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Ultrastructural observations on the phagocytic behavior of winter flounder *Pleuronectes americanus* peritoneal neutrophils and macrophages *in vivo*

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ABSTRACT: A qualitative electron microscopical examination of peritoneal phagocytes in winter flounder *Pleuronectes americanus* after brief (i.e. 30 or 60 min) exposure to formalin-killed *Bacillus cereus* or *Yersinia ruckeri* revealed that both neutrophils and macrophages had phagocytozed bacteria. Neutrophils obtained from both the peritoneal cavity and from the head kidney were identified on the basis of their overall cytological appearance and more specifically on the presence of elongated, striated (fibrillar) appearing granules in their cytoplasm which stained positively for peroxidase. Macrophages recovered from the peritoneal cavity were phagocytic like neutrophils; however, they were not observed to contain peroxidase. The surface membrane changes observed for 'activated' neutrophils and macrophages which had engulfed bacteria were consistent with those reported for other fish species and higher vertebrates. Images of aggregated cells suggestive of cellular communication between lymphocytes, macrophages, and/or neutrophils are provided.

KEY WORDS: Winter flounder · Ultrastructure · Peritoneal phagocytes · *In vivo*

INTRODUCTION

As indicated in a number of recent reviews (Blazer 1991, Ainsworth 1992, Hine 1992, Secombes & Fletcher 1992), the phagocytic behavior of both macrophages and neutrophils plays an important role in the non-specific defense mechanisms of teleost fish. Although the cytological characteristics and phagocytic nature of both these cell types have been extensively studied in certain groups of fishes (e.g. salmonids, cyprinids, and ictalurids), much less is known about their morphology and function among the pleuronectiformes. Presently, the plaice *Pleuronectes platessa* is the only flatfish species, the peritoneal macrophages and neutrophils of which have been studied with respect to their ultrastructure and function (MacArthur et al. 1984, 1985, MacArthur & Fletcher 1985). Regarding function, plaice macrophages and neutrophils were shown to engulf oyster glycogen and the marine bacterium *Vibrio alginolyticus*.

The winter flounder *Pleuronectes americanus* is an important commercial, recreational, and research species along the northeast coast of the United States. Reduction in stocks is being extensively investigated with respect to such factors as overfishing, habitat loss, and the effects of pollution (Murchelano 1989, Murchelano & Wolke 1991). With the exception of the earlier work of Bridges et al. (1976) on the basic hematology of winter flounder and an *in vitro* study on the phagocytic and bactericidal capability of their macrophages (Daniels 1988), there are no detailed studies on the leukocytes of this species. Our knowledge of the immune system in winter flounder is at present limited to studies on their antibody responses after exposure to

a variety of antigens under either natural (Robohm et al. 1979) or experimental conditions (Stolen et al. 1984, 1985, Laudan et al. 1987, 1989).

The purpose of this study was to define the *in vivo* ultrastructural and cytochemical (peroxidase staining) characteristics of peritoneal macrophages and neutrophils of this infrequently studied species after short periods (30 to 60 min) of exposure to formalin-fixed *Bacillus cereus* or *Yersinia ruckeri*.

MATERIALS AND METHODS

Fish. The 14 winter flounder (15 to 25 cm in total length) used in this study were collected by trawling in the Atlantic Ocean along the coast near Cape May, New Jersey (USA). The fish were transported to the National Marine Fisheries Service Laboratory at Oxford, Maryland (USA) where they were maintained for at least 1 wk before experimentation. Fish were housed in four 100 l aquaria receiving flowing seawater (12‰) at a temperature of 12 to 13°C. The fish were fed chopped clams (*Mya arenaria*) *ad libitum* every other day.

Leukocyte preparations. Peritoneal cells were harvested from all fish by anaesthetizing them with MS-222, killing them with a blow to the head, swabbing the abdomen with 70% alcohol, and incising the lateral body wall. The exposed abdominal cavity was vigorously rinsed with 3.0 ml of sterile tissue culture medium (STCM) and the harvested cells from 2 fish in each experiment were pooled. The pooled cells were pelleted by centrifugation at 1000 × *g* for 5 min before being fixed for electron microscopy as described below. In order to preserve the leukocytes as rapidly as possible, no attempt at washing or cell purification was made. Consequently, the cell preparations were moderately contaminated by red blood cells introduced from the edges of the abdominal incision.

Peritoneal leukocytes from control fish. Peritoneal leukocytes serving as controls were obtained from 2 fish after intraperitoneal (i.p.) injection with 0.5 ml of STCM at 0 and 30 min with the leukocytes being recovered 60 min after the initial injection (0 min). The STCM used in this set of experiments and for peritoneal leukocytes exposed to formalin-killed *Bacillus cereus* (see below) consisted of: 91% Minimal Essential Medium, Eagle-Earle Base, 22 mM sodium bicarbonate, 62 mM L-glutamine, 9.0% fetal bovine serum (heat inactivated at 56°C for 30 min), 20 USP units ml^{-1} heparin sodium salt, 62 mM additional sodium chloride, 100 I.U. ml^{-1} penicillin G potassium, and 100 I.U. ml^{-1} streptomycin sulfate.

Peritoneal leukocytes from fish exposed to *Bacillus cereus*. Eight flounder (4 fish per group) were injected i.p. with formalin-fixed, washed *Bacillus cereus* (1×10^8 cells ml^{-1} suspended in STCM. Group 1 consisted of 4 fish injected with 0.5 ml of bacterial suspension; leukocytes were collected from the fish at 30 min post-injection. Group 2 consisted of 4 fish injected with 0.5 ml of bacterial suspension at 0 min and again at 30 min; leukocytes were collected from these fish 60 min after the time of their first injection (0 min).

Cell preparation for conventional electron microscopy. All of the routine (non-peroxidase) preparations of leukocytes examined in this study were fixed by first pelleting the cells and then resuspending them in cold (0 to 4°C), 2% glutaraldehyde in 0.1 M phosphate buffer (ph 7.4) for 24 to 48 h. The cells were then washed (4×) in cold (0 to 4°C) buffer and allowed to adjust to room temperature. Post-fixation of the leukocytes was accomplished in 1% osmium tetroxide in 0.1 M phosphate buffer (ph 7.4) for 1 h at room temperature. Subsequently, the cells were stained *en bloc* in uranyl acetate following the method of Terzakis (1968) and dehydrated in a graded series of ethanols and propylene oxide. Infiltration and embedment of the cells was accomplished in resin (Spurr 1969). Thick sections (1 to 2 µm) were cut with glass knives and stained with toluidine blue dye. Selected areas were thin sectioned (60 to 90 nm) with diamond knives and stained with uranyl acetate and lead citrate before examination and photography with a Zeiss EM9 S2 electron microscope.

