

Neutralizing antibodies obtained in a persistent immune response are effective against deleterious effects induced by the *Thalassophryne nattereri* fish venom

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Abstract

Thalassophryne nattereri envenoming represents a great cost to North and Northeast Brazilian communities in terms of public healths, leisure and tourism. Victims rapidly develop symptoms as pain, local swelling, erythema followed by intense necrosis that persist for long days. The aim of this work was tested the immune competence of neutralizing antibodies in pre-immunized mice against principal toxic activities induced by venom. During the primary antibody response in mice, an elevation of IgG antibody levels was only observed on day 28. After boosting, high antibody levels were detected between days 49 and 70, with a 12-fold increase in IgG level over control values at day 49. We confirmed the *in vitro* neutralizing capacity of *T. nattereri* anti-venom against toxic effects and thereafter we show that neutralizing antibodies obtained in a persistent immune response are more effective, inclusive against edematous reaction. After boosting during the secondary response mice with high antibody levels do not present any alterations in venule or arteriole after topical application of venom on cremaster muscle. In addition, CK activity diminished in these mice with high neutralizing antibody levels corroborating the attenuation of the myonecrotic effect by venom. In addition, we determined the presence of high IgG antibodies levels in patients 6 months after injury by *T. nattereri*. In conclusion, the presence of neutralizing antibodies against to *T. nattereri* venom in the serum of pre-immunized mice could change the outcome of lesion at site of posterior envenoming. Antigen-specific antibodies of high affinity in consequence to specific immune response, dependent of *T* lymphocyte activation, could minimize the symptoms of intense and immediate inflammatory reaction caused by *T. nattereri* venom. These finding prompt us to the possibility of development of immune therapeutic strategies using specific anti-venom as an efficient intervention for protecting human victims.

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1. Introduction

Thalassophryne nattereri envenoming represents a great cost to North and Northeast Brazilian communities in terms of public healths, leisure and tourism (Fonseca and Lopes-Ferreira, 2000; Haddad Jr. et al., 2003; Faco et al., 2005). *T. nattereri* venomous fish are member of the Batrachoididae family, and in Brazil they are known by severity of accidents that provoke in fishermen and bathers. Victims rapidly develop symptoms as pain, local swelling, erythema followed by intense necrosis that persist for long days (Fonseca and Lopes-Ferreira, 2000). 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 (Fróes, 1933).

Experimental studies performed with the *T. nattereri* venom showed that low doses of the venom (0.3 µg/animal) induced local effects as the nociception and edema, similar to that described in humans, independently of the presence of hemorrhagic, phospholipase A₂ or coagulant activities (Lopes-Ferreira et al., 1998). The histological analysis of the lesion provoked by the venom in the gastrocnemius muscle evidenced acute myonecrosis, presence of thrombi, a scarce infiltrate of polymorphonuclear leukocytes and macrophages, and the skeletal muscle regeneration was partially impaired (Lopes-Ferreira et al., 2001). Recently, local acute inflammatory response induced by the venom was characterized (Lima et al., 2003). Cytokines as TNF-α, IL-1β and IL-6 and a weak leukocyte influx were detected on footpad of mice. Additionally, a cytotoxic effect of the venom on mononuclear cell was also observed.

Most accidents with *T. nattereri* venom occur in the fishing communities and, due to the lack of efficient therapy, victims may take weeks, or even months before returning to work. The immediate treatment for the fish venom accidents has been to place the wound region in hot water, or administration of local anaesthetics or analgesics, resulting in slight decrease of the symptoms of the envenomation (Sutherland, 1983). 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 in many cases the local lesion evolve to permanent sequela (Fonseca and Lopes-Ferreira, 2000). In addition, our laboratory showed recently

in an experimental model that nociception and edema induced by the venom were not reduced either by treatment with inhibitors of serotonin and histamine or by non-steroidal and steroidal anti-inflammatory drugs, but only by the administration of kallikrein-specific inhibitor (Lopes-Ferreira et al., 2004).

The capacity of venoms or toxins from venomous marine animals to induce humoral immune responses with neutralizing antibodies has been described previously (White, 1998; Currie, 2003). Evidence supporting the *in vitro* efficacy of *T. nattereri* antivenom was initially demonstrated by Lopes-Ferreira et al. (2000) using the antiserum produced in rabbits. In a recent study, we also reported that lower doses of *T. nattereri* venom mixed to alum for optimal conditions of T cell activation confer a strong and sustained active humoral immune response in mice (Grund et al., 2006). It is interesting to register that unlike other poisons that can provoke sensitization and shock (bee venoms) the fishermen habitual victims of the *T. nattereri* develop progressive resistance after successive attacks, with gradual decrease of the effect of the venom with minimization of the pain and of the inflammatory effects in the repeated lesions (Auto, 1992).

