**Premolis semirufa** (Walker, 1856) Envenomation, Disease Affecting Rubber Tappers of the Amazon: Searching for Caterpillar-Bristles Toxic Components

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**Abstract**

**Background:** The caterpillar of the moth *Premolis semirufa* (Lepidoptera: Arctiidae), commonly named *Pararama*, is endemic of the Amazon basin. Accidental contact with these caterpillar bristles causes local symptoms such as intense heat, pain, edema and itching which last for three to seven days; however, after multiples contacts, it may induce joint-space narrowing and bone alteration, as well as degeneration of the articular cartilage and immobilization of the affected joints. Specific treatment for this disease does not exist, but corticosteroids are frequently administered. Despite of the public health hazard of *Premolis semirufa* caterpillar poisoning, little is known about the nature of the toxic components involved in the induction of the pathology.

**Methodology/Principal Findings:** Here we have investigated the biological and immunochemical characteristics of the caterpillar’s bristles components. Analysis of the bristles extract in *in vitro* assays revealed the presence of proteolytic and hyaluronidase activities but no phospholipase A2 activity. In *vivo*, it was observed that the bristles extract is not lethal but can induce an intense inflammatory process, characterized by the presence of neutrophils in the paw tissues of injected mice. Furthermore, the bristles components stimulated an intense and specific antibody response but autoantibodies such as anti-DNA or anti-collagen type II were not detected.

**Conclusion:** The results suggest that *Premolis semirufa* caterpillar bristles secretion contains a mixture of different enzymes that may act together in the generation and development of the clinical manifestations of the Pararama envenomation. Moreover, the high immunogenicity of the caterpillar bristles components, as shown by the generation of high antibody titers, may also contribute to the induction and establishment of the inflammatory disease.

**Introduction**

Moths and butterflies are insects of the Lepidoptera order, of which the young stage is called larva. The larval form of some families of moths containing urticating hairs is known as caterpillar.

Although caterpillar venoms have not been analyzed as much as the venoms from snakes, spiders and scorpions, there are many reports on the characterization of bristles extracts from a variety of species. Coagulation disorders have been reported after contact with the Saturniidae caterpillars from *Lonomia* genus. Since 1989, accidents involving *Lonomia obliqua* species were reported in South of Brazil, Argentine, Paraguay and Uruguay [1,2]. The physical contact with this caterpillar induces a toxic secretion from bristle, which promotes local and systemic symptoms in the victim between 6 to 72 hours after contact, such as burning sensation, intense hematuria, disseminated intravascular coagulation-like reactions (severe depletion of the coagulation factors) and secondary fibrinolysis [2]. Serious clinical complications, such as acute renal failure and intracranial hemorrhage may also occur [1,3].

The Brazilian caterpillar of *Premolis semirufa* usually called as *Pararama*, belongs to the Arctiidae family. The genus *Premolis* contains four species: *P. semirufa*, recorded in the Amazon region in Brazil, French Guiana, Ecuador, Peru and Panama; *P. excavata* found in Panama; *P. rhyssa* in Peru and *P. amaryllis* in French Guiana.

*Premolis semirufa* feeds of *Hevea brasiliensis*, the rubber tree found in the Amazon forest (Figure 1). The tappers, when collecting the latex, can stick their fingers in the trunk of the rubber trees to
Materials and Methods

