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Development of Monoclonal Antibody Anti-African *Bitis arietans* Snake Toxin Phospholipase A_2

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Abstract

This work is part of long-term research to improve the quality of antivenom antibodies based on cumulative information gathered from established knowledge of the immune response cellular and molecular mechanisms as well as from molecular animal venom toxins. There are two main objectives: first, the introduction of punctual modifications to antitoxin antibody (Ab) production for immediate use; second, the use of Ab hyper variable regions to model scFvs that have high toxin neutralizing potency. The first objective has been successfully accomplished in recent years. Polyclonal Abs (pAbs) that has high antigen (Ag) potency recognition has been developed. For the second objective, the use of purified-characterized toxins as antigens to produce monoclonal Abs (mAbs) is ongoing. This manuscript contains data indicating that purified and enzymatically characterized PLA_2 from *Bitis arietans* venom induces IgA mAbs that recognize epitopes and neutralize toxic domains with desirable potency. The obtained mAb will serve as a hypervariable sequence source for single chain fragments variable (scFvs). The present study highlights the use of purified venom toxins as substitutes for complete venoms in immunization procedures.

Abbreviations

LD_{50}: Median Lethality Dose; Ab, Antibody; Ag, Antigen; D, Diversity Segments; ELISA: Enzyme-Linked Immunosorbent Assay; ED_{50}: Median Effective Dose ; $F(Ab')_2$: Ab Two Fragments; Igg: Immunoglobulin Gama; HAT: Hypoxantine Amino Transferase; HGPTR: Hypoxantine-Guanine Phoribosyltransferase; Ig: Immunoglobulin; J: Joining Segments; KSCN: Potassium Thiocyanate; Mab: Monoclonal Antibody; TK: Thymidine Kinase; Pabs: Polyclonal Antibodies; PLA_2: Phospholipase A_2; Scfvs: Single Chain Fragments Variable; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; V: Variable Light Chain; VH: Variable Heavy Chain

Introduction

The currently used snake antivenoms are produced in horses immunized with whole venoms, partially isolated and their neutralizing potencies evaluated according to World Health Organization guidelines [1]. Resulting immunoglobulin's are IgG / $F(ab')_2$ mixtures of anti-toxins and anti-non toxins Abs. Although their neutralizing potency expressed in terms of ED_{50}, is evaluated, other important Abs qualities, such as affinity and specific activity, are not. High doses of intravenously injected antivenom are used to treat snake bites". Although are therapeutically effectives, these antivenoms activate the complement system, resulting in anaphylatoxin formation solubilization of the venom component-antivenom immune aggregates and induction of adverse hypersensitivity reactions [2-10]. In addition, their cost of production is unaffordable for some developing countries [11]. Actual advances in antibody biotechnology largely used to produces therapeutic or immune diagnostic and images reagents were not yet incorporated into antivenom production. Therefore, incorporation of these advances in antivenom technology must be stimulated. To reach these objectives two decisions must be observed. First, introduction of punctual modifications into the immunization protocols aiming stimulating development of memory rather than naïve T and B cells. The longer life span of memory-immunologic T and B cells, compared to naïve cells, requires 10-50 less antigen to produce an effective immunological response that is characterized by the production of Abs with high affinity to specific epitopes [12-14].

The splenic naïve-B cells express cell surface antigen receptors, BCRs, in which the surrogate L-chain and definitive H-chain were constructed by somatic rearrangements in the absence of Ag contact. These rearrangements involve by chance associations of single regions from the multiple V-region, the five J-joining regions and the four D-regions, resulting in VJ surrogate L-chain and V(D) J definitive H-chain. Resulting BCRs upon contact with the specific Ag suffer punctual hyper mutations in both chains with substitution of the surrogate by definitive L-chain and increasing in both Ab specificity and affinity for the epitopes [15,16].

Second, substitute whole venom by purified relevant toxins as immunogens. Antitoxins Abs antitoxins predominate on unimportant Abs production. The therapeutically antivenom doses must be reduced.

Based on these known aspects of the immune response, we introduced strategic modifications into immunization protocols, such as reducing the venom dosage and increasing the number of immunizations as well as time between immunizations. Lower responding animals, identified by Ab titration using ELISA, were eliminated during immunization. Antivenom Abs with high specificity, affinity, and venom lethality-neutralizing potency against *Crotalus* spp. as well as snake African venoms were developed. These expected results stimulated further refinements in antivenom biotechnology. The elimination of antibodies against