Since systemic antibody levels could be induced by *T. nattereri* venom, we tested the immune competence of these neutralizing antibodies in pre-immunized mice. Our results showed that the immunization with *T. nattereri* venom induced not only a strong long-lasting B-cell memory against venom antigens but also neutralizing antibodies against the principal toxic effects.

2. Materials and methods

2.1. Patients

Seven patients injured by *T. nattereri* in Maceió (Alagoas State) were observed during 6 months, which allowed the collection of the blood for determination of antibody levels 6 months after accidents.

2.2. Animals and venom

Outbred Swiss male, weighing 18–22 g were provided by the Butantan Institute and used in accordance with the guidelines provided by the Brazilian College of Animal Experimentation, and

were authorized by the Ethics Committee for Animal Research of the Butantan Institute (002/2001). Specimens of adult *T. nattereri* fish were collected in the Brazilian state of Alagoas. The venom were obtained as previously described (Lopes-Ferreira et al., 1998). Protein concentrations were determined by the colorimetric method of Bradford (1976). Standard curves were constructed using bovine serum albumin (Sigma Chemicals, St Louis, MO, USA) diluted in duplicate.

2.3. Immunization of mice against *T. nattereri* venom

In order to characterize the immune responses to *T. nattereri* venom, mice ($n = 5/\text{group}$) were injected with $3\text{ }\mu\text{g}$ of protein venom at day 0. Forty-two days later after the first injection mice were boosted with the same dose of venom. Blood samples from the mice were obtained at days 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, and 77 after immunization by retroorbital bleeding, and individual mouse serum were tested for antibody response by enzyme-linked immunosorbent assay (ELISA).

2.4. ELISA for IgG determination

Sera from injected mice (serial dilution) or patients (diluted at 1:16,000) were titrated for their IgG antibody content against *T. nattereri* venom in ELISA plates (Polosorp, Nunc, Denmark) coated with $3\text{ }\mu\text{g}/\text{ml}$ of protein venom according to the technique described by Theakston et al. (1977). The plates were analyzed at 492 nm in an ELISA reader (Multiskan, Spectrophotometer, EFLAB, Helsinki, Finland). The negative control consisted of sera from mice injected with saline or sera from normal humans.

2.5. SDS–polyacrylamide gel electrophoresis

The proteins ($10\text{ }\mu\text{g}$) of *T. nattereri* venom were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (12% acrylamide resolution gels) under non-reducing conditions using the method of Laemmli (1970).

2.6. Western blotting

The proteins of *T. nattereri* venom ($10\text{ }\mu\text{g}$) fractionated by SDS–PAGE as described above were transferred to nitrocellulose membrane and

revealed using sera (1/10,000) from venom-immunized mice, as described by Towbin et al. (1979).

2.7. Neutralization assays

The sera collected at days 21, 28, 49, and 56 after the first immunization were used for the pre-incubation assays (nociception, edema, and necrosis). The dose of $3\text{ }\mu\text{g}$ of protein venom plus $30\text{ }\mu\text{l}$ of serum was used for nociception and edema, and $30\text{ }\mu\text{g}$ of protein venom plus $300\text{ }\mu\text{l}$ of serum for necrosis, based on previous dose–response experiments (Lopes-Ferreira et al., 1998). Mixtures of venom and sera were incubated for 30 min at 37°C , centrifuged and the supernatants injected into mice, according to proceedings of WHO (1981). Control studies used venom incubated with normal mouse serum or saline. For *in vivo* neutralization assays, pre-immunized mice were injected with venom at days 28 and 49 for evaluation of toxic activities.

2.7.1. Nociception activity

Mixtures of venom and sera were incubated for 30 min at 37°C , centrifuged and the supernatants collected. Supernatant from the mixture of venom plus serum or venom alone were injected into the intraplantar region of the hind foot paw in a fixed volume of $30\text{ }\mu\text{l}$. The control group was injected only with sterile saline. Each animal was then returned to the observation chamber and the amount of time spent licking or biting each hind paw was recorded for 30 min and taken as the index of nociception (Hunskar et al., 1985). Each point represents mean $\pm$ SEM of three independent experiments.

2.7.2. Edema-inducing activity

Mixtures of venom and sera were incubated for 30 min at 37°C , centrifuged and the supernatants collected. Supernatant from the mixture of venom plus serum or venom alone were injected into the intraplantar region of the hind foot paw in a fixed volume of $30\text{ }\mu\text{l}$. Local edema was quantified by measuring the thickness of injected paws with a paquimeter (Mytutoyo) in 2 h after injection. Mice injected with $30\text{ }\mu\text{l}$ of sterile saline were considered as control-group. The results were expressed by the difference between experimental and control foot-pad thickness. Each point represents mean $\pm$ SEM of three independent experiments.

