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Local and systemic adaptive immune responses toward viral infection via gills in ginbuna crucian carp

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ABSTRACT

Recent studies on fish immunity highlighted the significance of gills as mucosal immune tissues. To understand potential of gills as vaccination sites for inducing adaptive systemic immunity, we investigated virus-specific cell-mediated and humoral immune responses following a "per-gill infection method", which directly exposes virus only to gills. The viral load in crucian carp hematopoietic necrosis virus (CHNV)-infected gills decreased after peaking at a particular time point. Furthermore, the viral titers in the gills following the secondary infection were lower than that after the primary infection, indicating that local adaptive immunity helped the elimination of virus. Gene expression analysis demonstrated that IFN-γ in gills and perforin in kidney were increased after the gill infection. $CD8^+$ cells in kidney leukocytes increased after the secondary infection, whereas IgM^+ cells decreased. These results suggest that IFN-γ and CTL contribute in controlling CHNV-replication in gills and kidney. Gill infection could induce specific cell-mediated cytotoxicity of peripheral blood leukocytes (PBL) and secretion of CHNV-specific IgM in serum, indicating that local priming of the gill site can generate adaptive systemic immunity. Thus, the gills could be prospective antigen-sensitization sites for mucosal vaccination.

1. Introduction

There are several anatomical and physiological differences between mammals and fishes. Contrast in their environmental habitat has created significant differences in mucosal organs; for example, the presence of gills and mucous skins and the absence of Peyer's patches. The mucosal organs of fish prevent invasion of pathogens from their surrounding environment. Several recent reviews have suggested that fish have robust mucosal immune systems, and hence it is important to conduct further studies to improve our understanding of its functions (Gomez et al., 2013, 2014; Lazado and Caipang, 2014; Rombout et al., 2011, 2014; Salinas et al., 2011).

The gills are one of the first organs exposed to pathogens, and thus, is thought to be an important organ in fish mucosal immunity (Andrews et al., 2010; Murray et al., 2007). Transcriptional analysis has demonstrated that many genes that are involved in adaptive immunity are abundantly expressed in gills (Abdelkhalek et al., 2014; Aquilino et al., 2014; Koppang et al., 1998a,b; Rebl et al., 2014; Takizawa et al., 2011). In addition, antibody producing cells have been isolated from gills and IgM has been detected in gill mucus in several fish species (Salinas et al., 2011; von Gersdorff Jorgensen et al., 2011; Zilberg and Klesius, 1997). IgT, a unique immunoglobulin isotype in teleosts, appears to be specialized in mucosal immunity and is present in the gill mucosa (Aquilino et al., 2014; Fillatreau et al., 2013; Salinas et al., 2011). Castro et al (2014) have recently reported that the chemokine receptor CCR7, which represents an important determinant for circulating lymphocytes to enter lymph nodes in mammals, is mainly expressed in trout gills. Furthermore, a unique T-cell-rich intraepithelial structure in gills was identified in Atlantic salmon (Haugarvoll et al., 2008; Koppang et al., 2010; Aas et al., 2014; Weli et al., 2013). Thus, cellular, molecular, or histological analyses have provided evidence that the teleost gill is an important site to induce systemic adaptive immunity as a secondary lymphoid organ.

Ginbuna crucian carp, a naturally occurring gynogenetic fish, is a useful model animal for studying adaptive immunity (Fischer et al., 2013; Nakanishi et al., 2011; Somamoto et al., 2014a). We have investigated the antiviral functions of cytotoxic T-lymphocytes (CTLs) and helper T cells (Th-cells) using this clonal fish and the crucian carp hepatopoietic necrosis virus (CHNV), suggesting that their functions are basically similar to those in mammals (Somamoto et al., 2009 2013 2014b). Because these studies demonstrated that

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intraperitoneal (i.p.) injection with virus can induce systemic CTL and Th-cell responses, it is unclear whether mucosal sensitization can also effectively activate adaptive anti-viral immunity. The "per-gill infection method", which directly exposes virus to only gills, has previously been developed as a procedure to infect fish with koi herpesvirus (Miyazaki et al., 2008). We believe that this method enables focusing on immune responses induced by sensitization through gills. Therefore, the present study has examined the systemic and mucosal immune responses following infection with CHNV via the gill.

2. Materials and methods

2.1. Fish and virus

The S3n and OB1 strains of ginbuna crucian carp, *Carassius auratus langsdorfii*, were maintained at a temperature of 25 °C and were daily fed with commercial food pellets. CFS (Carassius fin from Lake Suwa) cells from the S3n strain of ginbuna crucian carp were used as syngeneic target cells and for propagation of the CHNV, as described by Somamoto et al. (2014a,b). The S3n strain was used in the cell-mediated cytotoxicity assay, because the target cell line was available. The OB1 strain was used in experiments other than the cytotoxic assay.

2.2. Infection with CHNV through gills

The method of infection was referred to as a "per-gill infection method", which was previously reported (Miyazaki et al., 2008), and it was modified for CHNV and ginbuna crucian carp. Clonal ginbuna crucian carp (OB1 and S3n strain) were anesthetized in 25 mg/L quinaldine and inoculated with 0.5 mL/50 g fish weight of CHNV solution ($10^{7.8}$ $TCID_{50}$/mL in PBS) into their gills (to both sides) in air. After the treatment, fish were wrapped with wet papers and kept in air for 5 min at 25 °C to allow the virus to adsorb into gill tissue. The fish were then returned to the tank and maintained at 25 °C. The control fish were treated with PBS instead of CHNV. Two weeks after the primary infection, the secondary infection was performed using the same procedure described earlier.