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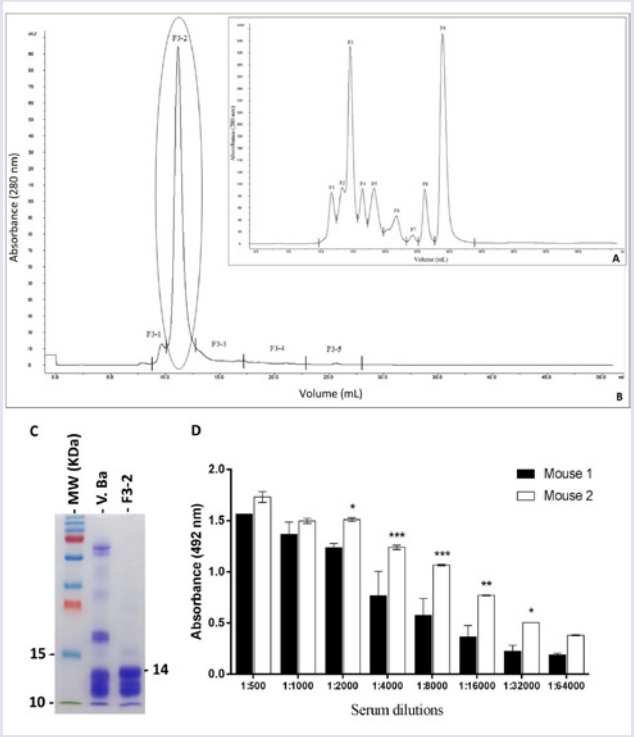

Figure 1: Purification of PLA_2 from crude *Bitis arietans* venom.
Figure 1A: First chromatographic run: Twenty milligrams of *Bitis arietans* venom were dissolved in five milliliters of 50 mM ammonium acetate, and applied to a Superose 12 HR (10/30, ÄKTA FPLC, Pharmacia, Uppsala, Swedish column. Nine protein fractions, F1 → F9 were obtained and PLA_2 activity, assayed by using a phospholipid FRET substrate kit, was restricted to the elution fraction F3.
Figure1B: First chromatographic run: The peak F3 was concentrated and submitted to a Superdex 75 10/300 GL column (GE Healthcare, Bio-Sciences AB, LC, UK) chromatography. Five proteins peaks, F3-1 →F3-5, were obtained, and PLA_2 activity was restricted to F3-2 peak.
Figure 1C: SDS-PAGE electrophoresis: Concentrated F3-2 protein peak exhibits one strong protein band, ± 14 kDa similar to one present in *Bitis arietans* crude venom.
Figure 1D: Generation anti-PLA_2 Abs: After the 3rd immunization with concentrated F3-2 protein fraction, significant antibody titers anti-PLA_2 was developed by BALB/c mice. Y-axis, Absorbance (492 nm); X-axis: 1→8, serum dilutions factor 2: 1:500 →64000. Data are displayed as means ± SD from assays in duplicates. Serum samples collected before immunization from both mice were running in parallel; Obtained Absorbance (492 nm) < 0.05 (data not shown).

non-toxic components from therapeutically used antivenoms and the identification of hypervariable amino acid sequences that are responsible for the anti-toxin activities of high-quality antibodies are the next objectives. The use of purified venom toxins as Ags to develop mAb antitoxins represents the next experimental approaches [17,18].

PLA_2, which has a molecular weight of approximately 14 kDa, is a toxin that belongs to the *Viperidae* family. This enzyme hydrolyses phospholipids and stimulates the release of arachidonic acid from the cell membrane [19]. The enzyme structure is characterized by a rigid three-dimensional structure that is composed of seven disulphide bridges, with an internal His-Asp dyad sequence. Ca^{++} is required for the interaction with and cleavage of specific substrates [20]. Snake venom PLA_2 leads to neurotoxicity, muscle cell lysis and anticoagulation [21,22]. Based on its molecular properties and wide distribution among snake venoms, PLA_2 from *Bitis arietans* venom was selected as the Ag for the immunization of mice to obtain mAbs.

Results

Purification of PLA_2

Representative *Bitis arietans* venom samples were submitted to a molecular exclusion Superose 12 HR chromatography column (10/30, ÄKTA FPLC, Pharmacia, Uppsala, Sweden). Nine protein fractions, P1, P2, P3, P4, P5, P6, P7, and P8 were obtained and PLA_2 activity, assayed by using a phospholipid FRET substrate kit, was restricted to the elution fraction P3 Figure 1A. The fraction P3 was concentrated and subjected to a second molecular exclusion Superdex 75 chromatography. Five protein peaks, F3-1, F3-2, F3-3, F3-4 and F3-5 were obtained Figure 1B. PLA_2 activity also assayed by the phospholipid FRET substrate kit was restricted to the peak fraction F3-2. After concentration, the peak F3-2 was analysed by SDS-PAGE and tested for PLA_2 activity. Figure 1C shows that F3-2 resulted in a single protein band with ± 14 kDa. This protein band corresponds in terms of molecular mass to a similar protein band present in *B. arietans* venom that was running in parallel. Upon assay