2.7.3. Necrotizing activity

Mixtures of venom and sera were incubated for 30 min at 37 °C, centrifuged and the supernatants collected. Supernatant from the mixture of venom plus serum or venom alone were injected into the shaved backs of the mice in a fixed volume of 300 µl. After 72 h, the animals were killed and the skin removed. The necrotic area was measured. Two diameters were determined for the necrotic spot by measuring the longest diameter and the one perpendicular to the longest. Results were expressed as the product of the diameters. Each point represents mean $\pm$ SEM of three independent experiments and the control-group was injected only with sterile saline.

2.8. Quantification of plasmatic Creatine Kinase activity

At days 28 and 49 pre-immunized mice were injected (i.m., thigh muscle) with 3 µg of protein venom. After 3 h, blood was collected from the ophthalmic plexus. Sera of mice were separated and immediately assayed for creatin kinase activity (Sigma Chemical Co. St. louis, MO, USA). CK activity was expressed in units/ml, one unit resulting in the phosphorylation of one nanomole of creatine per min at 25 °C. The results are reported as the mean $\pm$ SEM of three independent experiments and the control group was injected only with sterile saline.

2.9. Microcirculatory alterations

At day 49, pre-immunized mice were anaesthetized with an i.p. injection of sodium pentobarbital (20 mg/Kg body weight), placed on a water-heated bed (at 37 °C), and the cremaster muscle was exposed according to Lomonte et al. (1994) for topical application of venom (3 µg). Control experiments were performed by applying 30 µl saline under otherwise identical conditions. Muscle preparations were observed in a triocular microscope (Axioskope, Carl-Zeiss), and images were analysed using image analyzer software (KS 300, Kontron). The images were obtained using a x10/025 longitudinal distance objective/numeric aperture and 1.6 optovar. Five minutes of observation were recorded before application of the venom to analyze the dynamics in control tissue. Experiments were carried out and tissue reactions were observed for up to 40 min.

2.10. Statistical analysis

All results were presented as means $\pm$ SEM of at least 5 animals in each group. Differences among data were determined by one way analysis of variance (ANOVA) followed by Dunnett's test. Data were considered different at $p < 0.05$.

3. Results

3.1. *T. nattereri* venom induces sustained humoral response in patients

T. nattereri venomous fish are members of the Batrachoididae family, and in Brazil they are known by severity of accidents that provoke in fishermen and bathers. Their venomous apparatus present two dorsal fin and two gills cover hollow needlelike spines with connected venomous glands in the base (Fig. 1). Mainly, fishermen were injured stepping on the fish in shallow waters and while removing the fish from the fish-nets. The victims presented symptoms as pain, local swelling, erythema followed by intense necrosis that persist for long days, and 6 months after the accidents these patients presented high levels of specific IgG against *T. nattereri* venom (Fig. 2).

3.2. *T. nattereri* venom induces sustained humoral response in mice

For evaluating the time span of the antibody response induced by *T. nattereri* venom, Swiss mice were immunized with 3 µg of native venom on day 0 and an identical booster dose was give on day 42. The antigen was diluted in saline as vehicle, and injected i.pl. in the absence of adjuvant. As shown in Fig. 3, 7 days after the first immunization, the anti-venom IgG response was barely detectable in all mice. During the primary antibody response, an elevation of IgG antibody levels was only observed on day 28. After boosting, high antibody levels were detected between days 49 and 70, with a 12-fold increase in IgG level over control values at day 49. On day 77, IgG levels reached levels similar to those at day 7.

3.3. Recognition of principal toxins of the *T. nattereri* venom by antibodies

In order to identify the *T. nattereri* antigens recognition by the systemic antibody response of

