Fish & Shellfish Immunology 30 (2011) 1348–1353

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

# Development of a monoclonal antibody specific to granulocytes and its application for variation of granulocytes in scallop *Chlamys farreri* after acute viral necrobiotic virus (AVNV) infection

Tingting Lin, Jing Xing*, Xiuzhen Sheng, Xiaoqian Tang, Wenbin Zhan

*Laboratory of Pathology and Immunology of Aquatic Animals, LMMEC, Ocean University of China, Qingdao 266003, PR China*

ARTICLE INFO

*Article history:*
Received 26 November 2010
Received in revised form
24 January 2011
Accepted 9 March 2011
Available online 17 March 2011

*Keywords:*
Scallop *Chlamys farreri*
Monoclonal antibody
Granulocyte
Acute viral necrobiotic virus (AVNV)
Haemocyte

ABSTRACT

A monoclonal antibody (MAb 6H7) specific to granulocytes of scallop *Chlamys farreri* was produced by immunising mice with separated granulocytes as an antigen. Characterised using a flow cytometric immunofluorescence assay, MAb 6H7 reacted to granulocytes by 87.1% of total positive haemocytes. At the ultrastructural level, MAb 6H7 demonstrated epitope in cytoplasmic granules of granulocytes. Western blotting analysis indicated that a peptide of 155 kDa was recognised by MAb 6H7. It was therefore used to investigate granulocyte variation in *C. farreri* after acute viral necrobiotic virus (AVNV) infection using an enzyme-linked immunosorbent assay. The result illustrated that granulocytes varied greatly by AVNV infection, and their amount significantly increased on day 1 post-injection, then decreased on days 2, 3 and 4, thereafter, rebounded and approached to a second peak on day 6, finally went down gradually to the control level on day 8.



## 1. Introduction

Bivalve haemocytes play an important role in host immune defence, including nodule formation, phagocytosis, melanisation, wound healing and production of reactive oxygen species (ROS) [1–5]. Bivalve haemocytes are generally classified into two types, granulocytes and hyalinocytes, although a third type called as haemoblasts is often observed in some species [6–8]. Granulocytes generally contain many cytoplasmic granules and have the ability to phagocytose microbial pathogens. They contain a mixture of hydrolytic enzymes that contribute to intracellular killing and are more phagocytic than hyalinocytes [8–10]. Whereas hyalinocytes have few or no granules and are usually more morphologically heterogeneous than granulocytes [11,12]. Haemocyte populations and their morphology and function in some extent vary with bivalve species, life stages, pathogen infections or environmental factors. These results were documented that sudden temperature elevation could cause a general decrease in size of haemocytes and a higher percentage of dead hyalinocytes than granulocytes in oyster *Crassostrea virginica* [10,13]. Spawning could induce a significant decrease in phagocytic activity of granulocytes in oyster *Crassostrea gigas* [14]. Additionally, infection with *Vibrio tapetis* could result in a reduction in viability, phagocytic activity, size and cytoplasmic granularity of granulocytes in clam *Ruditapes philippinarum* [15]. Moreover, an infection with acute viral necrobiotic virus (AVNV) could cause a great variation in total haemocyte count of scallop *Chlamys farreri* [16].

Recently some studies on classification, ontogenesis and localisation of bivalve haemocytes using monoclonal antibodies (MAbs) have been reported [17–19]. In clam *Mya arenaria*, a MAb reacted with haemocytes and connective tissue cells (CTCs) lining haemolymph sinuses, suggesting that the CTCs may be the origin of haemocytes [20]. In oyster *Ostrea edulis*, a MAb specific to granulocytes was used to investigate granulocyte distribution and ontogenesis, illustrating that granulocytes were absent in early larval stages and mostly observed in connective tissues in different organs [18]. In mussel *Mytilus edulis*, haemocyte classification was carried out by MAbs [21]. This paper described the development of a MAb specific to granulocytes of scallop *C. farreri* and its application for variation of granulocytes after AVNV infection to get a better understanding of immune defence of granulocytes.

## 2. Materials and methods

### 2.1. Scallops and haemocyte preparation

Adult scallops *C. farreri* (6.5 ± 0.45 cm shell height) were collected from Qingdao Harbor, China, and processed immediately after arrival in the laboratory.

* Corresponding author. Tel.: +86 532 82031580; fax: +86 532 82032284.
*E-mail address:* xingjing@ouc.edu.cn (J. Xing).

1050-4648/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2011.03.010

Haemolymph from ten scallops was withdrawn from the adductor muscle sinus with sterilised syringes, simultaneously mixed (1:1, v/v) with phosphate buffered saline containing EDTA (PBS-E; 0.14 M NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 19.1 mM EDTA, pH 7.4), then pooled and centrifuged at 500 × g for 10 min at 4 °C. The pellet was washed twice, resuspended in PBS and the haemocyte suspension was adjusted to a final concentration of $10^7$ cells/ml.