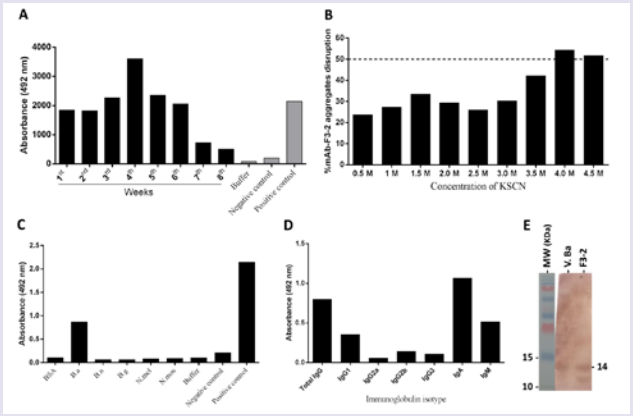

Figure 2: Imunochemical characterization of mAbs anti-purified PLA_2.
Antibody titers, **Figure 2A** shows by standard ELISA assay that secreted mAb anti-F3(2a) antibody into the culture medium when titrated by ELISA using either *Bitis arietans* venom or purified F3(2a) as antigens attained at maximal values after 4th week incubation. Y-axis: Absorbance (492 nm); X-axis: weeks of culture (1→8); 9, buffer; negative control; 10, negative control. 11, positive control.
Antibody affinity. **Figure 2B** shows by modified ELISA assay that mAb anti-purified F3(2a) preformed aggregates mAbs and purified PLA_2 or *Bitis arietans* venom need 4.5 M caotropic agent potassium KSCN concentration to be 50% disrupted. Y-axis: Y-axis: Absorbance (492 nm); X-axis: KSCN M concentrations.
Antibody inter Bits spp, species venom crossing-reacting. **Figure 2C** shows in assay by standard ELISA that the mAb anti-purified F3(2a) antibody ability of recognizing activity was restricted PLA_2 present in B. arietan. Y-axis: Absorbance (492 nm); X-axis: BSA; 2, *B. arietans*; 3, *Bitis nasicornis*; 4, *Bitis gabonica*; 5, *Naja melanoleucal*; 6. *Naja mossambica*;7, Buffer; 8, negative control; 9, positive control. F3(2a) fraction.
Identification of mAb anti-F3(2a) immunoglobulin isotype. Figure 2D: Immunoglobulin isotype identification. The figure shows by standard ELISA that mAbs are a heterogeneous mixture of IgA > IgM>IgG1. Y-axis: Absorbance (492 nm); X-axis, polyclonal IgG antibodies specifics to poly mice Igs, IgG1, IgG2, IgG2b, IgG3, IgA and IgM.
Recognition of PLA_2 by mAbs anti-F3(2a) PLA_2 purified fraction. Samples of *Bitis arietans* venom (center) and purified F3(2a) (right) were separated in SDA-PAGE, the separated protein bands were electrotransferred to membrane, and submitted immunoblotting assay. Molecular weight markers (left).

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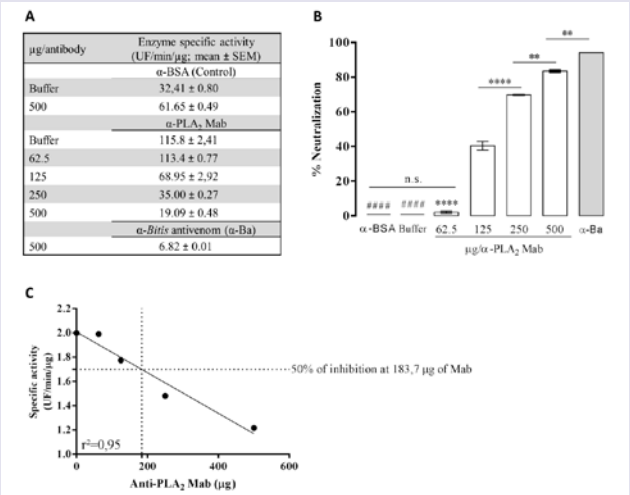

Figure 3: *In vitro* mAb anti-F3(2) characterization. Once immunochemically characterized the neutralizing properties of the mAb anti- PLA_2 (F3(2) fraction) from *Bitis arietans* were investigated. First, the PLA_2 enzyme specific activities of *Bitis arietans* venom were evaluated using the EnzChek® Phospholipase A_2 Assay kit. Next, samples of venom containing determined specific activities are treated with various amounts of mAb anti- PLA_2 and both residual and blocked enzyme specific activity determined or calculated. The Enzyme Specific Activity decreases progressively with the increase of the mAb anti- PLA_2 (**Figure 3A**, left) directly indicating its neutralizing action (**Figure 3B**) and specific activity (**Figure 3A**, right). Log10 linear interpolation of *Bitis arietans* venom PLA_2 neutralization by mAbs: Y-axis, PLA_2 activity; X-axis; anti-PLA_2 mAb (µg) (**Figure 3C**).

also by phospholipid FRET substrate kit this fraction exhibited PLA_2 activity (data not shown).

Mice immunization

Two adult male BALB/c mice were immunized three times at 21-day intervals with F3-2 purified protein fraction (20 µg) diluted in PBS, pH7.2-7,4 containing 4 mg of potassium hydroxide. The anti-PLA_2 Abs titers and affinities were measured using ELISA in samples of serum harvested five days after the 3rd immunization. As shown in Figure 1D, although with different titers, both mice developed anti-PLA_2 Abs. Splenic B cells were harvested from the mice 2 also after 3rd immunization and subjected to *in vitro* culture (Figure 1).

Hybridoma production

Step # 1: Activated B cells ($HGPRT^+$, V (D) J Ig^+, TK^+) were fused with myeloma cells ($HGPRT^-$, Ig^-, Tk^+) in the presence of HAT medium containing hypoxanthine, aminopterin, and thymidine (Sigma Aldrich). Aminopterin blocks the enzymes required for the synthesis of nucleotides by the *de novo* pathway, but nucleotide generation by the salvage pathway continues once activated B cells express HGPRT (hypoxanthine-guanine phosphoryltransferase) and TK (thymidine kinase).

