Studies on the development of a vaccine against *Mycobacterium* sp.

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ABSTRACT

Mycobacteriosis, caused by *Mycobacterium* sp., results in severe loss of fish production in Japan's aquaculture industry. Since the bacterium can survive and grow in side host cells, chemotherapeutic agents are not effective and potent vaccines are required to control the disease. In this study, the effects of two vaccine candidates, Bacillus Calmette and Guèrin (BCG, an attenuated strain of *Mycobacterium bovis*) and formalin-killed cells (FKC) of *Mycobacterium* sp. strain 012971, on the immediate and acquired immune response were evaluated in Japanese flounder, *Paralichthys olivaceus*. On 1, 3 and 7 days post treatments with these candidates, gene expression levels of inflammatory cytokines encoding interleukin-1β (IL-1β), IL-6 and tumor necrosis factor α (TNFα) increased. This suggests that the vaccine candidates stimulate immediate immune responses. To determine if specific defenses against mycobacteriosis were acquired, purified protein derivative (PPD) from *Mycobacterium* sp. strain 012971 was injected intramuscularly into fish at 4 weeks after vaccine treatments and the inflammatory cytokine gene expression levels were analyzed. Gene expression levels of the inflammatory cytokines were increased only for the fish treated with BCG, but not those with FKC. Subsequently, the vaccinated fishes were challenged with *Mycobacterium* sp. The relative percentage survival (RPS) of BCG and FKC vaccinated groups at 12 days post challenge were 37.5 and 18.1, respectively. These data indicate that BCG might be useful as a vaccine against fish mycobacteriosis.

Key words: mycobacteriosis, live attenuated vaccine, BCG, innate immunity, cell-mediated immunity

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INTRODUCTION

Mycobacterium species are gram positive, aerobic, acid-fast, non-motile bacteria that cause mycobacteriosis in various fish species. Three species of *Mycobacterium* that are major pathogens of fish are *M. marinum* (Aronson, 1926), *M. fortuitum* (Ross and Brancato, 1959) and *M. chelonae* (Bruno *et al*., 1998). However, mycobacteriosis also has been attributed to other *Mycobacterium* species and synonyms for the three major species (Decostere, Hermans and Haesebrouck, 2004).

In 1985, an epidemic caused by *Mycobacterium* sp. affected cultured yellowtail (*Seriola quinqueradiata*) in Sukumo bay, Japan (Kusuda, Kawakami and Kawai, 1987). The symptoms of the disease in yellowtail were haemorrhagic ascites, hypertrophy of the spleen and kidney with tubercles and visceral adhesions (Kusuda, Kawakami and Kawai, 1987). Later, the disease was also shown to affect striped jack (*Pseudogaranx dentex*) (Kusuda *et al.*, 1993) and amberjack (*Seriola dumerili*) and has, to date, resulted in severe loss of fish production in Japan's aquaculture industry. The species name of the bacteria has not been identified yet, although biochemical characteristic had been examined (Kusuda, Kawakami and Kawai, 1987).

Pathogenic mycobacteria are capable of intracellular parasitism. The bacteria can avoid initial degradation in the phagosome by producing superoxide dismutase that eliminates oxygen radicals (Yamamoto, 2006). Furthermore, they can survive inside macrophages by secreting the enzyme protein kinase G out of the phagosome, assisting in the prevention of phagosome-lysosome fusion (Nguyen and Pieters, 2005). The infected macrophages are enveloped by immune cells to avoid dissemination of the bacteria, resulting in the formation of tubercules or granuloma formation in the host.

The effects of chemotherapeutic agents are limited *in vivo*, because mycobacteria can survive within host macrophages. In fact, although rifampicin, streptomycin and erythromycin can inhibit the growth of *Mycobacterium* sp., the bacteria can survive in the fish and then proliferate after the antibiotic treatment has ceased (Kawakami and Kusuda, 1990). Therefore, it is important to develop a potent vaccine against the disease.

