Toxicon 71 (2013) 49–56

Contents lists available at SciVerse ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Bothropic antivenom based on monoclonal antibodies, is it possible?

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ARTICLE INFO

Article history:
Received 8 January 2013
Received in revised form 3 May 2013
Accepted 15 May 2013
Available online 31 May 2013

Keywords:
Monoclonal antibodies
Antivenom
Bothrops atrox
Snake venom

ABSTRACT

Neutralizing monoclonal antibodies against three major toxic components of *Bothrops atrox* venom were produced and tested. The mAbs against phospholipase A_2, hemorrhagic metalloprotease, and thrombin-like enzymes were produced in large amounts and purified with caprylic acid followed by ammonium sulfate precipitation. Purified mAbs were analyzed by SDS-PAGE and their ability to neutralize the respective toxins was tested. Five Swiss mice were injected i.p. with 13.5 mg of pooled mAbs and challenged via s.c. route with venom. Survival rate was recorded for the next 48 h. All mice treated and challenged with venom survived, whereas only one mouse in the control group survived. Bleeding time in mice treated with mAbs was similar to that observed in control mice. Our results show that monoclonal antibodies neutralized the lethal toxicity of *Bothrops* venom and indicate that there is a reasonable possibility of developing antivenoms based on humanized mAbs to treat victims of venomous animals in the future.

1. Introduction

Antivenom immunotherapy still is the most effective treatment for victims of venomous animals, particularly snakes and scorpions. The efficacy of conventional antivenoms in neutralizing most of the toxic properties of the venom, including their lethality, has been improved by the introduction of modifications in production protocols dictated by new discoveries in basic immunology and protein chemistry. Moreover, immunization schedules are continuously adapted, new adjuvants have been introduced, and the resulting antibodies are better isolated, assayed, and characterized. Despite the progress in the preparation of conventional antivenoms, the risk of adverse reactions remains (Cardoso et al., 1993; Otero-Patiño et al., 1998; FUNASA, 2001). Although $F(ab')_2$ rich antivenoms are just as effective as the rich intact IgG in neutralizing venom toxins, their side-effects and ability to activate the host complement system have not yet been eliminated (Chippaux and Goyffon, 1991; Chippaux, 1991).

In Brazil, of the 17,704 snake accidents (incidence rate: 10.4 accidents/100,000 inhabitants) reported in 1999, 21% (3697) snake bites occurred in the northern region, where only 7.6% of the Brazilian population live (28.6 accidents/100,000 inhabitants) (IBGE, 2004). In that region, *Bothrops atrox* is the major snake group responsible for accidents (FUNASA, 2001).

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[1] In memoriam for her deep involvement in basic immunological research and discovering young talent for science.

0041-0101/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxicon.2013.05.005

B. atrox snake venom, like the venom from other *Bothrops* species, is a complex mixture that includes proteins exhibiting proteolytic activity (Rosenfeld, 1971; Kamiguti and Cardoso, 1989). Some of these proteins are proteases, whose substrates include components of the blood clotting system such as factors XII, X, and fibrinogen (Nahas et al., 1964; Kamiguti et al., 1992). Other proteins in the venom are zinc-dependent metalloproteinases (SVMP), most of which have hemorrhagic-inducing properties (Ohsaka, 1979; Bjarnason and Fox, 1988), and phospholipase A_2 (PLA_2), which have been shown to be involved in myonecrosis and inflammation (Maragananore et al., 1984; Gutierrez and Ownby, 2003).

Conventional antivenoms are prepared by immunizing horses with venom from a single snake species or a mixture of venoms from different species. The aim of immunization is to elicit high levels of antibodies that bind to and neutralize most relevant toxins. Conversely, immunization also elicits undesirable antibodies directed to non-toxic venom components and irrelevant venom epitopes, according to Harrison et al. (2011) 95% of IgGs comprising current antivenoms are not therapeutic. All the irrelevant proteins contribute to some antivenom therapy side effects. For instance, even though immunoglobulin G(T) is effective in the treatment of envenomed patients, a high incidence (37–87%) of early anaphylatic reactions requiring urgent treatment with adrenalin and antihistamines have been observed (Cardoso et al., 1993). Mixtures containing monospecific antibodies against a repertoire of epitopes in toxic venoms could help achieve two desirable immunotherapy requirements: the use of smaller amounts of antivenom, and higher specificity. In addition, the development of bothropic antivenoms should consider the need to reduce components other than the desired venom-specific IgG or their $F(ab')_2$ fragments and the use of a mixture of antibodies restricted to the relevant toxic venom components.