Step # 2: Positive hybridomas, identified based on viability and the production and secretion of anti-F3-2 mAbs, were propagated. Representative hybridomas were submitted to three sequential rounds of cloning. The immunochemical and neutralizing properties of the anti-F3-2 Abs fractions were evaluated (Table 1).

Step # 3: Clonal propagation was performed by cultivation for 8 weeks in 25 cm^3 plastic bottle bioreactors containing DMEM-F12 medium supplemented with 10% FCS, 10% gentamicin and 5 mM of 2-β-mercaptoethanol at 35 °C under a 5% CO_2 atmosphere. The secreted mAbs were collected, and the titers and affinities of these mAbs were measured using ELISA. As shown in Figure 2A, antibody secretion peaked at the 4th week of culture. The resulting F3-2-mAb complexes formed at the maximal ELISA, Y-axis absorbance (492 nm) required 4.5 M of the chaotropic agent KSCN for 50% dissociation (Figure 2B). These mAbs were endowed with high specific activity, as indicated by their ability to exclusively recognize PLA_2, either against purified F3-2 or in *Bitis arietans* venom (Figure 2C).

Step # 4: The use of specific goat anti-mouse Ig isotypes in ELISA indicated that the anti-F3-2 mAbs belongs to the IgA isotype (Figure 2D). The specificity of the anti-F3-2 mAbs was further confirmed by Western blotting analysis, which showed that this antibody was able to recognize a single protein band corresponding to the F3-2 fraction in *Bitis arietans* venom (Figure 2E). $HGPRT^+$-TK^+ immortal hybridomas producing anti-F3-2 mAbs and culture supernatants were stored in liquid nitrogen.

Step # 5: After the fusion of activated B cells ($HGPRT^+$, V(D)J Ig^+, TK^+) with myeloma cells ($HGPRT^-$, Ig^-, Tk^+) in the presence of HAT medium containing hypoxanthine, aminopterin and thymidine, positive hybridomas were cloned by three successive limiting dilution steps. Clones resulting from the 3rd step were expanded and produced mAbs that were subsequently submitted to immunochemical and toxin neutralizing assays (Table 1).

Step # 6: The secreted mAbs were harvested from culture medium, and the antibody titers, affinity, specificity, immunoglobulin isotypes and antigen recognition were again assayed. Figure 2A indicates that secreted mAb anti-F3-2 antibodies in culture medium attained maximal values after the 4th week of incubation when titrated with either *Bitis arietans* venom or purified F3-2 as antigens by ELISA. Axis-Y: ABS 492 nm; Axis-X: weeks of culture (1→8); 9, negative control; 10, positive control. As shown in Figure 2B, preformed aggregates of anti-purified F3-2 mAbs required 4.5 M KSCN, a chaotropic agent, to achieve 50% disruption, as demonstrated using ELISA. Figure 2C shows that the anti-purified F3-2 mAb activity was restricted to *Bitis arietans* venom and the purified F3-2 fraction

Table 1: Cloning of hybridomas producing mAb anti-*Bitis arietans* venom F3(a2) PLA_2 purified fraction.

Table 1 After fusion of activated B cells ($HGPRT^+$,V(D)J Ig^+, TK^+) with myeloma cells ($HGPRT^-$, Ig^-, Tk^+) in the presence of HAT medium containing hypoxanthine, aminopterin and thymidine positive hybridomas were cloning by three successive limit dilutions steps. Clones resulting from the 3rd step were expanded and produced mAbs submitted to immunochemical and toxin neutralizing assays.

Activated B cells ($HGPRT^+$,V(O)J!I Ig^+, TK^+) myeloma cells + ($HGPRT^-$, Ig^-, Tk^+)	1st cloning	2nd cloning	3rd cloning
51 individual fused cells (three days cultured)	Clone P3B7	Clone P3B7 (F2)	Clone P3B7(F2/B7)
51 survived hybridomas	Six hybridomas individually cultutured	Six hybridomas individually cultutured	15 hybridomas individually cultured
100% secreting mAb anti-F3-2fraction	85% secreting mAb anti-F3-2fraction	6% secreting mAb anti-F3-2fraction	100% secreting mAb anti-F3-2fraction

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(positive control) according to ELISA. Y-axis: 492 nm; X-axis:1, BSA; 2, *Bitis arietans*; 3, *Bitis nasicornis*; 4, *Bitisgabonica*; 5, *Naja. melanoleuca*; 6. *Najamososambica*; 7, Buffer; 8, mice preimmune sera and 9, hyper immune mice sera. (D) ELISA of the anti-F3-2 immunoglobulin isotype mAbs. Axis-Y: ABS 492 nm; Axis-X: specific anti-isotypes: 1 and 10, anti-IgM; 2 and 11, anti-IgG2a; 3 and 12, anti-IgG1; 4 and 13, anti-IgA; 5 and total Igs; 6 and 15, anti-IgG2b; 7 and 16, anti-IgG3. (E) The results confirmed the mono specificity of the mAb based on its ability to recognize a single 14-kDa protein in both *Bitis arietans* venom (V. Ba) and the purified F3-2 fraction according to Western blotting. Table 1 schematizes the overall mAbs production (Table 1 and Figure 2).

