Particle size and traffic of phagocytes between the turbot peritoneal cavity and lymphoid organs

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ABSTRACT

New adjuvants based on microparticles are being developed for use in fish vaccines. The size of the microparticle may affect the immune response generated, as the adjuvant can either be retained at the site of injection or transported to lymphoid organs. The objectives of this study were to evaluate the maximum size of particles that can be exported out of the cavity, to determine the phagocytosis kinetics and to establish the routes whereby particle-containing cells move from the peritoneal cavity after injection. Fish were injected intraperitoneally with fluorescent cyclodextrins or with fluorescent particles of different size (0.1 - 10 µm). Phagocytes containing beads of size 4 µm or larger did not reach lymphoid organs, although some were able to cross the peritoneal mesothelium. The number of free peritoneal neutrophils and macrophage-like cells containing beads peaked at 6 and 24 h respectively, and the numbers then decreased quickly, indicating migration of cells to the peritoneum or other body areas. Migration of cells containing beads mainly occurs through the visceral peritoneum. Those cells were found in the latero-ventral surfaces of the peritoneal folds that connect the visceral organs. Except for some vascularised areas, the surfaces of liver, stomach and intestine were devoid of particle-containing cells. Some cells containing beads were also found attached to the parietal peritoneum, although in lower numbers than in the visceral peritoneum. Those were also found in high numbers in the spleen and kidney 6 h post injection. Because cells containing phagocytosed material quickly become attached to the peritoneum or migrate to lymphoid organs, the immune response generated by a vaccine or by an inflammatory stimulus should probably be evaluated in attached cells as well as in free peritoneal cells.

Keywords: Fish, peritoneum, adjuvant, microparticles, phagocytes, migration

1. Introduction

Vaccination of fish through the intraperitoneal pathway usually generates the strongest and longest-lasting immune responses. Although vaccines containing antigen alone are weakly immunogenic, adjuvants can enhance and also shape antigen-specific immune responses [1]. Several adjuvants have been tested in fish vaccines. Some of these, such as oil emulsions, have been shown to generate long-term immune responses with various antigens [2], but can also cause important lesions in the fish [3,4,5,6]. New adjuvants based on nanospheres, microspheres or microparticles have been developed, and some have induced a good immune response with less damage than associated with oil-based adjuvants [6,7,8,9]. Some oil-based adjuvants, such as those formed by Freund's adjuvant, Montanide ISA51 or ISA720, are used to form water-in-oil emulsions in which aqueous droplets containing the antigen are dispersed in the oily phase [10]. These droplets are of different sizes, and the larger ones cannot be phagocytosed and transported from the peritoneal cavity to lymphoid organs [6], remaining at the site of injection until they have disintegrated. In the case of particulate polymers, the antigen can be entrapped in the polymer, and particle size is important in terms of induction of the immune response [11]. Studies carried out in mammals have shown that mouse antigen-presenting cells can phagocytose poly(D,L-lactic-*co*-glycolic acid) microspheres (ranging from 0.7 - 5 μm in diameter) after intradermal or intraperitoneal injection, although the exact size of the microspheres phagocytosed was not determined [12]. Particle size may affect the efficiency of cellular uptake, the mode of endocytosis and the subsequent efficiency of particle processing throughout the endocytic pathway. Particle size may also affect the type of cells involved in endocytosis, as microspheres as large as 500 nm can be internalized by non-phagocytic cells [13]. Mammalian and fish B cells can phagocytose beads of at least 1 μm in size [14,15], and antigen presentation to T cells is drastically reduced when antigen is not engulfed [15]. For all those reasons, the size of the droplets/particles formed by the adjuvant is an important factor for consideration in designing an antigen delivery system.