Haemocytes were separated with haemocyte suspension by Percoll density gradient (20%, 30%, 40% and 50%) centrifugation at 700 × g for 30 min at 4 °C. The haemocytes at each resulting interface were collected and washed twice with PBS, and then identified using a flow cytometer (Becton Dickinson, France) and Giemsa staining. This experiment was performed in quadruplicate. Finally the separated granulocytes and hyalinocytes were adjusted to a final concentration of $10^7$ cells/ml respectively and stored at −20 °C until use.

### 2.2. Monoclonal antibody (MAb) production

Granulocytes mentioned above were emulsified (1:1, v/v) with Freund's complete adjuvant. Two female Balb/c mice were injected intraperitoneally with 100 μl of this emulsion. Two weeks later, the mice were inoculated using the same method with 100 μl of the antigen in Freund's incomplete adjuvant. Then booster injections of 100 μl of antigen without adjuvant were given intravenously to each mouse once a week for two weeks. Three days after the final immunisation, the mice were sacrificed and spleens were aseptically removed for cell fusion.

Splenocytes from the immunised mice were fused with P3-X63-Ag8U1 myeloma cells using polyethylene glycol as a fusogen as described in Xing and Zhan [22]. The hybridomas were cultured in GIT medium (Nihon Seiyaku Co., Japan) containing 1% (v/v) HAT (Gibco) in 96-well flat-bottom culture plates. Culture supernatants from each well were screened by indirect immunofluorescence assay (IIFA) to detect hybridomas producing anti-haemocyte antibodies. After that, positive hybridomas secreting anti-granulocyte antibody were cloned by limiting dilution.

### 2.3. Indirect immunofluorescence assay (IIFA)

One hundred microlitre of haemocyte suspension (see Section 2.1.) was applied to a 96-well flat-bottom culture plate and incubated with 100 μl of hybridoma culture supernatant as the primary antibody for 45 min at 37 °C. After three washes with PBS at 500 × g for 5 min in a microplate centrifuge (Sigma, Germany), haemocyte pellet was incubated with 200 μl of fluorescein isothiocyanate conjugated goat anti-mouse Ig (GAM-FITC, Sigma) diluted at 1:200 as the secondary antibody for 30 min at 37 °C, then washed again and resuspended in 50 μl of PBS. Finally, a drop of this suspension was applied to a clean glass slide and observed under a fluorescence microscope (Olympus, Japan). A negative control was incubated with a culture supernatant of myeloma cells as the primary antibody.

### 2.4. Flow cytometric immunofluorescence assay (FCIFA)

One millilitre of haemocyte suspension was applied to a 24-well culture plate and incubated successively with 1 ml of anti-granulocyte MAb and 0.5 ml of GAM-FITC in a similar procedure to IIFA, the resulting haemocyte suspension was analysed by a flow cytometer. Three parameters FS, SS and FL, corresponding to cell size, cytoplasmic granularity and fluorescence intensity respectively, were employed to evaluate the specificity of MAb. Data were analysed using WinMDI 2.9 software. Negative control was the same as IIFA. This experiment was performed in quadruplicate.

### 2.5. Immunoelectron microscopy (IEM)

Haemocyte suspension was immediately fixed (1:1) with 5% (v/v) glutaraldehyde in PBS for 2 h. After centrifugation, the haemocyte pellet was post-fixed with 1% (v/v) osmic acid (Sigma), then dehydrated with an ethanol series, subsequently infiltrated and embedded in Epon 812 resin (Sigma), finally ultrasectioned and transferred onto nickel grids.

The sections were immersed in 1% (v/v) hydrogen peroxide for 30 min to renature the antigens, then washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 3% (w/v) bovine serum albumin (BSA) in PBS for 45 min at 37 °C. The sections were washed a second time, incubated with anti-granulocyte MAb for 1 h at 37 °C and then incubated with 10 nm colloidal gold conjugated GAMIg (Sigma) diluted at 1:20 for 1 h at 37 °C after a third wash. Finally, the sections were washed with PBS-T and distilled water and then air-dried. The sections were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (Hitachi, Japan) at 100 kV. Negative control was the same as IIFA.

### 2.6. Western blot assay (WBA)

The separated granulocytes and hyalinocytes were sonicated for 4 min respectively and adjusted to the same protein concentration (2 mg $ml^{-1}$) by the Bradford method [23]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% polyacrylamide gels using standard proteins ranging from 29 to 205 kDa as molecular weight markers (Sigma). The gels were stained with Coomassie Blue R-250 for 45 min, then photographed and analysed using Quantity One software (Bio-Rad). This experiment was performed in triplicate.

For western blotting in wet transfer, the gels were blotted onto a nitrocellulose (NC) membrane at 200 mA for 5 h. The NC membrane was blocked with 3% BSA for 1 h at 37 °C, washed with PBS-T three times, and incubated with anti-granulocyte MAb for 1 h at 37 °C. MAb binding was detected with alkaline phosphatise (AP) conjugated goat anti-mouse Ig (GAM-AP, Sigma) diluted at 1:2000 for 1 h at 37 °C and washed three times with PBS-T. The reaction was developed with the substrate of NBT/BCIP in AP buffer (100 mM NaCl, 100 mM Tris−Cl, 5 mM $MgCl_2$, pH 9.5) for 5 min and observed for colour development on the NC membrane. Negative control was the same as IIFA.

