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CELLULAR IMMUNOLOGICAL PARAMETERS OF THE OCTOPUS, OCTOPUS VULGARIS

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ABSTRACT The white body is the main hematopoietic organ of cephalopods. In this study, we have investigated the capacity of the cephalopod (Octopus vulgaris) white body cells to perform common cellular defense parameters known to be done by hemocytes of other mollusks such as phagocytosis of zymosan particles, respiratory burst activity and nitric oxide (NO) production. White body cells were capable of respiratory burst and NO production, however, they exhibited a low phagocytic response. Similar capabilities were observed in hemocytes withdrawn from the hemolymph. We have studied the effects of in vitro incubation with bacterial lipopolysaccharide (LPS) or zymosan for 24 hours on these two functions. Incubation of the white body cells with zymosan, but not with LPS, resulted in a significantly increased respiratory burst activity and NO production.

We have also investigated the capacity of circulating hemocytes and white body cells to increase their thymidine uptake (indicative of DNA synthesis) in response to LPS and phytohemagglutinin (PHA). In some animals, both mitogens induced a significant increase in thymidine uptake. If this thymidine uptake correlates with cell proliferation, this will be the first report of any proliferation of hemocytes in mollusks.

In the hemolymph, we observed two different morphologies under the electron microscope, however, we cannot conclude that they correspond to two distinct cell types. Among white body cells different morphologies that may correspond to intermediate stages were observed. All these findings represent a baseline for future studies to elucidate mechanisms of host defense in this mollusk.

KEY WORDS: Octopus vulgaris, hemocytes, white body, respiratory burst, thymidine uptake, nitric oxide (NO), phagocytosis

INTRODUCTION

Due to the decrease in Spanish cephalopod fisheries, the interest in the commercial culture of cephalopod species has gained increasing attention. Diseases are one of the major obstacles in achieving this goal. Several pathogens have been identified in wild and aquarium-kept octopuses, including viruses, bacteria and fungi. Farley (1978) described the presence of viruses in the muscle of the octopus, Octopus vulgaris. Bacteria have been reported to cause several disease outbreaks in laboratory reared octopuses (Hanlon & Forsythe 1990; Hanlon et al. 1984). Parasites have been blamed for several pathogenic problems. Hootberg (1990) described a flagellated parasite in cultured octopuses. Fungi have also been described as causing problems in cephalopods maintained in captivity (Polglase 1980; Polglase et al. 1984). Despite the threat these pathogens may cause to cephalopod populations, elucidation of defense mechanisms in these species have received little attention.

Humoral defense factors have been identified and are reviewed by Ford (1992). Rogener et al. (1985) and Fisher and Diauzzo (1991) described hemaglutinating activity and agglutinins in cell free hemolymph of several molluscan species, including the octopus, Octopus vulgaris. In this species, an antiprotease of the α-macroglobulin family was also detected in the hemolymph (Thogersen et al. 1992). Malham et al. (1998) reported lysozyme and antiprotease activity in hemocytes and hemolymph of the lesser octopus Eledone cirrhosa. Like in other mollusks, cephalopod hemocytes are believed to play a role in host defense mechanisms, however, the function of the hemocytes has been poorly studied. Cowden and Curtis (1981) estimated that the phagocytic capacity of octopus hemocytes was low while high phagocytosis of carbon particles has been described in Eledone cirrhosa (Stuart 1968). Bayne (1983) reported a clearance of Serratia marcescens by hemocytes of the octopus, Octopus dofleini.

The generation of hemocytes of cephalopods is believed to take place in an organ situated around the optic nerve called the white body, gland of Hensen or gland of Faussek (Smitt 1968; Cowden 1983; Bolognini et al. 1980). Hence, the aim of this study was to determine whether white body cells from Octopus vulgaris are capable of performing certain functions believed to be of relevance to defense mechanisms like phagocytosis of zymosan, respiratory burst and nitric oxide (NO) production and to compare their functionality to that of circulating hemocytes. We have also examined the ability of two mitogens, bacterial lipopolysaccharide (LPS) and phytohemagglutinin (PHA), to stimulate thymidine uptake, an indicative of DNA synthesis. In the case of the respiratory burst and NO production, we have also determined the effect of in vitro activation with bacterial lipopolysaccharide (LPS) and zymosan.

MATERIALS AND METHODS

Sampling

Adult octopuses (Octopus vulgaris) of both sexes, weighing 2–5 kg were caught from a raft in the Ría de Vigo (Spain) and after a day of adaptation under laboratory conditions, were anesthetized with excess MS 222. When animals were fully anesthetized (identified by muscle relaxation and absence of movement), the visceral cavity was dissected. Once the heart and its associated vessels were reached, around 1 ml of hemolymph was drawn using a 27 gauge needle from the artery and subsequently from the heart, placed in an appendorf and kept on ice until used. The white body was removed and kept on ice until used.

