Phagocytosis and Peroxidase Release by Seabream (*Sparus aurata* L.) Leucocytes in Response to Yeast Cells

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ABSTRACT

A flow cytometric method was adapted to evaluate phagocytosis by gilthead seabream leucocytes after incubation with yeast cells (*Saccharomyces cerevisiae*). Head-kidney leucocytes were incubated in vitro for different times in different proportions with heat-killed fluorescein isothiocyanate (FITC)-labeled yeast cells to study the kinetics of phagocytosis. Attached and internalized yeast cells were differentiated by quenching FITC-labeled *S. cerevisiae* with trypan blue dye. Only internalized cells kept their FITC fluorescence after quenching. Monocyte-macrophages and acidophilic granulocytes showed phagocytic activity, as demonstrated by transmission electron microscopy (TEM). From the ultrastructural features of the phagocytic process, it was observed that cytoplasmic granule membranes fused with the phagocyte membrane at the point where the yeast cell was attached to the phagocyte surface. This observation led us to adapt a colorimetric method to study peroxidase (myeloperoxidase and eosinophil peroxidase) release, since both are considered to be markers of the degranulation that occurs in seabream head-kidney leucocytes in response to yeast cells. Head-kidney leucocytes were incubated with calcium ionophore (CaI), phorbol myristate acetate (PMA), or yeast cells for different periods of time (0–30 min) to study the kinetics of peroxidase release. The results obtained indicate that CaI and yeast cells, but not PMA, stimulate the degranulation (by about 44.51% and 21.04%, respectively, at 30 min) of seabream head-kidney leucocytes. Anat Rec Part A 272A:415–423, 2003.

Key words: phagocytosis; peroxidase release; gilthead seabream (*Sparus aurata* L.); teleosts; *Saccharomyces cerevisiae*; flow cytometry; transmission electron microscopy

Phagocytosis, the process whereby cells engulf, kill, and digest different particles (damaged or effete cells, microorganisms, etc.), plays an important role in the fish nonspecific immune response. For this reason, the study of phagocytic functions are of prime importance, particularly for those species used in aquaculture. Rapid and precise techniques to estimate leucocyte activities are required, to which end flow cytometry may offer an important contribution (Smits et al., 1997).

Although numerous flow cytometric methods for quantifying phagocytosis in mammals have been described (Ichinose et al., 1994; Van Amersfoort and Van Strijp, 1994; Smits et al., 1997; Malickova et al., 1999), only a few studies have applied such methods to the quantification of phagocytosis by fish leucocytes (Thuvander et al., 1987;

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Esteban et al., 1998). Both in vivo and in vitro studies have shown that fish monocyte-macrophages and granulocytes are phagocytic (Ainsworth, 1992; Steinhagen and Jendrysek, 1994; Esteban et al., 1998). However, ultrastructural studies of the phagocytosis process are particularly scarce, even though they represent a valuable tool for studying specific phagocytic stages, such as pseudopodia extension and phagolysosome formation (Esteban and Meseguer, 1994).

After phagocytosis of microorganisms, leucocytes release their granule content (including a wide variety of cytotoxic agents, such as lysosomal enzymes, oxygen metabolites, and cytotoxic proteins, used to kill pathogens) into the phagosomes (Torreilles et al., 1996; Ortuño et al., 2000). Among the lysosomal enzymes released by leucocytes, two peroxidases (myeloperoxidase (MPO) and eosinophil peroxidase (EPO)) have been widely reported to act as very important microbicidal agents in mammals. These peroxidases, in the presence of the H_2O_2 produced during respiratory burst and halide ions, give rise to chlorine and chloramines mediated by the MPO-H_2O_2-chloride or EPO-H_2O_2-chloride oxidative systems (Suzuki et al., 1983; Nauseef et al., 1983; Quade and Roth, 1997; King et al., 1997; Klebanoff, 1999; Ellis, 1999; Hachiya et al., 2000). In addition to this mechanism of intracellular toxicity, the granule content (including these peroxidases and H_2O_2) can be released by exocytosis from the cell, where they are toxic to adjacent microorganisms (Klebanoff, 1998). In spite of their importance for maintaining animal homeostasis, little is known about these peroxidase-H_2O_2-chloride systems of microbicidal activity in fish leucocytes (Ellis, 1999; Praveen et al., 2000).

MPO and EPO have been described as very abundant heme-proteins found in different leucocyte populations of invertebrate and vertebrate organisms. Thus, MPO is mainly found in the azurophilic granules of neutrophils and, in smaller amounts, in the lysosomes of monocytes (but not in macrophages), while EPO is found in the matrix of the cytoplasmic granules of eosinophils (Garavini and Martelli, 1981; Garavini et al., 1981; Suzuki et al., 1983; Zabucchi et al., 1989; Menegazzi et al., 1992; Meseguer et al., 1994; Quade and Roth, 1997; Torreilles et al., 1997; Klebanoff, 1998; Persson et al., 2001). In fish, it is known that monocyte-macrophages, which express low peroxidase activity, can receive MPO and/or EPO from granulocytes, which enhance their bactericidal activity (Meseguer et al., 1994; Klebanoff, 1998; Ellis, 1999).