### 2.7. Acute viral necrobiotic virus (AVNV) infection

The AVNV supernatant was provided by Prof. Chongming Wang (Yellow Sea Fisheries Research Institute, Qingdao, China). In order to ensure that scallops *C. farreri* were infected successfully, and enough individuals were survived for granulocyte measurement, AVNV supernatant diluted at 1:625 was used as suggested by Xing et al. [16]. Briefly, 150 AVNV-free scallops were injected individually in the adductor muscle with 100 μl of the diluted AVNV supernatant, six scallops were then randomly sampled daily for 9 days. Control scallop was injected with 100 μl of sterilised 2% NaCl. One millilitre of haemolymph from sampled scallops was withdrawn individually from the adductor muscle sinus, simultaneously mixed (1:1) with PBS-E and then centrifuged at 500 × g for 10 min at 4 °C. The haemocyte pellet was respectively resuspended in 1 ml of PBS, sonicated for 4 min, and frozen at −20 °C until use.

### 2.8. Detection of granulocytes using ELISA

Haemocyte samples collected from AVNV challenged scallops were coated in a microtiter plate (100 μl per well), at the same time, 50 μl of 0.5 M EDTA was added to each well in order to eliminate

endoenzyme activities. After incubation overnight at 4 °C and washes with PBS-T, each coated well was blocked with 200 μl of 3% BSA for 1 h at 37 °C, washed thrice with PBS-T, and incubated successively with 100 μl of anti-granulocyte MAb and 100 μl of GAM-AP in a similar procedure to WBA. After the last wash, colour reaction was developed in 100 μl of 0.1% (w/v) pNPP (Sigma) in 0.05 M carbonate–bicarbonate buffer containing 0.5 mM $MgCl_2$ for each well for 20 min, then stopped by the addition of 50 μl of 2 M NaOH. Finally absorbance values were measured at 405 nm with an automatic ELISA reader (Molecular Devices, US). Each measurement was performed in sextuplicate.

### 2.9. Statistical analysis

All data were presented as mean ± standard deviation. Statistical analysis was carried out using SPSS 16.0. The effects of experimental days and treatment (control vs. virus) on granulocyte amount were examined using univariate analysis based on a two-way ANOVA linear model: $y = \mu + \text{Day} + \text{Treatment} + \text{Day} \times \text{Treatment}$. If an interaction between main effects, i.e. day and treatment, was detected, the Student's *t*-test was conducted to examine the treatment effects on each specific experimental day. Factors detected to be of significant effects by ANOVAs were further analysed using a Bonferroni multiple comparison procedure which adjusted the critical significance level ($p < 0.05$) by dividing it by the number of comparisons being made.

## 3. Results

### 3.1. Separation of granulocytes and hyalinocytes

After centrifugation in Percoll gradients, the haemocyte suspension was separated into three layers present at 20–30%, 30–40% and 40–50% Percoll interface, respectively. Flow cytometric analysis showed that the suspension consisted of 54 ± 3.1% of hyalinocytes and 46 ± 2.8% of granulocytes at 20–30% interface (Fig. 1A), 89 ± 1.9% of hyalinocytes at 30–40% interface (Fig. 1B) and 91 ± 1.8% of granulocytes at 40–50% interface (Fig. 1C). Giemsa staining showed that hyalinocytes had few or no cytoplasmic granules (Fig. 1a and b), whereas granulocytes contained dense cytoplasmic granules (Fig. 1a and c).

### 3.2. Production and screening of monoclonal antibody

Screened by IIFA, more than fifty hybridomas secreting positive antibodies were obtained, however, most of the antibodies showed specificities for both hyalinocytes and granulocytes, among them one MAb designated as 6H7 presented a specificity only for granulocytes. As shown in Fig. 2, the fluorescent foci were presented all over the granulocytes, and no fluorescence was labelled on hyalinocytes.

### 3.3. Flow cytometric immunofluorescence assay

Analysed by FCIFA, hyalinocytes and granulocytes formed two areas according to their cell size and cytoplasmic granularity in the dotplots, with the respective percentage of 42 ± 2.9% and 58 ± 2.6% (Fig. 3A and B), additionally, presented their fluorescence intensity in the histograms (Fig. 3a and b). The results illustrated positive haemocytes recognised by MAb 6H7 accounted for 62 ± 2.8% of total haemocytes, comprising of 54 ± 3.0% granulocytes (marker PG) and 8 ± 2.3% hyalinocytes (marker PH) (Fig. 3b).

