Cell Tissue Res (1998) 293:133-141

REGULAR ARTICLE

M. A. Esteban · V. Mulero · J. Muñoz · J. Meseguer

Methodological aspects of assessing phagocytosis of *Vibrio anguillarum* by leucocytes of gilthead seabream (*Sparus aurata* L.) by flow cytometry and electron microscopy

Received: 21 July 1997 / Accepted: 7 January 1998

Abstract In this paper we optimize a flow cytometric method for evaluating the phagocytic activity of leucocytes in gilthead seabream *(Sparus aurata* L.) and characterize the phagocytic cells observed. Optimal conditions were established for the fluorescein-labelling and analysis of the bacterium *Vibrio anguillarum* by flow cytometry. Head-kidney leucocytes were incubated with the heat-killed fluorescein isothiocyanate (FITC)-labelled bacteria for different periods, during which the kinetics of phagocytosis was studied. Attached and interiorized bacteria were distinguished. Although phagocytic ability reached a maximum after 60 min, phagocytic capacity reached its maximum at 20 min. The amount of ingested bacteria per phagocyte was estimated from the mean fluorescence of the leucocytes. Cytochalasin B or colchicine was used to inhibit phagocytosis. Monocyte-macrophages and acidophilic granulocytes showed phagocytic activity as demonstrated by transmission electron microscopy. In conclusion, the technique presented allows the screening of thousands of cells, and individual cell evaluation, by quantifying interiorized particles in fish phagocytes. Our ultrastructural results demonstrate that *V. anguillarum* is actively phagocytized by seabream macrophages and acidophilic granulocytes.

Key words Phagocytosis · *Vibrio anguillarum* · Flow cytometry · Transmission electron microscopy · Seabream (*Sparus aurata* L.)

Introduction

In mammals, phagocytosis is carried out primarily by mononuclear phagocytes and neutrophils. At present, the different cell types involved in the fish phagocytic defence have not been elucidated because of the marked morphological heterogeneity of leucocytes, especially of granulocytes. The available results from both in vivo and in vitro studies (Rowley et al. 1988; Doggett and Harris 1989; Esteban and Meseguer 1997) indicate that fish monocyte-macrophages may be considered the most active phagocytic cell type. Nevertheless, the involvement of fish granulocytes in phagocytosis has not been unequivocally demonstrated, and it is not clear which granulocyte types are phagocytic. Reliable methods for quantifying phagocytosis as well as for identifying fish phagocytes are therefore important.

Vibrio anguillarum, the causative agent of vibriosis, causes serious losses in cultured seabream (Devesa et al. 1985; Toranzo et al. 1987; Toranzo and Barja 1990). The nature of the cellular immune response that must be elicited to protect fish against vibriosis is unknown, and so it is important to determine whether fish phagocytes engulf, kill and digest this bacterial species. To date, no direct evidence of this process has been reported. The importance of possessing a simple method to determine phagocytosis derives from the fact that phagocytic activity is an important immunological parameter in the study of bacterial infections in fish, since it is related to the nutritional status, age and race of the animals and to preventive or therapeutic treatment.

The aim of the present work was to study the phagocytic response of gilthead seabream (*Sparus aurata* L.) leucocytes against *V. anguillarum* using flow cytometry and transmission electron microscopy. The phagocytic activity of leucocytes and the kinetics of phagocytosis were evaluated by means of an easy to use technique. Concomitantly, the cell types involved in the process were characterized.

Materials and methods

Animals

Twenty-five specimens of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.), with an average body length of 20 cm and a weight of 200 g, were obtained from CULMAREX S.A. (Murcia, Spain) and kept in 260-l running seawa-

M. A. Esteban · V. Mulero · J. Muñoz · J. Meseguer (✉)
Department of Cell Biology, Faculty of Biology,
University of Murcia, E-30100 Murcia, Spain
Tel.: 34–68–307100; Fax: 34–68–363963;
e-mail: meseguer@fcu.um.es

ter aquaria (flow rate 100%/h) at 20°C with a natural photoperiod. The fish were fed a commercial pellet diet (Purina) at a rate of 2% body weight/day.

Isolation of head-kidney leucocytes

The specimens were anaesthetized with MS222 (Sandoz) (100 mg/l) and the head-kidney leucocytes were isolated under sterile conditions, as described previously (Esteban and Meseguer 1994). Briefly, the head-kidney was removed, cut into small fragments and transferred to 8 ml sRPMI-1640 culture medium [RPMI-1640 medium (Gibco) with 0.35% sodium chloride to adjust the medium's osmolarity to gilthead seabream plasma osmolarity] supplemented with 2% fetal calf serum (FCS) (Gibco), 100 IU/ml penicillin (Flow), 100 µg/ml streptomycin (Flow) and 10 IU/ml heparin (Sigma). Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 102 µm). Head-kidney cell suspensions were layered over a 34–51% Percoll density gradient (Pharmacia) and centrifuged at 400×*g* for 25–30 min at 4°C (Braun-Nesje et al. 1981). After centrifugation, the bands of leucocytes above the 34–51% interfaces were collected with a Pasteur pipette, washed twice, counted and adjusted to 10^7 cells/ml in sRPMI-1640 with 5% FCS. Cell viability was greater than 98%, as determined by the trypan blue exclusion test.

Bacteria

Vibrio anguillarum (now named *Listonella anguillarum*) strain R82 (serotype 01)(Toranzo and Barja 1990) was grown in trypticase soy broth (TSB) (Gibco) on agar plates from 1.0-ml aliquots of stock cultures that had been frozen at –80°C. To label bacteria with fluorescein isothiocyanate (FITC) (Sigma), an isolated colony from each culture was expanded in TSB with FITC (50 or 100 µg/ml) and grown overnight at 20°C in a light-protected microenvironment (Kalmar 1994). The optical density of *V. anguillarun* cell suspensions was measured at 600 nm, the number of cfu/ml being adjusted to 10^9 with standard curves of growth in phosphate-buffered saline solution (PBS). After labelling, free FITC was removed by washing 3 times in PBS, and the bacteria were heat-killed at 60°C for 15 min. After inactivation the bacteria were washed again and three different concentrations (10^8, 10^7 and 10^6 bacteria/ml in PBS) of FITC-labelled bacteria were acquired for flow cytometric study. The staining uniformity was examined and then the bacterial cell suspensions were aliquoted and stored at –80°C.

Samples of the FITC-labelled bacterial cell suspensions were fixed in 70% ethanol in PBS or in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 30 min, before being acquired and analysed.