Caterpillar bristles extract

Caterpillars from *Premolis semirufa* were collected in the city of São Francisco do Pará, Pará, Brazil, and maintained at the Immunochemistry Laboratory, Butantan Institute, SP, Brazil. The bristles extract was prepared after exposing the caterpillars to 4°C for few minutes; the bristles were cut off with scissors at their insertion in the tegument, avoiding any tegument incision and, then, suspended in cold phosphate-buffered saline - PBS (0.1 mM sodium phosphate, 1.5 mM potassium phosphate, 137 mM sodium chloride and 2.7 mM potassium chloride, pH 7.2). This suspension was macerated with the aid of a glass stick, homogenized and centrifuged at 560 × g for 20 min at 4°C. The supernatant was collected and its protein content was determined by using the BCA Protein Assay Kit (Pierce Biotechnology, MA, USA). Supernatant aliquots were stored at −80°C until use. Venoms from *Micrurus hemprichii* and *Bothrops jararaca* snakes, which were used as positive controls in the assays for determination of PLA₂ and hyaluronidase activities, respectively, were supplied by Herpetology Laboratory from Butantan Institute, SP, Brazil. The authorization to access the venoms of *Premolis semirufa* caterpillar, *Bothrops jararaca* and *Micrurus hemprichii* snakes were provided by the Brazilian Institute of Environment and Renewable Natural Resources - IBAMA - a Brazilian Ministry of the Environment’s enforcement agency (permission no. 01/2009).

Ethics statement

BALB/c strain male mice aged 2 months and weighing 18–22 g were obtained from Central Animal Breeding from Butantan Institute, SP, Brazil. All the procedures involving animals were in accordance with the ethical principles in animal research adopted by the Brazilian Society of Animal Science and the National Brazilian Legislation no. 11,794/08. Protocols were approved by Institutional Animal Care and Use Committee (protocol approval number 413/07).

Electrophoresis

The caterpillar bristles extract (10 µg of protein) was solubilized in sample buffer, using non-reducing and reducing conditions, and separated on 12% SDS-PAGE gel [15]. Molecular weight markers were included in all runs. Gels were stained with silver [16].

Phospholipase A₂ activity

The Phospholipase A₂ activity of *Premolis semirufa*’s bristles extract was determined as described by Price III [17], with some modifications. Samples of the extract (4 µg or 16 µg of protein), 20 µL HCl (positive control), or 20 µL PBS (negative control) were mixed in 96-well microtitre plates. 180 µL of an assay mixture containing 10 mM Trition X-100, 5 mM phosphatidylcholine, 1.5 mM HEPES, 10 mM calcium chloride, 0.9% sodium chloride Brilliant Blue R-250, phenylmethylsulfonyl fluoride (PMSE), 1,10-phenanthroline, hyaluronic acid, anti-mouse IgG horseradish labelled with peroxidase (IgG-HRPO), native salmon sperm DNA and collagen from bovine trachael cartilage were purchased from Sigma–Aldrich (Missouri, USA). Anti-mouse IgM, -IgG1, -IgG2a HRPO-conjugate and anti-mouse IgG2b, IgG3 biotin-conjugate were purchased from BD Bioscience (California, USA). Anti-mouse IgG labelled with alkaline phosphatase (IgG-AP), 5-bromo-4-chloro-3-indoly-phosphate (BCIP) and nitroblue tetrazolium (NBT) were from Promega Corp. (Wisconsin, USA). Brij-35 P was purchased from Fuka – BioChemika (Werdenberg, Switzerland). Fluorescence Resonance Energy Transfer (FRET) substrates were synthesized and purified according to Arasjo et al. [14].
and 0.03% (wt./vol.) bromothymol blue dye in water, at pH 7.5 and 37°C, were added. The plate was analyzed at λ 620 nm in a spectrophotometer (Multiskan EX, Labsystems, Finland) after 5 min of incubation and the linearity of the reaction was verified by linear regression (MSExcel 2007). All enzymatic assays were performed in duplicate and expressed as specific activity (nmol/min/mg). As positive control for PLA2 activity, venom of the snake *Micrurus hemprichii* (4.0 mg) was used.

**Hyaluronidase activity**

Hyaluronidase activity was measured as described by Pukrittayakamee [18], with slight modifications. In a microtitre plate, *Premolis semirufa*’s bristles extract (8.0 mg of protein) were mixed with 25 μL of the hyaluronic acid (0.5 mg/mL) and acetate buffer (0.2 M sodium acetate-acetic acid, pH 6.0, containing 0.15 M NaCl), in a final volume of 100 μL, and incubated for 30 min at 37°C. After incubation, 200 μL of CTAB 2.5% in NaOH 2% was added to the samples. The absorbances were measured at λ 405 nm in a spectrophotometer (Multiskan EX, Labsystems, Finland) against a blank containing hyaluronic acid, acetate buffer and 250 μL of CTAB. All assays were performed in duplicate. Results were expressed in units of turbidity reduction (UTR) per mg of extract. Bothrops jararaca snake venom (4.0 μg) was used as positive control.

