

Differential modulation of cell recruitment and acute edema in a model of *Polybia paulista* venom-induced inflammation

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Abstract

Hymenoptera stings are quite common and can cause inflammatory reactions (in nonallergic individuals) or serious reactions (in allergic individuals). Hymenoptera venom contains histamine, vasoactive kinins, serotonin, phospholipase A, phospholipase B, hyaluronidase, antigen 5 and mastoparans. Some of these substances are responsible for local pain, as well as for activation of complement and endothelial cells. *Polybia paulista* is a wasp typically found in the state of São Paulo, Brazil. In the present study, we evaluated inflammatory reactions in the peritoneal cavities of rats injected with *P. paulista* venom (PPV). We evaluated leukocyte recruitment and edema formation at the site of inflammation. After i.p. inoculation with PPV, there was dose-dependent and time-dependent recruitment of neutrophils, eosinophils and mononuclear cells. At 4 to 48 h after stimulus, administration of MK 886, a leukotriene synthesis inhibitor, completely abolished granulocyte recruitment to the peritoneal cavity. Therefore, leukotrienes seem to be the primary mediators of PPV-induced neutrophil and eosinophil recruitment. Inoculation with PPV also induced protein extravasation into the peritoneal cavity. This phenomenon was not inhibited by treatment with MK 886 or indomethacin (a prostaglandin synthesis inhibitor), demonstrating that neither leukotrienes nor prostaglandins are involved in this inflammatory reaction. However, edema formation was significantly inhibited by treatment with pyrilamine, indicating that the histamine H₁ receptor plays a critical role in PPV-induced formation of acute edema. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

Hymenoptera venoms are mixtures of various biochemical and pharmacologically active components such as proteins, small peptides and vasoactive amines.

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Venoms of the Hymenoptera family Vespidae (wasps) contain the phospholipases A and B, as well as hyaluronidases, acid phosphatases, proteases and mastoparans [1,2]. Exposure to wasp venom occurs through a sting. Some venom components are allergens [3], whereas others produce pain responses at the site of the sting [4]. In nonallergic individuals, who constitute the majority of the population, the sting site can be easily identified by the cutaneous reaction [5]. The local nonallergic reaction begins immediately after the sting in response to toxic venom components, such as phospholipase A₂ and mastoparans [2], that induce basophil degranulation, mast cell degranulation and secretion of IL-4 in the absence of specific IgE antibody [6], as well as complement and endothelial cell activation, resulting in edema formation [7–9]. Multiple stings from these insects can cause a reaction similar to systemic allergic or late-phase reactions followed by intravascular hemolysis or acute renal failure [10].

To date, there have been few studies evaluating the acute inflammatory response to insect stings in non-allergic subjects. In a study involving nonallergic individuals, an extensive inflammatory skin reaction to ant stings was described [11]. The authors found that the sting induced vasodilation followed by recruitment of lymphocytes, monocytes and neutrophils. The same authors showed that, by 24 h after papule formation, there was intense lymphocytic and polymorphonuclear infiltration and that, in some subjects, the papule contained eosinophils. Insect venoms are known to be strong inflammatory agents and some can induce the release of arachidonic acid from granulocyte and macrophage membranes. Leukotrienes and prostaglandins are products formed by the oxidation of arachidonic acid via lipoxygenase and cyclooxygenase pathways, respectively [12]. Leukotriene B₄ (LTB₄) is a potent inducer of leukocyte functions, including chemotaxis, chemokinesis and polymorphonuclear aggregation [13]. In addition, LTB₄, LTC₄, LTD₄ and LTE₄ are chemoattractants for eosinophils [13–16]. Furthermore, LTC₄ and LTD₄ induce wheal and flare responses [17,18] while production of prostaglandin E₂, a potent vasodilator, is enhanced in inflammatory reactions, causing edema, erythema and pain [12,19].

The various Vespidae venoms are similar in composition. However, there is little biological data regarding Polistinae venoms, especially regarding

that of the social wasp *Polybia paulista*, typically found in the state of São Paulo, Brazil. In view of these facts, the present study was carried out in order to describe the pattern of local release of acute inflammatory mediators after *P. paulista* venom (PPV) injection and to study the mechanisms that mediate such release in a rat model of PPV-induced tissue injury. We demonstrated leukocyte recruitment to the peritoneal cavity and acute edema formation.

