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ScienceDirect
Progress in Natural Science 19 (2009) 179–185
Progress in Natural Science
www.elsevier.com/locate/pnsc

Synergistic actions of complement and lysozyme in clearance of *Escherichia coli* from amphioxus *Branchiostoma belcheri*

Guangfeng Wang [a], Shicui Zhang [a,*], Zhimeng Zhuang [b], Zhiping Wang [a]

[a] *Department of Marine Biology, Ocean University of China, Qingdao 266003, China*
[b] *Yellow Sea Fisheries Research Institute, Qingdao 266071, China*

Received 25 March 2008; received in revised form 22 April 2008; accepted 22 April 2008

Abstract

Amphioxus appears to lack free circulating blood cells. How it clears invading pathogens from its body remains unknown to date. We demonstrate here that amphioxus *Branchiostoma belcheri* is capable of efficiently eliminating the invading bacterium *Escherichia coli* from its humoral fluid, and the complement and lysozyme are both involved in the elimination of the invading pathogen. Both the complement and lysozyme act in concert against the invading bacterium, but the complement appears to play a more dominant role than the lysozyme.

Keywords: Amphioxus; *Branchiostoma*; Complement; Lysozyme; Humoral fluids; Anti-bacterial activity

1. Introduction

Animals are exposed to millions of potential pathogens daily, via contact, ingestion and inhalation, but most of them are able to clear invading microbes from their body. In higher animals, microbial clearance depends upon the prompt recruitment of specialized cells like macrophages and extracellular proteins including antibodies and complement proteins from the blood to extravascular sites of invasion [1]. A major function of macrophages is to eliminate proliferating extracellular microbes, which is accomplished in large part by phagocytosis and intracellular destruction of the ingested microbes [2,3]. Usually, phagocytosis by macrophages demands the help of extracellular opsonins such as antibodies and complement component C3b [1,4]. Activation of the complement system may also contribute to the lysis of invading microbes via the membrane attack complex formed [1].

The complement system consists of approximately 35 plasma and membrane-bound proteins. 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 the 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 [5,6]. The AP is mainly triggered by certain structures on the microbial surface in an antibody-independent manner [7]. 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 [8]. The LP is activated by binding of microbial polysaccharides to circulating lectins, such as mannose-binding lectin (MBL) [9–12]. 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

* Corresponding author. Tel.: +86 532 82032787; fax: +86 532 82032276.
E-mail address: sczhang@ouc.edu.cn (S. Zhang).

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doi:10.1016/j.pnsc.2008.04.019

involving C3, a central complement component being a part of all the three pathways.

Besides complement, there are numerous molecules in the blood, such as lysozyme [13]. They act against bacteria either alone or in concert with each other. Lysozyme, originally described as 'bacteriolytic activity' by Fleming in 1922, is an antimicrobial enzyme existing in both prokaryotes and eukaryotes. It catalyzes the hydrolysis of bacterial cell walls and acts as a nonspecific innate immune molecule against the invasion of bacterial pathogens [14]. Although lysozyme is mainly active against Gram-positive bacteria [15], it is also active against Gram-negative bacteria including *Escherichia coli* [16].

Amphioxus or cephalochordate, an emerging model organism for gaining insights into the origin and evolution of vertebrates [17], is a marine filter-feeder, and filters large volumes of seawater containing various bacteria and single-celled algae during its daily feeding activities, and may concentrate some pathogenic microbes. How amphioxus removes invading potentially pathogenic microbes from its body has not yet been studied to date. Moreover, there is little information regarding the kinetics of the specific processes by which microbial invaders are eliminated. Ratcliffe et al. [18] have proposed that blood cells including macrophages are present in both vertebrates and invertebrates. However, the presence of freely circulating blood cells in amphioxus remains uncertain [19,20], suggesting the absence for migration of macrophages to the sites of invasion in amphioxus. It has also been demonstrated that a mammalian-like complement system operating via AP is present in amphioxus [21], which may lead to the formation of a membrane attack complex, resulting in microbial cell lysis. Besides, lysozyme has been found in amphioxus [22], which may also result in the lysis of invading microbes.