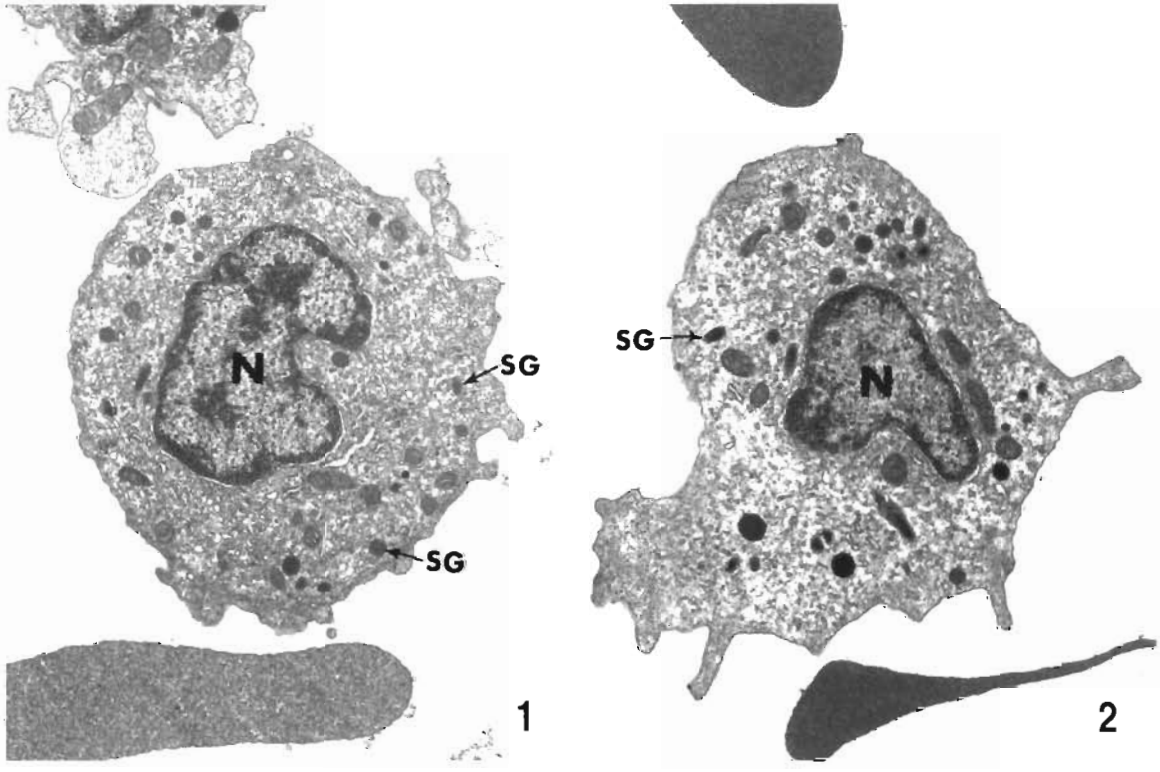
Peroxidase evaluation. Peritoneal leukocytes for peroxidase studies were obtained from 4 fish after injecting them i.p. with 1 ml (1×10^8 cells ml^{-1}) of formalin-killed, washed *Yersinia ruckeri* suspended in Earle's Minimal Essential Medium (EMEM) supplemented with 2% fetal bovine serum as per the recommendation of D. P. Anderson (pers. comm.). The leukocytes were collected from the peritoneal cavity after 60 min as described above. Cells were prepared for peroxidase staining at pH 7.6 by the diaminobenzidine (DAB) method of Bielek (1981). Peritoneal leukocytes exposed to *Y ruckeri* that served as controls for the cytochemical test were incubated without DAB. Although peritoneal leukocytes that had not been exposed to the bacteria were not available for peroxidase evaluation, head kidney tissue from the fish used in these experiments was removed immediately after collection and fixation of the peritoneal leukocytes. The kidney tissue was then minced into small pieces while still in fixative and subsequently processed for peroxidase evaluation (including controls incubated without DAB) as described above. Upon completion of the cytochemical procedures, all peritoneal cells and head kidney tissue were post-fixed and embedded as described above for conventional electron microscopy. The cytochemically treated leukocytes were lightly

stained after thin sectioning with alcoholic uranyl acetate to facilitate their identification.

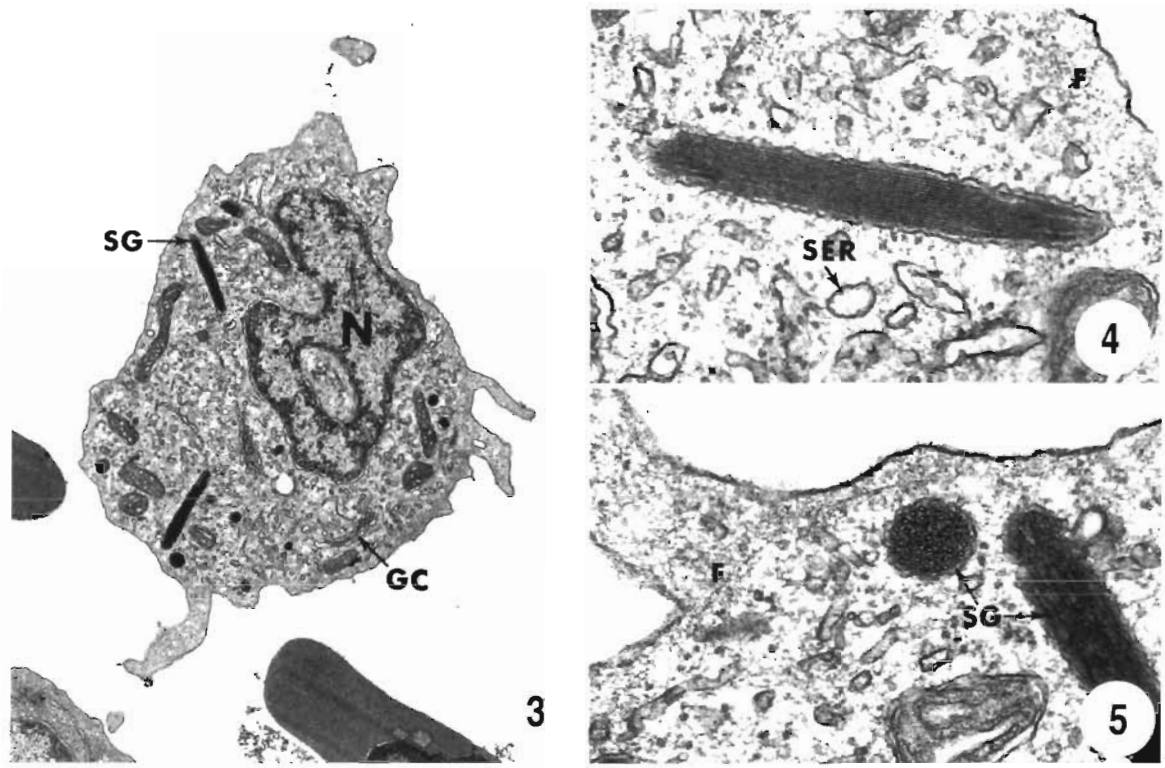
RESULTS

Peritoneal neutrophils from control fish injected i.p. with STCM (Figs. 1 & 2) and some of those recovered from fish (Fig. 3) at 30 min post-injection with STCM and *Bacillus cereus* had a quiescent appearance (i.e. lacked well developed pseudo- or lamellipodia or internalized bacteria); in addition, they were round to oval in form and demonstrated only slight undulations of the plasma membrane. Their nuclei were horseshoe-shaped and demonstrated a dense band of heterochromatin both peripherally and as islands within the nucleoplasm. The cells were approximately 7 to 8 µm in length and 4 to 6 µm in width, and their cytoplasm contained modest amounts of rough endoplasmic reticulum membranes, a readily recognized Golgi complex (GC; Fig. 3), dense, elongate mitochondria which varied considerably in form, numerous anastomosing tubules of smooth endoplasmic reticulum (SER; Fig. 4), and glycogen-like particles. Fine filaments (F; Figs. 4 & 5) were observed in the cells' moderate surface elaborations and subjacent to its plasma membrane. The striated granules (SG; Figs. 1 to 3) typically found within their cytoplasm were revealed in some planes of section (Figs. 4 & 5) to be composed of a microtubular array embedded in a dense matrix that in most instances gave the granules a filamentous or striated appearance when obliquely or longitudinally sectioned. As shall be demonstrated in a subsequent section of this report, the granules of these cells stained positively for peroxidase.