MPO and/or EPO activities are assumed to be good markers of leucocyte activation. Polymorphonuclear cells release a large amount of these peroxidases into the blood in response to infection, and elevated serum levels may identify many pathological situations (Zhao et al., 1997; Klebanoff, 1998; Abu-Soud and Hazen, 2000; Oruckaptan et al., 2000). However, nonregulated peroxidase release may cause substantial damage to host tissues (King et al., 1997; Klebanoff, 1998). To address this issue, peroxidase clearing mechanisms based on the recognition of the sugars linked to these enzymes by the mannose receptor on the macrophages have been developed (Shepherd and Hoidal, 1990).

To further elucidate the mechanism of phagocytosis in fish, we studied the phagocytosis of yeast cells by gilthead seabream (*Sparus aurata* L.) head-kidney leucocytes, as well as the way in which yeast cells may induce the release of the cytoplasmic granule content. Flow cytometric and spectrophotometric methods were adapted for this purpose, and the ultrastructural features of the phagocytosis process were studied by transmission electron microscopy (TEM).

MATERIALS AND METHODS

Animals

Thirty-five specimens (175 g mean weight) of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.), obtained from Culmarex S.A. (Murcia, Spain), were kept in running seawater aquaria (flow rate 1,500 L/hr) at 20°C with a 12 hr light/12 hr dark cycle. The fish were fed with a commercial pellet diet (ProAqua, ProAqua Nutrition SA, Palencia, Spain) at a rate of 2% body weight/day.

Isolation of Head-Kidney Leucocytes

Fish were anesthetized with MS-222 (Sandoz) (100 mg/L) and head-kidney leucocytes were isolated under sterile conditions, as described previously (Esteban and Meseguer, 1994). The head-kidney was dissected out by a ventral incision, cut into small fragments, and transferred to 4 ml sRPMI-1640 medium (RPMI-1640 medium (Gibco) supplemented with 10 IU/ml heparin (Sigma, St. Louis, MO), with 100 IU/ml penicillin (Flow), 100 μg/ml streptomycin (Flow), and 0.35% sodium chloride (Sigma) to adjust medium osmolarity to gilthead seabream plasma osmolarity (353.33 mOs)). Cell suspensions were obtained by forcing fragments of the organ through a 100-μm nylon mesh. Head-kidney cell suspensions were layered over a 51% Percoll density gradient (Pharmacia) and centrifuged at 400 *g* for 30 min at 4°C. After centrifugation, the band of leucocytes above the Percoll-medium interface was collected with a Pasteur pipette, washed twice, counted, and adjusted to 10^7 cells/ml in sRPMI-1640. Cell viability was greater than 98% as determined by the trypan blue exclusion test.

Yeast

Heat-killed (by autoclaving at 120°C for 45 min) and lyophilized *Saccharomyces cerevisiae* (strain S288C) were washed twice, counted, and adjusted to 10^8 yeast cells/ml in sRPMI-1640. To label the yeast cells with fluorescein isothiocyanate (FITC) (Sigma), they were stirred in a plate shaker (IUL) at 40 cycles/min and incubated with 5 μg/ml FITC at 22°C, in the dark, for 15 min. After the yeast cells were labeled, free FITC was removed by washing the cells twice in PBS. The yeast cells were resuspended in sRPMI-1640 and used for flow cytometric study. The staining uniformity was examined, and the yeast cell suspensions were then aliquoted and stored at 4°C.

Phagocytosis Assay

The phagocytic activity of gilthead seabream head-kidney leucocytes was studied by flow cytometry. Each phagocytic assay was carried out in triplicate (Esteban et al., 1998). Samples (125 μl) of medium containing FITC-labeled yeast cells (adjusted to 0.5×10^8 yeast cells/ml) were placed in 5-ml tubes (Falcon, Becton Dickinson). Afterwards, the samples were centrifuged (860 *g*, 5 min, 22°C) and the supernatants were removed. Then 100 μl of gilthead seabream head-kidney leucocytes (adjusted to 10^7 cells/ml in sRPMI-1640 medium) were added to each tube

to obtain a final leucocyte : yeast cell ratio of 1:6.25. The samples were then resuspended and incubated at 22°C for different periods of time ranging from 5 to 30 min. At the end of each incubation period the samples were placed on ice to stop phagocytosis and 600 μl of ice-cold PBS were added to each sample. The fluorescence of the extracellular yeast cells (i.e., free yeast cells and yeast cells adhered to phagocytes but not interiorized) was quenched by adding 40 μl ice-cold trypan blue (0.4% in PBS) per sample. Immediately, the samples were mixed gently, acquired, and analyzed in a FACScan (Becton Dickinson, Madrid, Spain) flow cytometer with an argon-ion laser adjusted to 488 nm. The instrument settings were adjusted to obtain optimal discrimination of the different cell populations present in gilthead seabream head-kidney leucocyte suspensions. DNA staining with propidium iodide (PI; Sigma) was carried out to exclude cell debris (Orpegen Pharma, 1995), according to the manufacturer's instructions. Data were collected on a computerized system in the form of two-parameter dot plots and histograms of side scatter (granularity) (SSC), forward scatter (size) (FSC), green fluorescence (FITC) (FL1), and red fluorescence (PI) (FL2). Flow cytometric analysis of seabream leucocyte provided FSC/SSC dot plots of three different populations, which corresponded to phagocytes (R1), lymphocytes and thrombocytes (R2), and cell debris (R3) (Esteban et al., 1998). A gating strategy allowed us to acquire only 10,000 R1 cells per sample at a rate of 300 cells/sec. Fluorescence histograms represented the relative fluorescence on a logarithmic scale. Standard samples of FITC-labeled yeast cells or seabream leucocytes were included in each phagocytosis assay. Samples incubated with FITC-labeled yeast cells for 0 min or incubated at 4°C were used as negative controls. Phagocytic ability was defined as the percentage of cells with one or more ingested yeast cells (green-FITC fluorescent cells) within the total cell population (10,000 cells).