**Proteolytic activity**

**Zymography.** Samples of the extract (0.5 μg of protein) were incubated, at 37°C for 30 min, in the presence or absence of 10 mM 1,10-phenanthroline or PMSF, metallo- and serineproteases inhibitors, respectively, solubilized in non-reducing sample buffer and separated on 10% SDS-PAGE gels containing 1 mg/mL gelatin. The gels were washed for 30 min at room temperature in 2.5% Triton X-100, and incubated for 12 h at 37°C in zymography buffer (50 mM Tris-HCl, 200 mM sodium chloride, 10 mM calcium chloride, 0.05% Brij-35 P; pH 8.3). Following incubation, the gels were stained with 0.2% Coomassie Brilliant Blue R-250. The gelatinolytic activity was detected as unstained bands and densitometry analysis of zymography gels was performed using the Kodak Molecular Imaging Software.

**Fluorimetric test.** The enzymatic activity of the caterpillar bristles extract was determined using the fluorescence resonance energy transfer (FRET) substrate peptide Abz-FRSSRQ-EDDnp. Samples of the extract (1 μg of protein) were mixed with 5 μM of FRET substrate, in cold phosphate-buffered saline (PBS). The relative inhibition was determined in parallel using in the assays 5 mM PMSF or 5 mM 1,10-phenanthroline, inhibitors of serine- and metalloproteases, respectively. The stock solutions and the work concentration of the synthetic inhibitors used in the characterization of the venoms proteolytic activity were made as described [19].

The reactions were monitored by measuring hydrolysis in a fluorescence spectrophotometer (Victor 3™, Perkin-Elmer, MA, USA) using 96-well microtitre plates (λex = 420 nm and λem = 320 nm) at 37°C, as described by Araújo et al. [14]. Control samples were prepared in the presence of an equal volume of ethanol, used

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**Figure 1.** *Premolis semirufa* caterpillar. Pararama in the trunk of *Hevea brasiliensis* in São Francisco do Pará, Pará, Brazil. Photo by Rosana de Fátima Shoji. doi:10.1371/journal.pntd.0001531.g001
in-inhibitors stock solutions. All assays were performed in duplicate and the specific proteolytic activity was expressed as units of free fluorescence of cleaved substrate per min per µg of extract (UF/min/µg).

**Determination of the median lethal dose (LD50)**

Lethality was assessed by intraperitoneal injection of increasing amounts of bristles extract in 200 µL of PBS into male BALB/c strain of mice. Four animals were used for each dose and the LD50 was calculated by probit analysis of death occurring within 72 h after extract injection [20].

**Evaluation of the edema**

The possible edematogenic activity of the caterpillar bristles extract was evaluated by BALB/c mice intraplantar injection of 50 µL of sterile PBS containing 10 µg (protein) of the extract into the left hind footpad. As control group, mice received 50 µL of sterile PBS into the left hind footpad. The animals were injected, seven times, at intervals of two weeks. Before extract or PBS inoculations, the thickness of each left footpad (Th0) was determined using a caliper measurement (Mitutoyo, Sul American Ltda.). Subsequent readings of the thickness (Th) after extract or PBS injections were carried out at 30, 60, 120 and 180 min, and compared to the initial readings. The edema (E) was calculated as follows: E [%] = [(Th − Th0)/Th0] × 100. Where Th is the thickness (mm) of the footpad at time “t” after the injection of the extract or PBS. Th0 is the thickness (mm) of the footpad before the injection of the extract or PBS.

