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Vibrio anguillarum bacterin uptake via the gills of Japanese flounder and subsequent immune responses

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ABSTRACT

The mucosal surfaces of fish allow for the introduction of foreign substances, including antigens, from the surrounding environment. In this study, uptake of *Vibrio anguillarum* J-O-3 serotype bacterin by Japanese flounder, and the subsequent immune responses were investigated. Immunohistochemistry revealed that the bacterin was taken up through the epithelial cells of gills. The transcription levels of inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor α were significantly up-regulated in the gills at 3 days following exposure to the bacterin. There was also a corresponding increase in IL-8 receptor, CD4-1, CD4-2 and CD8α transcript levels in the gills. Our findings suggest that the gills play a major role in the uptake of *V. anguillarum* bacterin and induction of inflammation, which results in an activation of the adaptive immune response in teleost fish.

1. Introduction

In animals, the digestive system, respiratory system and skin are potential areas of ingress by pathogens from the external environment. The immune system has evolved in mucosal tissues and plays an important role in the recognition and elimination of pathogens. In mammals, immune responses in mucosal tissues are initiated and induced by mucosa-associated lymphoid tissue (MALT). MALT consists of a germinal center, lymphoid follicle, T cell region, and includes antigen presenting cells (APCs) such as macrophages and dendritic cells [1]. M cells in the Peyer's patches [2], dendritic cell extensions into the lumen [3], and Goblet cells [4] uptakes antigens from the lumen and deliver them to APCs. The APCs phagocytize enteropathogens, and secrete pro-inflammatory cytokines and chemokines, such as interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α, to promote inflammation in mucosal tissues [5]. In contrast, commensals induce secretion of anti-inflammatory cytokines, such as transforming growth factor β and IL-10, resulting in T cell tolerance of the commensals [6]. Therefore, induction of an inflammatory response is important to induce an adaptive immune response against invasive pathogens in mucosal tissues. Finally, antigen presentation to T cells occurs, and adaptive immune responses such as production of IgA are induced in local mucosal tissue.

Fish habitat aquatic environments, in which microorganisms are more abundant compared with terrestrial environments. The entire body surface of fish (gill, intestine and skin) is surrounded by mucus, the initial immune barrier to prevent invasion of pathogens. The mucosal tissues of teleosts are abundant with immune cells such as B and T cells, macrophages and granulocytes. In rainbow trout (*Oncorhynchus mykiss*), IgT^+ B cells localize and play a major role in immune response against intestinal parasites in the gut, while IgM^+ B cells are the main subset of cells involved in the systemic immune response [7]. Gut intraepithelial lymphocytes account for 55% of total leukocytes in the gut of European sea bass, *Dicentrarchus labrax* [8]. $CD8\alpha^+$ cytotoxic T lymphocytes comprise up to 55 and 25% of all lymphocytes from the gut and gills, respectively, in rainbow trout [9]. Regarding antigen uptake,

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it has been reported that primary cultures of rainbow trout skin epithelial cells phagocityzed the bacterial pathogen, *Vibrio anguillarum* [10]. A limited number of antigen-sampling cells are located in the gut epithelium of Atlantic salmon (*Salmo salar*) and the cells have similar characteristics as mammalian M cells with respect to lectin-binding activities [11]. During adaptive immune responses in teleost mucosal tissues, IgT and IgM are secreted into the mucus by a polymeric Ig receptor that exists in the gut and skin epithelium [7,12].

The typical fish pathogen *V. anguillarum* affects many fish species, including salmonid fish, sea bass, sea bream *Sparus aurata*, ayu *Plecoglossus altivelis* [13] and Japanese flounder *Paralichthys olivaceus* [14]. To combat vibriosis, formalin-inactivated *V. anguillarum* bacterin, is used as a commercial vaccine. It is normally administered by intraperitoneal injection, immersion or orally [15]. Immersion and oral vaccines are considered mucosal vaccines in fish; they result in minimal pain and harm for fish and are cheaper to buy and administer compared with vaccines that require injection. These mucosal vaccines confer adaptive immune responses, such as increase in antibody titer in the serum, gut and skin mucus in many fish species [16–18]. Additionally, immersion vaccination of bacterin induces Mx gene expression in the liver of Atlantic salmon [19]. However, little is known about antigen uptake mechanisms and subsequent immune responses following mucosal administration of vaccines. In this study, *V. anguillarum* bacterin uptake and immune responses after immersion vaccination were investigated.

2. Materials and methods

2.1. Fish rearing

Japanese flounder (average body weight 12.5 g) were obtained from Marinetech (Japan) and kept in 60 L tanks using a free-flowing system with sand-filtered sea water. The water temperature was maintained at 20 °C and fish were fed every other day.