Phagocytosis

Flow cytometry

The phagocytic activity of gilthead seabream head-kidney leucocytes was studied by flow cytometry. Each phagocytic assay was carried out in triplicate (Rest and Speert 1994). Samples of 100 ml head-kidney leucocyte suspensions, previously adjusted to 10^7 cells/ml in sRPMI-1640 medium supplemented with 5% FCS, were placed in 5-ml tubes (Falcon, Becton Dickinson). To each tube was added 10 µl FITC-labelled bacteria (adjusted to 10^9 bacteria/ml), which was then centrifuged (400×g, 5 min, 20°C). Afterwards, the samples were resuspended and incubated at 25°C for different times ranging from 5 to 180 min. At the end of each incubation period the samples were placed on ice to stop phagocytosis and 500 µl ice-cold PBS was added to each sample. The fluorescence of the extracellular bacteria (i.e. free bacteria and bacteria adhered to phagocytes but not interiorized) was quenched by adding 8.5 µl ice-cold trypan blue (0.4% in PBS) per sample. Immediately, the samples were mixed gently, acquired and analysed in a FACScan (Becton Dickinson) flow cytometer with an argon-ion laser adjusted to 488 nm. The instrument's 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 performed to exclude cell debris (Orpegen Pharma 1995). Cell suspensions (10^6 cells/200 µl PBS) were fixed in ethanol for 30 min at 4°C, washed by centrifugation and resuspended in 800 µl fresh PBS. Then 100 µl RNase (Sigma) (l mg/ml) and 100 µl PI (400 mg/ml) were added to each sample, before the samples were incubated (37°C, 30 min), acquired and analysed. Data were collected 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) on a computerized system. Each of the analyses was performed on 10 000 cells, which were acquired at the rate of 300 cells/s. Fluorescence histograms represented the relative fluorescence on a logarithmic scale. Standard samples of FITC-labelled *V. anguillarum* cells or seabream leucocytes were included in each phagocytosis assay. Samples incubated at 4°C were used as negative controls of the phagocytosis which took place. Phagocytic ability was defined as the percentage of cells with one or more ingested bacteria (green-FITC fluorescent cells) within the total cell population (10,000 cells). The number of ingested bacteria per cell (phagocytic capacity) was assessed from the mean fluorescence intensity of the cells.

Inhibition of phagocytosis

Seabream leucocytes were incubated with 0.1, 1 or 10 µg/ml cytochalasin B (Sigma) or with 5, 50 or 500 µg/ml colchicine (Sigma) in sRPMI with 5% FCS, for 2 h at 25°C. They were then challenged with bacteria and phagocytosis assays were developed. Samples of

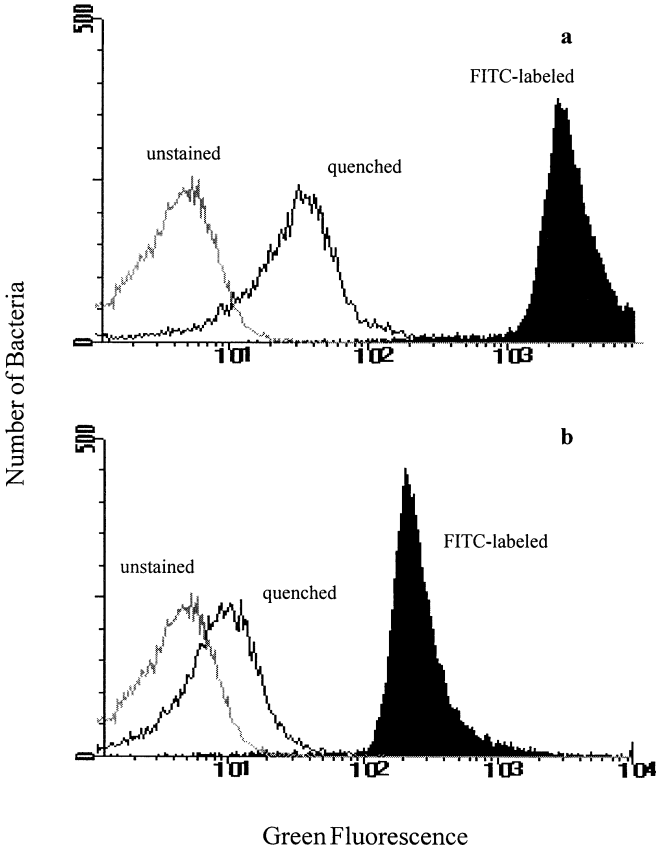

Fig. 1a,b Representative green fluorescence histograms of *V. anguillarum* unstained, FITC-stained and FITC-stained and quenched with trypan blue (125 µg/ml). Bacteria stained by incubation in TSB with 100 µg/ml (**a**) or 50 µg/ml (**b**) FITC

leucocytes incubated without cytochalasine B or colchicine were used as controls.

Transmission electron microscopy

To determine the phagocytic cell types present in gilthead seabream head-kidney leucocyte cell suspensions, 100 µl of the bacterial cell suspensions (10^9 bacteria/ml) were added to samples of 1 ml (10^7 cells/ml in sRPMI-1640 culture medium). The samples were centrifuged (400*g, 5 min, 4°C), resuspended and incubated at 25°C for periods ranging from 10 min to 180 min. Leucocyte samples not challenged with bacteria were used as control. After incubation the cells were pelleted (400×g, 10 min, 4°C) and the extracellular bacteria removed with the supernatants. Immediately, samples were fixed in 2.5% glutaraldehyde in 0.1 M cacocylate buffer, pH 7.2–7.4 for 1 h at 4°C, then postfixed in 1% OsO_4 and embedded in Epon prior to sectioning on a Reichert Jung ultramicrotome. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 10C electron microscope.

Statistical analysis

The quantitative study of flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson). Data were represented as means (±SE). Inhibition of phagocytosis was evaluated by dividing the phagocytic ability obtained with leucocytes which had been preincubated with cytochalasin B or colchicine by the values obtained with leucocytes incubated without the inhibitors (control), and multiplying the fraction by 100. Data were analysed by one-way analysis of variance (ANOVA) and the unpaired Student's *t*-test.