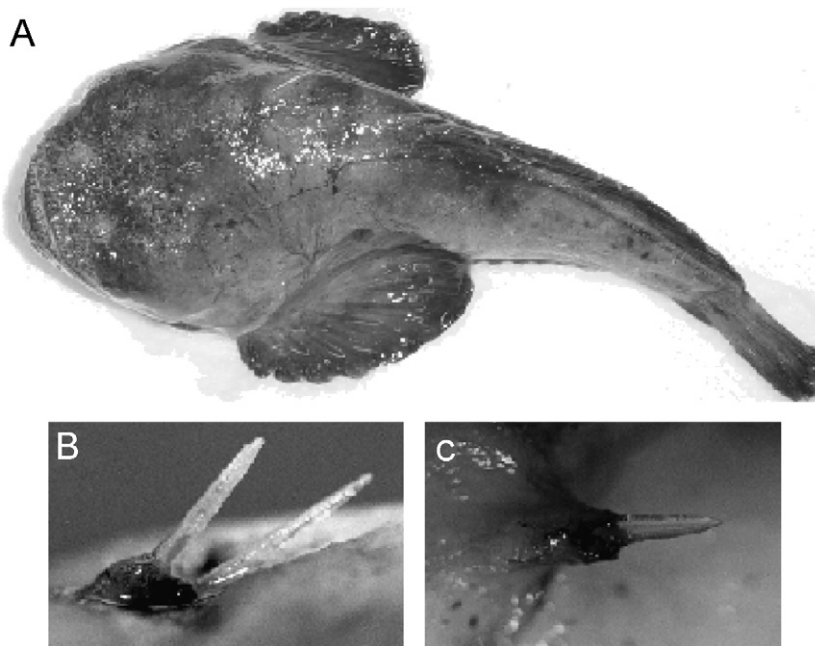


Fig. 1. *Thallasophryne nattereri*, the niquim or Brazilian toadfish (A). Dorsal (B) and lateral spines (C).

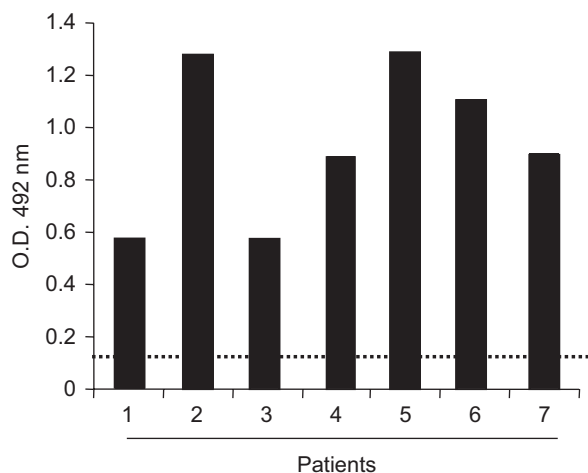


Fig. 2. Antibody levels in patients injured by *T. nattereri*. Seven patients injured by *T. nattereri* in Maceió (Alagoas State) were observed during a period of 6 months. Sera from patients (diluted at 1:16,000) were evaluated for their IgG antibody content against *T. nattereri* venom by ELISA. The dotted line represents the value for human normal serum.

immunized mice, western blots were performed with sera. Sera polyclonal antibody during a primary response (day 7–35) were most directed against all antigenic bands of the venom (above 68, between 46.9 and 68 and 24 and 46.9 kDa and around 14.3—Fig. 4). This antibody reactivity was strongly

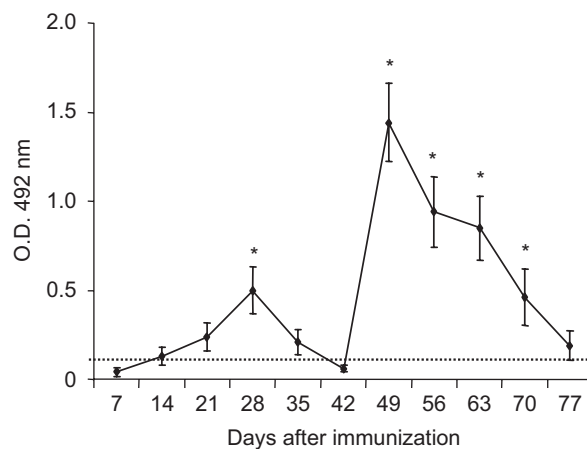


Fig. 3. Antibody levels in mice immunized with *T. nattereri* venom. Mice ($n = 5$ /group) were injected with 3 μ g of protein venom at day 0. Forty-two days later after the first immunization mice were boosted with the same dose of venom. Mice were bled by retroorbital bleeding at days 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, and 77 after immunization, and individual mouse serum were titrated for their IgG antibody content against *T. nattereri* venom by ELISA. Each point represents mean $\pm$ SEM $p < 0.05$ compared with control-group (dotted line).

detected in serum collected at day 28. Sera collected from boosted mice recognized all principal bands of the venom and this antibody recognition was clearly detected from days 49 to 70. Control sera from

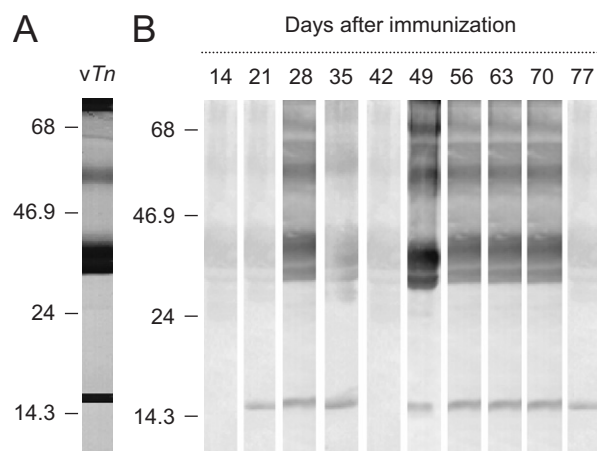


Fig. 4. Electrophoretal profile and Western blotting of *T. nattereri* venom. Venom of *T. nattereri* (VTn) was analyzed by SDS-PAGE using polyacrylamide resolution gel 12% under no-reduction conditions, and revealed by Comassie Blue (A) or transferred to nitrocellulose membranes by Western blotting using immunized mice serum diluted at 1:10,000 (B). Numbers at left corresponded to position of M_w markers.

saline injected mice did not exhibit any immunoreactivity (data not shown) (Fig. 5).

