

Characterisation of local inflammatory response induced by *Thalassophryne nattereri* fish venom in a mouse model of tissue injury

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Received 22 April 2003; accepted 23 July 2003

Abstract

The *Thalassophryne nattereri* fish venom induces a severe burning pain, oedema, and necrosis observed both clinically and experimentally. The present study was carried out in order to describe the pattern of local acute inflammatory response after *T. nattereri* venom injection. Our findings show that the edematogenic response induced by *T. nattereri* venom in footpad of mice was dose- and time dependent, and remained significantly elevated over 48 h after injection. Analysis of footpad homogenates were tested for the presence of TNF- α , IL-1 β and IL-6, and demonstrated augmented levels of these cytokines. Our results showed that the injection of venom developed an inadequate cellular inflammatory response evidenced by poor infiltration of mononuclear cells, preceded by decreased number of these cells in peripheral blood. In contrast, we observed an early intense recruitment of neutrophil to peritoneal cavity, accompanied by a significant decrease in the number of mononuclear cells. A drastic increase in the total amount of cells, mainly in neutrophils, followed by mononuclear cell recruitment was observed 24 h. In addition, we also demonstrated that *T. nattereri* venom affects the viability of mononuclear cells (J774A1) in culture. We conclude that the scarcity of inflammatory cellular influx into local lesions (intraplantar) induced by *T. nattereri* venom could be a consequence of an impaired blood flow in venules at injured tissue and cytotoxic effect of the venom on inflammatory cells can contribute to this impairment.

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Keywords: *Thalassophryne nattereri*; Oedema; Mononuclear cells; Blood supply; Cytotoxicity

1. Introduction

Approximately 200 species of marine fish, included in the *Scorpaenidae*, *Batrachoididae*, *Tetraodontidae*, and *Dasyatidae* families (scorpionfish, stonefish, weeverfish, and toadfish) are known to be venomous. The Brazilian venomous fish *Thalassophryne nattereri* (*Batrachoididae*)

is common on the shores of north-eastern areas, where it provoke a great number of accidents (Almeida and Rocha, 1989). Venom is delivered when the spine pierces the tissue of the victim, the integumentary sheath enclosing the spine and venom disrupted, and the venom injected into the victim. Envenomation by this fish is associated with severe burning pain, oedema, and necrosis observed both clinically (Auto, 1992) and experimentally (Lopes-Ferreira et al., 1998a).

Preliminary experimental observations in mice indicate that *T. nattereri* venom induces acute myonecrosis with histological features distinct from those characterising myonecrosis caused by myotoxins isolated from snake

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venoms. Pronounced alterations on microvascular hemodynamics, represented by fibrin depots and thrombus formation followed by complete venular and transient arteriolar stasis were visualised by intravital microscopy after venom application in mice. In ultra-structural studies, it was observed low numbers of phagocyte cells during the first 24 h, and the presence of necrotic material, which had not been cleared out seven days after envenomation (Lopes-Ferreira et al., 2001, 2002).

The immediate treatment for the fish toxin accidents has been to place the wound region in hot water, or administration of local anaesthetics and analgesics, resulting in slight decrease of the symptoms of the envenomation (Sutherland, 1992). The anti-inflammatory drugs used (dexamethasone and indomethacin) are not efficient in reducing the clinical symptoms. In the absence of an appropriate treatment, it is common the occurrence of concomitant bacterial infection and many cases of local lesion evolve to permanent sequela (Fonseca and Lopes-Ferreira, 2000). Using an experimental mouse model that reproduce human accidents, we showed that venom-induced nociception and oedema were not reduced neither by treatment with inhibitors of serotonin and histamine nor by non-steroidal and steroidal anti-inflammatory drugs, but by the administration of kallikrein specific inhibitor (Lopes-Ferreira et al., 1998b).

In the view of these facts, the present study was carried out in order to describe the pattern of local acute inflammatory response after *T. nattereri* venom injection, including the leukocyte recruitment into local tissue from the peripheral blood in a mouse model of tissue injury. A better understanding of the inflammatory events following envenomation could lead us to the development of new therapeutic strategies complementary to conventional therapy.

2. Materials and methods

2.1. Animals

Groups of five Swiss mice (18–22 g) of both sexes obtained from a colony at Institute Butantan (São Paulo, Brazil) were maintained at the animal house facilities of the Laboratory of Immunopathology, under specific pathogen-free conditions. All experiments were approved by the Experimental Animals Committee of Butantan Institute in accordance with the procedures laid down by the Universities Federation for Animal Welfare.