### 3.4. Immunoelectron microscopy

Observed under an electron microscope, hyalinocytes and granulocytes were ovoid, with central nucleus, and contained abundant cytoplasmic organelles, some of them had a thin pseudopodium. Differently, granulocytes contained many granules and

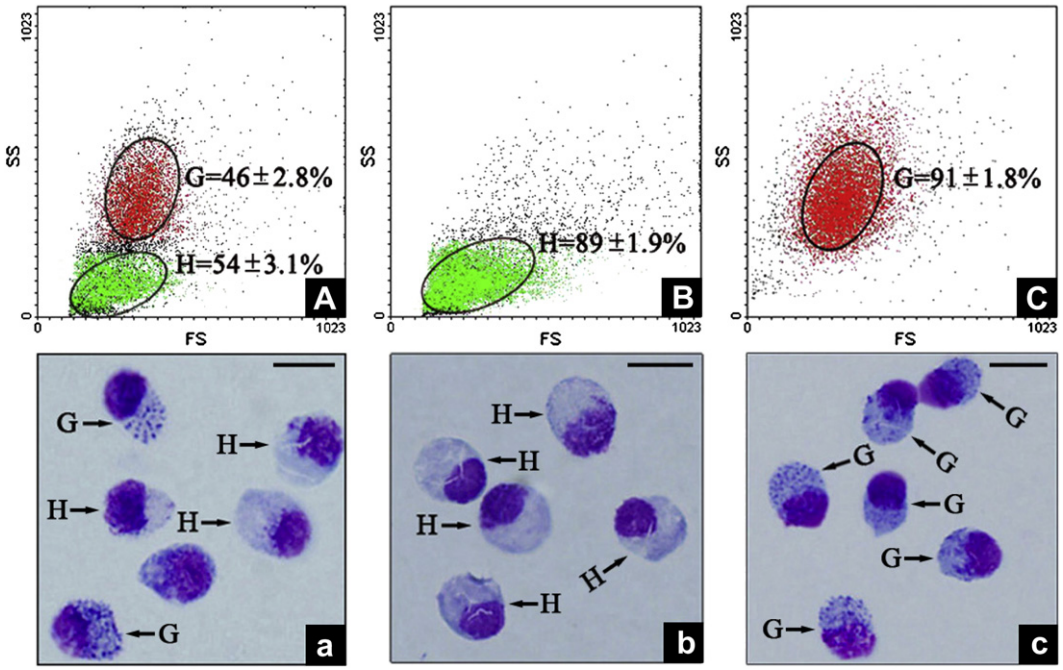


**Fig. 1.** Haemocytes separated using Percoll density gradient centrifugation were analysed by flow cytometry and Giemsa staining. (A), (B) and (C) show separated haemocytes measured by flow cytometry within the layer at 20–30, 30–40 and 40–50% interface, respectively; (a), (b) and (c) show separated haemocytes stained with Giemsa within the layer at 20–30, 30–40 and 40–50% interface, respectively. G: granulocytes; H: hyalinocytes; bar = 10 μm.

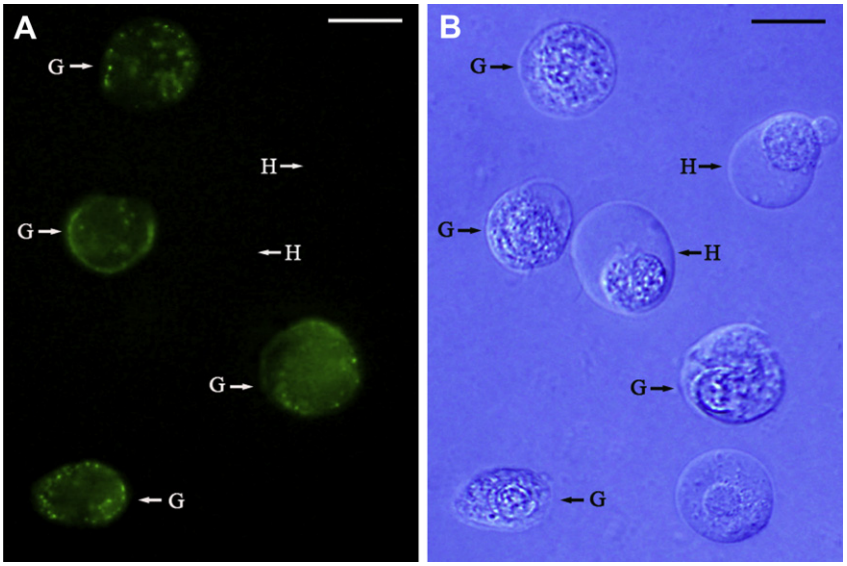


**Fig. 2.** Detection of MAb 6H7 reactive to the haemocytes of *C. farreri* using indirect immunofluorescence assay. (A) and (B) show identical views through fluorescence and differential interference contrast microscopes, respectively. G: granulocytes; H: hyalinocytes; bar = 10 μm.

few vacuoles compared with hyalinocytes (Fig. 4A). Fig. 4B–D revealed colloidal gold nanoparticles were principally in cytoplasmic granules of granulocytes, neither in elsewhere of granulocytes (Fig. 4E) nor in hyalinocytes.

