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# The role of hemocytes in the immunity of the spider *Acanthoscurria gomesiana*

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## Abstract

Invertebrates protect themselves against microbial infection through cellular and humoral immune defenses. Since the available information on the immune system of spiders is scarce, the main goal of the present study was to investigate the role of hemocytes and antimicrobial peptides (AMPs) in defense against microbes of spider *Acanthoscurria gomesiana*. We previously described the purification and characterization of two AMPs from the hemocytes of naïve spider *A. gomesiana*, gomesin and acanthoscurrin. Here we show that 57% of the hemocytes store both gomesin and acanthoscurrin, either in the same or in different granules. Progomesin labeling in hemocyte granules indicates that gomesin is addressed to those organelles as a propeptide. *In vivo* and *in vitro* experiments showed that lipopolysaccharide (LPS) and yeast caused the hemocytes to migrate. Once they have reached the infection site, hemocytes may secrete coagulation cascade components and AMPs to cell-free hemolymph. Furthermore, our results suggest that phagocytosis is not the major defense mechanism activated after microbial challenge. Therefore, the main reactions involved in the spider immune defense might be coagulation and AMP secretion. © 2007 Elsevier Ltd. All rights reserved.

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## 1. Introduction

Invertebrates are constantly exposed to microbial infections, and to protect themselves they have developed powerful immune defense mechanisms similar to those innate to vertebrates. These mechanisms involve cellular and humoral responses. The former consists of encapsulation, nodulation, and phagocytosis of microbes by hemocytes, while humoral response comprises factors related to the recognition of invading microorganisms, serine protease cascades participating in melanization and coagulation, and killing factors such as antimicrobial peptides (AMPs), reactive oxygen species, and reactive nitrogen intermediates, including nitric oxide [1]. Over 1000 AMPs produced by multicellular organisms (plants, invertebrates, and vertebrates) have been isolated and characterized at the primary structure level (<http://www.bbcm.univ.trieste.it/tossi/pag1.htm>) [2–4].

Several AMPs are synthesized as large precursors. In some cases co-translational removal of signal peptides releases the active molecule, but in others one or more anionic propieces are also commonly removed during proteolytic processing [5]. Depending on the organism and tissue considered, AMPs are either constitutively stored within secretory cells, or their synthesis is induced at the time of infection. In most insects, AMP synthesis starts a few hours after an infection has occurred. In other invertebrates, such as horseshoe crabs, mussels, and shrimps, AMPs are constitutively produced and stored in hemocyte granules [6–9].

AMPs stored in cell granules interact with and eliminate the invading organisms. Studies on invertebrates have demonstrated that this can be achieved by two different processes: (i) fusion of the granules where AMPs are stored within phagosomes [10], and/or (ii) release of these AMPs into the plasma by exocytosis [11,12]. Similar mechanisms are found in vertebrates [13–15].

Previously we reported on the purification and characterization of two AMPs, acanthoscurrin and gomesin, from the hemocytes of naïve mygalomorph spider *Acanthoscurria gomesiana* [16,17]. In addition, an acylpolyamine isolated from hemocytes of the same spider species presented antibacterial activity [18].

Acanthoscurrin is a glycine-rich peptide post-translationally processed by the removal of the signal peptide and C-terminal amidation. Acanthoscurrin is released into the plasma following immune challenge [17]. Gomesin is a small-sized antimicrobial peptide containing 18 amino acids, including pyroglutamic acid as the N-terminus, a C-terminal arginine  $\alpha$ -amide, and four cysteine residues forming two disulfide bridges [16]. This peptide presents a well-defined  $\beta$ -hairpin-like structure, as determined by 2-D NMR and molecular dynamics studies [19]. Gomesin presents marked sequence and structural similarities to horseshoe crab tachyplesin [20,21] and polyphemusin [22,23], and to scorpion androctonin [24]. Interestingly, gomesin also shares structural similarities with protegrins, porcine leukocyte AMPs [25,26]. In addition, gomesin is translated into a prepropeptide presenting 84 amino acid residues that contains a signal peptide with 23 amino acids, an 18 amino acids mature molecule, and a C-terminal prodomain with 43 amino acid residues. Immunolocalization studies have demonstrated that mature gomesin is stored within hemocyte granules [27].

Since the literature on the spider immune system is limited, the main goal of the present study was to investigate how spiders combat microbes, and the role of hemocytes and AMPs in this process. We show that gomesin is addressed to hemocyte granules as a propeptide and report on the relative distribution of gomesin and acanthoscurrin in hemocytes by double-immunolocalization on confocal microscopy. We demonstrate by means of *in vivo* and *in vitro* experiments that hemocytes may migrate to the microbial infection site. Once they have reached the site of infection, hemocytes may secrete coagulation cascade components and AMPs. Furthermore, our results suggest that phagocytosis is not the major defense mechanism activated after microbial challenge.