2.2. Venom

Venom was obtained from specimens of *T. nattereri* collected on the Mundaú Lake, Alagoas, Brazil. Venom was collected by pressuring the spines at their bases, and was

either used immediately or frozen at -20°C until use. Protein content was determined by the colorimetric method of Bradford (1976) using bovine serum albumin (Sigma) as standard protein.

2.3. Estimation of oedema-inducing activity

Samples of 30 μl containing different doses of venom (0.3, 3.0, 30 μg of protein/animal) were injected into the right footpad of mice. Local oedema was quantified by measuring the thickness of injected paws with a pachymeter (Mytutoyo) in 0.5, 2, 6, 9, 24 and 48 h after injection. Mice injected with 30 μl of sterile phosphate buffered saline (PBS) were considered as control-group. The results were expressed by the difference between experimental and control footpad thickness. Each point represents mean $\pm$ SEM.

2.4. Induction of local inflammatory reaction

T. nattereri venom (3.0 μg of protein in 30 μl of PBS) was injected in the intraplantar region of the right hind footpad (venom-group). Animals injected with 30 μl PBS were considered as control-group. Four or 24 h after injection blood samples were obtained by retroorbital bleeding. After that, animals were sacrificed and the right paws were amputated, the tissue was disrupted with scissor and homogenised with glass piston in 200 μl of PBS to reach a 1 ml of cell suspension.

2.5. Induction of peritoneal inflammation by *T. nattereri* venom

Venom (10 μg of protein/cavity) diluted in 1 ml of PBS was injected intraperitoneally (i.p.) in another group of mice according to protocols described elsewhere. Control mice received 1 ml of PBS alone. Four or 24 h after venom injection, blood samples were obtained by retroorbital bleeding. After that, animals were sacrificed and peritoneal cavities were washed with 3 ml of PBS.

2.6. Cell harvesting and counting

Leukocyte migration was assessed 4 or 24 h after venom or PBS administration in the footpad or peritoneal cavities. The samples were immediately centrifuged at 3000 rpm, 4°C , for 20 min. The supernatants were stored at -20°C for cytokine determination. The cell pellets were resuspended in 1 ml of PBS for cell counts. Total cell counts were performed in a hemocytometer and differential leukocyte counts in cytocentrifuge preparations stained with Wright–Giemsa. Cells were differentially counted by microscopy, evaluating 300 cells per slide. The results represent the mean $\pm$ SEM per millilitre of cell suspension.

2.7. Quantification of cytokines

Cytokines were measured in supernatant of footpad cell suspension by specific two-site sandwich ELISA, using the cytokine Duo Set kit for IL-1 β , TNF- α , and IL-6 (R&D Systems) according to the manufacturer's instructions. Binding of biotinylated monoclonal antibodies was detected using streptavidin-biotinylated horseradish peroxidase complex (Genzyme) and 3,3',5,5'-tetramethylbenzidine (Sigma). Samples were quantified by comparison with standard curves of recombinant mouse cytokines. The results were expressed as the arithmetic mean $\pm$ SEM for triplicate samples.

2.8. RNA extraction

Total RNA was extracted from the paw homogenised with 1 ml of Tri-ReagentTM (Sigma). The extraction method followed the manufacturer's instructions. The RNA extraction was carried out in an RNase-free environment. RNA was quantified by reading the absorbance at 260 nm according to the methods described by Sambrook and co-workers (1989).

2.9. RT-PCR

The reverse transcription of 1 μ g RNA was carried out using AMV reverse transcriptase (7.5 U), oligo (dT)₁₅ primer (2.5 μ g/ml), dNTP (0.5 μ M), MgCl₂ (2.5 nM) and 0.5 U RNase inhibitor (Reverse Transcription System from Promega). After incubation, at 42 °C for 30 min, AMV reverse transcriptase was inactivated by heating at 90 °C for 10 min. For the polymerase chain reaction (PCR) the cDNA obtained was incubated with 1.25 IU AmpliTaq Gold DNA polymerase (Perkin Elmer), 50 μ M 3' and 5' primers and 200 μ M dNTP in 200 mM Tris-HCl buffer, pH 8.4, containing 500 mM KCl and 1–4 mM MgCl₂ (depending on the primer used). The PCRs were run at 94 °C for 1 min (denaturing), 58 °C for 1 min (annealing temperature) and 72 °C for 1.5 min (extension). PCR products were detected on 2% agarose electrophoresis using as nucleotide size markers the 100 bp Ladder (Promega). The pair of primers used for IL-1 β , IL-6, TNF- α and β -actin were: GAGATT-GAGCTGTCTGCTCA (Forward) and AAGGAGAACC AAGCAACGAC (Reverse); GTACTCCAGAAGACCA GAGG (Forward) and TGCTGGTGACAACCACGGCC (Reverse); TTGACCTCAGCGCTGAGTTG (Forward) and CCTGTAGCCCCACGTCGTAGC (Reverse); CGTG GGCCGCCCTAGGCACAGGG (Forward) and CGGA GGAAGAGGATGCGGCAGTGG (Reverse), respectively.