Macrophages from either control fish (Fig. 6) injected with STCM and recovered from the peritoneal cavity of fish at 60 min post-injection as well as those from test fish receiving STCM containing *Bacillus cereus* and recovered from the peritoneal cavity at 30 min post-injection (Fig. 7) lacked the elongate, striated granules observed for the neutrophils, were approximately 8.3 to 11.5 µm in diameter and often had a spherical shape. The eccentrically placed nuclei of these cells were typically elongate, frequently indented and demonstrated a moderate amount of heterochromatin along their inner nuclear membrane or in islets. Typically the nucleoplasm of these cells was less electron dense than that observed for the neutrophils. Likewise, their cytoplasm also appeared to be less electron dense than the neutrophils and contained vacuoles, a well developed system of multiple Golgi organelles, prominent arrays of rough endoplasmic reticulum membranes, and numerous lysosome-



Figs. 1 & 2. *Pleuronectes americanus*. Electron micrographs of peritoneal neutrophils from control fish injected i.p. with sterile tissue culture medium (STCM) and recovered 60 min post-injection. SG: striate granule; N: nucleus. 10 065×



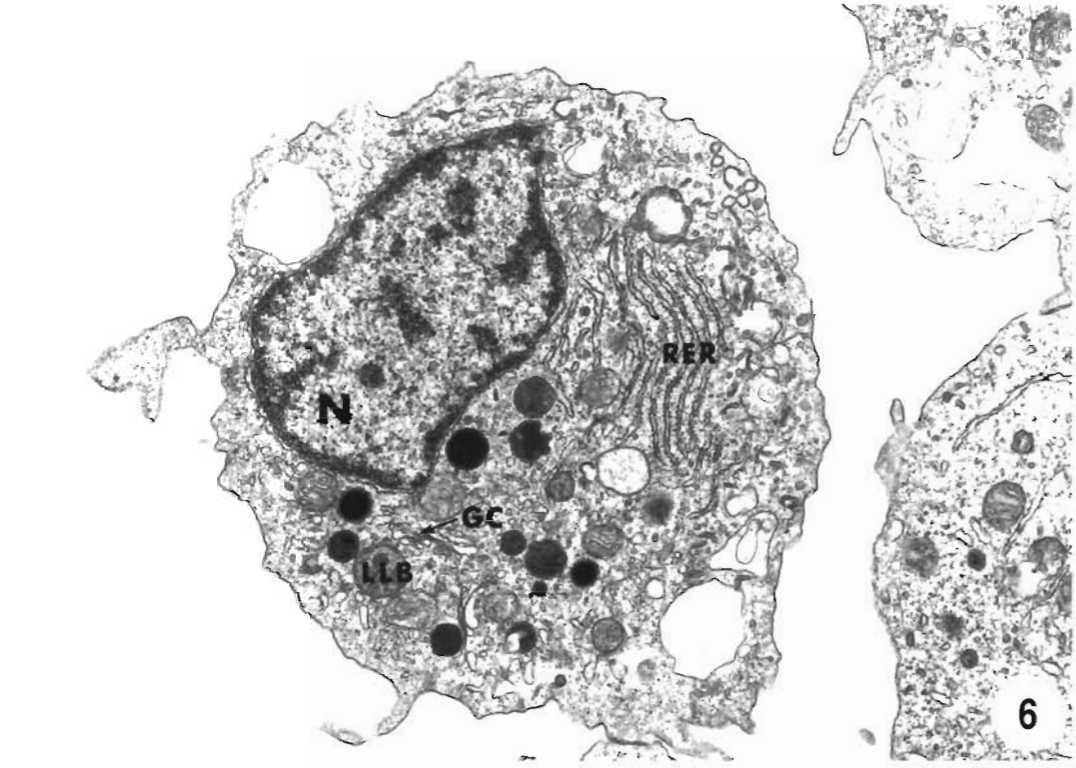
Figs. 3 to 5. *Pleuronectes americanus*. Fig. 3. Electron micrographs of a peritoneal neutrophil recovered 30 min post-injection with STCM containing *Bacillus cereus*. This cell more clearly reveals the elongate nature of the striated granules (SG) found within the neutrophil cytoplasm and at higher magnifications the striate (Fig. 4) and/or microtubular nature (Fig. 5) of the granules is readily observed. N: nucleus; GC: Golgi complex; SER: smooth endoplasmic reticulum; F: fine filaments. Fig. 3, 9840×; Figs. 4 & 5, 56 000×

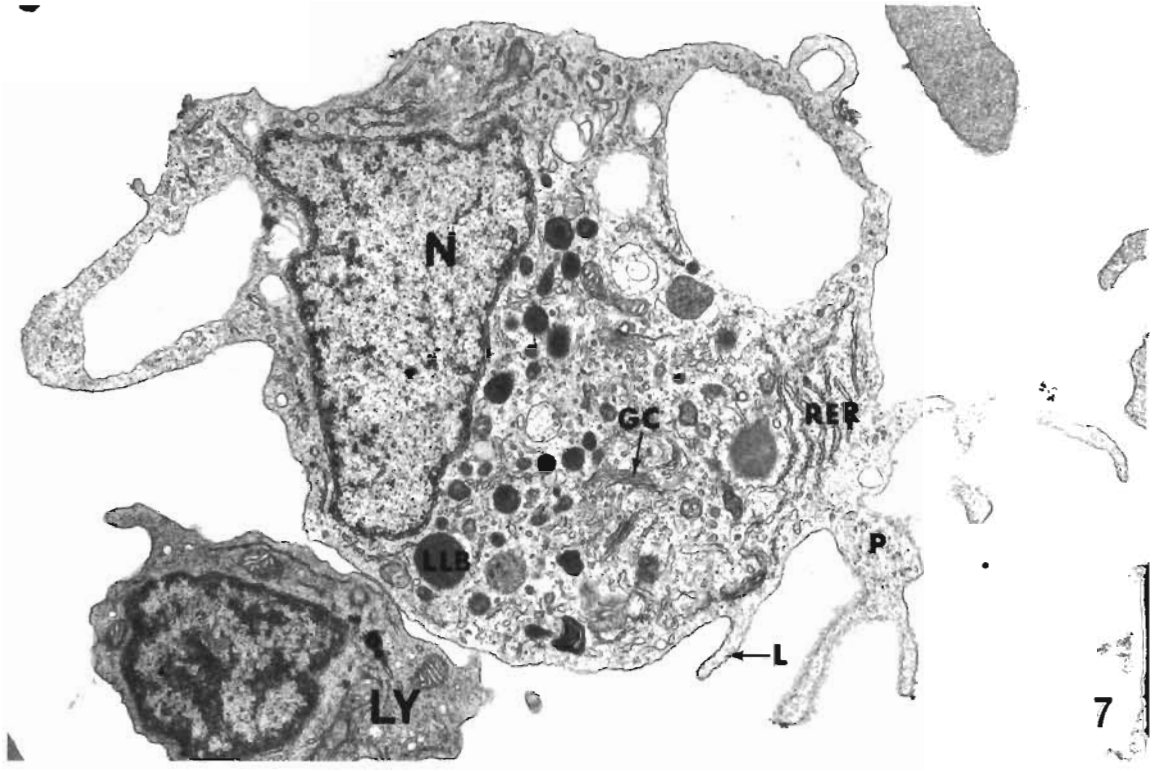
like and/or residual bodies. The macrophage cell membrane was elaborated in some regions to form pseudopodial (P) or lamellipodial (L) extensions which contained fine filaments (F).