Results

Flow cytometry

FITC-labelled Vibrio anguillarum

After incubation of *Vibrio anguillarum* overnight in TSB-containing FITC (50 or 100 µg/ml), a homogeneous population of FITC-labelled bacteria was obtained. Lower FITC

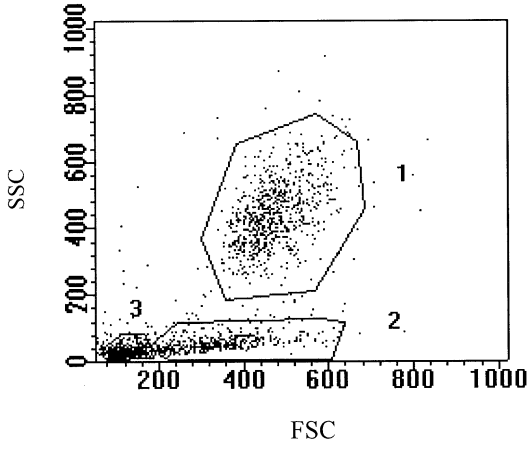

Fig. 2 Representative dot plot of FSC (cell size) vs SSC (cell granularity) of gilthead seabream head-kidney leucocytes. Each *dot* represents one cell. Note the presence of three main cell clusters

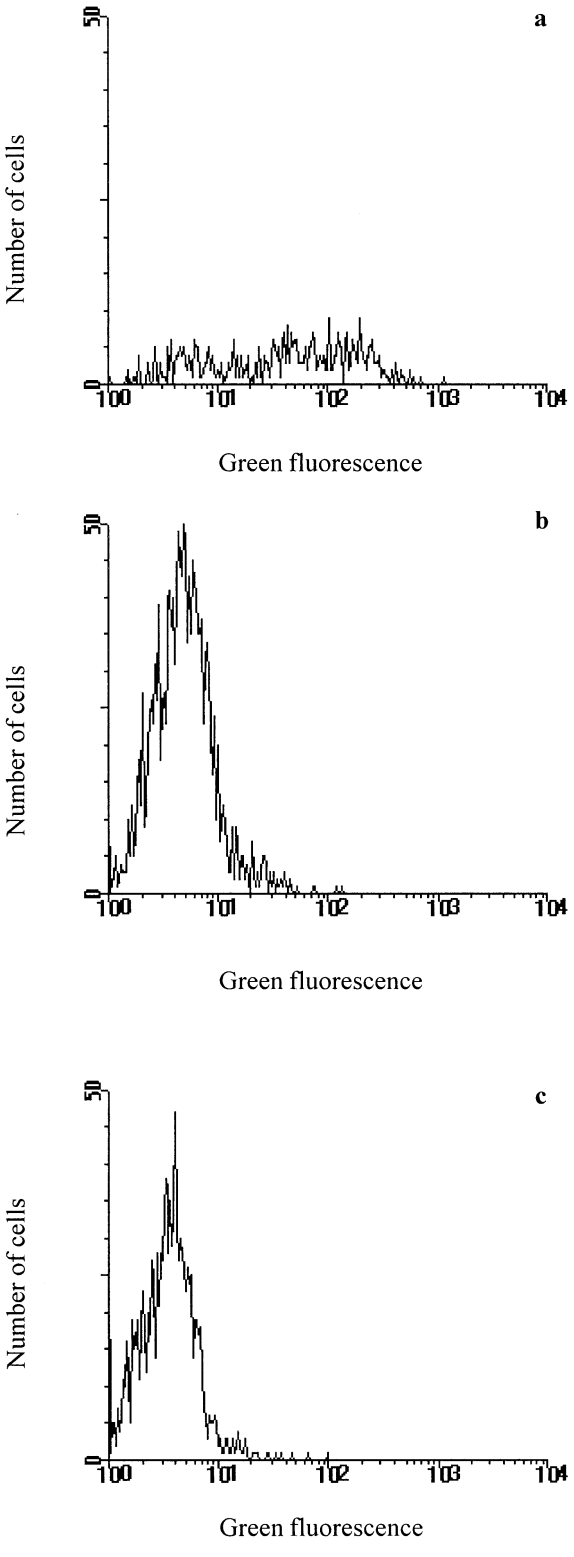

Fig. 3a–c Representative green fluorescence histograms of the three main cell clusters observed in a dot plot of gilthead seabream head-kidney leucocytes (represented in Fig. 2) after incubation for 1 h with *V. anguillarum*. The histograms correspond to cells present in cluster 1 (**a**), cluster 2 (**b**) and cluster 3 (**c**)

concentrations resulted in a lower and more variable labelling of the bacteria. All bacteria were labelled and showed similar mean fluorescence intensity, although slightly higher with 100 µg/ml FITC than with 50 µg/ml.

When acquiring FITC-labelled bacteria the best fluorescence signal detected by the FACs was obtained when they were adjusted to 10^8 bacteria/ml.

The percentage of 100 µg/ml FITC-labelled *V. anguillarum* bacteria showing fluorescence after quenching with trypan blue (Fig. 1a) was higher than when the bacteria were labelled using 50 µg/ml FITC (Fig. 1b). In further experiments, bacteria were labelled with 50 µg/ml FITC in TSB.

When FITC-labelled bacteria were fixed in ethanol and analysed by flow cytometry, a loss of fluorescence intensity was observed. When FITC-labelled bacteria were fixed in glutaraldehyde they exhibited both red and green fluorescences. In further experiments, therefore, unfixed samples were used.