2.10. Determination of cell viability by MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) assay was used to evaluate the cell viability after treatment with venom. The assay detects living, but not dead cells and the signal generated is dependent on the degree

of activation of the cells (Mosmann, 1983). J774A1 cells were cultivated in RPMI 1640 medium (Gibco[®]) containing 10% FCS (GibcoBRL), at 37 °C and 5% CO₂. To make the test, the concentration of J774A1 cells was adjusted and the cells were distributed in 96 well microplate (5 $\times$ 10⁴ cells per well). After 24 h, the medium was changed and the samples containing the venoms or sterile PBS, diluted in medium were added. Doses of 0.3, 1.0 and 3.0 μ g of protein venom were used in 100 μ l per well. After 6, 24, 48 and 72 h, the microplates were centrifuged (1000 rpm, 10 min, at 4 °C) and the medium was removed and 20 μ l of MTT (5 mg/ml) diluted in PBS per well was added. The microplates were incubated at 37 °C and 5% CO₂ for 3 h. Following that, 100 μ l of SDS 10% diluted in 0.01N HCl/PBS were put per well (18 h, at 37 °C and 5% CO₂) and the results were read on a multiwell scanning spectrophotometer (ELISA reader) containing a filter of 570 nm. The number of cells was estimated according with absorbance obtained in a standard curve of cells. The results were expressed as the percentage of viable cells compared with PBS treated cells. Similar results were obtained in at least three separated experiments.

2.11. Statistical analysis

Two-way analysis of variance was used to determine the levels of difference between all groups. Differences between cell numbers in the several groups were analysed by the Mann–Whitney test. All dates are reported as arithmetic means. Error bars represent 1 SEM. Differences were considered statistically significant at $p < 0.05$. The SPSS statistical package (Release 8.0, Standard Version, 1997) was employed.

3. Results

3.1. Effect of *T. nattereri* venom on mice footpad oedema

For determination of the edematogenic response induced by *T. nattereri* venom doses at 0.3, 3.0 and 30.0 μ g of venom/footpad were used. Fig. 1 shows that intraplantar injection of different doses of venom produced a dose-dependent oedema. Furthermore, the highest dose of venom (30.0 μ g of venom/footpad) induced an intense necrosis and the lower dose (0.3 μ g of venom/footpad) induced footpad thickness only in initial times returning to control-levels after 9 h. Then, for further experiments, the dose of 3.0 μ g of venom/footpad was routinely used. The maximal response was observed at 2 h after venom injection remaining significantly elevated compared with control-group over 48 h.

3.2. Release of acute phase cytokines by *T. nattereri* venom

In the view of the local (intraplantar) relationship between vessel permeability and protein extravasation,

