiroz et al., 2010; Tanaka et al., 2012).

The human complement system consists of more than 30 plasma and cell surface proteins that interact with each other in a highly regulated manner. This system can be activated by antigen-antibody complexes (classical pathway), by interaction

Abbreviations: MP, metalloprotease/Batroxase; PLA$_2$, phospholipase A$_2$/BatroxPLA$_2$; NHS, normal human serum; CP, classical pathway; LP, lectin pathway; AP, alternative pathway; FB, Factor B; C1-INH, C1-Inhibitor.

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of mannose-binding lectins with carbohydrates on the surface of pathogens (lectin pathway) or by direct activation induced by microorganisms in the absence of antibodies (alternative pathway) (Turner 1996; Carroll, 2004). Its activation initiates a cascade of reactions that lead to the formation of cleavage products and activation complexes with important biological actions, ranging from the initiation of inflammatory processes to the promotion of phagocytosis and lysis of pathogens and altered cells by opsonization and formation of membrane attack complexes (MAC) (Wagner and Frank, 2010).

The complement system plays an important role in several acute and chronic inflammatory reactions as well as in different pathogenic mechanisms of tissue damage associated with autoimmune and ischemic diseases (Kirschfink, 2001; Morgan and Harris, 2003). In many of these situations, the complement system is the main contributor to the initiation, amplification and perpetuation of tissue damage (Morgan and Harris, 2003). Common events to the three pathways include the generation of the anaphylatoxins C3a and C5a, which elicit various proinflammatory effects such as chemotaxis, activation of phagocytes and mast cell degranulation (Walport, 2001). These anaphylatoxins are also commonly associated with the proinflammatory effects induced by *Bothrops* venoms and toxins (Farsky et al., 2000; Pidde-Queiroz et al., 2013).

Snake venom metalloproteases comprise a subfamily of zinc-dependent enzymes that are the main responsible for the hemorrhagic effects characteristic of Crotalidae and Viperidae snake venoms. This class of enzymes is important in the pathogenesis of *Bothrops* envenomations mainly due to its effects on the hemostatic system, but it has also been assigned as responsible for several proinflammatory and anticomplementary effects (Farsky et al., 2000; Teixeira et al., 2005). Snake venom phospholipases A_2 (PLA_2s), on the other hand, constitute a family of enzymes that hydrolyze phospholipids at the sn-2 position in a calcium-dependent manner, and are considered key proteins in the inflammatory processes induced by snake venoms due to their effects on arachidonic acid, the substrate for the biosynthesis of several lipid mediators of inflammation (Teixeira et al., 2003). Nevertheless, the effects of PLA_2s on the complement system are still poorly investigated.

The evaluation of snake venom toxins on the complement system is important in order to validate or contest the use of these molecules as templates for agents with different applications as well as for better understanding the mechanisms of toxicity induced after snakebite envenomations. Thus, the present work aimed to investigate and characterize the actions of *Bothrops atrox* crude venom and two of its toxins (the P-I metalloprotease Batroxase and the acidic phospholipase A_2 BatroxPLA$_2$) on the human complement system, evaluating their effects on the classical (CP), lectin (LP) and alternative (AP) pathways, as well as on different complement components related to the formation of fragments and anaphylatoxins.

2. Materials and methods

2.1. Venom, toxins and other materials

Bothrops atrox venom was acquired from the Center for Extraction of Animal Toxins (CETA, Morungaba, SP). The toxins of interest, Batroxase (MP) and BatroxPLA$_2$ (PLA_2), were isolated and identified as described by Menaldo et al. (2015). Absence of bacterial endotoxins in toxin samples was evaluated using a limulus amebocyte lysate (LAL) quantification kit (Lonza), following the manufacturer's instructions. For that, Batroxase was previously inhibited with EDTA (ethylenediamine tetraacetic acid) for 30 min at 37 °C in order to assure that its proteolytic activity would not interfere in the analysis.

Reagents from Sigma-Aldrich included IgM (I8260), mannan (M7504), lipopolysaccharides (LPS, L4130), zymosan (Z4250), sodium polyanethole sulfonate (SPS, P2008), polyvinyl alcohol (PVA, 363170), bovine serum albumin (BSA, A2153) and o-phenylenediamine dihydrochloride (OPD, P9187). Anti-C3, anti-C4, anti-Factor B and horseradish peroxidase (HRP)-conjugated anti-sheep IgG antibodies were purchased from The Binding Site. Human purified complement components C3, C4 and Factor B (FB) were acquired from Quidel Corp. and C1-Inhibitor (C1-INH) from Genway Biotech Inc. Equipment and other materials used in this study are described throughout the article, and reagents not otherwise specified were of analytical grade.

2.2. Normal human serum (NHS)

NHS, used as a source of complement components, was obtained from blood of healthy volunteers of both sexes, aged 20 to 40 years, collected in the absence of anticoagulants. The blood was allowed to clot at room temperature for 1 h, and the serum was separated from the clot by centrifugation at 500 × *g* for 10 min at 4 °C. Then, serum was pooled, aliquoted and frozen at −80 °C until the experiments. All procedures involving humans were approved by the Research Ethics Committee of FCFRP-USP (CAAE n° 14762913.7.0000.5403) and were performed in accordance with ethical standards. Blood-donors gave their informed consent prior to the inclusion of their blood samples in this study.

2.3. Hemolytic assays

Preparation of erythrocyte suspensions from sheep blood (obtained from Boa Vista Bioterium, Valinhos-SP) and subsequent hemolytic assays were performed as previously described (Menaldo et al., 2013), using 96-well microplates and a kinetic method.

Initially, samples of *B. atrox* venom and toxins were evaluated with sheep erythrocytes in the absence of NHS to guarantee that they did not induce direct hemolysis. After that, different concentrations of *B. atrox* venom, Batroxase, BatroxPLA$_2$ and combinations of both toxins (MP + PLA_2) were prepared in TEA-Ca^{2+}-Mg^{2+} buffer and placed into wells with NHS, followed by incubation at 37 °C for 1 h. NHS diluted with buffer in the absence of samples was employed as negative control.

After the incubation period, hemolysin-sensitized sheep erythrocytes were added to each well and the absorbance at 700 nm was assayed for 15 min in a microplate reader (SpectraMax Plus, Molecular Devices). Residual CP activity was evaluated by t½ values (in s), which correspond to the time required so that the absorbance of the reaction mixtures is reduced by half as a result of erythrocyte lysis related to CP activation.

Hemolytic assays were also carried out using C1q-depleted human serum (Quidel Corp.) or human serum inactivated at 56 °C for 30 min (Kochi and Johnson, 1988), in order to assure that the hemolysis observed was in fact related to the CP of the complement system. For that, these serum samples were incubated at 37 °C for 1 h with *B. atrox* venom (50 μg/mL), Batroxase (20 μg/mL) or BatroxPLA$_2$ (50 μg/mL), followed by addition of sensitized erythrocytes and absorbance analysis, as described above.

2.4. Residual activity of complement pathways determined by enzymatic immunoassays

The determination of the residual activity of CP, LP and AP after incubation of NHS with *B. atrox* venom and toxins was standardized according to methods described by Petersen et al. (2001), Roos et al. (2003) and Pidde-Queiroz et al. (2013), which are based on the