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Maternal transfer and protective role of antibodies in zebrafish *Danio rerio*

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ABSTRACT

Maternal transfer of antibodies from mother to eggs has been reported in various species of fishes, and these antibodies have been proposed to play immune roles in developing embryos and larvae. However, firm evidence supporting this remains lacking. In this study, we clearly demonstrated that immunization of female zebrafish with the hapten–carrier complex, trinitrophenylated bovine serum albumin (TNP-BSA), induced a significant increase in anti-TNP antibody production in the mothers, which in turn induced a marked increase in anti-TNP antibody level in their eggs. Microinjection of anti-zebrafish IgM antibody into early embryos (to neutralize endogenous antibody action) resulted in a remarkable decrease in the resistance of recipient embryos to pathogenic *Aeromonas hydrophila*, whereas injection of BSA or anti-β-actin monoclonal antibody into the same stage embryos had little effect on their resistance to the pathogen. Moreover, the content of endogenous antibodies in eggs was clearly correlated with their antibacterial activity against *A. hydrophila*. This is the first report showing that maternally transferred antibodies in fish can protect early embyros/larvae from the attack of pathogens like *A. hydrophila*.

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1. Introduction

The immune system of zebrafish *Danio rerio* begins to develop in the early embryonic stages soon after fertilization. The expression of *ikaros*, which encodes a transcription factor involving in the development of hemato-lymphoid system, is observed as early as 16 h post fertilization (hpf) (Willett et al., 2001). The thymic anlage, albeit devoid of lymphocytes, forms at 54 hpf, and colonization of primary T cells takes place at 65 hpf (Willett et al., 1997, 1999, 2001). From 4 day post fertilization (dpf) onward, T cells differentiate in the thymus; at 21 dpf, the thymus becomes morphologically mature, full of numerous T lymphocytes (Willett et al., 1999). With respect to B lymphopoiesis, rearrangements of genes encoding B cell receptors are detected at 4 dpf in DNA extracted from whole larvae and *rag-1* is expressed in the pancreas; by day 10 dpf, immunoglobulin (Ig) heavy chain gene is expressed in the pancreatic tissue (Danilova and Steiner, 2002). Transcripts of membrane and secreted forms of IgM gene are detected at 7 and 10 dpf, respectively, and at protein levels, IgM is measured by 28 dpf, when zebrafish is believed to be able to respond to immunization by production of specific immunoglobulin (Lam et al., 2004). Clearly, there exists a window of around 28 days in which the adaptive immune response of zebrafish larvae is not yet fully competent.

Zebrafish eggs are released and fertilized externally, and the resulting embryos and larvae are therefore exposed to an aquatic environment full of potential pathogens capable of causing various types of diseases. Moreover, zebrafish eggs are cleidoic, i.e. closed free-living system following fertilization, which are supposed to solely rely on the maternal provision of immune-relevant molecules for protection against invading pathogens before full maturation of immune system. Previous studies on several fish species have shown that maternal transfer of the innate immune factors including the complement components C3 and Bf (Ellingsen et al., 2005; Huttenhuis et al., 2006; Løvoll et al., 2007; Wang et al., 2008, 2009), lectins (Bildfell et al., 1992; Tateno et al., 2002; Jung et al., 2003), lysozymes (Yousif et al., 1991, 1994; Wang and Zhang, 2010) and Vg-derived yolk proteins such as phosvitin (Pv) (Wang et al., 2011) are able to be transferred from mother to offspring. Likewise, maternal transfer of IgM to offspring has also been reported in different teleost species (Bly et al., 1986; Picchietti et al., 2004; Hanif et al., 2005). These maternally transferred molecules have been proposed to be involved in the immune defense against pathogens in developing fish embryos and larvae. Consistently, the maternal complement components C3 and Bf and yolk protein Pv have been demonstrated to be attributable to the protection of *D. rerio* embryos against microbial attack (Wang et al., 2008, 2009, 2011). Similarly, maternally transferred IgM has also been associated with the enhanced survival rates of carp and sea bream

Abbreviations: TNP-BSA, trinitrophenylated bovine serum albumin; BSA, bovine serum albumin; TNP-OVA, trinitrophenylated ovalbumin; OVA, ovalbumin; hpf, hour post fertilization; dpf, day post fertilization; C3, third complement factor; Bf, factor B; Vg, vitellogenin; Pv, phosvitin.