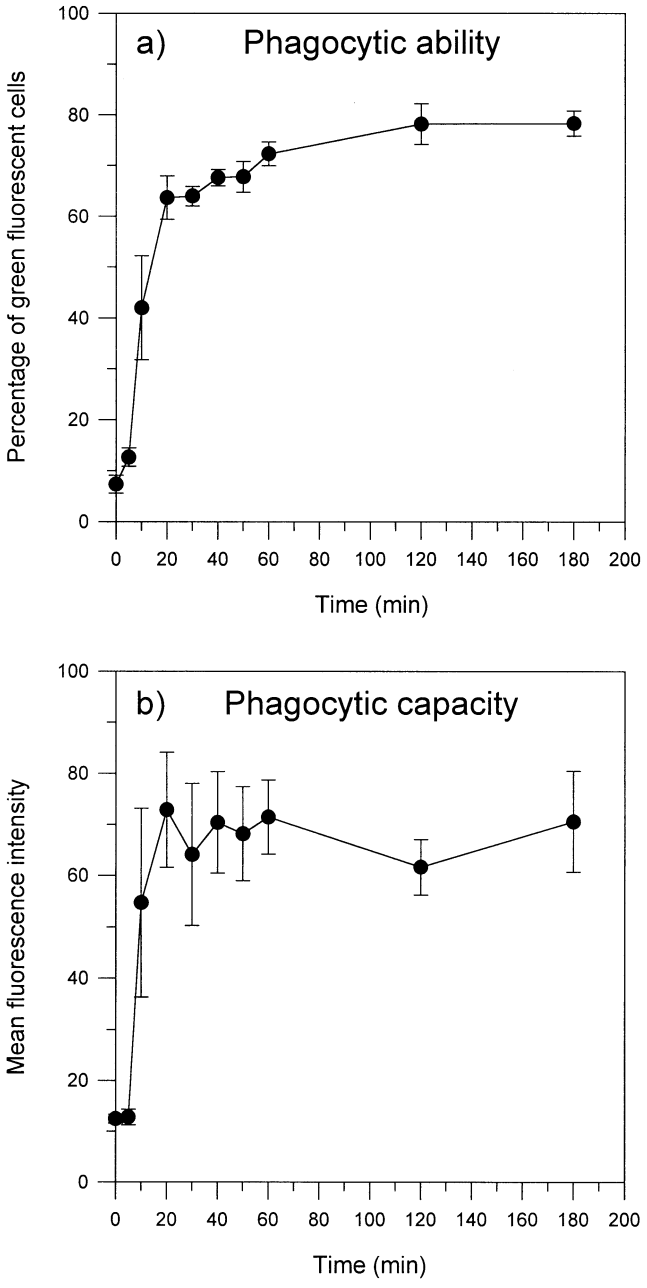

Fig. 4a,b Kinetics of the phagocytosis of FITC-labelled *V. anguillarum* by gilthead seabream head-kidney phagocytes. **a** Phagocytic ability. **b** Phagocytic capacity. Data represent means±standard error

Seabream leucocytes

The gilthead seabream head-kidney leucocytes above the 34–51% Percoll interface were studied by flow cytometry, propidium iodide stain being used to discriminate between viable cells and cell debris. Three main cell clusters were observed according to their FSC (size) and SSC (granularity) properties (Fig. 2).

Phagocytosis assay

Before incubation with bacteria, gilthead seabream leucocytes showed no fluorescence. After challenge with FITC-labelled *V. anguillarum*, the cells that had phagocytized bacteria, which were only present in cluster 1, ingested fluorescent microorganisms and then showed green (FITC) fluorescence (Fig. 3). Following phagocytosis, free

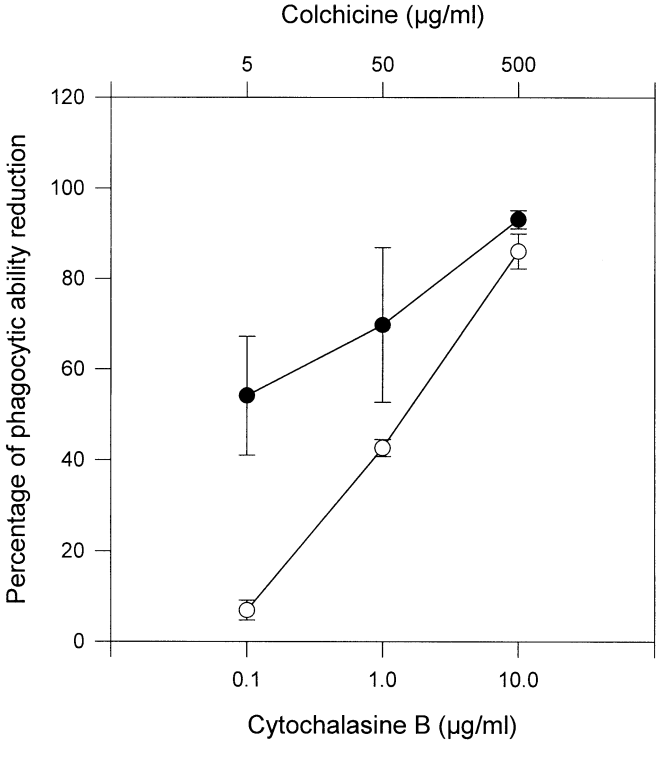

Fig. 5 Inhibition of the phagocytosis of FITC-labelled *V. anguillarum* by seabream head-kidney leucocytes by pretreatment with cytochalasin B (*open circles*) or colchicine (*solid circles*). Data represent means±standard error

bacteria, nonphagocytic leucocytes and phagocytes were discriminated by the combined measurement of their green (FITC) fluorescence and size (FSC).

Kinetics of phagocytosis

The kinetics of the phagocytosis carried out by gilthead seabream leucocytes was studied by incubation with the bacteria for 5, 10, 20, 30, 40, 60, 90, 120, 150 or 180 min. Phagocytic ability and phagocytic capacity increased in a time-dependent manner (Fig. 4). Phagocytic ability increased rapidly from the beginning of the incubation time until 20 min ($p<0.0001$) and peaked at 60 min ($p<0.0001$), maintaining this level until 180 min (Fig. 4a). Phagocytic capacity on the other hand reached a maximum at 20 min ($p<0.01$) and this level of activity was maintained, with small fluctuations, until 3 h of incubation (Fig. 4b).

Inhibition of phagocytosis

Gilthead seabream phagocytes did not ingest FITC-labelled bacteria when the incubation was made on ice (control). Preincubation of seabream leucocytes with cytochalasin B or colchicine before they were challenged with bacteria resulted in a marked dose-dependent reduction of their phagocytic ability, which was almost completely blocked at 10 µg/ml cytochalasin B ($p<0.01$) or 500 µg/ml colchicine ($p<0.0001$) (Fig. 5). Cell viability was unaffected by cytochalasin B or colchicine incubation as assayed by propidium iodide staining.

Transmission electron microscopy

The gilthead seabream head-kidney leucocytes present in the band above the 34–51% Percoll interface were ultratructurally characterized according to our previous results (López Ruiz et al. 1992; Meseguer et al. 1993). They were found to consist of monocyte-macrophages, melanomacrophages, granulocytes and lymphocytes. After challenge with *V. anguillarum* only two cell types showed phagocytic activity. These phagocytic cells were characterized by their ultrastructural features and matched those of the monocyte-macrophages and acidophilic granulocytes of the cells present in control samples.

Monocyte-macrophages not challenged with bacteria (control samples) were rounded cells with a large, round to kidney-shaped nucleus with a nucleolus. Some mitochondria, flat cisternae of rough endoplasmic reticulum, numerous free ribosomes, lysosomes and light vesicles were observed (Fig. 6a).

The acidohilic granulocytes present in control samples showed an elongated or rounded eccentric nucleus. Three different granule types (G1, G2 and G3) could be distinguished in the cytoplasm according to the ultrastructural features of their content. A Golgi apparatus, some cisternae of rough endoplasmic reticulum, free ribosomes and light vesicles were also seen (Fig. 6b).

