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Vaccine-induced modulation of gene expression in turbot peritoneal cells. A microarray approach

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ABSTRACT

We used a microarray approach to examine changes in gene expression in turbot peritoneal cells after injection of the fish with vaccines containing the ciliate parasite *Philasterides dicentrarchi* as antigen and one of the following adjuvants: chitosan-PVMMA microspheres, Freundís complete adjuvant, aluminium hydroxide gel or Matrix-Q (Isconova, Sweden). We identified 374 genes that were differentially expressed in all groups of fish. Forty-two genes related to tight junctions and focal adhesions and/or actin cytoskeleton were differentially expressed in free peritoneal cells. The profound changes in gene expression related to cell adherence and cytoskeleton may be associated with cell migration and also with the formation of cell-vaccine masses and their attachment to the peritoneal wall. Thirty-five genes related to apoptosis were differentially expressed. Although most of the proteins coded by these genes have a proapoptotic effect, others are antiapoptotic, indicating that both types of signals occur in peritoneal leukocytes of vaccinated fish. Interestingly, many of the genes related to lymphocytes and lymphocyte activity were downregulated in the groups injected with vaccine. We also observed decreased expression of genes related to antigen presentation, suggesting that macrophages (which were abundant in the peritoneal cavity after vaccination) did not express these during the early inflammatory response in the peritoneal cavity. Finally, several genes that participate in the inflammatory response were differentially expressed, and most participated in resolution of inflammation, indicating that an M2 macrophage response is generated in the peritoneal cavity of fish one day post vaccination.

1. Introduction

Injection of vaccines is the method that results in the best immune response and protection in fish (Lillehaug, 2014). Although vaccines can be administered via the intramuscular or intraperitoneal route, the latter is the most commonly used. Antigen by itself is usually poorly immunogenic and an adjuvant must be added to enhance the antigenicity (Tafalla et al., 2013). Most studies in fish injected with oil-based vaccines have evaluated the immune response generated (mainly by measuring the serum IgM levels) and the protection induced after experimental challenge. For example, in rainbow trout inoculated with a *Yersinia ruckeri* bacterin and the adjuvant Montanide™ ISA 763 A VG (Seppic), significantly enhanced serum IgM levels, increased numbers of IgM^+ cells in the spleen and significant upregulation of several immune genes in spleen, head kidney and liver were observed (Jaafar et al., 2015). Analysis of the immune response in salmonids suggests simultaneous expression of Th1, Th2, and Treg cytokines after administration of oil-based vaccines, with no clearly predominant response (Mutoloki et al., 2010; Kumari et al., 2013; Jaafar et al., 2015). Although aluminium-containing adjuvants are widely used in mammals, they are less commonly used in fish vaccines, probably because the immune response is narrower than that produced by the more commonly used oil-based adjuvants (Jiao et al., 2010). Very little is known about the immune responses generated by aluminium hydroxide adjuvants in fish, except in

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relation to the antibody levels generated and the protection induced. Although aluminium hydroxide-based adjuvants are generally considered to prime Th2-type immune responses preferentially, more recent studies in mammals suggest that aluminium hydroxide-based adjuvants can enhance both Th1 and Th2 cellular responses, depending on the vaccination route (He et al., 2015). Several vaccines containing microspheres have been shown to induce activation of innate and adaptive immune responses in fish (Behera and Swain, 2012; Harikrishnan et al., 2012; Leon-Rodriguez et al., 2012, 2013), although there is no information about how these vaccines affect the activity of peritoneal cells or cells located in lymphoid organs. It is not known what takes place in the peritoneal cavity of fish after administration of a vaccine. Vaccination induces expression of inflammatory genes in lymphoid organs (Fredriksen et al., 2011; Dan et al., 2013) and generation of Th1/Th2/Th17 immune responses has been suggested to occur; however, more sensitive tools are required to help us understand the type of response produced and thus enable more complex analysis of the immune response, comparable to that carried out in mammals.

Vaccines, particularly those containing oil-based adjuvants, can cause important lesions in the peritoneal cavity (Noia et al., 2014). The lesions, which include granulomas as well as adhesions between internal organs or between the organs and the peritoneal wall, vary depending on the adjuvant. Many of the free cells in the peritoneal cavity tend to adhere to the vaccine and thus form masses that become attached to the peritoneal wall. The cell-vaccine masses contain macrophage-like cells and apoptotic cells (Noia et al., 2014). Strong expression of genes related to cell–cell or cell-matrix adhesion and cell–cell junctions and also of genes related to actin cytoskeleton must occur in order to generate cell-vaccine masses. Differentiation, polarization, migration and adhesion processes are affected by the role of the cytoskeleton in innate immunity and cellular self-defence (Mostowy and Shenoy, 2015). In the present study, we used an oligo-microarray containing 43,803 spots and which is rich in genes involved in immunity and reproduction, but also in genes associated with cell adhesion and cytoskeleton (Ribas et al., 2013), to analyse the early cell response in the peritoneal cavity after vaccination. This is the first study of this type carried out in fish.