As previously described for neutrophils from control fish, macrophages (Fig. 6) recovered from fish injected i.p. only with STCM and recovered 60 min post-injection appeared to be only moderately activated, as evidenced by their lack of surface elaborations and limited vacuolation. While not studied quantitatively, macrophages (Fig. 7) were found less often than neutrophils in our peritoneal cell preparations collected at 30 min post-injection with STCM containing *Bacillus cereus*. When observed, they usually failed to contain bacteria within their cytoplasm, but they appeared to have more elaborate surface modifications and larger vacuoles than those recovered from the control fish injected only with STCM (Fig. 6). Exceptions to this generality did exist, however, because some macrophages collected from the peritoneal cavity of test fish at this time interval (30 min) were obviously capable of recognizing and engulfing the injected test bacterium (Fig 8).

Neutrophils (Fig. 9) from test fish injected i.p. with STCM containing *Bacillus cereus* and recovered after 30 min were observed to engulf the test bacteria (B). When compared with neutrophils lacking bacteria in their cytoplasm (Figs. 1 to 3), the nuclei of these cells were often found closer to the cell periphery and their surface membranes were extensively modified to form ruffles (lamellipodia, L) or pseudopodia (P) which contacted adjacent cells. The cytoplasm of these cells often contained a number of vacuoles; however, some of these may have resulted from sections through infoldings in the plasmalemma. Fusion of the cells' striated granules with phagosomes containing bacteria was not documented; however, the dense cap-like structure observed in direct apposition to the bacterial cell wall (CLS; Fig. 9) suggests that such fusion did occur. At 30 min post-injection, neutrophils containing bacteria within their cytoplasm were frequently found in aggregates. Neutrophils from control fish (Figs. 1 & 2) failed to form detectable aggregates, suggesting that aggregation was a response to the presence of internalized bacteria. Firm conclusions on this will, however, have to await the outcome of additional (quantitative) studies.

At 60 min post-injection with STCM containing *Bacillus cereus*, observations similar to those made at the earlier time interval regarding peritoneal cell iden-





Figs. 6 & 7. *Pleuronectes americanus*. Fig. 6. Electron micrograph of a macrophage from control fish injected with STCM and recovered 60 min post-injection. Fig. 7. Electron micrograph of a peritoneal macrophage recovered at 30 min post-injection with STCM containing *Bacillus cereus*. The cytoplasm of these cells contained well developed Golgi organelles (GC), abundant rough endoplasmic reticulum membranes (RER), and lysosome-like bodies (LLB). N nucleus, P pseudopod; L lamellipodium or ruffle; LY, lymphocyte Fig 6, 13 200×; Fig. 7, 9722×

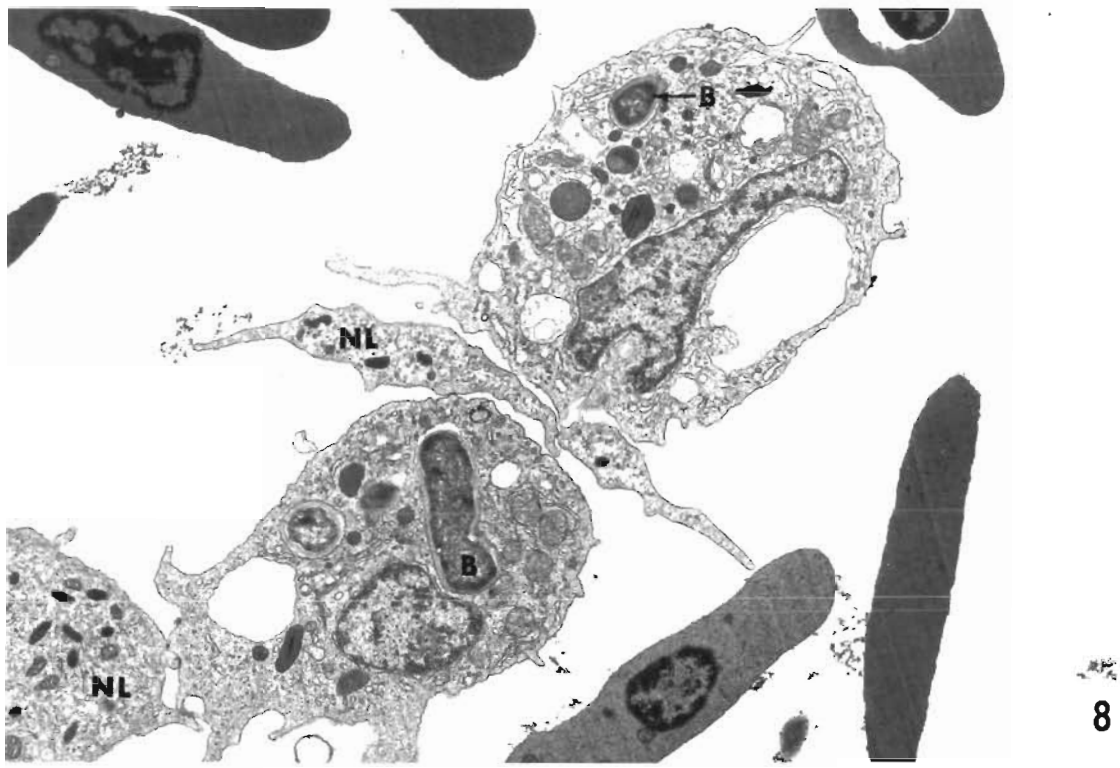


Fig 8 *Pleuronectes americanus*. Electron micrograph of 2 peritoneal macrophages recovered at 30 min post-injection with STCM containing *Bacillus cereus*. Both cells have phagocytozed bacteria (B) and their cytoplasmic characteristics are like the cells shown in Figs 6 & 7. NL: neutrophil; B. bacterium. 7333×

