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Morphologic, cytometric and functional characterisation of abalone (*Haliotis tuberculata*) haemocytes

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Abstract This work presents the first detailed microscopic and functional analysis of the haemocytes of an abalone; the European *Haliotis tuberculata*. It is shown that in contrast to the situation in bivalves, only very few basophilic ''granulocytes'' could be found and exclusively with a histological stain. Neither flow cytometry, phase contrast observation nor transmission electron microscopy were able to detect any granular cells. The large majority of cells was constituted of ''hyalinocytes'', which could be sorted by flow cytometry, for the first time, into small (blast-like) and large cells. This permits a detailed analysis of haemocytes and especially of the lowly represented blast-like cells. The differences in haemolymph cell composition between bivalves and gastropods is reviewed in depth and discussed in view of the new data we present. Most of the abalone haemocytes analysed harbour many vacuoles, large glycogen deposits, lipid inclusions and acidic compartments. However, although the number of these ''inclusions'' was rather variable in between individual hyalinocytes, these experiments did not allow to discern subpopulations using these criteria, and the population appears more

Abbreviations: AASH, Anti-Aggregant Solution; MAS, Modified Alsever's Solution; FSSW, Filtered and sterile seawater; PBS, Phosphate Buffered Saline; HCM, Haemolymph Cell Monolayer; MGG, May-Grünwald Giemsa; PAS, Positive Acid Schiff; FCM, Flow Cytometry; FSC, Forward Scatter; SSC, Side Scatter; TEM, Transmission Electron Microscopy.

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as a ''differentiation continuum''. Haemocytes adhere very rapidly and are immunologically active as they quickly phagocytose latex beads and zymozan particles. This study is the first step towards understanding the *H. tuberculata* immune system by adapting new tools to gastropods and in providing a first detailed morpho-functional study of their haemocytes.
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Introduction

Increasing prevalence of bacterial diseases such as vibrioses [1—3] has become an important impairment for the sustainable development of natural and cultured abalone stocks [4]. Investigation into the abalone immune system is becoming very important as only little is known about the cellular immune response of marine gastropods compared to that of bivalve molluscs. Haemocyte characterisation is the first step towards understanding their immune capacity and its potential failure during disease development.

Haemocytes are thought to be involved in many functions, including digestion, metabolite transport [1] and wound and shell repair [5]. However, their most important role resides in internal defence [6] as they are the main immune effector cells. They are responsible for chemotaxis, lectin-mediated pathogen recognition and phagocytosis. They are also implicated in encapsulation or elimination of invaders (reviewed in [6,7]) and are able to produce antimicrobial peptides [8—10].

A number of publications are available on mollusc haemocytes, but controversies still exist about their classification [11] which could, in part, be due to the differences in the methods used. The bivalves haemocyte classification established by Cheng in 1981, in which two main haemocyte types are described [6], is currently generally applied to most mollusc species. Hyalinocytes contain few or no granules, and a round well-centred nucleus, and granulocytes contain granules and an eccentric, round to ovoid nucleus [12]. Cytochemical properties allowed classification of bivalve granulocytes in eosinophils, basophils or neutrophils [13,14]. Functionally, both hyalinocytes and granulocytes can form pseudopodia, aggregate, phagocyte particles and produce reactive oxygen species [15,16]. However, granulocytes were found to be more efficient in the destruction of invading particles [14,17,18].

In gastropods, most studies have been done on the pulmonates *Biomphalaria glabrata* (the intermediate host of *Schistosoma mansoni*) and *Lymnaea stagnalis*. Two haemocyte types (Types I and II) have been described and suggested to correspond to the bivalve granulocyte and blast-like cell types [19,20], even if the authors recognized the difficulty to use the term granulocyte.

The immune response of abalone and especially their haemocyte composition has been poorly studied [21,22]. In 1996 Lebel, describing the effect of vertebrate growth factors on haemocytes in primary culture, divided *H. tuberculata* haemocytes in two categories: adhering fibroblast-like cells and non-adherent epithelial-like cells [21]. However, no proper morphological classification has ever been proposed for *H. tuberculata*, or any other abalone.

Flow cytometry has been applied to study morphologically and functionally bivalve haemocytes [23—28]. This technique has contributed to improve investigation in this field because of its potential for rapid screening of large amounts of cells and for measuring various immune parameters on a single individual. Here, we present a detailed morphological and functional characterisation of the haemocytes of the abalone *H. tuberculata*.

Material and methods

Animals

Thirty abalone, *Haliotis tuberculata* (60—80 mm shell length) were collected from natural populations in the Bay of Roscoff, Brittany (3°58′ W, 48°43′ N) in November 2006, and 30 in the Bay of Brest, Brittany (4°33′ W, 48°21′ N) in February 2007. The animals were acclimatised in the laboratory in 110-L tanks in an open seawater circuit with continuous aeration at 15 ± 2 °C, for at least 2 weeks prior to the experiments. During the acclimation period, animals were fed on a marine macroalgae diet of *Laminaria digitata* and *Palmaria palmata*.

Haemolymph collection

Haemolymph was withdrawn from the cephalic arterial sinus located at the anterior part of the muscle using a 25-gauge needle attached to a 2-ml syringe containing different solutions depending on the procedure (see below). Haemolymph from each abalone was transferred into a vial and kept on ice.

Light microscopy

Fresh and fixed haemocytes

Cells in suspension

A fixed volume of haemolymph was withdrawn into syringes containing an equal volume of either filtered sterilized seawater (FSSW), anti-aggregant solution AASH (1.5% EDTA in 0.1 M phosphate buffer, pH 7.4 [29]), Hemofix® fixative (Becton Dickinson) or 6% glutaraldehyde solution (in 0.1 M phosphate-buffered saline (PBS), pH 7.4). These preparations were immediately observed on a phase contrast microscope (Leica) or a differential interference microscope (Zeiss Apotome) equipped with Nomarsky contrast.

Haemolymph cell monolayers (HCM)

HCMs were prepared in two different ways: cyto-centrifugation or cell adhesion. Haemolymph, for both procedures, was withdrawn directly in a same volume of modified Alsever's solution (MAS, 20.8 g L^{-1} glucose, 8 g L^{-1} sodium citrate, 3.36 g L^{-1} EDTA adjusted with NaCl to 1100 mOsm) [30], washed twice and re-suspended. To

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