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Interplay Between Amphioxus Complement with Sea Bass Macrophages: Opsonic Activity of Amphioxus Humoral Fluids

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Abstract Previous studies have shown the existence of a complement system in the amphioxus *Branchiostoma japonicum.* However, whether it has an opsonic activity similar to that of vertebrates remains unknown. We demonstrated that the humoral fluid (HF) of amphioxus promoted the phagocytosis of yeast cells with sea bass (*Lateolabrax japonicus*) macrophages, whereas the C3-depleted and heated HF significantly lost the phagocytosis-promoting capacity. In addition, the precipitation of factor B (Bf) led to a marked loss of opsonic activity. Moreover, C3 fragments in the HF were found to bind to yeast cell surfaces. The results indicate that the amphioxus complement system is an important element involved in the opsonic activity, which promotes the sea bass macrophage phagocytosis by tagging yeast cells with C3 fragments *via* the activation of alternative complement pathway.

Key words amphioxus; complement; opsonin; phagocytosis; sea bass

1 Introduction

Mammalian complement system is composed of about 35 humoral and cell surface proteins. It can be activated by three pathways including the classical pathway (CP), the alternative pathway (AP) and the lectin pathway (LP). The CP is activated by an antigen-antibody complex, while AP is an antibody-independent route initiated by C3 as well as certain molecules on the surface of microbes. In contrast, LP is triggered by the binding of mannose-binding lectin to carbohydrates on microbial surfaces (Abbas and Lichtman, 2003). Finally these three pathways merge at a common step involving the central component C3, which is cleaved into C3a and C3b. C3b covalently binds to the surface of the microbes *via* a thiolester bond, which can also be attacked by small nucleophiles such as methylamine (Nagar *et al*., 1998). The bound C3 fragments tag pathogens as non-self, act as a focus for further complement activation and then lead to several activities such as immune cell activation, chemotaxis, opsonization, and lysis of pathogens, protecting host from infection (Abbas *et al*., 2003).

Recent discoveries of complement components from corals and sea anemones demonstrate that the complement system has emerged at an early stage of protostome evolution. Molecular cloning and analysis have identified several components such as C3 and factor B (Bf) from the corals (Hemmrich *et al*., 2007), sea anemones (Hemmrich *et al*., 2007; Kimura *et al*., 2009; Fujito *et al*., 2010), sea urchin (Al-Sharif *et al*., 1998; Smith *et al*., 1998), ascidian (Nonaka *et al*., 1999; Yoshizaki *et al*., 2005) and amphioxus (Suzuki *et al*., 2002; He *et al*., 2008), as well as mannan binding protein-associated serine proteases (MASPs) from the ascidian (Ji *et al*., 1997) and amphioxus (Endo *et al*., 2003) and C6, a terminal component, from the amphioxus (He *et al*., 2008). These findings suggest that the primitive complement system in lower animals has activation pathways similar to mammalian alternative and lectin pathways (Smith *et al*., 1999). But its physiological functions are poorly explored. One physiological role of the primitive complement system, as revealed in ascidian (Nonaka *et al*., 1999) and sea urchin (Clow *et al*., 2004), is to enhance the phagocytic activity of hemocytes through C3 deposition on invading microbes. Our previous studies showed that the complement system of the amphioxus *Branchiostoma japonicum* (formerly known as *B. belcheri tsingtauense*) is involved in the lysis of erythrocytes and microbes *via* activating alternative pathway (Zhang *et al*., 2003; Li *et al*., 2008). However, whether the complement system of the amphioxus has the opsonic activity to enhance phagocytosis by phagocytes remains to be determined.

Amphioxus, as a cephalochordate, is the closest extant

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relative of the vertebrates. It possesses a circulation system with an organization resembling that of vertebrates and a genome uncomplicated by extensive genomic duplication (Rähr, 1979; Putnam *et al*., 2008), which makes it an ideal organism for the study of comparative immunology (Zhang *et al*., 2009). The aims of this study were to test if the complement system of the amphioxus facilitates the phagocytosis by macrophages, and if so, to examine the activation pathway.

