Differential phagocytic ability of the circulating haemocyte types of the carpet shell clam

*Ruditapes decussatus* (Mollusca: Bivalvia)

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**ABSTRACT:** Phagocytosis of foreign materials by haemocytes is an important aspect of the internal defence of bivalve molluscs. Two main haemocyte types can be distinguished in the haemolymph of the clam *Ruditapes decussatus*: granulocytes and hyalinocytes. The ability of clam haemocytes to phagocytose zymosan particles, *Vibrio P1* cells and trophozoites of the protistan parasite *Perkinsus atlanticus* was demonstrated by means of in vitro assays. However, clam haemocytes did not phagocytose *P. atlanticus* zoospores in the assays. Granulocytes showed the highest phagocytic capacity in each assay. Phagocytic capacity of haemocytes was not significantly affected by clam age. An ultrastructural study of phagocytosis showed the following sequence of events: engulfment of particles by pseudopods, formation of a phagocytic vacuole, fusion of lysosomes with the phagocytic vacuole, and digestion of the particles giving rise to residual bodies that might be discharged.

**KEY WORDS:** *Ruditapes decussatus* · Haemocytes · Phagocytosis · *Vibrio P1* · *Perkinsus atlanticus*

**INTRODUCTION**

It is well-known that haemocytes are involved in the immune system of bivalve molluscs. Bivalve haemocytes are usually classified into 2 types, granulocytes and hyalinocytes (Cheng 1975, Auffret 1988). Nevertheless, heterogeneous cell populations are not precisely characterized in terms of their respective defence functions.

Phagocytosis of non-self materials by haemocytes is one of the defence mechanisms of bivalve molluscs (Feng 1988). It is known that haemocytes are the phagocytic cells in bivalve molluscs.

Infectious diseases are a very important problem in mollusc aquaculture. Among bivalve molluscs commercial losses occur as a result of mortalities caused by the presence of protozoa, rickettsia and chlamydia, and bacteria and viruses (Lauckner 1983). The availability of pathogen purification protocols (Mialhe et al. 1985, 1988, Le Gall & Mialhe 1992) has permitted the study of different aspects of defence mechanisms against a number of pathogens.

Several authors have studied the phagocytosis process and associated cytotoxic mechanisms in species belonging to the Veneridae family: e.g. *Mercenaria mercenaria* (Anderson 1994) and *Ruditapes decussatus* (López et al. 1994). *R. decussatus* is one of the most important species of this family in Europe, from a commercial point of view. Populations of this species are affected by 2 important epizootics caused by *Perkinsus atlanticus* (Azevedo 1989) and *Vibrio P1* (Paillard et al. 1994).

Morphological characterization of the circulating haemocytes of *Ruditapes decussatus* was studied as a first step of research on cellular defence and 2 cell types were found, granulocytes and hyalinocytes (López et al. 1997).

The purpose of the present study was to evaluate the phagocytic capacity of the haemocyte types of *Ruditapes decussatus* through in vitro assays using live...
cells of the clam pathogens mentioned above and inert particles as phagocytosis stimulants.

MATERIALS AND METHODS

Haemolymph collection. Haemolymph was taken from the posterior adductor muscle of clams *Ruditapes decussatus* using a syringe, through a hole made in the shell margin. It was collected diluted 1:3 in a modified anti-aggregant Alsever solution (MAS) (20.80 g l⁻¹ glucose, 8.00 g l⁻¹ sodium citrate, 3.36 g l⁻¹ EDTA, 22.50 g l⁻¹ sodium chloride in distilled water). Haemolymph of 5 specimens was pooled and the number of haemocytes was estimated with a Malassez haemocytometer.

For phagocytosis assays, the concentration in MAS was reduced to 3% by centrifugation, supernatant removal, and resuspension of haemocytes in sterile filtered seawater (FSW).

Phagocytosis assays. Light microscopy. In phagocytosis assays *zymosan*, *Vibrio* P1 bacteria (VP1), trophozoites and zoospores of *Perkinsus atlanticus* were used as phagocytosis stimulants.

In the *in vitro* phagocytosis assays, 2 × 10⁵ haemocytes were incubated in suspension with different particle stimulants for 1 h at room temperature (Fries & Tripp 1980), in Eppendorf tubes. The particle-to-haemocyte ratio was 5:1 for *zymosan*, trophozoites and zoospores of *Perkinsus atlanticus* and 20:1 for VP1. After the incubation, cytospin slides were performed, fixed, stained with rapid Hemacolor kit (Merck) and mounted.