The aim of our work was to develop in mice monoclonal antibodies against some *B. atrox* venom components. Their neutralizing properties were analyzed using some well known pathological process induced by venom components as indicators. Three specific neutralizing mAbs (thrombin-like 6AD2-G5 clone, PLA_2 A85/9-4 clone, and Zn-metalloprotease 59/2-E4 clone) were prepared and tested by their ability to neutralize the main *B. atrox* venom toxins. These monoclonal antibodies will be used in the future as raw reagents to prepare hybrid antibodies expressing the mouse L_V and H_V regions molecular linked into human L_C and H_C regions.

2. Material and methods

2.1. Venom and antivenom

B. atrox venom was kindly provided by the Laboratório de Hepertologia, Instituto Butantan, São Paulo, SP, Brazil, in the lyophilized form. The venom used in this work is a pool of snake venom from Tucuruí, Pará, Brazil. Venom was weighed, diluted in distilled water to a final concentration of 10 mg/ml, and stored at −20 °C. Bothropic antivenom (batch 0512219/B, expiry date April 2009) was provided by Instituto Butantan.

2.2. Animals

Swiss mice weighing between 18 and 22 g were used throughout this study. Male and female adult BALB/c mice were also used. Animals were bred at the Vivarium of Isogenic Mice at the Center for Biosciences and Biotechnology of Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF). All animals were housed in controlled rooms and received water and food *ad libitum* until used. When necessary, 250 μg of ketamine chloridrate were used to anesthetize mice. This study was approved by the Experimental Animals Committee of UENF.

2.3. Monoclonal antibody production

2.3.1. Hybridoma and ascitic fluid

Hybridoma cells producing neutralizing mAbs against serineproteinase (thrombin-like) clone 6AD2-G5 (Petretski et al., 2000), PLA_2 clone A85/9-4 (Kanashiro et al., 2002), and hemorrhagin (Zn-metalloproteinase) clone 59/2-E4 (Barros et al., 1998) of *B. atrox* snake venom were cultured with DMEN-F12 medium, supplemented with 10% FCS and 10 μg/mL gentamicin. Each culture was expanded and 1×10^6 cells were inoculated i.p. in adult BALB/c mice previously i.p. injected with 400 μl mineral oil. After ten days, mice were euthanized by CO_2 inhalation, and the ascitic fluid was collected by abdominal puncture.

2.3.2. Monoclonal antibody purification

Monoclonal antibodies were purified with caprylic acid followed by ammonium sulfate precipitation (Steinbuch et al., 1970). Briefly, ascitic fluid was diluted 1:3 in 60 mM sodium acetate buffer, pH 4.0, and 0.4 mL caprylic acid was added under agitation for 30 min at room temperature for each 10 mL of ascitic fluid. The mixture was centrifuged at 5000× *g* for 1 h and the supernatant was collected. After centrifugation, the pH of supernatant was adjusted to 7.0 and ammonium sulfate was added under agitation to achieve a 45% concentration (w/v), and the mixture allowed to stand at 4 °C overnight. Precipitates were recovered by centrifugation at 5000× *g* for 30 min, redissolved and dialyzed against saline 0.9%, and immunochemically analyzed by SDS-PAGE and Western blot.

2.3.3. Immunochemical analysis

Samples of dialyzed mAbs were subjected to 12% polyacrylamide gel electrophoresis (SDS-PAGE), according to the method described by Laemmli (1970) with modifications. The samples were dissolved in sample buffer (0.5 M Tris–HCl buffer, pH 6.8 plus 10% SDS, 10% 2-β-mercaptoethanol, and 0.5% bromophenol blue dye), boiled at 100 °C, loaded on 12% polyacrylamide gel, and run at 150 v. Protein bands were stained with Coomassie brilliant blue and subjected to computerized densitometric analysis (Bozzo and Retamal, 1991). Western blot was performed, according to a previously described method (Towbin et al., 1979).

2.3.4. Monoclonal antibody titration by ELISA

Binding ability of the purified mAbs to the respective antigen was evaluated by ELISA test, according to the methodology described by Almeida et al. (1998). Briefly, *B.*

atrox venom (10 μg/mL) or enriched fraction of thrombin-like toxin (10 μg/mL) was diluted in 0.1 M carbonate/bicarbonate buffer (pH 9.6) and adsorbed to the ELISA plate. After a blocking step with gelatin, mAbs were diluted and added to wells. ELISA plates were incubated at 37 °C for 45 min followed by the addition of secondary antibody. The reaction was developed with o-phenylenediamine plus hydrogen peroxide, and color development was stopped with 50 μL 3 N H_2SO_4. Plates were read spectrophotometrically at 490 nm.