## 2. Material and methods

### 2.1. Animals and sample collection

The common spider (*A. gomesiana*) was obtained from Instituto Butantan (São Paulo, Brazil). The hemolymph was collected from pre-chilled animals by cardiac puncture with an apyrogenic syringe. To avoid hemocyte degranulation and coagulation, the hemolymph was collected in the presence of sodium citrate buffer, pH 4.6 (2:1; v:v) [28]. The hemocytes were removed from plasma by centrifugation at 800g for 10 min at 4 °C.

### 2.2. Production and purification of the C-terminal prodomain of gomesin

To obtain the peptide corresponding to the C-terminal domain, PCR was performed using the cDNA of gomesin [27] as a template with 5' primer (AGT TTA GAT GAG ACC) and 3' primer (CTT AGT CGA AAA TAA). The PCR product was digested with *Bam*HI and *Eco*RI for subsequent cloning.

To express the recombinant C-terminal prodomain, the PCR product was inserted into the vector pGEX-2T (Amersham Biosciences). This vector produces recombinant protein in fusion with glutathione S-transferase (GST). The construct was inserted into *Escherichia coli* DH5 $\alpha$ , which was maintained in LB containing 100  $\mu$ g/mL ampicillin. To induce protein production, 0.4 mM IPTG was added to the culture medium, kept at 37 °C for 3 h, and centrifuged for 10 min at 12,000g. The cells were resuspended in phosphate-buffered saline (PBS) and disrupted by sonication using a Vibra Cell sonicator (Branson Digital Sonifier, Model 450, USA) at 30 W, 5  $\times$  30 s. After that, Triton-X100 was added to a final concentration of 1%. The cells were kept under agitation for 30 min. The homogenate was centrifuged at 12,000g for 10 min at 4 °C and the recombinant protein was purified from the supernatant.

The C-terminal prodomain was purified using a Glutathione Sepharose 4B (Amersham Biosciences) column and eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione. The C-terminal prodomain was cleaved from GST with thrombin (Amersham Biosciences) for 16 h at 22 °C following the manufacturer's instructions. After cleavage, the proteins were separated by reversed-phase chromatography on an analytical C<sub>18</sub> (Vydac<sup>TM</sup>, 300 Å, 5  $\mu$ m, 4.6 mm  $\times$  250 mm) column equilibrated with 2% acetonitrile

(ACN) in acidified water (0.046% trifluoroacetic acid (TFA)). Elution was performed with a linear 2–95% ACN gradient in acidified water over 60 min at a flow rate of 1.0 mL/min.

HPLC purification was carried out at room temperature on a Shimadzu LC-10 HPLC system with a Shimadzu diode array detector (SPD-M10AV). The column effluent was monitored for absorbance at 225 nm. Fractions corresponding to absorbance peaks were hand-collected, concentrated in a vacuum centrifuge (Savant Instruments, Inc.), and reconstituted in ultrapure (Milli-Q) water.

The presence of the C-terminal prodomain in the chromatographic fractions was determined by electrospray ionization-mass spectrometry (ESI-MS) on a Finnigan LCQ<sup>TM</sup>Duo mass spectrometer (ThermoQuest, EUA) under the same previous conditions [17].

## 2.3. Antiserum production

### 2.3.1. Anti-acanthoscurrin

Three BALB/c mice were intraperitoneally immunized with 100  $\mu$ L of PBS containing 35  $\mu$ g of acanthoscurrin purified as previously described [17] and emulsified with Lipid A from *Bordetella pertussis* (Almeida, I.C.; unpublished data). The first immunization was followed by two boosts at 2-week intervals. Antiserum was collected 15 days after the last injection.

### 2.3.2. Anti-C-terminal prodomain

The C-terminal prodomain of gomesin was coupled to bovine serum albumin (BSA) with reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce Biotechnology) following the manufacturer's protocol. Three BALB/c mice were intraperitoneally immunized with 100  $\mu$ L of PBS containing 30  $\mu$ g of the C-terminal prodomain emulsified in complete Freund adjuvant. The first immunization was followed by two boosts at 2-week intervals with aliquots emulsified in incomplete Freund adjuvant. Antiserum was collected 15 days after the last injection. The C-terminal prodomain concentration was calculated with the standard curve obtained from absorbance at 255 nm of phenylalanine at known concentrations.

### 2.3.3. Anti-gomesin

The anti-gomesin antibody was obtained from a rabbit as described in a previous study [27].