3.4. Ability of sera to neutralize principal toxic effects induced by *T. nattereri* venom in pre-incubation assays

The immune competence of the antibody directed against *T. nattereri* venom was estimated by measuring the level of protection in pre-incubation assays (Table 1). Therefore, we assessed the neutralizing activity of the sera from mice at days 21, 28, 49 and 56 after mixture with *T. nattereri* venom. The supernatant were injected in naive mice for evaluation of toxic activities. Primary anti-venom antibodies (days 21 and 28) suppressed the nociception (189.4 ± 32.3 vs. 90.3 ± 19.6 and 189.4 ± 32.3 vs. 47.6 ± 10.5 , respectively) and necrosis (only serum from day 28 to 3.3 ± 0.9 vs. 1.3 ± 0.3) when compared with animals injected only with venom. Antibodies elicited in the secondary response (days 49 and 56) showed higher neutralizing activity than in the primary response, mainly on nociceptive and necrotizing activity (100%). Neutralizing antibodies from primary responses do not protected the naive mice against edema formation, this activity was only partially protected by secondary antibodies (3.2 ± 0.5 vs. 2.4 ± 0.3).

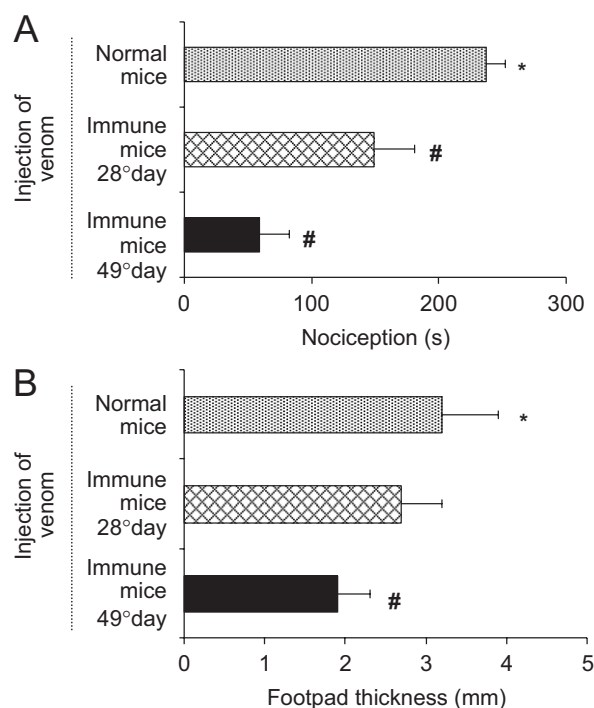


Fig. 5. Neutralization of nociception and edema in pre-immunized mice. Samples of 30 μ l containing 3 μ g protein were injected into the right footpad of pre-immunized mice at days 28 and 49. Mice were placed in a chamber and the amount of time spent licking or biting each hind paw was recorded for 30 min and taken as the index of nociception (A). Local edema was quantified by measuring the thickness of injected paws with a pachymeter (Mytutoyo) in 2 h after injection (B). Each bars represent mean $\pm$ SEM of three independent experiments. * $p < 0.001$ compared with the saline-group; # $p < 0.01$ compared with the venom-group.

3.5. Protective efficacy of the humoral immune responses elicited by *T. nattereri* venom

To evaluate the protective efficacy of the humoral immune responses elicited by *T. nattereri* venom, pre-immunized mice were challenged with a injection of toxic dose of venom at days 28 and 49, time with high plasmatic antibody levels. When the venom was administered at a dose of 3 μ g i.p. in pre-immunized mice at day 28 the nociception and edema response was partially inhibited (149.3 ± 31 and 2.7 ± 0.5 , respectively) when compared with naive mice without circulating antibodies (257 ± 23.5 and 3.2 ± 0.4 , respectively). At day 49, the protective efficacy of the neutralizing antibodies in pre-immunized mice reached 75% and 40.6%, respectively.