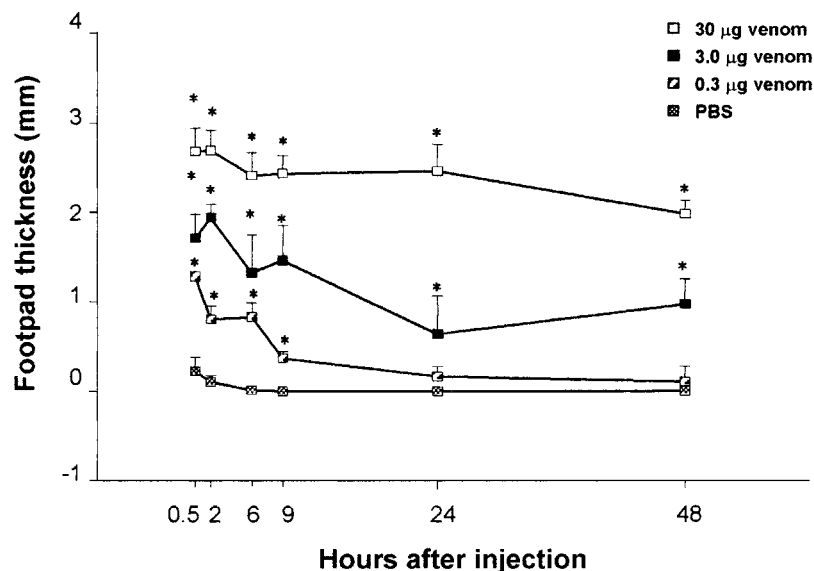


Fig. 1. Estimation of oedema-inducing activity. Samples of 30 µl containing different doses of venom (0.3, 3.0, 30 µg protein/animal) were injected into the right footpad of mice. Local oedema was quantified by measuring the thickness of injected paw in 0.5, 2, 6, 9, 24 and 48 h after injection. Mice only injected with PBS were considered as control-group. The results were expressed by the difference between injected paw and contralateral paw. Each point represents mean $\pm$ SEM. $p < 0.05$ compared with control-group.

footpad homogenates were tested for the presence of TNF- α , IL-1 β and IL-6 cytokines as shown in Fig. 2. Maximal levels (40 pg/footpad) of TNF- α were recorded 2 h after injection of venom, but elevated levels of TNF- α compared with control-group were observed 4 and 12 h, returning to the normal levels in 24 h.

We also assessed IL-1 β and IL-6 levels, which were maximal and significantly higher than control-group at 2 and 4 h (320 and 2500 pg/footpad, respectively). The IL-1 β levels returned to normal levels after 24 h and IL-6, 12 h after injection. To confirm that this effect was produced locally, the production of mRNA encoding for TNF- α , IL-1 β , and IL-6 was evaluated in the footpad injury model. Tissue was sampled at 2, 4, 12 and 24 h following injection of 3 µg of *T. nattereri* venom (in 30 µl) or PBS alone. TNF- α , IL-6 and IL-1 β were upregulated at 2 h in footpads injected with venom and PBS, however, at 4–24 h after injection the mRNA expression encoding for these cytokines was upregulated only in the venom injected footpad (data not shown).

3.3. Modulation of leukocyte migration by *T. nattereri* venom

Cytokines are typically associated with oedema and acute inflammatory response. Thus, leukocyte recruitment to the site of injury after *T. nattereri* venom injection was evaluated. Fig. 3A shows no difference in cell recruitment into footpad tissue of venom-group 4 h after injection, when compared with control-group. At this time, a significant

decrease was observed in the circulation of leukocytes in peripheral blood, mainly in mononuclear cells (Table 1).

As it can be observed in Fig. 3B, 24 h after *T. nattereri* venom injection in the footpad there was a significant decrease in the total amount of cells, mainly in mononuclear cells (approximately 41%). However, the accumulation of neutrophils was significantly higher compared with control-group (an increase of 100%). At this time, we detected no difference in the circulating cells in peripheral blood between venom- and control-group (Table 1). To verify the delayed effect of *T. nattereri* on cellular influx to footpad other group of mice was evaluated 48 h after venom injection. In contrast to 24 h no alterations were observed in leukocyte recruitment to footpad tissue at this time. In addition, the number of leukocytes in peripheral blood was similar in both groups (data not shown).

The results depicted in Fig. 4A shows that 4 h after the venom injection in the peritoneal cavity the total amount of cells recruited was unaltered, but neutrophils recruitment was 3-fold higher than the number recovered from control-group, and there was a significant decrease in the number of mononuclear cells. The number of circulating cells in peripheral blood remained similar to control-group (Table 2).

Analyses of the cell suspension recovered from the peritoneal cavity 24 h after the venom injection reveal a drastic increase in the total amount of cells, mainly in neutrophils (23-fold), followed by a 2-fold mononuclear cells recruitment (Fig. 4B). Significant neutrophilia was also observed in peripheral blood, but mononuclear cells did not occur (Table 2).