**Histopathological analysis**

BALB/c mice, injected as described above, were euthanized 24 h after the 7th extract inoculation, their hind limbs removed and processed for histological analysis. The paws were immersed in 10% neutral buffered formaldehyde solution for 24 h. After decalcification, the tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The H&E preparations were microscopically observed and examined for the presence of inflammatory cell infiltration. As control, paws injected with an equal volume of PBS, were collected, processed and analyzed as described above. All tissue sections were examined under a light microscope (Leica DM2500; Wetzlar, Germany).

**Anti-bristles extract mouse serum**

The antiserum against *Premolis semirufa*’s bristles extract was obtained from mice inoculated with 10 µg (protein) of the extract or PBS, into the left hind footpad, seven times at two weekly intervals, without adjuvant. Bleeding was carried out, by retro-orbital plexus with a Pasteur pipette, 48 h after the injection. The blood was allowed to clot at room temperature for 15 min and then was left at 4°C for 6 h. After centrifugation at 560 × g for 15 min at 4°C, the serum was collected and immediately frozen at −20°C until use.

**Enzyme linked immunosorbent assay (ELISA)**

Detection of antibodies against bristles extract components. ELISA plates (Costar®, Corning Inc., USA) were coated with 100 µL of the caterpillar bristles extract (10 µg protein/mL; overnight at 4°C). Plates were blocked with 5% BSA in PBS and incubated with dilutions of the anti-caterpillar bristles extract or normal sera obtained from BALB/c mice. After 1 h of incubation at 37°C, plates were washed with PBS/0.05% Tween 20 and incubated with the specific anti-mouse IgG, -IgM, -IgG1 or -IgG2a HRPO-conjugate or with anti-mouse IgG2b, -IgG3 biotin-conjugate for 1 h at 37°C. For biotin conjugated antibodies, an additional step of incubation with HRP-conjugated streptavidin for 30 min at room temperature was carried out. Plates were washed and the reactions developed with OPD substrate, according to the manufacturers conditions (Sigma). The absorbances were recorded in a spectrophotometer (Multiskan EX, Labsystems, Finland) at λ 492 nm. The titer was established as the highest antiserum dilution, which produced an absorbance twice greater than that determined for the normal serum, and expressed as log2.

For the detection of anti-DNA or anti-Collagen type II antibodies. The presence of anti-DNA or anti-collagen type II IgM and IgG antibodies in sera obtained from mice inoculated or not with the caterpillar bristles extract were also determined by ELISA. Briefly, Immulon 2-HB 96-well flat bottom microtiter plate (Thermo Scientific, NY, USA) were coated with native salmon sperm DNA (1 µg/well), diluted in 50 µL of 10 mM TRIS-HCl, 1 mM EDTA, pH 7.5 or with collagen from bovine tracheal cartilage (3 µg/well), diluted in 100 µL of PBS, and incubated overnight at 4°C. Plates were blocked with 5% BSA in PBS at 37°C for 2 h and dilutions of the sera were added and incubated for 2 h at room temperature. Sera from [NZBxNZW]F1 mice with systemic lupus erythematosus autoimmune disease were obtained as described [21] and used as positive control for the presence of autoantibodies. Plates were washed with PBS/0.05% Tween 20 and incubated with the specific anti-IgG or anti-IgM HRPO-conjugates for 1 h at room temperature. Plates were washed and the reactions developed with OPD substrate, according to the manufacturers conditions (Sigma). The absorbances were recorded in a spectrophotometer (Multiskan EX, Labsystems, Finland) at λ 492 nm.

**Statistical analysis**

Statistical analysis was performed by Students’s test using GraphPad Prism software. Differences were considered statistically significant when P values were P<0.05, P<0.01 and P<0.0001.

**Results**

Electrophoretic characterization of the *Premolis semirufa*’s bristles extract

Extract samples collected from Pararama bristles were prepared in PBS and analyzed for protein composition using SDS-PAGE, under reducing and non-reducing conditions. Figure 2 shows that the electrophoretic profiles of the extract, analyzed under both conditions, were similar, showing components with Mrs between 20 and 200 kDa and the presence of an intense band with Mr around 82 kDa.