2. Materials and methods

2.1. Animals

Outbred male Wistar rats weighing 180–200 g were obtained from the animal facilities of the *Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo* (University of São Paulo at Ribeirão Preto School of Pharmaceutical Sciences). The animals received food and water ad libitum and were maintained in a temperature-controlled environment (25 °C). All experiments were approved by, and conducted in accordance with, guidelines established by the Animal Care Committee of the institution.

2.2. PPV extraction

P. paulista workers were captured on the university campus, which is located in the city of Ribeirão Preto, in the state of São Paulo, Brazil. The freshly collected wasps were immediately frozen and dissected. In order to minimize contamination from extraneous tissues, the venom sacs were removed from the sting apparatus under a stereomicroscope, using forceps and microscissors. The venom sacs were carefully washed in a small volume of isotonic solution, then thawed and punctured. This was followed by several washings with distilled water to extract the venom, which was then centrifuged at 12,000 ×g for 15 min at 4 °C. The supernatant was freeze-dried and stored at –80 °C until use. Protein concentrations in venom solutions were determined by the Lowry method [20], using bovine serum albumin as the standard. The solution containing PPV was filtered through a 0.2 µm filter (Sartorius, Goettingen, Germany).

2.3. Dose–response curve of leukocyte recruitment induced by PPV injection into the peritoneal cavity

Rats were injected i.p. with 0.22, 2.2 or 22 µg/cavity of PPV in 1 mL of phosphate-buffered saline (PBS). Control group animals received PBS only. At 24 h after PPV injection, the animals were killed with an overdose of sodium

pentobarbitone. Cells were harvested from the peritoneal cavities by injecting 5 mL of PBS containing 0.5% of sodium citrate. The abdomens were gently massaged and the blood-free cell suspension carefully withdrawn with a syringe. Abdominal lavage fluid was placed in plastic tubes and total cell counts were performed in a Neubauer chamber. Cell counts were performed in blinded fashion by an independent observer unaware of the identity of the samples. Differential counts were obtained using Rosenfeld-stained cytopsin preparations [14].

2.4. Time course of leukocyte recruitment induced by PPV injection into the peritoneal cavity

Inflammatory cell recruitment into the peritoneal cavity was determined at 4, 24 and 48 h after PPV injection. Rats were injected i.p. with 22 µg of PPV in 1 mL of PBS and control group rats received PBS only. Subsequently, the animals were killed and the cell numbers determined as described above.

2.5. Evaluation of peritoneal cavity edema

Inflammation was induced in the peritoneal cavities by i.p. injection of 22 µg of PPV in 1 mL of PBS. Control group animals were injected with the same volume of PBS or were not injected (naïve controls). At 4, 24 and 48 h after injection, the animals were killed, abdominal lavage fluid was collected as described above and centrifuged at $3000 \times g$ for 10 min at 4 °C. The supernatant was freeze-dried and stored at –80 °C until use. Protein extravasation was determined by Coomassie Protein Assay Reagent Kit (PIERCE, Rockford, IL, USA) and standard curves for albumin were prepared according to manufacturer instructions.

2.6. Treatment with MK 886, indomethacin, cimetidine and pyrilamine

At 1 h before PPV injection, animals were treated with MK 886 (1, 3 or 10 mg/kg, p.o.), a leukotriene biosynthesis inhibitor. To evaluate the role of prostaglandins, animals were treated with indomethacin (2 mg/kg, p.o.). Histamine receptor antagonists, cimetidine (10 mg/kg, i.p.) or pyrilamine (10 mg/kg, i.p.) were employed to evaluate the edema formation. Control group animals received water p.o. and were i.p. injected with PPV (22 µg) or PBS. At 30 min after PPV injection, the animals were killed as previously described.

