Complement Activity in the Egg Cytosol of Zebrafish

Danio rerio: Evidence for the Defense Role of Maternal Complement Components

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Most fish embryos that develop externally are exposed to an environment full of microbes. How they survive microbial attacks are not understood to date. Here we demonstrated that the egg cytosol prepared from the newly fertilized eggs of zebrafish Danio rerio is capable of killing the Gram-negative bacterium Escherichia coli, via in vitro assay system of the complement activity established. All findings indicate that it is the complement system operating via the alternative pathway that is attributable to the bacteriolytic activity. This is the first report providing the evidence for the functional role of the maternal complement components in fish eggs, paving the way for study of maternal immunity in other organisms whose eggs are fertilized in vitro.

INTRODUCTION

Most fish embryos that develop externally are exposed to an environment full of microbes, many of which are pathogens capable of killing other organisms. Furthermore, maturation of immunocompetence in fish develops quite late although both lymphoid organs and T- and B-lymphocytes may appear early in embryogenesis [1]. Therefore, developing fish embryos and larvae have little or only limited ability to synthesize immune-related molecules endogenously [2,3]. How developing fish embryos and larvae survive microbial attacks is one of the central problems for reproductive and developmental immunology, but little information as such is available to date.

Complement system consisting of approximately 35 plasma and membrane-bound proteins comprises one of the first lines of defense against pathogenic infection by alerting host the presence of potential pathogens as well as clearing pathogens. There are three pathways by which the complement system can be activated: the classical pathway (CP), the alternative pathway (AP) and the lectin pathway (LP). The CP activation is initiated by binding of antibody to the C1 complex, formed by C1q and two serine proteases (C1r and C1s), or by direct binding of the C1q component to the pathogen surface, and requires both Ca2+ and Mg2+ [4–6]. The AP is mainly triggered by the certain structures on microbial surface in an antibody-independent manner, and requires Mg2+ alone [7,8]. The C3 is cleaved spontaneously in plasma to yield C3b which interacts non-covalently with factor B (Bf) and factor D, resulting in the formation of the alternative C3 convertase [9]. The LP is activated by binding of microbial polysaccharides to circulating lectins, such as mannose-binding lectin (MBL), and requires Ca2+ [10–13]. MBL binds to mannose residues which then results in the cleavage of C4 via mannose-binding protein-associated serine esterase. All the three pathways merge at a common amplification step involving C3, a central complement component being a part of all the three pathways, and proceed through a terminal pathway that leads to the formation of a membrane attack complex, which can directly lyse microbial cells. Maternal transfer of complement components such as C3, C4, C5 and Bf to offspring has recently been demonstrated in rainbow trout [14]. Maternal transmission of C3 has also been reported in carp [15] and spotted wolffish [16]. Likewise, immunoglobulins (IgM) has been shown to be present in eggs and early embryos of several fish species including carp [17,18], tilapia [19,20], sea bream [21,22], sea bass [23,24], rainbow trout [25], salmon [26] and plaice [27]. These transferred maternal molecules have been proposed to be involved in the early defense against pathogens in developing fish embryos and larvae. It has recently been shown that the homogenates of Atlantic salmon embryos have very low hemolytic activities [28]. However, whether the maternal complement components function during early embryogenesis remains uncertain.

In this study, we have sought to establish an in vitro system to assay the complement-mediated bacteriolysis in the extracts of fertilized eggs of zebrafish Danio rerio, and thereby to examine which complement pathway is responsible for the bacteriolytic activity in the early developing embryos.

RESULTS

Bacteriolytic activity of egg cytosol

The protein concentration of the cytosols prepared from the fertilized eggs of zebrafish D. rerio ranged from 13.8 mg/ml to 15.9 mg/ml, with an average of 14.9 mg/ml. The egg cytosol exhibited a conspicuous bacteriolytic activity to Escherichia coli in a temperature-dependent manner (Fig. 1A and B). Although the egg cytosol had only slight bacteriolytic activity at low temperature (4°C), the lytic activity increased markedly as temperature rose, with the

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optimum temperature being 25°C. When the cytosol was pre-incubated at 37°C for 2 h, it lost the bacteriolytic activity completely.

Complement activity
Pre-incubation of anti-C3 antibody with the egg cytosol was capable of abrogating the bacteriolytic activity to E. coli in a concentration-dependent manner (Fig. 2A), which strongly suggested a role for complement in the lytic activity of the egg cytosol. This was further supported by the fact that heating the cytosol (45°C, 30 min) significantly reduced (p<0.01) the bacteriolytic activity, with an inhibition rate of 24.3% observed contrasting to that of 48.8% in control (Fig. 2B).