In order to obtain single cell suspensions, individual white bodies were passed through a 100 μm nylon mesh using Leibovitz medium (L-15, Gibco) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL) and 2% fetal calf serum (FCS). The resulting cell suspension was centrifuged (500 × g for 15 min at 4°C) and the cells were resuspended in L-15 supplemented with penicillin, streptomycin and 2% (FCS). Cell viability was determined by Trypan blue exclusion. Cells were resuspended in L-15 supplemented with penicillin, streptomycin and 0.1% FCS at a density of 1 × 10^6 cells/mL.
Hemocytes were obtained by centrifuging octopus hemolymph (500 x g for 15 min at 4°C) and hemocytes were resuspended in L-15 supplemented with penicillin, streptomycin and 2% FCS. The cell viability was determined by Trypan blue exclusion test. Cells were resuspended in L-15 supplemented with penicillin, streptomycin and 0.1% FCS at a concentration of 1 x 10⁶ cells/mL.

Electron Microscopy

Pelleted cells (from white body and hemolymph) were fixed for one hour in 1% osmium tetroxide in cacodilate buffer 0.1 M (pH 7.3). In some cases, a previous step of fixation with 1% glutaraldehyde in cacodilate buffer 0.1 M (pH 7.3) for 15 minutes was performed. Following three washes in 0.1 M cacodilate, the cells were dehydrated with increasing percentages of ethanol and embedded in Araldite/Poly Bed (Polyscience). Ultrathin sections (50-70 nm) were stained with uranyl acetate and lead citrate and examined using a Phillips electron microscope CM100.

Phagocytosis Assay

To measure the phagocytic ability of octopus cells, 200 µL of cell suspensions (derived from white body or hemolymph of six animals) were incubated in chamber slides (Nunc) for 2 h at 18°C in moist incubation chambers to allow the cells to adhere. Medium was removed and the adherent cell layer was washed twice with L-15. Zymosan A (Sigma) resuspended in sterile phosphate buffered saline, PBS, at a concentration of 1 mg/mL, was heated at 100°C for 30 min, washed twice, and resuspended in the same PBS volume. Zymosan was added to the cells at a final concentration of 250 µg/mL, and the same volume of L-15 was added to the controls. Slides were incubated in a moist chamber for one hour at room temperature or 18°C to allow phagocytosis. Some slides were kept up to three hours. Slides were then washed twice in PBS, fixed in absolute ethanol, stained with Hemacolor, and mounted with DePex. Two replicates were made for each octopus and at least 150 cells were observed in each replicate.

Respiratory Burst Activity

Respiratory burst activity of octopus cells was assayed by the reduction of ferricytochrome C (Ct C, Sigma) by released superoxide anion (O₂⁻), following stimulation of the cells with phorbol myristate acetate (PMA, Sigma) (Secombes 1990) in 4 octopuses. White body adherent cells and circulating hemocytes were obtained as described above, resuspended in L-15 supplemented with penicillin, streptomycin and 0.1% FCS and dispensed into 96-well tissue culture plates (Iwaki) at a concentration of 1 x 10⁶ cells/mL (100 µL per well).

In a preliminary experiment, we determined the specificity of the respiratory burst by assaying the response of the cells to PMA. After 24 hours of incubation at 18°C, octopus cell monolayers were washed twice in phenol red-free Hank’s balanced salt solution (HBSS, Gibco). One hundred µL of HBSS containing Ct C (2 mg/mL) and PMA (1 µg/mL) were added to each well. As a control for specificity, 300 IU/mL superoxide dismutase (SOD, Sigma) was added to some wells. The optical density (O.D.) was measured at 550 nm after 30 min in a multispan spectrophotometer (Labsystems). Triplicate wells were used in all the experiments for each octopus and the mean ± SD was calculated.

Once the responsiveness of octopus cells to PMA had been determined, the effects of stimulation with zymosan or Escherichia coli serotype 0111: B4 lipopolysaccharide (LPS) on the respiratory burst of octopus cells triggered by PMA was also studied. After three hours incubation of the cell monolayers at 18°C, LPS and zymosan were added to a final concentration of 50 and 250 µg/mL respectively. After an additional 24 hours of incubation at 18°C with these substances, the respiratory burst activity was measured by adding 100 µL of HBSS containing Ct C (2 mg/mL) and PMA (1 µg/mL) were added to each well. The O.D. at 550 nm was then determined as described above.