The mAb inhibits F3-2 enzymatic activity

Once immunochemically characterized, the neutralizing properties of the anti-F3-2 mAb against *Bitis arietans* venom were investigated. The PLA_2 enzyme-specific activities (SA) of *Bitis arietans* venom were evaluated using EnzChek® Phospholipase A_2 Assay kit (Molecular Probes®, Invitrogen, MA, USA) according to the manufacturer's instructions and expressed as UF/min/µg. SA of *Bitis arietans* venom = 115.82 ± 2.41.Subsequently, samples of *Bitis arietans* venom were treated 30 min at room temperature, with different mAbs concentration and residual PLA_2 activity evaluated and the results expressed as % of inhibition. Samples of fixed venom amount (0,5 µg) containing 115. 8 ± 2.41 of PLA_2 activity were treated with various amounts of mAb anti- PLA_2, and the residual PLA_2 activity was assayed on synthetic substrates as described. Figure 3A, shows the residual PLA_2 activity in venom samples treated with mAb. Figure 3B shows the corresponding of percentage of inhibition. Stimulated by the general principle orienting evaluation the snake venom lethality neutralization, basing in data Figure 3A and B the mAb neutralizing potency was evaluated by calculating the amounts of mAb anti-purified PLA_2 that blocked 50% of the enzyme activity . The Figure 3C indicates that 183.7 µg of specific mAb inhibit 50% of SA of PLA_2 present in *Bitis arietans* venom (Figure 3).

Discussion

Abs play an important role in interfering with complex cross talk involving toxin molecular domains and soluble or cell-fixed receptor protein targets. These antibodies exert this role by first specifically recognizing and combining with epitopes on toxic domains through complementarity-determining regions and then by presenting the resulting aggregates to phagocyte cell receptors via their Fc domains. Prompt epitope recognition and strong interactions confer efficient toxin neutralization by Abs.

Horses immunized with African snake venom from *Bitis arietans, Bitis gabonica rhinoceros* and *Bitis nasicornis* respond by producing neutralizing IgG antitoxins against PLA_2, hyaluronidases, and fibrinolytic enzymes. Inhibition assays were performed *in vitro* using specific synthetic substrates [18]. Horse anti-*Bitis spp* and anti-*Naja spp* antivenoms exhibit high specificity and affinity, positive qualities that are responsible for the high neutralizing potency of the antivenoms. Although these antivenoms are currently used to treat snakebite victims in Mozambique, further improvements to their antivenom properties are needed. The reduction or even elimination of activity against nontoxic venom components in antivenoms would help to potentiate their toxin-neutralizing specific activity and reduce the effective therapeutic dosage. Patients would be helped by a reduction in undesirable adverse reactions [9,10]. Additionally, a reduction in the therapeutic doses currently used to treat snakebite victims could positively affect snakebite treatment costs.

In the present study, we demonstrated that substituting purified toxins for crude snake venoms in immunization procedures is reliable. Correct selection of venom and toxins must be the first step. *B. arietans* is representative of sub-Saharan African snake fauna and is a broadly important contributor to snake bites in the region, which were decisive facts in selecting this snake [23-27]. PLA_2 was selected because it is a well-characterized enzyme from the molecular perspective and is widely distributed among animal tissues and venoms.

Since the main purpose of the present study was to develop specific mAbs against *Bitis arietans* PLA_2, the first step was to purify this enzyme and measure its activity. A purified fraction with high PLA_2 specific activity was obtained using a combination of molecular exclusion chromatography and a phospholipid FRET substrate kit. This fraction had desirable immunogenicity as evaluated by the emergence of reasonable Abs titers in immunized BALB/c mice.

B cells isolated from the spleen at peak antibody production can synthesize nucleotides through the *de novo* pathway because they are capable of multiplying in an *in vitro* culture medium containing hypoxantine. Therefore, these cells are $HGPTR^+$, Ig^+, and TK^+, exhibiting the first set of conditions required for use of these cells as counterparts in the fusion step with myeloma cells, which are $HGPTR^-$, Ig^-, TK^+, to obtain hybridomas that use purine and pyrimidine bases to synthetize nucleotides via the salvage pathway. These enzymes are centrally involved in recycling the building blocks of nucleic acids by attaching the original or adenine-modified forms to ribose, generating a 5-ribosyl-phosphoryl [28]. Hybridomas secreting stable mAbs were essentially developed as previously described, selected by HAT, cloned and used as sources for anti-F3-2 mAbs.m [29].

Recent in vitro analysis using *Bothrops atrox* venom as phospholipase A_2 source reinforced previous observations that Asp49 and Lys49 myotoxinsare synergics in their action. The assay was performed using murine myogenic cell line C2C12 (ATCC-CRL1772) as targets and lactate dehydrogenase release assay as indicators [30].