In order to control mycobacteriosis, a potent vaccine is required to induce cell-mediated immunity in the host. *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) is a live attenuated vaccine developed from the *M. bovis* strain almost a century ago, and is still commonly used worldwide. Besides its efficacy against human tuberculosis caused by *M. tuberculosis*, BCG is also known to provide protection against leprosy caused by *M. leprae* (Ponnighaus *et al.*, 1992) and Buruli ulcer caused by *M. ulcerans* (Portaels *et al.*, 2002, Portaels *et al.*, 2004 and Nackers *et al.*, 2006) in human. Live attenuated vaccine causes chronic, but weak infection in the host. Hence, these vaccines can confer specific cell-mediated immunity and long protective efficacy. Nevertheless, the exact mechanisms of BCG-induced protection in human and animal models are still unclear (Sander and McShane, 2007).

The tuberculin response, a delayed-type hypersensitivity (DTH) response, is used to test whether the host has previously been exposed to mycobacterial antigens, including BCG vaccination. Intradermal injection of mycobacterial purified protein derivative (PPD) as an antigen elicits hall mark responses including indurations, swelling and monocytic infiltration into the site of the lesion within 24 to 72 hours (Black *et al*., 1999). Upon injection of the antigen, Langerhans cells process and present the antigen to the local memory T cells. These T cells secrete numerous cytokines and chemokines including interleukin-1 (IL-1), IL-6, IL-8, IL-12, interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα) (Grabbe and Schwarz., 1998 and Black *et al.*, 1999) that result in vasodilatation, an influx of immune cells and activation of macrophages. As a result, granulomatous inflammation occurs at the site of PPD injection, ceasing only after degradation of the antigen.

The innate immune response, the first line of defense against pathogenic microbes, plays a critical role during mycobacterial infection. Toll-like receptor 2 (TLR2) which exists on the surface of the phagocyte recognizes pathogen-associated molecular patterns (PAMPs) of mycobacteria and activates MyD88, resulting in the induction of inflammatory cytokines (Underhill *et al.*, 1999). The activation of the innate immune response mediated by TLRs is a vital step for triggering acquired immunity. For example, TLR2 signaling leads to the release of an instructive cytokine, such as IL-12 which drives the differentiation of naïve T cells to Th1 cells (Akira, Takeda and Kaisho, 2001). Therefore, their ligands such as mycobacterial cell wall, lipopolysaccharide (LPS) and CpG-rich DNA are often used as vaccine adjuvants.

In order to develop an effective vaccine against *Mycobacterium* sp. infection, two vaccine candidates, BCG and the formalin-killed cells of *Mycobacterium* sp. strain 012971 were tested in Japanese flounder (*Paralichthys olivaceus*) as a model. Fish treated with the candidates were evaluated by quantification of the inflammatory cytokine gene expression levels during the immediate immune response and DTH response. Furthermore, protection efficacy of the candidates was confirmed by intramuscular challenge with live *Mycobacterium* sp.

MATERIALS AND METHODS

Fish rearing and bacteria propagation

Japanese flounder (*Paralichthys olivaceus*) weighing approximately 10 g were kept in 60 L tank with circulation systems maintained at 20 ºC. They were fed every other day until use.

Mycobacterium sp. strain 012971, which was isolated from cultured yellowtail (*Seriola quinqueradiata*), was grown on 1 % Ogawa medium (Nissui, Japan) at 25 ºC for 3 weeks. *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) was grown on 1 % Ogawa medium at 37 ºC for 3 weeks.

Vaccine preparation

To prepare the live attenuated vaccine, *M. bovis* BCG was suspended in sterilized phosphate buffered saline (PBS) using a glass homogenizer. One hundred microliters of the bacterial suspension was spread on Middlebrook 7H10 agar (Becton, Dickinson and Company, USA) supplemented with OADC Enrichment (Becton, Dickinson and Company, USA) and cultured at 25 ºC for 4 weeks to calculate the number of colony forming units (CFU). For the preparation of formalin-killed cells of *Mycobacterium* sp. as an inactivated vaccine, the bacteria was suspended in 10 ml of PBS containing 5 % formaldehyde (Wako, Japan) and incubated at 25 ºC for 48 h. Cell count for *Mycobacterium* sp. was done as described above. To confirm the complete inactivation of the bacteria, 100 µl of suspension was spread on Middlebrook 7H10B agar with OADC Enrichment and incubated at 25 ºC for 4 weeks.