NO Production

The ability of octopus cells to produce NO in response to LPS and zymosan was also determined in 4 animals. Cells resuspended in L-15 with 0.1% FCS were disposed into 96-well plates at a concentration of 1 x 10⁶ cells/mL. After 3 h of incubation at 18°C, LPS and zymosan were added to a final concentration 50 and 250 µg/mL respectively. After additional 24 h of incubation at 18°C, the NO concentration present in the cell supernatants was assayed through the Griess reaction (Green et al. 1982) that quantifies the nitrite content of the cell supernatants, since NO is an unstable molecule and degrades to nitrite and nitrate. Fifty µL of hemocyte supernatants were removed from individual wells and placed in a separate 96-well plate. One hundred microliters of 1% sulfanilamide (Sigma) in 2.5% phosphoric acid were added to each well, followed by the addition of 100 µL of 0.1% N-naphthylethylene diamine (Sigma) in 2.5% phosphoric acid. Optical density at 540 nm was determined using a multispan spectrophotometer. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite (100, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 µM).

Effect of Mitogens on [³H]-Thymidine Uptake by Octopus Cells

The thymidine uptake by octopus cells was assayed following a modification of the method described by Marsden et al. (1994) in six octopuses. Briefly, hemocytes derived from the hemolymph or the white body were adjusted to a density of 5 x 10⁶ cells/mL in RPMI 1640 medium (Gibco) supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL), 25 mM NaHCO₃, and 1 x 10⁻⁵ M 2-mercaptoethanol. Aliquots of 100 µL of cell suspensions were added to wells of 96-well plate containing 100 µL of LPS or PHA dilutions (Sigma) to make final concentrations of 50, 25 and 12.5 µg/mL or 5, 2.5, and 1.25 µg/mL, respectively. Controls without mitogens were also included. After 3 h of incubation at 18°C, FCS was added to the wells to give a final concentration of 10%. Following 48 h of incubation, the cells were pulsed with 0.5 µCi of [³H]-thymidine (Amersham). After additional 24 h incubation at 18°C, DNA was harvested onto glass filter mats. One mL Xyloflour scintillant (Packard) was added to dried filter circles in vials and counts per minute (cpm) were recorded using a Packard liquid scintillation counter. Triplicate cultures were used in all cases.

Statistics

The data were compared using a Student’s t test. Results are expressed as the mean ± standard deviation and differences were considered statistically significant at P < 0.05.

RESULTS

Cell Morphology

After two hours of incubation of the cell suspensions at 18°C, the white body adherent cells were adhered to the bottom of the
wells, flatten and spread over the surface by extending pseudopodia. Circulating hemocytes presented the same aspect.

When visualized under the electron microscope (Fig. 1), what seems as two different morphologies, that were distinct under the inverted microscope, were observed among circulating hemocytes. No differences were found in the quality of fixation when the previous glutaraldehyde step was omitted and therefore the cells were always directly fixed in osmium tetroxide. We identified in the first cell type a kidney-shaped nucleus that occupied about 2/3 of the cell volume with a well-defined nucleolus and abundant heterochromatin in peripheral positions. Their cytoplasm was rich in vacuoles and electron-dense granules of various sizes. The second cell type had a nucleus with faint chromatin, a round nucleus that occupied about 1/3 of the cell volume. The cytoplasm was rich in vacuoles, but had only scarce granules. However, these results are not conclusive and we cannot assure that what looks as two different morphologies corresponds to two distinct cell types.

In the case of white body cells, we were able of identifying cells showing other morphologies that may correspond to intermediate stages between the two cell types found in the hemolymph.

**Phagocytosis Activity of White Body Adherent Cells and Circulating Hemocytes**

The phagocytic activity detected after incubation of octopus hemocytes and white body cells with zymosan was low. The percentage of phagocytosis observed in circulating hemocytes was 19.3% (SD = 14), while only 9.3% (SD = 8) of white body cells contained zymosan particles in their cytoplasm. In all cases, variations among individuals were high, since in some samples no phagocytosis was observed (0% of phagocytosis). The same results were obtained with the two incubation temperatures (18°C or room temperature) in both circulating and white body cells. No differences were observed when the hemocytes were incubated in their own hemolymph (data not shown).

**Respiratory Burst Activity**

Octopus white body cells showed a significant increase in the release of superoxide anion after stimulation with PMA compared with controls, as depicted in Figure 2A. The specificity of the reaction was demonstrated since SOD completely inhibited the respiratory burst response of octopus cells in all cases. This response was also observed with circulating hemocytes. The respiratory burst activity of hemocytes incubated directly in their own hemolymph was also assayed. In these conditions, some octopuses did not respond to PMA and did not elicit a respiratory burst response.