Intraperitoneal injection of a vaccine or an inflammatory agent induces bidirectional cell traffic between the peritoneal cavity and lymphoid organs. Some cells migrate to the peritoneal cavity, where cell numbers increase considerably during the first day and then decrease [6,16,17]. The intraperitoneally-administered antigen or

particulate material is endocytosed by cells that can migrate to lymphoid organs such as the kidney and spleen, where the presence of the injected material can be detected within a few hours of injection and be observed for weeks [6,17,18,19,20,21]. However, it is not clear how intraperitoneally administered antigen or particulate material reaches lymphoid organs in fish. In mammals, omental milky spots have been shown to play a role in initial bacterial clearance from the peritoneal cavity [22]. Spleen lymphocytes introduced in the peritoneal cavity migrate rapidly to the omentum [23], although other authors have concluded that the major route of removal of inflammatory cells and fluid from the peritoneal cavity is through diaphragmatic lymphatics [24].

The main aims of the present study were to determine the maximum size of microparticles that can migrate from the peritoneal cavity to lymphoid organs and to establish the routes whereby the cells containing macromolecules or microparticles migrate from the peritoneal cavity to those organs. As well as elucidating these routes, the information obtained may be important for determining which cells (apart from kidney, spleen and free peritoneal cells) should be used to evaluate the early immune response to vaccination.

2. Materials and methods

2.1. Fish

Specimens of the turbot *Scophthalmus maximus* (L.), of approximately 30 g body weight, were obtained from a local fish farm. The fish were maintained in 250-L tanks with aerated recirculated sea water, at 16°C, and were fed daily with commercial pellets. Fish were acclimatized to laboratory conditions for two weeks before the experiments began. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela (Spain). For all procedures, the fish were anaesthetized with benzocaine (50 mg/l). Anaesthetized turbot were killed by cervical dislocation.

2.2. Preparation of the β-cyclodextrins, beads and microparticles

Fluorescent beta-cyclodextrins were prepared following a modification of the method described elsewhere [25, 26]. A solution of 6-monodeoxy-6-monoamino-beta-

cyclodextrin (Sigma-Aldrich, M2314) was prepared at a concentration of 1 mg/mL in 0.1 M Na_2HPO_4 pH 9.0. Fluorescein isothiocyanate (FITC, Sigma-Aldrich) was added to this solution at a concentration of 50 µg/mL, from a stock solution of 1 mg/mL prepared in dimethyl sulphoxide, and incubated for 1 h at room temperature. Unconjugated FITC was removed by ultrafiltration with NMWL Ultracel YM membranes, cut-off 1 kD (Millipore), in a Amicon ultrafiltration cell (Millipore) pressurized with N_2, by adding phosphate buffered saline until no fluorescence was detected in samples of the eluate. Fluorescence of the eluate was measured in a fluorescence reader (Bioteck) at excitation and emission wavelengths of 494 nm and 518 nm, respectively. The fluorescent cyclodextrins used measured between 100-250 nm (measured by scanning electron microscopy) and were diluted in PBS to a concentration of 8,5 mg/ml. Beads, carboxylate-modified polystyrene (diameter 0.1 µm, fluorescent orange, L9904; diameter 0.5 µm, fluorescent green, L2153; and 2 µm, fluorescent red, L3030), microparticles based on polystyrene (3 µm, 79166), microparticles based on melamine resin (4 µm, rhodamine B-marked, 80462), and microparticles based on polystyrene dark red (10 µm, 61946) were obtained from Sigma-Aldrich. The beads (2.5 or 10% stock suspensions) were washed and resuspended in PBS to a concentration of 1%; approximately 1×10^{13} (0,1 µm), 5×10^{11} (0,5 µm), 1×10^{9} (2 µm), 4×10^{8} (3 µm), $1,5 \times 10^{8}$ (4 µm) and 1×10^{7} (10 µm) beads ml^{-1}.

2.3. Fish injection and sample collection

Seven groups of fish (12 fish each) were injected intraperitoneally (i.p.) with 100 µl of PBS containing fluorescent cyclodextrins or with beads or microparticles of different sizes (0.1, 0.5, 2, 3, 4 and 10 µm in diameter). Uninjected fish were used as controls. The fish were injected in the central part of the peritoneal cavity. Three fish in each group were then sampled at 6 h, 1, 3 and 7 days post injection. The peritoneal cavity was washed carefully with cold L-15 medium containing heparin (10U ml^{-1}). The cells obtained were washed twice with L-15 and counted in a haemocytometer. Smears of the cell suspensions were stained with hemacolor (Merck) or diaminobenzidine (Sigma-Aldrich) (for peroxidase activity) and counterstained with haematoxylin, according to [27]. To establish the percentage of phagocytosis, cell smears were mounted in Mowiol and studied at 100X by using both bright-field and

fluorescent microscopy. Two hundred cells were counted per sample and the results are shown as the mean of the phagocytosis activity of peroxidase positive cells (neutrophils) and peroxidase negative cells per group.