**Toxic activities of the *Premolis semirufa*’s bristles extract**

In order to assess *Premolis semirufa*’s bristles extract toxicity, the extract was tested using a variety of functional biochemical assays, to identify if it contained activities frequently found in animal venoms. The lethal toxicity of the bristles extract was determined in groups of BALB/c mice, after intraperitoneal injection of increasing protein concentrations of the extracts (1.2 mg/kg, 2.3 mg/kg and 6.8 mg/kg) and no death was observed after 72 hours of the inoculation (data not shown). Moreover, in this condition, no manifestation of discomfort was observed in any of the envenomated animals.

The phospholipase A2 (PLA2) activity of *P. semirufa* caterpillar bristles extract was assessed by a colorimetric method after incubating samples of 4 or 16 µg of the extract with phosphatid-
The proteolytic activity of the bristles extract (1 μg of protein) was tested using the fluorescence resonance energy transfer (FRET) peptide Abz-FRSSRQ-EDDnp as substrate. Figure 3C shows that the extract efficiently hydrolyzed the FRET peptide, and that this activity was strongly inhibited by the serine protease inhibitor PMSF (88%) and partially by the metalloprotease inhibitor phenanthroline (50%).

Figure 3D shows that a 82 kDa component, the Mr corresponding the intense protein band observed after silver staining (Figure 2), has a high gelatinolytic activity, as measured by zymography (Fig. 3 - line 1). This activity was significantly inhibited by PMSF (Fig. 3 - line 3), a serineprotease inhibitor, and poorly blocked by phenanthroline (Fig. 3 - line 2), a metalloprotease inhibitor.

Edema inducing activity

BALB/c mice were injected seven times, at intervals of two weeks, with 10 μg of the extract proteins into the foot pad of the left hind leg. Controls animals were injected with PBS. The intraplantar injection of Premolis semirufa caterpillar bristles extract, caused discomfort to the animals (pain) and a significant increase in the paw volume, as compared to that induced by injection of the vehicle, i.e., PBS (Figure 4A and B). The edema induced by both, extract and PBS, was detected as early as 5 min post-injection and peaked at 30 min. The increase in paw volume was observed until 300 min after injection of the extract, while the increase induced by PBS was resolved 120 min after injection. The comparison of the edematogenic responses, along the seven inoculations of the extract or PBS, determined at the peak of the reaction, i.e., at 30 min is shown in Figure 4C. The edematogenic responses were significant and successively more intense after the inoculations of the extract compared to PBS and reached a maximum after the 4th injection.

Histopathological analysis

BALB/c mice, injected as described above, were euthanized 24 h after the 7th extract or PBS inoculations, their hind limbs removed and processed for histological analysis. Figure 5B shows that the bristles extract injection resulted in the establishment of a pronounced inflammatory reaction, characterized by the presence of mixed inflammatory cellular infiltrate distributed throughout the tissue. Furthermore, the connective tissue was increased, partially occupying areas where, in normal tissues, structures such as sweat glands and fat tissue were found (Figures 5A and C), and initiating a fibrotic process (Figure 5D).

Immunogenicity of the Pararama bristles extract antiserum

The immunogenicity of Premolis semirufa caterpillar bristles extract was assessed by ELISA, using sera obtained from BALB/c mice subcutaneously inoculated with 10 μg of the extract proteins or PBS. Figure 6A shows that the repeated inoculations of the extract, but not of PBS, induced a high IgG antibody response. In addition, analysis of antibody classes and subclasses revealed that the sera obtained from animal injected with the extract presented higher IgG1, IgG2a, IgG2b and IgM titers as compared to the sera collected from PBS injected mice. IgG1 sera titer, determined for envenomated animals, were higher than the others antibodies isotype/substrclasses and no IgG3 antibodies could be detected in these samples (Figure 6B).

Detection of the anti-DNA and anti-Collagen type II autoantibodies

The presence of anti-DNA or anti-Collagen type II IgM and IgG autoantibodies in sera from BALB/c mice inoculated with extract or PBS, was evaluated by ELISA. Anti-DNA or anti-Collagen type II IgG antibodies were not detected in sera from bristles extract inoculated animals, while high titers of these antibodies were detected in the serum of mice with systemic lupus erythematosus (SLE) autoimmune disease. Anti-DNA and anti-Collagen type II IgM antibodies were not detected in bristles extract injected or SLE mice (data not shown).