2.7. Materials

MK 886 (L-663,536 (3-[1*p*-chlorobenzyl]-5-isopropyl-3-*tert*-butylthioindo-2-yl]-2,2-dimethylpropanoic acid)) was a

generous gift from Merck Frosst, Canada, Inc. Indomethacin, pyrilamine and cimetidine were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.8. Statistical analysis

The experiments were performed in duplicate, and the data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis of the differences between groups was performed using the Mann–Whitney *U*-test and analysis of variance (ANOVA) for nonparametric data. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Dose–response curve of leukocyte recruitment induced by PPV

In a preliminary set of experiments, we assessed the dose dependence of PPV on leukocyte recruitment into the peritoneal cavity. The dose–response curve of leukocyte recruitment evaluated at 24 h after injection is shown in Fig. 1. Compared to control animals (receiving PBS only), animals receiving 0.2 and 2.2 µg doses of PPV did not present significant cell recruitment into the peritoneal cavity. However, the 22 µg PPV dose induced significant ($p < 0.05$) recruitment of neutrophils, eosinophils and mononuclear cells to the peritoneal cavity. Following analysis of the effect that the various PPV concentrations had on the recruitment of leukocytes to the peritoneal cavity, subsequent experiments were conducted using 22 µg/cavity of PPV in 1 mL of PBS, a dose equivalent to the quantity of proteins produced by a single venom gland.

3.2. Time course of leukocyte recruitment induced by PPV

The time course of PPV-induced leukocyte recruitment to the peritoneal cavity at 4, 24 and 48 h after injection is shown in Fig. 2. Compared to PBS alone (control), PPV injection induced significant ($p < 0.05$) neutrophil recruitment to the peritoneal cavity at all time points analyzed, peaking at 4 h after injection and progressively decreasing by 24 and 48 h after injection (Fig. 2A). Administration of PPV also induced significant ($p < 0.05$) eosinophil recruitment to the peritoneal cavity at all time points analyzed, peaking at 24 h after injection and remaining elevated at 48 h after injection (Fig. 2B). In addition, PPV-induced mononuclear cell recruitment was similar at all time points analyzed and was significantly higher ($p < 0.05$) than that seen in control group animals (Fig. 2C). Furthermore, mast

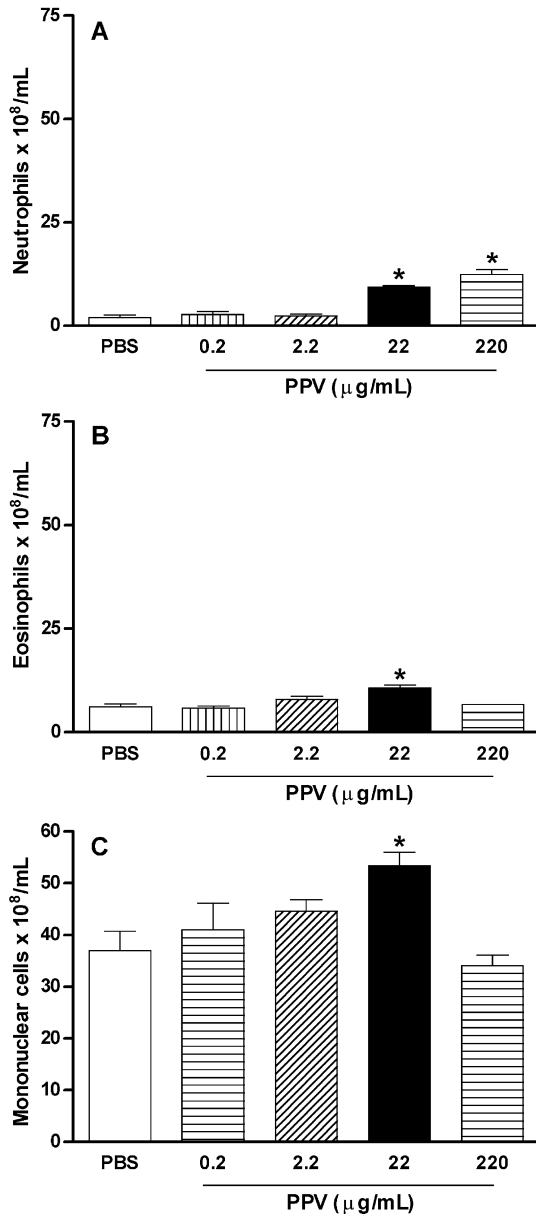


Fig. 1. Dose–response curve of neutrophil, eosinophil and mononuclear cell recruitment induced by i.p. injection of 0.22, 2.2 and 22 $\mu\text{g}/\text{cavity}$ of PPV in 1 mL of PBS. Control groups were injected with 1 mL of PBS. Cell recruitment was evaluated at 24 h after injection. Values represent the mean $\pm$ SEM ($n=12$) obtained in two independent experiments. * $p<0.05$ in comparison with controls.

cell recruitment was also significantly greater in animals receiving PPV than in control group animals (data not shown).