The organs in the abdominal cavity were extracted by sectioning the digestive tube at the level of oesophagus and anus. The organs were washed with cold PBS and placed in a Petri dish filled with ice. The ventral and dorsal sides of the digestive system were then observed and photographed in a Leica stereo fluorescence microscope equipped with green, red and orange fluorescence filters. The visceral organs are bound by peritoneal folds, which contain blood vessels surrounded by the pancreas and mesothelium. The visceral peritoneal folds were cut into several pieces with scissors, and the pieces were used for light or scanning electron microscopy studies. The spleen and the anterior kidney were also sectioned and processed for fluorescence and light microscopy. Finally, the parietal surface of the peritoneal cavity was washed carefully with cold PBS, before being examined and photographed in a stereo fluorescence microscope.

2.4. Light and fluorescence microscopy

Pieces of the peritoneal folds containing blood vessels and pancreas, dorsal abdominal wall, intestine, liver, spleen, head kidney or gills of injected fish were fixed in 10% neutral-buffered formalin. Some of the pieces were processed by standard paraffin wax and plastic histology. Sections of wax (5 µm) or plastic (1 µm) embedded samples were stained with haematoxylin and eosin (H&E) or with toluidine blue and examined under light microscopy. Other pieces were maintained at 4^{o}C in the dark for 24 h, before being immersed in 30 % sucrose, sectioned (15 µm) on a cryostat, and examined by fluorescence or confocal microscopy. In the sections examined by fluorescence microscopy, the number of fluorescent beads per field was counted, using the 60X objective, in 3 sections of spleen and kidney from each fish (two fields were counted per section). The results of the counts are shown as the mean number of fluorescent beads per field.

2.5. Scanning electron microscopy

Pieces of intestine and pieces of peritoneal folds containing blood vessels and pancreas were fixed overnight at 4°C in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and postfixed for 1.5 h in 1% osmium tetroxide in the same buffer. The samples were then washed three times in dH_2O, dehydrated in increasing concentrations of acetone, critical point-dried with liquid carbon dioxide, sputter-coated with gold, before finally being viewed and photographed in a Leica scanning electron microscope.

2.6. Statistical analysis

Data were analysed by analysis of variance followed by Duncan's test, with SPSS for MS Windows.

3. Results

3.1. Phagocytosis of beads by free peritoneal cells

Macromolecules, such as β-cyclodextrins and beads of different sizes (0.1, 0.5, 2, 3, 4 and 10 µm in diameter), were used to monitor the migration of cells from the peritoneal cavity to internal organs. The total number of free peritoneal cells peaked at 24 h post-injection in fish injected with 0,5 µm fluorescent beads and at 3 days in fish injected with 2 µm or 4 µm beads. (Fig. 1). Turbot peritoneal cells were able to phagocytose 4 µm but not 10 µm beads (Fig. 2). In the case of fish injected with 0,5 µm beads, the percentage of neutrophils with endocytosed beads peaked at 6 h (about 6 %) and then decreased quickly (Fig. 1). The percentage of peroxidase negative cells with phagocytosed beads peaked at 24 h post-injection (about 14 %) and then decreased quickly. Those cells had mainly a macrophage-like morphology and appeared isolated or forming small groups (Figs. 1, 2). After 3 and 7 days, less than 1% of the whole free peritoneal cell population had phagocytosed beads (Fig. 1), indicating that most of the cells with phagocytosed material had migrated to the peritoneum or to other body areas.