Table 1

Ability of antiserum to neutralize *T. nattererivenom* toxic activities by pre-incubation

	Nociception (s)	Edema (mm)	Necrosis (mm ²)
Saline	10.0 ± 1	0	0
Venom plus saline	189.4 ± 32.3 ^a	3.2 ± 0.5 ^a	3.3 ± 0.9 ^a
Venom plus normal serum	183.5 ± 31.3 ^a	3.0 ± 0.6 ^a	3.0 ± 0.9 ^a
Venom plus antivenom (21° d)	90.3 ± 19.6 ^b	3.1 ± 0.2	2.3 ± 0.4
Venom plus antivenom (28° d)	47.6 ± 10.5 ^b	3.0 ± 0.1	1.3 ± 0.3 ^b
Venom plus antivenom (49° d)	0 ^b	2.4 ± 0.3 ^b	0 ^b
Venom plus antivenom (56° d)	0 ^b	2.4 ± 0.3 ^b	0 ^b

Venom and antivenom (21, 28, 49 or 56 d) were incubated for 30 min at 37 °C before injection into mice. A dose of 30 µg to induced edema and nociception. In the appropriate studies these amounts of venom were incubated with 300 or 30 µl of antivenom.

^a*p* < 0.001 compared with the saline-group.

^b*p* < 0.01 compared with the venom-group.

Plasma levels of creatine kinase (CK) are usually applied to monitor rhabdomyolysis (De Meijer et al., 2003; Olivei et al., 2004). Pre-immunized mice (day 28) presented a reduction of 53% in CK activity in relation to naive mice injected only with venom. The percentage of CK activity reduction augmented after booster (day 49), showing in pre-immunized mice a small concentration of CK activity in plasma after venom injection, indicating low degree of rhabdomyolysis (Fig. 6).

3.6. Neutralization of microvascular alterations in pre-immunized mice

The dynamics of alterations in the microcirculatory network were determined using intravital microscopy by transillumination of mice cremaster muscle after topical application of 3 µg of venom during 40 min. Immediately after topic application of the venom (1 min), it was observed in non-immunized mice, the presence of abundant thrombi of varying sizes in venules, which accumulated in the vessel walls, and in arterioles a peculiar vascular constriction was observed (Fig. 7A and B). When the venom was administered in pre-immunized mice at day 49 these alterations were completely inhibited (Fig. 7C and D).

4. Discussion

The capacity of venoms or toxins from venomous marine animals to induce humoral immune responses with neutralizing antibodies has been described previously (White, 1998; Currie, 2003). In this work we have demonstrated that the harmful effect of *T. nattereri* envenoming could be prevented

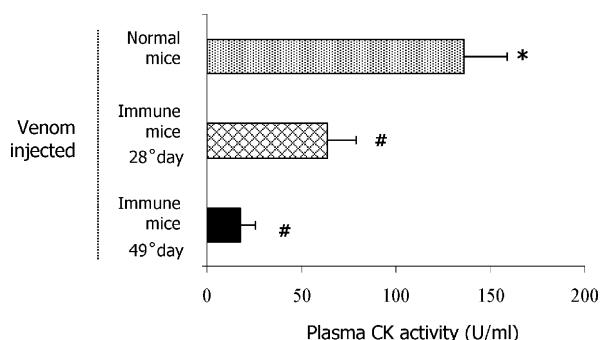


Fig. 6. Neutralization of CK activity in pre-immunized mice. At days 28 and 49 pre-immunized mice were injected (i.m., thigh muscle) with 3 µg of protein venom. After 3 h, blood was collected and sera were separated and immediately assayed for creatin kinase activity. CK activity was expressed in units/ml, one unit resulting in the phosphorylation of one nanomole of creatine per min at 25 °C. The results are reported as the mean ± SEM of three independent experiments and the control group was injected only with sterile saline. **p* < 0.001 compared with the saline-group; #*p* < 0.01 compared with the venom-group.

in previously immunized mice in a persistent way. Our results indicated that venom-specific memory IgG antibodies directly for all proteins in the venom are committed with this protection through its potent neutralizing role.

After bits of venomous animals, the passive transfer of heterologous-specific antibody allows that immediate toxic effect (edema, ischemia, pain, hemorrhage) could be neutralized in the victims (Hawgood, 1992). Regarding to *T. nattereri* venom, only *in vitro* neutralization assays were performed (Lopes-Ferreira et al., 2000). Nociception and necrosis were completely impaired when venom was previously incubated with *T. nattereri* antivenom, but not edema.