## 2.4. Immunocytochemistry

Hemocytes were resuspended and fixed in a sodium citrate buffer [28] containing 3.7% formaldehyde for 15 min on ice. The hemocytes were transferred to poly-L-lysine-coated glass slides and stored at  $-20^{\circ}\text{C}$ . The slides were incubated for 10 min in 25 mM Tris-HCl, pH 7, containing 50 mM ammonium chloride, 0.2% gelatin, and 0.5% Triton X-100. After extensive wash with PBS containing 0.1% Tween-20 (PBS-T), the slides were incubated for 1 h at room temperature with 5% gelatin/PBS-T. Next, they were washed again with PBS-T and incubated for 1 h at room temperature in 5% gelatin/PBS-T with either rabbit anti-gomesin antibodies (1:500), or mouse anti-acanthoscurrin antiserum (1:200), or mouse anti-C-terminal prodomain antiserum

(1:200). Control experiments were performed using either mouse or rabbit pre-immune antiserum. Following another washing step, the slides were incubated for 20 min with either 1:500 Alexa Fluor 488 conjugated anti-rabbit IgG (Molecular Probes) or 1:200 Rhodamine conjugated anti-mouse IgG (Molecular Probes), both diluted in 5% gelatin/PBS-T. Finally, the slides were washed with PBS-T, incubated with 10 mM DAPI for 1 min, and washed with PBS-T. Samples were imaged on a BioRad 1024-UV confocal system attached to a Zeiss Axiovert 100 microscope, using a  $63\times$  N.A. 1.4 Plan-Apochromatic (with Nomarski Differential Interference Contrast optics) oil immersion objective.

## 2.5. *In vivo* immune challenge

For experimental immune challenge, 50  $\mu$ L of fluorescein isothiocyanate (FITC) coupled latex beads ( $10^5$  particles) were injected into the spider legs. Twenty-four hours later, the legs were dissected and the cuticle was carefully removed. Muscle fiber slides were analyzed under a fluorescence microscope (Axiophot Zeiss). In other experiment, 50  $\mu$ L of yeast *Saccharomyces cerevisiae* ( $10^3$  cells) resuspended in PBS were injected into the spider heart. Three hours later, 50  $\mu$ L of ice-cold 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, was injected into the heart. After 15 min, the heart was dissected and maintained in a polypropylene tube with the fixative solution described above for 24 h at  $4^{\circ}\text{C}$ . The same procedure was performed with 50  $\mu$ L of sterile PBS inoculated into the spider heart. Fixed hearts were longitudinally divided and processed for optical and electron microscopy analysis.

## 2.6. Optical microscopy

Fixed hearts were included into a Leica resin, following the manufacturer's protocol. Sections of 1  $\mu$ m were placed onto glass slides and stained by the May-Grünwald-Giemsa method (modified Giemsa) [29]. Slides were examined in a Zeiss Axiophot microscope.

## 2.7. Transmission electron microscopy (TEM)

Fixed hearts were post-fixed in 1.0% OsO<sub>4</sub> in ultrapure water and dehydrated prior to Spurr resin embedding [30]. Ultrathin sections of 70 nm were gathered onto copper grids and stained with 0.5% uranyl acetate in ultrapure water for 5 min, washed in ultrapure water, and stained again in 0.5% lead citrate in ultrapure water. Ultrastructure was examined on a JEOL 100 CX-II electron microscope.

## 2.8. Migration assay

The migration ability of hemocytes was measured in a 3- $\mu$ m Transwell filter coupled to a 24-well plate (Corning). Hemolymph was diluted in 100  $\mu$ L of serum-free modified L15 medium [31] to a final concentration of  $10^5$  cells per well. The hemocyte suspension was placed into the upper chamber. Lipopolysaccharide (LPS) (1  $\mu$ g/mL), yeast in a proportion of 5:1 (yeast: hemocyte) or 10  $\mu$ M gomesin was

placed into the lower chamber as chemoattractant. The preparation was kept at 37 °C for 5 h. Cells that did not penetrate the filter were wiped out with cotton swabs, and the cells that migrated to the filter's lower surface were fixed with 3.7% paraformaldehyde for 15 min at 37 °C. After washing with PBS, the cells were stained with 0.1% toluidine blue. The filters were extensively washed with ultrapure water and incubated with 150  $\mu$ L of 1% SDS for 1 h at 37 °C to promote cell lyses. After that, 100  $\mu$ L of the SDS solution was collected and distributed in a 96-well plate and absorbance was determined at 630 nm. Two independent experiments were performed (groups A and B), one in duplicate and the other in triplicate. The experimental absorbance was corrected through the equation: Corrected value = (Experimental absorbance – Average absorbance of non-treated samples from either group A or group B)/standard deviation of non-treated samples from either group A or B. The average of the corrected value and the corresponding standard mean error (SME) were plotted. The differences between treatments and control were statistically analyzed by Student's *t*-test for independent samples using the software SPSS for Windows. Differences were considered significant when  $P < 0.05$ .

### 2.9. Phagocytic assay

Hemolymph was diluted in 100  $\mu$ L of L15-modified medium [31] supplemented with 20% bovine fetal serum to a final concentration of  $10^5$  cells per well. The hemocyte suspension was distributed through a 24-well plate. Either live or heat-killed *S. cerevisiae* ( $5 \times 10^5$  per well) were added. After 1 h at 30 °C, the cells were fixed with PBS containing 3.7% formaldehyde for 15 min on ice and placed onto slides. The preparation was analyzed on a Zeiss Axiophot fluorescence microscope. Experiments were performed in triplicate.