3.2. Distribution of bead-containing cells in the peritoneal cavity

To determine the sites of cell migration in the peritoneal cavity, the distribution of cells with fluorescent material was analysed by stereo fluorescence microscopy. After six hours, and independently of their size, beads containing cells had attached to the mesothelium, mainly on the peritoneal folds located between the visceral organs (Fig. 3A, B, C). The surface of liver, intestine and stomach were devoid of cells with beads, except in some areas where blood vessels appeared on the surface of the organ. In this case, cells containing beads appeared adjacent to those vascularised areas (Fig. 3D). Some cells with beads were also attached to the parietal peritoneum, although in very low numbers. One day post injection, the intensity of fluorescence in the visceral peritoneum increased in all groups and areas with high fluorescence intensity were observed; this effect was particularly obvious in fish injected with 4 µm beads (Figs. 3B and 4A). The number of cells also increased in areas close to blood vessels located on the surface of visceral organs (Fig. 4B). Cells with beads were also dispersed over the parietal peritoneum, although at low concentrations (Fig. 4C). After 3 and 7 days, cells containing beads mainly formed groups located on the lateral-ventral sides of the folds of the visceral peritoneum (Figs. 4D, E, F and 5A). Those cells also appeared beside vascularised areas located on the surface of the digestive tract where the intensity of fluorescence increased in the fish injected with 4 µm beads, but decreased in fish injected with smaller beads (Fig. 5B, C, D). The intensity of fluorescence increased in the parietal peritoneum of fish injected with 4 µm beads (Figs. 4C, 5E), particularly beside the site of injection; however, the increase was not as evident in fish injected with smaller beads.

The surface of the folds of visceral peritoneum was studied by scanning electron microscopy to determine where the cells were attached. The peritoneum of control fish did not contain cells attached to the mesothelium (Fig. 5F). However, peritoneal cells were attached to the mesothelium located on the latero-ventral sides of the peritoneal folds in fish injected with beads (Fig. 6A). Leukocytes were distributed in small groups throughout the peritoneum, usually located in small cavities (Fig. 6B, C). In the areas of contact, the mesothelial cells and the peritoneal cells developed large cytoplasmic extensions (Fig. 6D).

The results indicate that migration of peritoneal cells to lymphoid organs mainly occurs through the visceral peritoneum. For confirmation of this, sections of the peritoneal folds were studied in histological sections. The peritoneal folds are covered by a mesothelium and contain blood vessels and pancreas. We found that control fish

contain very few leucocytes between the pancreatic acini and had peritoneal cells attached to the visceral mesothelium. However, numerous leucocytes were located between the pancreatic acini, beside or attached to the wall of blood vessels, or attached to the mesothelium in fish injected with beads, and the number of these increased considerably 3 and 7 days after injection (Fig. 7A, B), indicating that cells with beads migrated to internal organs through these areas.

3.3. Only cells containing beads smaller than 4 μM were able to reach the internal organs

Fluorescence and light microscopy were used to determine whether cells containing particles could cross the parietal and visceral peritoneum. Cells containing beads smaller than 4 μm were found attached to the visceral mesothelium and also in internal areas of the connective tissue distributed between the pancreatic acini, which are rich in small blood vessels (Fig. 8 A, B, C, D). Phagocytes containing beads of 4 μm were observed attached to the mesothelial cell layer, but not between the pancreatic acini (Fig. 9A). Regarding the parietal endothelium, phagocytes containing beads of 4 μm were able to cross the parietal mesothelium, although they were only observed beside the mesothelial cell layer and not in internal areas of connective tissue (Fig. 9B).

We also examined the presence of latex beads in internal organs, by studying sections of anterior kidney, spleen, liver, intestine and gills. No beads of diameter 4 μm were found in internal organs. Cells containing cyclodextrins or smaller beads were abundant in the spleen and kidney, but were observed only occasionally in the other organs (Fig. 9C, D). Counting the number of beads per field under a 60X objective lens revealed higher numbers of beads in the kidney than in the spleen at 6h, 1 d and 3 d, and it was similar at 7 days post injection (Fig. 10). The number of beads per area of spleen and kidney was higher at 6 h than at 24 h post injection, and the number of beads per field increased thereafter.