Anti-F3-2 mAbs produced at a large scale exhibit high titers, affinity, and PLA_{2-} neutralizing potency, thus satisfying the three objectives of the present study. First, anti-F3-2 mAbs provide support for the use of purified snake venom toxins as immunogens. Second, the results of the present study indicated that it is important to obtain mAbs that are specific for the most relevant toxins present in the venom, and third, pooled mAb anti-toxins were submitted as regulatory quality controls to treat snakebite victims.

Materials and Methods

Reagents

-AP buffer (Tris-HCl, 100 mM; sodium chloride, 100 mM; and magnesium chloride, 5 mM; pH 9.5).

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-BSA, bovine serum albumin.

-Complete MMT80 (MarcolMontanide ISA 50, 2 mL; sodium chloride, 0.15 M, 5 mL; Tween 80, 1 mL; and lyophilized BCG, 1 mg).

-Citrate buffer (citric acid, 0.1 M, and monobasic sodium phosphate, 0.2 M; pH 5.0).

-Developing solution for Western/dot blotting (AP buffer, 5 mL; NBT solution, 33 µL; and BCIP solution, 16.5 µL).

-Dulbecco's Modified Eagles Medium F-12 (DMEM-F12, GIBCO, Invitrogen Corp., CA, EUA)

- Incomplete MMT80 (MarcolMontanide ISA 50, 2 mL; sodium chloride, 0.15 M, 5 mL; and Tween 80, 1 mL).

-NBT solution (NBT, 50 mg; dimethylformamide, 700 µL; and H2O, 300 µL), BCIP solution (BCIP, 50 mg, and dimethylformamide, 1 mL).

- Solution A for SDS buffer (Tris, 6.25 mM, and SDS, 6.94 mM; pH 6.8).

- OPD solution (OPD, 20 mg, and citric acid, 1 mL) and substrate buffer for ELISA (citrate buffer, 5 mL.

- OPD solution, 100 µL; and H_2O_2 30 volumes, 5 µL).

-SDS buffer for non-reducing conditions (solution A, 8.5 mL; glycerol, 1 mL; and bromophenol blue 1%, 2 mL).

-PBS buffer (potassium chloride, 2.6 mM; monobasic potassium phosphate, 1.5 mM; sodium chloride, 76 mM; and disodium phosphate, 8.2 mM; pH 7.2-7.4).

- Tris buffer (Tris-HCl, 25 mM-pH 7.4).

Other than the NBT/BCIP solution system, obtained from Molecular Probes (USA), all reagents were obtained from Sigma-Aldrich (USA).

-The chaotropic agent was potassium thiocyanate (KSCN).

Mice

BALB/c and Swiss male mice, 4-6 weeks old, were obtained from "Biotério do CBB/UENF"; maintained in propylene boxes; and receiving food and water ad libitum. All animal experiments were performed in accordance with WHO guidelines using protocols approved by the Ethics Committee of Animal Usage in Research of "Centro de Biociências e Biotecnologia – UniversidadeEstadual do NorteFluminense" - Darcy Ribeiro, Campos dos Goytacazes, RJ, Brazil [31].

Snake venom

African snake venoms were purchased from Venom Supplies Pty Ltd. (Murray Street, Tanunda, Australia). Brazilian snake venoms were supplied by the "Laboratório de Venenos, Instituto Butantan, São Paulo, SP, Brazil". Venom lethality was evaluated after injecting (i. p.) 100 µL of 0.15 M NaCl with increasing dosages of venom into groups of five Swiss mice. The death/survivals ratios were recorded during 48 h of observation and the LD_{50} values were estimated using the Spearman-Karber method [32].

PLA_2 purification

Twenty milligrams of *Bitis arietans* venom dissolved in five milliliters of column eluent (ammonium acetate 50 mM) were applied to a Superose 12 HR (10/30, Amersham Pharmacia Biotech AB, Uppsala, Sweden) molecular exclusion chromatography column that was previously equilibrated with 50 mM ammonium acetate. Proteins were progressively eluted at a flow rate of 24 ml/h. The eluted protein content was estimated based on absorbance (280 nm) using a UPC-900 spectrophotometer (Amersham Pharmacia Biotech). The enzymatic activity and protein content of the eluted fractions were determined as described below. Fractions exhibiting enzymatic activity were concentrated and submitted to another cycle of molecular exclusion using a Superdex 75 10/300 GL column (GE Healthcare, Bio-Sciences AB, equilibrated and eluted with 50 mM ammonium acetate. Eluted fractions corresponding to protein peaks were lyophilized, diluted in PBS (pH 7.4) and centrifuged at 3000 rpm/min to eliminate precipitated. This step was essential for calculating the PLA_2 specific activity. The PLA_2 activity was assayed on a synthetic substrate (structure described below). The purified PLA_2 protein concentration was measured using the bicinchoninic acid method [33]. Specific activity was calculated using the PLA_2 activity/mg of protein ratio. Purified PLA_2, peak F3-2, was stored at -80 °C until use to immunize mice.

Protein quantification

The protein concentrations of the venoms, purified PLA_2, pAbs and mAbs were assessed using the bicinchoninic acid method with a Pierce BCA Protein Assay kit (Rockford, IL, USA), using BSA as the standard protein [33].