### 2.10. Exocytosis assay

Fresh hemolymph was diluted in 100  $\mu$ L of L15-modified medium [31] supplemented with 20% bovine fetal serum, and distributed into a 96-well plate at a concentration of  $10^5$  cells per well. Different concentrations of LPS (1, 0.1, 0.05, 0.02, 0.01  $\mu$ g/mL) were added to the medium. The preparation was kept at 30 °C for 1 h. The supernatants were then collected from the wells, pooled, and subjected to solid phase extraction on Sep-Pak C<sub>18</sub> cartridges (Waters Associates) equilibrated with acidified water. Three step-wise elutions were performed with 5%, 40%, and 80% ACN in acidified water. The 40% Sep-Pak fraction was concentrated in a vacuum centrifuge, reconstituted with 50 mM sodium bicarbonate, pH 9.6, and subjected to ELISA assay. Three independent experiments were performed. Data are expressed as means  $\pm$  SME.

### 2.11. ELISA

The quantity of gomesin in the exocytosed fluid was determined with polyclonal anti-gomesin [27]. Microtiter plates were coated with the pre-purified exocytosed Sep-Pak fraction and incubated at 4 °C for 18 h. The wells were

blocked with 5% of non-fat dry milk in PBS for 1 h at room temperature. The plates were washed with PBS containing 0.1% Tween-20 and incubated with anti-gomesin (1/500) diluted in 5% of non-fat dry milk in PBS for 1 h at room temperature. Following another washing step, horseradish peroxidase-conjugated goat anti-rabbit (1/1000) (Amersham Biosciences) was added and the plates were kept at room temperature for 1 h. After another washing step, the enzyme activity of horseradish peroxidase was detected with *o*-phenylenediamine at 490 nm. Secreted gomesin was quantified using a standard curve with different concentrations of synthetic gomesin, which was produced as previously described [16].

## 3. Results

### 3.1. Localization of gomesin precursor

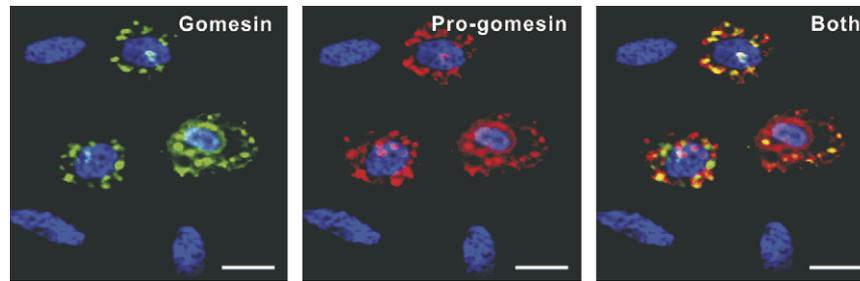
The translation of gomesin into a prepropeptide raised our interest to determine the subcellular localization of gomesin processing. To address this question, the C-terminal prodomain localization was determined by immunofluorescence. The C-terminal prodomain labeling was localized in hemocyte granules, and, most often, co-localized with anti-gomesin labeling (Figure 1). This indicates that gomesin is addressed to the hemocyte granules as a propeptide.

### 3.2. Comparative distribution of gomesin and acanthoscurrin in hemocytes

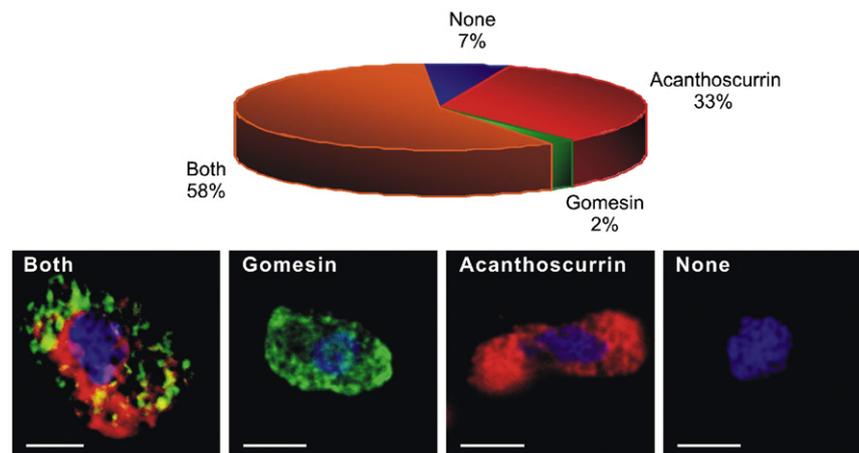
We previously showed that gomesin is stored in hemocyte granules [27]. Herein, the distributions of gomesin and acanthoscurrin were compared in the same preparations using Alexa Fluor 488 and rhodamine-conjugated antibodies, respectively, and examined by confocal microscopy (Figure 2). Both gomesin and acanthoscurrin labeling were detected in hemocyte granules. Different hemocytes were positive for only either gomesin (2%) or acanthoscurrin (33%), but both immune reactivities often appeared within the same cell (58%). In the latter, merged confocal microscope images suggested that gomesin and acanthoscurrin could be packed in either different or within the same cell granule. In addition, 7% of the cells were not labeled for either of the AMPs. A control experiment was performed by replacing anti-gomesin and anti-acanthoscurrin with either rabbit or mouse pre-immune serum, respectively. No fluorescence was detected in these preparations (results not shown).

