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Application of zebrafish as a model for evaluation of vaccine efficacy against *Philasterides dicentrarchi* (Ciliphora: Scuticociliatia)

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Zebrafish was firstly applied to an experimental model for scuticociliatosis caused by *Philasterides dicentrarchi*, a facultative parasitic ciliate in cultured marine fish. The susceptibility of zebrafish to infection of *P. dicentrarchi* was assessed by intraperitoneal injection of the ciliates, which produced typical symptoms of scuticociliatosis and significant mortality. The potential use of zebrafish as a model to evaluate the vaccine efficacy against scuticociliatosis was analyzed by immunization of zebrafish with the ciliates lysate. Furthermore, the effect of different adjuvants, such as Quillaja saponin (QS), Montanide, and Freund' s incomplete adjuvant (FIA) on the protective efficacy of the vaccine was investigated. Groups of zebrafish injected with QS or Montanide alone showed higher survival of fish against challenge test compared to control fish. The results suggest that adjuvant-mediated enhancement of innate immune responses play important roles in protection of fish against scuticociliatosis. The considerably high survival in the fish immunized with the antigen alone indicates that the ciliate lysate itself is highly immunogenic to zebrafish, which can elicit protective immune responses. The protective potential of the antigen, ciliate lysate, was enforced through combined administration with adjuvants including QS, Montinide and FIA. No or low mortalities in the groups of fish immunized with the antigen plus adjuvants suggests that the adaptive immune responses of zebrafish might be accelerated by the adjuvants or the protective potential of the antigen and adjuvants might synergistically interact. In spite of several shortcomings such as difficulties in sampling of serum and leucocytes enough to routine immunological analyses, zebrafsih might be the most convenient experimental animal for scuticociliatosis.

Key words: *Philasterides dicentrarchi*, Zebrafish, Scuticociliatosis model, Vaccine, Adjuvant

The facultative parasitic marine ciliate, *Philasterides dicentrarchi*, is a notorious culprit of scuticociliatosis in diverse cultured marine fish species (Dragesco *et al*., 1995; Iglesias *et al*., 2001; Kim *et al*., 2004). Although attempts to control scuticociliatosis using various chrmotherapeutants have been made (Iglesias *et al*., 2002; Quintela *et al*., 2003; Paramá *et al*., 2007), there is at present no effective in vivo chemotherapeutant especially for systemic infections. Therefore, development of preventive measures using vaccines or immunostimulants would be highly desirable.

During the last decades, the zebrafish, *Danio rerio*, has been a choice of experimental animal model in various fields of the life science. Recently, zebrafish has been utilized as an infection model for several viral and bacterial diseases in cultured fish, such as infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) (LaPatra *et al*., 2000; Garner *et al*., 2003; Wang *et al*., 2006), spring viremia of carp virus (SVCV) (Sanders *et al*., 2003; Wang *et al*., 2006), Snakehead rhabdovirus (SHRV) (Phelan *et al*., 2005), nervous necrosis virus (NNV) (Lu *et al*.,

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2008), viral haemorrhagic septicemia virus (VHSV) (Novoa *et al*., 2006), infectious spleen and kidney necrosis virus (ISKNV) (Xu *et al*., 2008), *Listonella anguillarum* (O' Toole *et al*., 2004; Rojo *et al*., 2007), *Edwardsiella tarda* (Pressley *et al*., 2005), *Aeromonas salmonicida* (Lin *et al*., 2007), *Aeromonas hydrophila* (Rodríguez *et al*., 2008), *Flavobacterium columnare* (Moyer and Hunnicutt, 2007), *Streptococcus iniae* (Neely *et al*., 2002), and *Mycobacterium marinum* (Pouty *et al*., 2003; Meijer *et al*., 2005; Swaim *et al*., 2006; Haeeiff *et al*., 2007; Watral and Kent, 2007). However, to our knowledge, there is no report on the use of zebrafish as a model for fish parasitic diseases.

The aim of the present study was to establish a zebrafish model of *Philasterides dicentrarchi* infection that could be helpful to investigate the efficacy of vaccines or immunostimulants. The susceptibility of zebrafish to infection of *P. dicentrarchi* was assessed by intraperitoneal injection of the ciliates, which produced typical symptoms of scuticociliatosis and significant mortality. The potential use of zebrafish as a model to evaluate the vaccine efficacy against scuticociliatosis was analyzed by immunization of zebrafish with the ciliates lysate. Furthermore, the effect of different adjuvants, such as Quillaja saponin, Montanide, and Freund' s incomplete adjuvant on the protective efficacy of the vaccine was investigated.