2.4. Neutralizing properties of mAbs

2.4.1. Neutralization of myonecrosis

Forty micrograms of myotoxic PLA_2 from *B. atrox* venom, purified according to the method described by Kanashiro et al. (2002), were preincubated with 140 μg A85/9-4 mAb, and then aliquots of the mixtures were injected into the gastrocnemius muscle of five Swiss mice. Similarly, samples of myotoxic PLA_2 were preincubated with non-related mouse IgG, instead of mAbs, and injected into five control mice. Blood samples were collected 1 h later and serum creatine kinase (CK) activity was measured using Merck Granutest 2.5.

2.4.2. Neutralization of hemorrhagic activity

Concentrations of 0, 25, 50, and 100 μg of purified 59/2-E4 mAb were incubated with 5 μg of *B. atrox* venom and injected i.d. into the shaved back of three Swiss mice. After 30 min, animals were euthanized and the size and intensity of subcutaneous hemorrhage in injected areas was estimated.

2.4.3. Neutralization of bleeding

3.5 mg samples of purified mAb 6AD2-G5 were pre-incubated with 150 μg of venom for 30 min at ambient temperature and i.p. injected into five Swiss mice (18–22 g). One hour after inoculation, the tips of tails were cut and immersed in 10 mL of distilled water until bleeding stopped (Assafim et al. 2006; Greene et al. 2010). The optical density of samples was determined in a spectrophotometer at 410 nm. In addition, 500 μL of horse $F(ab')_2$ bothropic antivenom was used as positive control group, whereas venom plus saline was injected into the mice as negative control.

2.4.4. Neutralization of lethality

Groups of five Swiss mice (18–20 g) were injected i.p. with 500 μL saline containing 5 mg 59/2-E4, 5 mg A85/9-4, and 3.5 mg 6AD2-G5 mAb. After 30 min, mice were challenged s.c. with 350 μg of crude venom. Controls were injected i.p. with 500 μL saline and challenged s.c. with 350 μg of venom. In another experiment 10.5 mg of mAbs (3.5 mg of each mAbs) were incubated with 200 μg of venom for 30 min at 37 °C followed i.p. injection into the mice. The control group received 200 μg of venom. Survival/death rates were recorded at 24 and 48 h.

2.4.5. Histological examination

A mixture containing 3.45 mg each of mAb 59/2-E4, A85/9-4, and 6AD2-G5 incubated with 200 μg of venom was injected i.p. in groups of six Swiss mice. Controls received only saline and venom. After 2, 24, and 48 h, two mice from each group were euthanized by CO_2 inhalation and their tissues and organs removed and fixed in 10% neutral *p*-formaldehyde. Tissues were dehydrated in ascending concentrations of ethanol (70–100%) and embedded in paraffin using an automatic tissue processor (TP 1020, Leica, Germany). Then, 5 μm sections were stained with hematoxylin-eosin and tissue sections were observed using a digital image analysis system coupled to a microscope (Zeiss axioplan/axiocam, Germany).

3. Results and discussion

We evaluated the lethality neutralization by monoclonal antibodies against three major toxic components of *B. atrox* venom to test the prospects of developing bothropic antivenom based on monoclonal antibodies. General features of purified mAb specific to serineproteinase (thrombin-like 6AD2-G5 clone), PLA_2 (A85/9-4 clone), and hemorrhagin (Zn-metalloproteinase 59/2-E4 clone) are shown in Fig. 1. When submitted to SDS-PAGE analysis, all three mAb preparations demonstrated two major protein bands, one of around 55 kDa and one of approximately 29 kDa, suggestive of immunoglobulin heavy and light chains, in addition to several minor contaminant bands (Fig. 1A). Western blot analyses, using anti-mouse IgG as the primary antibody, confirmed that the two major protein bands correspond to the mouse IgG heavy and light chains (Fig. 1B). In addition, densitometric analysis of the SDS-PAGE was performed to estimate the purity of the mAb preparations. The purity grade for mAbs A85/9-4 (46%), 59/2-E4 (45%), and 6AD2-G5 (37%) is shown in Fig. 1C.