### 3.3. Cellular-mediated response to immune challenge

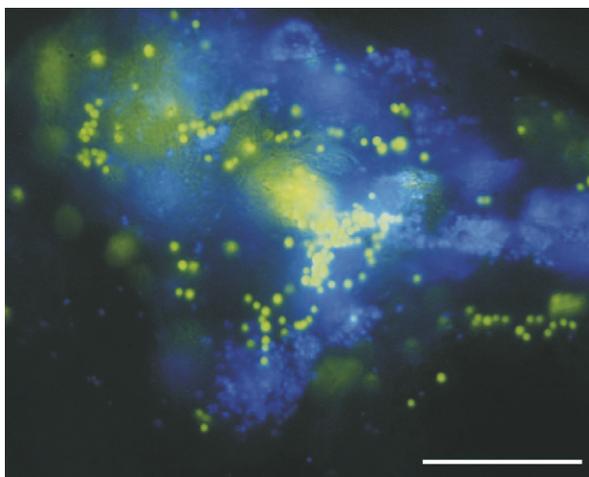
*In vivo* immune challenge was performed through injection of fluorescent beads into the spider leg. Twenty-four hours later, numerous fluorescent beads and hemocytes exhibiting autofluorescence in blue were visualized at 470 nm. Both were held in a gelatinous material, which was probably related to a coagulation response activated by non-self particles (Figure 3).



**Figure 1** Intracellular distribution of mature gomesin (green) and C-terminal prodomain of gomesin (red) in spider circulating hemocytes. Double immunofluorescence-labeling assay was performed using anti-gomesin and anti-C-terminal prodomain as primary antibodies and Alexa Fluor 488 conjugated anti-rabbit IgG and Rhodamine conjugated anti-mouse IgG, respectively, as secondary antibodies. Nuclei were DAPI-stained. Merged confocal images indicate that C-terminal prodomain and mature gomesin are stored in hemocyte granules as a propeptide. Bar, 5  $\mu\text{m}$ .



**Figure 2** Intracellular distribution of gomesin (green) and acanthoscurrin (red) in double-labeled circulating spider hemocytes. Hemocytes were submitted to anti-gomesin and anti-acanthoscurrin immunofluorescence assay. Hemocytes were then incubated with Alexa Fluor 488 conjugated anti-rabbit IgG and Rhodamine conjugated anti-mouse IgG, respectively. Nuclei were DAPI-stained. Most of the cells (upper panel) presented both gomesin and acanthoscurrin either in the same (yellow granules) or in different granules (red or green isolated granules). Bar, 5  $\mu\text{m}$ .

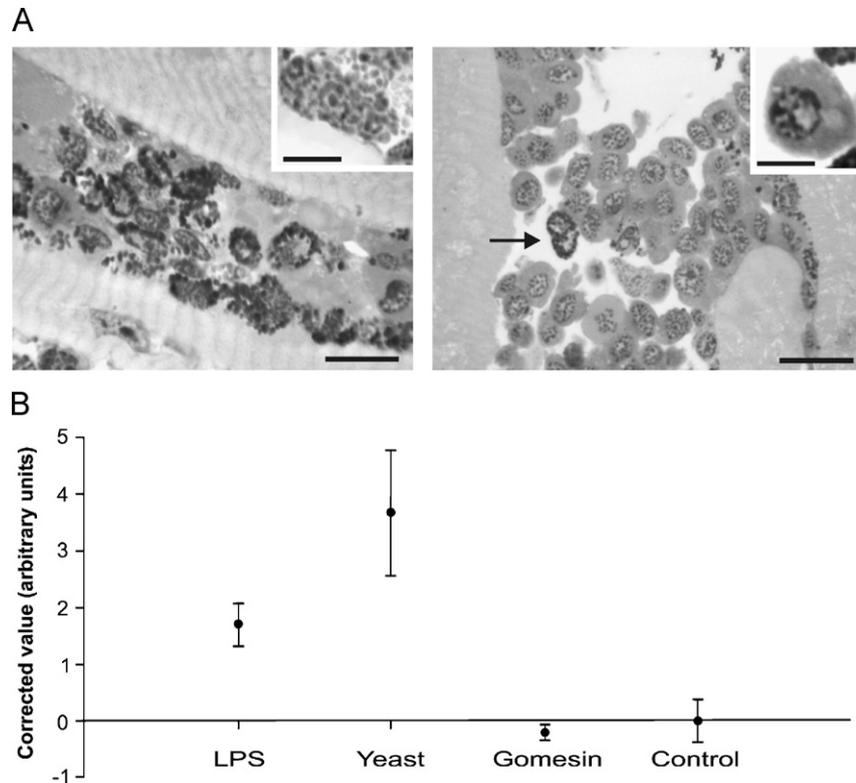


**Figure 3** Clot formation. FITC-coupled latex beads ( $10^5$  particles) were injected into the spider leg. The muscle fibers of the dissected leg were observed by microscopy 24 h later. The presence of beads (green) and hemocytes exhibiting autofluorescence in blue held in gelatinous material indicates the activation of the coagulation cascade. Bar, 50  $\mu\text{m}$ .