2 Materials and Methods

2.1 Preparation of Humoral Fluids (HF)

Amphioxus *B. japonicum* were collected from the 'amphioxus ground' near Shazikou, Qingdao, China. The humoral fluids were prepared as described previously (Wang *et al*., 2002).

2.2 Isolation of Macrophages

The macrophages were prepared using the density gradient centrifugation method from the head kidneys of *Lateolabrax japonicus* as described by Li *et al*. (2008). The discontinuous gradient was formed by two layers of 32% and 45% percoll (Solarbio). The macrophages were collected from 32%–45% interface, washed 3 times with DMEM containing 10% NCS, and adjusted to 5×10^6 cells mL^{-1}. The cell viability was assessed by trypan blue exclusion.

2.3 Labeling of Yeasts with Fluorescein Isothiocyanate (FITC)

The yeast (*Pichia pastoris*) cells were labeled with FITC as described by Li *et al*. (2008) and adjusted to 1.25×10^8 cells mL^{-1}.

2.4 Phagocytosis Assay

Amphioxus HF was diluted 2-fold serially (up to 4 times) with 10 mmolL^{-1} PBS (pH 7.4). A total of 200 μL FITC-labeled *P. pastoris* suspension was centrifuged at 3000 *g* for 5 min, and the pellet was resuspended in 200 μL of the serially diluted HFs and incubated at room temperature in the dark on a shaker for 30 min.

Both HF-treated and non-treated *P. pastoris* cells were washed 3 times with 10 mmol L^{-1} PBS and then mixed with 200μL of macrophage suspension (5×10^6 cells mL^{-1}). The mixture was incubated at room temperature in the dark for 90 min, shaking every 5 min. After centrifugation at 200 *g* at 4℃ for 5 min, the macrophage pellet was fixed by being resuspended in 300 μL of 10 mmol L^{-1} PBS with 2% glutaraldehyde. For each smear, more than 100 macrophages were examined. The phagocytic ability (PA) was defined as the percentage of macrophages with one or more ingested FITC-labeled *P. pastoris* cells within the total macrophage population.

2.5 Complement Activity Assay

HF was heated at 45℃ for 30 min. Then the aliquots of 200 μL heated HF were mixed with the FITC-labeled *P. pastoris* cells pelleted by centrifuging 200 μL of *P. pastoris* suspension (1.25×10^8 cells mL^{-1}) at 3000 *g*, and incubated at room temperature in the dark on a shaker for 30 min. For control, the normal HF was used instead of heated HF. The phagocytosis assays were then conducted as above.

The capacities of the antibodies against C3 (ab23891, Abcam Inc., USA), Bf (AF2739, R&D systems Inc., USA), and C4 (BA2383, Boster Inc., China) to inhibit the opsonic activity of HF were measured by the method of Nonaka *et al*. (1999). Briefly, aliquots of 200 μL HF were pre-incubated with anti-C3, anti-Bf and anti-C4 IgG (used as control) at concentrations 0.20, 0.01 and 0.24 μg mL^{-1} (Wang *et al*., 2008; Liang *et al*., 2009), respectively, and then treated with protein A/G PLUS-agarose (sc2003, Santa Cruz, Europe) to remove the IgG and Ag-Ab complexes. The phagocytosis assays were then performed as above.

2.6 Binding of C3 to Yeast Cells Assay

Aliquots of 1 mL of unlabelled and heat-inactivated yeast cell suspension (1.25×10^8 cells mL^{-1}) were centrifuged at 3000 *g* for 5 min. The pellets were resuspended in 1 mL HF and heated HF separately and incubated at room temperature for 30 min, followed by 3 washes with 10 mmol L^{-1} PBS. The treated yeast cells were then incubated in 40 μL of the elution buffer (10 mmol L^{-1} Tris-HCl buffer containing 1% SDS, 10 mmol L^{-1} EDTA and 100 mmol L^{-1} methylamine hydrochloride, pH8.5) at room temperature for 30 min (Gröndahl *et al*., 2001). The mixtures were centrifuged at 3000 g and the supernatant was collected and subjected to SDS-PAGE (12%) and Western blotting analyses as described previously (Zhang *et al*., 2003). Human serum was used as a positive control and processed similarly.