The aim of this study was thus to examine if amphioxus can efficiently remove invading microbes, and if so, to pinpoint the main elements responsible for the removal of invaders from amphioxus.

2. Materials and methods

2.1. Preparation of bacterium

The bacterium *E. coli* (P8760) with transgenic green fluorescent protein (GFP) gene was provided by Dr. Xiao Tian, Institute of Oceanology, Chinese Academy of Sciences. *E. coli* was incubated in LB broth for 3 h, and GFP expression was induced by the addition of isopropyl-*b*-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM to the medium. The bacterium was then grown to logarithmic growth phase and was harvested by centrifugation at 3000*g* at 4 °C for 10 min. The bacterial pellets were washed three times with 0.9% saline, re-suspended in the sterile saline at a density of 10^9 cells/ml, and used for the following experiments.

2.2. Preparation of humoral fluids

The humoral fluids were prepared from amphioxus *Branchiostoma belcheri* by the method described in Ref. [23]. Briefly, about 1000 amphioxi with an average body length of 4 cm were rinsed with distilled water, wiped out thoroughly with sterile gauze, and severed into about 2 mm^3 pieces on ice to bleed. After centrifugation at 12,000*g* at 4 °C for 30 min, the supernatant was pooled and stored at −70 °C.

The protein concentration was determined by the method of Bradford [24] with bovine serum albumin as the standard.

2.3. Assay for in vivo clearance of E. coli

The assay for *in vivo* clearance of *E. coli* by amphioxus was performed at 25 °C. Totally, 200 amphioxi were exposed to *E. coli* suspension with a density of 10^8 cells/ml (in 2 l of sterile seawater) for 1 h. They were then washed three times with seawater and transferred to sterile seawater. After half an hour, the animals were washed again to remove the bacterium attached to the surface [25]. Soon after the final washing, 10 amphioxi were sampled at 0 (control), 1, 3, 6, 12, 18, 24, 36, 48 and 72 h, respectively. The treated amphioxi were cut with a blade at the middle of their bodies, and the humoral fluids were smeared onto a slide. The number of *E. coli* was countered under a fluorescence microscope (Olympus U-TV1X-2), and the percentage of bacterial survival was inferred from the difference between the numbers of *E. coli* in the test and control.

2.4. Assay for in vitro lysis of E. coli

The humoral fluids were filtered through a 0.22 μm Millipore filter, and diluted two-fold serially up to 16 times with sterile saline. An aliquot of 50 μl diluted humoral fluids was mixed with an equal volume of *E. coli* suspensions containing 10^5 cells/ml, and the mixture was pre-incubated, with gentle stirring, at 25 °Cfor 2 h. Subsequently, a volume of 20 μl of the mixture was sampled at 30, 60, 90 and 120 min, respectively, diluted to 100 μl with sterile saline, and plated onto 3 LB agar plates (30 μl/plate). After incubation at 37 °C for 16 h, the resulting bacterial colonies in each plate were counted. The control was processed similarly except that the humoral fluids were replaced by sterile saline. Total bacterial survival was calculated, and the percentage of bacterial survival was inferred from the difference between the numbers of colonies in the test and control.

2.5. Assays for inhibition of lytic activity

To test which element may be involved in the lysis of *E. coli*, rabbit anti-human C3α, goat anti-human C1q, rabbit anti-mouse C4 (Boster, China), goat anti-human factor

B (Bf) (R&D, USA) and goat anti-human lysozyme (Santa Cruz, USA) antibodies were used to inhibit the bacteriolytic activity of amphioxus humoral fluids. An aliquot of 50 μl of the humoral fluids diluted (1:2) was pre-incubated with an equal volume of anti-C3, anti-C1q, anti-C4, anti-Bf and anti-lysozyme antibodies at different concentrations at 25 °C for 30 min, followed by the addition of 50 μl of *E. coli* suspension with 2×10^5 cells/ml. The mixtures were adjusted to a volume of 200 μl with sterile saline, and incubated at 25 °C for 2 h. The bacteriolytic activities were measured as described above. For the control, the antibodies were withdrawn.

