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Intestinal T cells of *Dicentrarchus labrax* (L.): Gene expression and functional studies

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ABSTRACT

Cellular and molecular data have evidenced a gut-associated lymphoid tissue in a variety of teleost species, abundantly containing T cells, whose origin, selection and functions are still unclear. This study reports *CD4*, *CD8-α*, *MHCI-α*, *MHCII-β*, *rag-1* and *TCR-β* gene transcription along the intestine (anterior, middle and posterior segments) and in the thymus of one year-old *Dicentrarchus labrax* (L.).

Real-time PCR findings depicted a main role of the thymus in T-cell development, but also *rag-1* and *CD8-α* transcripts are detected in the intestine, having significant expression in the posterior segment. In the whole intestine *TCR-β* and *CD8-α* exceeded *CD4* transcripts. RNA ISH confirmed these data and detailed that mucosal CD8-α^+ cells were especially numerous in the epithelium and in aggregates in the lamina propria. Regional differences in T-cell-specific gene expressions are first described in the intestine of a bony fish.

High non-specific cytotoxic activity against xenogeneic and allogeneic cells was found in lymphocytes purified from the intestinal mucosa, providing further insight into their local defence roles.

1. Introduction

The intestinal mucosa of vertebrates is exposed to both potential pathogens and beneficial commensal microorganisms, therefore the central challenge of its immune system is a local defence towards pathogens, while tolerating resident bacteria and food antigens [1]. A continuous and sensitive balance thus occurs between differentiation into regulatory T-cell subsets and effector T cells. The functional requirements to fight against different pathogens led to the identification of distinct CD4$^+$ and CD8$^+$ T-cell populations dealing with active defence and characterized by specific cytokine profiles [2].

The intestine is a relevant and peculiar site of interactions with the external world that appear essential in the development of the adaptive immune responses of jawed vertebrates [3]. The basal position of fish in vertebrate phylogeny makes them very attractive for genomic, anatomical and functional comparative studies. A gut-associated lymphoid tissue (GALT) has been described in different teleost species, although its role in immune development and response is still poorly elucidated [4]. The GALT of teleosts differs from tetrapode vertebrate counterparts in that it only comprises leucocytes scattered throughout the mucosa [5–8], whereas neither aggregated structures akin to Peyer's patches nor mesenteric lymph nodes have been identified. Indeed fish body apparently lacks specialized lymph nodes as those described in more evolved vertebrates. Findings obtained in European sea bass [7], carp [9] and rainbow trout [10] have suggested that the lymphocytes located in the intestinal epithelium were mostly T cells as in some mammalian species.

T cells have a relevant role in teleost cellular immunity as recently proved by a few studies with antibodies and extensive cloning of genes encoding for T-cell receptor and co-receptors [11]. After the first cloning of a fish *CD8-α* gene in rainbow trout [12], gene sequences (also for *CD8-β*) from many other species, including the sea bass [13], were discovered showing a fairly well preserved structure of the *CD8* genes throughout vertebrate evolution. In addition, antigen-specific cytotoxicity has been recently attributed to teleost *CD8-α*$^+$ leucocytes sorted using monoclonal antibodies [14]. Otherwise, after the first cloning of a fish *CD4*-like gene in fugu (*Takifugu rubripes*) [15], subsequent cloning in other teleost species

Abbreviations: DLEC, *Dicentrarchus labrax* embryonic cell; E/T, effector:target; FCS, foetal calf serum; GALT, gut-associated lymphoid tissue; IEL, intraepithelial lymphocytes; ISH, *in situ* hybridization; mAb, monoclonal antibody; LDH, lactate dehydrogenase; NCC, non-specific cytotoxic cells; PCR, polymerase chain reaction; RT, room temperature.

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revealed CD4 heterogeneity: a four Ig-like domain molecule (CD4-1), present in all species studied, and a second one (CD4-2) containing two (salmonids and fugu) or three Ig-like domains (catfish). In addition, two CD4-2 molecules were found in salmonids: CD4-2a (also named as CD4-REL for CD4-related) and CD4-2b [16]. Thus the complex phylogenetic relationships among fish CD4-like molecules do not appear fully established. The sea bass *CD4* cDNA consists of 2071 bp that translates in one reading frame to give the entire molecule (480 amino acids), with four Ig-like domains [17].

Intestinal lymphocytes immunopurified from the sea bass were shown to transcribe *CD4*, *CD8-α*, *rag-1*, *TCR-β* and *TCR-γ* [18,19], while in rainbow trout intraepithelial lymphocytes (IEL) transcribed homologous of various T-cell marker genes (*CD3-ε*, *CD4*, *CD8*, *CD28*, *TCR-β*, *TCR-γ* and *TCR-ζ*) [10]. However, rainbow trout IEL seem not to home specifically to the gut mucosa [10], at difference with mammals where this behaviour led to define them as a common mucosal population.

The present study aimed to improve knowledge about the cell-mediated intestinal immunity in the European sea bass, *Dicentrarchus labrax* (L.), a teleost fish species that has gained great relevance for basic and applied research. Transcripts of selected genes (*TCR-β*, *CD4*, *CD8-α*, *rag-1*, *MHCI-α* and *MHCII-β*) are quantified along the intestine of sexually immature sea bass. Gene expressions in the thymus of the same specimens are also determined to yield comparative indications about T-cell storage, somatic rearrangement and expected function/s. Based on earlier findings about the predominance of T cells in the sea bass posterior intestine [7,8,20], as well as on high amount of *CD8-α* transcripts here reported, RNA *in situ* hybridization (ISH) studies are performed to localize expression of *CD4*, *CD8-α*, *MHCII-β* and *TCR-β* genes. Furthermore, considering that a consistent amount of monoclonal antibody (mAb) DLT15^{+} cells, whether or not expressing *TCR-β*, have been located in the intestinal mucosa [21], lymphoid cells were isolated from the tissue to analyse the cell-mediated cytotoxic response against allogeneic or xenogeneic cells.