Immediate immune response after vaccination

BCG (1.2×10^8 CFU/fish) or FKC (2.0×10^8 CFU/fish) suspended in PBS were intramuscularly injected into Japanese flounder. The kidneys of the vaccinated fish (n=3) were collected at 1, 3 and 7 days post-vaccination. Total RNAs were extracted from the kidney using RNA iso (TAKARA, Japan), following manufacturer's instructions. RNA concentration was determined by Gene Quant (Pharmacia, USA) and stored in DEPC treated water at -80 ºC until used.

First strand cDNA was synthesized with 2 µg of total RNA using MMLV reverse transcriptase (Invitrogen, USA), following manufacturer's instruction. The changes in mRNA levels for Japanese flounder inflammatory cytokines including IL-1β (GenBank Accession number AB070835), IL-6 (DQ267937) and TNFα (AB040448) in fish at 1, 3 and 7 day post-vaccination were determined by quantitative RT-PCR (qPCR). Primers for qPCR were designed using Primer Express Software Version 3.0 (Applied Biosystems, USA) including ribosomal protein L10 (RPL10, AU050650) used as internal control (Table 1). The reaction mixture contained 5 µl of the diluted cDNA sample, 10 µl of Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and 0.4 µl of the forward and

Table 1
Primers used in this study.

Primer	Sequence
IL-1β short F	5'-CAGCACATCAGAGCAAGACAACA-3'
IL-1β short R	5'-TGGTAGCACCGGGCATTCT-3'
IL-6 short F	5'-CAGCTGCTGCAAGACATGGA-3'
IL-6 short R	5'-GATGTTGTGCGCCGTCATC-3'
TNF α short F	5'-CGAAGGCCTAGCATTCACTCA-3'
TNF α short R	5'-TCGTGGGATGATGATGTGGTT-3'
RPL10 short F	5'-GCTCCTCTGGTGCAGTTTGTGA-3'
RPL10 short R	5'-TGGTGTTTGCTGGCGTCACTCT-3'

reverse primer (10 μM) designed for each gene were adjusted up to 20 μl with sterilized water. qPCR was performed using 7300 Real Time PCR System (Applied Biosystems, USA), according to manufacturer's instruction.

Preparation of purified protein derivative (PPD)

The purified protein derivative (PPD) was prepared from *Mycobacterium* sp. strain 012971. The bacteria were cultured in 200 ml of Middlebrook 7H9 broth (Becton, Dickinson and Company, USA) supplemented with OADC Enrichment for 8 weeks at 25 ºC. After heat sterilization at 105 ºC for 3 h, bacterial cells were removed by centrifugation at 200,000×g for 1 h and the supernatant was passed through Syringe Driven Filter Unit (Millipore, USA). The filtrate was then concentrated by boiling for 30 min and this was followed by the addition of 40% (w/v) trichloroethanoic acid (Wako, Japan). The precipitate was collected by centrifugation at 6,500×g for 15 min. After washing with 4% (w/v) trichloroethanoic acid, the precipitate was resuspended in sterilized PBS and stored at -80 ºC until used.

Tuberculin response

BCG at 2.1×10^7 CFU/fish or FKC at 2.0×10^8 CFU/fish were used for this vaccine study. Four weeks after vaccination, the vaccinated fish were injected with PPD intramuscularly and the kidneys were sampled at 1 and 3 days post-injection. Total RNA was extracted and cDNA was synthesized as described above and the cDNA was used for qPCR.