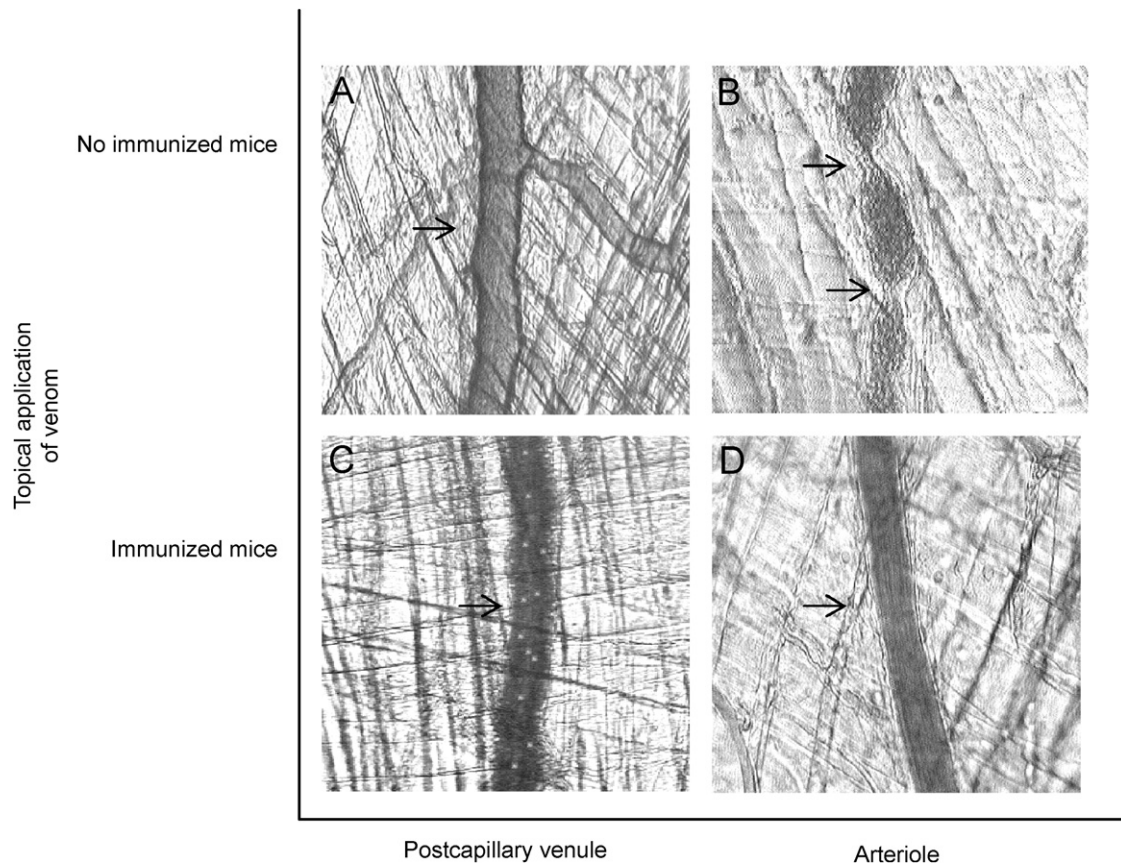


Fig. 7. Neutralization of microcirculatory alterations in pre-immunized mice. At day 49, pre-immunized mice were anaesthetized with an i.p. injection of sodium pentobarbital (20 mg/Kg body weight), placed on a water-heated bed (at 37 °C), and the cremaster muscle was exposed for topical application of venom (3 µg). Control experiments were performed using non-immunized mice by applying 3 µg venom. Stasis in a postcapillary venule (A) and arteriolar constriction (B) were observed 1 min after venom administration in non-immunized mice. In pre-immunized mice no alterations were seen in postcapillary venule (C) or in arteriole (D). The images were obtained using a x10/025 longitudinal distance objective/numeric aperture and 1.6 optovar.

Here, we confirmed the *in vitro* neutralizing capacity of *T. nattereri* anti-venom for toxic effects and thereafter we show that neutralizing antibodies obtained in a persistent immune response are more effective, inclusive for edematous reaction. These findings are in agreement with the great amount of antibody produced in a secondary response, beyond strength high of the interaction conferred to antibody from memory cell after the phenomenon of affinity maturation (Muramatsu et al., 2000).

Lopes-Ferreira et al. (2002) evidenced that the venom, in injured tissue, lead to an intense vascular congestion, stasis of blood flow in postcapillary venule and capillaries, beyond focal transient constrictions in arterioles. In addition, Lima et al. (2003) observed an inadequate leukocyte influx in venom-induced lesions, which was attributed to impaired blood flow, instead of an impairment in a

pro-inflammatory stimulus by venom. Lack of leukocytes influx delayed the restoration of injured tissue promoting necrotic process. Our result showed that after boosting (i.e., during the secondary response, Fig. 3) mice with high antibody levels do not present any alterations in venule or arteriole after topical application of venom on cremaster muscle. In addition, CK activity diminished in these mice with high neutralizing antibody levels corroborating the attenuation of the myonecrotic effect by venom. These findings suggest that the circulating antibodies could avoid the tissue necrosis for neutralizing venom toxins and allowing an efficient blood flux and development of local cellular inflammatory response (Fig. 7C).