It has been shown that C3b can covalently bind to zymosan particles [26], and thus the AP-mediated bacteriolytic activity can be selectively inhibited by the addition of zymosan. Therefore, to further understand if the AP is related to the killing of *E. coli*, zymosan was used to inhibit the lytic activity. A total of 150 μl (1.5 mg of zymosan) of 10 mg/ml zymosan A stock solution was centrifuged at 16,000*g* for 5 min at 4 °C, and the zymosan pellet was resuspended in 50 μl of the humoral fluids (diluted 1:4). After pre-incubation at 25 °C for 30 min, the zymosan in the reaction medium was removed by centrifugation at 16,000*g* for 5 min at 4 °C. The resulting humoral fluid was mixed with 50 μl of *E. coli* suspension with 2×10^5 cells/ml. The mixture was incubated at 25 °C for 30 min, and the bacteriolytic activity was tested.

2.6. Assay for response of C3 and lysozyme to E. coli challenge

To confirm the presence of C3 and lysozyme in the humoral fluids, Western blotting analysis was carried out as described previously [27]. The humoral fluids and human serum were both electrophoresed on 12% SDS–polyacrylamide gel (SDS–PAGE) using the buffer system of Laemmli [28]. The proteins separated were blotted on the nitrocellulose membrane (Hybond, Amersham Pharmacia), and immunostained with rabbit anti-human C3α antibody (1:200) or goat anti-human lysozyme (1:100) antibody, followed by staining with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Zhongshan, China) or HRP-labeled rabbit anti-goat IgG (Boster, China).

To examine the response of C3 and lysozyme to bacterial infection, amphioxi were exposed to *E. coli* suspension, and sampled at 0 (control), 6, 12, 24, and 48 h, as described before. The humoral fluids were prepared, run on 12% SDS–PAGE, and immunostained as described. To compare the differences in C3 and lysozyme levels at different time points, the blots were scanned in the RGB mode with Founder Super T35 Color Scanner, saved in gray scale, and semi-quantitatively analyzed with the Gel-Pro Express 4.0 (American SIM International Co., Ltd).

The molecular mass standards used were *β*-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp981 (25.0 kDa), β-lactoglobuin (18.4 kDa) and lysozyme (14.4 kDa).

2.7. Statistical analysis

All experiments were performed at least three times. Data were subjected to statistical evaluation with ANOVA, and difference at $p < 0.05$ was considered significant. All data were expressed as mean ± standard deviation (SD).

3. Results

3.1. In vivo clearance of E. coli by amphioxus

Fig. 1 shows the *in vivo* clearance curve of *E. coli* by amphioxus over a period of 72 h at 25 °C. Nearly 80% of *E. coli* was cleared in the initial 6 h after the challenge, and no bacterium was detected after 72 h.

3.2. In vitro killing of E. coli by amphioxus humoral fluids

The protein concentrations of the humoral fluids prepared from amphioxus *B. belcheri* ranged from 13.62 to

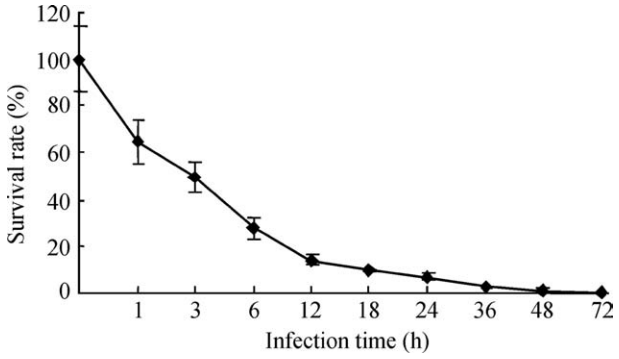

Fig. 1. *In vivo* clearance of *E. coli* from the humoral fluids of amphioxus. Values shown are mean ± standard deviation ($n = 3$).