The changes in mRNA levels of IL-1β, IL-6 and TNFα genes after PPD injection were determined by qPCR, as described above.

Challenge test

The fish were vaccinated with BCG at 3.0×10^8 CFU/fish (23 individuals) or FKC at 1.6×10^7 CFU/fish (27 individuals) for 4 weeks before challenging with live *Mycobacterium* sp. strain 012971 (1.5×10^7 CFU/fish). The number of fish used in this experiment was shown in Table 2. A colony of strain 012971 on the 1 % Ogawa medium were scraped and suspended in sterilized PBS using grass homogenizer and CFU was determined as described above. One hundred microliters of the bacterial suspension was injected into the fish intramuscularly. The fish were then maintained in 60 L tanks with circulation systems kept at 25 ºC and fed every other day. Cumulative mortality of the fishes were recorded for 12 days and relative percent survival (RPS) was calculated using the following equation; RPS = (1-[% loss of vaccinated fish/% loss of PBS control fish]) ×100.

Data analysis

Inflammatory cytokine gene expression levels during the immediate immune response and tuberculin response experiments were normalized with ribosomal protein L10 expression. In addition, the gene expression values for each group were represented as fold change relative to the value of the PBS injected group at day 1. Statistical analysis was performed with *t*-test between gene expression levels of each vaccinated groups and those of the PBS control groups at day 1.

Results

Immediate immune response after vaccination

Gene expression levels of inflammatory cytokines including IL-1β, IL-6 and TNFα in the fish injected with BCG, FKC and PBS were determined to analyze the immediate immune response at 1, 3 and 7 days post-vaccination by qPCR (Fig. 1). Gene expression levels of the inflammatory cytokines were up-regulated by BCG or FKC vaccinated fish. The IL-1β gene expression level increased by 12-fold in the BCG-vaccinated group and 8-fold in the FKC-vaccinated group at 3 days post-vaccination. The expression level of IL-6 increased 3-fold in the BCG-vaccinated group at 1 and 3 days post-vaccination. In contrast, the FKC-vaccinated group showed 3-fold increase in IL-6 gene expression level at only 3 days post-vaccination. The expression level of TNFα increased 4-fold and 3-fold in the BCG-vaccinated group at 3 and 7 days post-vaccination, respectively. In contrast, the FKC vaccinated-group showed 3-fold increase in gene expression level only at 3 days post-vaccination.

Tuberculin response

Cytokine gene expression levels were significantly up-regulated in only the BCG-vaccinated group (Fig. 2). In the BCG-vaccinated group at 1 and 3 days post-injection of PPD, IL-1β gene expression levels increased by 15-fold and 8-fold, respectively, and TNFα gene expression levels increased by 4-fold and 2-fold, respectively. In contrast, IL-6 gene expression level in BCG-vaccinated group increased by 5-fold at only 3 days post-injection of PPD.

Challenge test

Challenge experiments were performed using strain 012971 (2.4×10^8 CFU/fish), 4 weeks after the fish were injected with BCG, FKC or PBS. Twelve days after the challenge, cumulative mortality of BCG and FKC vaccinated fish and PBS injected fish were 56.5 %, 74.1 % and 90.5 %, respectively. RPS of the BCG and FKC vaccinated groups were 37.5 and 18.1, respectively (Table 2).

Table 2
Cumulative percent mortality (%) and calculated RPS values of BCG- and FKC- vaccinated fish.

Group	No. of dead fish/total fish	Mortality (%)	RPS[a]
BCG	**13/23**	**56.5**	**37.5**
FKC	**20/27**	**74.1**	**18.1**
PBS	**19/21**	**90.5**	**—**

[a] Relative percent survival = (1-[% loss of vaccinated fish/%loss of PBS control fish]) ×100

Discussion

The Japanese flounder's immediate immune response to the two vaccine candidates against *Mycobacterium* sp. were evaluated by quantifying the inflammatory cytokine gene expression levels. In general, the inflammatory cytokine gene expression levels in the BCG-vaccinated group were higher than those in the FKC-vaccinated group during the immediate