Figure 2B shows the effect of incubation for 24 hours with LPS or zymosan on the respiratory burst triggered by PMA of white body cells, compared to the respiratory burst observed in cells that had been incubated with L-15 only. The pre-incubation of the cells with zymosan, but not LPS, significantly increased the respiratory burst of white body cells in response to PMA. The same response was observed in cells obtained from hemolymph, showing a higher respiratory burst after zymosan treatment than controls (Fig. 2C).

**NO Production**

The NO production of white body cells in response to LPS and zymosan is shown in Figure 3. Zymosan significantly stimulated the cells for NO production in all individuals, however, as in the case of the respiratory burst activity, LPS failed to stimulate the cells.

In the case of hemolymph cells, a similar response was observed. The NO production in the cultures treated with zymosan (8.4; SD = 0.8) was higher than the response observed in controls (6.7; SD = 0.3).
DISCUSSION

The findings in this study suggest that both octopus white body cells and circulating hemocytes are capable of performing functions associated with host defense mechanisms. This is particularly important to determine since scanty data is available on the immune response of this octopus species. This is the first work in which reagents and techniques usually used in vertebrate immunology have been successfully applied to study cellular responses of cephalopods.

Under the electron microscope, two distinct morphologies among circulating hemocytes were identified, although it had been described as only one cell type of the hemolymph of Octopus vulgaris (Bidder et al. 1989). Our results are not conclusive and more work should be done to determine whether these two morphologies correspond to different states of activation or they constitute two different cell types. Previous studies in bivalve mollusks have identified two main hemocyte types in the hemolymph (Fisher 1986; Lopez et al. 1997) that have been subdivided (Auffret 1988; Nakayama et al. 1997). In the white body, it is well known that there are cells, referred to as hemocytoblasts, with a large cytoplasmic volume and abundant rough endoplasmic reticulum and nucleioli (Ford 1992). Hemocytoblasts transform to leukoblasts by reducing its cytoplasmic volume and decreasing its nuclear size, whereas secondary leukoblasts (mature hemocytes) are cells with a larger size and a folded nucleus. Both under the light and electron microscope, we were able to identify different morphologies that may correspond to these intermediate stages among white body cells. As well, the two different morphologies that we describe in this work among circulating hemocytes seem to correspond to these two cell types (hemocytoblasts and leukoblasts). It may be possible that in these individuals maturation ends in the hemolymph or as will be discussed later, that these two cell types are functionally different.

The first cell type may correspond to what has been called granulocytes in bivalves (Auffret 1988). These cells possess a kidney-shaped nucleus that resembles those of vertebrate granulocytes. The second cell type had a round nucleus and a cytoplasm that was also rich in vacuoles, but had only scarce granules. This cell type may correspond to what in bivalves has been called hyalinocytes (agranular hemocytes) (Auffret 1988).

Results concerning intravitro phagocytosis by octopus hemocytes are in accordance with those previously reported (Crowden & Curtis 1981) where it was suggested that gill tissue or phagocytes from gill tissue could be responsible for clearing foreign substances and that circulating hemocytes had low activity (Crowden & Curtis 1981; Bayne 1983). However, studies in other cephalopod species, like Loligo pealei, reported bacterial phagocytic rates of even 40% (Malham et al. 1997). The low phagocytic activity that is observed in the octopus, Octopus vulgaris, circulating hemocytes also contrasts with the high phagocytic rate detected in bivalves (Mortensen & Glette 1996; Ordas et al. 1999), animals in which phagocytosis is a critical defense mechanism. Many factors can affect phagocytic rates in mollusks such as temperature (Carballal et al. 1997), time and pH (Abdul-Salam & Michelson 1980), size and nature of the particle presented for phagocytosis (Bayne 1983). In some cephalopod species, phagocytosis is not possible in the absence of hemolymph (Stuart 1968). It may be possible that on the contrary to what happens with the respiratory burst (that is inhibited by the presence of hemolymph),

TABLE 1.

<table>
<thead>
<tr>
<th>Mitogens</th>
<th>Octopus 1</th>
<th>Octopus 2</th>
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<tbody>
<tr>
<td></td>
<td>Circulating Hemocytes</td>
<td>White Body Cells</td>
</tr>
<tr>
<td>LPS 50 µg/ml</td>
<td>630 ± 242</td>
<td>694 ± 351</td>
</tr>
<tr>
<td>LPS 25 µg/ml</td>
<td>340 ± 118</td>
<td>766 ± 144</td>
</tr>
<tr>
<td>LPS 12.5 µg/ml</td>
<td>458 ± 98</td>
<td>761 ± 148</td>
</tr>
<tr>
<td>PHA 5 µg/ml</td>
<td>340 ± 92</td>
<td>241 ± 171</td>
</tr>
<tr>
<td>PHA 2.5 µg/ml</td>
<td>238 ± 12</td>
<td>475 ± 147</td>
</tr>
<tr>
<td>PHA 1.125 µg/ml</td>
<td>178 ± 52</td>
<td>313 ± 79</td>
</tr>
<tr>
<td>Control</td>
<td>204 ± 64</td>
<td>381 ± 105</td>
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</table>
phagocytosis in this species needs humoral factors present in the hemolymph.