TEM

Pellets from a yeast cell suspension and the phagocytosis assays (1:6.25 leucocyte : yeast ratio) were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2–7.4, for 1 hr at 4°C and then postfixed in 1% OsO_4 (in 0.1 M cacodylate buffer, pH 7.2–7.4, for 1 hr at 4°C in the dark), embedded in Epon, and sectioned on a Reichert Jung ultramicrotome. Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined with a Zeiss EM 10C electron microscope.

Peroxidase Activity Release

Peroxidase activity release by leucocytes in response to calcium ionophore A23187 (CaI; Sigma), phorbol myristate acetate (PMA; Sigma), and yeast cells (*S. cerevisiae*), preincubated with and without 5 μg/ml cytochalasin B (Sigma) was determined by adapting a colorimetric assay (Quade and Roth, 1997). For this, leucocytes and yeast cells were resuspended in a phenol-red free sRPMI-1640 medium and adjusted to 1.11×10^7 cells/ml and 6×10^8 yeast/ml, respectively. Cytochalasin B (1 mg/ml) was dissolved in phenol-red free sRPMI-1640 medium, CaI (2 mg/ml) in dimethyl sulfoxide (DMSO) and PMA (1 mg/ml) in ethanol absolute. Ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA; Sigma) was dissolved in phenol-red free sRPMI-1640 medium, obtaining a final concentration of 50 mM, and the pH of the solution was adjusted to 7. Each degranulation assay was carried out in triplicate.

Next, 450 μl of the suspension of leucocytes were placed in 5-ml tubes, which were treated or not with 2.5 μl cytochalasin B for 0 or 15 min, and then incubated with 1.25 μl of CaI (to obtain a final concetration of 5 μg/ml), 0.5 μl PMA (to obtain a final concentration of 1 μg/ml), or 50 μl yeast cells (in order to obtain a final leucocyte : yeast cell ratio of 1:6). After 0, 1, 2, 3, 10, 20, or 30 min of incubation at 25°C, the tubes were centrifuged (860 *g*, 5 min, 4°C) and maintained in ice to stop degranulation. The supernatants were carefully withdrawn and stored at 4°C until the colorimetric assay was carried out.

In order to determine total peroxidase activity, the leucocytes present in 450 μl were lysed by incubation with 50 μl of cetyltrimethylammonium bromide (CTAB) (0.02% in water) (Sigma) for 2 min, and phenol-red free sRPMI-1640 medium was added to obtain a final volume of 502.5 μl. Background tubes containing 5×10^6 leucocytes in 502.5 μl of phenol-red free sRPMI-1640 medium were also included in each assay. The total peroxidase and background activity for each animal and for each time were determined with the leucocytes exposed to stimuli, centrifuging at 860 *g* (5 min, 4°C), and after carefully withdrawing the supernatants and storing them at 4°C. Tubes containing stimulants but no leucocytes were used as controls.

The optimal concentrations of the substrates (TMB and H_2O_2) were tested. For this procedure, 50 μl of supernatant obtained from tubes containing 450 μl leucocyte suspensions (1.11×10^7) and 50 μl CTAB were dispensed into flat-bottomed 96-well plates. Then 50 μl of 2.5, 5, 10, 20, or 30 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride and 1′25, 2′5 or 5 mM H_2O_2 (both substrates of peroxidases and prepared daily) were assayed. The color-change reaction was read at 650 nm for 45 min in a multiscan spectrophotometer (model 450; Bio-Rad, Richmond, CA). Standard samples without supernatant were also analyzed. The maximum slope of each curve was determined and the optimal concentrations of TMB and H_2O_2, were used in degranulation assays. The percentage release of peroxidase activity was calculated using the following formula: % peroxidase activity release = [(maximum slope of stimulated samples – maximum slope of background)/(maximum slope of lysed samples – maximum slope of background)].

To study the involvement of Ca^{2+} in CaI-provoked degranulation, samples were preincubated with 50 μl EGTA (final concentration 5 mM) and cytochalasin B for 15 min, and then stimulated with CaI for 30 min.

In order to verify the inhibitory effect of sodium azide (Sigma) on peroxidase activity, 50 μl of supernatant were placed in each well and incubated with 1 μl of sodium azide (50 mM) dissolved in phenol-red free sRPMI-1640 medium, for 5 min, and then the colorimetric assay was carried out as described above.

To ensure that the the extracellular peroxidase activity was due to degranulation and not to leucocyte lysis, the viability of leucocytes exposed to each one of the stimulants for 30 min was determined by the propidium iodide test.

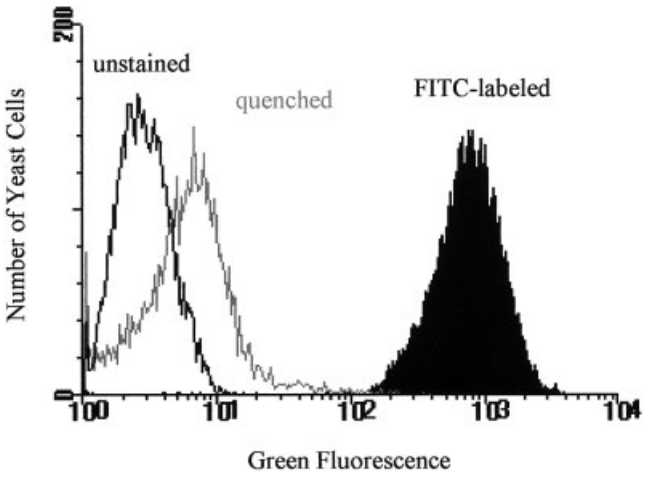


Fig. 1. Representative green fluorescence histogram of unlabeled, FITC-labeled (5 μg/ml), and trypan blue-quenched *S. cerevisiae*.