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larvae (Hanif et al., 2005; Swain et al., 2006), but firm evidence supporting this remains lacking. Zebrafish is a widely used model organism, whose immune system has been extensively studied (Trede et al., 2004), but little information is available regarding the maternal transfer of antibodies in this important model animal. This study was therefore undertaken to address these issues via injection of the hapten–carrier complex, trinitrophenylated bovine serum albumin (TNP-BSA), into *D. rerio*, to elicit an anti-hapten antibody response. TNP has a single antigenic determinant, but it must be conjugated to a carrier protein to provoke the production of antibodies. If maternal transfer of antibodies occurs in *D. rerio*, then anti-TNP antibodies will be present in their eggs. To test if the maternally transferred antibodies can protect the developing embryos against pathogenic *Aeromonas hydrophila*, anti-zebrafish IgM antibody was injected into the embryos to inactivate the action of the maternally transferred antibodies, and to examine the susceptibility of recipient embryos to *A. hydrophila*.

2. Materials and methods

2.1. Fish

Wild type zebrafish *D. rerio* were purchased from a local fish dealer and maintained in containers with well-aerated tap water at $27 \pm 1\,^{\circ}$C. The fishes were fed on live bloodworms and fish flakes (Tetramin, Germany) twice a day.

2.2. Fish immunization

TNP-BSA (Biosearch Technologies, Novato, CA) was dissolved in 10 mM PBS (pH 7.4), yielding a concentration of 1.0 mg/ml. A total of 40 sexually matured female *D. rerio* at age of about 5 months old were divided into two groups: experimental group and control group. Individuals of *D. rerio* in the experimental group were anesthetized with 120 μg/ml MS222 and injected abdominally with 20 μl of PBS-buffered TNP-BSA as a primary immunization. A secondary immunization was conducted with the same volume of PBS-buffered TNP-BSA 8 days after the primary immunization (Poorten and Kuhn, 2009). Similarly, individuals of *D. rerio* in the control group were injected with 20 μl of sterile PBS as a primary immunization and with the same volume of sterile PBS as a secondary immunization. After immunization, all the fishes were maintained in two separate tanks with well-aerated tap water at $27 \pm 1\,^{\circ}$C.

2.3. Preparation of egg cytosol and serum

Female *D. rerio* from both immunized and non-immunized groups were mated with the healthy males at day 10 after the secondary immunization. In brief, female and male *D. rerio* were placed in the late evening in a 10 l tank at a female to male ratio of 2:1, and maintained at $27 \pm 1\,^{\circ}$C. The naturally fertilized eggs, which were usually at 2- to 8-cell stage, were collected in the next early morning. The unhealthy eggs were removed, and the healthy fertilized eggs were rinsed three times with double-distilled H_2O and then once with ice-cold double-distilled H_2O. After the excess H_2O was withdrawn, the eggs were immediately homogenized on ice for 30 s, and centrifuged at $15{,}000 \times g$ at 4 °C for 30 min. The supernatant, egg cytosol, was pooled and added with protease inhibitor cocktail (Roche, Indianapolis, IN), aliquoted and stored at −70 °C until used.

After spawning, female *D. rerio* from both immunized and non-immunized groups were sampled, respectively, to collect the blood by the method of Jagadeeswaran and Sheehan (2003) at day 10 post secondary immunization. The blood collected was allowed to clot for 2 h at 4 °C and the serum was pooled after centrifugation for 10 min at $3000 \times g$ for 10 min. The sera were aliquoted and stored at −20 °C until use.