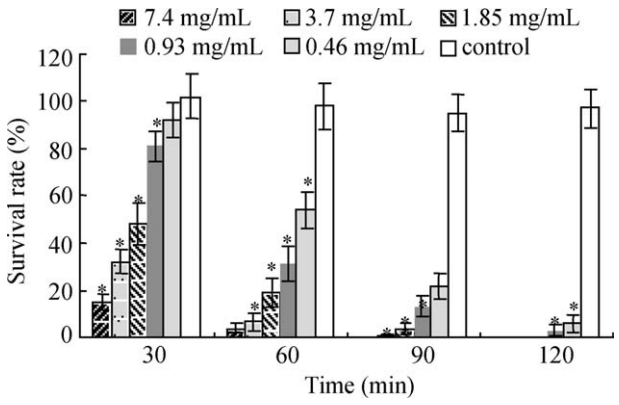

Fig. 2. Bacteriolytic effects of humoral fluids at different concentrations. The humoral fluids were diluted 1:2, 1:4, 1:8, 1:16 and 1:32 in the final volumes, the protein concentrations were 7.4, 3.7, 1.85, 0.93 and 0.46 mg/ml, respectively. Values shown are mean ± standard deviation ($n = 3$). $^*p < 0.05$ compared with controls.

15.94 mg/ml, with an average of 14.80 mg/ml. It was found that the humoral fluids were capable of lysing *E. coli* efficiently, and the lytic activity of the fluids was in a dose-dependent manner (Fig. 2). When the protein concentration of humoral fluids was 1.85 mg/ml, they could kill all the bacteria within 2 h, therefore, the fluids that were diluted 8-fold were used in all the following experiments.

3.3. Involvement of both complement and lysozyme in killing of E. coli

Pre-incubation of the antibodies against C3 and Bf with amphioxus humoral fluids was capable of inhibiting the lytic activity to *E. coli* in a dose-dependent manner (Fig. 3(a) and (b)), whereas pre-incubation of anti-C1q and anti-C4 antibodies at the same concentrations with the humoral fluids did not show any inhibitory effect (Fig. 3(c) and (d)). In addition, it was found that using zymosan A to deplete the AP also resulted in a significant increase in *E. coli* survival (Fig. 3(e)). On the other hand, pre-incubation of anti-lysozyme antibody with amphioxus humoral fluids was able to lessen the lytic activity to *E. coli* in a dose-dependent manner (Fig. 3(f)). In comparison, the complement showed a stronger efficiency against *E. coli* than the lysozyme. When both anti-C3 and anti-lysozyme antibodies were co-incubated with the humoral fluids, a synergistic inhibitory effect was observed (Fig. 4). All these strongly suggested a role for both complement operating via the AP