It has been shown that C3b can covalently bind to zymosan particles [29], and thus the AP-mediated bacteriolytic activity can be selectively inhibited by addition of zymosan. It was found that the lytic activity of the egg cytosol was significantly decreased by pre-incubation with zymosan, with an inhibition rate of 24.9% contrasting to that of 32.1% in control (Fig. 3). Similarly, pre-incubation with the antibody against Bf, a key enzyme in the AP activation, also resulted in a marked reduction of the lytic activity of the egg cytosol (23.8%; Fig. 3). In contrast, pre-incubation with either mammalian CP inactivator L-lysine [30,31] or anti-C1q antibody or anti-C4 antibody had little effects on the bacteriolytic activity to E. coli (Fig. 3). All these suggested that activation of the AP contributed to the bacteriolytic activity. In agreement, addition of 0.3 mM EGTA to the egg cytosol did not impair the bacteriolytic activity to E. coli, while addition of 0.5 mM EDTA to the cytosol significantly depleted its lytic activity to E. coli (Fig. 4). Moreover, the bacteriolytic activity of the EDTA-treated egg cytosol was able to be restored by addition of Mg²⁺, but not by addition of Ca²⁺ (Fig. 4).

Presence of C3 and Bf in egg cytosol
It was shown by Western blotting analyses that both rabbit anti-human C3 and goat anti-human Bf antibodies reacted with human serum and with the egg cytosol. The cytosol was reactive with rabbit anti-human C3 antibody, forming a main band (~185 kDa) equivalent to C3 and two minor bands (~115 kDa; ~70 kDa) resembling C3α and C3β chains, respectively (Fig. 5A). Similarly, the egg cytosol reacted with goat anti-human Bf...
antibody, producing a single positive band of ~93 kDa, matching that of human Bf (Fig. 5B).

**DISCUSSION**

The existence of complement components like C3 and Bf has recently been confirmed in rainbow trout, carp and spotted wolffish. In this study, we found that both C3 and Bf, the key factors functioning in the AP, are also present in the newly fertilized eggs of *D. rerio*, providing the first evidence for a maternal transfer of the complement proteins in zebrafish.

The role of maternal complement components in fish eggs has been proposed to be associated with the early defense against pathogens in developing fish embryos and larvae. However, little functional studies have been conducted thus far. Here we showed for the first time that the Gram-negative bacterium *E. coli* is sensitive to lysis by the cytosol prepared from the fertilized eggs of zebrafish *D. rerio*, and all the findings point to complement system being one of the most important factors involved in the bacteriolytic activity observed. First, the bacteriolytic activity was abolished by pre-incubation of anti-C3 antibody with the egg cytosol, a process that would cause the precipitation of the central component of all known complement pathways, C3. Second, the lytic activity was depleted by heating at 45°C, a temperature known to inactivate fish complement [32]. It has been observed that 2-day-old zebrafish embryos possess special macrophages, which have the ability to migrate to sites of infection and engulf bacteria injected [33,34]. The maternal complement components appear to function in the developing embryos earlier than the embryonic macrophages. It is possible that both macrophages and maternally-derived complement factors can protect the developing zebrafish embryos from microbial attacks.

To determine which pathway of complement activation might be involved in the bacteriolytic activity of the egg cytosol, the antibodies against C1q (a key component of CP), C4 (a key component of both CP and LP) and Bf (a key component of AP) were utilized to block the CP, LP or AP, respectively. It is found that precipitation of C1q and C4 causes little loss of the bacteriolytic activity of the egg cytosol, whereas precipitation of Bf results in a significant reduction of the lytic activity. Furthermore, addition of EGTA to remove Ca^{2+} from the egg cytosol, which can inhibit both CP and LP, induces little decrease in the bacteriolytic activity. In contrast, pre-incubation of EDTA with the egg cytosol leads to a substantial reduction of the bacteriolytic activity, and saturation of the chelator with Mg^{2+} is capable of restoring the lytic activity, but not by addition of Ca^{2+}. Moreover, selective inhibition of the AP by zymosan A induces marked loss of bacteriolytic activity, while addition of L-lysine, an inactivator of the CP, is not inhibitory. Taken together, all these undoubtedly indicate that activation of the AP is responsible for the bacteriolytic activity of the egg cytosol, but the CP and LP have little contribution to the lytic activity.