2.4. Assay for specific antibody titer in egg

The specific antibody titer in eggs was determined by an ELISA assay according to the method of Poorten and Kuhn (2009). Aliquots of 40 μl of 50 μg/ml TNP-BSA, trinitrophenylated ovalbumin (TNP-OVA, Biosearch Technologies, Novato, CA), bovine serum albumin (BSA, Roche, Indianapolis, IN) and ovalbumin (OVA, Sigma, St. Louis, MO) solved in PBS (pH 7.4) were coated onto the wells in a 96-well microplate (Costar). The wells were air-dried overnight at room temperature, blocked with 200 μl of 4% non-fat dry milk in PBS (pH 7.4) at 37 °C for 2 h, and then washed four times with PBS containing 0.1% Tween-20. The egg cytosol from TNP-BSA-immunized females was serially diluted in PBS, giving (protein) concentrations of 600, 400, 200 and 100 μg/ml, respectively. For negative control, PBS was used instead of the egg cytosol. A total of 50 μl diluted egg cytosol or PBS was added into each well. After incubation at room temperature for 1 h, the wells were each washed three times with 200 μl PBS containing 0.1% Tween-20. The anti-zebrafish IgM antibody, which was produced in rabbits immunized with IgM of *D. rerio* and affinity-purified (Gong et al., 2009), was diluted to 1:4000 in PBS with 1% non-fat dry milk, and then aliquots of 100 μl diluted anti-zebrafish IgM antibody were added into each well, and incubated at room temperature for 2 h. After washing four times in PBS containing 0.1% Tween-20, a total of 100 μl horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) diluted to 1:4000 in PBS with 1% non-fat dry milk was added into each well and incubated at room temperature for 1 h. Subsequently, 100 μl of 400 μg/ml O-phenylenediamine (OPD; Amresco) dissolved in 51.4 mM Na_2HPO_4, 24.3 mM citric acid and 0.045% H_2O_2 (pH 5.0) was added into each well. After incubation at 37 °C for 20 min, 25 μl of 2 mM H_2SO_4 was added into each well to terminate the reactions, and the absorbance (OD) at 492 nm was obtained using a microplate reader (GENios Plus, Tecan). The binding affinity of the antibodies in eggs to each antigen was calculated by subtraction of the OD of negative control from the OD of each dilution.

2.5. Assay for specific antibody levels in serum and egg

To test the level of specific antibody against TNP in sera and eggs in immunized *D. rerio*, aliquots of 40 μl of 50 μg/ml TNP-BSA and TNP-OVA in PBS (pH 7.4) were coated onto the wells in a 96-well plate and air-dried overnight at room temperature. After blocking with 200 μl of 4% non-fat dry milk in PBS (pH 7.4) at 37 °C for 2 h, the wells were washed four times with PBS containing 0.1% Tween-20. PBS was incubated with each coated antigen as negative control. The sera and egg cytosols from each TNP-BSA-immunized *D. rerio* were adjusted with PBS to the concentrations of 100 and 300 μg/ml, respectively. A total of 50 μl diluted sera or egg cytosols was added into each well and incubated at room temperature for 1 h. This experiment was repeated for three times. For control, the sera or egg cytosols from non-immunized *D. rerio* were processed similarly. The wells were then washed three times with 200 μl PBS containing 0.1% Tween-20, and the ELISA was carried out as above.

2.6. Assays for protective role of antibody in developing embryo

Gram-negative bacterium *A. hydrophila* LSA 20 (pathogenic to *D. rerio*) was incubated in the tryptic soy broth (pH 7.4) medium (which consists of 1.7% tryptone, 0.3% soy peptone, 0.5% NaCl, 0.25% K_2HPO_4 and 0.25% glucose) at 28 °C for 16 h and then harvested by centrifugation at $3500 \times g$ at 4 °C for 15 min. The bacterial pellet was resuspended in 10 mM PBS (pH 7.4) and adjusted to a density of 1×10^8 cells/ml.

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