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Comparison between intestinal and non-mucosal immune functions of rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

Since mucosal surfaces represent major portals of entry for pathogens, its associated immune system is important to protect the organism. In this paper, we compared at the cellular and molecular levels intestinal leukocyte suspensions with their head kidney (HK) or peripheral blood (PBL) counterparts to highlight characteristics of intestinal immune functions in healthy rainbow trout. These studies show that intestinal phagocytes are less activated by yeast cells but when they are activated they can ingest as many yeast cells as their HK counterparts. A natural cytotoxic activity could be detected which is twice higher in intestinal than in HK leukocyte preparations. This natural cytotoxic activity is correlated with the expression of transcripts encoding the natural killer enhancement factor (NKEF). Intestinal leukocytes did not respond to an *in vitro* mitogenic stimulation performed under classical culture conditions. And finally, a high expression of CD8α transcripts was observed in gut leukocyte preparations, suggesting that the intestine could contain a high proportion of T cells expressing the αα homodimeric form of CD8. This kind of comparison on nonimmunized fish provides better knowledge on basal immune functions in the intestine to, analyze later on, immune responses induced by an antigenic stimulation.

1. Introduction

The intestinal mucosa is constantly in contact with a wide variety of antigens, including potential pathogens and food antigens present in the lumen. The large surface of this mucosa, further amplified by the numerous villi and microvilli in mammals or by mucosal plicae in fish, increases the risk of pathogen entry thereby highlighting the importance of the immune system associated to the intestinal mucosa. Histological studies have identified this immunological tissue in teleost fish [1–4]. Although it does not contain lymphoid structures such as mammalian germinal centers [5], it is composed of a diffuse population of leukocytes comprising phagocytes, natural cytotoxic cells and lymphocytes present in the epithelium and the lamina propria of the fish intestine [6–10]. Several authors reported phagocytic activity of rainbow trout, *Oncorhynchus mykiss*, intestinal macrophages against fluorescent latex microspheres, yeast and radioactively labeled live or inactivated bacteria *in vitro* [11–13]. McMillan and Secombes [8] detected a nonspecific cytotoxic response of intestinal leukocytes against the EL4 mouse thymoma cell line. Zhang et al. [14] demonstrated that rainbow trout express NKEF, a factor enhancing the action of natural killer cells in mammals. Furthermore, it was shown that fish intestine contains a large population of T cells as shown by the expression of specific T cells markers [15,16], most of them expressing CD8 [16]. CD8 can be homodimers of two alpha chains or heterodimers of one alpha and one beta chain. In mammals, 90% of circulating T lymphocytes express the heterodimeric forms of CD8 whereas homodimeric CD8 molecules are predominantly expressed by natural killer and intraepithelial lymphocytes (IEL). As in mammals, trout CD8 is a dimeric transmembrane glycoprotein [17]. Moreover, Wang et al. and Takizawa et al. [18,19] recently demonstrated the existence of the T-bet and GATA3 transcription factors and the IL4/13A cytokine in rainbow trout which suggest the presence of Th1 and Th2 responses. The discovery of IgT, an immunoglobulin isotype associated to the intestinal mucosa of rainbow trout [20,21], confirms the presence of B cells in this tissue. Furthermore, molecular investigations reported constitutive expressions of inflammatory cytokines, IL-1β, TNF-α and IL-8, in the

Abbreviations: NKEF, natural killer enhancing factor; HK, head kidney; IEL, intraepithelial lymphocyte; NCC, natural cytotoxic cell; PBL, peripheral blood leukocyte; AUC, area under the curve.

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intestine of rainbow trout [22–25]. These cytokines have properties similar to the ones of their mammalian orthologs [26]. Based on this similarity, it could be hypothesized that resident cells of fish intestinal mucosa could modulate local immunity via the secretion of various cytokines, as seen in mammals [27].

In this study, we used cellular and molecular approaches to study rainbow trout's intestinal immune functions. Based on prior knowledge of the rainbow trout systemic immune response, we studied major functions linked to actors of the innate and adaptive components of the immune system at the level of the intestine and compared them with results obtained at the level of HK or peripheral blood. For the innate immunity, we studied the oxidative burst, the phagocytic and cytotoxic activities in relation to the expression of the NKEF gene and the expression of pro-inflammatory cytokine (IL-1β, TNF-α and IL-8) transcripts. For the adaptive immunity, we studied lymphocyte proliferative responses, apoptosis, and the expression of CD3, CD8α, CD8β, GATA3, T-bet, IL4/13A, Pax5, IgM and IgT heavy chain transcripts. We believe that, in the future, this kind of comparative study is interesting to analyze fish immune responses induced by an antigenic stimulation.

2. Materials and methods

2.1. Animals

Female rainbow trout (*O. mykiss*) of body weight ranging from 130 to 230 g were raised from the egg stage at the Research Centre for Animal Nutrition & Health of DSM Nutritional Products in Village-Neuf, France. Animals were treated in accordance with the national legislation and the European community directive (86/609/EEC). Fish were maintained in 250 L sub-square tanks, part of a recirculating unit supplied with tap water at 15 °C ± 1 °C with partial renewal. Maximum biomass per tank was 25 kg/m^3. Fish were fed with a standard trout diet.