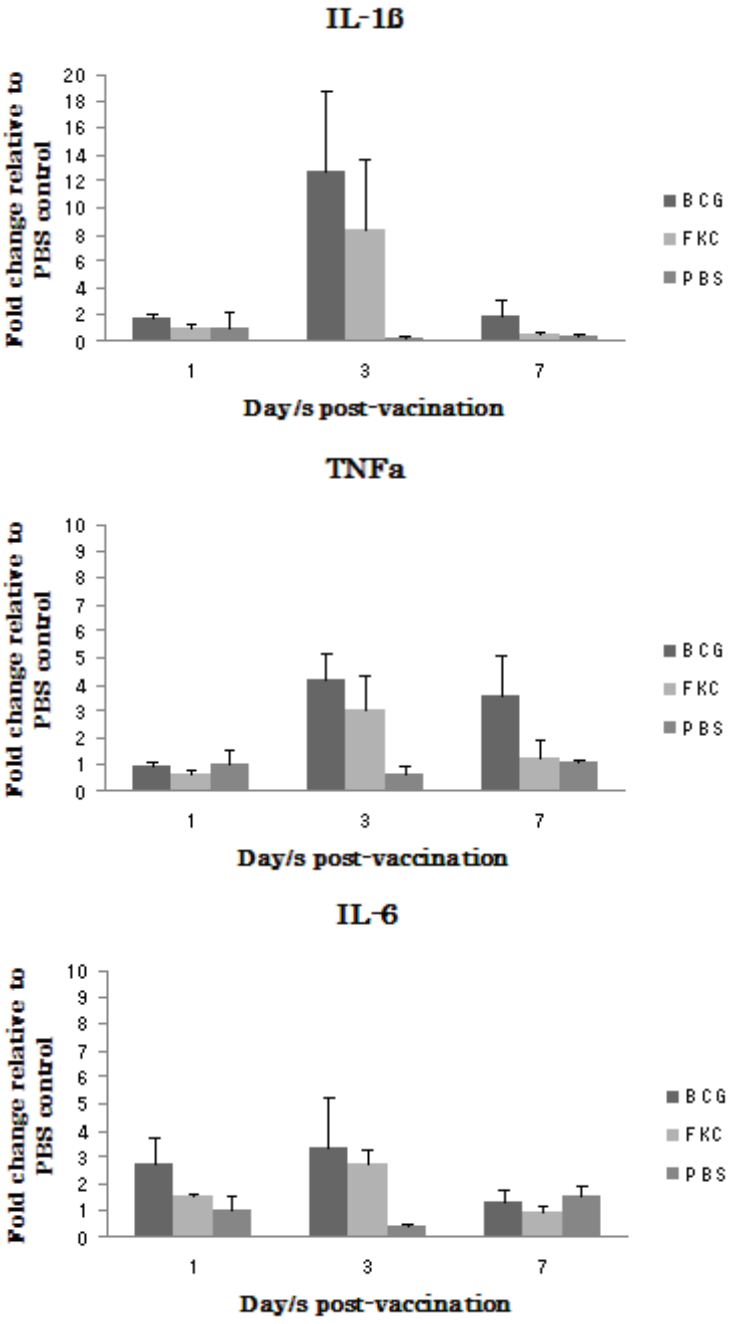

Figure 1 Immediate immune response after vaccination. Gene expression levels of (A) IL-1β, (B) IL-6 and (C) TNFα in vaccination groups (BCG and FKC) and PBS injected (control) group at 1, 3 and 7 days post-vaccination were shown as fold change relative to PBS injected group at 1 day post-vaccination. Data are expressed as mean ± S.D. of three individuals and the asterisk indicates gene expression levels are significantly higher (P<0.05) than those of PBS injected groups at day 1. Japanese flounder ribosomal protein L10 was used to normalize the data.