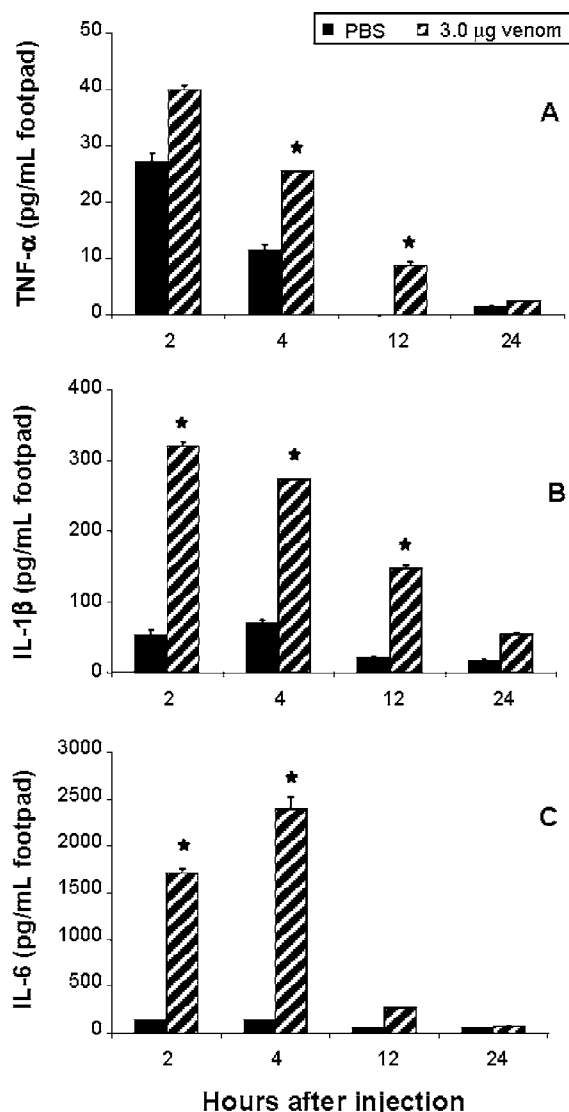


Fig. 2. Quantification of cytokines in homogenates of footpad from mice injected with *T. nattereri* venom. Venom (3.0 μg protein in 30 μl of PBS) was injected in the intraplantar region of the right hind footpad (venom-group). Mice only injected with PBS were considered as control-group. After several times, animals were sacrificed and the right paws were amputated, and homogenised for ELISA determinations. Each bar represents mean $\pm$ SEM. $p < 0.05$ compared with control-group.

3.4. *T. nattereri* venom affects cell viability of cell line *in vitro*

To investigate the cytotoxic effect of venom, J774A1 cells were incubated for several period of time with doses at 0.3, 1.0, and 3.0 μg protein of venom (100 μl) or medium alone, at 37 °C, in 5% CO₂. The results depicted in Table 3 show that 24 h was the critical period for the cytotoxic effect induced by the venom. In this time, all doses used were

effective to dead the majority of mononuclear cells in culture. In contrast, in 48 or 72 h only the highest doses (1.0 or 3.0 μg) were cytotoxic for mononuclear cells.

4. Discussion

In this study we have demonstrated for the first time the dissociation between the acute inflammatory response and the leukocyte recruitment in a footpad of mice injured with *T. nattereri* venom.

Our findings show that the edematogenic response induced by *T. nattereri* venom in footpad of mice was dose- and time dependent, and remained significantly elevated over 48 h after injection. Based on our previous study that show the defective effect of anti-inflammatory drugs (dexamethasone and indomethacin) on reducing the clinical symptoms as pain and oedema (Fonseca and Lopes-Ferreira, 2000) we ruled out cyclooxygenase and lipoxygenase products from the mediators involved in this inflammatory reaction.

In several animal models of inflammation using agents as complete Freund's adjuvant (CFA), capsaicin, and venom from *Phoneutria nigriventer* spider it is observed local plasma protein efflux and oedema formation. These alterations are due, at least in part, to activation of the tissue kallikrein–kininogen–kinin system leading to local generation of kinins (Marangoni et al., 1993; Blais et al., 2000; Calixto et al., 2000; Emin et al., 2000; Costa et al., 2001). According to our previous results, this is the major mechanism involved in *T. nattereri* venom-induced oedema and nociception (Lopes-Ferreira et al., 1998c).

Kinins are released during tissue damage, causing increased microvascular permeability, vasodilatation, pain and accumulation of leukocytes into inflammatory sites (Bhoola et al., 1992). These molecules exert their major actions by the activation of two distinct membrane receptors, B1 and B2 (Regoli et al., 1993; Marceau et al., 1998). Several studies demonstrated that B2 receptors are constitutively present throughout the peripheral and central nervous system and that they are responsible for carrying out most of the physiological responses attributed to kinins. On the other hand, B1 receptors are rarely present in normal tissues, but they can be expressed under special stimuli including tissue trauma or injury, or following treatment with certain agents, such as endotoxins, CFA, cytokines, or *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Recently, Ferreira and co-workers (2001) demonstrated in an elegant study, using mice deficient in B1 or B2 kinin receptors, the distinct pattern of biological roles of these receptors during CFA-induced inflammatory and nociceptive responses. The authors confirmed the critical role of kinin B1 receptor, but not B2 receptor, in controlling the persistent inflammatory nociception, and partial relevance of B1 receptor in CFA-induced mouse paw oedema.