Octopus hemocytes, however, were able to respond to PMA and release significant amounts of superoxide anion. Respiratory burst activity is an interesting non-specific defense mechanism that vertebrate macrophages use to avoid bacterial and parasitic infections due to the potent microbicidal effects of oxygen radicals. The reason why the respiratory burst activity was not detected in some of the octopus samples while incubated in their own hemolymph is unknown. The immune system is regulated by multiple signals that can up or down modulate the animal responses. Maybe regulating factors, affecting this immune response, are present in the hemolymph of these animals, in the same way that other humoral factors such as lysozyme or proteases have been detected in cephalopod hemolymph (Malham et al. 1998).

We have also demonstrated that octopus cells derived from hemolymph and white blood produced NO. In vertebrates, NO mediates many functions including neurotransmission, vasodilatation, as well as several immune functions. It is known that this molecule when secreted by macrophages is microbicidal against viruses, parasites and bacteria (Nathan & Hibbs 1991; Tafall & al. 1999). In the octopus, NO production was significantly increased when the cells were incubated with zymosan for 24 hours. However, incubation with LPS did not have an effect on the NO secretion. This is the first report on NO production by cells of any cephalopod. NO production in other mollusks has been shown, in which NO production was demonstrated by indirect methods (Martinez 1995; Otaviani & Franchini 1995).

Octopus hemocytes were also able to increase their thymidine uptake after stimulation with LPS and PHA, although this response was not found in all individuals, maybe because this immune function is more related to a specific response not known to be present in cephalopods. Thymidine uptake implies DNA synthesis, so this increase may mean that the hemocytes are proliferating. If so, this will be the first report of any proliferation in mollusks, outside the hematopoietic organ. More work must be done to determine if this thymidine uptake detected correlates with cell proliferation. In higher vertebrates, these two mitogens correlate to B and T lymphocyte mitogens, respectively. Since we observed ultrastructurally two morphologies among hemocytes in responsive animals, it may be possible that LPS and PHA-induced proliferation are being supported by different hemocyte populations, and therefore imply a certain grade of heterogeneity, as in vertebrate immune cells.

In conclusion, we have effectively applied immunological techniques designed for vertebrates to evaluate cephalopod hemocyte functions. Among mollusks, cephalopods are highly evolved animals with a body design, nervous system and sense organs that often reach vertebrate standards (Budelmann et al. 1997). It seems possible that immune system may also be more developed than other mollusks in which modulation of immune responses by pathological agents or other factors has been reported (Ordas et al. 1999). We have set up different in vitro techniques, such us determination of respiratory burst activity, NO production and cell proliferation, which will let us further evaluate the octopus immune response against infections or the influence of environmental conditions. This latter aspect is especially relevant because of increasing interest that cephalopod aquaculture has had in the past years (Osako & Murata 1983; Guerra & Rocha 1994; Guerra et al. 1994). More work must be done to clarify the regulation of these immune functions in cephalopods and determine their role in the defense against pathogens.

ACKNOWLEDGMENTS

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LITERATURE CITED


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**INTRODUCTION**

Due to the decrease in Spanish cephalopod fisheries, the interest in the commercial culture of cephalopod species has gained increasing attention. Diseases are one of the major obstacles in achieving this goal. Several pathogens have been identified in wild and aquarium-kept octopuses, including viruses, bacteria and fungi, Farley (1978) described the presence of viruses in the muscle of the octopus. Octopus vulgaris. Bacteria have been reported to cause several disease outbreaks in laboratory reared octopuses (Hanlon & Forsythe 1990; Hanlon et al. 1984). Parasites have been blamed for several pathogenic problems. Hochberg (1990) described a flagellated parasite in cultured octopuses. Fungi have been also described as causing problems in cephalopods maintained in captivity (Polglase 1980; Polglase et al. 1984). Despite the threat these pathogens may cause to octopus populations, elucidation of defense mechanisms in these species have received
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zymosan were added to a final concentration of 50 and 250 p-g/mL respectively. After an additional 24 hours of incubation at 18°C with these substances, the respiratory burst activity was measured by adding 100 p.L of HBSS containing Cit C (2 mg/mL) and PMA (1 ng/mL) were added to each well. The O.D. at 550 nm was then determined as described above.

