Fish and Shellfish Immunology 81 (2018) 73–82

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Proliferation and differentiation of circulating haemocytes of *Ruditapes philippinarum* as a response to bacterial challenge

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ARTICLE INFO

Keywords:
Bacterial phagocytosis
Bivalves
Haemoblast proliferation
Haemocytes
Lectin affinity
Manila clam
Molluscs
Ruditapes philippinarum
Stem cells
Ultrastructure

ABSTRACT

Ultrastructural investigation confirmed the presence of four cell types (granulocytes, hyalinocytes, serous cells, and haemoblasts) in the haemolymph of the Manila clam, *Ruditapes philippinarum*. Granulocytes were characterised by numerous electron-dense granules, whereas hyalinocytes had a considerable number of small clear vesicles. Serous cells exhibited large vacuoles, which filled the cytoplasm, and haemoblasts (the undifferentiated cells) were small roundish cells characterised by a high nucleus/cytoplasm ratio. The presence of circulating haemoblasts was observed at various phases of mitosis. Updated data concerning the proliferation and differentiation of circulating haemocytes were obtained after both *in vitro* and *in vivo* bacterial challenge. The results demonstrated that cell proliferation occurred within 15 h of exposure, and most haemocyte types responded to the stimuli. The number of granulocytes significantly decreased after massive phagocytosis and ultrastructural observations confirmed that they were active phagocytic cells against both Gram-positive and Gram-negative bacteria, which were rapidly engulfed into large phagosomes. Granulocyte lysis may represent a protection response against bacterial proliferation inside phagosomes. The number of serous cells significantly increased, suggesting a previously unreported pivotal immune role during bacterial infection. A panel of lectins was used as probes to further characterise haemocytes and their relationships. Only hyalinocytes were not positive for the lectins assayed, whereas all lectins labelled serous cells, suggesting that these cells have a variety of specific carbohydrates, which are shared with certain haemoblasts. The hypothesis of the existence of a prospective haemoblast for serous cell origin is discussed.

1. Introduction

Ruditapes philippinarum (formerly *Tapes* or *Venerupis philippinarum* or *semidecussatus*) is a bivalve species, known as the Manila clam, which is widespread in several marine coastal areas, extending from the Pacific Ocean to the coasts of North America and the Mediterranean Sea [1]. This species has largely been used as a model organism to study the role of haemocytes in innate immune responses and to assess the effects of stressors (pollutants in particular) on cell parameters of bivalves [2].

In a previous study, we characterised the circulating haemocytes of this species from a morpho-functional point of view [3]. Four cell types were identified, namely, granulocytes (the most abundant cell type, approximately 50%), hyalinocytes (approximately 30%), haemoblasts (approximately 20%), and serous cells (approximately 1%) by means of differing cytochemical assays. The granulocytes were further divided in basophils, neutrophils and acidophils. Granulocytes and hyalinocytes were competent phagocytes towards yeast as experimental target and were able to produce hydrolytic and oxidative enzymes (mostly after stimulation with yeast) and superoxide anions. Interestingly, the presence of both granulocytes and hyalinocytes has been confirmed in the haemolymph of the congenus species *Ruditapes decussatus* [2,4,5].

Regarding the other haemocyte types of *R. philippinarum*, we suggested that the chemical composition of cell pigments contained in the large vacuoles of serous cells resembled acid mucopolysaccharides [3] and other authors hypothesised that these cells may release their large granules during aggregation [6]. However, the origin and role of this cell type remain unknown.

We demonstrated that haemoblasts had the typical features of small undifferentiated cells and were positive to the anti-CD34 antibody, suggesting that they could be haematopoietic stem cells freely circulating in the haemolymph [3]. Although the origin of haemocytes in bivalve molluscs represents a controversial issue [7], we observed that haemoblasts of *R. philippinarum* are capable of dividing in the haemolymph [8]. In 1981, Cheng [9] first theorised that the three differentiated cell types (granulocytes, hyalinocytes, and serous cells) derived from three separate cell lines: granuloblasts and hyalinoblasts for

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https://doi.org/10.1016/j.fsi.2018.07.010
Received 8 March 2018; Received in revised form 27 June 2018; Accepted 4 July 2018
Available online 05 July 2018

granulocytes and hyalinocytes, respectively, and large serous cells derived from small serous cells. The presence of a single cell line of haemoblasts was successively proposed [10], that would mature first into hyalinocytes, the juvenile stage, and subsequently into granulocytes, considered the final stage of differentiation. Conversely, it was hypothesised [2,11,12] that haemocytes originate from a haematopoietic process in the connective tissues and the mantle, from which circulating haemoblasts move into the haemolymph where they complete the differentiation process. A recent study demonstrated that certain prohaemocytes arise from the basal tissue of the oyster gills [13]. Nevertheless, in the circulating haemocytes of the scallop, *Chlamys farreri*, *runt* gene family molecules have been found [14], which encode proteins that are necessary for haematopoiesis in mammals. These proteins are associated with the proliferation and differentiation and the recruitment of new haemocytes to the circulation after immune challenge.

In this study, the morphological and functional features of haemocytes from *R. philippinarum* were studied in depth by means of various techniques after *in vitro* and *in vivo* bacteria challenge experiments in order i) to demonstrate the multipotency of the circulating haemoblasts previously proposed as haematopoietic stem cells by evaluating the haemocyte differentiation after non-self stimuli, ii) to distinguish the various cell types in the haemolymph, in particular those involved in cell differentiation during bacterial challenge at both light and electron microscopy through lectin cytochemistry as a labelling tool and iii) to increase the knowledge on the roles of haemocyte types in immune response of bivalves.