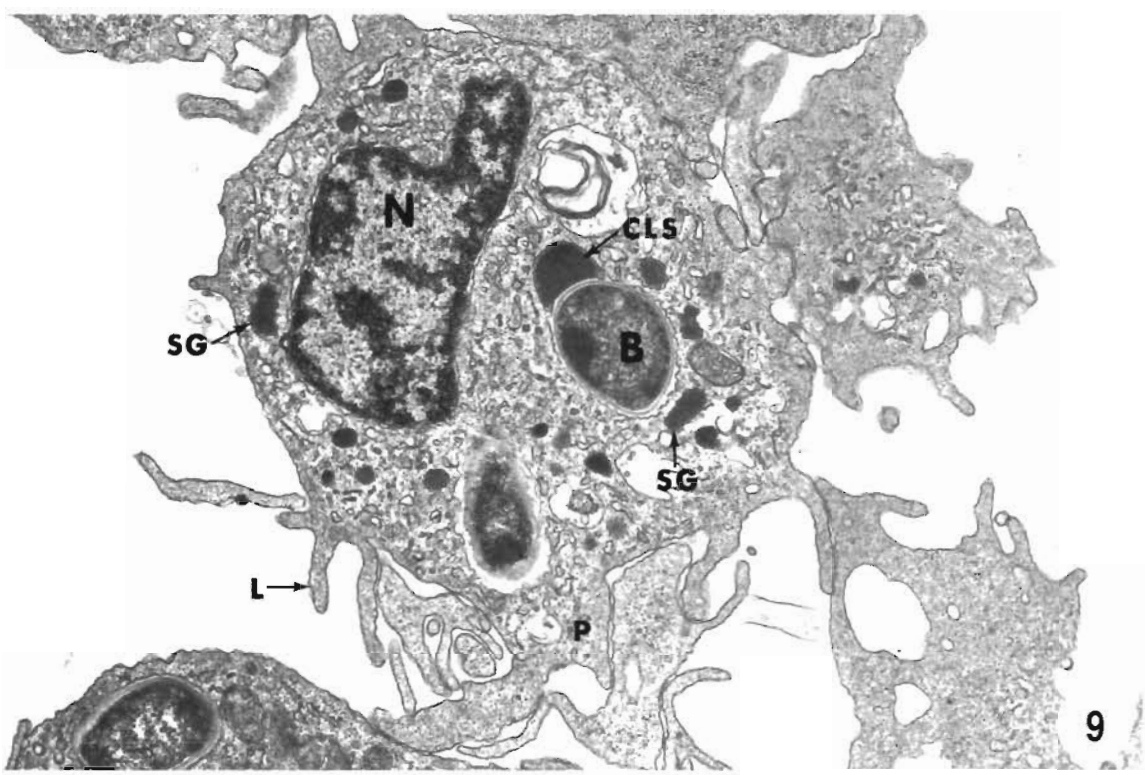


Fig 9 *Pleuronectes americanus* Electron micrograph of an activated neutrophil recovered from the peritoneal cavity 30 min post-injection with STCM containing *Bacillus cereus* This cell has taken up several bacteria (B), and its striated granules (SG) can be clearly seen The dense cap-like structure (CLS) suggests a point of granule fusion with a phagosome. P: pseudopod; L: lamellipod or ruffle, N nucleus. 13 333×

tification and behavior were repeated, but the following new information on peritoneal leukocytes was obtained. First, there appeared to be a greater tendency for the leukocytes either to aggregate in mixed groups of neutrophils and macrophages (Fig 10) between which extensive membranous contact could be observed, or to be segregated into groups consisting mainly of macrophages (Fig. 11). Second, phagocytes, the identity of which was uncertain (Fig 12), were found at this stage. These cells appeared hypertrophied perhaps due to an abundance of degenerating engulfed bacteria and the remnants of other cell organelles (e.g. lysosomes, myelin figures, and residual bodies) within their cytoplasm. Unlike other cells containing bacteria, the surfaces of these cells were often smoother and had fewer membranous elaborations (i.e. projections).

The granules in neutrophils (Fig. 13) recovered from the peritoneum of test fish which lacked engulfed *Yersinia ruckeri* and those from the head kidney neutrophils lacking *Y. ruckeri* (Fig. 14) exhibited a readily observed positive peroxidase reaction which was not seen in either peritoneal or head kidney neutrophils (cytochemical controls) incubated without DAB. These cells were similar in appearance to those described by routine electron microscopy (Figs. 1 to 3); however, the required incubations and treatment with DAB involved in peroxidase staining altered the typical elongate appearance of the striate granules, rendering them more oval to spherical in shape, as well as resulting in overall poor preservation of mitochondrial and other cellular membranes (i.e. plasmalemma, nuclear and vesicular membranes, and those of the endoplasmic reticulum system). In peritoneal neutrophils that had phagocytozed *Y. ruckeri* (Figs. 15 & 16), a less intense peroxidase reaction product was observed for the granules, within phagosomes, and around bacterial cell walls. Macrophages (Fig. 17) with or without engulfed bacteria failed to demonstrate a peroxidase reaction product within their cytoplasm.

DISCUSSION

The fine structural cytology of winter flounder neutrophils observed in this study is in agreement with that found for these types of cells in plaice *Pleuronectes platessa* (Ferguson 1976, MacArthur et al. 1984, 1985). The horseshoe-shaped nuclear configuration observed for winter flounder neutrophils has also been reported for the neutrophils of other teleosts (Bielek 1981, Bodammer 1986, Doggett & Harris 1989, Bodammer et al. 1990). Except for its granules, the cytoplasm of winter flounder neutrophils was unspec-

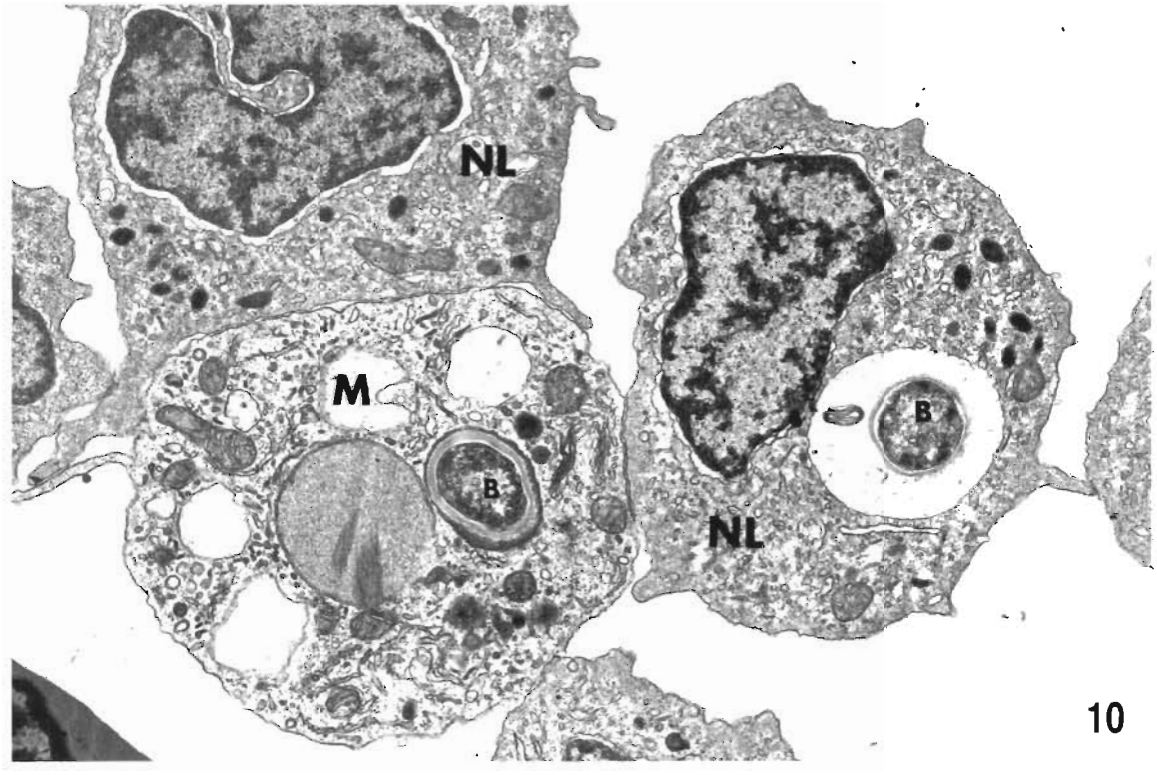


Fig. 10. *Pleuronectes americanus*. Electron micrograph of an aggregate of 2 neutrophils (NL) and a macrophage (M) recovered from the peritoneal cavity 60 min post-injection with STCM containing *Bacillus cereus* B: bacterium 12 000×