NO Production

The ability of octopus cells to produce NO in response to LPS and zymosan was also determined in 4 animals. Cells resuspended in L-15 with 0.1% FCS were disposed into 96-well plates at a concentration of 1 x 10⁶ cells/mL. After 3 h of incubation at 18°C, LPS and zymosan were added at a final concentration 50 and 250 ng/mL respectively. After additional 24 h of incubation at 18°C, the NO concentration present in the cell supernatants was assayed through the Griess reaction (Green et al. 1982) that quantifies the nitrite content of the cell supernatants. since NO is an unstable molecule and degrades to nitrite and nitrate. Fifty µL of hemocyte supernatants were removed from individual wells and placed in a separate 96-well plate. One hundred microliters of 1% sulfanilamide (Sigma) in 2.5% phosphoric acid were added to each well, followed by the addition of 100 µL of 0.1% N-naphthyl-ethylenediamine (Sigma) in 2.5% phosphoric acid. Optical density at 540 nm was determined using a multiscan spectrophotometer. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite (100, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 µM).
The thymidine uptake by octopus cells was assayed following a modification of the method described by Marsden et al. (1994) in six octopuses. Briefly, hemocytes derived from the hemolymph or the white body were adjusted to a density of $5 \times 10^6$ cells/mL in RPMI 1640 medium (Gibco) supplemented with penicillin (100 IU/mL), streptomycin (100 |jg/mL), 25 mM NaHCO₃, and $1 \times 10^{-5}$ M 2-mercaptoethanol. Aliquots of 100 |jL of cell suspensions were added to wells of 96-well plates containing 100 |jL of LPS or PHA dilutions (Sigma) to make final concentrations of 50, 25 and 12.5 |jg/mL or 5, 2.5, and 1.25 |jg/mL, respectively. Controls without mitogens were also included. After 3 h of incubation at 18°C, FCS was added to the wells to give a final concentration of 10%. Following 48 h of incubation, the cells were pulsed with 0.5 |jL of (H)-thymidine (Amersham). After additional 24 h incubation at 18°C, DNA was harvested onto glass filter mats. One |jL Xylofluor scintillant (Packard) was added to dried filter circles in vials and counts per minute (cpm) were recorded using a Packard liquid scintillation counter. Triplicate cultures were used in all cases.

Statistics

The data were compared using a Student’s t test. Results are expressed as the mean ± standard deviation and differences were considered statistically significant at $P < 0.05$. 

Effect of Mitogens on (H)-Thymidine L’ptake by Octopus Cells
RESULTS

Cell Morphology

After two hours of incubation of the cell suspensions at 18°C, the white bod\ adherent cells were adhered to the bottom of the wells, slitten and spread over the surface by extending pseudopodia. Circulating hemocytes presented the same aspect.

When visualized under the electron microscope (Fig. 1), what seems as two different morphologies, that were distinct under the inverted microscope, were observed among circulating hemocytes. No differences were found in the quality of fixation when the previous glutaraldehyde step was omitted and therefore the cells were always directly fixed in osmium tetroxide. We identified in the first cell type a kidney-shaped nucleus that occupied about 2/3 of the cell volume with a well-defined nucleolus and abundant heterochromatin in peripheral positions. Their cytoplasm was rich in vacuoles and electron-dense granules of various sizes. The second cell type had a nucleus with faint chromatin, a round nucleus
that occupied about 1/3 of the cell volume. The cytoplasm was rich in vacuoles, but had only scarce granules. However, these results are not conclusive and we cannot assure that what looks as two different morphologies corresponds to two distinct cell types.

In the case of white body cells, we were able of identifying cells showing other morphologies that may correspond to intermediate stages between the two cell types found in the hemolymph.

Phagocytosis Activity of White Body Adherent Cells and Circulating Hemocytes

The phagocytic activity detected after incubation of octopus hemocytes and white body cells with zymosan was low. The percentage of phagocytosis observed in circulating hemocytes was 19.3% (SD = 14), while only 9.3% (SD = 8) of white body cells contained zymosan particles in their cytoplasm. In all cases, variations among individuals were high, since in some samples no phagocytosis was observed (0% of phagocytosis). The same results were obtained with the two incubation temperatures (18°C or room temperature) in both circulating and white body cells. No differences were observed when the hemocytes were incubated in their own hemolymph (data not shown).

Respiratory Burst Activity

Octopus white body cells showed a significant increase in the release of superoxide anion after stimulation with PMA compared with controls, as depicted in Figure 2A. The specificity of the
Figure 1. Under the electron microscope, two distinct morphologies were identified among circulating hemocytes. The first cell type (I) is characterized by its kidney-shaped nucleus and the high number of electron-dense granules. The second cell type (III) possessed a round nucleus with a few electron dense granules. Bar = 5 μm.