2.2. Anesthesia and dissection

Trout were anesthetized with MS222 (Tricaine methane sulfonate MS222, 100 g/L; Thomson, Ref: Vm 01360/4000) and euthanized by a blow to the head. Blood samples were taken from anesthetized fish with a heparinised syringe. Fish were bled and ventrally dissected to remove the entire gastrointestinal tract and the HK. After being carefully unrolled, the portion of the intestine from the pyloric caeca to the anus was cleared from its perivisceral fat, incised in its length, and feces and undigested food were washed off. Tissues were then kept in culture medium (RPMI 1640, Sigma, Ref. R7638), on ice until further processing. All experiments were performed using four to twelve individuals.

2.3. Cell isolation

2.3.1. Isolation of intestinal leukocytes

Intestinal leukocytes were isolated following the chemical and enzymatic method of Salinas et al. [28] and Davidson et al. [11] except that all steps were performed at 4 °C and all reagents were diluted in culture medium. Gut samples from individual fish were washed twice in fresh culture medium and cut into small pieces in 15 mL of DTT solution (0.145 mg/mL, Sigma, Ref. D5545) + EDTA (0.37 mg/mL, Sigma, Ref. E-9884). After 10 min of incubation at 22 °C with gentle agitation, supernatants were removed and samples were washed three times with fresh culture medium before being incubated for 60 min at 22 °C under vigorous agitation in 25 mL of medium containing collagenase type II (0.37 mg/mL, Sigma, Ref. C6885), DNAse I (0.05 mg/mL, Sigma, Ref. DN-25), fish serum (0.5%, Interchim, Ref. N82800F), penicillin and streptomycin (25 units/mL + 25 μg/mL respectively, Sigma, Ref. P0781). Cells contained in the supernatant were collected by filtration of the digested samples through a 100 μm nylon mesh. Tissues remaining in the filtration mesh were further grinded and rinsed with 5 mL of culture medium. Cells collected in the culture medium after this grinding step, were added to those present in the supernatant to maximize cell recovery. These cell suspensions were centrifuged (10 min, 488 *g*). The pellets were resuspended in 5 mL of culture medium and passed through a 10 mL syringe filled with 2.5 g of nylon wool. Columns were rinsed with 5 mL of culture medium and drained to obtain the final cell suspensions that were kept at 4 °C until further processed (density gradient). During the optimization of this protocol we determined its impact on cellular profile and viability using flow cytometry. Moreover, after each isolation step, the viability of our cells was determined with trypan blue exclusion test. Cell suspensions did always present viability above 90%.

2.3.2. Isolation of head kidney leukocytes

The HK from individual fish was crushed through a metallic mesh in 5 mL of culture medium and filtered through a 30 μm nylon mesh, rinsed with 10 mL of culture medium and kept at 4 °C until further processed (density gradient).

2.3.3. Density gradient centrifugation

The Ficoll solution (10–15 mL Ficoll-Paque PLUS, 1.077 g/mL Amersham, Ref. 17-1440-03) was carefully layered under the diluted cell suspensions obtained from intestine, HK or peripheral blood (1 mL blood in 15 mL of culture medium). After centrifugation (40 min, 1356 *g*, 4 °C) the interface between the Ficoll solution and the sample was collected. Leukocyte-enriched suspensions were centrifuged (10 min, 488 *g*, 4 °C) and filtered through a 30 μm nylon mesh. This operation was repeated a second time for the HK but not for the intestine because of the low yield of cell isolation. Cells were resuspended in 1 mL of culture medium, counted using a Thoma hemacytometer and their viability evaluated using Trypan blue. May–Grünwald/Giemsa cytospin staining of the obtained intestinal, HK and peripheral blood leukocyte suspensions were performed to determine the percentages of the different cell types present in each suspension of leukocytes. In all cases, we counted 200 cells in each of 4 cytospins.

2.4. Analysis of cellular parameters

2.4.1. Oxidative burst

Two methods were used to study oxidative burst, the first one was based on chemiluminescence and the second one on flow cytometry. In each case suspensions of intestine or HK leukocytes were adjusted at 1×10^7 cells/mL in fresh RPMI 1640 culture medium.

Chemiluminescence was performed using a modification of the Scott & Klesius [29] and the Stave et al. [30] method. Briefly, 100 μL of cell suspension were added to each well of a white microplate (VWR/Greiner, Ref. 736-0228). Working solutions of luminol (Sigma, Ref. A8511) and zymosan (Sigma, Ref. Z4250) opsonised with chicken serum were placed in the reservoirs of the luminometer (Tristar® LB941, Berthold Technologies) for injection of 50 μL of luminol and 100 μL of zymosan in each well. All reagents were diluted in Cortland solution (7.25 g NaCl, 0.23 g $CaCl_2 \cdot H_2O$, 0.38 g KCl, 0.41 g $NaH_2PO_4 \cdot H_2O$, 1 g $NaHCO_3$, 0.23 g $MgSO_4 \cdot 7H_2O$, 1 g glucose/1 L H_2O, Sigma). Light emissions were measured over 180 min, generating a kinetic curve for each sample. The reaction started as soon as zymosan was added. The proportion of phagocytic cells present in each well was determined by flow cytometry