### 3.5. Western blot assay

Hyalinocytes and granulocytes presented many protein bands after SDS-PAGE, their protein profiles were very similar, showing

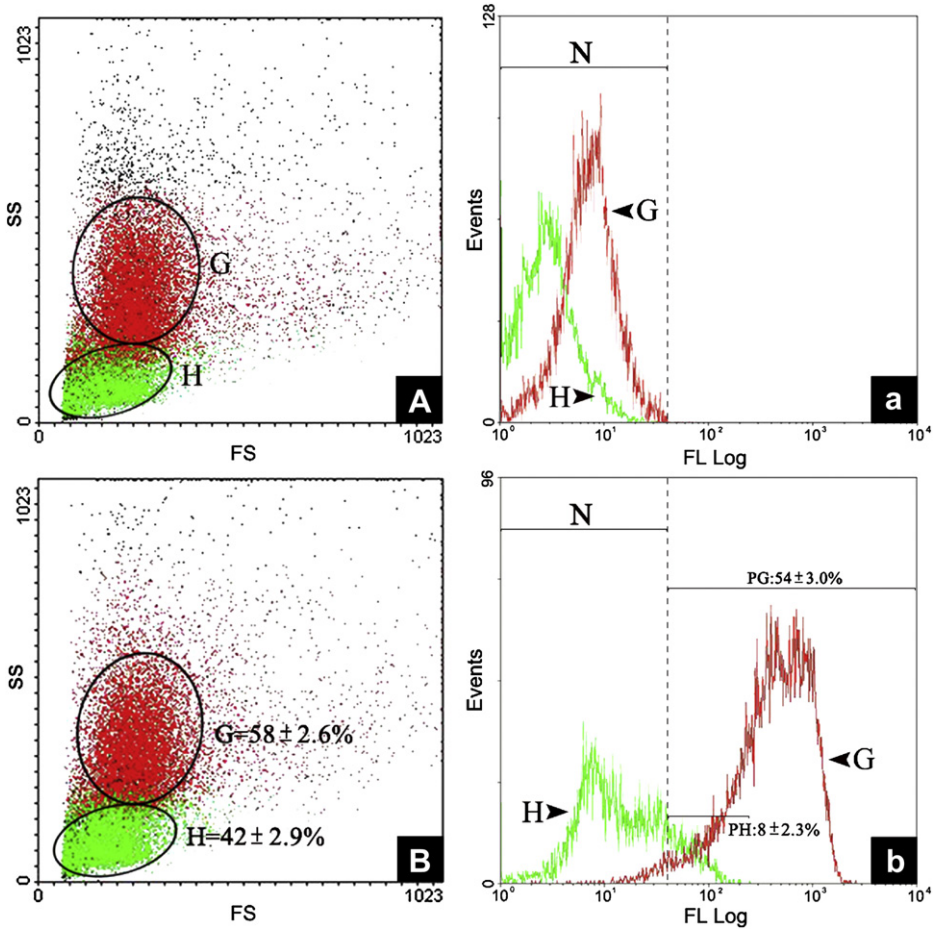


**Fig. 3.** Detection of MAb 6H7 reactive to the haemocytes of *C. farreri* using flow cytometric immunofluorescence assay. (A) and (a): negative control; (B) and (b): MAb 6H7. G: granulocytes; H: hyalinocytes; N: negative; PG: positive granulocytes; PH: positive hyalinocytes.

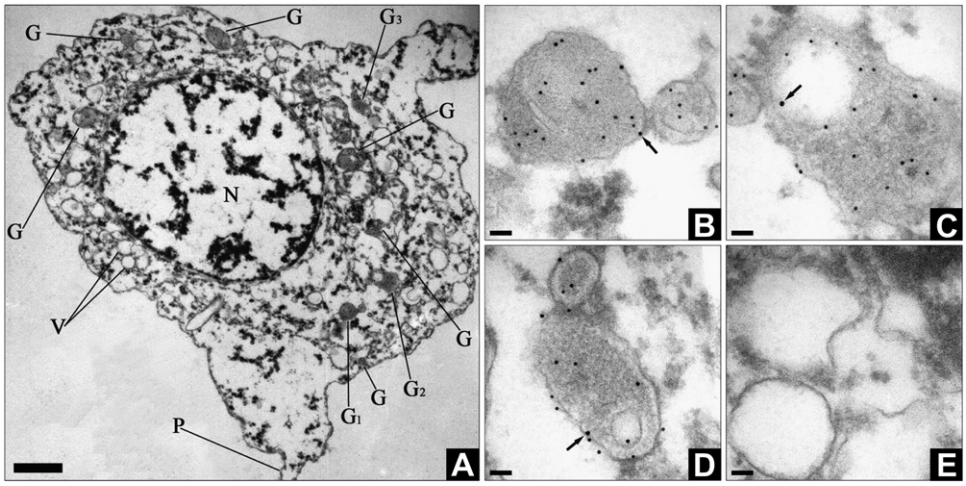


**Fig. 4.** Detection of MAb 6H7 reactive to the haemocytes of *C. farreri* using immunoelectron microscopy. (A) shows overall view of MAb 6H7 positive granulocytes, bar = 1 μm, ×6000; (B), (C) and (D) show magnifications of positive granules of G1, G2, and G3, respectively, bar = 50 nm, ×100,000; (E) shows magnifications of vacuole (negative), bar = 50 nm, ×100,000. G: granule; N: nucleus; P: pseudopodium; V: vacuole.