3.3. Effect of MK 886 on leukocyte recruitment induced by PPV

The 1 and 3 mg/kg doses of MK 886 significantly ($p<0.05$) inhibited PPV-induced neutrophil (Fig. 3A) and eosinophil (Fig. 3B) recruitment to the peritoneal cavity in a dose-dependent manner. However, PPV-induced mononuc-

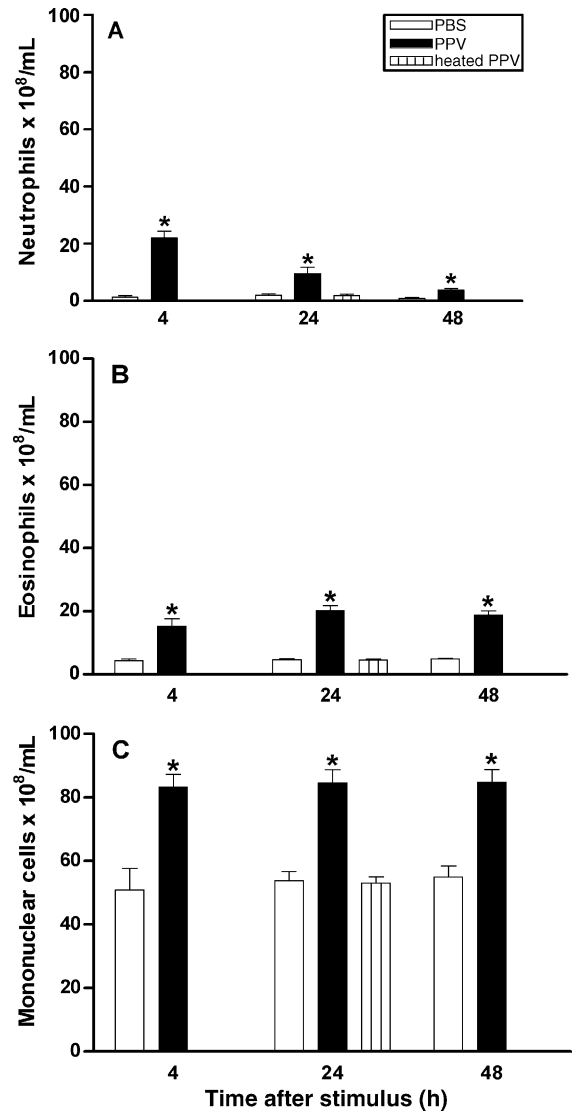


Fig. 2. Time-course curve of neutrophil, eosinophil and mononuclear cell recruitment induced by i.p. injection of 22 μg of PPV in 1 mL of PBS. Cell recruitment was evaluated at 4, 24 and 48 h after PPV injection. Controls were injected with 1 mL of PBS. Values represent the mean $\pm$ SEM ($n=12$) obtained in two independent experiments at each time. * $p<0.05$ in comparison with controls.