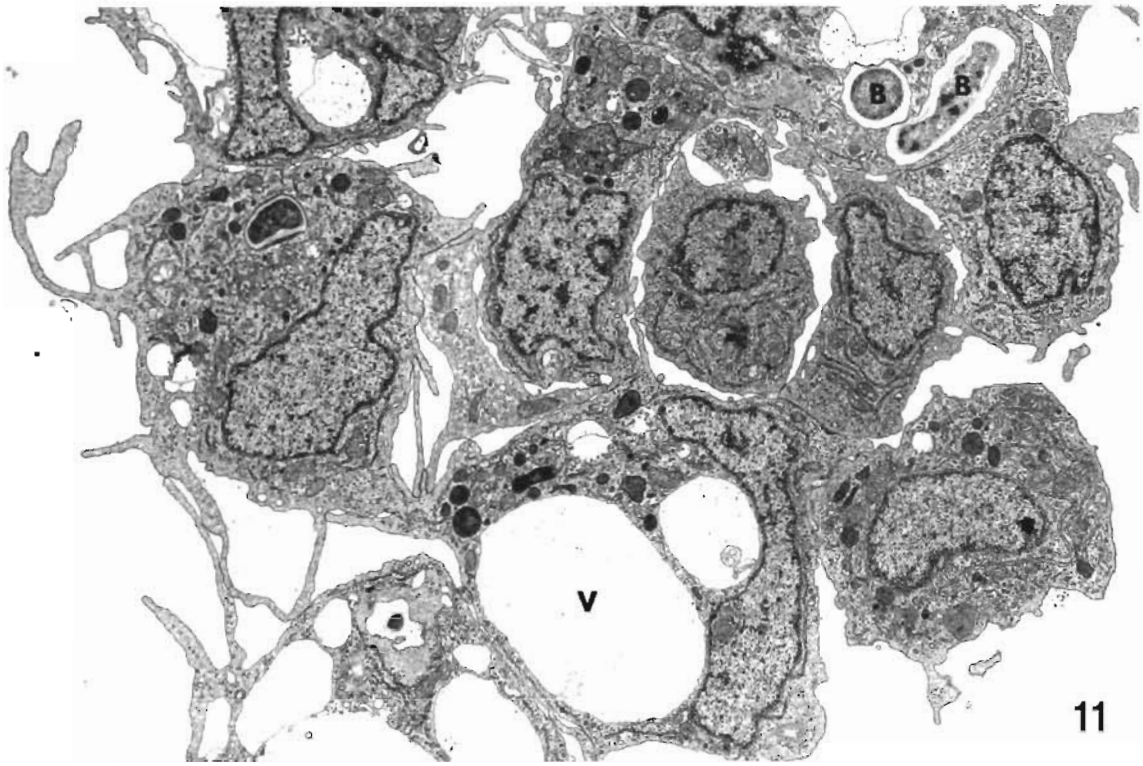


Fig 11 *Pleuronectes americanus* Electron micrograph of part of an aggregate of activated macrophages recovered from the peritoneal cavity 60 min post-injection with STCM containing *Bacillus cereus* Profiles of degenerating bacteria (B) and large vacuoles (V) were observed within the cytoplasm of these cells. 5700×

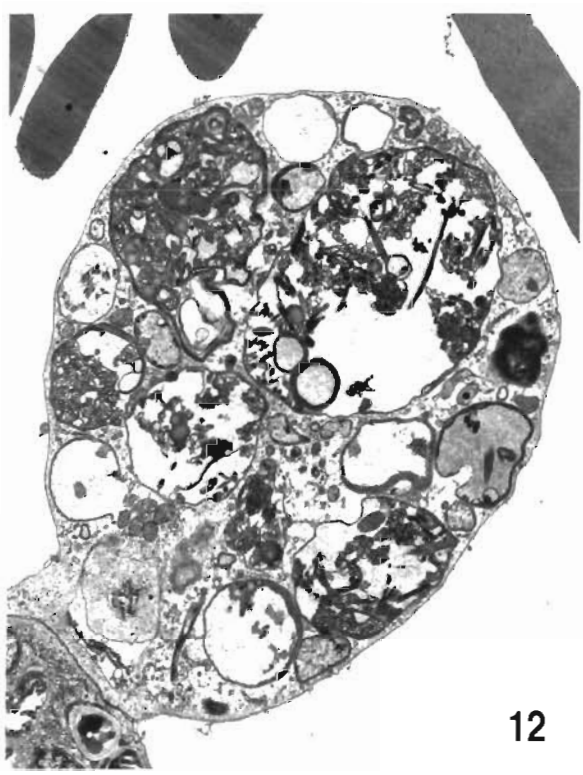


Fig 12. *Pleuronectes americanus.* Electron micrograph of a hypertrophied phagocyte recovered from the peritoneum 60 min post-injection with STCM containing *Bacillus cereus* Cell type could not be identified owing to the large numbers of degenerate elements (e g residual bodies, myelin figures, bacterial cells) found within its cytoplasm 6000×

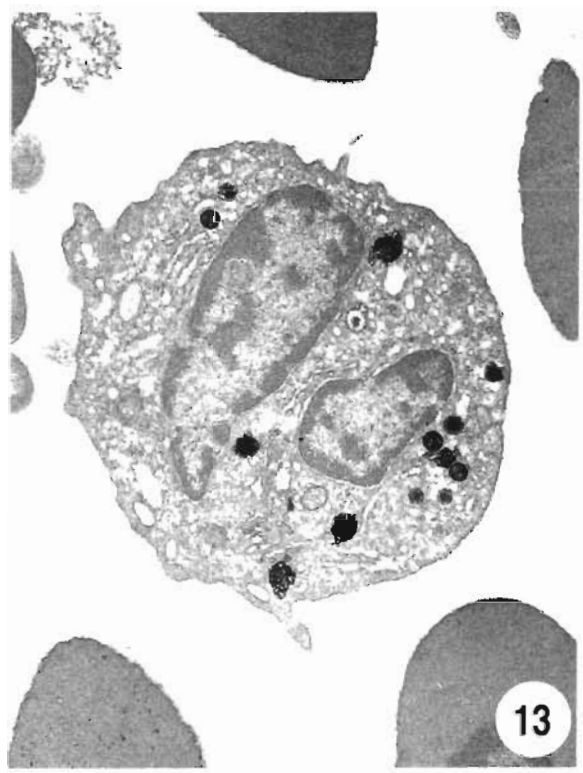


Fig 13 *Pleuronectes americanus.* Electron micrograph of a peroxidase stained neutrophil recovered from the peritoneum 60 min post-injection with EMEM containing *Yersinia ruckeri.* This cell which appears to lack bacteria within its cytoplasm contains a number of peroxidase positive granules. 14 400×