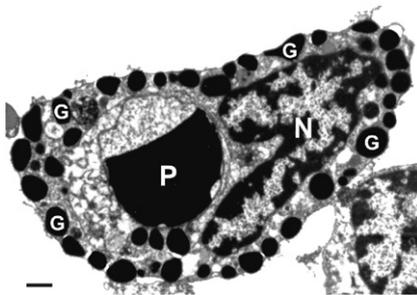
We also compared heart sections from a yeast-challenged spider with those of an unchallenged spider. In contrast to the heart inoculated with saline buffer whose prohemocytes predominated, the yeast-inoculated heart showed an increased number of mature hemocytes at the inoculation site (Figure 4A). This result suggests that these cells present a migratory response. However, we cannot discard the possibility that yeast challenge caused an increase in the differentiation of prohemocytes or a new production of the hemocytes.

The major morphological difference between prohemocytes and mature hemocytes is the presence of numerous granules in the latter. These granules present different sizes and shapes and completely fill the cytoplasm. Non-granular cells (prohemocytes) predominated in the heart of the unchallenged spider, while granular cells (mature hemocytes) predominated in circulating hemolymph.

The migratory capacity of hemocytes was confirmed *in vitro* as both yeast and LPS stimulated the migration response, which was significantly different from that of unstimulated cells ( $P < 0.014$  and  $P < 0.013$ , respectively). Gomesin did not act as a chemoattractant at the concentration of 10  $\mu\text{M}$  (Figure 4B).



**Figure 4** Migratory response of spider hemocytes. (A) *Saccharomyces cerevisiae* ( $10^3$  cells) were injected into the spider heart. Three hours later, the heart was dissected and prepared for optical microscopy observation. The same procedure was performed with PBS-inoculated spider heart. Heart sections of yeast-challenged (left panel) and unchallenged spider (right panel) are shown. In the first image, most of the hemocytes present granules that fill the cytoplasm completely, being impossible to observe the nuclei. One enlarged cell and parts of other two cells are shown in the inset. The second image (right) shows one mature hemocyte (arrow) prohemocytes without granules with visible nuclei (inset). Our results suggest a migratory response from mature hemocytes to the yeast injection site. Bar,  $20\ \mu\text{m}$  (inset bar,  $10\ \mu\text{m}$ ). (B) Hemolymph was distributed into  $3\text{-}\mu\text{m}$  Transwell filters coupled to a 24-well plate (upper chamber). LPS, yeast, and gomesin were placed in the lower chamber as chemoattractants. The control corresponds to non-treated cells. Cells that migrated to the lower surface of the filter were stained with 0.1% toluidine blue. The absorbance of SDS-treated cells was determined at  $630\ \text{nm}$ . Absorbance was corrected by the equation described in Section 2.8. The average corrected values and the corresponding standard mean error (SME) were plotted. Yeast and LPS attracted hemocytes to the lower chamber ( $P < 0.014$  and  $P < 0.013$ , respectively). Gomesin did not act as a hemocyte chemoattractant at  $10\ \mu\text{M}$  ( $P < 0.646$ ).



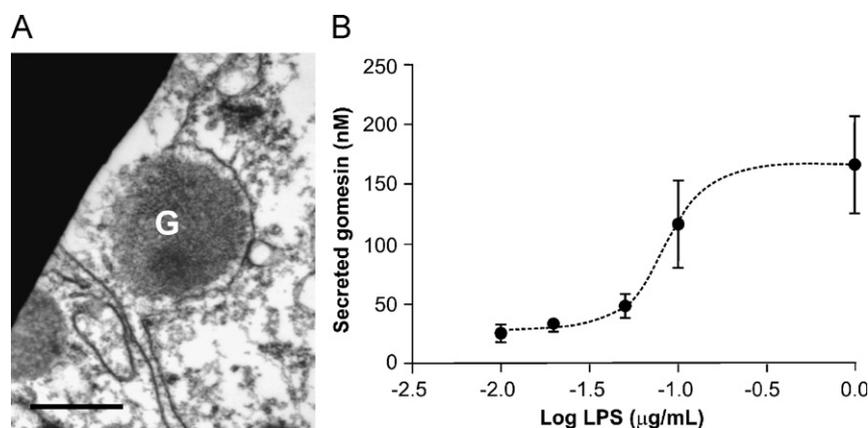
**Figure 5** Hemocyte phagocytic activity. *Saccharomyces cerevisiae* ( $10^3$  cells) were inoculated into the spider heart. Three hours later, the heart was dissected and prepared for TEM observations. The electron micrograph shows one hemocyte containing phagocytosed yeast (P), nucleus (N), and granules (G). Bar,  $1\ \mu\text{m}$ .