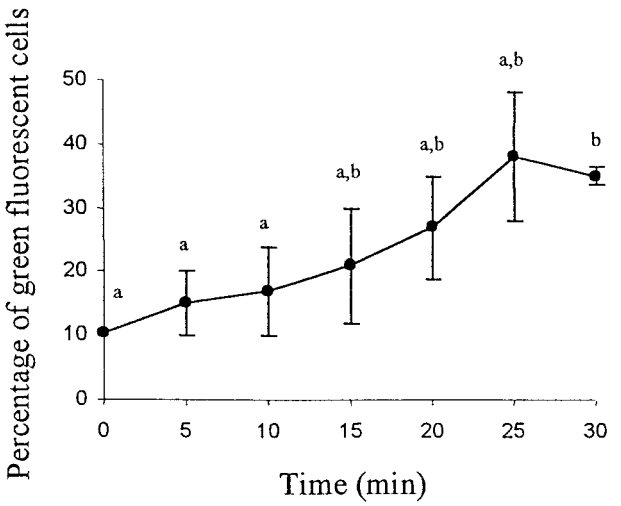


Fig. 2. Kinetics of phagocytic ability (percentage of cells with one or more ingested yeast cells within the total cell population) of gilthead seabream head-kidney leucocytes after incubation with FITC-labeled *S. cerevisiae*, at a 1:6.25 leucocyte : yeast cell ratio. Data represent mean ± S.E. The letters a and b denote statistically significant differences ($P < 0.05$; N = 3).

Statistical Analysis

Data from the flow cytometric assays were studied by using the statistical option of the Lysis Software Package (Becton Dickinson). Data were represented as mean ± S.E. Data were analyzed by one-way analysis of variance (ANOVA) and the unpaired Tukey test.

RESULTS

Phagocytosis Study

FITC-labeled S. cerevisiae. After the yeast cells were incubated in sRPMI-1640 containing 5 μg/ml FITC for 15 min, a homogeneous population of FITC-labeled yeast cells was obtained. Lower FITC concentrations resulted in a more variable level of labeling. After the yeast cells were quenched with trypan blue, the percentage of green fluorescent yeast cells was almost 0% (Fig. 1). In further experiments, therefore, yeast cells were labeled using 5 μg/ml FITC in sRPMI-1640 medium with a 15-min incubation time.

Phagocytosis Assay

Before they were incubated with the yeast cells, the gilthead seabream head-kidney leucocytes showed no fluorescence. The cells that contained phagocytized yeast cells after challenge with FITC-labeled *S. cerevisiae* ingested fluorescent microorganisms and then showed green (FITC) fluorescence. Following phagocytosis, the free yeast cells, nonphagocytic leucocytes, and phagocytes were discriminated by the combined measurement of their green (FITC) fluorescence and size (FSC) in dot plots and histograms.

The kinetics of the phagocytosis carried out by gilthead seabream leucocytes was studied by incubation with FITC-labeled yeast cells for several periods of time ranging from 5 to 30 min. Phagocytic ability (the percentage of cells phagocytizing yeast cells) increased with increasing incubation time (Fig. 2).

TEM Study

The seabream head-kidney consisted of monocyte-macrophages, granulocytes, thrombocytes, and lymphocytes. Only the monocyte-macrophages and acidophilic granulocytes showed phagocytic ability (Fig. 3), as demonstrated previously by Meseguer et al. (1993, 1994).

The main stages of the phagocytic process (yeast cell-phagocyte contact, yeast cell engulfment, endocytic vesicle formation, phagolysosome formation, and intracellular digestion) carried out by monocyte-macrophages and acidophilic granulocytes of gilthead seabream were ultrastructurally characterized. The surface of the yeast cell appeared bound to the phagocyte membrane. The phagocyte surface showed a slight depression or local membrane invagination, and usually showed one or two thin cell processes surrounding one yeast cell (Fig. 4a).

Some cytoplasmatic granules (lysosomes) were seen in the cytoplasm of acidophilic granulocytes close to the endocytic vesicles, fusing their membranes with those of endocytic vesicles and giving rise to phagolysosomes (Fig. 4b). Of note was the fusion of cytoplasmic granule membrane with the phagocyte membrane at the site where the yeast cell was attached to the phagocyte surface (Fig. 4c), and with the endosome membrane (Fig. 4d). In both cases the granule content formed an electron-dense cap over the enclosed yeast cells.

Peroxidase Activity Release

To determine the optimum concentrations of the two peroxidase substrates to be used in further assays, colorimetric assays were carried out using supernatants obtained from lysed leucocytes. TMB and H_2O_2 concentrations of 20 and 5 mM, respectively, were established as the optimum concentrations. The kinetics obtained using these concentrations are shown in Figure 5.