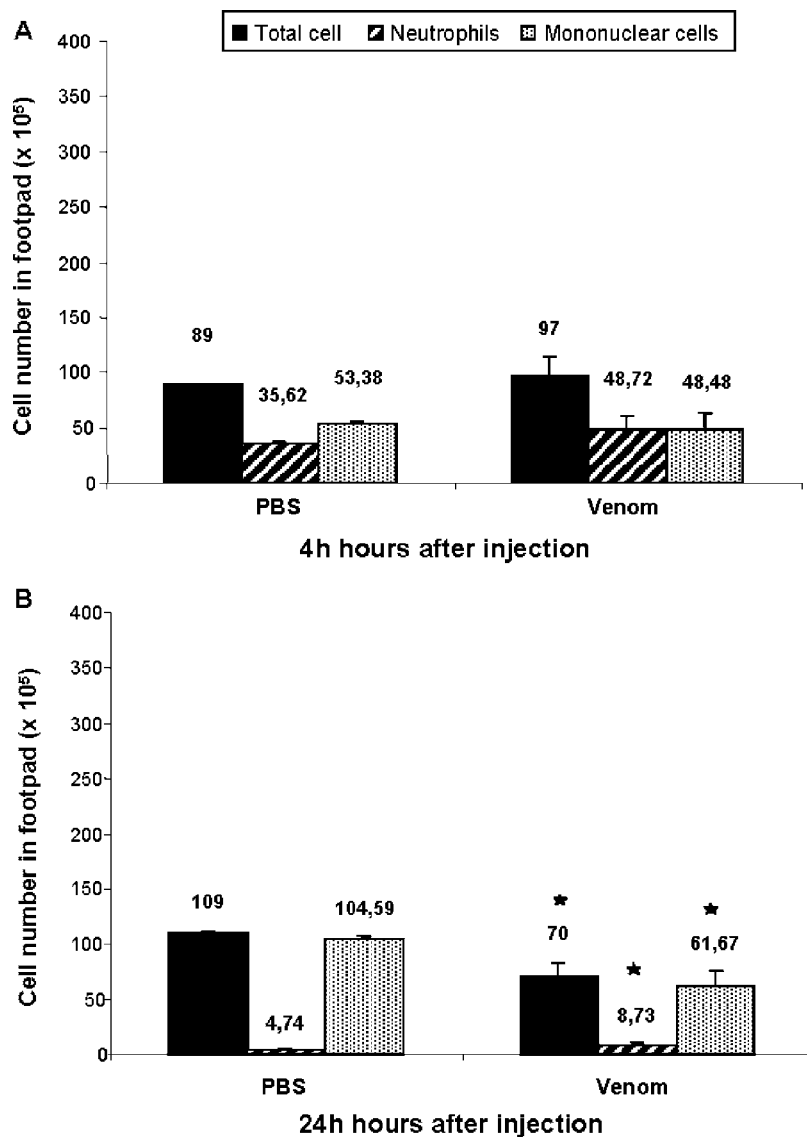


Fig. 3. Effect of *T. nattereri* venom on leukocyte recruitment to footpad. Four or 24 h after injection of venom (3.0 μ g protein in 30 μ l PBS) animals were sacrificed, and tissue footpad was processed for cell count. Mice only injected with PBS were considered as control-group. The results represent the mean $\pm$ SEM. $p < 0.05$ compared with control-group.

Table 1
Leukocyte numbers in the peripheral blood after *T. nattereri* venom injection in footpad

	4 h		24 h	
	PBS	Venom	PBS	Venom
Total cell number	143.00 $\pm$ 7.54	111.00 $\pm$ 18.02*	172.00 $\pm$ 6.00	157.00 $\pm$ 26.32
Neutrophils	4.23 $\pm$ 1.96	4.95 $\pm$ 2.59	33.51 $\pm$ 0.31	24.80 $\pm$ 5.48
Mononuclear cells	138.44 $\pm$ 8.48	105.57 $\pm$ 16.20*	138.49 $\pm$ 5.69	131.71 $\pm$ 21.69

Four or 24 h after injection of 3.0 μ g protein of venom in 30 μ l PBS in the intraplantar region of the right hind footpad, mice were bleeding by retroorbital plexus for cell count. The results represent the mean $\pm$ SEM. $p < 0.05$ compared with control-group.