2.7 Statistical Analysis

The phagocytosis experiments were performed in triplicate and conducted three times. Statistical evaluation was performed by Student's t-test and $P<0.05$ was considered significant. All data were described as means ± standard deviation.

3 Results

3.1 Enhancement of the Phagocytosis of Sea Bass Macrophages by Amphioxus HF

To test the opsonic activity of amphioxus HF, phagocytosis was determined after yeast cells were treated with HF isolated from amphioxus. It was found that amphioxus HF could markedly promote the phagocytosis of sea bass macrophages, compared to non-opsonized control (Fig.1). The opsonic activity of amphioxus HF was in a dose-dependent manner. Significant enhancement of phagocytosis was still detectable even when the HF was diluted 4 folds.

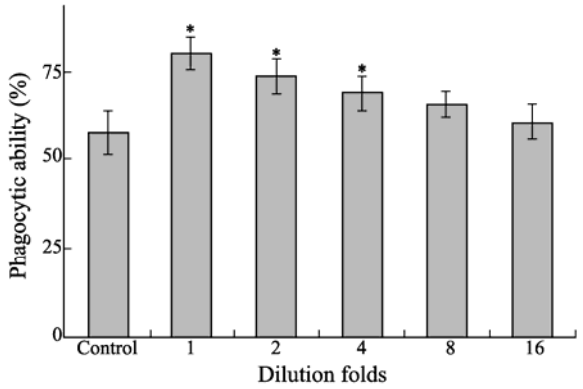

Fig.1 Opsonic activity of amphioxus HF. The FITC-labeled yeast cells were pre-incubated with serially-diluted HF for 30 min, and then incubated with sea bass macrophages for 90 min. For each example, at least 100 macrophages were tested. The opsonic activity was evaluated by the phagocytic ability (PA) (see Materials and Methods). The values are means ± S.D. from three experiments. The bars represent the standard deviations of the mean, and the star * denotes statistically significant differences (P<0.05).

3.2 Complement Activity

The phagocytosis of sea bass macrophages was significantly reduced when yeast cells were opsonized with amphioxus HF, which was heated at 45℃ for 30 min (Fig.2). This indicated the role of complement in the opsonic activity of amphioxus HF. Moreover, the pre-incubation of HF with anti-C3 antibody resulted in a significant reduction of phagocytosis of the macrophages. Similarly, the pre-incubation of HF with the antibody against Bf, a key enzyme involved in the AP activation, also led to a remarkable decrease in the opsonic activity of HF. In contrast, the pre-incubation of HF with anti-C4 antibody (used as control) had little influence on the opsonic activity of HF. These data suggested that the activation of the AP is associated with the opsonic activity in amphioxus HF.

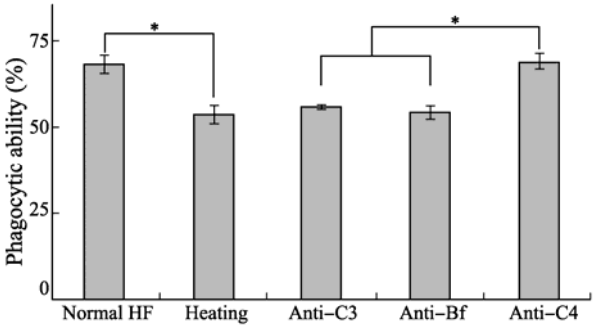

Fig.2 Effects of heating and antibodies against C3, Bf and C4 on the opsonic activity. HF was heated or pre-incubated with excess antibodies against C3, Bf and C4, and then treated with protein A/G PLUS-agarose to remove IgG and Ag-Ab complexes. The FITC-labeled yeast cells were pre-incubated with the treated HF and normal HF, respectively, for 30 min, and then incubated with sea bass macrophages for 90 min. For each example, at least 100 macrophages were tested. The opsonic activity was evaluated by the phagocytic ability (PA) (see Materials and Methods). The values are means ± S.D. from three experiments. The bars represent the standard deviations of the mean, and the star * denotes statistically significant differences (P<0.05).