minor differences in the content of some bands (Fig. 5, Lane 1 and 2). The proteins of 40 kDa, 74.5 kDa and 155 kDa in granulocytes were more abundant than those in hyalinocytes, whereas the protein of 44 kDa in granulocytes was less abundant than that in hyalinocytes. Probing with MAb 6H7 resulted in a clear positive band in the granulocyte lysate at 155 kDa (Fig. 5, Lane 3). No band was observed in the hyalinocyte lysate (Fig. 5, Lane 4).

### 3.6. Variation of granulocytes after AVNV infection

After AVNV injection, scallops began to die on day 2 post-injection, mortality on days 3 and 4 was the highest in the infection period, and no death was found after day 7. For variation of granulocytes, their amount significantly increased on day 1 post-injection ($A_{405nm} = 0.5033 \pm 0.011$, ANOVA, $p < 0.05$), then significantly decreased to the nadir ($A_{405nm} = 0.4338 \pm 0.012$, ANOVA, $p < 0.05$) on day 3, thereafter, rebounded and approached to a second peak on day 6 ($A_{405nm} = 0.5111 \pm 0.014$, ANOVA, $p < 0.05$), finally went down gradually to the control level till day 8 and 9. In control group, the amount of granulocytes maintained at a constant level during the entire experimental period, with a mean of $0.4754 \pm 0.016$ (Fig. 6).

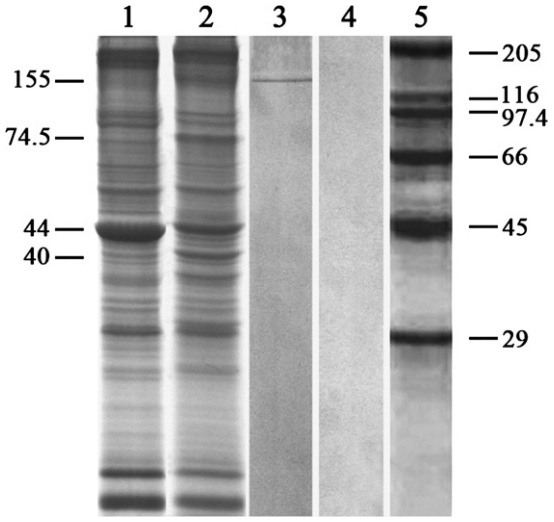


**Fig. 5.** Detection of MAb 6H7 reactive to the granulocytes of *C. farreri* using western blot assay. Lane 1: protein profile of hyalinocytes; Lane 2: protein profile of granulocytes; Lane 3: western blotting result of MAb 6H7 to granulocytes; Lane 4: western blotting result of MAb 6H7 to hyalinocytes; Lane 5: marker (kDa).

## 4. Discussion

Our previous work on the classification of *C. farreri* haemocytes into hyalinocytes and granulocytes was carried out [24], continuously, this paper described the development of anti-granulocyte MAb (6H7) confirmed by FCIFA, IEM and WBA, and the MAb was used in variation of granulocytes in *C. farreri* after AVNV infection.

In IIFA, freshly prepared haemocyte suspension was used as a detection antigen in order to increase the effectiveness of screening anti-granulocyte MAbs because fresh haemocyte suspension contained intact and undamaged granulocytes. Our primary trials on fixed cells using several fixative agents like acetone, ethanol, methanol and paraformaldehyde resulted a failure in anti-granulocyte MAb screening. The reason may be that although fixation could increase the permeability of haemocyte membranes [25], thereby allow antibody access to the cell interior, it may alter the internal structure and membrane protein of haemocytes, for example, fixation with formalin could cause an increase in size and cytoplasmic complexity of haemocytes in oyster *C. virginica* [13]. Similar result was observed in

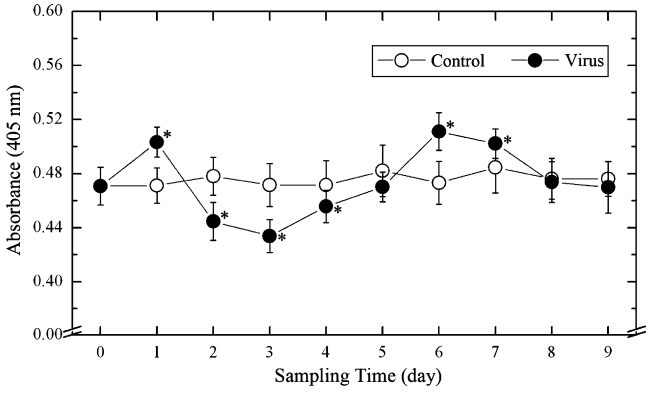


**Fig. 6.** Variation of granulocytes in *C. farreri* haemolymph after AVNV infection using enzyme-linked immunosorbent assay ($n = 6$). Asterisks "*" indicate statistical significance ($p < 0.05$) compared to the control.