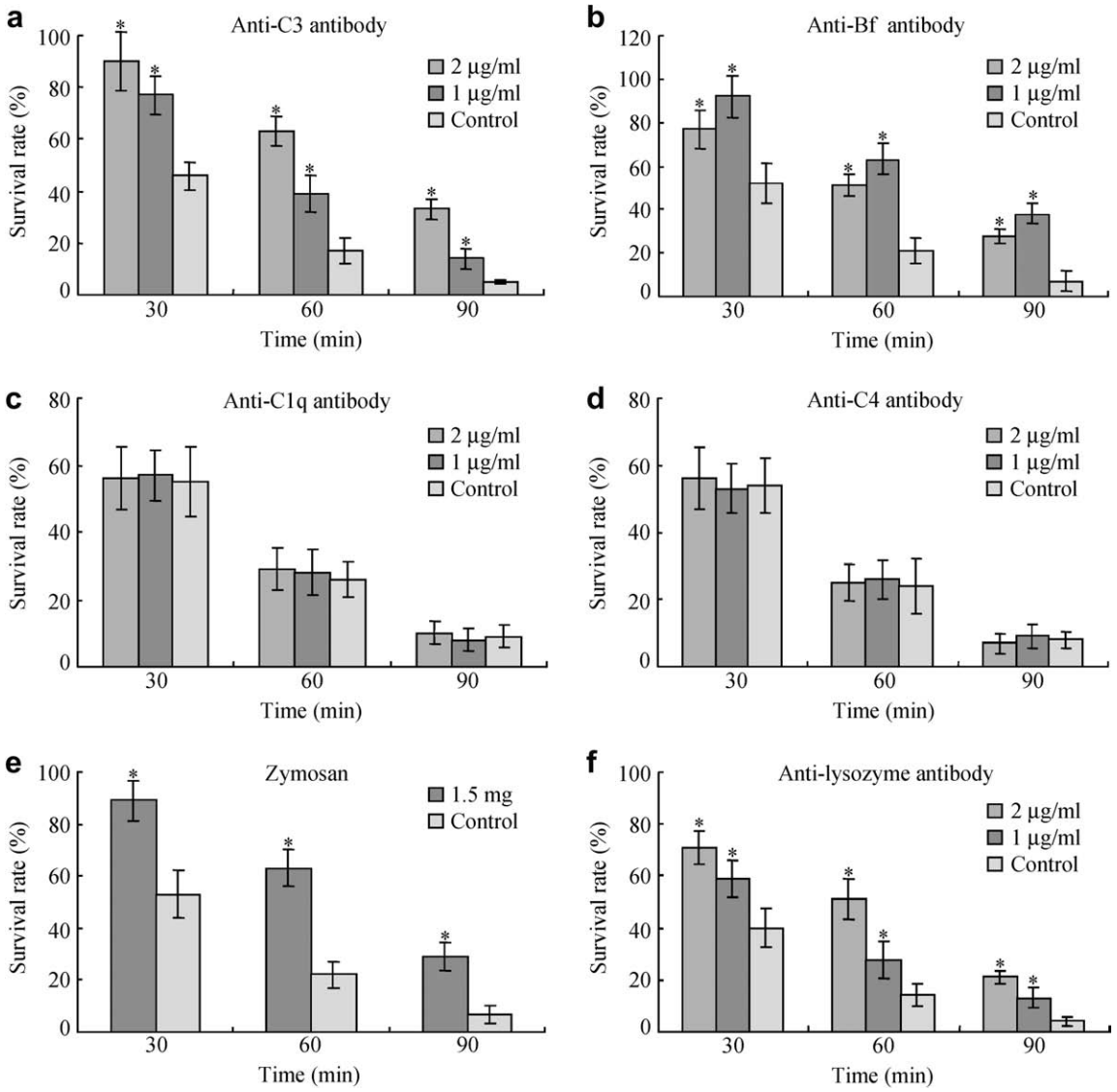

Fig. 3. Inhibitory effects of antibodies and zymosan A on the bacteriolytic activity of amphioxus humoral fluids. The bacteriolytic activity was diminished when the humoral fluids pre-incubated with zymosan A or the antibodies against C3, Bf and lysozyme. But pre-incubation of anti-C1q and anti-C4 antibodies with the humoral fluids did not show any inhibitory effect. Values shown are mean ± standard deviation ($n = 3$). $^{*}P < 0.05$ compared with the controls.

