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Insight on cellular and humoral components of innate immunity in *Squilla mantis* (Crustacea, Stomatopoda)

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ABSTRACT

For deeper insights into the function of crustacean haemocytes in immune responses, we studied the morphology and enzyme content of circulating cells of the mantis shrimp *Squilla mantis* from the North Adriatic Sea, together with their ability to phagocytose foreign cells. We also assayed the enzyme content and the agglutinating and haemolytic activities of cell-free haemolymph.

Three haemocyte types, i.e., hyalinocytes, semigranulocytes and granulocytes, can be distinguished, according to cell and nuclear morphology and the presence of cytoplasmic granules. All of them share the same patterns of enzyme activities and are recognised by the same lectins. Spreading cells (hyalinocytes and semigranulocytes) can ingest foreign cells; granules of semigranular and granular cells have similar cytochemical properties. Injection of *Micrococcus luteus* into the heart sinus results in an increase in the frequency of hyaline cells and a decrease in the frequency of granulocytes. After 24 h from the injection, a decrease in the number of phagocytosing hyalinocytes, and a general decrease in the frequency of acid phosphatase-positive cells was reported.

Our data match previous results and suggest the existence of a single differentiation pathway for *Squilla* haemocytes with the three haemocyte morphs as different stages of cell differentiation. Results also indicate that *Squilla* haemolymph performs immunosurveillance, through rapid changes in haemocyte distribution, increase of antimicrobial and antioxidant enzymes and secretion of lectins stimulating agglutination, phagocytosis and encapsulation.

1. Introduction

As invertebrates, crustaceans rely only on innate immunity to cope with potentially pathogenic non-self particles or cells which have entered the organism. Circulating haemocytes play a major role in crustacean immune defence, being involved in prevention of blood loss, clearance of non-self particles through phagocytosis and encapsulation, recognition and confinement of invasive organisms by clot formation [1,2]. Most of the published data on crustacean immunity refers to members of the order Decapoda, where at least three haemocyte types have been described: hyaline, semigranular and granular cells [1]. However, the classification of crustacean circulating cells is still uncertain and many doubts still exist on the origin of the various cell types and their mutual relationships. Bodammer [3] suggested that the various blood cells represent distinct stages of cytogenesis of a single cell type, and this hypothesis was corroborated by Bauchau and De Brouwer [4], who stated that semigranular or intermediate cells represent transitional forms between hyaline and granular cells.

Within the class Malacostraca, the members of the order Stomatopoda have been less frequently studied than Decapoda, and there are relatively few papers in the literature dealing with their haemocytes and their role in immune responses [5–7].

Squilla mantis is a stomatopod shrimp, widespread along European coasts, which represents a common by-catch product, of important economical value, in various fishing techniques. Three haemocyte types have been described in *S. mantis* by Barracco and Amirante [5], who classified circulating cells into hyaline cells, small granule haemocytes and large granule haemocytes. According to their view, hyaline cells lack large distinctive cytoplasmic

Abbreviations: CB, cacodylate buffer; CFH, cell-free haemolymph; ConA, concanavalin A; DAB, 3-3′ diaminobenzidine; DMF, dimethylformamide; DSL, *Datura stramonium* lectin; EDTA, ethylene diamine tetraacetic acid; FSW, filtered seawater; HL, haemocyte lysate; HT, haemagglutination titre; HPA, *Helix pomatia* agglutinin; L-DOPA, 3,4-dihydroxy-L-phenyl-alanine; LFA, *Limax flavus* agglutinin; LM, light microscope; NPA, *Narcissus pseudonarcissus* agglutinin; PBS, phosphate-buffered saline; PNA, peanut agglutinin; RCA, *Ricinus communis* agglutinin; RU, relative unit; SAB, sodium acetate buffer; SOD, superoxide dismutase; UEA-I, *Ulex europaeus* agglutinin I; VVA, *Vicia villosa* agglutinin; WGA, wheat germ agglutinin.

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granules and can adhere and spread on glass surfaces; they also play an active role in phagocytosis. Conversely, the semigranular and granular cells contain large granules and are mainly involved in non-self recognition and clearance, induction of coagulation and toxic effects [7]. Granulocytes represent one of the first barriers against foreign bodies, as they are responsible for secreting lectins, with specificity for β-glycosides, able to trigger the encapsulation of foreign corpuscles [8].

In the present work, we re-examined the haemocytes of *S. mantis*, with particular reference to their morphology, enzyme content and phagocytosis capability, with the aim of gaining deeper insights into their functions in immune responses and differentiation pathways. We also studied some properties of the haemolymph related to its immune role, such as enzyme content, agglutinating and haemolytic capabilities. In agreement with previous results [5], our data suggest the existence of a single differentiation pathway for *Squilla* haemocytes with the three, classically described, haemocyte morphs as different stages in the cell differentiation pathway. Results also indicate that haemolymph exerts its immunosurveillance role through quick changes in haemocyte distribution, increase of antimicrobial and antioxidant enzymes and secretion of lectins stimulating agglutination, phagocytosis and encapsulation.