The ability of purified mAbs to recognize the respective toxins by ELISA is shown in Fig. 2. The mAbs A85/9-4 and 59/2-E4, which recognize phospholipase A_2 and Zn-metalloproteinase, respectively, were able to bind the antigen at the lowest concentration tested (10 ng/mL) at a relatively high optical density when compared to the control sample (Fig. 2A, B). However, mAb 6AD2-G5 was not as effective as the other two, as the final dilution that recognized the antigen was 8 μg/mL (Fig. 2C). In a previous study from our group, Petretski et al. (2000) showed that mAb 6AD2-G5 was very effective in neutralizing the catalytic activity of the thrombin-like enzyme and also it recognized a conformational epitope of the toxin. In fact, this could explain why mAb 6AD2-G5 weakly binds the target antigen adsorbed to the solid phase of the ELISA plate, as the adsorption of the antigen to the plastic surface could result in slight changes in the antigen epitope structure.

The neutralizing properties of the mAbs against their respective toxins are shown in Fig. 3. The ability of three different mAb 59/2-E4 concentrations to neutralize hemorrhage induced by 5 μg of venom is shown in Fig. 3A. Hemorrhage neutralization by the mAb 59/2-E4 was seen in a dose-dependent manner from 25 μg to 100 μg of antibody tested. Conversely, the ability of mAb 59/2-E4 to neutralize the enzyme's catalytic activity was negligible (data not shown) when azocasein was used as substrate. This result indicates that mAb 59/2-E4 does not bind to the

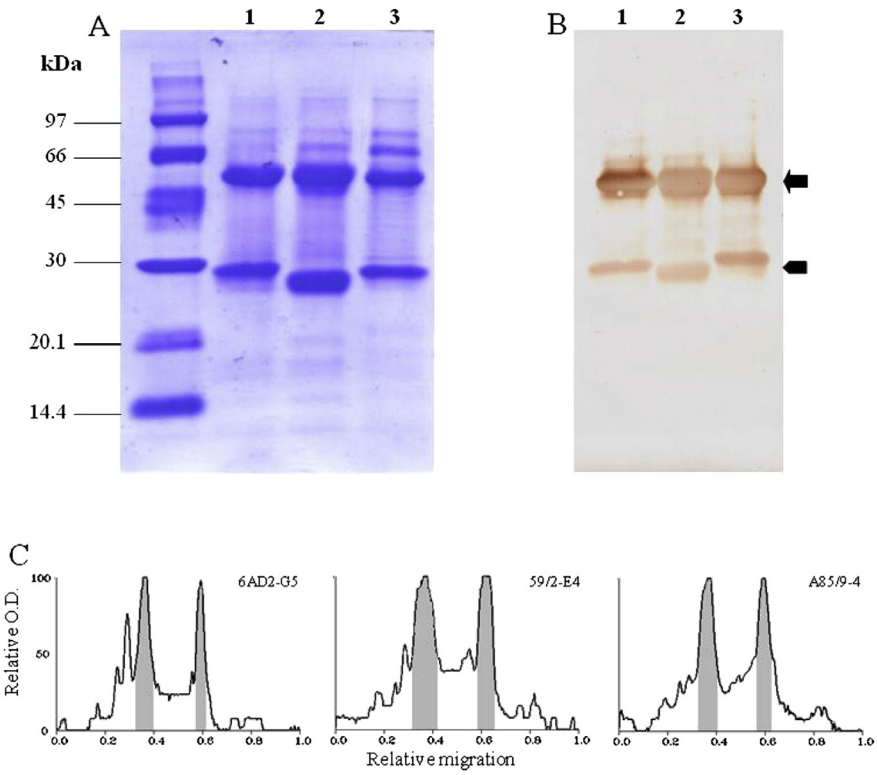

Fig. 1. SDS-PAGE and Western blot analysis of purified mAbs. A – SDS-PAGE analysis of the mAbs purified with caprylic acid and ammonium sulfate precipitation. Lane 1: purified A85/9-4 mAb (15 μg); lane 2: purified 59/2-E4 mAb (15 μg); lane 3: purified 6AD2-G5 mAb (15 μg). Left lane: molecular weight marker. B – mAb heavy and light chain identification by Western blot analysis. SDS-PAGE, run according to Figure 1A, was transferred to nitrocellulose membrane and incubated with anti-mouse IgG conjugated to peroxidase. Lane 1: A85/9-4 mAb (15 μg); lane 2: 59/2-E4 mAb (15 μg); lane 3: 6AD2-G5 mAb (15 μg). C – Densitometry analysis of the SDS-PAGE.

catalytic domain of *B. atrox* metalloproteinase. The same pattern was observed for mAb MAJar 3 against jararhagin, a *Bothrops jararaca* PIII metalloproteinase (Tanjoni et al., 2003). MAJar 3 efficiently neutralized the hemorrhagic activity of jararhagin without blocking the catalytic activity of the enzyme and was shown to bind to the C-terminal portion of the disintegrin domain, which could be in conformational proximity to the catalytic domain or functionally modulate the hemorrhagic activity of the snake venom metalloproteinase. Because mAb 59/2-E4 neutralized the biological activity of hemorrhagin, which has properties similar to those of MAJar 3, it is possible that both mAbs recognize the same epitope.