The percentage of haemocytes corresponding to each cell type in the cytopins was estimated after evaluation of 300 cells by light microscopy.

A similar *in vitro* assay, with *zymosan* as phagocytosis stimulant, was performed from 2 groups of 15 clams of different ages (31.00 ± 0.31 mm and 23.93 ± 0.27 mm in longest axis) to test the influence of age on phagocytic capacity.

Phagocytosis assays. Electron microscopy. Similar *in vitro* assays were performed to study ultrastructural aspects of the process of phagocytosis. The number of haemocytes in these assays was 3 × 10⁶ and *zymosan* and VP1 were used as phagocytosis stimulant. No stage of *Perkinsus atlanticus* was used in these assays. After incubation, the suspension was fixed with 2.5% glutaraldehyde in PIPES buffer (0.1 M, at pH 7.2 and 7% sucrose) for 1 h at 4°C, and centrifuged (750 × g, 10 min). The pellet was washed in PIPES buffer with sucrose for 2 h at 4°C, and post-fixed in 1% osmium tetroxide in the buffer for 75 min at 4°C. The cells were then embedded in 1.5% agar at 40°C, and quickly centrifuged (1700 × g, 5 min). The pellet was dehydrated and embedded in Epon resin. Ultrathin sections (50 to 70 nm) were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope (Jeol100CXII).

Production of *Perkinsus atlanticus* zoospores. Gills of clams which were heavily infected with *P. atlanticus* were incubated in fluid thioglycollate medium (FTM) at room temperature, in the dark, for 3 d (Ray 1966).

Gills were then removed from FTM, blotted on filter paper, transferred to a Petri dish with FSW containing antibiotics (Penicillin G 500 units ml⁻¹ and streptomycin 500 mg ml⁻¹) and chopped with a scalpel. After attachment of the zoosporangia to the base of Petri dish, the supernatant was poured into another dish (carefully, to prevent the detachment of zoosporangia). Then, the first Petri dish was filled with FSW with antibiotics and this was repeated in the other Petri dishes. Petri dishes were kept at 28°C and it was possible to see zoospores in the FSW on the third day. Zoospores were observed to be actively motile just before the performance of *in vitro* phagocytosis assays.

**Purification of trophozoites of *Perkinsus atlanticus***.

The following procedure was adapted from the procedure for the purification of *Bonamia ostreae* from *Ostrea edulis* (Mialhe et al. 1988).

Only gills from *Ruditapes decussatus* heavily infected with *Perkinsus atlanticus* were used in the purification. Clams that were heavily infected could be distinguished by naked eye because white pustules were visible in the gills and mantle. Up to 12 clams were used in each purification.

Gills were homogenised with FSW with the addition of 0.5% Tween 80 (FSWT). The homogenate was then sieved progressively through 100, 75 and 20 μm nylon meshes. The resultant filtrate was spun at 2500 × g for 30 min at 8°C. The pellet was suspended in FSWT and layered onto a cushion of 15% sucrose in FSWT. After centrifugation, the supernatant was discarded and the pellet diluted in FSWT and layered onto a gradient of sucrose in FSWT (35% and 15%), and centrifuged again. Trophozoites were found at the interface 15%/35%. This fraction was resuspended in FSW and spun, and the pellet was resuspended in 1 ml of FSW and layered onto a Percoll gradient (10, 20, 30, 40 and 50%), containing 0.5 M NaCl, to prevent osmotic shock in trophozoites. This gradient was spun and trophozoites were found at the interfaces between 20%/30% and 30%/40%. These fractions were collected with a syringe. An equivalent volume of FSW was added and the solution layered onto a cushion of 15% sucrose in FSW (to remove the Percoll) and centrifuged. The supernatant was discarded and the trophozoites which were found in the pellets were resuspended in FSW and centrifuged as a final rinse.

**Vibrio P1 culture**. The strain *Vibrio* P1 used in this study was provided by the IFREMER laboratory (La
Tremblade, France). The bacteria were maintained in marine agar (Difco) at 4°C. For phagocytosis assays VP1 was seeded in marine broth (Difco). The number of VP1 was determined by measuring the optical density (1.25 OD is equivalent to 1500 × 10^8 VP1 ml^-1). The marine broth was eliminated by centrifugation (2000 × g, 15 min); VP1 was resuspended in FSW and measured again.

Zymosan. Preparation of a suspension of yeast cell walls using zymosan A (Sigma, USA) followed that described by Dikkeboom et al. (1987).