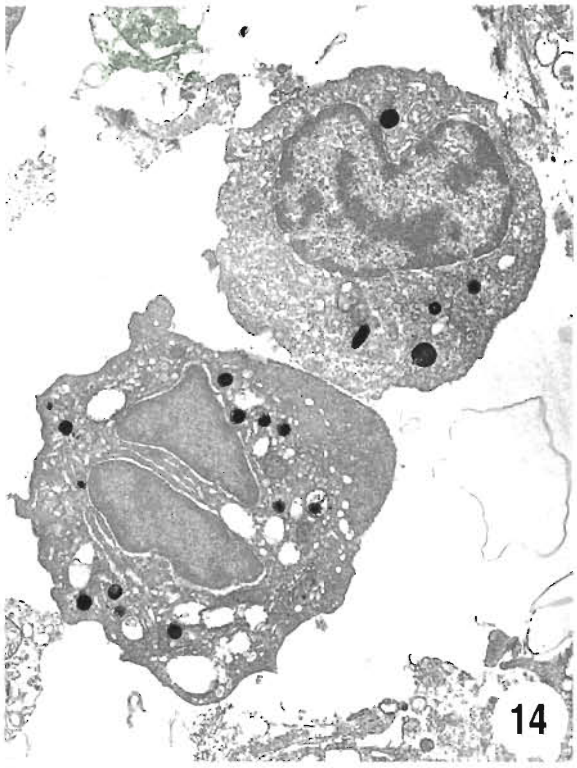
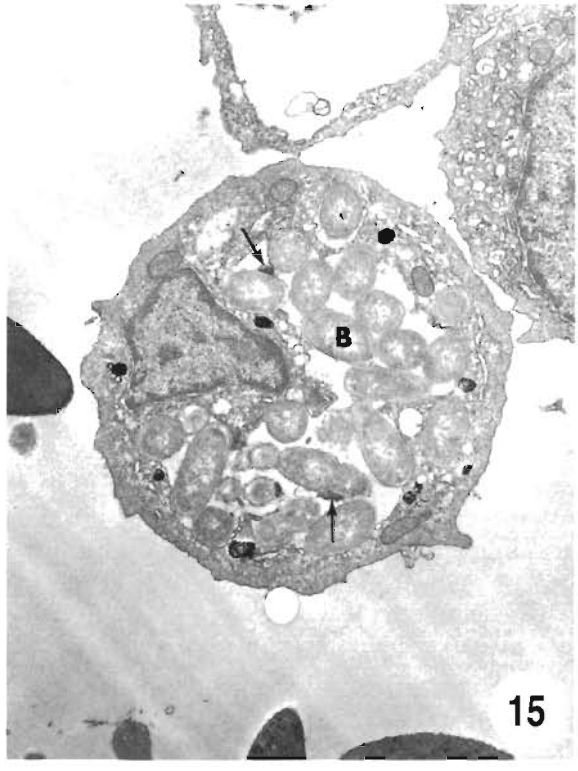
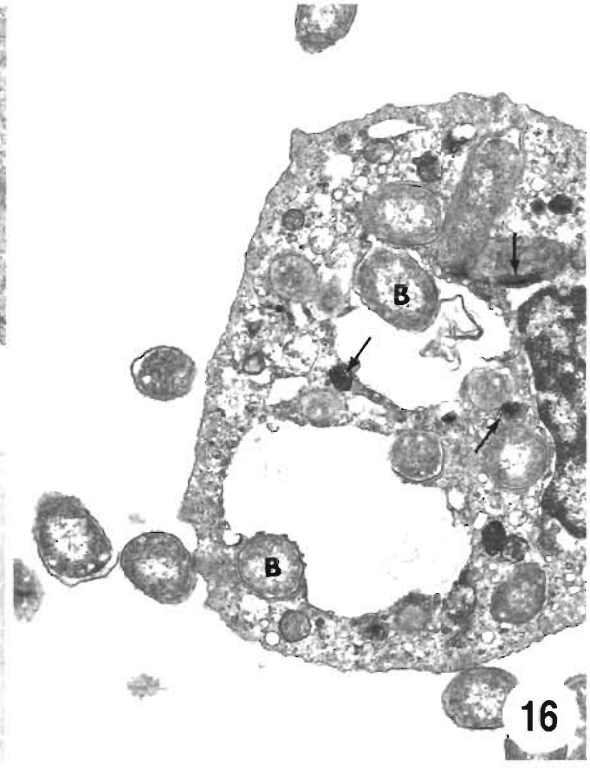


Fig 14 *Pleuronectes americanus*. Electron micrograph of 2 neutrophils from the head kidney of fish that served as positive control for the peroxidase method as applied to neutrophils from the peritoneum of fish exposed to EMEM containing *Yersinia ruckeri* Note the dense peroxidase staining reaction of the granules in these cells compared to that for granules contained in the cell depicted in Fig 13 8400×

tacular, its only other organelles were the numerous tubular profiles of smooth endoplasmic reticulum, glycogen-like particles, a prominent Golgi complex, a modest amount of rough endoplasmic reticulum, and fine filaments in the cytoplasm subjacent to the cell's plasmalemma. A similar description of neutrophil cytoplasm has been provided for the channel catfish *Ictalurus punctatus* (Cannon et al. 1980b) and the striped bass *Morone saxatilis* (Bodammer 1986) Winter flounder neutrophils that appeared to be in an 'activated or stimulated' state, particularly those that had taken up *Bacillus cereus* (Fig. 9), demonstrated numerous lamellipodial (ruffles) and pseudopodial projections on their surfaces similar in appearance to those of stimulated neutrophils in mammals immediately before and/or during phagocytosis (Berlin & Oliver 1978, Moore et





Figs. 15 & 16. *Pleuronectes americanus* Electron micrographs of peritoneal neutrophils recovered from from fish 60 min post-injection with EMEM containing *Yersinia ruckeri* These bacteria-laden cells demonstrated a weaker positive reaction for peroxidase within their granules than did peritoneal neutrophils lacking bacteria (see Fig 13) or head kidney neutrophils lacking bacteria (see Fig. 14). In addition, a modest peroxidase reaction product was observed at the level of their phagosomes (arrows) containing the test bacterium (B) Fig. 15, 9230×, Fig 16, 14 400×