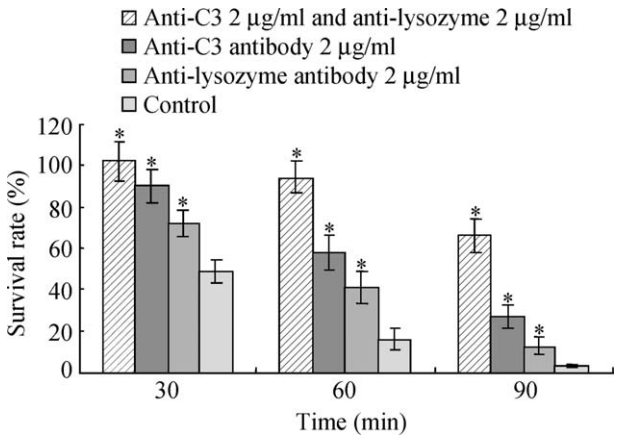

Fig. 4. Inhibitory effects on the bacteriolytic activity by anti-C3 and anti-lysozyme antibodies. The complement showed a stronger efficiency against *E. coli* than the lysozyme. When both anti-C3 and anti-lysozyme antibodies were co-incubated with the humoral fluids, a synergistic inhibitory effect was observed. Values shown are mean ± standard deviation ($n = 3$). $^{*}P < 0.05$ compared with the controls.

and lysozyme in the lytic activity of amphioxus humoral fluids.

3.4. Increase in C3 and lysozyme levels following *E. coli* infection

Western blotting revealed that rabbit anti-human C3α reacted with both human serum and amphioxus humoral fluids, forming a band of approximately 44 kDa equivalent to the size of degraded fragment C3α of human C3 (Fig. 5(a)). Similarly, goat anti-lysozyme antibody reacted with both human serum and amphioxus humoral fluids, producing a positive band of about 16.0 kDa matching that of human lysozyme (Fig. 5(c)). In response to *E. coli* challenge, both C3α and lysozyme levels increased (Fig. 5(b) and (d)). The C3α level reached a peak (2.9-fold) at 24 h after the bacterial challenge, while the lysozyme content maximized (2.4-fold) at 24 h following the challenge. This indicates that variation in C3α and lysozyme levels is correlated with *E. coli* infection.

4. Discussion

Phagocytosis by blood cells is regarded as a central and important way to remove bacteria in invertebrates including decapods [29,30] and clam [25]. We demon-