The myotoxic activity induced by PLA_2 was inhibited when the enzyme was incubated with mAb A85/9-4 followed by injection into the gastrocnemius muscle of mice (Fig. 3B). The CK serum level was drastically reduced in mice treated with the specific mAb when compared to control mice, treated with the non-specific IgG. Moreover, mAb A85/9-4 was not able to neutralize the catalytic activity of the enzyme (data not shown). In fact, a lack of correlation between the enzymatic activity of snake venom PLA_2 and myotoxic activity has been shown in several studies (Kini and Evans, 1989; Diaz-Oreiro and Gutiérrez, 1997; Kanashiro et al., 2002).

The effective neutralization of mAb 6AD2-G5 was previously assessed *in vivo* in a murine tail bleeding model (Greene et al., 2010). Fig. 3C summarizes bleeding time of a group of mice injected i.p. with a mixture of mAb 6AD2-G5 or antivenom with *B. atrox* venom. Mouse-tail bleeding time indicated no significant differences in blood loss between mice treated with mAb and antivenom. Petretski et al. (2000) showed that mAb 6AD2-G5 was also very effective in neutralizing fibrinogen-clotting and catalytic activities of the thrombin-like enzyme of *B. atrox* venom. In addition, it also neutralized the thrombin-like enzyme from other *Bothrops* species. These results indicate that the neutralizing properties of mAb 6AD2-G5 could be used for new therapeutic approaches in bothropic accidents. Interestingly, we easily succeeded in neutralizing the catalytic activity of the thrombin-like enzyme in the venom using mAb 6AD2-G5. We then immunized rabbit, chicken, rat, and guinea pig to obtain sera to neutralize the catalytic activity of PLA_2 and Zn-metalloproteinase from *B. atrox* venom. The resulting sera recognized the enzymes, but could not block their catalytic activity (data not shown).

Lethality assay performed in mice pretreated with mAb mixture showed 100% survival and venom control group of mice experienced an 80% death rate. When mAbs mixture plus venom were incubated before injection into the mice 80% of animals survived and the control group of venom 100% of death was observed (Table 1), showing that mAbs assayed by both methods neutralize lethality of venom. Although the protein concentrations in those experiments

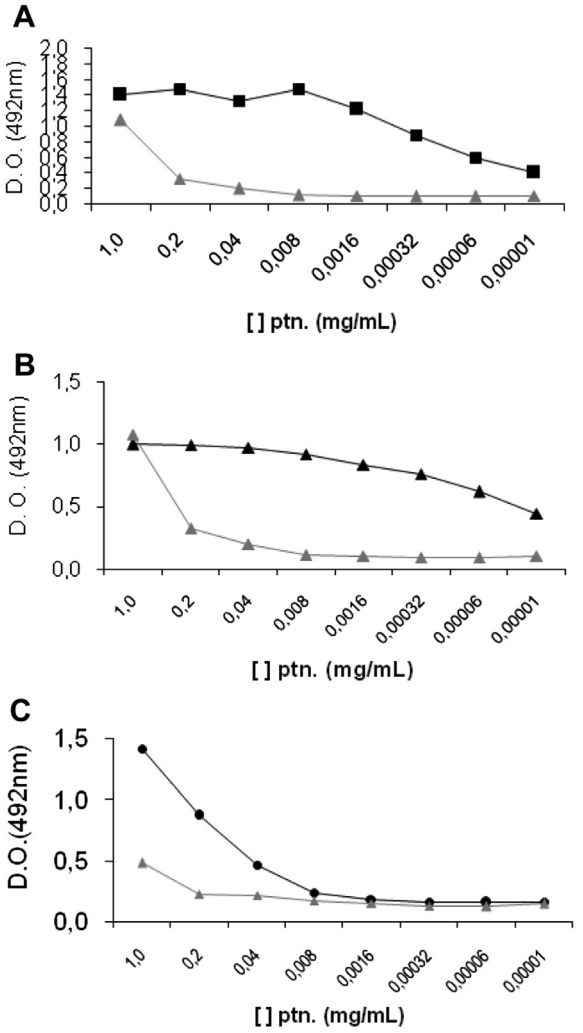

Fig. 2. Titration of monoclonal antibodies by ELISA. Nunc maxisorp ELISA plate was activated with 10 μg/mL of antigens and incubated with several dilutions of monoclonal antibodies. A – mAb A85/9-4; B – mAb 59/2-E4; C – mAb 6AD2-G5.