2. Materials and methods

2.1. Animals

Specimens of *S. mantis* were caught with creels or drift nets within two miles of the coast, in the North Adriatic Sea off the Lagoon of Venice, during summer 2010 (June–September) and spring 2011 (March–May). Their weight ranged between 33 and 64 g. They were kept in aerated aquaria provided with sandy bottoms and filled with filtered seawater (FSW), with a salinity of 35‰, at the temperature of 16 °C, for a period of at least 3 days before their use in experiments. Water was changed every day. The animals were fed with small shrimps of the genus *Palaemon* or soft parts of *Mytilus galloprovincialis*.

2.2. Collection of haemocytes and preparation of haemocyte lysate and cell-free haemolymph

Haemolymph was collected from the dorsal, tubular heart of at least 6 specimens (3 males, 3 females) for each experimental condition, with a sterile 1-ml syringe and immediately centrifuged at 800 × g for 10 min at 4 °C: the supernatant represented the cell-free haemolymph (CFH). In order to collect haemocytes, haemolymph was immediately diluted 1:1 with EDTA–citrate buffer (NaCl 0.45 M, glucose 0.1 M, sodium citrate 30 mM, citric acid 26 mM, EDTA 10 mM, pH 4.6) as anticoagulant [9] and the pellet was then resuspended in FSW at a concentration of 10^5 cells/ml. Blood samples were not pooled. For haemocyte lysate (HL) preparation, haemocytes were resuspended in 100 μl of distilled water, sonicated at 0 °C for 2 min with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant, corresponding to HL, was then collected and stored at −80 °C until use. Protein concentration in CFH and HL was quantified according to Bradford [10], with bovine serum albumin as standard.

2.3. Determination of haemocyte concentration and morphological studies

Total haemocyte count (THC) was performed with a Bürker haemocytometer. Haemocytes, collected as described above, were centrifuged at 800 × g and resuspended in FSW at a concentration of 10^5 cells/ml. Sixty microlitres of haemocyte suspension were placed in the centre of a clean glass slide and cells were left to adhere for 30 min at room temperature in a humid chamber before their observation under a Leitz Dialux 22 light microscope. Haemocytes were classified on the basis of their granular content, according to Bauchau [1]. The cell shape factor, defined as in Ottaviani et al. [11], was evaluated: lower shape factors indicate larger perimeters with respect to the areas and, therefore, an increased amoeboid shape.

3. Effects of *Micrococcus* injection

One hundred microlitres of a lyophilised *Micrococcus luteus* (strain ATCC 4698; Sigma) suspension (10^8 cells/ml) in FSW, were injected into the heart sinus of healthy specimens with a 1-ml sterile syringe; FSW was injected into controls. Similar concentrations were reported to affect immune parameters in various invertebrate species [12,13]. The animals were then re-introduced into the aquaria and samples of haemolymph were collected after 1, 3 and 24 h from the injection. Haemolymph from untreated specimens was used as reference control. No animal died in the 3 days following the injection.

3.1. Electron microscopy

Pelleted haemocytes were fixed in 1.5% glutaraldehyde buffered with 0.2 M sodium cacodylate buffer (CB), pH 7.4, plus 0.29 M NaCl. After washing in CB and postfixation in 1% OsO_4 in CB, cells were dehydrated and embedded in Epon Araldite. Serial sections (1 μm) of haemocytes were counterstained with Toluidine blue; thin sections (90 nm) were stained with uranyl acetate and lead citrate. Micrographs were taken with a Hitachi H-600 electron microscope operating at 75 kV. Photographs were digitalised with an Epson Perfections Scanner 1200S and were collected and typeset in Corel Draw X3.

3.2. Cytochemical assays

Once stuck to the glass slides, cells were fixed for 30 min at 4 °C with a solution of 1% sucrose and 1% glutaraldehyde in FSW and stained according to the cytochemical methods reported below. Slides were then mounted with Acquovitrex (Carlo Erba) and observed under an Olimpus CX31 light microscope (LM) equipped with camera and image analysis system.

3.2.1. Giemsa's stain

Haemocytes were stained for 5 min in 10% Giemsa's solution (Fluka) and then washed in distilled water. Nuclei appear blue and cytoplasm pale blue, or violet in the case of metachromasia.

3.2.2. Periodic acid Schiff (PAS) reaction for polysaccharides

Fixed haemocytes were incubated in 1% periodic acid for 10 min, rinsed in tap water and stained with Schiff's reagent for 30 min at 37 °C. Coverslips were then dipped in a solution of 0.6% sodium metabisulphite in 0.02 M HCl for 6 min, washed in tap water for 10 min, and then rinsed in distilled water. Positive sites appear pink.

3.2.3. Sudan black stain for lipids

After adhesion of haemocytes, coverslips were dipped in 70% ethanol for 30 s and stained with a saturated solution of Sudan Black (Sigma) in 70% ethanol for 15 min at 70 °C. They were then rinsed in 70% ethanol and washed in distilled water. Black spots reveal the presence of lipids.