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The lethality test used for estimating the potency of antivenoms against *Bothrops asper* snake venom: Pathophysiological mechanisms, prophylactic analgesia, and a surrogate *in vitro* assay

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ABSTRACT

The potency of antivenoms is assessed by analyzing the neutralization of venom-induced lethality, and is expressed as the Median Effective Dose (ED_{50}). The present study was designed to investigate the pathophysiological mechanisms responsible for lethality induced by the venom of *Bothrops asper*, in the experimental conditions used for the evaluation of the neutralizing potency of antivenoms. Mice injected with 4 LD_{50}s of venom by the intraperitoneal route died within ~25 min with drastic alterations in the abdominal organs, characterized by hemorrhage, increment in plasma extravasation, and hemoconcentration, thus leading to hypovolemia and cardiovascular collapse. Snake venom metalloproteinases (SVMPs) play a predominat role in lethality, as judged by partial inhibition by the chelating agent $CaNa_2EDTA$. When venom was mixed with antivenom, there was a venom/antivenom ratio at which hemorrhage was significantly reduced, but mice died at later time intervals with evident hemoconcentration, indicating that other components in addition to SVMPs also contribute to plasma extravasation and lethality. Pretreatment with the analgesic tramadol did not affect the outcome of the neutralization test, thus suggesting that prophylactic (precautionary) analgesia can be introduced in this assay. Neutralization of lethality in mice correlated with neutralization of *in vitro* coagulant activity in human plasma.

1. Introduction

The most traditional way to assess the overall toxicity of venoms or toxins is based on the study of their capacity to induce death in experimental animals, which is performed by the estimation of the Median Lethal Dose (LD_{50}). For this, various doses of venom, or toxin, are injected in animals, usually mice, by intravenous (i.v.), intraperitoneal (i.p.), or subcutaneous (s.c.) routes. Deaths occurring during a defined time span, usually 24 or 48 h, are recorded, and LD_{50} is estimated by using appropriate statistical methods such as probits, Spearman-Karber or non-parametric tests (WHO, 1981, 2010). On the other hand, the gold standard in the assessment of the preclinical efficacy of antivenoms or antitoxins is the analysis of the neutralization of lethality. In this case, a fixed amount of venom, or toxin, is incubated with various dilutions of antivenom. Then, aliquots of the mixtures, containing a 'challenge dose' of venom, i.e. a number of LD_{50}s (usually 3–6), are injected in experimental animals and lethality is recorded. Neutralization is generally expressed as the Median Effective Dose (ED_{50}), defined as the venom/antivenom ratio at which 50% of the injected animals survive (WHO, 2010).

Despite the widespread use of these experimental protocols in the analysis of venom toxicity and in the assessment of the neutralizing potency of antivenoms, in many cases the lethality test remains a 'black box', since the main pathological and pathophysiological mechanisms involved in lethality in these assays have not been explored in detail. In the case of predominantly neurotoxic venoms, such as those of the majority of elapid snake species, neuromuscular paralysis leading to respiratory arrest is the mechanism of death. In contrast, in the case of viperid snake venoms, which induce a complex pattern of systemic pathophysiological effects, the study of the mechanisms of death in experimental models has received little attention (Gutiérrez et al., 2013). This is relevant for understanding the test itself and the neutralizing ability of antivenoms, i.e. which systemic venom effects are being abrogated by antivenoms in the experimental conditions in which these tests are performed.

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In Central America, a polyspecific antivenom ('suero antiofídico polivalente') is used in the treatment of envenomings caused by viperid snakes (Bolaños and Cerdas, 1980; Gutiérrez, 2010). It is produced by immunizing horses with a mixture of the venoms of *Bothrops asper*, *Crotalus simus* and *Lachesis stenophrys* (Bolaños and Cerdas, 1980; Angulo et al., 1997). The quality control of this antivenom includes a test for the neutralization of lethality induced by these venoms in mice, using the i.p. route and a challenge dose of venom corresponding to 4 LD_{50}s. The design and analytical properties of this test have been previously characterized (Solano et al., 2010). The present investigation was designed to study the pathological and pathophysiological alterations occurring in mice by this dose of *B. asper* venom, in order to understand the mechanisms of lethality induced by venom alone and by mixtures of venom and antivenom, as applied to the lethality neutralization assay. In addition, an *in vitro* test for the study of neutralization of venom by this antivenom was investigated, with the aim of finding a surrogate laboratory alternative that could replace the mouse lethality test and, consequently, reduce the number of animals used in the quality control of this antivenom. Finally, the introduction of precautionary (prophylactic) analgesia in this test, based on the administration of tramadol, was analyzed, as an attempt to decrease the suffering and distress induced in mice in this acute toxicity assay.

