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Differential involvement of mussel hemocyte sub-populations in the clearance of bacteria

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ABSTRACT

Mussels are filter-feeders living in a bacteria-rich environment. We have previously found that numerous bacterial species are naturally present within the cell-free hemolymph, including several of the *Vibrio* genus, whereas the intra-cellular content of hemocytes was sterile. When bacteria were injected into the circulation of the mussel, the number of living intra-hemocyte bacteria dramatically increased in less than an hour, suggesting intense phagocytosis, then gradually decreased, with no viable bacteria remaining 12 h post-injection for *Micrococcus lysodeikticus*, 24 h for *Vibrio splendidus* and more than 48 h for *V ibrio anguillarum*. The total hemocyte count (THC) was dramatically lowered by the bacterial injections, as quantified by flow cytometry. *V. splendidus* induced the strongest decreases with −66% 9 h post-injection of living bacteria and −56% 3 h post-injection of heat-killed bacteria. Flow cytometry was used to identify three main sub-populations of hemocytes, namely hyalinocytes, small granulocytes and large granulocytes. When THC was minimal, i.e. within the first 9 h post-injection, proportions of the three cell categories varied dramatically, suggesting differential involvement according to the targets, but small granulocytes remained the majority. According to a decrease in their number followed by an increase (+90% at 12 h with living *V. splendidus*), hyalinocytes also appeared to be involved as cellular effectors of antibacterial immunity, despite possessing little capacity for phagocytosis and not containing antimicrobial peptides.

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1. Introduction

Bivalve hemocytes are responsible for cell-mediated immunity through a panel of activities such as phagocytosis [1], the release of cytotoxic and agglutinating molecules [2], and the production of several reactive oxygen intermediates ROIs) [3,4] and nitric oxide (NO) [5,6]. In addition, lysosomal enzymes [7], phenoloxidase/peroxidase activities [8–10] and lysozyme [11] were found within the granules of eosinophils [7,8]. Also associated with different granules are the antimicrobial peptides (AMPs) from *Mytilus edulis* and *Mytilus galloprovincialis*, acting against bacteria inside phagolysosomes, then released into the circulation [12,13]. Complex cell-signaling pathways occurring during the immune response emerged in different invertebrates, including the mussels [14], indicating close similarities with the mammalian kinase-mediated cascades.

The different cell types found in mussel hemolymph were described in detail in the early 1990s. The general assertion was that two cell categories existed in *M. edulis*: (i) hyalinocytes and (ii) granulocytes which might be further subdivided according to granule size [15]. Staining capacities also resulted in two cell types: (i) basophils (about 40% of the total hemocytes) including a large majority of hyalinocytes and (ii) eosinophils (about 60%) including small and large granules [16,17]. Also in the mussel, *M. galloprovincialis*, two cell types have been described on the basis of staining properties: (i) hyalinocytes with characteristics of undifferentiated cells, and (ii) granulocytes being acidophils, basophils or both [18]. Finally, monoclonal antibodies revealed three cell sub-groups in *M. edulis*: (i) basophilic granulocytes, (ii) a sub-group including basophilic granular and hyaline cells and (iii) eosinophilic granular cells [19].

Although granulocytes were largely suspected to play a prominent role in defense, few reports aimed to establish functional relationships between mussel hemocyte sub-populations and immune capabilities [20–22]. In previous studies on immune gene expressions in *M. galloprovincialis*, we showed that *HSP70* and *AMP*

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genes responded specifically to the challenges, confirming that at least some of the innate immune mechanisms are specifically orientated [23,24]. In addition, hemocyte sub-populations are capable of discriminating between two *Vibrio* species [25]. To compliment this, the present report addressed functional aspects bridging (i) differential clearance of Gram positive *versus* Gram negative bacteria, with (ii) variations in the total number of hemocytes, and (iii) the behavior of hemocyte sub-populations in response to various bacteria. To achieve such goals, mussels have been challenged with one injection of either *Vibrio splendidus* LGP32, *Vibrio anguillarum* or *Micrococcus lysodeikticus*. The remaining living bacteria have been quantified in hemocytes using the colony-forming unit (CFU) technique. Total circulating hemocytes and three sub-populations have been quantified by flow cytometry, considering both cell size and granularity.

2. Material and methods

2.1. Mussels and bacterial growth

Adult mussels, *Mytilus galloprovincialis* (6–7 cm shell length), were purchased from the marine farm Les Compagnons de Maguelone (Palavas-France). They were maintained in the laboratory in oxygenated sea water at 20 °C for 1–3 days prior to experimentation.