Cit PMA PMA+SOD

Treatment

Conml LPS Zymosan

Ta-.itinenl

c

Control LPS Z'nisosai

Treatment

Figure 2. Respiratory burst activity of octopus white body cells. First, the capacity of PMA to stimulate the respiratory burst activity and the specificity of the reaction was assayed (A) (N = 2). Once, this was determined, the effects of LPS and zymosan on the respiratory burst activity of white body hemocytes (B) (N = 4) and circulating hemocytes (C) were studied through the reduction of Cit C, stimulating the cells with PMA. Data are shown as the mean O.I, at 550 nm. *Respiratory
burst significantly higher than the respiratory burst obtained in controls. \( P < 0.05 \).

reaction was demonstrated since SOD completely inhibited the respiratory burst response of octopus cells in all cases. This response was also observed with circulating hemocytes. The respiratory burst activity of hemocytes incubated directly in their own hemolymph was also assayed. In these conditions, some octopuses did not respond to PMA and did not elicit a respiratory burst response.

Figure 2B shows the effect of incubation for 24 hours with LPS or zymosan on the respiratory burst triggered by PMA of white body cells, compared to the respiratory burst observed in cells that had been incubated with L-15 only. The pre-incubation of the cells with zymosan, but not LPS, significantly increased the respiratory burst of white body cells in response to PMA. The same response was observed in cells obtained from hemolymph, showing a higher respiratory burst after zymosan treatment than controls (Fig. 2C).

NO Production

The NO production of white body cells in response to LPS and zymosan is shown in Figure 3. Zymosan significantly stimulated the cells for NO production in all individuals, however, as in the case of the respiratory burst activity, LPS failed to stimulate the cells.

In the case of hemolymph cells, a similar response was ob-
served. The NO production in the cultures treated with zyinosan (S.4; SD = 0.8) was higher than the response observed in controls (6.7; SD = 0.3).

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Control LPS Zymosan

Treatment

Figure 3. NO production of cells from the wliile bodj after incubation with LPS or zymosan. Data are presented as the mean nitrite concentration obtained with 4 octopus. 'Nitrite concentration si)irieantl> hifjher than the one observed in controls onlj treated with 1,-15. P < 0.05.

('H)-Thymidiiae Uptake

Not all animals studied showed a significant response. Only two out of six individuals assayed responded with a significant increase of the thymidine uptake in response to mitogens. The individual responses observed in these two animals are shown in Table I. When means were compared, concerning white body
cells, a maximum thymidine uptake was observed with the higher LPS dose (687.3: SD = 6.7). In this case, the PHA dose that significantly stimulated the thymidine uptake was 2.5 pg/ml (495; SD = 20) in comparison to controls (78.3; SD = 2.5).

In these two responsive animals, hemocyte thymidine uptake was also significantly affected by the mitogens. All concentrations of LPS significantly increased ('H)-thymidine uptake compared to non-stimulated controls, although the higher response was observed with the highest LPS dose. However, hemocyte thymidine uptake was only significantly increased with the higher dose of PHA.

**TABLE 1.**

| Thymidine uptake by circulating hcmocytes and white body cells in two responsive animals. Data are presented as the mean cpmi obtained in the replicas (N = 31 ± SD. |

**DISCUSSION**

The findings in this study suggest that both octopus white body cells and circulating hemocytes are capable of performing functions associated with host defense mechanisms. This is particularly important to determine since scanty data is available on the immune response of this octopus species. This is the first work in
which reagents and techniques usually used in vertebrate immunology have been successfully applied to study cellular responses of cephalopods.

Under the electron microscope, two distinct morphologies among circulating hemocytes were identified, although it had been described as only one cell type of the hemolymph of Oclopus vulgaris (Bidder et al. 1989). Our results are not conclusive and more work should be done to determine whether these two morphologies correspond to different states of activation or they constitute two different cell types. Previous studies in bivalve mollusks have identified two main hemocyte types in the hemolymph (Fisher 1986; Lopez et al. 1997) that have been subdivided (Auffret 1988; Nakayama et al. 1997). In the white body, it is well known that there are cells, referred to as hemocytoblasts, with a large cytoplasmic volume and abundant rough endoplasmic reticulum and nucleoli (Ford 1992). Hemocytoblasts transform to leukoblasts by reducing its cytoplasmic volume and decreasing its nuclear size, whereas secondary leukoblasts (mature hemocytes) are cells with a larger size and a folded nucleus. Both under the light and electron microscope, we were able to identify different morphologies that may correspond to these intermediate stages among white body cells. As well, the two different morphologies that we describe in this work among circulating hemocytes seem to correspond to these two cell types (hemocytoblasts and leukoblasts). It may be possible that in these individuals maturation ends in the hemolymph or as will be discussed later, that these two cell types are functionally different.
The first cell type may correspond to what has been called granulocytes in bivalves (Auffret 1988). These cells possess a kidney-shaped nucleus that resembles those of vertebrate granulocytes. The second cell type had a round nucleus and a cytoplasm that was also rich in vacuoles, but had only scarce granules. This cell type may correspond to what in bivalves has been called hyalinocytes (agranular hemocytes) (Auffret 1988).