4. Discussion

A better understanding of which cell populations remain free, become attached to tissues in the peritoneal cavity or migrate to lymphoid organs after vaccination or during an inflammatory response may be important for evaluating the immune response

to stimuli administered by intraperitoneal injection. Several studies have described the types and the number of free peritoneal cells in the unstimulated peritoneal cavity of fish and the kinetics of the peritoneal cell populations after injection of an inflammatory agent into the peritoneal cavity. Such studies have shown that under inflammatory conditions cells are recruited to the peritoneal cavity [28,29,30,31,32]. In addition, the inflammatory agent injected in the peritoneal cavity appears in the kidney and spleen a few hours/days later, indicating traffic of cells from the cavity to these organs [6]. In the present study, we determined the areas to which cells attach and the routes whereby they move out of the peritoneal cavity after injection of a stimulus. We show that peritoneal cells containing macromolecules or phagocytosed beads became attached to the peritoneal folds that connect the visceral organs. These folds, which are surrounded by the visceral mesothelium, contain blood vessels, connective tissue and pancreas, as also described in other teleost fish species [33]. Phagocytes containing beads were found attached to the mesothelium, but also in regions of connective tissue that surround small vessels, which are located among the pancreatic acini, indicating that migration of cells from the mesothelial surface to those vessels. Cells containing beads were also observed in other sites, usually close to the blood vessels that are attached or penetrate the visceral organs. Although studies carried out in zebrafish have suggested that the lymphatic system extends over the entire intestine and runs alongside the anterior mesenteric artery, the supraintestinal artery and the pancreas [34], it is not known whether any of those vessels belong to the vascular or lymphatic system. Cells also became attached to the parietal peritoneum, but in lower numbers than in the peritoneal folds. In mammals, efflux of peritoneal cells during peritonitis mainly occurs through milky spots found on the omentum and on the sub-diaphragmatic surface to the draining lymph nodes [35]. Milky spots are also involved in the clearance of antigen from the peritoneal cavity [22], but leucocytes can also use this route on resolution of inflammation [24,35]. Omental milky spots are formed by small accumulations of leucocytes, mainly containing macrophages and B cells [36]. We searched for similar structures in the mesenteries of control fish and although we found small accumulations of leukocytes near some of the blood vessels, usually in areas of connective tissue, these areas could not be described as milky spot-like structures. However, there are certain similarities between the migration of cells in mammals and in fish. Although migration of peritoneal turbot cells through the peritoneal folds is common, cell infiltration only occurs in some areas close to the blood vessels, where clusters of leucocytes are

observed by light and scanning electron microscopy. We found that the leucocytes and the mesothelium that these are in contact with were highly activated, as both had large cell projections, which were usually connected, as described in mammals for activated peritoneal mesothelium and leucocytes after injection of an inflammatory agent into the peritoneal cavity [37]. Migration of leucocytes to these areas is probably due to some type of attraction, such as the release of chemokines. As the visceral peritoneum is covered by mesothelial cells, chemoattractants may be released from other types of cells or from the blood, as the cells migrated to regions rich in blood vessels. Injection of an inflammatory agent into the peritoneal cavity generates the release of some chemoattractants that recruit cells to the peritoneal cavity and the production of others that attractant cells to the peritoneal folds. In mammals, traffic occurs through the omentum from the blood to the peritoneal cavity and also from the peritoneal cavity to the omentum [23], and different molecules mediate cell migration in and out of the peritoneal cavity as well as being retained at this site [38]. A similar process probably takes place in the turbot peritoneal cavity, although the molecules involved in the process have not been identified. Moreover, it is not known whether the cells that migrate in and out the peritoneal cavity follow the same route, as alternative routes have also been described in mammals [38].