After incubation with *V. anguillarum*, gilthead seabream head-kidney phagocytes, monocyte-macrophages and acidophilic granulocytes showed an irregular shape as a consequence of their cytoplasmic processes, which varied in size and shape, and some bacteria inside endocytic vesicles. The main stages of the bacteria's phagocytic process were ultrastructurally identified (Figs. 7, 8). Some cytoplasmic granules (lysosomes) appeared near the endocytic vesicles, some showing small electron-dense material with an ultrastructural appearance similar to that of the granule content (Figs. 7a,b, 8a,d,e).

The number of bacteria interiorized per phagocyte varied greatly. When only one bacterium was found inside an endocytic vesicle this was small and usually the bacterium was surrounded by a narrow light halo (Fig. 7b). When the number of interiorized bacteria was low (from one to six), they appeared inside a medium-sized endocytic vesicle (Fig. 7a,b). However, when this number was higher, the cell nucleus was pushed towards the cell periphery, the cytoplasm was reduced to a peripheral rim and most of the cell appeared filled by one or more endocytic vesicles containing the bacteria (Fig. 8a–c). A great number of bacteria per phagocyte were observed, especially in samples incubated for longer periods.

When incubation was for 1 h or longer, the bacteria inside phagolysosomes had a variable ultrastructural appearance. Most of them appeared similar to those which had not been ingested, while others showed membrane and cell wall disruptions and a heterogeneous granular bacterial content (Fig. 8d,e).

Discussion

The ideal assay for evaluating phagocytosis would use small samples, analyse a great number of cells, be technically simple, provide quantitative results, allow discrete cell evaluation, and provide a clear distinction between adhered and ingested particles. Several methods have been used to study and quantify phagocytosis by fish leucocytes although none has permitted a clear distinction between attached and interiorized particles. Without microscopic examination it is impossible to make a cell by cell evaluation. Using flow cytometry in a way similar to that used in mammals, it is possible to study phagocytosis in fish with the above advantages, although there are very few studies on the fish immune system using such a method (Ellsaesser et al. 1985; Thuvander et al. 1987; Kodama et al. 1993; Fisher et al. 1994).

The flow cytometric assay described in the present study seems to satisfy all the above requirements for a good evaluation of phagocytosis. Furthermore it does not involve any washing step and so avoids cell losses. It can easily be perfomed using small cell numbers and involves a technically simple procedure. Phagocytosis is studied under con-

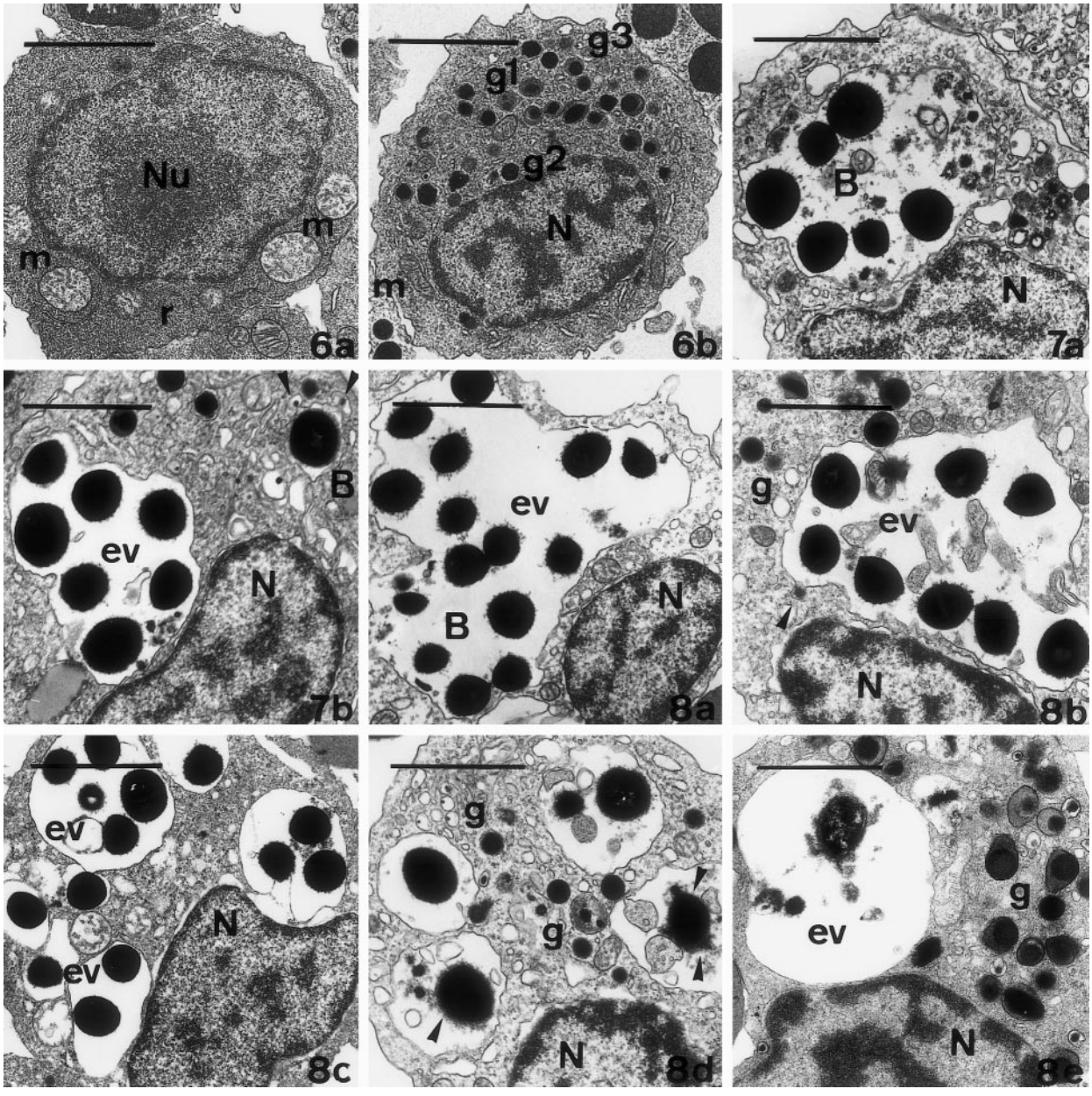

Fig. 6a,b Gilthead seabream head-kidney leucocytes not challenged with bacteria (control). **a** Macrophage. *Nu* Nucleolus, *m* mitochondria, *r* free ribosomes. ×9 000. *Bar* 2.22 μm. **b** Acidophilic granulocyte showing numerous cytoplasmic granules corresponding to three granule populations (*g1, g2, g3*). *N* Nucleus, *m* mitochondria. ×7 500. *Bar* 2.66 μm