In summary, the complement system operating via the AP plays a crucial role in the developing zebrafish embryos and larvae. As the maternal transfer of immune factors to piscine offspring is widespread, it is highly likely that the complement-mediated killing of pathogens may generally occur in the early developmental stages of fishes. It will be of interest in the future to study if the maternal IgM can aid activation of the AP in the developing fish embryos.
MATERIALS AND METHODS

Reagents
Ethylene-diamine tetraacetic acid (EDTA), ethyleneglycol-bis (β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA), zymosan A, L-lysine and bovine serum albumin (BSA) were purchased from Sigma (USA), and peptone and yeast extract were from OXOID (Japan). Rabbit anti-human C3 antibody was procured from Abcam (UK), and goat anti-human factor B (Bf) antibody from R & D (America). Goat anti-human C1q antibody, Rabbit anti-mouse C4 antibody and horseradish peroxidase (HRP)-labeled rabbit anti-goat IgG were from Boster (China), and HRP-labeled goat anti-rabbit IgG from Zhongshan (China). All other chemicals used were analytical reagents.

Preparation of Escherichia coli
The Gram-negative bacterium E. coli (P8760) was incubated in LB broth to logarithmic growth phase, and harvested by centrifugation at 3,000 g at 4°C for 10 min. The pellets were washed three times with sterilized 0.9% saline, re-suspended in the saline at a density of 10⁶ cells/ml, and used for the following experiments.

Preparation of egg cytosol
Mature male and female fishes D. rerio were placed in the late evening in a 10 litre tank at a female to male ratio of 2:1, and maintained at 26±1°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 H2O and then once with ice-cold double-distilled H2O. After the excess H2O was withdrawn, the eggs were immediately homogenized on ice for 30 seconds, and centrifuged at 15,000 g at 4°C for 30 min. The supernatant, egg cytosol, was pooled, aliquoted and stored at −70°C until used.

The protein concentrations were determined by the method of Bradford [35] with BSA as standard.

Assays for bacteriolytic activity

The egg cytosol was filtered through 0.22 µm filter (Millipore) before use. An aliquot of 120 µl of the egg cytosol was mixed with 6 µl of E. coli suspension with 10⁵ cells/ml, and the mixture was pre-incubated, with gentle stirring, at 25°C for 2 h. Subsequently, an aliquot of 20 µl of the mixture was sampled at 30, 60, 90, 120 and 180 min, respectively, diluted to a volume of 100 µl with sterilized 0.9% saline, and plated onto 3 LB agar plates (30 µl each plate). After incubation at 37°C for 24 h, the resulting bacterial colonies in each plate were counted. The control was processed similarly except that the cytosol was replaced with sterilized saline. The percent of bacterial growth inhibition by the cytosol was inferred from the difference between the numbers of colonies in the test and control.

To determine the optimum temperature for the bacteriolytic activity, 40 µl of the egg cytosol was mixed with 2 µl of E. coli suspension. The mixtures were pre-incubated at 4°C, 20°C, 25°C and 37°C, respectively, for 2 h, and the bacteriolytic activity was then assayed as described above.

Assays for inhibition of complement

The capacity of the antibodies against C3, Bf, C1q and C4 to inhibit the bacteriolytic activity of the egg cytosol was analyzed by the method of Nonaka et al. [36]. Briefly, 40 µl of the egg cytosol was pre-incubated with anti-C3, C1q, C4 and Bf antibodies at final concentrations of 0.12—0.28, 0.01, 0.08 and 0.24 µg/ml, respectively, at 25°C for 30 min, followed by addition of 2 µl of E. coli suspension. The mixtures were adjusted to a volume of 50 µl with sterilized saline, and incubated at 25°C for 2 h. The bacteriolytic activities were measured as described above. For control, the antibodies were replaced by sterilized saline. Prior to use, aliquots of 40 µl of the egg cytosol were inactivated by heating at 45°C for 30 min [8,37], and mixed with 2 µl of E. coli suspension. The mixtures were adjusted to 50 µl with sterilized saline, incubated at 25°C for 2 h, and the remaining bacteriolytic activities assayed. The egg cytosol without heating was used in control.

For chelation experiments, aliquots of 40 µl of the egg cytosol were mixed with 1.5 µl of 10 mM EGTA, 2.5 µl of 10 mM EDTA, 2.5 µl of 10 mM EDTA with 2.5 µl of 10 mM MgCl₂, and 2.5 µl of 10 mM with 2.5 µl of 10 mM CaCl₂, respectively, and pre-incubated at 25°C for 30 min. The mixtures were then combined with 2 µl of E. coli suspension, and adjusted to 50 µl with sterilized saline. After incubation at 25°C for 2 h, the bacteriolytic activity was determined. Control was processed similarly except that the chelator solutions were replaced by sterilized saline.