Vibrio splendidus LGP32 is a Gram negative marine bacterium isolated from juvenile oysters, *Crassostrea gigas*, during 2001 summer mortalities [26]. *V. anguillarum* was from the Institut Pasteur-France (ATCC 19264). Both *Vibrio* species (50 µl of overnight-cultured inoculum) were grown at 20 °C in 10 ml of trypsin–casein–soya (TCS, AES Laboratoire, Bruz-France) for 4–6 h to ensure bacteria were in exponential growth phase, centrifuged for 10 min at $500 \times g$, then adjusted to 10^8 bacteria/ml with phosphate buffered solution isotonic to sea water (PBS–NaCl: 2 mM KH_2HPO_4, 10 mM Na_2HPO_4, 3 mM KCl, 600 mM NaCl in distilled water, pH 7.4) according to $1\ OD_{600\ nm} = 5 \times 10^8$ bacteria/ml as established by counting the number of CFU. Gram positive *Micrococcus lysodeikticus* from Institut Pasteur-France (ATCC 4698) was grown at 37 °C in Luria Broth (LB, Sigma Chemical Co, St Louis, MO, USA) until the bacteria were in exponential growth phase, centrifuged for 10 min at $500 \times g$, then adjusted to 10^8 bacteria/ml with PBS–NaCl according to $1\ OD_{600\ nm} = 0.36 \times 10^8$ bacteria/ml as established by counting the number of CFU.

2.2. Bacteria injection and hemocyte sampling

Four batches of 10 mussels each per each sampling time point were injected with 100 µl (10^7 bacteria) into the posterior adductor muscle, through a hole created by light filing on their shells. After injection, mussels were returned to 20 °C sea water. Control injections consisted of 100 µl of PBS–NaCl. Four batches of 10 unchallenged mussels each (referred to as controls) were sampled at the time of the corresponding injection to minimize batch variations. The full experiment involved a grand total of 2520 mussels.

Hemolymph was collected from the posterior adductor muscle with a 1 ml syringe containing 120 µl of anti aggregate Alsever's solution, 0.5, 1, 3, 6, 9, 12, 24 and 48 h post-injection. To ensure the same quantity of hemolymph was collected from each mussel, we limited the sampling to 580 µl per mussel. Samples from 10 mussels were pooled and 3 ml were added to 3 ml of 3.7% formaldehyde in PBS–NaCl for later flow cytometry analysis. The remaining 4 ml were centrifuged ($500 \times g$, 6 min, 4 °C), and the pellet of hemocytes was resuspended in 4 ml of PBS–NaCl for clearance measurement.

2.3. Clearance measurement

Aliquots of 50 µl of undiluted hemolymph or of hemolymph diluted 10- and 100-fold in PBS–NaCl, were plated in duplicate Petri dishes containing either TCS or LB agarose medium. CFU were counted after 24 h incubation at 20 °C for both *Vibrio* and 37 °C for *M. lysodeikticus*. Data from at least 2 consecutive dilutions (4 Petri dishes) were combined and presented as the arithmetic mean ± SEM. Statistically significant differences between some time point values were inferred from Student's *t*-test with $p < 0.05$.

2.4. Flow cytometry analysis

Aliquots (200 µl) from the formaldehyde-fixed hemocyte suspensions were added to 800 µl of PBS–NaCl. The samples were analyzed by flow cytometry (Cyflow® SL, data acquisition with Partec FloMax® software) using the parameters of relative size (FSC) and granularity (SSC). Each of the four different pools per time point were analyzed at least in duplicate. Data analysis and 3D graphs were generated with the software WinMDI 2.9 (Windows Multiple Document Interface for flow cytometry: http://facs.scripps.edu). A lower limit threshold was set up to exclude small events (<3 µm). Data are presented as the arithmetic mean ± SEM, with statistically significant differences between controls and selected time points values inferred from Student's *t*-test with $p < 0.05$.

3. Results

3.1. Clearance of bacteria

Previously, we have observed that no living bacteria could be recovered from circulating hemocytes collected from unchallenged mussels. In contrast, living bacteria from different species, including *Vibrio*, are normally present in the cell-free hemolymph as revealed by their capacity to form CFU of various morphologies (not shown). Consequently, CFU counts from cell-free hemolymph appeared unreliable. In our assays, the use of *Vibrio* selective thiosulfate citrate bile salts sucrose (TCBS) medium resulted in highly variable and largely under-estimated numbers of CFUs compared with the number of CFU with *Vibrio*-like morphology obtained with non-selective TCS medium (unpublished data). As a consequence, the present report considers only intra-hemocyte bacteria.

Following injection, both the quantities of living intra-hemocyte bacteria and the kinetics of clearance were dependent on the nature of the injected bacteria (Fig. 1). The three bacteria species were found inside hemocytes within the first hour post-injection. However, the number of recovered, living *V. anguillarum* 1 h post-injection (463 CFU) appeared about four times higher than the number of living *V. splendidus* (121 CFU, $p = 0.00011$) or *M. lysodeikticus* (133 CFU, $p = 0.00014$). Then, the number of CFU from the three bacteria species gradually decreased. *M. lysodeikticus* was the quickest to be eliminated as no living bacteria could be detected from 12 h post-injection onwards. Numerous CFU obtained from 6 h (239 CFU) to 24 h (261 CFU) post-injection samples revealed that injected *V. anguillarum* remained alive inside hemocytes but were reduced to 79 CFU at 48 h post-injection. Behavior of *V. splendidus* appeared different as, after a first minimum observed 3 h post-injection (60 CFU), a peak of living bacteria was observed 6 h post-injection (359 CFU, $p = 0.0008$) followed by a rapid decrease. Few living *V. splendidus* could be detected at 24 h post-injection (26 CFU).

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