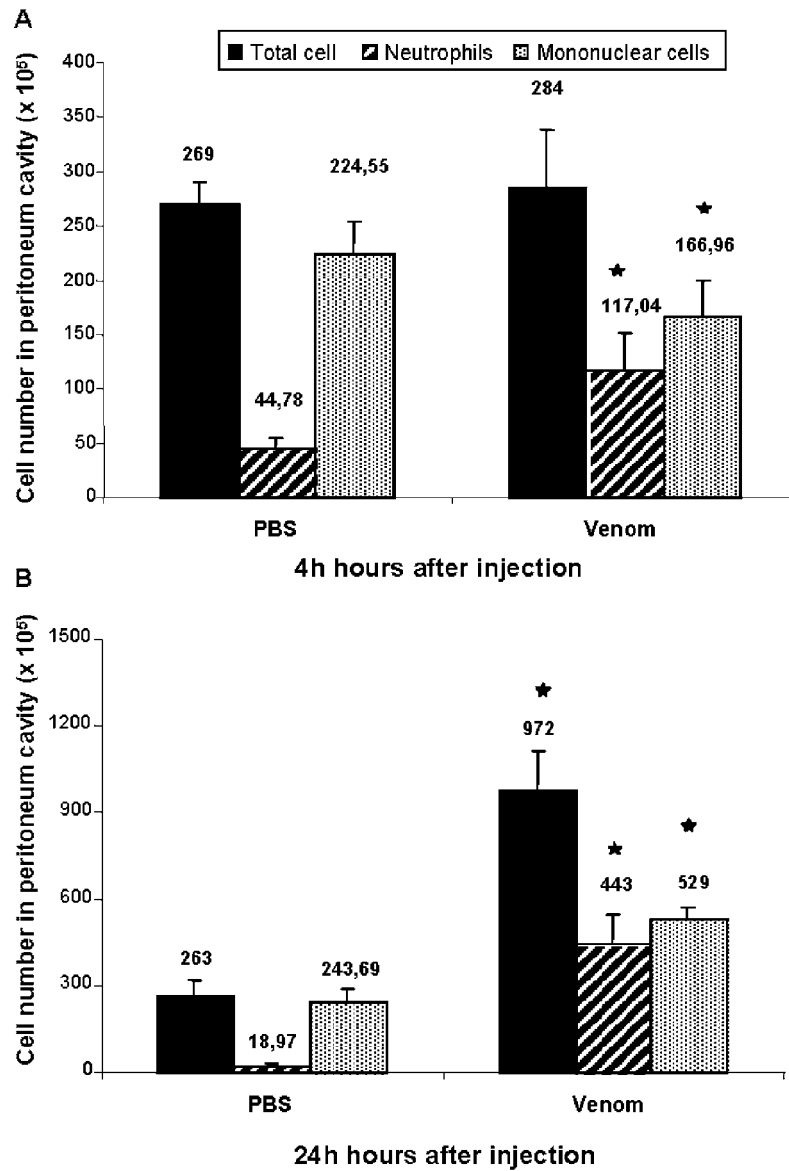


Fig. 4. Effect of *T. nattereri* venom on leukocyte recruitment to peritoneal cavity. Four or 24 h after injection of venom (10.0 μ g protein in 1 ml PBS) animals were sacrificed and peritoneal cavities were washed for cell count. Mice only injected with PBS were considered as control-group. The results represent the mean $\pm$ SEM. $p < 0.05$ compared with control-group.

Table 2
Leukocyte numbers in the peripheral blood after *T. nattereri* venom injection in peritoneal cavity

	4 h		24 h	
	PBS	Venom	PBS	Venom
Total cell number	199.00 $\pm$ 20.22	150.00 $\pm$ 41.81	113.00 $\pm$ 15.00	159.00 $\pm$ 28.13
Neutrophils	73.96 $\pm$ 25.45	64.85 $\pm$ 24.28	13.56 $\pm$ 1.80	35.95 $\pm$ 13.94*
Mononuclear cells	125.05 $\pm$ 7.55	85.15 $\pm$ 30.65	99.44 $\pm$ 13.2	123.25 $\pm$ 32.95

Four or 24 h after injection of 10.0 μ g protein of venom in 1 ml PBS in the peritoneal cavity, mice were bleeding by retroorbital plexus for cell count. The results represent the mean $\pm$ SEM. $p < 0.05$ compared with control-group.