The percentage of peroxidase activity release from seabream head-kidney leucocytes exposed to CaI, PMA, or yeast cells (*S. cerevisiae*) for 30 min preincubated for 15 min or coincubated with cytochalasin B is shown in Figure 6. Samples containing only 5 μg/ml cytochalasin B pro-

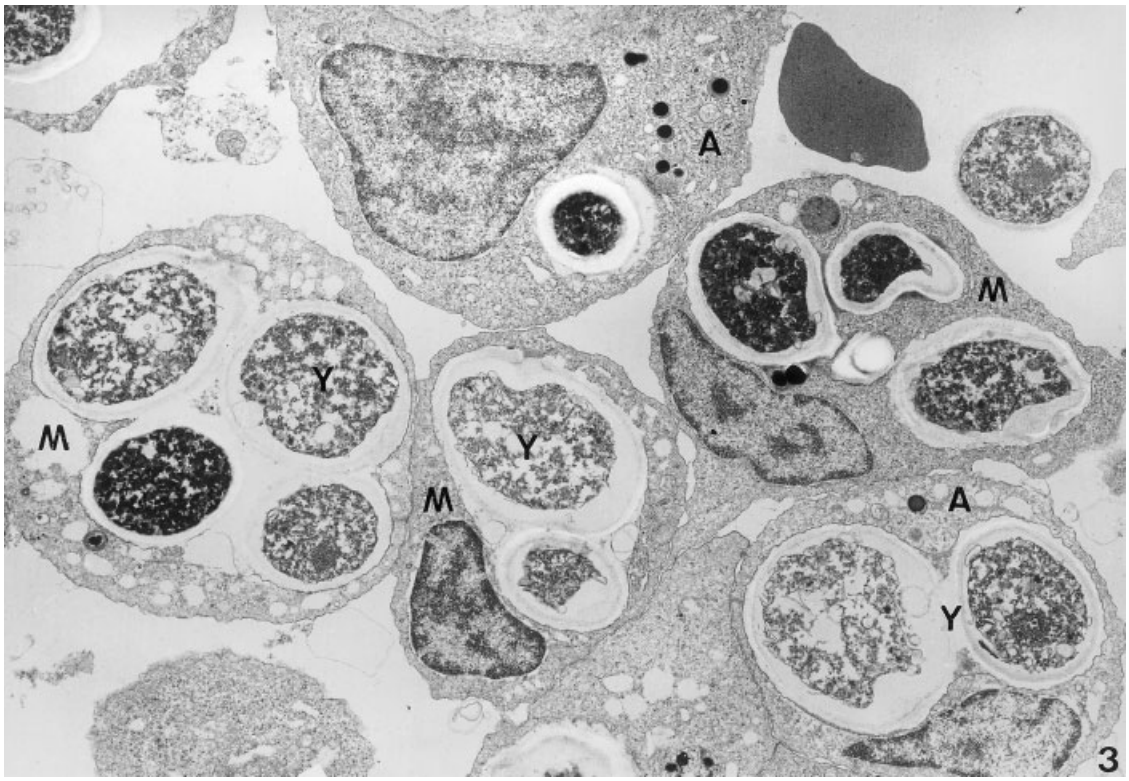


Fig. 3. General view of samples containing gilthead seabream head-kidney leucocytes incubated with *S. cerevisiae* (Y), in which are shown two cell types with phagocytic activity. A, acidophilic granulocytes; M, monocyte-macrophages. ×8,000.

voked around 4.9% ± 3.54% peroxidase activity release. Preincubation with cytochalasin B was neccessary for degranulation, since only low levels of peroxidase activity exocytosis were observed in its absence. In the presence of cytochalasin B, the addition of CaI or yeast cells significantly increased the percentage of peroxidase release (about 48% and 20%, respectively, compared to cytochalasin B alone). No statistical differences were found among the samples preincubated for 15 min or coincubated with cytochalasin B. The addition of PMA did not significantly increase peroxidase release compared with cytochalasin B alone.

The addition of 5 mM EGTA to samples consisting of leucocytes preincubated with cytochalasin B for 15 min and stimulated with CaI totally inhibited CaI-induced degranulation (3.14% ± 2.61% release). The addition of 1 μl EGTA (250 mM) to 50 μl supernatant of samples stimulated with CaI did not affect the colorimetric measurement (43.54 ± 6.53) compared with samples without EGTA. When supernatants obtained from samples consisting of leucocytes preincubated with cytochalasin B and incubated with CaI, PMA, or yeast cells were treated with sodium azide, the peroxidase activity was almost completely inhibited, resulting in 3.2% ± 1.12%, 5.6% ± 1.63%, or 2.2% ± 0.80% activity, respectively.

Leucocyte viability, as measured by the propidium iodide test, was unaffected by incubation with CaI, PMA, yeast cells, cytochalasin B, or EGTA for 1 hr.