2. Materials and methods

2.1. Animals

Specimens of *R. philippinarum* (approximately 3.5 cm mean shell length) were collected in the Lagoon of Venice and kept in the laboratory in large aquaria provided with a natural sandy bottom (sand was sieved accurately before use) and natural aerated seawater (salinity of 35 ± 1 psu, temperature of 17 ± 0.5 °C). A semi-static system was used for clam maintenance, with seawater renewal every other day. Clams were fed daily with microalgae (*Isochrysis galbana*) or Liquifry Marine (Interpet, Dorking, England). Before proceeding with experiments, the animals were left to acclimate for at least a week.

2.2. Haemolymph collection

The haemolymph was collected from the anterior adductor muscle with a 1-mL syringe following the partial opening of the valves using a scalpel. The haemolymph was stored in 1.5-mL Eppendorf tubes at 4 °C with an equivalent volume of a solution of 0.38% sodium citrate in 0.45-μm-filtered seawater (FSW), pH 7.5, to avoid cell clotting. The cell suspensions were centrifuged at 800 x *g* for 15 min, the supernatant with the anticoagulant was discharged, and the pellet was resuspended in an equal volume of FSW.

2.3. Light microscopy

Observations, counting and micrographs of haemocytes were performed at 1000x with an Olympus CX31 light microscope (LM) equipped with a DV Lumenera Infinity 2 and the Infinity Capture Application software version 5.0.0 (Lumenera Co. 2002–2009). Observations with differential interference contrast (DIC) or Nomarski microscopy of living cells were performed with a polarising Leitz Orthoplan LM.

2.4. Cytochemical assays for rapid haemocyte characterisation

2.4.1. Neutral red

Sixty microlitres of haemocyte suspension were placed on a Superfrost™ Plus (Thermo Scientific) slide with an electrostatic surface, leaving the haemocytes to adhere spontaneously to it for 30 min and to form a monolayer. After adhesion, the drop was replaced with an 8 mg L^{-1} of Neutral Red (Merck) solution in FSW. Living cells were incubated for 20 min, the staining solution was replaced by an equal volume of FSW and the sample was directly observed by LM with a coverslip held slightly raised at the corners by plasticine to avoid cell crushing. Neutral Red is a lipophilic dye, which crosses cell membranes. The low pH inside lysosomes and other compartments with acid content causes the dissociation of the dye not allowing its release into the cytoplasm and making it visible under LM. Due to this technique of vital staining, the serous cells and granulocytes could be rapidly recognised.

2.4.2. Modified Pappenheim's panoptic staining method

The adhered haemocytes were fixed for 30 min at 4 °C in a fixative mixture for marine tunicates [15] consisting of 4% paraformaldehyde plus 0.2% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, with the addition of 1% NaCl and 1% sucrose. After 2–3 washes of 10 min each in a 0.1 M phosphate buffered saline (PBS: 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 0.2 g L^{-1} KH_2PO_4, 1.15 g L^{-1} Na_2HPO_4, pH 7.2), the haemocytes were stained for 10 s in a filtered aqueous solution of 0.5% Toluidine Blue (Fluka) and 0.5% sodium tetraborate. After washing in tap water, the haemocytes were stained with May-Grünwald solution (Fluka) for 3 min, quickly washed with tap water and stained with a 5% Giemsa aqueous solution for 10 min. After a rapid wash in distilled water, the monolayers were mounted with Acquovitrex (Carlo Erba). This method reveals the granules of granulocytes and the vacuolar content of serous cells, with the latter exhibiting a typical pink-violet metachromasia of Toluidine Blue. In this way, the various cell types could be easily recognised and distinguished for counts.

2.4.3. Staining methods for mitotic activity characterisation

This method [16] characterises non-condensed chromatin, which turns violet with haematoxylin, from condensed chromatin into chromosomes, which turns red-orange with Pfitzner's safranin. Cells in various mitotic phases can be distinguished. The haemocytes were previously fixed with Sanfelice's solution (1% chromic acid in seawater, 32% formalin, 4% acetic acid) for 30 min at room temperature. After washing with distilled water, the haemocytes were stained with haematoxylin for 1 min and differentiated in tap water for 15 min. The haemocytes were successively stained with Pfitzner's safranin alcoholic solution (1% safranin in absolute ethanol diluted with 150 mL of distilled water) for 1 h, washed in absolute ethanol and dehydrated in xylene. Slides were mounted in Eukitt (O. Kindler Gmbh & Co).

2.5. Bacterial strains

Two bacterial strains were used: *Bacillus clausii* (strain Enterogermina®, Sanofi-Aventis) as Gram-positive bacteria and *Escherichia coli* O4 as Gram-negative bacteria. They grew for 24 h at 37 °C in Broth Brain Heart Infusion (BHI, Fluka Analytical 53286). The concentration of bacteria was determined by reading the optical density (OD) at 600 nm; appropriate bacteria dilutions were made with the growth medium.

2.6. In vitro immune stimulation assay

After haemolymph collection from 30 individuals, a total volume of 6 mL of haemocyte suspension was obtained and divided into 12 tubes containing 500 μL each. The haemocyte suspensions were centrifuged at 800 x *g* for 15 min and resuspended in equal volume of FSW. Bacterial pellets were obtained separately after centrifugation at 12500