PLA_2 activity measurements

Bitis arietans PLA_2 activity was determined by the fluorimetric assay using the EnzChek Phospholipase A_2 Assay Kit (Invitrogen, MA, EUA) according to the manufacturer's instructions [34]. Briefly, 2.5 µg samples of venom or purified PLA_2 in 50 µL of PBS, pH 7.2-7.4 were mixed with 50 µL of the phospholipid FRET substrate provided in the kit in white 96-well microtitre plates and immediately analysed on a filter-based multi-mode microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). The emission wavelength was measured at absorbance 515 nm with an excitation wavelength of 460 nm at 37 °C. All enzyme assays were performed in duplicate, and specific activity was expressed as arbitrary fluorescent units per minute per microgram of venom (UF/min/µg). Venom from Crotalus durissus terrificus (0.5 µg) or PBS, pH 7.-2 - 7.4 with the phospholipid FRET substrate was used as positive or negative controls, respectively.

Production of anti-PLA_2 hybridomas

Hybridomas were produced according previously described method [29].

Step # 1: Peritoneal feeder layer cell preparation: Feeder layer cells were prepared by injection of 5 ml of Dulbecco's Modified Eagles Medium F-12 (DMEM-F12, GIBCO, Invitrogen Corp., CA, EUA) into the peritoneal cavity of 5 week-old mice. After 15 min, peritoneal fluid was collected, and the cells were centrifuged at 1200 x g for 5 min. The pellets were recovered and suspended in 10 mL of DMEM-F12 medium plus 10% Foetal Calf Serum (FCS, GIBCO).

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Aliquots of these cells were distributed in 96-well plates (100 µL / well) and incubated for 24 h at 37°C under 5% CO_2 atmosphere.

Step # 2: Mice immunization: Two adult male BALB/c mice were immunized three times at 21-day intervals with purified *Bitis arietans* PLA_2 (20 µg) diluted in PBS, pH 7.4 containing 4 mg of potassium hydroxide. The anti-PLA_2 Ab titers and affinities were measured using ELISA in serum samples harvested five days after the third immunization.

Cell fusion: Spleen B cells positive for $HGPTR^+$, Ig^+, and TK^+ from mice producing significant anti-PLA_2 Abs titers were harvested and fused with myeloma (A2 HGPTR), Ig^- and TK^+ immortal cells using PEG 4000 (Gibco). The resulting mixed cell population included the hybrids, heterokarion-unfused B and myeloma cells, and fused dead cells.

Fused heterokarions cell isolation: The cell mixtures were suspended in DMEM-F12 (Gibco) containing 10% FCS, 20 µg/mL gentamicin (Gibco), 50 µg/mL β-mercaptoethanol, and B cell-myeloma hybrid selection agent HAT (hypoxanthine-aminopterin-thymidine; Sigma). Samples of cells (2x105)were seeded onto 96-well plates (Corning, USA) pre-coated with a monolayer of mice peritoneal feeder cells and incubated for ten days at 37 °C. Anti-PLA_2 Abs in the culture medium was measured using ELISA.

Step # 3: Myeloma screening: After HAT selection of hybridomas grown in the presence of hypoxanthine, aminopterin, and thymidine, potent hybridomas expressing anti-PLA_2 immunoglobulin were cloned by limiting dilution. Samples containing 1-2 fused cells were seeded onto 96-well plates (Corning, USA) pre-coated with a monolayer of mice peritoneal feeder layer cells and incubated at 37 °C, and anti-PLA_2 Abs in the culture medium were titrated using ELISA.

Myeloma propagation: Screened hybridomas producing high-affinity anti-PLA_2 mAbs were propagated by cultivation in 25-cm^3 plastic bottles containing DMEM-F12 medium (Gibco) supplemented with 10% FCS (Gibco), 10% gentamycin and 5 mM 2-β-mercaptoethanol for 3 days at 35 °C under 5% CO_2 atmosphere. Viable hybridomas secreting anti-PLA_2 Abs were stored in liquid nitrogen, and anti-PLA_2 was isolated from the supernatants.

mAbs Anti-PLA_2 identification: The resulting volume of hybridoma culture medium was concentrated, immunoglobulins were purified, isotypes were identified, and ability to neutralize enzymatic and haemorrhagic activity was evaluated.

mAb anti-PLA_2 concentration: Immunoglobulins secreted by the hybridomas were precipitated by slowly adding an equal volume of a saturated ammonium sulphate solution with stirring to 500 ml of culture medium. After 3 h, the solution was placed at 4°C and incubated overnight without stirring. The solution was centrifuged at 3000 rpm for 30 min at 4°C, and the resulting pellet was suspended in a 50% saturated ammonium sulphate solution, followed by centrifugation under the same conditions. The recovered precipitates were dissolved in 25 mL of 0.15 M NaCl. Ammonium sulphate was removed by passing the solution through a 10 mL SEFACRYL S100 column equilibrated with 1.5 M PBS buffer (0.1 M sodium phosphate and 0.1 M NaCl, pH 7.4), and 1.0 mL samples were eluted with 0.1 M α-D-galactose. After protein content determination using the bicinchoninic acid method (BCA Protein Assay Kit, Pierce, Rockford, IL), representative samples were analysed using SDS-PAGE [32]. Subsequently, the immunoglobulin isotype was determined, and the inhibition of PLA_2 enzyme activity and haemorrhagic action were evaluated in vitro and *in vivo*.