Materials and Methods

Zebrafish

Zebrafish *Danio rerio* (approximately 3 cm in body length) were obtained from a local aquarium and maintained at 28° C in dechlorinated tap water. Fish were fed at least twice a day with commercially available dry flake food and bit food, and were acclimated for more than two weeks before experiments.

Ciliates

Ciliates were isolated from ascitic fluid of a diseased olive flounder *Paralichthys olivaceus* collected from a local fish farm in Korea, and were identified as *Philasterides decentrarchi* using species specific oligonucleotide primers (Kim *et al*., 2004). Chinook salmon epithelia (CHSE)-214 cells, incubated at 20° C in Eagle' s minimum essential medium (MEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum, were used to grow the ciliates.

Infection of zebrafish

Zebrafish were anesthetized with MS222 (Sigma), and were injected intraperitoneally (i.p.) with 1 x 10^4 - 1 x 10^5 cells of *P. dicentrarchi* in a 10 $\mu\ell$ volume. Control groups were similarly anesthetized and injected with phosphate buffered saline (PBS). The external symptoms of the fish following infection were examined and mortality was monitored for two weeks.

For histological examination, dying fish were removed, anesthetized and fixed in Bouin' s fixative solution for two days. After embedding in paraffin, at least 9 serial longitudinal sections per fish with 5 μm thick were made and stained with hematoxylin and eosin (H&E).

Antigen preparation

The cultured ciliates were collected by centrifugation at 200 g for 5 min and washed three times in Hank' s balanced salt solution (HBSS, Sigma). The number of cells was counted using a hemocytometer after the final washing. The cell pellets were lysed through two rounds of freeze-thaw cycle and

resuspended in PBS.

Immunization

Prior to immunization and challenge infection, zebrafish were anesthetized with MS222. The antigen dose administered to each zebrafish was corresponding to 2 x 10^4 cells of *P. dicentrarchi*. Fish were divided into eight experimental groups (20 fish/group), and were i.p. injected with 10 $\mu\ell$ of following formulations. Group 1 received PBS alone, and group 2 received the antigen alone. Fish in group 3 were injected with 10 μg of Quillaja saponin (QS, Sigma) suspended in PBS, and group 4 were immunized with the antigen plus 10 μg QS. Group 5 and group 7 received Montanide ISA 70 (Montanide, Seppic) and Freund's incomplete adjuvant (FIA, Sigma) emulsified in same volume of PBS, respectively. Group 6 received the antigen plus Mon, and group 8 received the antigen plus FIA. All the fish were boost-injected with the same formulations two weeks after the first injection.

Challenge test

Two weeks after the boost immunization, fish of each group were challenged by i.p. injection of 4.5 x 10^4 CHSE-cultured *P. dicentrarchi*. Dead fish during the experiment were immediately removed and necropsied to confirm the presence of ciliates in the ascitic fluid and internal organs. The mortalities were recorded for 2 weeks, and all survived fish were also necropsied to confirm the presence or absence of the ciliates.

Results

Symptoms and Mortality

Zebrafish infected with *P. dicentrarchi* showed lethargic swimming behavior with an increased rate of respiration and darkness of the body surface. Fish also showed severe abdominal dropsy with ascites and local hemorrhages (Fig. 1). The ciliates were found in the ascitic fluid and even in the brain of dead fish. Zebrafish i.p. injected with 1 x 10^5 ciliates showed 100% mortality within one day after the injection. The cumulative mortality was lowered in proportion to decrease of the challenged ciliates number, and about 20-50% of fish was died within 5 days post challenge by i.p. injection with 1 x 10^4

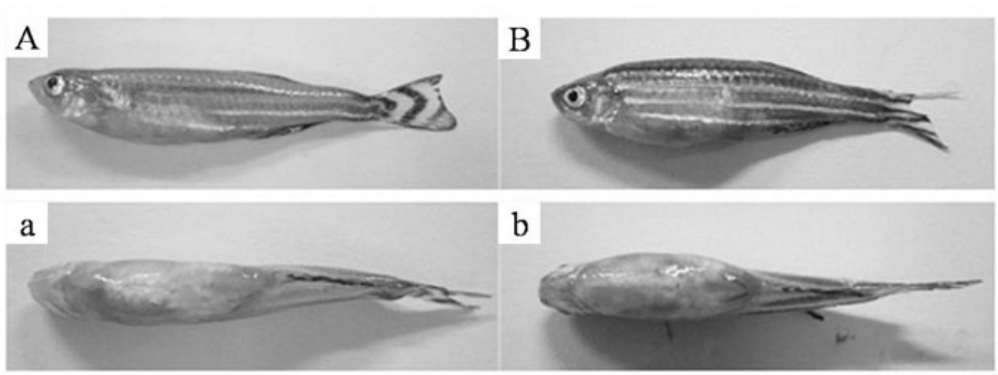

Fig. 1. External symptoms of zebrafish 48 h post intraperitoneal injection with *Philasterides dicentrarchi*. Male (A, a) and female (B, b) zebrafish showed similar external symptoms, such as severe abdominal dropsy with ascites and local hemorrhages.

ciliates.