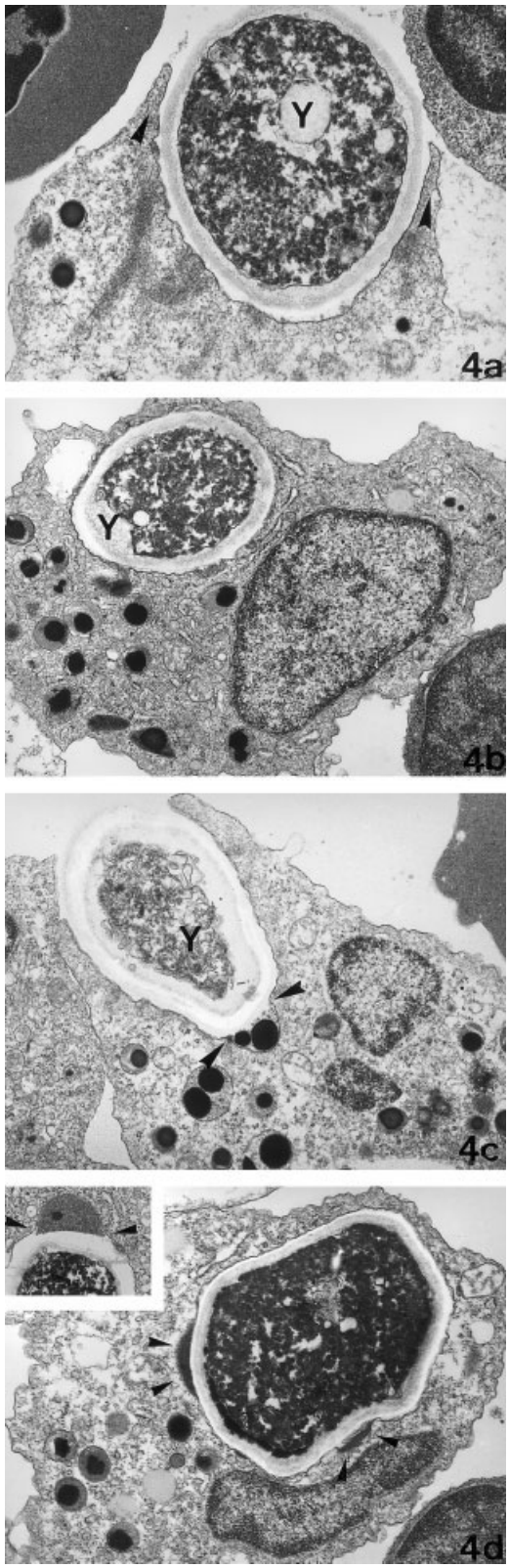
The kinetics of peroxidase activity release in response to CaI, PMA, or yeast cells in samples previously incubated with cytochalasin B for 15 min were studied (Fig. 7). CaI significantly increased the peroxidase activity release compared with nonstimulated samples, after incubation for periods longer than 3 min. Maximum peroxidase activity values (44.51%) were obtained at 10–30 min, although no significant differences were found between these time periods. PMA provoked a nonstatistically significant increase of peroxidase activity release during the incubation time. When yeast cells were used as stimulant, peroxidase activity release increased with the time, but statistically significant differences were only detected at 30 min of incubation time (21.04%).

DISCUSSION

Phagocytosis is considered to be the main cellular process involved in the elimination of damaged cells and microorganisms (Olivier et al., 1985; Finco-Kent and Thune, 1987; Esteban and Meseguer, 1994). Phagocytosis by fish cells has been evaluated, both in vivo and in vitro, using different test particles (Suzuki, 1986; Rowley et al., 1988; Esteban et al., 1998), including yeast cells (Hyder et al., 1983; Bayne, 1986; Saggers and Gould, 1989).

A flow cytometric method that was previously developed for evaluating the phagocytosis of *Vibrio anguillarum* by leucocytes from the same fish species (Esteban et al., 1998) was adapted for use with yeast cells in the present study.

After in vitro coincubation of leucocytes and yeast cells, the leucocytes exhibited phagocytic properties, as deduced from the kinetics of the process. It has been demonstrated



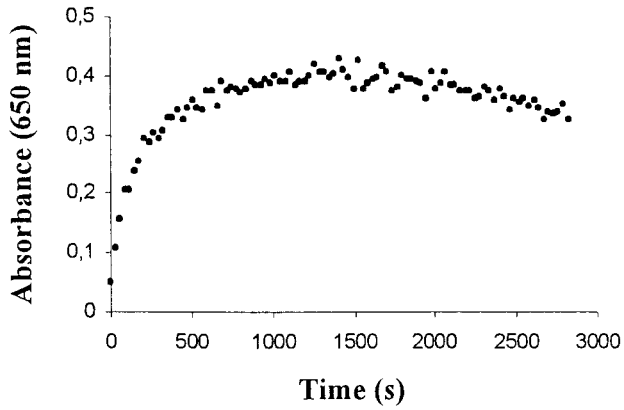


Fig. 5. Peroxidase activity from lysed seabream leucocytes measured as absorbance (650 nm) after addition of 20 mM TMB and 5 mM H_2O_2.

that incubation time is one of the strongest influences on phagocytosis (Leiro et al., 1995; Esteban and Meseguer, 1997), and this is supported by the present results. During the initial phases of in vitro ingestion, uptake increases sharply and then slows down before reaching the maximum. However, we think that the large size of yeast cells compared with leucocytes should be taken into account. The influence of particle size on phagocytosis has previously been reported in studies carried out using bacterial aggregates as test particles, when it was demonstrated that the aggregates formed by a smaller number of cells were more easily phagocytized than the larger ones (Galdiero et al., 1988). The phagocytic ability of seabream leucocytes was approximately 80% when *V. anguillarum* was used as a test particle (Esteban et al., 1998). In the present study, in which yeast cells were used, the phagocytic ability was lower (≈50%), indicating that fewer cells were capable of interiorizing yeast cells. This finding may also be related to the size of the test particle.