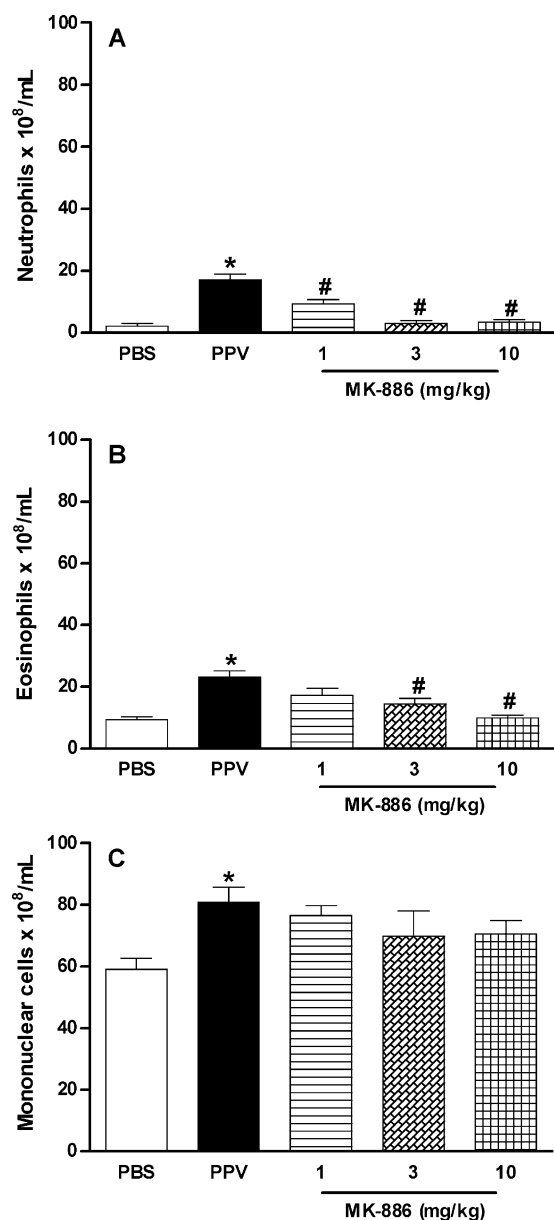


Fig. 3. Dose-response curve of MK-886 effect on neutrophil, eosinophil and mononuclear cell recruitment. Rats were injected i.p. of 22 μ g of PPV. MK 886 was given p.o. at 1 h before stimulus. Values represent the mean $\pm$ SEM ($n=12$) obtained in two different experiments at 24 h after injection. Controls were injected with 1 mL of PBS. * $p<0.05$ in comparison with controls; # $p<0.05$ in comparison with the PPV group.

leukocyte cell recruitment was unaffected by treatment with MK 886 (Fig. 3C). In addition, PPV-induced cell recruitment to the peritoneal cavity in animals treated with MK 886 was

similar to that induced by PBS alone. Since no significant differences in inhibition of cell recruitment were observed between animals receiving 3 mg/kg of MK 886 and those receiving 10 mg/kg of MK 886, the 3 mg/kg dose was employed in the subsequent experiments. The time-course

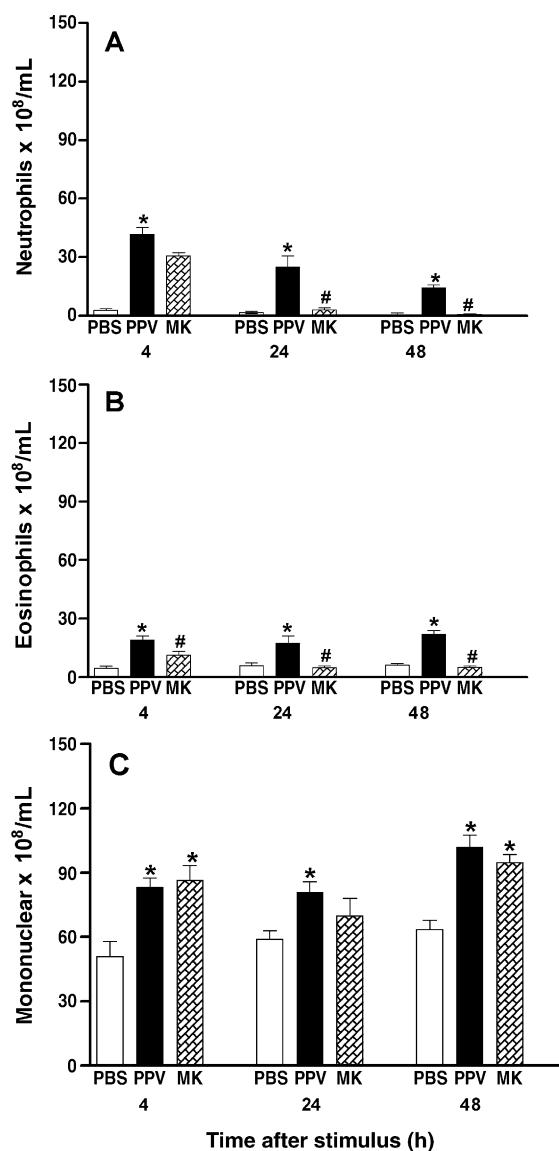


Fig. 4. Effect of pretreatment with 3 mg/kg of MK886 on the time course of neutrophil, eosinophil and mononuclear cell recruitment. Cell recruitment was evaluated at 4, 24 and 48 h after PPV injection. Controls were injected with 1 mL of PBS. Values represent the mean $\pm$ SEM ($n=12$) obtained in two different experiments conducted at each time point. * $p<0.05$ in comparison with controls; # $p<0.05$ in comparison with the PPV group.