Fig. 7a,b Gilthead seabream head-kidney leucocytes incubated for 30 min with *V. anguillarum.* **a** Macrophage with an endocytic vesicle containing bacteria (*B*) and a material of heterogeneous appearance. *N* Nucleus. ×9 500. *Bar* 2.10 μm. **b** Acidophilic granulocyte showing a bacterium (*B*) inside a small endocytic vesicle and some cytoplasmic granules in its vicinity (*arrowheads*). Note the presence of another bigger endocytic vesicle (*ev*) containing several bacteria. *N* Nucleus. ×9 500. *Bar* 2.22 μm

Fig. 8 **a** Macrophage incubated for 2 h with *V. anguillarum.* The cell shows a large endocytic vesicle (*ev*) containing numerous bacteria (*B*). *N* Nucleus. ×7 500. *Bar* 2.66 μm. **b** Acidophilic granulocyte incubated for 1 h with *V. anguillarum*. Note the presence of an endocytic vesicle (*ev*) containing bacteria and cytoplasmic granules (*g*). One of these granules shows the membrane fused to that of the endocytic vesicle (*arrowhead*). *N* Nucleus. ×10 000. *Bar* 2 μm. **c** Macrophage incubated for 2 h with *V. anguillarum.* The cell shows several sizes of endocytic vesicle *(ev)* enclosing a different number of bacteria. *N* Nucleus. ×9 500. *Bar* 2.22 μm. **d** Acidophilic granulocyte incubated for 2 h with *V. anguillarum*. Note the damaged cell wall of the bacteria inside the endocytic vesicles (*arrowheads*). *N* Nucleus, *g* granules. ×7 500. *Bar* 2.66 μm. **e** Acidophilic granulocyte incubated for 3 h with *V. anguillarum.* The cell shows an endocytic vesicle (*ev*) enclosing a damaged bacterium. *N* Nucleus, *g* granules. ×10 000. *Bar* 2 μm

trolled conditions (incubation temperature and time, bacteria/leucocyte ratio, volume) and both the percentage of cells which have phagocytized one or more particles (phagocytic ability) and the variable number of interiorized particles per cell (phagocytic capacity) are easily analysed. The electron-microscopic study used alongside the flow cytometric study enabled us to identify the phagocytic leucocyte types present in gilthead seabream head-kidney.

To study flow cytometric phagocytosis by mammalian leucocytes, a variety of fluorescent-labelled particles have been used including bacterial species (Bassoe 1984; Bjerknes et al. 1989; Ichinose et al. 1994; Van Amersfoort and Van Strijp 1994; Drevets and Elliot 1995). One of the most commonly used fluorochromes is fluorescein isothiocyanate (FITC) (Bassoe et al. 1983; Bassoe and Bjerknes 1985; Bjerknes et al. 1989; Cantinieaux et al. 1989; White-Owen et al. 1992). In the present work *V. anguillarum* were homogeneously labelled by growing in TSB containing 50 or 100 μg/ml FITC, the fluorescence remaining stable for at least 3 months when cell suspensions were stored at –80°C in a light-protected environment. Furthermore, FITC-labelled *V. anguillarum* were bright enough after phagocytosis by seabream leucocytes for phagocytic cells to be distinguished from nonphagocytic cells by flow cytometry. Our assays were based on the ingestion of heat-killed FITC-labelled bacteria by gilthead seabream leucocytes, and on the discrimination between phagocytes and nonphagocytes depending on differences in FITC fluorescence. The two parameters estimated were the number of cells taking up FITC-labelled bacteria (phagocytic ability) and the amount of FITC-labelled ingested bacteria per cell, expressed as mean fluorescence intensity of the cells which had ingested bacteria (phagocytic capacity). The counting of nonphagocytic cells was avoided and the study focused only on the region of the phagocytic cell population (Thwaits and Kadis 1993). In our work a total of 10 000 events per assay were analysed, which is very high compared with the 100–250 cells usually counted in light microscopy studies. This results in a considerable increase in precision without great intra- or interassay variations being observed.

FITC-labelled bacteria were fixed with ethanol or glutaraldehyde to establish the possibility of stopping the phagocytic process and to understand the effect of the treatment on fluorescence. Changes in the bacterial fluorescence intensity were observed when these fixative solutions were used. The phagocytic process was stopped by placing the samples on ice. The cells were acquired and analysed. This proved to be a useful technique since their fluorescence always remained minimal compared with that of the samples incubated at 25°C. The phagocytosis of FITC-labelled *Staphylococcus aureus* by human neutrophils (Cantinieaux et al. 1989) was stopped by the addition of paraformaldehyde, no change in bacterial fluorescence due to the fixation procedure being observed.

The phagocytic process of gilthead seabream head-kidney leucocytes showed similar ultrastructural features to those described for head-kidney sea bass leucocytes (Esteban and Meseguer 1994). We ultrastructurally characterized the following phagocytic stages: bacteria-phagocyte contact, bacteria engulfment, endocytic vesicle formation, phagolysosome formation and intracellular digestion in seabream phagocytes. The process begins with the adherence of a particle onto a phagocytic membrane. Discrimination between adhered and ingested particles by flow cytometry can be achieved in different ways (Bassoe et al. 1983; Fattorossi et al. 1989), although the quenching of the fluorescence of extracellular particles with a dye is the most frequently used (Hed 1977; Sahlin et al. 1983; Bjerknes 1984; Cantinieaux et al. 1989; Sjursen et al. 1989; Giaimis et al. 1994). In the present work bacteria ingestion was demonstrated by quenching the fluorescence of extracellular bacteria by trypan blue.

The study of the kinetics of the phagocytosis carried out by seabream phagocytes allows us to deduce that the phagocytic process occurs in a short period of time. Both phagocytic ability and capacity increased between 5 and 20 min of incubation. These results suggest that the in vitro phagocytic activity of gilthead seabream head-kidney leucocytes becomes saturated, in agreement with results obtained for the in vivo clearance of *Staphylococcus aureus* by blood leucocytes of *Cyprinus carpio* (Avtalion and Shahrabani 1975). Incubation periods shorter than 30 min resulted in a great variation of the phagocytic ability and phagocytic capacity among specimens, although there were no such variations when the incubation periods were 30 min or longer. Incubation periods of 1 h were used in the standard procedure.