The understanding of the pharmacological mechanisms of the *T. nattereri* venom is useful for our investigation. Nociception and edema induced by

venom appear relying on unlike inflammatory response. We have demonstrated that phospholipase A₂ or coagulant activities were not committed with nociception or edema induced by venom (Lopes-Ferreira et al., 1998). Furthermore, eicosanoids generated from the arachidonic acid by cyclooxygenase or lipoxygenase, as well as serotonin and nitric oxide were not related with these inflammatory symptoms (Lopes-Ferreira et al., 2004). Interestingly, it was verified that proteases of the venom acted like tissue-kallikrein leading to production of kinins (calidin), known mediator of inflammation. It was pointed that the kallikrein–kininogen–kinin system could be the major mechanism of nociception and an important component of edematous response, since the use of kallikrein-specific antagonist inhibited 80% of nociceptive response and 24% of edematogenic response (Lopes-Ferreira et al., 2004). According to these finding, a group of toxin from *T. nattereri*, denominated Natterins, was characterized and identified as endowed of kininogenase activity (Magalhaes et al., 2005).

The above-mentioned mechanism support the hypothesis that antigen-specific antibodies of high affinity neutralize the kininogenase activity of venom proteins impeding kinins production and consequently the nociception intensity and partially the edema. Additional mechanisms appear to be required for edematogenic reaction, which can be unrelated of protein components, therefore less impaired by antibodies. For example, lectins components. Lectins in the serum when complexed to sugar can activate enzymatic cascade of complement system, resulting in anaphylatoxins production like C5a, C4a, and C3a. These fragments from complement system can increase vascular permeability by provoking the histamine liberation by mast cells or acting directly in endothelial cells (Hugli, 1986; Wetsel, 1995). These data could explain the difficulty in reverting the edema caused by *T. nattereri* envenomation.

We accomplished an *in vivo* experimental model of immunization to conceive an accident in previously immunized victims. Our results suggested that more than one re-stimulus with venom is required to provide a drastic decreased sera CK activity and nociception, while the edema was partially impaired. Indeed, in animals only once sensitized the neutralizing antibodies were less potent, diminished the deleterious effects induced by the venom and did not affect edema. These

findings could explain the less severity of envenoming symptoms in victims that suffered successive attacks by *T. nattereri* fish (Auto, 1992). Interestingly, patients injured by *T. nattereri* presented high levels of specific IgG antibodies against the venom 6 months after the accident.

Our experiments point out that the affinity maturation and class-switch recombination of B cells and consequently the production of antibodies with neutralizing capacity could play an important role in the protection against envenoming by the venom of *T. nattereri* fish. It is known that very close contacts between CD4⁺ T and B cells are required to a sustainable humoral secondary response. CD4⁺ T cells through membrane molecules signaling (CD40-ligand) and cytokines action trigger the B cell proliferation, switching of antibody classes and B memory cell development (Mosmann and Coffman, 1989; Kawabe et al., 1994; Abbas et al., 1996). Following re-exposure to the same antigen, memory B cells undergo rapid proliferation, culminating in differentiation into plasma cells and in the secretion of high-affinity IgG, which might persist in the circulation for years, thus providing long-term antibody production (Zinkernagel et al., 1996; Gourley et al., 2004). However, because the half-life of serum IgG is <3 weeks (Vieira and Rajewsky, 1988), continuous antibody production is necessary to sustain IgG antibody titers for long-term antibody-mediated immunity. Thus, memory B-cell differentiation into short- or long-lived plasma cells represents an important source of protective antibodies for long-term immunity (Ochsenbein et al., 2000; Gourley et al., 2004; Manz et al., 2005). Although some studies have demonstrated that high systemic antibody titers measured by ELISA correlate with immunity against pathogens or toxins, efficient B-cell memory and protection depend critically on the neutralizing activity of these antibodies (Ochsenbein et al., 2000; Zinkernagel, 2003; Gourley et al., 2004). In fact, intraperitoneal immunization of BALB/c mice with venom plus adjuvant stimulated a mixed Th1/Th2 response, with remarkable IL-5 production and venom-specific memory IgG antibodies secretion that could be detected several months after priming (Grund et al., 2006).

In conclusion, the presence of neutralizing antibodies against *T. nattereri* venom in the serum of pre-immunized mice could change the outcome of lesion at site of posterior envenoming. Antibodies in consequence to specific immune response,

dependent of T lymphocyte activation, could minimize the symptoms of intense and immediate inflammatory reaction caused by *T. nattereri* venom. These findings prompt us to the possibility of development of immune therapeutic strategies using specific anti-venom as an efficient intervention for protecting human victims.

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