Discussion

We have investigated activities of the bristles extract of Pararama, a caterpillar responsible for the occupational disease ‘Pararama associated phalangeal periartthritis’. Until now nothing was know about the composition of its venom.
In this paper we present, for the first time, some of the biochemical and biological properties of *Premolis semirufa*’s bristles extract. Electrophoretic analysis of the extract showed that it contained a great diversity of proteins, with Mr ranging from 20 to 200 kDa, with a major protein band of 82 kDa, contributing to over 90% of the protein content. No significant difference in the protein profile was observed in the extracts submitted to reducing or non-reducing conditions.

We subjected the venom to a variety of functional biochemical assays, to identify if it contained activities frequently found in animal venoms. Hyaluronidase activity is present in many animals venoms and its activity potentiates the toxicity of the venom, promoting loss of extracellular matrix integrity of soft connective tissues, surrounding the blood vessels, increasing the systemic influx of toxins and, thus, facilitating the dispersion of the toxic components [22]. *Premolis semirufa*’s bristles extract showed significant hyaluronidase activity, suggesting that this enzyme may participate in the genesis of the joint immobility, since hyaluronic acid is an abundant component of the intercellular matrix of the skin, cartilage and synovial fluid, playing an important role as stabilizer and lubricant of the joints [23]. The hyaluronic acid degradation may explain, in part, the changes in the joint and loss of the cartilage and bone structure, seen in the pararana induced disease.

Zymography analysis showed that the 82 kDa component found in the caterpillar bristles extract possesses gelatinolytic activity. Gelatinases are capable of degrading types IV, V, VII and XI collagens, present in bone and articular cartilage, and may regulate their remodeling [24]. Using specific inhibitors for metallo- and serine- proteases we identified the gelatinase as a serine protease. The bristles extract also demonstrated high proteolytic activity towards the FRET peptide Abz-FRSSRQ-EDDnp. The use of PMSF showed that serineproteases were largely responsible for this while using the metalloproteases...
inhibitor phenanthroline demonstrated that metalloproteases were involved as well. Venom serineproteinases have a highly diverse pharmacological potential, including actions on proteins of the coagulation cascade, activation of factor V, activation of protein C, fibrinogenolysis, activation of the plasminogen and induction of platelet aggregation [25]. Thus, it is possible to propose that serineproteinases, with gelatinolytic activity and other proteolytic activities, may be involved in the process of cartilage and joint degradation produced by contact with the bristles of Pararama [26]. Metalloproteinases, abundant molecules in snake venoms, are responsible for the development of local tissue injury and the occurrence of bleeding [27], being able to degrade important components of the matrix, such as laminin and type IV collagen [28]. Thus, the presence of different classes of proteases in the Pararama bristles extract may contribute to the tissue injury seen in the caterpillar human accidents.

In many animal venoms, Phospholipase A\textsubscript{2} (PLA\textsubscript{2}) is important for digestion and immobilization of the prey, as well as responsible for some pathologies observed in humans stung/bitten by bees, wasps, spiders and snakes [29–31]. Phospholipase A\textsubscript{2} activity has also been described in crude bristles extract of the Euproctis (Lymantriidae) caterpillar [32] and more recently in bristles crude extract of Lonomia obliqua (Saturniidae) [33]. However, Premolis semirufa’s bristles extract did not show phospholipase A\textsubscript{2} activity.

The present study also aimed to evaluate the toxicity of the bristles extract using a murine model and, under the experimental conditions used, no discomfort or death was observed. On the other hand, the intraplantar injection of the bristle extract, as used in histopathological/edema studies, caused a strong discomfort to the animals, suggesting that they were feeling pain. Further studies will be conducted in order to analyze the possible hyperalgesic properties of the pararama venom. Toxic venom proteins serve in a number of adaptive functions such as immobilizing, paralyzing, killing, liquefying prey and deterring competitors. Other proteins may act synergistically by enhancing the activity or spreading of toxins. In contrast to animals such as snakes and scorpions, which use venoms to immobilize prey and to facilitate its digestion, caterpillars feed on leaves; their venoms are used solely for defense [34] and, therefore, has not to be necessarily lethal.