2. Materials and methods

2.1. Fish

Twenty one sea bass specimens (one year-old; 200 g mean body weight) maintained at 15 ± 1 °C were reared and provided by the Aquaculture station "Bonello" (Porto Tolle, Italy).

Six of these specimens were anaesthetised with 2-phenoxyethanol (2% in sea water, v/v) before sampling. The dissected tissues were used for real-time PCR and *in situ* hybridization analysis as reported in the specific sections.

Other fifteen sea bass specimens were distributed into 300 l tanks containing filtered, aerated sea water (pH 8.6, salinity 36 ppth, mean temperature 15 °C) at the Dept. Biology & Evolution (University of Ferrara) fish facility. Fish were fed with commercial dry pellets (Inve, Belgium) at a 2% (diet/biomass) daily ration and acclimatised for 3 weeks before the cytotoxicity assays (see specific section). Neither mortalities nor disease symptoms were observed throughout the experiments, and at inspection of internal organs during final dissections.

2.2. Real-time PCR

Total RNA was extracted with Tripure (Roche), following the manufacturer's instructions, from the thymus, muscle, brain and intestine (anterior, middle and posterior segments) of three sea bass specimens. RNA was suspended in DEPC-treated water and used for real-time PCR. RNA integrity was verified by ethidium bromide staining of the ribosomal RNA bands on a denaturing 1% (w/v) agarose gel.

The absence of DNA contamination was checked using sea bass *β-actin* primers that bracketed an intron. For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used following this protocol: 2 μg of total RNA was mixed with 1 μl of random hexamer (0.2 μg/μl; Amersham Pharmacia) and nuclease free water was added to a 12 μl final volume. This mixture was incubated at 70 °C for 5 min, then cooled on ice. Later, 0.4 μl of a reaction mix containing 100 mM dNTPs (25 mM each; Promega), 4 μl of 5× Reaction buffer, nuclease free water to a 19.75 μl final volume and 0.25 μl of BioScript at 200 u/μl were added. The solution was incubated at 25 °C for 10 min, then at 37 °C for 60 min. Finally, the reaction was stopped by heating at 70 °C for 10 min.

The transcripts of *TCR-β*, *CD4*, *CD8-α*, *rag-1*, *MHCI-α*, *MHCII-β* and *β-actin* (house-keeping gene) were determined with a Mx3000P™ real-time PCR system (Stratagene) equipped with version 2.02 software and using the Brilliant SYBR Green Q-PCR Master Mix (Stratagene) following the manufacturer's instructions. ROX was used as internal passive reference dye since it is not reactive during real-time PCR and therefore can be used to normalize slight differences in the volume of the added reaction mix, transparency of the plastic caps and other sources of well-to-well differences.

Specific PCR primers (Table 1) were designed for the amplification of products (~200 bp) from conserved regions of the immune-related genes analysed. For *MHCI-α*, a gene region not highly polymorphic was selected. Ten nanogram of cDNA template were used in each PCR reaction. The PCR conditions were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. Reactions were performed in triplicate for each template cDNA, that was replaced with water in all blank reactions. Each run was terminated with a melting curve analysis which resulted in a melting peak profile specific for the amplified target DNA. The analysis was carried out using the endpoints method option of the Mx3000P™ software that allows the fluorescence data at the end of each extension stage of amplification to be collected.

A relative quantification was performed comparing the levels of the target transcript with a reference transcript. Tissues with expected very low expression of the genes under evaluation were chosen as calibrators. The brain sample with the lowest *CD8-α* transcript was used as calibrator (defined as 1.0) for the simultaneous quantification of *TCR-β*, *CD4* and *CD8-α* transcripts. The muscle was used as calibrator (defined as 1.0) for the quantification of *MHCI-α*, *MHCII-β* and *rag-1* transcripts, that was performed in separate runs for each gene of interest.

The same normalizer target (*β-actin*) was included in the analysis to correct for differences in total cDNA input among the

Table 1
Specific primers for real-time PCR.

Gene	Forward and reverse primer sequences (5′–3′)	Accession numbers
β-actin	ATGTACGTTGCCATCC GAGATGCCACGCTCTC	AJ493428
CD4	GTGATAACGCTGAAGATCGAGCC GAGGTGTGTCATCTTCCGTTG	AM849811
CD8-α	CTAAGATTCGGCAAAATAACTCGAC GATGAGGAGTAGAAGAAGAAGGCC	AJ846849
MHC-α class I	AAGCAATACCTCACCCAGA CTCCATCTTTCCTCCAGAT	AM943118
MHC-β class II	CAGAGACGGACAGGAAG CAAGATCAGACCCAGGA	AM113466
Rag-1	GGATGAGTCAGACCATGAG GTGCAGATATGGGTGGAC	AH008179
TCR-β	GACGGACGAAGCTGCCCA TGGCAGCCTGTGTGATCTTCA	AJ493441