To inhibit the classical pathway, 40 µl of the egg cytosol was pre-incubated with 0.6 mM L-lysine at 25°C for 30 min, and then mixed with 2 µl of E. coli suspension. The mixtures were adjusted to 50 µl with sterilized saline. After incubation at 25°C for 2 h, the bacteriolytic activity was assayed.

The stock solution of 10 mg/ml zymosan A was prepared by boiling 10 mg zymosan A in 1 ml of 14 mM NaCl for 30 min, followed by centrifugation at 16,000 g for 5 min. To inhibit the alternative pathway, 100 µl (1 mg of zymosan) of the stock solution was centrifuged at 16,000 g for 5 min, and the zymosan pellet was washed twice in 40 µl of 0.9% saline, and re-suspended in 50 µl of sterilized saline. After incubation at 25°C for 2 h, the bacteriolytic activity was assayed.

The result of egg cytosol was mixed with 2 µl of E. coli suspension, and adjusted to 30 µl with sterilized saline. The mixture was incubated at 25°C for 2 h, and the bacteriolytic activity was tested.

Western blotting
To confirm the presence of C3 and Bf in the egg cytosol, Western blotting analysis was carried out. The egg cytosol and human serum were electrophoresed on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel using the buffer system of Laemmli [38]. The proteins separated were blotted onto nitrocellulose membrane (Hybond, Amersham Pharmacia), and immunostained with rabbit anti-human C3 antibody (1:1000) and goat anti-human Bf (1:400) antibody, followed by staining with HRP-labeled anti-rabbit IgG and HRP-labeled anti-goat IgG, respectively [39]. The molecular mass standards used were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97.2 kDa), serum albumin (66.4 kDa) and ovalbumin (44.3 kDa).

Statistical analysis
All experiments were performed in triplicate, and repeated at least three times. Data were subjected to statistical evaluation with ANOVA, and differences at p<0.05 were considered significant. All data were expressed as a mean±standard deviation (SD).

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Author Contributions
Conceived and designed the experiments: SZ. Performed the experiments: ZW GW YA. Analyzed the data: ZW. Contributed reagents/materials/analysis tools: SZ. Wrote the paper: ZW. Other: Modified the paper: SZ.
REFERENCES