The recruitment of mature hemocytes to the injection site corroborated the importance of hemocytic reactions in response to microbial challenge. However, high hemocyte

density in the infection site made it difficult to investigate the interaction between hemocytes and the microorganisms. To address this question, sections of challenged spider hearts were examined by transmission electron microscopy. Only 2% of the cells contained internalized yeast (Figure 5). Hemocyte phagocytic activity was also evaluated by *in vitro* experiments. The presence of either live or heat-killed yeasts in the culture media did not activate phagocytic response. Some yeast was found in contact with hemocytes, but none were internalized after 1 h. Moreover, some hemocytes in these preparations had no cytoplasmic granules after 30 min, which suggests that they had been secreted. In fact, the secretion of granular contents by exocytosis was detected by TEM (Figure 6A).

### 3.4. Gomesin secretion after microbial challenge

Gomesin exocytosis was quantitatively assayed by ELISA (Figure 6B). Under the assay conditions, LPS induced gomesin secretion by the hemocytes in a concentration-dependent



**Figure 6** Gomesin secretion by hemocytes. Heart sections of *Saccharomyces cerevisiae* ( $10^3$  cells)-challenged spider were observed by TEM. (A) Electron micrograph showing exocytosis of contents from hemocyte granule (G). Bar, 1 μm. (B) The amount of gomesin released into the culture media after LPS stimulation measured by ELISA showed that LPS induced gomesin secretion by hemocytes in a concentration-dependent manner. Data expressed as means  $\pm$  SME.

manner from 0.01 to 1.0 μg/mL. The highest amount of gomesin (160 nM) was observed when the hemocytes were exposed to 1 μg/mL of LPS. The hemocytes presented the same secretory pattern in all three independent experiments performed.

#### 4. Discussion

The main goal of the present work was to investigate the involvement of hemocytes in spider immunity. In contrast to the literature on insects [1], few reports are available on how spiders mount an immune response. Most reports are concerned with the morphological characterization of hemocytes. Their specific functions are not known; however, they probably are involved in blood clotting, wound healing and fighting off infections [32]. Spider hemocytes are originated from the heart cell wall. Prohemocytes detach from the hematopoietic tissue and undergo mitotic divisions until maturation [32]. It was observed that the granulocytes are the most abundant cell type in spiders. They present numerous dense granules in cytoplasm [32,33]. Some hemocytes called leberidiocytes have glycogen inclusions. The increase of these cells number during the moulting indicated that they might play a role in energy supply during this process [34]. Cyanocytes are another type of hemocytes that are responsible for hemocyanin production and storage [32]. This cell type was firstly described in limulus. Different from other types of hemocytes, the origin of cyanocytes is not known [35].

Sherman has classified spider's hemocytes following the insect's nomenclature [33]. He observed sections of a spider heart by TEM and found at least three types of hemocytes. In agreement with Foelix [32], granulocytes were the most common cell type. Plasmacytoids were quite abundant while oenocytoids represented only 5% of the hemocytes population. In addition, some hemocytes appeared to be in transition from plasmacytoids to both oenocytoids and granular hemocytes.

During the development of this work, we observed mostly granulocytes in circulating hemocytes from the spider

*A. gomesiana*. In the heart sections, we also observed cells without cytoplasmic granules; according to Foelix [32], we called them prohemocytes. In addition, some hemocytes presenting crystalline inclusions (cyanocytes) were detected but they were rarely observed.

Recent work has shown that hemocyanin, the oxygen carrier in spider plasma, also displays phenoloxidase activity after limited proteolysis with either trypsin or chymotrypsin [36]. It is well known that the final product of the phenoloxidase system is melanin, which is deposited either onto the surface of the microorganisms or onto a capsule of microorganisms during the immune response of insects [37] and crustacea [38].

In previous reports, we described the purification and characterization of two AMPs from hemocytes of spider *A. gomesiana*: gomesin and acanthoscurrin [16,17]. Here we showed that they are often stored in the same cell (58%). Nevertheless, they can also be stored in different cells: 33% of the hemocytes contained only acanthoscurrin and 2% only gomesin. Two mussel AMPs, defensins and mytilin, are stored either in the same cell or in different ones. Hemocytes that store only mytilin seem to be involved in phagocytosis, but the role of hemocytes that store only defensins is not clear. Hemocytes that present both AMPs are activated later and might be responsible for AMP secretion [7]. In spiders, the differences in AMP distribution might be connected with either the distinct immunity roles played by hemocytes or differences in the cell maturation stages.

The injection of fluorescent particles into the spider leg clearly activated a coagulation cascade. A similar response occurs during hemolymph collection in the absence of an anticoagulation solution. Besides, except for coagulogen, all coagulation cascade components from horseshoe crab [39] are found in the cDNA library of spider hemocytes [40].

Coagulation is a well-conserved response in innate immunity and it is especially important for invertebrates, which have open circulatory systems. Generally, in wound repair it prevents the loss of hemolymph [41]. In horseshoe crabs, the presence of either LPS or  $\beta$ -1,3 glucan triggers the serine protease cascade that converts the soluble protein coagulogen into the insoluble coagulin [6].