3.3 Binding of C3 Fragments to Yeast Cells

Western blotting was done to test the ability of C3 in HF to bind the yeast cells. As shown in Fig.3, anti-C3 antibody reacted with both amphioxus HF and human serum, both yielding two main bands (~110 kDa; ~70 kDa) matching C3α and C3β chains, respectively, and two minor bands (~40 kDa; ~10 kDa) which could not be defined precisely. This confirmed the presence of C3 in amphioxus HF reported by Zhang *et al*. (2003) and Liang *et al*. (2009). Western blotting also revealed that two bands with molecular masses of ~66 and ~20 kDa were monitored in the eluates from yeast cells which had been incubated with amphioxus HF (Lane A in Fig.3), whereas no matching fragments were detected in the eluates derived from the yeast cells incubated with heated HF (Lane B in Fig.3), suggesting that the positive bands were cleaved fragments of C3. This implicated that C3 fragments in amphioxus HF were able to bind to yeast cell surfaces.

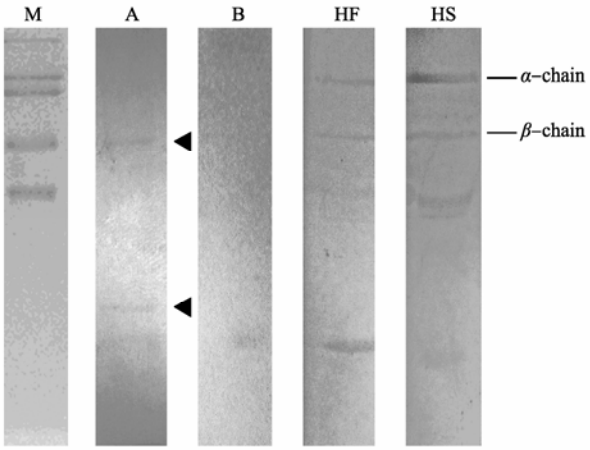

Fig.3 Western blot. Yeast cells were incubated with nomal HF (lane A) and heat-inactivated HF (lane B), respectively, and the proteins eluted from yeast cells were analyzed by SDS-PAGE and Western blot. Lane HS, Human serum; Lane HF, amphioxus humoral fluid. M shows the protein marker with molecular mass of 200 kD, 116 kD, 97.2 kD, 66.4 kD and 44.3 kD from top to bottom.

4 Discussion

Molecular cloning and analysis have demonstrated the existence of a primitive complement system in the protostome including corals and sea anemones and the deuterostome invertebrates such as sea urchin, ascidian and amphioxus. However, whether this system existing in the above animals could function as that in vertebrates remains largely unknown. One physiological role of the primitive complement system of ascidian (Nonaka *et al*., 1999) and sea urchin (Clow *et al*., 2004) is to enhance the

phagocytic activity of hemocytes through C3 deposition on microbes. As amphioxus lacks free circulating blood cells (Zhang *et al*., 2009) and pilot experiments have shown that amphioxus HF was able to enhance the phagocytosis by the macrophages of sea bass (data not shown), we therefore used sea bass macrophages to test the opsonic activity of HF in this study. We clearly demonstrated that the amphioxus HF has opsonic activity in a dose-dependent manner (Fig.1).

From the results it can be concluded that the complement system is an important element involved in the interplay between primitive chordate amphioxus HF and macrophages of sea bass. First, the phagocytic ability of the macrophages was significantly reduced by heating the HF at 45℃, a temperature known to inactivate amphioxus complement (Zhang *et al*., 2003). Second, the phagocytic ability was markedly decreased by the pre-incubation of the HF with anti-C3 antibody, a process leading to the precipitation of C3, the central component of all known complement pathways. It has been reported that C3-cleaved product C3b can bind to the surface of the microbes and function as an opsonin in both mammals and fish (van Lookeren Campagne *et al*., 2007). In agreement with this, the western blot result clearly demonstrated the binding of cleaved fragments of C3 to yeast cell surfaces, providing additional evidence that C3 in the HF acts as an opsonin.