2.3. Virus titer from organs

Eighteen ginbuna crucian carp (OB1 strain), weighing 18–23 g, was used in this experiment. The gills and trunk kidney were collected at 1, 12, and 24 h post-primary and -secondary infection. The respective organs were placed in MEM (1:10, weight: volume) containing 10% FBS, homogenized and then centrifuged at 1000 × *g* for 20 min. The supernatants were passed through a 0.45-μm membrane filter and the filtrate was then stored at –80 °C until further required. Virus titers were determined by a $TCID_{50}$ endpoint titration in CFS cells incubated for 21 days at 25 °C. Three fish were sampled at each time point in both primary and secondary infections. The results were expressed as $\log_{10}$ $TCID_{50}$ per gram of organ.

2.4. Leukocyte composition in the gill and kidney from infected fish

The infection procedure described in the previous section was followed in this study too. Three fish were sampled at 24 h after the infection and PBS treatment. The trunk kidneys from control and infected fish were disaggregated by passing them through a 150-gauge mesh stainless steel sieve in MEM supplemented with 10% heat-inactivated FBS (MEM-10) (Nissui Pharmaceutical Co., Tokyo, Japan). Whole gills including gill arch were excised and only the lamellae were disaggregated through the stainless steel sieve. The cells were washed with MEM-10, applied to a Histopaque density of 1.083 g/mL (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 400 × *g* for 40 min at 4 °C. The cells at the top of the Histopaque were collected and washed twice with MEM-10. The cell number from each cell was adjusted to 0.5–1.0 × 10^6 cells/mL with MEM-10. The cell suspensions were equally divided into three parts (5 × 10^5 cells/each suspension) and incubated with a 1:10,000 dilution of either rat anti-ginbuna CD8α mAb (mouse ascites), anti-ginbuna CD4 mAb, or anti-ginbuna IgM mAb for 30 min on ice (Somamoto et al., 2013; Toda et al., 2011a). The cells were then washed twice with medium, incubated for 30 min at 4 °C with a 1:100 dilution of anti-rat IgG goat antibody conjugated with phycoerythrin (PE; Abcam, Cambridge, MA, USA) and then washed additionally three times. The cell percentages were assessed by flow cytometry (EPICS XL; Beckman Coulter, Brea, CA, USA).

2.5. Real-time quantitative PCR

Three fish from the control and infected groups sampled at 1, 12, and 24 h post-primary and post-secondary infections were employed for assessing the gene expression in the cells of interest that were collected as described in Section 2.4. Total RNA was extracted from 1.0 × 10^6 cells of the gill and kidney leukocytes using the NucleoSpin RNA II (Machery-Nagel. GmbH Co KG, Duren, Germany), according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA) with an oligo (dT) primer, according to the manufacturer's instructions. Primers used for real-time PCR were designed to amplify cDNA fragments encoding the following cytokines and perforin: IFN-γ1 and IFN-γ2 (Yamasaki et al., 2014), IL-10 (accession HQ259106), and Perforin-1 (Toda et al., 2011b). An internal control for normalization was EF-1α (Toda et al., 2011b). The sequences or each primer sets of IFN-γ, perforin and EF-1α are indicated in the reference articles. The primer sequences for IL-10 were as follows: forward primer (5′-TTGGCACCATTACTCGATGA-3′) and reverse primer (5′-TCCAAGTAGAAGCGCAGGAT-3′). Quantitative real-time PCR was performed in duplicate on a M × 3000P System (Stratagene, La Jolla, CA, USA) in 16 μL reaction mixtures containing 2 μL of template cDNA, 0.5 μM primers, and other reagent components from the Fast Start DNA Master SYBR_Green (Roche Applied Science, Mannheim, Germany). Thermal cycling was performed using a two-step thermal cycling mode composed of an initial denaturation for 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 or 40 s at 55 °C (EF-1α, IFN-γ1, and IFN-γ2) or 60 °C (IL-10 and perforin). The relative quantitative value of each gene was calculated according to the standard curve from a serial dilution of a reference cDNA in the same plates and normalized with the level of EF1α.

2.6. Cell-mediated cytotoxic assay

The S3n strains of ginbuna crucain carp, weighing 63–80 g, were used in the cell-mediated cytotoxic assays. CHNV-infected fish (n = 3) were sampled 2 and 4 days post-secondary infection and were bled from the caudal vein into heparinized syringes. PBLs were isolated using a Percoll gradient method as previously described (Somamoto et al., 2013, 2014a,b). Blood were centrifuged (400 × *g*, 5 min, 4 °C), and the buffy coat was collected and diluted in OPTI-MEM (Invitrogen, Carlsbad, CA). These cells were applied to a Percoll (Sigma Chemical Co., St. Louis, MO, USA) at a density gradient of 1.08 g/mL and centrifuged at 450 × *g* for 30 min at 4 °C. The top layer of cells in the Percoll was collected and the cells were washed twice with OPTI-MEM by centrifugation (450 × *g*, 5 min, 4 °C). Cells were then re-suspended in DMEM/F-12 medium (Invitrogen, Carlsbad, CA) without phenol red and with 1% FBS (DMEM/F12-1) and then used as effector cells for a cytotoxicity assay.