effect of MK 886 treatment on PPV-induced cell recruitment is shown in Fig. 4. Although MK 886 was effective in inhibiting recruitment of neutrophils (Fig. 4A) and eosinophils (Fig. 4B) at all time points analyzed, this effect was partial at 4 h after PPV injection, peaking at 24 h after PPV injection and remaining elevated at 48 h after injection. Similarly to what was found in the dose-dependence experiment (Fig. 3C), MK 886 did not inhibit mononuclear cell recruitment at any of the time points analyzed (Fig. 4C).

3.4. Evaluation of PPV-induced edema

The edema formation induced by PPV injection was assessed by determining the degree of protein extravasation

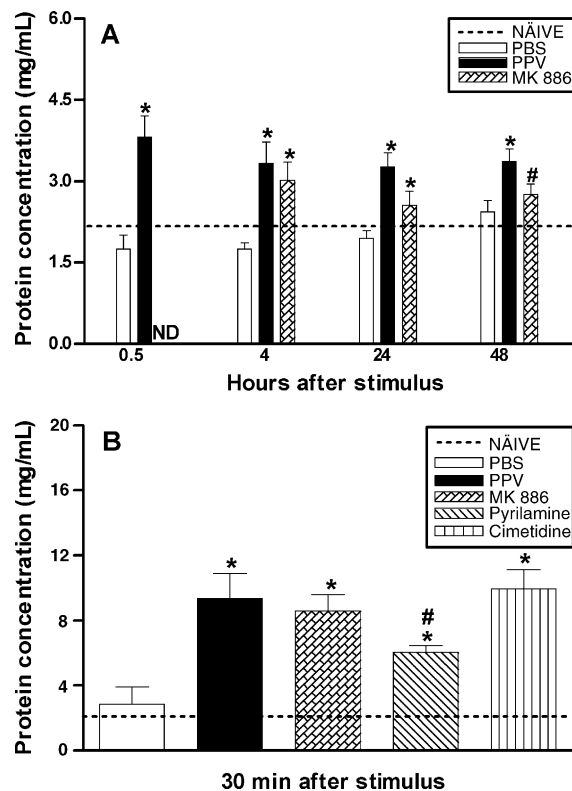


Fig. 5. Effect of pretreatment with MK 886, pyrilamine and cimetidine on the acute edema induced by PPV. Local edema was quantified by measuring the protein extravasation in peritoneal washings at 0.5, 4, 24 and 48 h (A) or only at 0.5 h (B) after PPV injection. Rats were treated with MK 886 (3 mg/kg), pyrilamine (10 mg/kg) and cimetidine (10 mg/kg) at 1 h prior to injection with 22 μ g of PPV. Controls were injected with 1 mL of PBS. Values represent the mean $\pm$ SEM ($n = 12$) obtained in two different experiments conducted at each time point. * $p < 0.05$ in comparison with controls; # $p < 0.05$ in comparison with the PPV group. Dotted line represents naïve rats (unstimulated). ND: not determined.

into the peritoneal cavity of PPV-injected animals in comparison to that seen in those injected with PBS alone or in those not stimulated (naïve). Intracavity edema formation was observed at 30 min after PPV injection and, when compared with the other groups, the edema remained significant until 48 h after injection. In mice treated with MK 886 and subsequently injected with PPV, no edema formation was observed at 4 and 24 h after injection, although slight edema was seen at 48 h (Fig. 5A). Since no differences in protein extravasation were observed between the groups at 7, 15 or 30 days after injection (data not shown), the PPV-induced edema was classified as acute.