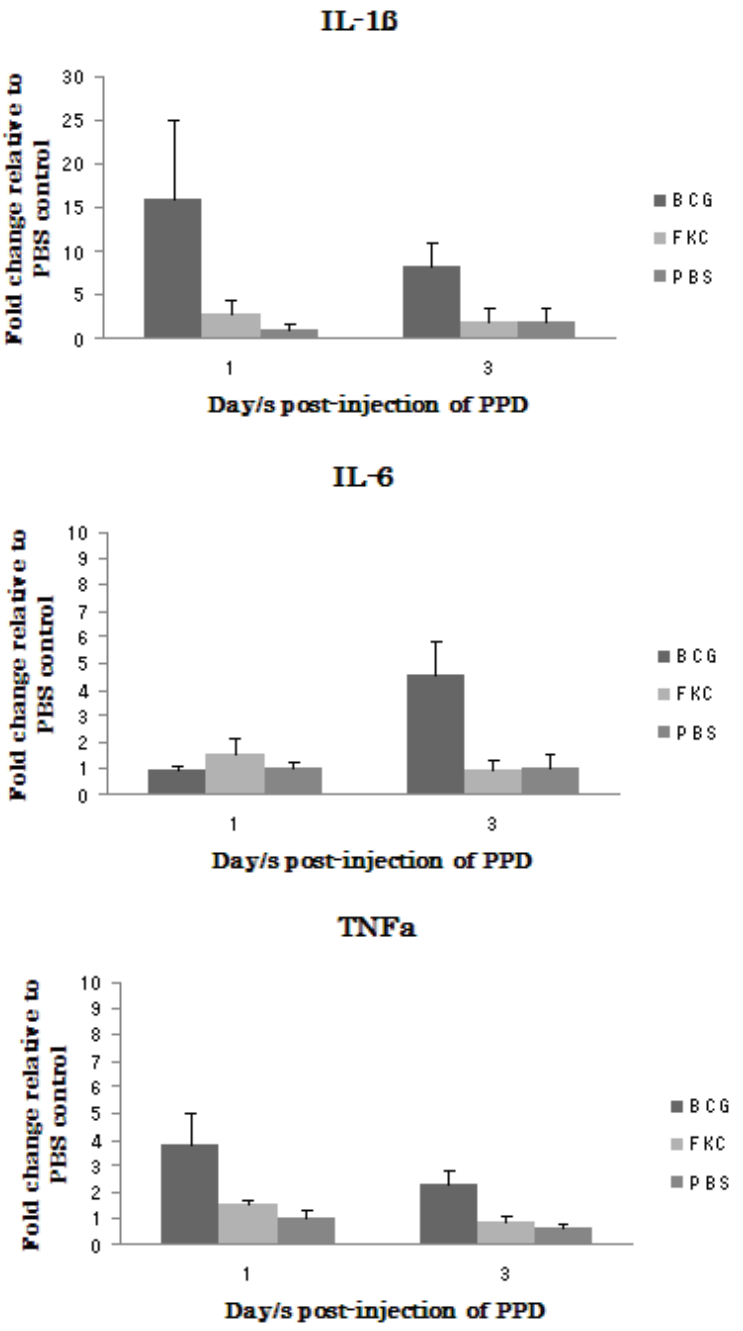

Figure 2 Tuberculin response in vaccinated fish after PPD injection. Gene expression levels of (A) IL-1β, (B) IL-6 and (C) TNFα in vaccination groups (BCG and FKC) and PBS injected (control) group at 1 and 3 days post-injection of PPD were shown as fold change relative to PBS injected group at 1 day post-injection of PPD. Data are expressed as mean ± S.D. of three individuals and the asterisk indicates gene expression levels are significantly higher (P<0.05) than those of PBS injected groups at day 1. Japanese flounder ribosomal protein L10 was used to normalize data.

immune response. Akira (2003) claimed that mycobacterial secreted components such as 19-kDa lipoprotein are important for the activation of the innate immune system in mammals through TLR2. These components are only produced and secreted by live bacteria such as BCG, but not by FKC. Thus, this might explain the greater up-regulation of cytokines in the BCG-vaccinated group.