The migratory response of hemocytes to the yeast injection site in the spider heart was confirmed by *in vitro* experiments, in which LPS and yeast acted as chemoattractants, whereas 10  $\mu$ M gomesin did not. In contrast to our results, human neutrophils can be attracted by human defensins [42]. Temporin, an AMP found on the skin of the European frog, also promoted leukocyte chemotaxis in mice and humans [43].

Sections of yeast-challenged spider heart revealed a very low percentage of ingested yeast. *In vitro* experiments performed with heat-killed and live *S. cerevisiae* and *E. coli* did not activate the hemocyte phagocytic response. These results suggest that phagocytosis is not the major defense mechanism activated upon microbial challenge. In contrast, when *S. cerevisiae* was injected into the cattle tick hemocoel, approximately 20% of the hemocytes internalized yeast [44]. Interestingly, phagocytosis in horseshoe crab was only observed after hemocyte incubation with endotoxin-free iron particles [45].

Exocytosis of contents of hemocytic granules was observed by TEM in heart sections of yeast-challenged spiders. Moreover, optical microscopy revealed that hemocytes incubated *in vitro* with yeast degranulated after 30 min. It was also shown that hemocyte incubation with LPS induced dose-dependent gomesin secretion. Furthermore, acanthoscurrin was also secreted after *in vivo* challenge, as previously demonstrated [17]. In all, these results strongly indicate that AMP secretion by spider hemocytes and coagulation might be the major defense mechanisms activated after infection.

Horseshoe crab hemocytes contain several defense molecules stored in two types of secretory granules: large granules and small granules. The former selectively accumulates more than 25 defense components, such as clotting factors, a clottable protein, coagulogen, proteinase inhibitors, lectins, and antimicrobial proteins. In contrast, small granules contain at least six AMP and several proteins <30 kDa [6].

In the case of horseshoe crabs, LPS induces secretion of 100% of the contents of hemocyte granules in less than 20 min. Exocytosis is mediated through protein G activation. This transmembrane receptor activates phospholipase C, which is responsible for increasing the concentration of inositol 1,4,5-triphosphate. Consequently, intracellular calcium is mobilized, triggering exocytosis [46]. We believe that the secretion of the AMPs, gomesin and acanthoscurrin, by hemocytes from challenged spiders is probably due to the activation of G protein as shown for limulus. Ozaki et al. [47] showed that tachyplesin, a horseshoe crab hemocyte AMP, stimulates LPS-induced exocytosis amplification through the same pathway, the activation of the G protein. More studies are needed to determine if spider AMPs can amplify the exocytosis response.

The presence of the C-terminal prodomain labeling in hemocyte granules suggests that gomesin is addressed to the granules as a propeptide. Progomesin processing might take place intra- or extracellularly after propeptide secretion. Gomesin was purified as a mature peptide using the procedure previously described [16] through acidic homogenate of the hemocytes. After removing the plasma from the hemocytes by centrifugation, the processing enzyme, which is responsible for progomesin cleavage, must be

localized inside the hemocytes. The sequence for gomesin shows a glycine residue followed by two basic amino acid residues (Lys-Arg) [27]. This dibasic site is specific for endopeptidases of the subtilisin family. These endopeptidases are known for processing several peptides, such as the yeast  $\alpha$  factor and prorenin [48]. Interestingly, sequences in the cDNA library of spider hemocytes were similar to two endoproteases of the subtilisin family: SPC3 from *Bachios-toma californiensis* (847761) and PC5 from *Rana esculenta* (23266416) [40]. Therefore, after cleavage by subtilisin, the two remaining basic gomesin precursor residues can be removed by carboxypeptidase and the glycine residue can provide the amide group to form an amidated C-terminal end through the peptidylglycine  $\alpha$ -amidating monooxygenase activity [49].

Peptides of the cathelicidin family are stored as precursors in the granules. After microbial infection, propeptide is secreted into the plasma, where proteases of the elastase family cleave the cathelin domain, resulting in a mature moiety [50]. Defensins in Paneth cells are also stored as propeptides. Although processing occurs extracellularly, both the processing enzyme and the propeptide are stored within hemocyte granules. The processing enzyme, a trypsin, is stored in the granules as a zymogen. After reaching the plasma, it is activated and converts prodefensin into the mature peptide [14].

In conclusion, our results indicate that gomesin is addressed to the hemocyte granules as a propeptide. The processing enzyme may be stored in hemocytes, probably in the granules. Gomesin processing may take place in either hemocyte granules or the plasma after secretion of both progomesin and the processing enzyme. Moreover, our results strongly suggest that the spider defensive mechanisms are very similar to those of horseshoe crab. After microbial infection, hemocytes may migrate to the infection site and release not only components of the coagulation cascade but also AMPs to trap and eliminate the invading microorganisms. Phagocytosis probably plays a secondary role, being responsible for clearing cellular debris and remodeling damaged tissues.

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