Table 3

T. nattereri venom affects cell viability of cell line in vitro

Time of culture (h)	0.3 µg (100 µl/well) (%)	1.0 µg (100 µl/well) (%)	3.0 µg (100 µl/well) (%)
6	84.0	62.5	60.5
24	55.5	35.9	35.9
48	86.6	36.9	36.4
72	84.8	71.2	25.7

Cell viability was determined 6, 24, 48 or 72 h after J774A1 cells cultured with 0.3, 1.0 and 3.0 µg protein of venom per 100 µl/well. The cell viability was estimated by a colorimetric test with MTT and expressed as percentage of viable cells compared with cells cultured with medium alone. Similar results were obtained in at least three separated experiments.

The expression of the B1 receptor in CFA-induced mouse paw oedema can be up regulated by alarm cytokines produced by resident inflammatory cells or epithelial cells located at site of acute inflammation and released into circulation (Baumann and Gauldie, 1994). In this context, we analysed the levels of TNF- α , IL-1, and IL-6 in homogenates of footpad tissue after injection of *T. nattereri* venom, and we observed that these cytokine levels were elevated in venom-group. Moreover, the presence of mRNA for all cytokines in homogenates of tissue footpad confirms the parallel production of these cytokines at local of injury.

Another consequence of altered capillary permeability in local inflammation is the extravasation of leukocytes into tissue. The pattern of leukocyte recruitment (early predominant neutrophil influx replaced later by mononuclear cell recruitment) appears to dictate the process of successful clearance of the offending agent and restoration of tissue homeostasis. Surprisingly, our results showed that the injection of venom developed an inadequate cellular inflammatory response, evidenced 24 h after injection by poor infiltration of leukocytes, mainly mononuclear cells. In addition, decreased number of mononuclear cell circulating in peripheral blood preceded this phenomenon (4 h after injection of venom). Even so, significant influx of neutrophils into tissue footpad 24 h after injection of venom was evidenced. The inflammatory reaction induced by *T. nattereri* venom in gastrocnemius muscle was also characterised by a scarce infiltrate of macrophages and polymorphonuclear leukocytes, particularly evident at 24 h after injection of the venom (Lopes-Ferreira et al., 2001).

The defective leukocyte infiltration in tissue footpad induced by *T. nattereri* venom and our previous observations of slow process of regeneration of the injured tissue, including areas of abundant necrotic fibres but very few phagocytic cells (Lopes-Ferreira et al., 2001) could be associated with an inadequate blood supply to muscle tissue. Such hypothesis was supported by the observation that *T. nattereri* venom-induced microvascular alterations characterised by blood stasis and thrombosis (Lopes-Ferreira

et al., 2002). To confirm this hypothesis, the recruitment of leukocytes by *T. nattereri* venom was evaluated in a highly vascularized inoculation site as peritoneal cavity. Analyses of the cell suspension recovered from the peritoneal cavity 24 h after the venom injection revealed a drastic increase in the total amount of cells, mainly in neutrophils (23-fold), followed by 2-fold mononuclear cells recruitment. Significant neutrophilia was also observed in peripheral blood, but increase in mononuclear cells was not observed. Based on the number and the leukocyte sub-populations recovered from the peritoneal cavity, leukocyte influx from the vasculature into the peritoneal space was enhanced by *T. nattereri* venom, which is actually acting as a pro-inflammatory stimulus.

The results observed in a murine model suggest that impaired blood supply might be responsible for the poor leukocyte influx observed in *T. nattereri* venom-induced lesions. However, venom cytotoxicity has also to be considered. In previous studies, we showed that very low doses of *T. nattereri* venom were cytotoxic to myoblasts, endothelial cells and platelets (Lopes-Ferreira et al., 2001, 2002). In this work we also demonstrated the cytotoxic effect of *T. nattereri* on mononuclear cell line (J774A1) in an in vitro assay. Therefore, cytotoxicity of local inflammatory cells may also be responsible for the reduced local numbers of leukocytes.

Therefore, our results support that the scarcity of inflammatory cellular influx into local lesions induced by *T. nattereri* venom could be a consequence of an impaired blood flow in venules at injured tissue and cytotoxic effect of the venom on inflammatory cells. Further studies with toxins from *T. nattereri* venom will be necessary for the elucidation of the mechanisms involved in the inflammatory reaction induced by the venom.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Brazilian Research Council (CNPq).

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