*C. farreri*, fixation could induce an alteration in cell size and granularity, which resulted in rarely differentiating two distinct populations using flow cytometry.

Recognised by MAb 6H7, the epitope was present on granulocyte membrane in IIFA, however, it occurred in cytoplasmic granules of granulocytes using IEM, which suggested that similar epitopes might exist both on granulocyte membranes and in cytoplasmic granules. The explanation might be that this epitope was a membrane protein, or an active protein secreted by granules but adhered on granulocyte membranes, because most cytoplasmic granules are membranes enclosed and contain abundant active proteins which can be exocytosed into haemolymph associated with phagocytosis [26–29]. Moreover, as to the reason for absence of colloidal gold in granulocyte membranes, it may be due to ultrasectioned angle, or low abundance of epitopes on haemocyte surface as opposed to within granules.

During FCIFA, in addition to positive granulocytes, 8 $\pm$ 2.3% of haemocytes with low FS and SS was recognised by MAb 6H7 showing they possessed epitope of granulocytes but had morphological features of hyalinocytes, indicating an inconsistence in morphology and antigenicity of haemocyte population, which was originated from different classification criteria used in immunological technique and flow cytometry [30,31].

ELISA was used to investigate granulocyte variation after AVNV infection because of its sensitivity, specificity and high throughput [32], in the meantime, it is suitable for frozen samples, which allows numerous and multiple samples from continuous sampling time to be assayed simultaneously [33,34]. The ELISA results reflected the general variation trend of granulocytes after AVNV infection, which proved that granulocytes varied greatly by the pathogens. Similar results were conducted in clam *Ruditapes decussates*, granulocytes increased when infection with *V. tapetis* [35], while in oyster *O. edulis*, granulocytes decreased when challenge by protozoan *Bonamia ostreae* [36]. Increase or decrease in granulocyte amount in response to pathogenic stress may be attributable to induced or reduced haemopoiesis, or cell migration between tissues and haemolymph [37–39].

In this paper, MAb 6H7 recognised a 155 kDa protein in granulocytes of *C. farreri*, for further work on the characterisation of this protein and the cross-reaction of this antibody to other bivalves will be helpful to understand granulocyte diversity.

## Acknowledgements

We thank Prof. Chongming Wang for kindly providing the AVNV supernatant. This research was supported by the NSFC (grant No. 30901112) and the National 863 Project (grant No. 2006AA100307).