Additionally, the precipitation of Bf led to a significant reduction of opsonic activity. It denotes that the activation of the AP pathway is primarily responsible for the opsonic activity of the HF, which is in line with our previous observation showing that the complement system of amphioxus is able to lyse erythrocytes and microbes *via* activation of the AP (Zhang *et al*., 2003; Li *et al*., 2008). This finding of a complement function in a deuterostome invertebrate species adds substantially to the accumulating evidence of a common ancestry of immune responses in invertebrates and vertebrates.

In conclusion, this study highlights the interplay between the complement systems of the lower animals (a deuterostome invertebrate) and the macrophages of the vertebrates (bony fish) *via* demonstrating the enhancement of the phagocytosis of amphioxus C3-opsonized yeast cells by sea bass macrophages. This bolsters the notion that the complement system in both invertebrates and vertebrates shares a common ancestry.

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References

Abbas, A., and Lichtman, A., 2003. *Cellular and Molecular Immunology*. 5th edition, Saunders, Philadelphia, 563pp.

Al-Sharif, W. Z., Sunyer, J. O., Lambris, J. D., and Smith, L. C., 1998. Sea urchin coelomocytes specifically express a homologue of the complement component C3. *Journal of Immunology*, **160**: 2983-2997.

Clow, L. A., Raftos, D. A., Gross, P. S., and Smith, L. C., 2004. The sea urchin complement homologue, SpC3, functions as an opsonin. *Journal of Experimental Biology*, **207**: 2147-2155.

Endo, Y., Nonaka, M., Saiga, H., Kakinuma, Y., Matsushita, A., Takahashi, M., Matsushita, M., and Fujita, T., 2003. Origin of mannose-binding lectin-associated serine protease (MASP)-1 and MASP-3 involved in the lectin complement pathway traced back to the invertebrate, amphioxus. *Journal of Immunology*, **170**: 4701-4707.

Fujito, N. T., Sugimoto, S., and Nonaka, M., 2010. Evolution of thioester-containing proteins revealed by cloning and characterization of their genes from a cnidarian sea anemone, *Haliplanella lineate*. *Developmental and Comparative Immunology*, **34**: 775-784.

Gröndahl, G., Johannisson, A., Jensen-Waern, M., and Nilsson Ekdahl, K., 2001. Opsonization of yeast cells with equine iC3b, C3b, and IgG. *Veterinary Immunology and Immunopathology*, **80**: 209-223.

He, Y., Tang, B., Zhang, S., Liu, Z., Zhao, B., and Chen, L., 2008. Molecular and immunochemical demonstration of a novel member of Bf/C2 homolog in amphioxus *Branchiostoma belcheri*: implications for involvement of hepatic cecum in acute phase response. *Fish and Shellfish Immunology*, **24**: 768-778.

Hemmrich, G., Miller, D. J., and Bosch, T. C., 2007. The evolution of immunity: a low-life perspective. *Trends in Immunology*, **28**: 449-454.

Ji, X., Azumi, K., Sasaki, M., and Nonaka, M., 1997. Ancient origin of the complement lectin pathway revealed by molecular cloning of mannan binding protein-associated serine protease from a urochordate, the Japanese ascidian, *Halocynthia roretzi*. *Proceedings of the National Academy of Sciences of the United States of America*, **94**: 6340-6345.

Kimura, A., Sakaguchi, E., and Nonaka, M., 2009. Multi-component complement system of Cnidaria: C3, Bf, and MASP genes expressed in the endodermal tissues of a sea anemone, *Nematostella vectensis*. *Immunobiology*, **214**: 165-178.

Li, Z., Zhang, S., and Liu, Q., 2008. Vitellogenin functions as a multivalent pattern recognition receptor with an opsonic activity. *PloS ONE*, **3** (4): e1940.

Li, Z., Zhang, S., Wang, C., and Pang, Q., 2008. Complement-mediated killing of *Vibrio* species by the humoral fluids of amphioxus *Branchiostoma belcheri*: implications for a dual role of O-antigens in the resistance to bactericidal activity. *Fish and Shellfish Immunology*, **24**: 215-222.