2.2. Bacterial propagation, bacterin preparation and administration

Vibrio anguillarum strain TC311, isolated from diseased yellowtail (*Seriola quinqueradiata*), was cultured in heart infusion broth (Difco, USA) containing 2% NaCl for 48 h at 25 °C. Bacterial cultures were serially diluted and incubated on heart infusion agar (Difco, USA) at 25 °C for 24 h, to count colony-forming units (cfu). Cultured bacteria were treated with 0.3% formalin for 24 h at room temperature, and inactivation of the bacteria was confirmed by incubating the solution on heart infusion agar at 25 °C. Formalin-killed cells (FKC) of the bacteria were washed with phosphate-buffered saline (PBS) and re-suspended in PBS. This FKC suspension was stored at 4 °C until required. Japanese flounder were immersed into FKC (1.8×10^8 cfu/ml) suspended in 10 L of seawater for 30 min with appropriate aeration. For quantitative RT-PCR (qPCR) and ELISA analysis, fish immersed into PBS that was diluted with seawater were used as negative controls. Fish were transferred and kept in 60 L tanks following vaccination.

2.3. Immunohistochemistry

Following exposure to FKC, fish were euthanized and dissected at 1, 3, 6 and 12 h post-immersion to collect the gills, intestine and skin. Tissues were fixed overnight in Davidson's solution (30% ethanol, 10% formaldehyde and 10% acetic acid). The fixed tissues were then decalcified in 0.1 M PBS containing 0.3 M EDTA for 48 h and embedded in paraffin. The tissue blocks were sectioned (3-μm thickness) and analyzed by immunohistochemistry for *V. anguillarum* antigens. Polyclonal rabbit antiserum raised against the J-O-3 strain of *V. anguillarum* was provided by the Japan Fisheries Resource Conservation Association. Deparaffinized sections were treated with normal goat serum and a VECTASTAIN ABC Rabbit IgG Kit (VECTOR Laboratories, USA), following the manufacturer's instructions. Sections were then incubated with primary antiserum against the J-O-3 strain of *V. anguillarum* at a 1:1000 dilution with Tris-buffered saline (TBS) containing 0.4% BLOCK ACE (DS Pharma Biomedical, Japan) for 1 h. Slides were washed with TBS and incubated with goat anti-rabbit IgG labeled with horseradish peroxidase. Slides were washed again with PBS and treated with 50 mM Tris–HCl (pH 7.6) containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Dojindo, Japan) and 0.03% H_2O_2 for 3 min, then lightly counterstained with hematoxylin. Inactivation of endogenous peroxidase activity was confirmed by immunohistochemistry without 1st antibody.

2.4. qPCR

Vaccinated and control fish ($n = 5$ fish per group) were euthanized and dissected at 1, 3 and 5 days post-immersion. Gills, intestine, kidney and skin were collected and tissues immediately homogenized in TRIzol Reagent (Invitrogen) and total RNAs extracted following the manufacturer's instructions. First-strand cDNAs were synthesized using 2 μg of total RNA from each sample using MMLV reverse transcriptase (Invitrogen, USA), following the manufacturer's instructions.

The mRNA levels of inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the gills, intestine, kidney and skin were determined by qPCR. The mRNA levels of cell surface receptors (CD4-1, CD4-2, CD8α and IL-8R) in the gills and kidney were also determined. Primers specific for these genes were designed using Primer Express Software Ver 3.0 (Applied Biosystems, USA) (Table 1). The reaction mixture contained 5 μl of diluted cDNA, 10 μl of THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan), 0.4 μl of Rox reference dye and 0.6 μl each of the sense and antisense primer (10 μM) made up to 20 μl with distilled water. The qPCRs were conducted using a Mx3005P Real-Time PCR System (STRATAGENE, USA) with MxPro QPCR Software (STRATAGENE, USA), following the manufacturer's instructions. The mRNA levels of target genes were normalized to those of an internal control (EF1α). Expressions levels were taken as the average values from three fish and presented as the fold-change

Table 1
Oligonucleotide primer sequences used in this study.

Primer name	Sequence of oligonucleotides (5′–3′)	GenBank accession No.
CD4-1 S[a]	CCAGTGGTCCCCACCTAAAA	AB643634
CD4-1 AS[b]	CACTTCTGGGACGGTGAGATG	
CD4-2 S	CACAGCGAGGACGTCAGAAA	AB640684
CD4-2 AS	TCTCTCCCATCACTCCTTTAGCA	
CD8α S	CCTCTCCCCATACATTGATTCC	AB082957
CD8α AS	CCGAGCTTTGCTGAAGGACTT	
EF1α S	CTCGGGCATAGACTCGTGGT	AU090803
EF1α AS	CATGGTCGTGACCTTCGCTC	
IL-1β S	CAGCACATCAGAGCAAGACAACA	AB070835
IL-1β AS	TGGTAGCACCGGGCATTCT	
IL-6 S	CAGCTGCTGCAAGACATGGA	DQ267937
IL-6 AS	GATGTTGTGCGCCGTCATC	
IL-8R S	CATCGACCGCTACCTTGTGA	AB079600
IL-8R AS	CTCTGGCGGCTCTTGAGAGT	
TNFα S	CGAAGGCCTAGCATTCACTCA	AB040448
TNFα AS	TCGTGGGATGATGATGTGGTT	

[a] S indicates sense primer.
[b] AS indicates anti-sense primer.