## References

[1] Cheng TC. Hemocytes: forms and functions. In: Kennedy VS, Newell RIE, Eble AF, editors. The eastern oyster *Crassostrea virginica*. College Park, MD, USA: Maryland Sea Grant; 1996. p. 299–333.
[2] Bayne CJ. Phagocytosis and non-self recognition in invertebrates. Bioscience 1990;40:723–31.
[3] Pipe RK. Generation of reactive oxygen metabolites by the haemocytes of the mussel *Mytilus edulis*. Dev Comp Immunol 1992;16:111–22.
[4] Söderhäll K, Cerenius L. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr Opin Immunol 1998;10:23–8.
[5] Franchini A, Ottaviani E. Repair of molluscan tissue injury: role of PDGF and TGF-b. Tissue Cell 2000;32:312–21.
[6] Hine PM. The inter-relationships of bivalve haemocytes. Fish Shellfish Immunol 1999;9:367–85.
[7] Cheng TC. A classification of molluscan hemocytes based on functional evidences. Comp Pathobiol 1984;6:111–46.
[8] Auffret M. Bivalve hemocyte morphology. Am Fish Soc Spec Publ 1988;18:169–77.
[9] Carballal MJ, López C, Azevedo C, Villalba A. In vitro study of phagocytic ability of *Mytilus galloprovincialis* Lmk haemocytes. Fish Shellfish Immunol 1997;7:403–16.
[10] Hégaret H, Wikfors GH, Soudant P. Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation II. Haemocyte functions: aggregation, viability, phagocytosis, and respiratory burst. J Exp Mar Biol Ecol 2003;293:249–65.
[11] Aladaileh S, Nair SV, Birch D, Raftos DA. Sydney rock oyster (*Saccostrea glomerata*) hemocytes: morphology and function. J Invertebr Pathol 2007;96:48–53.
[12] Anderson RS, Oliver LM, Brubacher LL. Superoxide anion generation by *Crassostrea virginica* hemocytes as measured by nitroblue tetrazolium reduction. J Invertebr Pathol 1992;59:303–7.
[13] Hégaret H, Wikfors GH, Soudant P. Flow-cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation I. Haemocyte types and morphology. J Exp Mar Biol Ecol 2003;293:237–48.
[14] Duchemin MB, Fournier M, Auffret M. Seasonal variations of immune parameters in diploid and triploid Pacific oysters, *Crassostrea gigas* (Thunberg). Aquaculture 2007;264:73–81.
[15] Allam B, Ford SE. Effects of the pathogenic *Vibrio tapetis* on defence factors of susceptible and non-susceptible bivalve species: I. Haemocyte changes following in vitro challenge. Fish Shellfish Immunol 2006;20:374–83.
[16] Xing J, Lin TT, Zhan WB. Variations of enzyme activities in the haemocytes of scallop *Chlamys farreri* after infection with the acute virus necrobiotic virus (AVNV). Fish Shellfish Immunol 2008;25:847–52.
[17] Dyrynda EA, Pipe RK, Ratcliffe NA. Sub-populations of haemocytes in the adult and developing marine mussel, *Mytilus edulis*, identified by use of monoclonal antibodies. Cell Tissue Res 1997;289:527–36.
[18] Xue QG, Renault T. Monoclonal antibodies to European flat oyster *Ostrea edulis* hemocytes: characterization and tissue distribution of granulocytes in adult and developing animals. Dev Comp Immunol 2001;25:187–94.
[19] Xing J, Zhan WB. Comparison of antigenicity among haemocytes of seven bivalve species by monoclonal antibodies against haemocytes of scallop (*Chlamys farreri*). Fish Shellfish Immunol 2006;20:528–35.
[20] Smolowitz RM, Miosky D, Reinisch CL. Ontogeny of leukemic cells of the soft shell clam. J Invertebr Pathol 1989;53:41–51.
[21] Noël D, Pipe R, Elston R, Bachère E, Miahle E. Antigenic characterization of hemocyte subpopulations in the mussel *Mytilus edulis* by means of monoclonal antibodies. Mar Biol 1994;119:549–56.
[22] Xing J, Zhan WB. Characterisation of monoclonal antibodies to haemocyte types of scallop (*Chlamys farreri*). Fish Shellfish Immunol 2005;19:17–25.
[23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[24] Xing J, Zhan WB. Hemocyte type of Chinese scallop *Chlamys farreri*. Am Fish Soc Symp 2003;38:301–7.
[25] Vaara M. Agents that increase the permeability of the outer membrane. Microbiol Rev 1992;56:395–411.
[26] Cheng TC, Foley DA. A scanning electron microscope study of the cytoplasmic granules of *Crassostrea virginica* granulocytes. J Invertebr Pathol 1972;20:372–4.
[27] Foley DA, Cheng TC. Degranulation and other changes of molluscan granulocytes associated with phagocytosis. J Invertebr Pathol 1977;29:321–5.
[28] Carballal MJ, López C, Azevedo C, Villalba A. Enzymes involved in defense functions of haemocytes of mussel *Mytilus galloprovincialis*. J Invertebr Pathol 1997;70:96–105.
[29] Davis JE, Sutton VR, Browne KA, Trapani JA. Purification of natural killer cell cytotoxic granules for assaying target cell apoptosis. J Immunol Methods 2003;276:59–68.
[30] Salimi L, Jamili S, Motalebi A, Eghtesadi-Araghi P, Rabbani M, Rostami-Beshman M. Morphological characterization and size of hemocytes in *Anodonta cygnea*. J Invertebr Pathol 2009;101:81–5.
[31] Matozzo V, Rova G, Marin MG. Haemocytes of the cockle *Cerastoderma glaucum*: morphological characterisation and involvement in immune responses. Fish Shellfish Immunol 2007;23:732–46.
[32] Voller A, Bartlett A, Bidwell D. Enzyme immunoassays with special reference to ELISA techniques. J Clin Pathol 1978;31:507–20.
[33] Smith JL, Allen SJ, Cherrington JM. A rapid antiviral in situ enzyme-linked immunosorbent assay for feline immunodeficiency virus. J Virol Methods 1995;54:29–38.
[34] Vidal MI, Stiene M, Henkel J, Bilitewski U, Costa JV, Oliva AG. A solid-phase enzyme linked immunosorbent assay using monoclonal antibodies, for the detection of african swine fever virus antigens and antibodies. J Virol Methods 1997;66:211–8.
[35] Allam B, Ashton-Alcox KA, Ford SE. Haemocyte parameters associated with resistance to brown ring disease in *Ruditapes* spp. clams. Dev Comp Immunol 2001;25:365–75.
[36] Mirella da Silva P, Comesaña P, Fuentes J, Villalba A. Variability of haemocyte and haemolymph parameters in European flat oyster *Ostrea edulis* families obtained from brood stocks of different geographical origins and relation with infection by the protozoan *Bonamia ostreae*. Fish Shellfish Immunol 2008;24:551–63.
[37] Solon E, Gupta AP, Gaugler R. Signal transduction during exocytosis in *Limulus polyphemus* granulocytes. Dev Comp Immunol 1996;20:307–27.
[38] Parry HE, Pipe RK. Interactive effects of temperature and copper on immunocompetence and disease susceptibility in mussels (*Mytilus edulis*). Aquat Toxicol 2004;69:311–25.
[39] Matozzo V, Marin MG, Cima F, Ballarin L. First evidence of cell division in circulating haemocytes from the manila clam *Tapes philippinarum*. Cell Biol Int 2008;32:865–8.