2. Materials and methods

2.1. Venom and antivenom

A pool of venom was prepared from more than 40 adult specimens of *B. asper* collected in the Pacific versant of Costa Rica and maintained at the serpentarium of Instituto Clodomiro Picado. Once obtained, venom was freeze-dried and stored at −20 °C. The polyspecific ('polyvalent') antivenom ('suero antiofídico polivalente', batch 5361213POLQ), manufactured at Instituto Clodomiro Picado, was used. In addition, the following batches were utilized for the study of the correlation between neutralization of lethality and coagulant effect: 4030906POLQ, 4051106POLQ, 4061106POLQ, 4070107POLQ, 4120507POLQ, 4180707POLQ, 4190807POLQ, and 4250208POLQ. In some cases, in-process samples of two batches of this antivenom were also used. Polyvalent antivenom is prepared by fractionating the plasma of horses immunized with a mixture of venoms of *B. asper*, *C. simus* and *L. stenophrys*. It is composed of whole IgG molecules purified by caprylic acid precipitation of non-immunoglobulin plasma proteins (Rojas et al., 1994).

2.2. Animals

CD-1 mice of 16–18 g were used throughout the study. All experiments involving mice were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of Universidad de Costa Rica.

2.3. Pathological and pathophysiological alterations in mice receiving a challenge dose of 4 LD_{50}s of venom

The LD_{50} of *B. asper* venom in mice, using the i.p. route as routinely performed in the Quality Control Laboratory of Instituto Clodomiro Picado, is 62.5 μg/16–18 g mice. For the study on the mechanisms of lethality, groups of mice (16–18 g) were injected i.p. with 250 μg venom (corresponding to 4 LD_{50}s), dissolved in 0.5 mL of 0.14 M NaCl, 0.04 M phosphate, pH 7.2 (PBS). The time of death of mice was recorded, and the pathological and pathophysiological alterations at the time of death were analyzed, as described below.

2.3.1. Macroscopic observations and histological analysis

Immediately after death, the peritoneal cavity of mice was opened and observed for macroscopic alterations. Then, samples of the following organs were collected: mesentery, small intestine, large intestine, liver, kidneys, diaphragm, lungs, heart and brain. Tissue samples were immediately placed in 10% formalin fixative solution, and were processed routinely for embedding in paraffin. Then, 4 μm sections were collected, placed in slides and stained with hematoxylin and eosin for microscopic observation. Control mice injected with 0.5 mL of PBS were sacrificed by an overdose of ketamine and xylazine at approximately 20 min after injection, which corresponds to the time of death of mice receiving venom. Upon sacrifice, samples of the various organs were obtained and processed as described.

2.3.2. Quantification of peritoneal hemorrhage and changes in hematocrit, coagulation time and plasma creatine kinase (CK) activity

Groups of mice were injected with 4 LD_{50}s of venom, as described. Twenty min after injection, animals were sacrificed by CO_2 inhalation. Immediately afterwards, 1.0 mL of PBS was injected in the peritoneal cavity and, after a mild massage, an incision was made in the abdominal wall and a sample of peritoneal fluid was collected from this cavity. Samples were diluted 1:2 with PBS-1% Triton X-100 to induce erythrocyte lysis. After centrifugation for 5 min at 1000 g, the absorbance of the supernatant at 540 nm was recorded as a quantitative estimation of the amount of hemoglobin present in the lavage fluid, which correlates with the extent of hemorrhage. In another group of mice injected with 4 LD_{50}s of venom, a blood sample was collected by cardiac puncture, under anesthesia, 20 min after injection. Blood was immediately placed in heparinized microcapillary tubes and centrifuged for estimating the hematocrit. Another blood sample was placed in dry glass tubes and allowed to stand at room temperature, and clotting times were recorded. In parallel, the creatine kinase (CK) activity of plasma, obtained by centrifugation of blood collected in heparinized microcapillaries, was quantified as an index of myonecrosis (Gutiérrez et al., 1980), using a commercial kit (CK LIQUI-UV, Stanbio Lab., Texas, USA). In all cases, a control group of mice was injected with 0.5 mL of PBS, and analyses were performed as described. In order to identify venom components responsible for hemoconcentration, similar experiments were performed by injecting i.p. a purified PI metalloproteinase (SVMP) (BaP1) (Gutiérrez et al., 1995) and a fraction containing a mixture of myotoxic Asp49 phospholipase A_2 (PLA_2) and Lys49 PLA_2 homologue, isolated from the venom of *B. asper* by ion-exchange chromatography on CM-Sepharose, using a KCl gradient (Lomonte and Gutiérrez, 1989). The amounts injected were 75 μg of BaP1 and 100 μg of PLA_2 myotoxin fraction, which roughly correspond to the amounts of PI SVMPs and myotoxic Asp49 and Lys49 PLA_2s present in 250 μg of *B. asper* venom, on the basis of the proteomic analysis of the venom of adult specimens from the Pacific population of Costa Rica (Alape-Girón et al., 2008).

2.3.3. Quantification of increase in vascular permeability in the peritoneal cavity

Groups of mice were injected with 250 μg venom dissolved in 0.5 mL PBS, as described. Immediately before venom injection, mice received an i.v. injection of 200 μL of a solution of 2 mg/mL Evans Blue (Sigma–Aldrich, Missouri, USA), dissolved in PBS. Twenty min after venom administration, mice were sacrificed by CO_2 inhalation, and 1.0 mL of PBS was injected in the peritoneal cavity. After a mild massage, an incision was performed in the abdominal wall and a sample of peritoneal fluid was collected. Samples were diluted 1:4 with PBS and, after 5 min centrifugation at 1000 g, the absorbance