Step # 4: mAb anti-PLA_2 immunoglobulin isotype identification: Ab isotyping was performed using a rapid ELISA mouse mAbs Isotyping Kit (Pierce Biotechnology, IL, EUA) according to the manufacturer's instructions. Briefly, ninety-six well ELISA plates (Costar) were coated with 5 µg of purified PLA2 overnight. After blocking with PBS plus 5% nonfat milk for 1 h, the wells were incubated with mAb dilutions that were previously shown to obtain an absorbance plateau at 492 nm via ELISA. After blocking with PBS plus 5% nonfat milk plates for 1 h, the wells were incubated with secondary Ab, and after washing, ELISA was performed and the mAb isotypes were identified.

Measurement of mAbs anti-PLA_2 titers using ELISA: Ninety-six well ELISA plates (Costar) were coated overnight with 5 µg of *Bitis arietans* venom or purified PLA_2. After blocking for 1 h with PBS buffer containing 5% nonfat milk, the wells were incubated with diluted antivenom serum or myeloma culture medium for 2 h, and the bound pAbs or mAbs were detected using peroxidase-conjugated rabbit anti-mouse IgA Abs and revealed with the peroxidase substrate OPD (o-phenylenediamine; Sigma Aldrich, St. Louis, MO, USA). The reactions were stopped with 4N sulphuric acid, the absorbance (ABS) was measured at 492 nm, and the results were plotted graphically: Y-axis, ABS 492 nm values, and X-axis, assayed samples dilutions. Maximum ABS 492 nm values were used as reference titers.

Step # 5: Measuring anti-PLA_2 mAbs affinity by modified ELISA: Ninety-six well ELISA plates (Costar) were coated with 5 µg of purified PLA_2 overnight. After blocking for 1 h with PBS containing 5% nonfat milk, samples of hybridoma culture media collected after 4 weeks of incubation, which were previously shown to reach an absorbance plateau of 492 nm via ELISA, were added and the plates were incubated for 2 h. After 1 h of blocking with PBS plus 5% nonfat milk, 0.5 to 4.5 M concentrations of the chaotropic agent KSCN (Sigma Aldrich, St. Louis, MO, USA) were added, and the plates were subsequently incubated for 10 min, washed and then ELISA assays were continued. The mAbs affinity index was calculated as the ratio of the KSCN-treated antibody to the previously determined reference titer and multiplied by 100.

SDS-PAGE and Western blot analyses

SDS-PAGE was performed as previously described [35]. Purified PLA_2 and *Bitis arietans* venom (10 µg) in SDS buffer (0.15 M, pH 8.0 Tris-Glycine plus SDS and 2-mercaptoethanol) was applied to 15% SDS-PAGE gels. After electrophoresis for 80-100 min in a Power Pac Basic (Bio-Rad), the protein bands were stained with Coomassie blue, and protein migration was evaluated using molecular mass standards run in parallel. The protein bands recognized by the developed Abs were detected using Western blotting, after immunoblotting on nitrocellulose. The bands were detected using peroxidase-conjugated rabbit Abs anti-mouse IgA secondary Abs and the peroxidase enzyme substrate DAB (3, 3'-Diaminobenzidine, Sigma Aldrich, St. Louis,

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MO, USA) [36].

Evaluation of the neutralizing ability of mAbs

To determine the ability of mAbs to neutralize PLA_2 activity, venom was pre-incubated with different concentrations of mAbs or with anti-BSA IgG and Butantan-specific antivenom (negative and positive control, respectively) for 30 min at room temperature. These mixtures (50 µL in PBS, pH 7.2 - 7.4) were added to white 96-well microtitre plates containing 50 µL of a phospholipid FRET substrate. After mixing, the reaction was immediately analysed in a filter-based multi-mode microplate reader at 37 °C (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) using an emission wavelength of 515 nm and excitation wavelength of 460 nm. All enzyme assays were performed in duplicate, and specific activity was expressed in UF/min/µg. Wells with 0.5 µg of *Bitis arietans* venom in PBS buffer were assayed in parallel as a negative control.

Statistical Analysis

The data were analysed using one-way ANOVA and Dunnett's Multiple Comparison Test or two-way ANOVA and Bonferroni's Post Test (using standard antivenom for comparison). P values < 0.05 were considered significant. Analysis was performed using Graphic PAD Prism 5 for Windows (Graph Pad Software, San Diego, USA).

Conclusion

In this work we attempted to obtain data aiming at introducing punctual modifications on actual snake antivenoms production. First, to avoid including of other Abs which are not related to the real important toxins, the *Bitis arietans* whole venom was substituted by purified PLA_2 one of its well recognized important toxins, as an Ag source. Second, based on established knowledge of immune response mechanisms, high specific, affinity and neutralizing mAbs were developed. This experimental information might be useful in production of mAbs to neutralize other important venom toxins. Adequate mixtures of mAbs against relevant toxins are putative substitutes for actually used polyclonal antivenom Abs".

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