In concordance with previous studies that have demonstrated the transport of antigen from the peritoneal cavity to the kidney and spleen [21], we have also found that peritoneal cells containing fluorescent beads migrate to lymphoid organs. Cells containing endocytosed beads were found in the kidney and spleen at 6 h post injection. The number of particle-containing cells in those organs, especially in the head kidney, was lower at 24 h than at 6 h and then increased progressively until day seven. It is possible that large numbers of cells containing fluorescent particles were observed in the head kidney at 6 h because cells may be retained temporarily before moving to other parts of the kidney or other organs. We did not identify the cells that transported the fluorescent macromolecules and beads to the lymphoid organs, although peritoneal neutrophils and macrophages with phagocytosed beads gradually disappeared from the peritoneal cavity, suggesting that at least these cells are involved in transportation of the particles. If cells such as B lymphocytes, which have phagocytosis activity in some fish species [14], or if other cell types, as particles as large as 0,5 µm can be internalized by pinocytosis [13], are also involved in bead transportation is unknown. The involvement of macrophages and fish neutrophils in the transport of antigen to lymphoid organs has

been demonstrated in fish [17,39], and studies in mammals have shown that both cell types are involved in the transport of phagocytosed material from the peritoneal cavity to lymph nodes and the spleen [12,40,41]. Studies in mammals have shown that inflammatory macrophages always disappear faster from the peritoneal cavity than non-stimulated macrophages [42]. Although further functional studies are required, the results of the present study also suggest that the activated cells in the peritoneal cavity are not free cells but are attached to the peritoneum. Therefore, the early immune response generated by a vaccine or an inflammatory agent in the peritoneal cavity should probably be evaluated in both free and attached peritoneal cells. The differences in activity of both cell groups will be analyzed in posterior studies.

We also determined the maximum size of particle that can be transported into lymphoid organs, as this may influence the immune response generated. Particles smaller than 4 µm were quickly transported to lymphoid organs, while particles of 4 µm or larger remained in the peritoneal cavity. If an antigen does not reach lymphoid organs in minimum doses, it is immunologically ignored [43]. However, the importance of transportation of the adjuvant to lymphoid organs remains to be determined. The size of the beads is known to be crucial to their adjuvant activity, although reports concerning the size of particle-based adjuvants and the resulting immune responses have been conflicting [44]. Some studies have suggested that small particles used to deliver antigen and adjuvants generate stronger antigen-specific cytotoxic T cell response than larger particles [45]. Others have shown that beads of size 2–8 µm elicited higher antibody titres than larger beads [46], although improved immune response to large particles have also been reported [47]. Other aspects such as antigen release and depot effect of the microparticle may also be important in the immune response. In this sense, poly-(D,L-lactic-co-glycolic) acid microparticles (about 4 µm) were mainly retained at the injection site where had a depot effect in fish injected intramuscularly, in contrast with nanoparticles that were readily transported to internal organs such as the head kidney [48]. For each case, and depending of the characteristics of the beads, it may be necessary to determine the optimal system in terms of type, strength and duration of the immune response generated.

We conclude that injection of particulate material into the peritoneal cavity generates rapid migration of cells containing particles to lymphoid organs. Cells migrate through the parietal and visceral peritoneum, although the preferred sites for cell migration appear to be the folds of visceral peritoneum located between visceral organs,

probably because of the higher vascularization of the areas. Cells containing phagocytosed material became attached to the peritoneum or migrated to internal organs in a short period of time, and therefore the inflammatory/immune responses should probably be analysed in attached cells as well as free peritoneal cells. Finally, the fact that only particles smaller than 4 µm are able to leave the peritoneal cavity should be taken into account when designing microparticles as adjuvant in turbot, and probably in other fish species.

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Figures

Fig. 1. Total number of peritoneal exudate cells (Total), total number of peroxidase negative (perox-) and positive (perox+) cells, and number of peritoneal cells containing phagocytosed beads at 2, 24, 72 and 168 h after i.p. injection with 0.5 µm beads. In brackets, the percentages of perox- (green), perox+ (red) cells and the percentages of perox- (purple) and perox+ (blue) cells with phagocytosed beads are shown.

Fig. 2. Peritoneal exudate cells of fish injected with beads of diameter (µm) (A) 0.5, (B) 4, (C) 2 and (D) 4; at (A) 6 h , (B, C) 1 day, and (D) 3 days after injection. A. Group of macrophages (arrow) containing numerous phagocytosed beads of size 0.5 µm. B, C. Macrophages containing 4 and 2 µm beads (arrows) surrounded by a group of peroxidase positive neutrophils. D. Peroxidase positive neutrophil (arrowhead) containing phagocytosed 4 µm beads. A, B, and D: peroxidase and haematoxylin staining. C: Merging of fluorescence and peroxidase and haematoxylin staining.