Histopathology

Histopathologically, typical inflammation responses were elicited by the infection of *P. dicentrarchi*. As shown in Fig.2, ciliates were found in the internal organs including kidney (Fig. 2 A), liver (Fig. 2 E,F), body cavity (Fig. 2 C) and gonad (Fig. 2 B). Furthermore, penetration of the ciliates into the beneath (Fig.2 D) or in the brain (Fig.2 G, H, I) was observed.

Immunization experiment

All experimental groups administered each adjuvant alone or each adjuvant plus the ciliates lysate showed lower cumulative mortalities than the control PBS injected group (Fig. 3). The relative percentage survival (RPS) of fish injected each adjuvant alone was 37% in QS, 68% in Mon, and 53% in FIA. The RPS of fish in the group immunized with ciliates lysate plus QS or FIA was 81% and 94%, respectively. No mortality was detected in the group immunized with ciliates lysate plus Montanide.

Discussion

In the present study, artificial infection of zebrafish with *Philasterides dicentrarchi* elicited typical symptoms of systemic scuticociliatosis and high mortalities, suggesting that zebrafish could be used as an infection model for scuticociliatosis caused by *P. dicentrarchi*. Although the intraperitoneal challenge did not reflect the natural infection routes, the exact mechanism of systemic infection of the ciliates even in marine fish is not elucidated. Therefore, i.p. injection has been the main method for inducing systemic infection of *P. dicentrarchi* in cultured marine fish (Iglesias *et al*., 2003; Sitjà-Bobadilla *et al*., 2008; Lee and Kim, 2008; Sanmartín *et al*., 2008). The external symptom of zebrafish infected with *P. dicentrarchi* was similar to that of olive flounder fingerling infected experimentally with the ciliates in that lethargy, severe

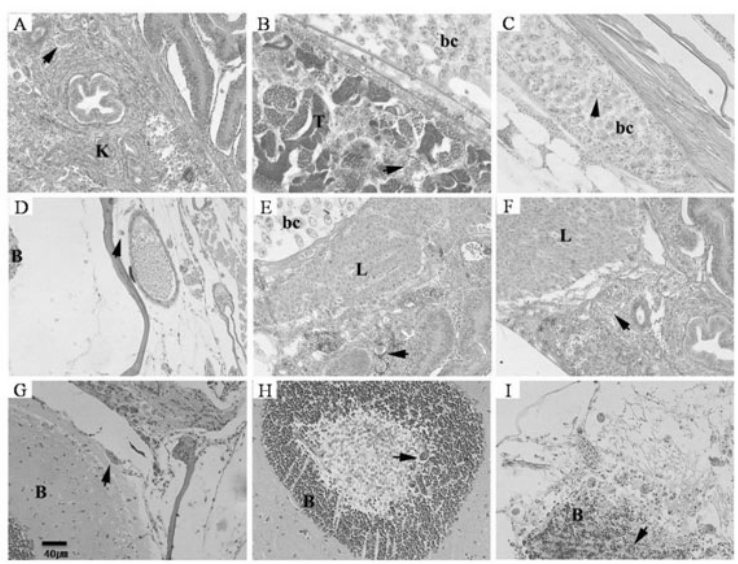

Fig. 2. Histological examination of zebrafish infected with *P. dicentrarchi* by intraperitoneal injection. Paraffin embedded samples were stained with hematoxylin and eosin. Ciliates (black arrows) were found frequently in spaces of body cavity (bc), kidney (K), liver (L), testis (T), brain (B). Numerous small inflammatory cells were appeared around ciliates.

abdominal dropsy, and hemorrhages. Inflammation in almost all internal organs, which was demonstrated in the present histopathological observation, and active devouring of fish tissues by the ciliates might be responsible for the symptom and acute mortality of the present experimentally infected zebrafish.

Recently, several studies have demonstrated that immunization of cultured marine fish with *P. dicentrarchi* lysate or formalin fixed ciliates was able to confer a certain degree of protection against challenge infection (Iglesias *et al*., 2003; Sitjà-Bobadilla *et al*., 2008; Lee and Kim, 2008; Sanmartín *et al*., 2008). In this study, zebrafish was chosen as a model for assessment of vaccine efficacy against scuticociliatosis. The advantages of zebrafish model in comparison with marine fish such as olive flounder, turbot or sea bass, are i) easy maintenance in laboratory conditions, which allows reduction of experimental space and cost; ii) no need for sea water, which can alleviate cost and labor for water quality control; iii) completely naive to *P. dicentrarchi*, which is important to assess vaccine efficacy; and iv) convenient to analysis of gene function in relation to immune responses.