In the BCG-vaccinated group, the up-regulation of TNFα was maintained until 7 days post-vaccination. TNFα plays several critical roles during mycobacterial infection. In mice infected with *M. bovis* BCG, TNF was found to inhibit the spread of the bacteria (Kindler *et al.*, 1989). It has also been reported that TNF and lymphotoxin α (LT α) signaling are required to activate cells of the immune system (Jacobs *et al.*, 2000). The loss of TNF-LTα genes in mice resulted in high susceptibility to BCG infection (Jacobs *et al.*, 2006). Therefore, the long term up-regulation of TNFα gene in Japanese flounder suggested that the host may have induced cell-mediated immune response against *M. bovis* BCG.

DTH reactions to bacterial antigens have been observed in lamprey, elasmobranches, chondrosteans and teleosts (Nakanishi *et al.*, 1999). Bartos and Sommer (1981) have also reported on DTH skin reactions against *M. tuberculosis* and *M. salmoniphilum* in rainbow trout (*Oncorhynchus mykiss*). In this study, DTH reaction was used to test whether vaccinated fish achieved specific cell-mediated immunity against *Mycobacterium* sp. antigens. Only BCG vaccinated fish showed up-regulation of the inflammatory cytokines gene expression at 1 or 3 days post-injection of PPD. During the elicitation phase of DTH, IL-1β and TNFα play major role in the efficient priming of T cells and the activation of dendritic cell (Nambu, Nakae and Iwakura, 2006), inducing chemokine production leading to an influx of other inflammatory cells (Harumi and Goldman, 2007). In contrast, IL-6 has a pivotal role in decreasing swelling at the local site to suppress or heal inflammation during the late phase of DTH (Mihara *et al.*, 1991). These results suggested that a DTH response similar to the DTH response in mammals occurred in Japanese flounder and that the BCG vaccine induced specific cell-mediated immunity against the antigen of *Mycobacterium* sp.

BCG can protect against various mycobacteriosises such as tuberculosis, leprosy, Buruli ulcer and paratuberculosis (Heinzmann *et al.*, 2008) in mammals. All mycobacteria possess mycolic acid, a component of the cell wall. CD1 molecules present mycolic acid as a lipid antigen to restricted $CD8^+$ T cells (Beckman *et al.*, 1994). Sugita (2006) suggested that the wide-spectrum efficacy of BCG is dependent on the response of CD1-restricted T cells to the lipid antigen. In this study, BCG vaccine was more effective at protecting the fish from *Mycobacterium* sp. infection than FKC vaccine. Hence, our data suggest that the Japanese flounder immune systems recognize and present mycobacterial common antigens such as mycolic acid and protect fish from *Mycobacterium* sp.

Although a return to virulence might have been concerned, live attenuated vaccine is useful for fish disease which is difficult to be eradicated by inactivated vaccine. Some mutant strains of pathogenic bacteria and some species related to pathogenic bacteria have been studied as live attenuated vaccines in fish. In fact, a mutant strain of *Edwardsiella ictaluri*

has been licensed as live attenuated vaccine against enteric septicemia of channel catfish in U.S.A. (Klesius and Shoemaker, 1999). In addition, *Arthrobacter* spp. has been also licensed as a live vaccine against bacterial kidney disease of salmonids in North America and Chile (Griffiths, Melville and Salonius, 1998). BCG vaccine has been used in mammals for a long time and shown stability of the avirulence (Calmette and Plotz, 1929), BCG vaccine may be useful against mycobacteriosis also in aquaculture industry.

In conclusion, intramuscular vaccination of Japanese flounder with BCG vaccine induced an immediate immune response and specific cell-mediated immune response against the antigens of *Mycobacterium* sp. Furthermore, BCG confers some protection efficacy against *Mycobacterium* sp. infection for Japanese flounder. It is likely that BCG will also be useful as a vaccine for other aquaculture species.

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