1. Zapata A, Diez B, Cejalvo T, Friaas CG, Cortés A (2006) Ontogeny of the immune system of fish. Fish Shellfish Immunol 20: 126–136.
2. Magnadottir B, Lange S, Steinarsson A, Gudmundsdottir S (2004) The ontogenetic development of innate immune parameters of cod (Gadus morhua L.). Comp Biochem Physiol B Biochem Mol Biol 139: 217–224.
3. Ellis AE (1968) Ontogeny of the immune system in teleost fish. In: Ellis AE, ed (1980) Fish vaccination. London: Academic Press. p 20–31.
4. Kishore U, Reid KB (2000) C1q: structure, function and receptors. Immunopharmacology 49: 159–170.
5. Robertson M (1998) Innate immunity. Curr Biol 8: 595–597.
6. Zhang S, Wang C, Wang Y, Wei R, Jiang G, et al. (2003) Presence and characterization of complement-like activity in the amphioxus Branchiostoma belcheri tsingtauense. Zoolog Sci 20: 1207–1214.
7. Bly JE, Grimm AS, Morris RG (1986) Transfer of passive immunity from mother to young in a cestog fish: haemagglutinating activity in the serum and eggs of plaice, Pleuronectes platessa L. Comp Biochem Physiol A 84: 309–313.
8. Mor A, Avtalion RR (1990) Transfer of antibody activity from immunised mother to embryos in tilapias. J Fish Biol 36: 294–305.
9. Schwaeble W, Laursen SB, Thiel S, Vorup-Jensen T, Stover CM, et al. (1997) A survey on the complement system of the Atlantic salmon, Salmo salar L. Fish Shellfish Immunol 7: 29–43.
10. Picciotti S, Taddei AR, Scapigliati G, Buonocore F, Fausto AM, et al. (2004) Immunoglobulin protein and gene transcripts in ovarian follicles throughout oogenesis in the teleost Dicentrarchus labrax. Cell Tissue Res 315: 259–270.
11. Kuo MM, Lane RS, Giclas PC (2000) A comparative study of mammalian and reptilian alternative pathway of complement-mediated killing of Lyme disease spirochete (Borrelia burgdorferi). J Parasitol 86: 1223–1228.
12. Morgan BP (1995) Physiology and pathophysiology of complement: progress and prospects. Trans Proc 6: 1–7.
13. Kono MM, Lane RS, Giclas PC (2000) A comparative study of mammalian and reptilian alternative pathway of complement-mediated killing of Lyme disease spirochete (Borrelia burgdorferi). J Parasitol 86: 1223–1228.
14. Yano T (1996) The nonspecific immune system: Humoral defence. In: Iwama G, ed (1996) The nonspecific immune system of fish. Tokyo: Kinokuniya. p 3–15.
15. Swain P, Dash S, Bal J, Routray P, Sahoo PK, et al. (2006) Passive transfer of maternal IgM to larvae in tilapia, Oreochromis mossambicus. Fish Shellfish Immunol 7: 353–363.
16. Thiel S, Vorup-Jensen T, Stover CM, Schwaeble W, Laursen SB, et al. (1997) A survey on the complement system of the Atlantic salmon, Salmo salar L. Fish Shellfish Immunol 7: 29–43.
17. Sunyer JQ, Tort L (1995) Natural hemolytic and bactericidal activities of sea bream Sparus aurata serum are affected by the alternative complement pathway. Vet Immunol Immunopathol 45: 333–345.
18. Sunyer JQ, Tort L (1995) Natural hemolytic and bactericidal activities of sea bream Sparus aurata serum are affected by the alternative complement pathway. Vet Immunol Immunopathol 45: 333–345.
19. Kanlis G, Suzuki Y, Tauchi M, Numata T, Shirojo Y, et al. (1995) Natural hemolytic and bactericidal activities of sea bream Sparus aurata serum are affected by the alternative complement pathway. Vet Immunol Immunopathol 45: 333–345.
20. Picciotti S, Taddei AR, Scapigliati G, Buonocore F, Fausto AM, et al. (2004) Immunoglobulin protein and gene transcripts in ovarian follicles throughout oogenesis in the teleost Dicentrarchus labrax. Cell Tissue Res 315: 259–270.
21. Kishore U, Reid KB (2000) C1q: structure, function and receptors. Immunopharmacology 49: 159–170.
22. Magaudotir B, Lange S, Steinarsson A, Gudmundsdotir S (2004) The ontogenetic development of innate immune parameters of cod (Gadus morhua L.). Comp Biochem Physiol B Biochem Mol Biol 139: 217–224.
23. Ellis AE (1968) Ontogeny of the immune system in teleost fish. In: Ellis AE, ed (1980) Fish vaccination. London: Academic Press. p 20–31.
24. Kono MM, Lane RS, Giclas PC (2000) A comparative study of mammalian and reptilian alternative pathway of complement-mediated killing of Lyme disease spirochete (Borrelia burgdorferi). J Parasitol 86: 1223–1228.
25. Morgan BP (1995) Physiology and pathophysiology of complement: progress and prospects. Trans Proc 6: 1–7.
26. Thiel S, Vorup-Jensen T, Stover CM, Schwaeble W, Laursen SB, et al. (1997) A survey on the complement system of the Atlantic salmon, Salmo salar L. Fish Shellfish Immunol 7: 29–43.
27. Kono MM, Lane RS, Giclas PC (2000) A comparative study of mammalian and reptilian alternative pathway of complement-mediated killing of Lyme disease spirochete (Borrelia burgdorferi). J Parasitol 86: 1223–1228.
28. Sunyer JQ, Tort L (1995) Natural hemolytic and bactericidal activities of sea bream Sparus aurata serum are affected by the alternative complement pathway. Vet Immunol Immunopathol 45: 333–345.
29. Sunyer JQ, Tort L (1995) Natural hemolytic and bactericidal activities of sea bream Sparus aurata serum are affected by the alternative complement pathway. Vet Immunol Immunopathol 45: 333–345.
30. Koppenheffer TL, Spong KD, Falvo HM (1998) The complement system of the marsupial Monodelphis domestica. Dev Comp Immunol 22: 231–232.
31. Van Dijk VH, Rademaker PM, Klerk JPAM, Williers JMN (1985) Study of the ontogeny and extrahepatic expression of complement factor C3 in Atlantic salmon (Salmo salar). Fish Shellfish Immunol 23: 542–552.
32. Zarkadis IK, Mastellos D, Lambris JD (2001) Phylogenetic aspects of the complement system. Dev Comp Immunol 25: 745–762.
33. Herbolom P, Thise B, Thise C (1999) Ontogeny and behaviour of early macropahages in the zebrafish embryo. Development 126: 3735–3745.
34. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE (2001) Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. Blood 98: 3087–3096.
35. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. Anal Biochem 72: 248–254.
36. Nonaka M, Azumi K, Ji X, Namikawa-Yamada C, Sasaki M, et al. (1999) Osmotic complement component C3 in the spotted wolffish (Anaspidea minus Olfater). Fish Shellfish Immunol 10: 351–358.