Fig. 3. Stereo fluorescence microscope images of fish injected with fluorescent beads at 6 h post injection. Fluorescent peritoneal folds (arrows) that attach visceral organs (ventral side) in fish injected with (A) 0.5 µm or with (B) 4 µm beads . C) Detail of B, showing the cells with fluorescent 4 µm beads located on one side of the peritoneal fold (arrow). D) Cells containing fluorescent 4 µm beads (arrow) located on each side of a blood vessel (V) at the surface of the stomach.

Fig. 4. Stereo fluorescence microscope images of fish injected with fluorescent beads at (A, B, C) 24 h and (D, E, F) 72 h post injection. A) Peritoneal folds of fish injected with 4 µm beads showing fluorescence (arrowheads). Intestine (I), Rectum (R). The fluorescence was more intense in some areas (arrows). B) Blood vessels (V) surrounded by numerous cells containing 4 µm beads (arrowheads). C) Parietal peritoneum with a few cells containing 4 µm beads (arrow). D) Peritoneal folds (arrows) of fish injected with 0.5 µm beads. E) Detail of D, showing groups of cells containing 0.5 µm fluorescent beads that are located on the lateral sides of the folds. F) Fluorescent peritoneal folds (arrow) located on top of the pyloric caeca (C). Fluorescent cells containing 4 µm beads can also be observed on the sides of blood vessels (arrowhead).

Fig. 5. Stereo fluorescence microscope images of fish injected with fluorescent beads at (C) 3 and (A, B, D, E) 7 days post injection. A) Peritoneal folds showing groups of phagocytes containing 4 µm beads (arrow) located on the lateral sides of the folds. B, C) Small groups of leucocytes containing 0.5 µm fluorescent beads (arrow) beside blood vessels. D) Blood vessel surrounded by a large number of phagocytes containing 4 µm beads (arrow). E) Parietal peritoneum showing numerous cells containing 4 µm beads beside the site of injection. The density of cells in other areas of the parietal peritoneum was much lower. F) Intestine (I) and peritoneal fold (P) of control fish (not injected) observed by scanning electron microscopy.

Fig. 6. Scanning electron microscopy of peritoneal folds 3 days after injection with 0.5 µm beads. A) Groups of cells with beads were observed mainly on the lateroventral

sides of the peritoneal folds (arrow). B, C) Cells were mainly located in small invaginations of the peritoneal fold and attached to the mesothelium. D) The mesothelial cells and the phagocytes developed large cytoplasmic extensions that were in contact with each other (arrows).

Fig. 7. Semithin sections of a peritoneal fold (A) 3 and (B) 7 days after injection with 2 µm beads, showing the mesothelium (arrows), the pancreatic acini (Pa) and the blood vessels (V). Numerous peritoneal cells (Pe) were attached to the mesothelium. Leucocytes were also located between the acini and surrounding the blood vessels.

Fig. 8. Fluorescence microscopy sections of the peritoneal folds, 3 days after injection with 0.5 µm beads (A, B). In the insets (C, D), cells containing phagocytosed fluorescent beads can be observed on the surface of the mesothelium and also internally, among pancreatic acini and near blood vessels.

Fig. 9. A) Fluorescence microscopy section of the peritoneal folds 3 days after injection with 4 µm beads. Beads were observed on the surface of mesothelim but not internally between the pancreatic acini. B) Light microscopy section of the abdominal wall showing that two 4 µm beads (white arrow) had crossed the mesothelium (black arrow), at 3 days post injection. Confocal microscopy images of (C) kidney sections showing numerous cells containing endocytosed cyclodextrins (arrow), and (D) phagocytes containing 0.5 µm beads (arrow), 7 days post injection.

Fig. 10. Number of fluorescent latex beads per field in the kidney and spleen of fish injected with 0.5 µm beads. *Significant difference between kidney and spleen.