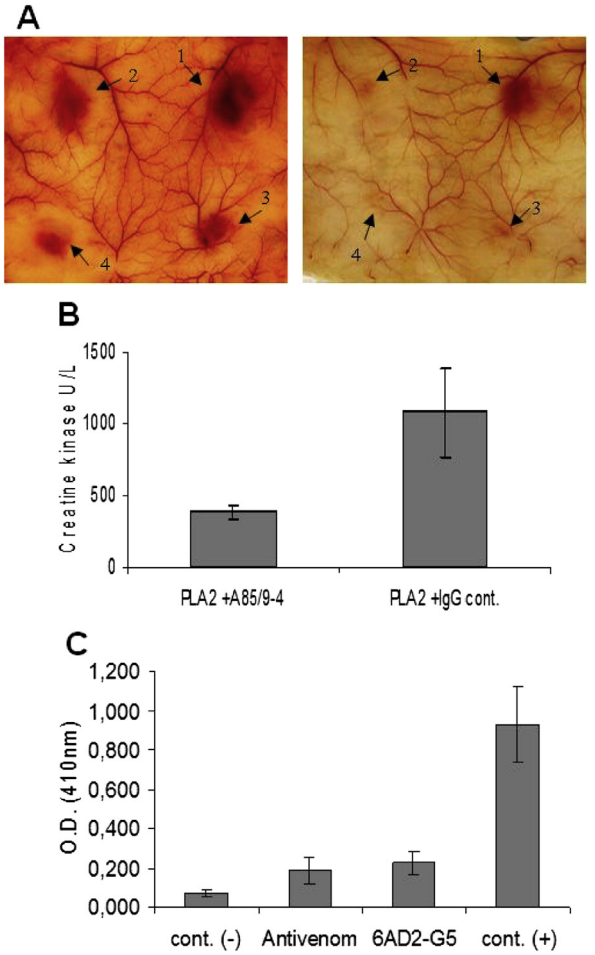

Fig. 3. Neutralization of the toxic components of *Bothrops atrox* venom by monoclonal antibodies. A – Neutralization of hemorrhagic activity. Concentrations of 59/2-E4 mAb (2) 25 μg, (3) 50 μg, and (4) 100 μg, (1) venom positive control, were incubated with 5 μg of the venom followed by i.d. injection into the mice. Hemorrhagic area was evaluated 30 min later. B – Inhibition of myotoxic activity by mAb A85/9-4. 140 μg of mAb were incubated with 40 μg of purified PLA_2, followed by IM injection into the mouse gastrocnemius muscle. Myotoxicity was evaluated by serum CK level. C – Bleeding time induced by the venom.

were high, our antibody preparations were not free from contaminants (55–63% impurity). Therefore, from the total protein administered to the animals, less than 40% could be considered specific antibodies. A similar experiment performed by da Silva et al. (2007) using polyvalent antivenom also showed lower antivenom efficiency when antivenom was injected into the animal prior to local challenging with venom, when compared to antivenom and venom pre-incubation followed by local injection into the mouse. We believe that antivenom administration by i.p. or i.v. route and venom challenge performed subcutaneously are more similar to the natural mechanism of ophydic accidents.

Mouse tissues used in lethality neutralization assays underwent histopathological analysis. Two hours after inoculation, the animals presented bristled hair, dyspnea, and exhaustion, in contrast to animals treated with the mAb pool, whose clinical signs were less evident. During necropsy, euthanized animals exhibited severe blood collection in the peritoneal cavity (hemoperitoneum). This observation was much more evident in the positive control group than in the group treated with the mAbs. Histopathology of peritoneal wall sections (serous membrane and skeletal muscle of the floor of the dorsal cavity) in mAb-treated animals (2 h)

Table 1
Mice protection assay.