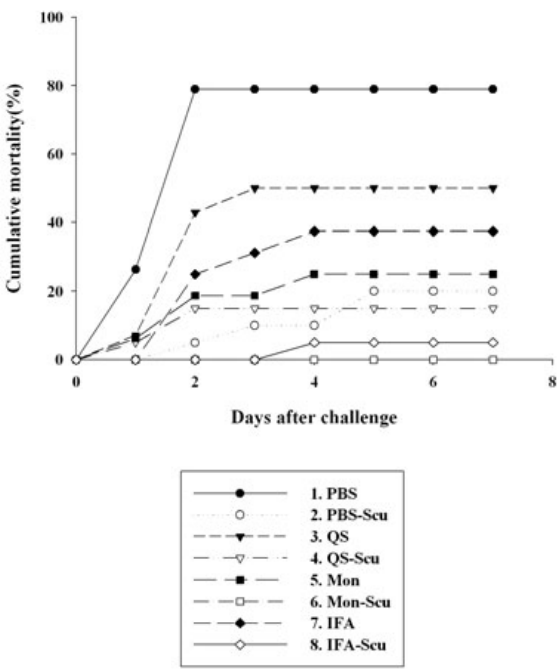

Fig. 3. Cumulative mortality of zebrafish challenged with *Philasterides dicentrarchi* after immunization. Zebrafish were immunized with the lysate of *P. dicentrarchi* (PBS-Scu) or mixed with different adjuvants including Quillaja saponin (QS-Scu), Montanide ISA 70 VG (Mon-Scu), and Freund's incomplete adjuvant (IFA-Scu). As controls, fish were injected with Phosphate buffered saline (PBS), Quillaja saponin (QS), Montanide (Mon), or Freund's incomplete adjuvant (IFA) alone. Each fish in each group was challenged by i.p. injection of 4.5 x 10^4 of *P. diecentrarchi* two weeks after booster injection.

Adjuvants have been proven effective in the enhancement of adaptive immune responses through stimulating innate immune factors. Recently, Lee and Kim (2008) demonstrated the reduced mortality of olive flounder injected with Freund's adjuvant alone against *P. dicentrarchi* challenge. In the present study, zebrafish injected with FIA alone also showed higher survival rates than fish injected with PBS alone. However, as Freund's adjuvants have been known to have side effects in immunized fish (Koppang *et al*., 2004, 2005), QS and Montanide were used as adjuvants alternative for Freund's adjuvants in the present scuticociliatosis vaccine experiment. Groups of zebrafish injected with QS or Montanide alone showed higher survival of fish against challenge test compared to control fish. The results suggest that adjuvant-mediated enhancement of innate immune responses play important roles in protection of fish against scuticociliatosis.

Iglesias *et al*. (2003) reported that turbot immunized with the ciliate lysate plus Freund's complete adjuvant (FCA) showed greatly higher survival rate (73.7%) than PBS control group (0%), while no fish were survived when immunized with the ciliate lysate alone. Similarly, Sanmartin *et al*. (2008) reported that turbot immunized with formalin fixed

P. dicentrarchi alone did not increase the survival rate of fish against challenge infection. Only fish immunized with the antigen plus adjuvant showed higher survival rate than control PBS injected fish. Whereas Sitjà-Bobadilla *et al*. (2008) reported that no mortality was registered in turbot immunized with *P. dicentrarchi* antigen alone or antigen plus adjuvant (Montanide) against challenge infection. At present, although the causes of discrepancies among those results are not known, we got a result similar to that of Sitjà-Bobadilla *et al*. (2008) in this study. The considerably higher survival in the fish immunized with the antigen alone indicates that the ciliate lysate itself is highly immunogenic to zebrafish, which can elicit protective immune responses. The protective potential of the ciliates lysate antigen was enforced through combined administration with adjuvants including QS, Montinide and FIA. No or low mortalities in the groups of fish immunized with the antigen plus adjuvants suggests that the adaptive immune responses of zebrafish might be accelerated by the adjuvants or the protective potential of the antigen and adjuvants might synergistically interact.

In the present study, we firstly applied zebrafish in an experimental model for scuticociliatosis caused by *P. dicentrarchi*. In spite of several shortcomings such as difficulties in sampling of serum and leucocytes enough to routine immunological analyses, zebrafsih might be the most convenient experimental animal for preliminary assessment of vaccine efficacy against scuticociliatosis. To elucidate the protective mechanism of the vaccines in zebrafish, further studies on the responses of immune-related genes are needed.

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