It is known that phagocytosis is the principal function of macrophages in teleosts. Granulocytes and thrombocytes have also been described as phagocytic cells in some fish species (Rowley et al. 1988). The morphofunctional results reported here demonstrate that monocytes/macrophages and acidophilic granulocytes are the leucocytes responsible for phagocytic ability in the head-kidney of this fish species, in agreement with results from other fish species (MacArthur and Fletcher 1985; Parish et al. 1985; Temmink and Bayne 1987; Bandin et al. 1993; Lamas and Ellis 1994). We have previously characterized these leucocytes of gilthead seabream using morphological and cytochemical criteria (López-Ruiz et al. 1992; Meseguer et al. 1993, 1994). A high level of phagocytic activity has been reported for acidophils from various fish species (Bodammer 1986; Parish et al. 1985; Temmink and Bayne 1987; Doggett and Harris 1989).

The distribution of fluorescence was studied in individual cells containing ingested bacteria. Although the cell fluorescent signal intensity was directly correlated with the number of bacteria interiorized per cell, it was not possible to ascertain the exact number of these bacteria. This was also the case when FITC-labelled particles were used for phagocytosis assays (Cantinieaux et al. 1989; Giaimis et al. 1994; Harvath and Terle 1994), although when small particles of relatively uniform size, such as latex spheres, were used such discrimination was possible (Dunn and Tyrer 1981; Steinkamp et al. 1982; Harvath and Terle 1994).

Cytochalasin B or colchicine have been used to determine the role of microfilaments and microtubules on cell motility in the phagocytosis mechanism of seabream

leucocytes (Rosenshine et al. 1994). Our results demonstrated that phagocytosis was inhibited in a dose-dependent manner by these substances. It is clear then that microfilaments and microtubules are involved in phagocytosis in seabream leucocytes, the process representing an active uptake of microorganisms and not a passive interiorization process.

To conclude, the assay described in the present paper can be standarized in a reproducible manner and used in further research to study the effect of different parameters on phagocytosis. The present ultrastructural results demonstrate that *V. anguillarum*, although pathogenic for this fish species, is actively ingested by seabream macrophages and acidophilic granulocytes.

Acknowledgements We thank Dr. A.E. Toranzo and Dr. J.L. Barja for providing the bacteria. This work was supported by the CICYT (reference project AGF-94–1360-CO3-02 and 95–0051-OP) and Fundación Séneca (PB/9/FS/97). Dra. M. A. Esteban had a grant from the Fundación Séneca, Murcia Spain. Dr. V. Mulero had a grant from the Ministry of Education and Science of Spain.

References

Ainsworth AJ, Dexiang C (1990) Differences on the phagocytosis of four bacteria by channel catfish neutrophils. Dev Comp Immunol 14:201–209

Avtalion RR, Shahrabani R (1975) Studies on phagocytosis in fish. I. In vitro uptake and killing of living *Staphylococcus aureus* by peripheral leucocytes of carp (*Cyprinus carpio*). Immunology 29:1181–1187

Bandin I, Ellis AE, Barja JL, Secombes CJ (1993) Interaction between rainbow trout macrophages and *Renibacterium salmoninarum* in vitro. Fish Shellfish Immunol 3:25–33

Bassoe CF (1984) Processing of *Staphylococcus aureus* and zymosan particles by human leukocytes measured by flow cytometry. Cytometry 5:86–91

Bassoe CF, Bjerknes R (1985) Phagocytosis by human leukocytes phagososomal pH, and degradation of seven species of bacteria measured by flow cytometry. J Med Microbiol 19:115–125

Bassoe CF, Laerum OD, Solberg CO, Haneberg B (1983) Phagocytosis of bacteria by leucocytes measured by flow cytometry. Proc Soc Exp Biol Med 174:182–186

Bjerknes R (1984) Flow cytometric assay for combined measurement of phagocytosis and intracellular killing of *Candida albicans*. J Immunol Methods 72:229–241

Bjerknes R, Bassoe CF, Sjursen H, Laerum OD, Solberg CO (1989) Flow cytometry for the study of the phagocyte functions. Rev Infect Dis II: 16–33

Bodammer JE (1986) Ultrastructural observations on peritoneal exudate cells from the striped bass. Vet Immunol Immunopathol 12:127–140

Braun-Nesje R, Bertheussen K, Kaplan K, Seljelid R (1981) Salmonid macrophages: separation, in vitro culture and characterization. J Fish Dis 4:141–151

Cantinieaux B, Hariga C, Courtoy P, Hupin J, Fondu P (1989) *Staphylococcus aureus* phagocytosis. A new cytofluorometric method using FITC and paraformaldehyde. J Immunol Methods 121:203–208

Caspi RR, Shahrabani R, Avtalion RR (1980) The cells involved in the immune response of fish. I. The separation and study of lymphocyte subpopulations in carp. A new approach. In: J Manning (ed) Phylogeny of the immunological memory. Elsevier, North Holland, Amsterdam, pp 131–180

Devesa S, Toranzo AE, Barja JL (1985) First report of vibriosis in turbot (*Scophthalmus maximus*) cultured in Northwestern Spain. In: Ellis AE (ed) Fish and shellfish pathology. Academic Press, London, pp 131–140

Doggett TA, Harris JE (1989) Ultrastructure of the peripheral blood leuocytes of *Oreochromis mossambicus*. J Fish Biol 33:747–756

Drevets DA, Elliott AM (1995) Fluorescence labeling of bacteria for studies of intracellular pathogenesis. J Immunol Methods 187:69–79

Dunn PA, Tyrer HW (1981) Quantitation of neutrophil phagocytosis, using fluorescent latex beads. Correlation of microscopy and flow cytometry. J Lab Clin Med 98:374–381

Ellis AE (1977) The leukocytes of fish: a review. J Fish Biol 11:453–491

Ellsaesser CF, Miller NW, Cuchens MA, Lobb CJ, Clem LW (1985) Analysis of channel catfish peripheral blood leucocytes by bright-field microscopy and flow cytometry. Trans Amer Fish Soc 114:279–285

Esteban MA, Meseguer J (1994) Phagocytic defence mechanism in sea bass (*Dicentrarchus labrax* L.): an ultrastructural study. Anat Rec 240:589–597

Esteban MA, Meseguer J (1997) Factors influencing phagocytic response of macrophages from the sea bass (*Dicentrarchus labrax* L.): an ultrastructural study. Anat Rec 248:533–541

Fänge R (1992) Fish blood cells. In: Nilsson N, Holmgren (eds) Fish physiology, vol XIIB. Academic Press, London, pp 1–53

Fattorossi A, Nisini R, Pizzolo JG, D'Amelio R (1989) New, simple flow cytometry technique to discriminate between internalized and membrane-bound particles in phagocytosis. Cytometry 10:320–325