2. Materials and methods

2.1. Fish

Specimens of 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 and recirculated sea water held 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 hydrochloride (50 mg/l) and killed by pithing.

2.2. Philasterides dicentrarchi: culture and preparation of membrane antigen

Philasterides dicentrarchi (isolate C1) was obtained from ascitic fluid of naturally infected turbot and maintained and cultured in the laboratory, as previously described (Piazzon et al., 2008). The membrane fraction of the ciliate was obtained as described in Budiño et al. (2012). Briefly, the ciliates (1.5×10^7) were resuspended in HEPES buffer containing dibucaine and incubated for 15 min at room temperature (to induce deciliation). After verification (by microscopic examination) that deciliation had occurred, the parasites were washed three times by centrifugation for 5 min at 600*g* in PBS at 4 °C. The pellet containing the ciliates was then resuspended in PBS with 0.25 M sucrose, disrupted ultrasonically and centrifuged at 8.000*g* for 5 min. The pellet was dissolved in 3 ml of HEPES buffer and added to a tube containing a sucrose gradient (0.5 M, 1 M and 0.15 M). After centrifugation at 6.000*g*, the 0.1 M sucrose phase was removed and centrifuged at 11,000*g* for 20 min. Finally, the pellet was resuspended in HEPES buffer with 0.5% Triton X-114 for 15 h at 0 °C, to solubilise the membrane proteins. The suspension was centrifuged at 8.000*g* for 10 min, and the supernatant was then concentrated by amicon ultrafiltration with 1 kDa nominal molecular weight limit membranes (Millipore). The concentration of protein in the samples was determined by the Bradford method (Bio-Rad Protein Assay kit: Bio-Rad Laboratories, Germany), with BSA (Sigma–Aldrich, Spain) as standard. *Philasterides dicentrarchi* membrane proteins (10 μg) were separated by SDS-PAGE on 10% linear polyacrylamide gels (Fig. S1), as previously described (Budiño et al., 2012).

2.3. Vaccine preparation and administration

Six groups of fish (in duplicate, 114 fish in total) were used. The control group (n = 24) was injected intraperitoneally (i.p.) with 0.1 ml of phosphate-buffered saline (PBS). The remaining groups (n = 18 fish per group) were injected i.p. with 230 μg of antigen in PBS, with 5 mg of microspheres containing 230 μg of covalently linked antigen to their surface in PBS (Leon-Rodriguez et al., 2012; Noia et al., 2014), or with a mixture of antigen and one of the following adjuvants (1:1, v:v): Freund's complete adjuvant (FCA), aluminium hydroxide gel (Alhydrogel, Sigma) or Matrix-Q (Isconova, Sweden) (5 μg/fish). Four of the fish in each of the duplicate control group and three of the fish in each of the other duplicate groups were then sampled at 1, 3 and 5 days after injection.

To determine the effects of vaccination on serum antibody levels, six groups of fish (each n = 10) were injected intraperitoneally (ip) on days 0 and 30 with 0.1 ml of PBS, membrane antigen, or antigen and one of the following adjuvants: microspheres, Matrix-Q, Alhydrogel or Freund's, as indicated above. The water temperature was 17 °C. One month after the second dose, the serum antibody levels were determined in immunized fish. Blood samples for antibody analysis were obtained by caudal vein puncture and were allowed to clot for 16 h at 4 °C. The samples were then centrifuged and the serum was collected. The serum antibody levels were determined by double indirect enzyme-linked immunosorbent assay (ELISA), as described by Piazzon et al. (2008).

2.4. Isolation of peritoneal cells

Fish were bled by caudal vein puncture to prevent the presence of erythrocytes in the peritoneal cavity. The peritoneal cavity was then washed carefully with 5 ml of cold PBS and the cells were counted with a haemocytometer. Some cell samples were also smeared onto glass slides for staining with hemacolor (Merck) or diaminobenzidine (Sigma–Aldrich) (for peroxidase activity) and counterstained with haematoxylin, according to Kiernan (1981). Finally, the cell suspensions were washed twice with cold PBS by centrifugation at 300*g* for 5 min, and the tubes containing the pellets were frozen in liquid nitrogen and held at −80 °C until RNA extraction.

2.5. RNA extractions and microarray hybridizations

The stimulated cells were pelleted, and total RNA was extracted using the RNAeasy Mini kit (Qiagen) according to the manufacturer's recommendations. All extractions were performed by