When the gilthead seabream head-kidney leucocytes present in the band above the 51% Percoll interface were ultrastructurally characterized (López-Ruiz et al., 1992; Meseguer et al., 1993), they were found to consist of monocyte-macrophages, melanomacrophages, granulocytes, and lymphocytes. The morphofunctional results reported here demonstrate that only monocyte-macrophages and acidophilic granulocytes showed phagocytic ability. Therefore, acidophilic granulocytes in seabream can be considered to be the functional counterparts of neutrophils, in

Fig. 4. Gilthead seabream head-kidney leucocytes incubated with *S. cerevisiae*. **a:** Acidophilic granulocyte showing two thin cell processes (arrowheads) surrounding a yeast cell (Y). Note the participation of cell cytoskeletal members in the process. ×10,500. **b:** Acidophilic granulocyte showing an endocytic vesicle enclosing a yeast cell. Observe the presence of some cytoplasmic granules close to the endocytic vesicle. ×10,000. **c:** Detail of the fusion of a cytoplasmic granule with the phagocyte membrane (arrowheads) at the site of contact with a yeast cell (Y). ×8,820. **d:** Observe the presence of electrondense caps (arrowheads) against the endocytic vesicle membrane. ×10,500. Inset: Detail of a cytoplasmic granule fusing with an endocytic vesicle (arrowheads). ×4,800.

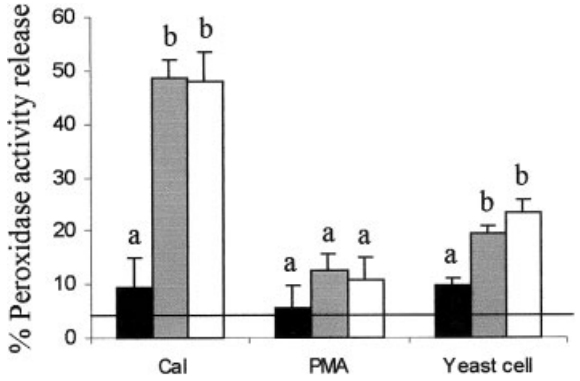


Fig. 6. Effect of incubation of gilthead seabream leucocytes with CaI (5 μg/ml), PMA (1 μg/ml), or yeast cells (1:6 leucocyte : yeast cell ratio) for 30 min on peroxidase activity release. The leucocytes were not preincubated with cytochalasin B (5 μg/ml) (■), or were preincubated for 0 (□) or 15 min (□). % peroxidase activity release = [(maximum slope of stimulated samples – maximum slope of background)/(maximum slope of lysed samples – maximum slope of background)]. Horizontal line denotes peroxidase activity release provoked by incubation of leucocytes with cytochalasin B for 45 min. The letter b represents statistically significant differences ($P < 0.05$; N = 3).

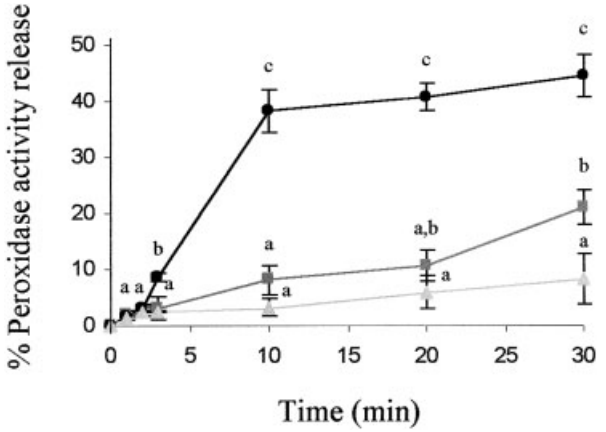


Fig. 7. Kinetics of peroxidase activity release from gilthead seabream leucocytes preincubated with cytochalasin B for 15 min and incubated for 30 min with CaI (5 μg/ml) (—●—); PMA (1 μg/ml) (–△–); or yeast cell (1:6 leucocyte : yeast cell ratio) (–□–). Different letters represent statistically significant differences ($P < 0.05$; N = 3).

agreement with results obtained in other fish species (Lamas and Ellis, 1994; Esteban et al., 1998).

The phagocytic process of gilthead seabream head-kidney leucocytes after incubation with yeast cells showed ultrastructural features similar to those described for head-kidney sea bass leucocytes after incubation with bacteria (Esteban and Meseguer, 1994).

In the present study the main stages of the phagocytosis process were ultrastructurally characterized. The most notable ultrastructural finding was that cytoplasmic granules of acidophils attached and fused to the phagosome membrane, releasing the granule content into endocytic vesicles and giving rise to phagolysosomes. However, occasionally the cytoplasmic granule membrane attached and fused to the plasma membrane before the yeast cell particle was completely engulfed. This process of exocytosis has not been reported previously. Phagolysosome formation in monocyte-macrophages was not observed, perhaps because granulocyte cytoplasmic granules are more evident than lysosomes, or because phagolysosome formation in monocyte-macrophages requires a longer coincubation time of leucocytes and yeast cells.

