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Preliminary study on the phagocytic ability of *Octopus vulgaris* Cuvier, 1797 (Mollusca: Cephalopoda) haemocytes in vitro

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Abstract

Phagocytosis by molluscan haemocytes is the major line of defence against invading micro-organisms and foreign materials, and a great deal of effort has been directed in recent years toward elucidating different aspects of this process. Despite this, there is a great lack of information about phagocytosis in a very economically and ecologically important group of molluscs: cephalopods. The phagocytic capacities of the common octopus, *Octopus vulgaris*, haemocytes were studied in vitro, using zymosan as test particle. Particle binding index and particle binding capacity were calculated in a variety of experimental conditions. Results showed that incubation time and temperature had no influence over those indexes, and only the ratio zymosan:haemocyte influenced significantly the amount of phagocytosis. The effect of preopsonization of zymosan and presence of haemolymph during phagocytosis were also tested employing a microplate assay with neutral red-stained zymosan. Data confirm not only that haemolymph was not necessary for the occurrence of the phagocytic process, but also that haemolymph interfered with in vitro phagocytosis.

Keywords: Octopus; Phagocytosis; Haemocyte; Opsonization

1. Introduction

Aquaculture of marine invertebrates is an economically important activity in both developing and developed countries. In any case, infectious diseases are considered as a chief limitation to production, in terms of quality, quantity, regularity as well as of continuity. In this context, research must be focused not only on the diagnosis for zoosanitary controls, but also on obtaining resistant animals. The latter depends heavily on the development of knowledge about marine invertebrate immunology (Bachère et al., 1995). The selection of the most resistant animals could be based on a screening of parameters reflective of immune status, such as phagocytosis, associated oxidative radical production, anti-microbial peptide levels, etc. (Roch, 1999).

Molluscan blood cells (haemocytes) play a prominent role in the internal defence of the animals against pathogens as well as in other biological and physiological functions. Haemocyte-mediated protective mechanisms (mainly phagocytosis) are of central importance to invertebrates that lack immunoglobulins and other serum components typical of the humoral immune system of higher vertebrates (Auffret, 1988).

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The study of the invertebrate immune system is also being considered of growing importance in the field of toxicology. There is growing awareness that contaminants may be partly responsible for the observed increase in disease in marine organisms by adversely affecting their immunity (Pipe and Coles, 1995; Sung et al., 2003; Wootton et al., 2003). In this sense, phagocytic activity has been shown to be a good biomarker of immunotoxicity in wildlife species (Fournier et al., 2000, 2002).

Scientific studies have been focused on bivalve molluscs that are commercially important and on molluscs that serve as vectors of human and animal diseases, and little emphasis has been placed on the defence mechanisms of cephalopods, even though they are highly valued animals, not only for commercial fishery, but also as possible aquaculture species (Ford, 1992; Iglesias et al., 2000; Vaz-Pires et al., 2004). In addition, cephalopods are regarded as key species in many marine ecosystems, representing an essential link in marine trophic chains and being eaten by many marine top predators, fish, birds and mammals (Amaratunga, 1983).

In the present study, we have taken the first steps towards characterizing the internal defence systems of the common octopus, *O. vulgaris*, by studying in vitro phagocytic processes.

2. Material and methods

2.1. Octopuses and haemocyte obtention

Octopuses (28 specimens weighing between 1 and 1.5 kg) were sampled by local fishermen at the same location (Cies Island coast) in the Ria de Vigo (Galicia, NW Spain), over several weeks between January and April. Animals were immediately carried to the laboratory (in 25 l tanks, and in the dark), where they were maintained in a closed seawater system at 15 °C until bleeding (usually no more than 1 h). Animals were anaesthetised by immersion in freshly prepared 2.5% ethanol in cold seawater. When the octopus was totally relaxed, the mantle was partially reflexed to expose the branchial blood vessel used for blood sampling (Malham et al., 1995). Haemolymph was extracted with anticoagulant buffer (AB) (0.05 M Tris–HCl, supplemented with 2% NaCl, 2% glucose and 0.5% ethylenediaminetetraacetic acid –EDTA – pH 7.6), to avoid cell clumping. Haemolymph used for preopsonization of neutral red-stained zymosan particles (see below) was extracted without buffer, and haemocytes were eliminated by centrifugation.

2.2. Cell counting and haemocyte viability

Immediately following bleeding, cell counting was carried out using a Neubauer chamber. Viability of octopus haemocytes (hcs) was determined using two methods: the Eosin-Y (Parrinello and Cammarata, 1995) and the Trypan blue exclusion techniques (Weeks-Perkins et al., 1995). Both assays were performed for every incubation time (45, 90 and 120 min) and temperature conditions (4, 15, 22 and 37 °C) used in the phagocytosis experiments.

2.3. Phagocytosis assays

The phagocytic ability of *O. vulgaris* hc was studied by light microscopy techniques, as described by Carballal et al. (1997), with some modifications, using zymosan (zy) as test particle. Previous experiments carried out in our laboratory showed that *O. vulgaris* hcs were able to phagocytose different kind of particles, including bacteria, yeast, and zy (unpublished data). Zy was elected as test particle because it is easily prepared in large amounts (Dikkeboom et al., 1987) and can be stored frozen until use.

Briefly, 36 eppendorf vials containing 1×10^6 hc in AB were prepared for each individual. The vials were then centrifuged (250×*g*, 10 min, 4 °C) to remove the AB and 1 ml of Tris buffer saline (TBS) (0.05 M Tris–HCl, containing 2% NaCl, pH 7.6) was added to each vial.

Phagocytosis assays were carried out by adding zy suspensions to the eppendorf vials. Three particle:hc ratios (2 zy:hc, 5 zy:hc and 10 zy:hc) were tested. The assays were carried out at room temperature (22 °C) for 45, 90 and 120 min. Each combination of particle ratio and incubation time was assayed in triplicate. Cytospins (92×*g*, 5 min, 4 °C) were performed at the end of each phagocytosis assay. Slides were fixed in methanol and stained with the Hemacolor kit (Merck, Darmstadt, Germany). Then we evaluated the Particle Binding Index (PBI, percent of phagocytic haemocytes) and the Particle Binding Capacity (PBC, average number of particles/hc) as described by Oliver and Fisher (1995) in 300 hc per sample (100 hc per replicate) in random selected microscope fields at × 1000 magnification.

To study the effects of temperature on phagocytosis, assays were performed by adding 10 zy:hc to vials. Then hc were incubated 90 min at 4, 15, 22 and 37 °C. Three vials were prepared for each temperature. Cytospins were performed and then fixed and stained as above, in order to estimate PBI and PBC.