Results concerning in vitro phagocytosis by octopus hemocytes are in accordance with those previously reported (Crowden & Curtis 1981) where it was suggested that gill tissue or phagocytes from gill tissue could be responsible for clearing foreign substances and that circulating hemocytes had low activity (Crowden & Curtis 1981: Bayne 1983). However, studies in other cephalopod species, like Eledone cirrhosa reported bacterial phagocytic rates of even 40% (Malham et al. 1997). The low phagocytic activity that is observed in the octopus, Octopus vulgaris, circulating hemocytes also contrasts with the high phagocytic rate detected in bivalves (Mortensen & Glette 1996; Ordas et al. 1999). Animals in which phagocytosis is a critical defense mechanism.

Many factors can affect phagocytic rates in mollusks such as temperature (Carballal et al. 1997), time and pH (Abdul-Salam & Michelson 1980), size and nature of the particle presented for phagocytosis (Bayne 1983). In some cephalopod species, phagocytosis is not possible in the absence of hemolymph (Stuart 1968). It may be possible that on the contrary to what happens with the respiratory burst (that is inhibited by the presence of hemolymph).
phagocytosis in this species needs humoral factors present in the hemolymph.

Octopus hemocytes, however, were able to respond to PMA and release significant amounts of superoxide anion. Respiratory burst activity is an interesting non-specific defense mechanism that vertebrate macrophages use to avoid bacterial and parasitic infections due to the potent microbiocidal effects of oxygen radicals. The reason why the respiratory burst activity was not detected in some of the octopuses while incubated in their own hemolymph is unknown. The immune system is regulated by multiple signals that can up or down modulate the animal responses. Maybe regulating factors, affecting this immune response, are present in the hemolymph of these animals, in the same way that other humoral factors such as lysozyme or antiproteases have been detected in cephalopod hemolymph (Malham et al. 1998).

We have also demonstrated that octopus cells derived from hemolymph and white body produced NO. In vertebrates, NO mediates many functions including neurotransmission, vasodilation, as well as several immune functions. It is known that this
molecule when secreted by macrophages is microbicidal against viruses, parasites and bacteria (Nathan & Hibbs 1991; Tafalla et al. 1999). In the octopus, NO production was significantly increased when the cells were incubated with zymosan for 24 hours. However, incubation with LPS did not have an effect on the NO secretion. This is the first report on NO production by cells of any cephalopod. NO production in other mollusks has been shown, in which NO production was demonstrated by indirect methods (Maninez 1995; Otaviani & Franchini 1995).

Octopus hemocytes were also able to increase their thymidine uptake after stimulation with LPS and PHA, although this response was not found in all individuals. Maybe because this immune function is more related to a specific response not known to be present in cephalopods. Thymidine uptake implies DNA synthesis, so this increase may mean that the hemocytes are proliferating. If so, this will be the first report of any proliferation in mollusks. Outside the hematopoietic organ. More work must be done to determine if this thymidine uptake detected correlates with cell proliferation. In higher vertebrates, these two mitogens correlate to B and T lymphocyte mitogens, respectively. Since we observed ultrastructurally two morphologies among hemocytes in responsive animals, it may be possible that LPS and PHA-induced proliferation are being supported by different hemocyte populations, and therefore imply a certain grade of heterogeneity, as in vertebrate immune cells.

In conclusion, we have effectively applied immunological tech-
niques designed for vertebrates to evaluate cephalopod hemocyte functions. Among mollusks, cephalopods are highly evolved animals with a body design, nervous system and sense organs that often reach vertebrate standards (Budelmann et al. 1997). It seems possible that immune system may also be more developed than other mollusks in which modulation of immune responses by pathological agents or other factors has been reported (Ordas et al. 1999). We have set up different in vitro techniques, such us determination of respiratory burst activity, NO production and cell proliferation, which will let us further evaluate the octopus immune response against infections or the influence of environmental conditions. This latter aspect is especially relevant because of increasing interest that cephalopod aquaculture has had in the past years (Osako & Murata 1983; Guerra & Rocha 1994; Guerra et al. 1994). More work must be done to clarify the regulation of these immune functions in cephalopods and determine their role in the defense against pathogens.

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