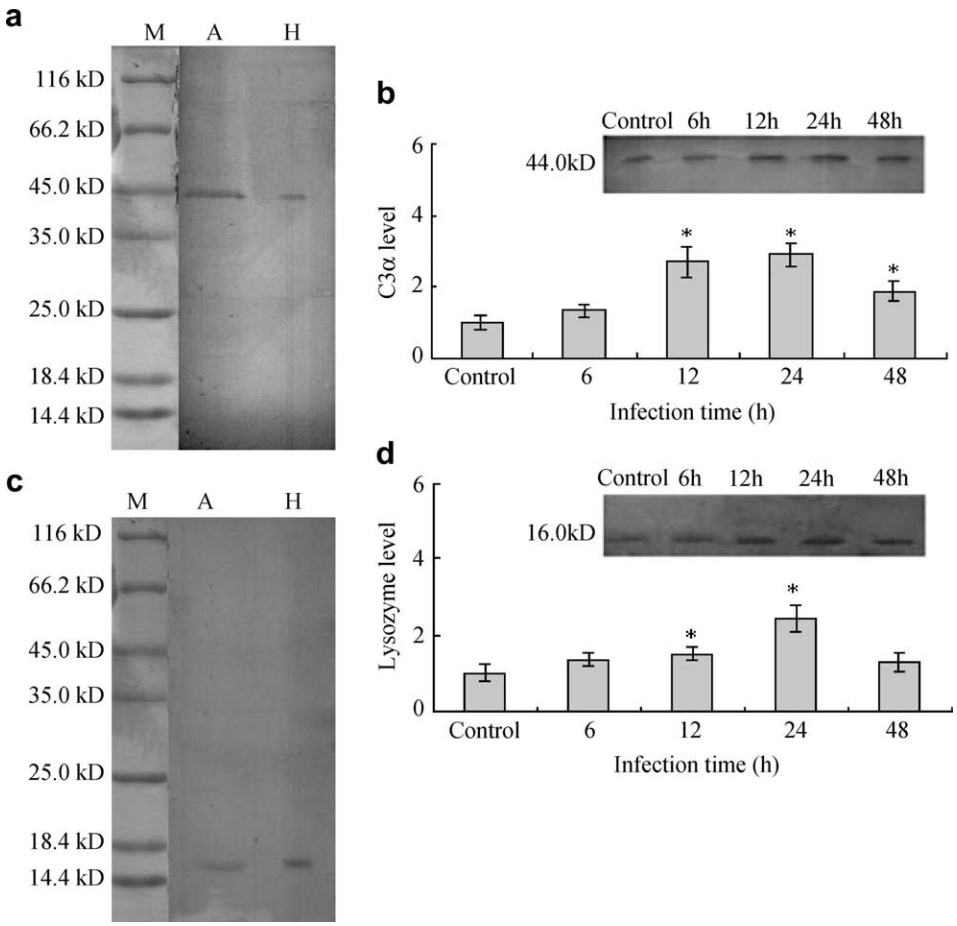

Fig. 5. Western blotting of C3 and lysozyme in amphioxus humoral fluids and the different levels at different time points in response to challenge with *E. coli* (Lane M, molecular marker; H, human serum; A, amphioxus humoral fluids). All values shown are mean ± standard deviation ($n = 3$). $^{*}P < 0.05$ compared with the controls.

strate here for the first time that the circulating haemocyte-free amphioxus *B. belcheri* is able to efficiently clear the invading bacteria like *E. coli* from its humoral fluids. We also establish the kinetics of the process by which the invading bacterium *E. coli* are eliminated. To find out the major elements responsible for the clearance of infected invaders, we have first sought to search for the cells which may engulf *E. coli* in amphioxus. The amphioxus infected with *E. coli* was cut at its middle into two parts, and the blood was smeared onto a slide. The smears were air-dried, fixed in absolute methanol, stained with Wright-Giemsa solution, and observed under a microscope. Not a single haemocyte-like cell was seen among at least 2000 samples examined (unpublished data; not shown), suggesting that free circulating macrophages, if any, are not the main elements for the elimination of invading bacterium *E. coli* in amphioxus.

Further, we have turned to the humoral fluids for screening of the factors attributable to the removal of invaders from amphioxus. As expected, the humoral fluids can readily lyse the bacterium *E. coli in vitro*. The complement operating via the AP appears to be one of the humoral fluid components involved in the lytic activity observed. First, the fluid bacteriolytic activity was abolished by pre-incubation of amphioxus humoral fluids with the antibodies against C3 and Bf (they are both key components of AP), but not by pre-incubation with antibody against C1q (a key component of CP) and antibody against C4 (a key component of both CP and LP). Second, selective inhibition of the AP by zymosan induced marked loss of the bacteriolytic activity. In addition to the complement, lysozyme appears to be another component in amphioxus humoral fluids responsible for the bacteriolytic activity because pre-incubation of the fluids with anti-lysozyme antibody markedly reduced the lytic activity. Taken together, all these suggest that the complement and lysozyme may both be responsible for *in vivo* removal of invading bacteria from amphioxus. This is further supported by the fact that both C3α and lysozyme levels increased with the challenge of *E. coli*. The complement seems to play a relatively dominant role against invading bacteria in amphioxus.

In summary, this study demonstrates that the circulating haemocyte-free amphioxus is able to eliminate invading bacteria like *E. coli* efficiently; both the complement and lysozyme are the main factors acting in concert to eliminate the invaders *in vivo*, the first such data in the protochordates. Further investigations on the synergistic actions of seemingly distinct molecules of the innate immune system in amphioxus will shed more light on the elimination *in vivo* of invading bacteria.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (Grant No. 30730072).

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