Ferguson HW, Claxton MJ, Moccia RD, Wilkie EJ (1982) The quantitative clearance of bacteria from the bloodstream of rainbow trout (*Salmo gairdneri*). Vet Pathol 19:687–699

Finco-Kent D, Thune RL (1987) Phagocytosis by catfish neutrophils. J Fish Biol 31(A):41–49

Fisher SK, Dallas CE, Jagoe C, Smith MH, Brisbin IL, Chesser RK (1994) Sources of error associated with sample collection and preparation of nucleated blood cells for flow cytometric analysis. Cell Biol Toxicol 10:145–153

Furth RV, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL (1972) The mononuclear phagocyte system: a new classification of macrophages, monocytes and their precursor cells. Bull Wld Health Org 46:845–852

Giaimis J, Lombard Y, Poindron P, Muller CD (1994) Flow cytometry distinction between adherent and phagocytized yeast particles. Cytometry 17:173–178

Harvath L, Terle DA (1994) Assay for phagocytosis. In: Javois LC (ed) Methods in molecular biology. Immunocytochemical methods and protocols, vol 34. Humana Press, Totowa, New York, pp 249–259

Hed J (1977) The extinction of fluorescence by crystal violet and its use to differentiate between attached and ingested microorganisms in phagocytosis. FEMS Lett 1:357–361

Ichinose M, Hara N, Sawada M, Maeno T (1994) A flow cytometric assay reveals an enhancement of phagocytosis by platelet activating factor in murine peritoneal macrophages. Cell Immunol 156:508–518

Kalmar JR (1994) Measurement of opsonic phagocytosis by human polymorphonuclear neutrophils. Methods Enzymol 236:108–119

Kodama H, Hirota Y, Mukamoto M, Baba T, Azuma Y (1993) Activation of rainbow trout (*Oncorhynchus mykiss*) phagocytes by muramyl dipeptide. Dev Comp Immunol 17:129–140

Lamas J, Ellis A (1994) Atlantic salmon (*Salmo salar*) neutrophil responses to *Aeromonas salmonicida*. Fish Shellfish Immunol 4:201–219

López Ruiz A, Esteban MA, Meseguer J (1992) Blood cells of the gilthead seabream (*Sparus aurata* L.): light and electron microscopic studies. Anat Rec 234:261–171

MacArthur JI, Fletcher TC (1985) Phagocytosis in fish. In: Tatner MF, Manning MJ (eds) Fish immunology. Academic Press, London, pp 29-46

Meseguer J, Esteban MA, Muñoz J, López-Ruiz A (1993) Ultrastructure of the peritoneal exudate cells of seawater teleosts, seabream (*Sparus aurata* L.) and sea bass (*Dicentrarchus labrax* L.). Cell Tissue Res 273:301–307

Meseguer J, López-Ruiz A, Esteban MA (1994) Cytochemical characterization of leucocytes from the seawater teleost, gilthead seabream (*Sparus aurata* L.). Histochemistry 102:37–44

Neumüller J, Tohidast-Akrad M (1994) Comparative *in vitro* investigations of the influence of mofebutazone, phenilbutazone and diclofenac on phagocytosis and respiratory burst of human peripheral blood leucocytes. Arzneim Forsch Drug Res 5:636–641

Orpegen Pharma (1995) Phagotest. Test kit for the quantification of phagocytic activity of monocytes and granulocytes in heparinized whole blood. 2/95:1–13

Parish N, Wrathmell A, Harris JE (1985) Phagocytic cells in the dogfish (*Scyliorhinus canicula* L.). In: Maning MJ, Tatner MF (eds) Fish immunology. Academic Press, London, pp 71-83

Pedrera IM, Collazos ME, Ortega E, Barriga C (1992) In vitro study of the phagocytic process in splenic granulocytes of the tench (*Tinca tinca*, L.). Dev Comp Immunol 16:431–439

Rest RT, Speert DP (1994) Measurement of nonopsonic phagocytic killing by human and mouse phagocytes. Methods Enzymol 236:91–108

Rosenshine Y, Ruschkowski S, Finlay BB (1994) Inhibitors of cytoskeletal function and signal transduction to study bacterial invasion. Methods Enzymol 236:467–476

Rowley AF, Hunt TC, Page M, Mainwaring G (1988) Fish. In: Rowley AF, Ratcliffe NA (eds) Vertebrate blood cells. Cambridge University Press, Cambridge, pp 19–127

Sahlin S, Hed J, Rundquist I (1983) Differentiation between attached and ingested immune complexes by a fluorescence quenching cytofluorometric assay. J Immunol Methods 60:115–124

Sjursen H, Bjerknes R, Halstensen A, Naess A, Sornes S, Solberg CO (1989) Flow cytometry for the measurement of serum opsonins to *Neisseria meningitidis* serogroup B, serotype 15. J Immunol Methods 116:235–243

Steinkamp JA, Wilson JS, Saunders GC, Stewart CC (1982) Phagocytosis: flow cytometric quantitation with fluorescent microspheres. Science 215:64

Temmink JHM, Bayne CJ (1987) Ultrastructural characterization of leucocytes in the pronephros of carp (*Cyprinus carpio* L.). Dev Comp Immunol 11:125–137

Thuvander A, Norrgren L, Fossum C (1987) Phagocytic cells in blood from rainbow trout, *Salmo gairdneri* (Richardson), characterized by flow cytometry and electron microscopy. J Fish Biol 31:197–208

Thwaits RN, Kadis S (1993) Purification of surface-exposed integral outer membrane proteins of *Actinobacillus pleuropneumoniae* and their role in opsonophagocytosis. Am J Vet Res 54:1462–1470

Toranzo AE, Barja JL (1990) A review of the taxonomy and seroepizootiology of *Vibrio anguillarum*, with special reference to aquaculture in the northwest of Spain. Dis Aquat Org 9:73–82

Toranzo AE, Santos Y, Lemos ML, Ledo A, Bolinches J (1987) Homology of strains causing epizootics in turbot, salmon and trout reared on the Atlantic coast of Spain. Aquaculture 67:41–52

Van Amersfoort ES, Van Strijp JAG (1994) Evaluation of a flow cytometric fluorescence quenching assay of phagocytosis of sensitized sheep erythrocytes by polymorphonuclear leukocytes. Cytometry 17:294–301

White-Owen C, Alexander JW, Sramkoski RM, Babcock GF (1992) Rapid whole-blood microassay using flow cytometry for measuring neutrophil phagocytosis. J Clin Microbiol 30:2071–2076