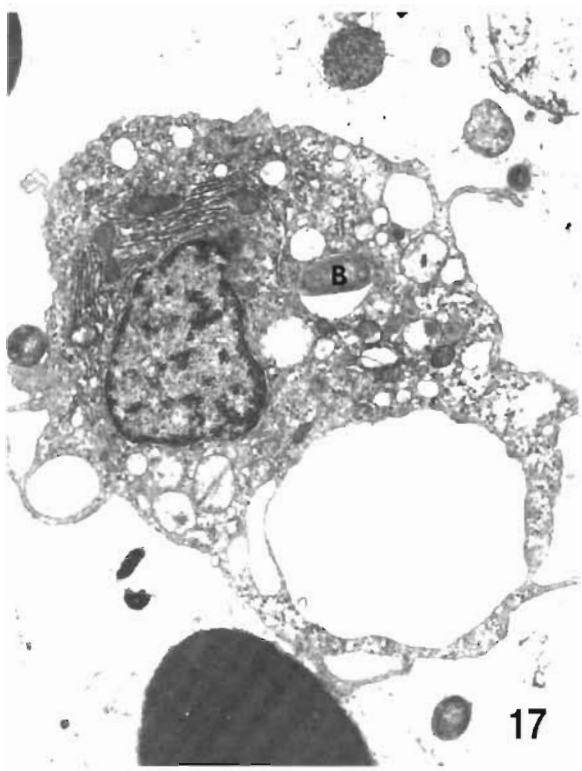


Fig. 17. *Pleuronectes americanus*. Electron micrograph of a macrophage recovered from the peritoneum 60 min post-injection with EMEM containing *Yersinia ruckeri* and stained for peroxidase. The cell was observed to contain a test bacterium (B) but demonstrated no evidence of a peroxidase reaction product within its cytoplasm. 9120×

al. 1978, MacRae et al. 1980, Hoffstein et al. 1982). Membrane elaborations associated with the surface of 'activated or stimulated' neutrophils were not seen on cells that lacked intracellular bacteria; such cells appeared to be quiescent (see Figs. 1 to 3). Although we were unable to identify the cell lineage of the hypertrophied smooth-surfaced phagocytes (Fig. 12), they were morphologically similar to the scanning electron microscopic images of bacteria-laden neutrophils of humans after the *in vitro* uptake of large numbers of *Escherichia coli* (MacRae et al. 1980).

The striate or filamentous-appearing granules we observed in winter flounder neutrophils were similar in shape and substructure to those observed in the heterophils of plaice (Ferguson 1976) and other teleost species (Cannon et al. 1980b, Bodammer 1986, Hine et al. 1986, Roubal 1986, Suzuki 1986, Hine & Wain 1988, Doggett & Harris 1989, Fujimaka & Isoda 1990). As mentioned earlier, because of the orientation of most of the neutrophil granules our images failed to reveal their microtubular nature most of the time (Figs. 4 & 5); thus, they most often resembled the striate or filamentous granules described by others.

The positive peroxidase reaction we found in the granules of winter flounder peritoneal and head kidney neutrophils (Figs. 13 & 14) is important in their identification and supports the hypothesis that the neutrophils are capable of killing microbial pathogens. The presence of peroxidase in the bacteria-containing phagosomes of winter flounder neutrophils provides supportive evidence that these cells have microbicidal capability via the H_2O_2-peroxidase-halide system thought to be operant in the neutrophils of other fish species (Nash et al. 1987, Ainsworth 1992). Peroxidase positive granules have also been observed in both developing and mature neutrophils in plaice (Ellis 1976) and in other species of teleosts (Cannon et al. 1980a, Bielek 1981, Suzuki 1984, Bayne 1986, Hine et al. 1986, Doggett et al. 1987, Finco-Kent & Thune 1987, Bodammer et al. 1990). Presently, the only explanation we can provide for the modest peroxidase reaction we observed in the granules or phagosomes (Figs. 15 & 16) of neutrophils containing bacteria was that the discharge of their granules' contents into phagosomes and/or surrounding cytoplasm resulted in enzyme quantities that were below the detection limits of the cytochemical method employed. Peroxidase-bearing phagosomes in teleost neutrophils have been reported in both the rockfish *Sebastes schlegli* and rainbow trout *Oncorhynchus mykiss* by Suzuki (1984) and in channel catfish (Finco-Kent & Thune 1987).

As reviewed by Ainsworth (1992), most observations on the phagocytic ability of neutrophils in teleost fish species have resulted from *in vitro* studies utilizing various inert particles and/or bacteria. *In vivo* observations on the phagocytic behavior of teleost neutrophils have been reported for rainbow trout during the inflammatory response to intramuscular, intraperitoneal, or periorbital injections of heat-killed *Staphylococcus aureus* (Finn & Nielson 1971), in the peripheral blood neutrophils of the sockeye salmon *Oncorhynchus nerka* after engulfment of *Lactobacillus brevis* (Bell 1976), and by systemic neutrophils in tilapia *Oreochromis niloticus* Trewavas that had taken up both attenuated *Aeromonas salmonicida* and colloidal carbon (Doggett & Harris 1989). In *in vivo* studies similar to the one reported here, electron microscopic observations of peritoneal neutrophils from tilapia and carp *Cyprinus carpio* L. have clearly shown them to be phagocytic after 24 h exposure to zymosan particles (Suzuki 1986), and as mentioned before, MacArthur et al. (1984) have observed that neutrophils in plaice engulfed oyster glycogen and *Vibrio alginolyticus* after 24 h. While they have not identified cell type, Noya et al. (1995) have shown that peritoneal granulocytes in the gilthead seabream *Sparus aurata* take up the pathogen *Pasteurella piscicida* within 60 min after i.p. injection. Our observations on the

phagocytic capability of winter flounder peritoneal neutrophils are in good accord with the 3 previously cited studies.

As we have shown, winter flounder peritoneal macrophages phagocytozed both types of test bacteria (Figs. 8, 10, 11 & 17). This is not surprising because recent reviews by Blazer (1991) and Secombes & Fletcher (1992) have indicated that macrophages are consistently observed among various teleosts to be the most efficient type of leukocyte involved in the engulfment of pathogens and necrotic debris resulting from inflammatory responses or other types of degenerative processes (e.g. cellular necrosis or apoptosis). In addition, Daniels (1988) has clearly demonstrated the *in vitro* capability of winter flounder macrophages to engulf and kill *Vibrio alginolyticus* under a variety of culture conditions.

The lamellipodia and pseudopodia we observed in our 30 and 60 min preparations of winter flounder peritoneal macrophages were ultrastructurally similar to those reported for activated trout peritoneal macrophages during the phagocytosis of formalin-killed *Aeromonas salmonicida* (Zelikoff & Enane 1991) and for those of activated murine peritoneal macrophages during the phagocytosis of rabbit erythrocytes and latex spheres (Polliack & Gordon 1975) or sheep red blood cells as reported by Orenstein & Shelton (1977). The cytoplasmic and nuclear features of the macrophages studied herein were comparable to those reported for peritoneal macrophages in plaice (MacArthur et al. 1984) and striped bass (Bodammer 1986).

In summary, we have demonstrated that winter flounder neutrophils and monocytes/macrophages are capable of bacterial engulfment after short periods of exposure to the test organisms within the peritoneal cavity. Although we have not quantified the results of our work nor studied the kinetics in a detailed manner, our results are in keeping with the *in vivo* studies performed by MacArthur et al. (1984) who found that neutrophils were the first cells to phagocytoze marine vibrios in the peritoneal cavity of plaice and that the response of macrophages to the test organism was less rapid. Further, MacArthur et al. (1985) showed that plaice monocytes migrated rapidly (after only 2 h) into the vascular system when stimulated by foreign erythrocytes in the blood stream. Despite the latter results, we were surprised to observe the speed of the phagocytic response of peritoneal neutrophils and macrophages in the winter flounder (it occurred in only 30 min). We intend to fully characterize the kinetics of these and other leukocyte responses in the future. Until further information is available, we must presume that the neutrophils and macrophages studied herein were resident in the peritoneal cavity at the time the test bacteria were introduced.

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