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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 microparticles 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. These cells were found on 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. Such cells 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.

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 [3–6]. 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 [6–9]. 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 to 5 μm in diameter) after intradermal or intraperitoneal injection, although the exact size of the microspheres phagocytosed was not determined

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[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 of these 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 can be observed during several weeks [6,17–21]. 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 start of the experiments. 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 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 measured between 100 and 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, 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), 1.5×10^{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). Non injected 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 (10 U 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 phagocytic activity, cell smears were mounted in Mowiol and examined at 100×, by both bright-field and fluorescent microscopy. Two hundred cells were counted per sample and the results are shown as the mean number and percentage of peroxidase positive (neutrophils) and peroxidase negative cells with phagocytosed beads 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 °C in the dark for 24 h, before being immersed in 30% sucrose, sectioned (15 μm) on a cryostat,