The first intraplantar injection of Premolis semirufa’s bristles extract produced a swelling which was detected after 5 min, peaked at 30 min and disappeared within 300 min after injection, while the response to PBS disappeared within 120 min. The prolonged and increased induction of the edema upon 1st exposure was likely to be mediated by the action of the venom, being pro-inflammatory itself or by inducing inflammatory mediators, locally released or synthesized in the course of the envenomation, all of which would increase the permeability of the microvessels. The response induced 30 min after extract injection, gradually increased over the inoculations and reached a maximum after 4 injections. This response was significantly higher than the response to PBS. In addition, the multiple extract injections in mice

Figure 4. Edematogenic action of Premolis semirufa’s bristles extract. Edema was induced by intraplantar administration of 0.05 ml of sterile PBS [A] or samples of 10μg of protein of the caterpillar bristles extract [B] into the left hind footpad. Paw edema was determined, by measuring paw thickness using a caliper at 0, 30, 60, 120 and 180 min after administration of extract or buffer. [C] Comparison of the edematogenic responses, after 30 min of PBS or extract injections, during the seven inoculations. Results were calculated by the formula: 

\[
E(\%) = \left(\frac{T_{ht} - T_{h0}}{T_{h0}}\right) \times 100;
\]

where \(T_{ht}\) is the thickness (mm) of the rear left footpad at "t" time after the injection of the extract or PBS, \(T_{h0}\) is the thickness (mm) of the rear left footpad before the injection of the extract or PBS. \*P<0.05 and ** P<0.01: significant differences between the mean values of buffer group and the mean values of P. semirufa group.

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footpads induced a pronounced inflammatory reaction, characterized by the presence of mixed inflammatory infiltrate, with increase of the conjunctive tissue and beginning of the fibrosis process. In a previous study using a rat model, Costa and collaborators [8] have shown that inflammation was induced by the injection of saline extract of pararama bristles, with the presence of a large number of inflammatory cells around the site of injury.

Investigation on the *P. semirufa*’s bristles extract immunogenicity revealed that the repeated inoculations of the extract, in the absence of adjuvants, induced a strong immune response, with high antibody titers. Moreover, data showed that the sera obtained from animal injected with the extract presented higher IgG1 titers than other IgG subclasses, indicating the predominance of a Th2 immune response, since this particular antibody subclass is mainly induced by the presence of Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13 [35].

The disease caused by the contact with the *Premolis semirufa*’s bristles shares many features with those found in patients with rheumatoid arthritis (RA), a systemic and chronic illness, characterized by severe synovial inflammation and cartilage and/or bone destruction [36]. Autoantibodies, such as anti-collagen type II and anti-DNA, are found in the vast majority of patients [37]. Analysis of the presence of anti-DNA or anti-collagen type II antibodies revealed that these autoantibodies were not present in the sera obtained from mice inoculated with the *Premolis semirufa*’s bristles extract.

Together, these data show the existence, in the *Premolis semirufa*’s bristles extract, of a mixture of different enzymes that may be acting together in the generation and development of clinical

![Figure 5. Analysis of the inflammatory process induced by *Premolis semirufa*’s bristles extract.](image-url)
disease manifestations. Moreover, this study demonstrates the production of high antibody titers in mice inoculated with the extract, which may also contribute to genesis of inflammatory reactions observed in the envenomation. The absence of autoantibodies indicate that the molecular mechanisms causing disease after multiple contact with the *Prenolis semirufa*’s bristles differ from that observed in chronic synovitis, such as the rheumatoid arthritis. The bristles toxic action, high antibody response with the formation of immune complexes and complement activation may also play a role in the establishment of the disease. These aspects will be further investigated in future studies.

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