To evaluate the roles of other mediators in PPV-induced edema formation at 30 min after injection, animals were treated with drugs of various types. At this time point, protein extravasation into the peritoneal cavity was not reduced by administration of either MK 886 (Fig. 5B) or indomethacin (data not shown). At the same time point, PPV-induced edema was also unaffected by administration of an H_2 histamine receptor antagonist (cimetidine — 10 mg/kg). In contrast, the H_1 histamine receptor antagonist pyrilamine (10 mg/kg) significantly inhibited ($p < 0.05$) PPV-induced protein extravasation into the peritoneal cavity.

4. Discussion

Our results demonstrate the inflammatory effect of PPV, which induced cell recruitment, protein extravasation and edema formation when injected into the peritoneal cavities of normal rats. In models of inflammation induced by the venoms of various species, leukocyte recruitment [21–23], protein extravasation and edema formation [24–26] have also been observed.

In this study, we demonstrated that PPV induces acute recruitment of neutrophils, eosinophils and mononuclear cells to the peritoneal cavity. The group of four phospholipases A_2 (PLA $_2$ s) found in PPV has been designated polybitoxins [27]. It has been shown that PLA $_2$ s release arachidonic acid from the cellular and nuclear membranes, leading to formation of eicosanoids such as leukotrienes, which contribute to increased vascular permeability, edema formation and leukocyte recruitment [28–30]. In addition, preformed leukotrienes are found in wasp venoms [31]. Such leukotrienes are inflammatory mediators and have been shown to recruit leukocytes, such as neutrophils and eosinophils, in various animal

and human models [13–15,32,33]. In a previous study, we demonstrated that MK 886 inhibited recruitment of neutrophils and eosinophils to the peritoneal cavity after administration of the alkali-insoluble fraction 1 from the cell wall of *Histoplasma capsulatum*, a dimorphic pathogenic fungus [34]. In the present study, treatment with MK 886 resulted in dose-dependent inhibition of neutrophil and eosinophil recruitment, although no effect on mononuclear cell recruitment was observed (Fig. 2). Leukotrienes synthesized after PPV injection seem to be principally involved in late cell recruitment since MK 886 had only a partial effect on the recruitment of these cells at 4 h after PPV injection (Fig. 4). However, we cannot rule out the possibility that preformed leukotrienes present in the PPV were responsible for the early neutrophil and eosinophil recruitment. This hypothesis is reinforced by the fact that leukotrienes have been shown to be components of both bee and wasp venoms [31].

We have also shown that PPV is a potent edematogenic factor that increases protein extravasation into the peritoneal cavity at 30 min after injection. This acute increase in protein extravasation after PPV injection suggests early release of edematogenic mediators, which has been demonstrated to occur in response to *Bothrops* venoms [9]. Our data are also in agreement with those described by Eno [35], who showed that edematogenic mediators are released after inoculation of *Polistes fuscatus* venom in rat paws. The fact that neither MK 886 nor indomethacin had any effect on PPV-induced edema formation suggests that neither leukotrienes nor prostaglandins play a role in this phenomenon. Similarly, cimetidine did not block edema formation in this model. However, pyrilamine partially blocked PPV-induced edema formation, demonstrating the role of histamine in this acute reaction. Previously published works have shown that wasp and snake venoms contain not only histamine [3,36] but also serotonin and prostaglandin E_2 [37]. Although the participation of these other mediators was not tested herein, they likely participate in the PPV-induced increase in vascular permeability and, together with histamine, induce edema formation.

In conclusion, this study highlights some interesting aspects of the inflammatory reaction induced by PPV in normal animals and of the molecules that participate in the inflammatory effect of PPV. First,

we demonstrated that PPV causes acute inflammation, characterized by recruitment of neutrophils and eosinophils, as well as by edema formation. Treatment with MK 886 differentially blocked PPV-induced neutrophil and eosinophil recruitment, suggesting that early recruitment (4 h after injection) is partially dependent upon leukotrienes, and that the late phase is totally leukotriene-dependent. In addition, we showed that leukotrienes do not participate in the onset of edema formation, but that histamine does, and that an H_1 histamine receptor-dependent mechanism is involved. These results contribute to the understanding of the local reaction induced by PPV and by other wasp venoms in normal nonallergic humans. A better understanding of the inflammatory events following envenomation could lead to the development of new therapeutic strategies that would complement conventional therapy in the future treatment of wasp stings.

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