RESULTS

Phagocytosis of zymosan assays

Both granulocytes and hyalinocytes showed phagocytic capacity in the in vitro phagocytosis assays with zymosan, although this capacity was higher in granulocytes. Of total haemocytes, 49% phagocytosed zymosan particles (Fig. 1). Of this population, 72.4% of the acidophilic granulocytes and 40.9% of the basophilic granulocytes had internalized zymosan particles at the end of the assays, whereas only 3.4% of the hyalinocytes had internalized particles. In most of the cells that had phagocytosed, 1 or 2 zymosan particles were observed regardless of cell type. Higher numbers of phagocytosed particles (up to 8) were occasionally observed.

The ultrastructural study allowed observation of entire zymosan particles inside primary phagosomes and partially digested particles in secondary phagosomes (Fig. 2). The number of cytoplasmic granules was lower in granulocytes with internalized zymosan, compared to those granulocytes without phagocytosed zymosan. This difference was probably due to the fusion of granules with primary phagosomes after zymosan internalization. In advanced stages of the phagocytosis process, haemocytes showed a vacuolated appearance with glycogen particles in the cytoplasm and highly degraded material in secondary phagosomes. Images of residual material discharge from haemocytes could be observed.

Phagocytosis of VP1 assays

In this case, 55.3% of the haemocytes had phagocytosed bacteria *Vibrio* P1 at the end of the assay. As in the previous case, each haemocyte type was able to phagocytose bacteria. Acidophilic granulocytes appeared as the most phagocytic (76.6% of them internalized bacteria) followed by basophilic granulocytes (39.7%) and hyalinocytes (33.0%). Up to 10 phagocytosed bacteria were observed in a single cell (Fig. 3).

Electron microscopy confirmed the capacity of haemocytes to phagocytose VP1 bacteria. Partially degraded VP1 bacteria were observed inside secondary phagosomes (Fig. 4), and rarely inside primary phagosomes. Haemocytes with phagocytosed bacteria showed some vacuoles with residual materials. The presence of small vesicles detached from the cytoplasmic membrane was very common in phagocytic haemocytes.

Phagocytosis of trophozoites and zoospores of *Perkinsus atlanticus*

It was not possible to distinguish the haemocyte type after trophozoites had been internalized because of the large size of the latter. However, it was possible to distinguish the haemocyte type in the trophozoite engulfment stage. Internalized trophozoites were found in 43.7% of the haemocytes at the end of the assays. At the engulfment stage, 41.1% of the acidophilic granulocytes, 37.2% of the basophilic granulocytes and 19.0% of the hyalinocytes were found to be engaged in the process.

In contrast, clam haemocytes were not able to phagocytose zoospores of *Perkinsus atlanticus* in the in vitro assays.

Influence of clam age in phagocytic capacity

Table 1 shows the percentages of haemocytes with phagocytosed Zymosan in 2 groups of different age clams. The difference found between the groups was not significant.

DISCUSSION

The results of in vitro assays showed that all clam haemocyte types had phagocytic capacity, although granulocytes showed a greater ability. These findings are consistent with results reported from *Crassostrea*

Table 1. Percentage of haemocyte types of *Ruditapes decussatus* with phagocytosed zymosan particles corresponding to 2 groups of clams of different ages. Mean size ± standard error corresponding to each clam group are indicated in parentheses.

<table>
<thead>
<tr>
<th>Haemocyte type</th>
<th>Older clams (31.00 ± 0.31 mm)</th>
<th>Younger clams (23.93 ± 0.27 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyalinocytes</td>
<td>4.71%</td>
<td>8.81%</td>
</tr>
<tr>
<td>Basophilic granulocytes</td>
<td>38.89%</td>
<td>41.18%</td>
</tr>
<tr>
<td>Acidophilic granulocytes</td>
<td>74.68%</td>
<td>73.98%</td>
</tr>
<tr>
<td>Total</td>
<td>47.76%</td>
<td>51.33%</td>
</tr>
</tbody>
</table>
Among granulocytes, the acidophilic granulocytes of *Ruditapes decussatus* were found to be more phagocytic, which is in agreement with the observations of Pipe et al. (1994). The higher phagocytic capacity observed in *K. decussatus* granulocytes is consistent with the presence of higher levels of hydrolytic enzymes in these cells (López et al. in press). The function of bivalve hyalinocytes is not known but it is possible that they have another specific function different to phagocytosis. Hose et al. (1990) and Clare & Lumb (1994) indicated that crustacean hyalinocytes have a role in the clotting process. Bivalves do not have humoral clotting factors but Suzuki et al. (1991) indicated that agranular haemocytes of pearl oyster *Pinctada fucata* show the ability to form a cellular sheath and produce an extracellular matrix in the wound healing process.