Groups	mAbs/route	Venom/route	Survived/total
[a]Antibody	[c]13.5 mg/i.p.	350 μg/s.c.	5/5
[a]Venom	Saline/i.p.	350 μg/s.c.	1/5
[b]Antibody	[d]10.5 mg/i.p.	200 μg/i.p.	4/5
[b]Venom	Saline/i.p.	200 μg/i.p.	0/5

[a] mAbs and saline was injected i.p. and 30 min later venom was injected via s.c.
[b] Pool of mAbs and venom or saline and venom were incubated for 30 min and injected i.p.
[c] mAbs mixture: 5 mg of mAbs A85/9-4 and 59/2-E4, and 3.5 mg of mAb 6AD2-G5.
[d] mAbs mixture: 3.5 mg of each mAbs A85/9-4, 59/2-E4 and 6AD2-G5.

showed vasodilatation signs with expressive numbers of intravascular leukocytes (leukocytosis), edema, and discreet hemorrhage (Fig. 4A). Cavity samples from control animals were represented by accentuated endomisial edema with muscular fiber dissociation and moderate hemorrhage (Fig. 4B). In addition, some muscle fibers exhibited coagulation necrosis (hyalinized: without striations and slightly eosinophilic). The pancreas from mice treated with mAbs exhibited hemorrhage and discreet edema in the intestine/pancreas interface (Fig. 4C). Conversely, controls that received only *B. atrox* venom showed evidence of extensive solid hemorrhage and acinar cell dissociation in pancreatic samples using conventional microscopy (Fig. 4D). Although Camargo et al. (2005) observed acute pancreatitis induced by phospholipase A_2 from *Bothrops* venom in rats, the changes in the peritoneal cavity and pancreas found in our study are probably associated to the direct contact between the mAb and venom mixture injected into the peritoneal cavity.