In the present study a direct, quick, and quantitative colorimetric assay, previously described for mammalian neutrophils (Menegazzi et al., 1992; Quade and Roth, 1997), was adapted to study the possible degranulation of seabream head-kidney leucocytes after different stimuli, such as CaI, PMA, and yeast cells (*S. cerevisiae*). The assay measures the exocytosis of peroxidase using TMB and H_2O_2 as substrates. The above-mentioned authors used this technique to measure myeloperoxidase (MPO) release from blood neutrophils that had been isolated from human or bovine specimens, respectively. It is known that neutrophils have large amounts of peroxidase stored in their azurophilic granules. In vertebrates, it is known that the lysosomes of eosinophils also contain great amounts of a peroxidase called eosinophil peroxidase (EPO), which is reported to be released by degranulation in response to several stimulants (Jong et al., 1980; Garavini and Martelli, 1981; Zabucchi et al., 1989; Kralj-Klobucar, 1991; Menegazzi et al., 1992; Klebanoff, 1998; Persson et al., 2001). In mammals, monocytes (but not macrophages) have been reported to possess great amounts of MPO stored in their lysosomes (Garavini et al., 1981; Dodon et al., 1984; Klebanoff, 1998; Abu-Soud and Hazen, 2000; Hachiya et al., 2000). In fish, myeloperoxidase activity has been detected in goldfish (*Carassius auratus* L.) head-kidney monocyte-macrophages (Neumann et al., 2000); however, other fish species, such as *Sparus aurata* and *Ictalurus punctatus*, show little or no such activity (Meseguer et al., 1994; Calduch-Giner et al., 1997; Praveen at al., 2000). Furthermore, unlike in mammalian platelets, peroxidase activity appears to be lacking in fish thrombocytes (Daimon et al., 1985). In this study, the assays were carried out using seabream head-kidney leucocyte suspensions containing granulocytes (heterophils, basophils, and mainly acidophils), monocyte-macrophages, thrombocytes, and lymphocytes. For this reason, the peroxidase activity released was the sum of MPO and EPO.

TMB is one of the most commonly used peroxidase substrates because of its high sensitivity (compared to other substrates such as ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)), OPD (0-phenylenediamine), and o-dianisidine), and because it is not mutagenic or carcinogenic (in contrast with the aforementioned substrates). DAB (3′,3′ diaminobenzidine) is the most sensitive chromogenic peroxidase substrate, but it is carcinogenic. Degranulation can be measured by iodination assay; however, a disadvantage of that technique is that it requires handling radioactive material (Suzuki et al., 1983; Bertram et al., 1986; Menegazzi et al., 1992; Quade and Roth, 1997).

The concentrations of TMB and H_2O_2, both peroxidase substrates, were optimized for use in the colorimetric assay, and 20 mM and 5 mM, respectively, were found to be optimal. We could not detect the color-change reaction with lower TMB concentrations (2.5 mM), as used in previous studies (Menegazzi et al., 1992; Quade and Roth, 1997). This may have been due to the lower number of cells per sample used in our study and/or our use of heterogeneous leucocyte suspensions.

It is known that primary granule exocytosis in human and bovine neutrophils is minimal unless cytochalasin B is present (Smolen, 1989; Quade and Roth, 1997). Although this compound inhibits actin polymerization, it does not by itself induce the degranulation process. However, it is widely used to enhance leucocyte responses to a variety of stimuli. How cytochalasin B acts in leucocytes is not clear (Zabucchi et al., 1981; Britigan et al., 1989; Bengtsson et al., 1991; Quade and Roth, 1997; Hamilton et al., 1998; Elola and Fink, 1999; Mocsai et al., 1999). Similarly, cytochalasin B was necessary to induce seabream head-kidney leucocytes to degranulate extracellularly in response to stimuli. However, in agreement with previous results (Quade and Roth, 1997), no statistical differences were observed between the levels of degranulation obtained in leucocytes preincubated with cytochalasin B for 0 and 15 min.

In the present work, CaI (5 μg/ml) stimulated extracellular peroxidase release (44.51%) by head-kidney leucocytes, and CaI was used as a positive control for peroxidase release. These results agree with those from previous experiments with mammalian leucocytes (Abrams et al., 1983; King et al., 1997; Quade and Roth, 1997; Nagaji, 1999). CaI induces an influx of extracellular Ca^{2+}, provoking an increase in the intracellular Ca^{2+} concentration, which may lead to exocytosis of granules through activation of protein kinase C and microfilaments (Nagaji, 1999; Kirino et al., 2000). We think that similar processes must be involved in seabream leucocytes, because the presence of EGTA (a chelator of extracellular Ca^{2+} (Forrest et al., 1991)) inhibited peroxidase release, i.e., the degranulation provoked by CaI.

While some authors observed that PMA increases human neutrophil degranulation (King et al., 1997; Nagaji, 1999), others reported (Praveen et al., 2000) that it has little effect on peroxidase activity exocytosis in catfish head-kidney or blood leucocytes. The results obtained in our study suggest that PMA has no effect on seabream leucocyte peroxidase release, in agreement with previous findings (Suematsu et al., 1988; Quade and Roth, 1997).

The present study demonstrates that after seabream leucocytes are incubated with yeast they degranulate, which may be related to the large size of the yeast cells compared to the leucocytes. The kinetics of such a peroxidase activity release point to a statistically significant increase after 30 min of incubation. In mammals, two yeast species (*Candida parapsilosis* and *C. albicans*) also trigger the release of peroxidase activity from human monocytes and murine eosinophils (Marodi et al., 1991; Lefkowitz et al., 1997). Moreover, zymosan, a yeast cell wall extract, is known to stimulate peroxidase release from human (Suematsu et al., 1988; King et al., 1997; Rebecchi et al., 2000) and bovine (Quade and Roth, 1997) blood neutrophils.

To conclude, the results of the present study demonstrate that gilthead seabream leucocytes are able to phagocytose yeast cells, and, after joint incubation, some of their cytoplasmic granules degranulate through exocytosis. Further studies are needed before we can completely understand the intracellular events associated with this process.

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