Around 50% of *Ruditapes decussatus* haemocytes showed phagocytic ability in our assays. Fries & Tripp (1980) noticed phagocytic ability in 100% of *Mercenaria mercenaria* haemocytes. Differences could be due to experimental procedures or to intrinsic properties of the bivalve species. La Peyre et al. (1995) reported a significant difference in the percentage of phagocytic haemocytes between *Crassostrea gigas* and *C. virginica* using the same experimental procedure with both species.

*Ruditapes decussatus* haemocytes showed the ability to phagocyte all stimulant particles used in the assays, except zoospores of *Perkinsus atlanticus*. In contrast, haemocytes of *Crassostrea virginica* are able to phagocyte zoospores of *P. marinus*. Different experimental conditions should be used to further study the inability of clam haemocytes to phagocyte *P. atlanticus* zoospores. Feng (1966, 1967) and Feng & Stauber (1968) found that *Haplosporidium nelsoni*, *Hexamita nelsoni* and *Staphylococcus aureus* induced little or no phagocytosis by *C. virginica* haemocytes. Some differences were found in the percentage of phagocytic hyalinocytes depending on the phagocytosis stimulant used. However, the percentage of phagocytic granulocytes was similar in all the assays. La Peyre et al. (1995) found a significant difference in the percentage of phagocytic haemocytes between assays using *P. marinus* trophozoites and those using zymosan.

According to Cheng (1983) and Rennwatz (1990), the phagocytic process can be subdivided into 4 steps: (1) chemotaxis, involving the attraction of the phagocyte to the non-self material, (2) recognition, involving the attachment of the foreign material to the surface of the phagocyte, (3) internalization, and (4) intracellular degradation. Light and electron microscopy studies of the phagocytic process by clam haemocytes demonstrated internalization and degradation but chemotaxis and recognition phases were not considered.

Internalization of foreign particles by clam haemocytes occurred by invagination of the cell surface followed by pseudopod formation and particle internalization into endocytotic vacuoles (primary phagosome). A similar model was described by Cheng (1975). Two more models of the same event were described by Banq (1961) and Rennwatz et al. (1979). Our fine structural observations indicate that intracellular degradation of internalized material occurs in secondary phagosomes of the clam haemocytes. In the phagocytosis assays, fusion between granules and the primary phagosome was not observed, but haemocytes with secondary phagosomes contained fewer granules. It is possible that secondary phagosomes result from the fusion of lysosomes with primary phagosomes. Auffret (1986) suggested that the formation of secondary phagosomes results from the fusion of granules and primary phagosomes in *Ostrea edulis*. Nevertheless, the loss of granules from granulocytes of *Ruditapes decussatus* during the in vitro phagocytosis assays could also be due to the release of lysosomal enzymes from granulocytes into the serum (degranulation). A degranulation process associated with phagocytosis was reported by Foley et al. (1977) in *Mercenaria mercenaria* and Hinsch & Hunte (1990) in *Crassostrea virginica*.

In this phase, lysosomal enzymes play a very important role. Mohandas et al. (1985) and Oubella et al. (1994) demonstrated that challenge with bacteria produced a hypersynthesis of lysosomal enzymes by haemocytes. A number of lysosomal enzymes were de-
ected in *Ruditapes decussatus* haemocytes and the presence of acid phosphatase in the granules suggests that these granules are lysosomes (López et al. in press). The presence of lysosomal enzymes in bivalve mollusc haemocytes was cited by several authors (Moore & Gelder 1985, Feng 1988. Beckmann et al. 1992). The absence of oxidative metabolism coupled to phagocytosis in *R. decussatus* haemocytes was previously reported (López et al. 1994).

Cheng (1981) described 2 models of intracellular degradation of phagocytosed materials in bivalve haemocytes. One of them involved diffusion of molecules through the phagosomal membrane into the cytoplasm after enzyme digestion. Glucose is then polymerized into glycogen in the cytoplasm. Indigestible material remains as residual bodies which are eventually discharged from the cell. The other model suggests digestion of bacteria beginning within a primary phagosome. The material is transferred from primary to secondary phagosomes and glycogen granules are synthesized from glucose inside these phagosomes. Then, the secondary phagosomal wall disintegrates and the glycogen granules are freed into the cytoplasm.

In our study, transference of digested material from primary to secondary phagosomes was not detected. However, glycogen granules were detected inside secondary phagosomes. Discharged residual bodies were also observed in *Ruditapes decussatus* haemocytes.

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