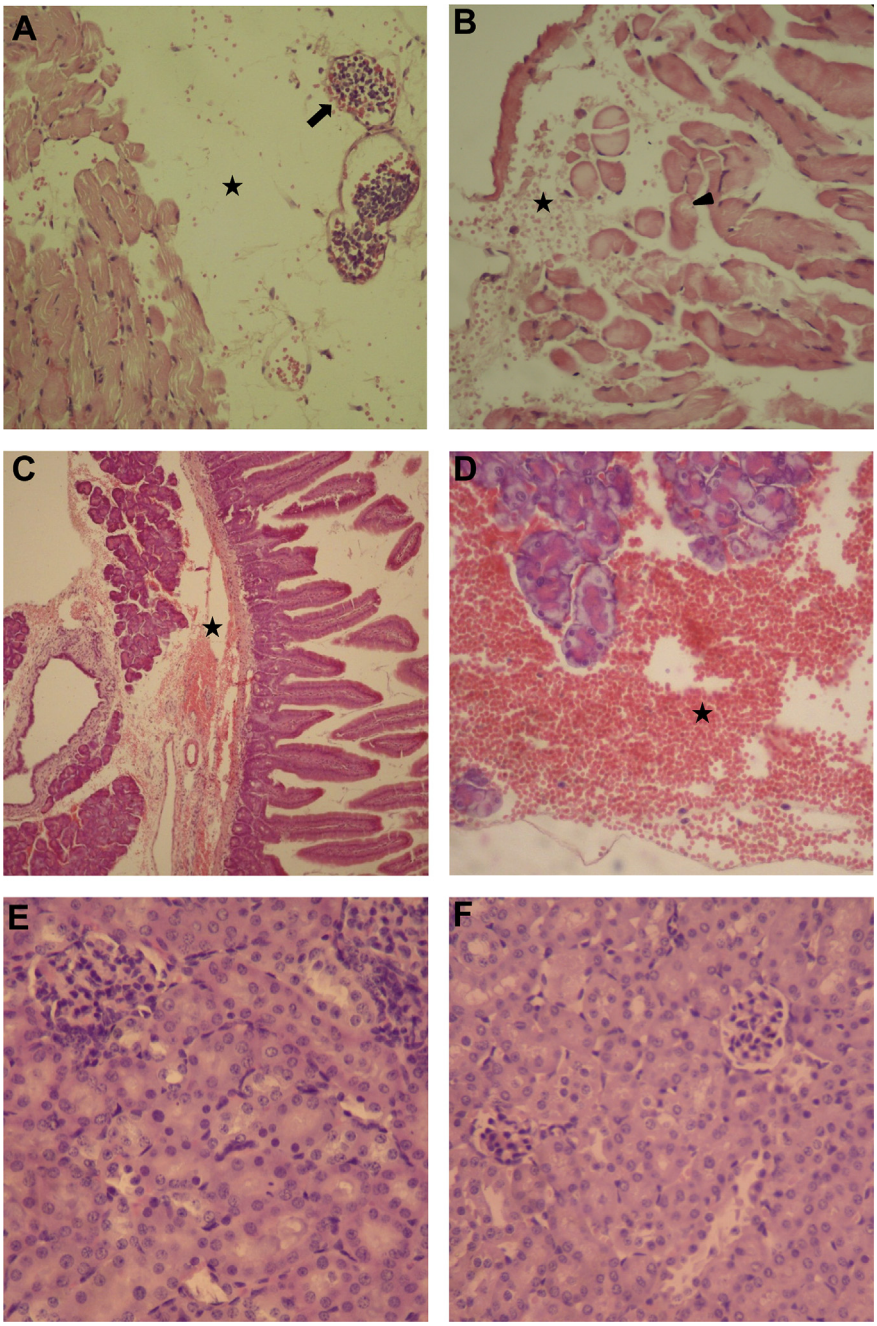

Fig. 4. Histopathological analysis of mice inoculated i.p. with a mixture of mAbs and venom. A, B: peritoneum wall; C, D: pancreas; E, F: kidney. A, C, and E: mice treated with mAb mixture; B and D: mice injected with venom; F: normal mice. Arrow – intravascular leukocytosis; star – edema and hemorrhage; arrowhead – muscle fiber hyalinization. Magnification A, F – ×200; B, D, E – ×400; C – ×100.

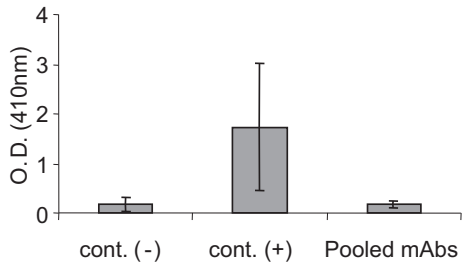

Fig. 5. Neutralization of the hemorrhage induced by *Bothrops atrox* venom. Anesthetized mice were inoculated i.p. with the previously incubated mixture of venom and mAb pool. Bleeding time was determined 30 min later.

Kidney histopathology from animals treated with mAbs (2 h) was not significantly different from that of control animals (Fig. 4E, F). Although human deaths by *Bothrops* envenomation are generally associated to acute renal failure (Milani Jr. et al., 1997), renal failure was not well reproduced in murine models. Moreover, several studies that evaluated renal alterations caused by bothropic venom in rats were performed using i.v. injection or ex-vivo renal perfusion (Gutiérrez et al., 2009; Boer-Lima et al., 1999), and this could explain the lack of alterations in kidney samples evaluated in this study.

Mice inoculated with the mAb and venom mixture lost the same quantity of blood as negative controls when bleeding time was determined (Fig. 5). In contrast, high blood loss was observed in mice given venom only. To our knowledge this is the first study to show that neutralizing monoclonal antibodies against three major *Bothrops* venom toxins abrogates the venom activity.

Our results show that a pool of three mAbs neutralizes the lethal activity of *B. atrox* venom. Nevertheless, we believe that the action of toxins present in minor concentration in the venom (Neiva et al., 2009), which could act alone or synergistically with other toxins, must also be considered. Moreover, intraspecific (Núñez et al., 2009) and interspecific (Queiroz et al., 2008) variation in venom characteristics should also be investigated when developing antivenoms based on monoclonal antibodies.

Monoclonal antibodies similarly to polyclonal antibodies when injected into xenogeneic animals induce antibody production against either their constant and variable regions resulting in a short circulating life. Owing to these restrictions monoclonal antibodies were developed to serve as raw materials for design different smaller antivenom antibody formats Fab and scFv (Holliger and Hudson, 2005). The aims of our work were therefore to obtain monoclonal antibodies directed to biologically significant toxin epitopes expressed on *B. atrox* lethal toxins. The corresponding hybridomas will be used to develop humanized or antibody fragments as nonimmunogenic *in vivo* biopharmaceutical endowed with superior biodistribution and blood clearance properties.

Financial support

This work was supported by FAPERJ, CNPq. WDS is supported by grants from the following agencies: CNPq, Bolsa de Produtividade, Nível A, Proc. No: 301836/2005 – 1; FAPERJ "Programa – Cientistas de Nosso Estado", Proc. No: E – 26/100.628/200; FAPESP, Proc. No: 09/52804 – 0 and INCTTOX program of the CNPq and FAPESP.

Acknowledgments

The authors are grateful to Instituto Butantan for providing *B. atrox* venom and horse $F(ab')_2$ anti-bothropic antivenom.

This manuscript was reviewed by a professional science editor and by a native English-speaking copy editor to improve readability.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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