Liang, Y., Zhang, S., and Wang, Z., 2009. Alternative complement activity in the egg cytosol of amphioxus *Branchiostoma belcheri*: evidence for the defense role of maternal complement components. *PloS ONE*, **4** (1): e4234.

Nagar, B., Jones, R. G., Diefenbach, R. J., Isenman, D. E., and Rini, J. M., 1998. X-ray crystal structure of C3d: a C3 fragment and ligand for complement receptor 2. *Science*, **280**: 1277-1281.

Nonaka, M., Azumi, K., Ji, X., Namikawa-Yamada, C., Sasaki, M., Saiga, H., Dodds, A. W., Sekine, H., Homma, M. K., Matsushita, M., Endo, Y., and Fujita, T., 1999. Opsonic complement component C3 in the solitary ascidian, *Halocynthia roretzi*. *Journal of Immunology*, **162**: 387-391.

Putnam, N. H., Butts, T., Ferrier, D. E., Furlong, R. F., Hellsten, U., Kawashima, T., Robinson-Rechavi, M., Shoguchi, E., Terry, A., Yu, J. K., Benito-Gutierrez, E. L., Dubchak, I., Garcia-Fernandez, J., Gibson-Brown, J. J., Grigoriev, I. V., Horton, A. C., de Jong, P. J., Jurka, J., Kapitonov, V. V., Kohara, Y., Kuroki, Y., Lindquist, E., Lucas, S., Osoegawa, K., Pennacchio, L. A., Salamov, A. A., Satou, Y., Sauka-Spengler, T., Schmutz, J., Shin, I. T., Toyoda, A., Bronner-Fraser, M., Fujiyama, A., Holland, L. Z., Holland, P. W., Satoh, N., and Rokhsar, D. S., 2008. The amphioxus genome and the evolution of the chordate karyotype. *Nature*, **453**: 1064-1071.

Rähr, H., 1979. The circulatory system of amphioxus (*Branchiostoma lanceolatum* (Pallas)).A light-microscope investigation based on intravascular injection technique. *Acta Zoologica (Stockholm)*, **60**: 1-18.

Smith, L. C., Azumi, K., and Nonaka, M., 1999. Complement systems in invertebrates. The ancient alternative and lectin pathways. *Immunopharmacology*, **42**: 107-120.

Smith, L. C., Shih, C. S., and Dachenhausen, S. G., 1998. Coelomocytes express SpBf, a homologue of factor B, the second component in the sea urchin complement system. *Journal of Immunology*, **161**: 6784-6793.

Suzuki, M. M., Satoh, N., and Nonaka, M., 2002. C6-like and C3-like molecules from the cephalochordate, amphioxus, suggest a cytolytic complement system in invertebrates. *Journal of Molecular Evolution*, **54**: 671-679.

Wang, C., Zhang, S., Luo, Y., and Xu, T., 2002. Presence and induction by bacteria of D-galactoside-specific lectins in the humoral fluids of amphioxus *Branchiostoma belcheri tsingtauense*. *Inflammopharmacology*, **9**: 241-248.

Wang, Z., Zhang, S., Wang, G., and An, Y., 2008. Complement activity in the egg cytosol of zebrafish *Danio rerio*: evidence for the defense role of maternal complement components. *PloS ONE*, **3** (1): e1463.

Yoshizaki, F. Y., Ikawa, S., Satake, M., Satoh, N., and Nonaka, M., 2005. Structure and the evolutionary implication of the triplicated complement factor B genes of a urochordate ascidian, *Ciona intestinalis*. *Immunogenetics*, **56**: 930-942.

Zhang, S., Liang, Y., Jia, G., and Zhuang, Z., 2009. Protochordate amphioxus is an emerging model organism for comparative immunology. *Progress in Natural Science*, **19** (8): 923-929.

Zhang, S., Wang, C., Wang, Y., Wei, R., Jiang, G., and Ju, H., 2003. Presence and characterization of complement-like activity in the amphioxus *Branchiostoma belcheri tsingtauense*. *Zoological Science*, **20**: 1207-1214.

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