Production, characterization and application of monoclonal antibody to spherulocytes: A subpopulation of coelomocytes of *Apostichopus japonicus*

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**A R T I C L E   I N F O**

Article history:
Received 15 April 2010
Received in revised form 4 July 2010
Accepted 19 July 2010
Available online 24 July 2010

Keywords:
*Apostichopus japonicus*
Coelomocyte
Spherulocyte
Monoclonal antibody

**A B S T R A C T**

One monoclonal antibody (mAb 3F6) against coelomocytes of sea cucumber *Apostichopus japonicus* was developed by immunization of Balb/C mice. Analyzed by indirect immunofluorescence assay test (IIFAT), immunocytochemical assay (ICA), Western blotting and fluorescence-activated cell sorter (FACS), mAb 3F6 showed specific for spherulocytes of *A. japonicus*. The mAb 3F6 recognized an antigen of molecular weight 136 kDa in Western blotting. Isotype analysis revealed mAb 3F6 as IgG type. The flow cytometry assay confirmed the microscopy observations and showed coelomocytes positive to mAb 3F6. The antigenicity of haemocytes or coelomocytes of *Hemicentrotus pulcherrimus*, *Scapharca subcrenata*, *Asterina pectinifera*, *Asterias rollestoni*, *Ruditapes philippinarum*, *Patinopsecten yessoensis* and *Mytilus edulis* was compared and the result showed that none of them was positive with mAb 3F6. Most of cells free in polian vesicle were positive with mAb 3F6. The positive cells are in spherical shape, 5–7 μm in diameter, smaller than coelomic spherulocytes of *A. japonicus*. The result of immunofluorescent staining with cells in hemal vessel showed that there were strong positive signals on cytoplasm of some spherical cells with diameter of 7–8 μm. Some other cells with higher nucleo-plasmatic ratio, about 5–6 μm in diameter showed weak positive signals on membrane. Immunohistochemistry assay revealed that positive signals were mostly observed in the lumen structure of rete mirabile and haemocoel of the respiratory tree. In addition, the outer epidermis of body wall and tentacle also showed positive.

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**1. Introduction**

Sea cucumbers, especially *Apostichopus japonicus*, are economically important organisms in East Asia. Currently, there is a real risk of over-capture of wild sea cucumbers with increasing market demand. High mortality in cultivated sea cucumbers has resulted in heavy economic losses in China. Most of studies on sea cucumbers have focused on culture and management, but studies on cytology and histology received little attention. A systematic understanding of the basic biology of *A. japonicus* is needed in order to protect sea cucumbers in the wild [18]. Coelomocytes in sea cucumbers have a number of functions including gas exchange, nutrient transportation and storage, clot formation, excretion, production of some components of the connective tissue, and immune defense [3,11]. Up to date, studies on coelomocytes of *A. japonicus* mainly focused on the morphological characterization for different types of cells. However, different research groups conducted studies using various methods and evaluation criteria so the published results were incomparable. Eliseikina and Magarlamov [3] described nine types of coelomocytes in *A. japonicus*: progenitor cells, amoeboocytes, vacuolated cells, small morula cells, morula cells of type I, type II and type III, crystal cells and vibratile cells using light microscopy, transmission electron microscopy and histochemistry. Xing et al. [18] distinguished six coelomocyte types in *A. japonicus*: lymphocytes, morula cells, amoeboocytes, crystal cells, fusiform cells and vibratile cells based on cell morphological and ultrastructural features. We identified lymphoid cells, spherulocytes, amoeboocytes, hyaline cells, fusiform cells and crystal cells in a previous study [9] based on microstructural and ultrastructural characteristics of coelomocytes in *A. japonicus*.

Monoclonal antibodies (mAbs) have been frequently used as essential probes for identification of cell types and determining functions of immune cells in vertebrate. Recently, mAbs have begun to be used in invertebrate immune studies. Rodriguez et al. [13] developed three mAbs against shrimp *Penaeus japonicus* haemocytes and identified various haemolymph components of the shrimp using those mAbs. Dyrynda et al. [2] produced three groups of mAbs to identify subpopulations of haemocytes in marine mussel *Mytilus edulis*. Xue and Renault [22] produced six mAbs against
haemocytes of European flat oyster Ostrea edulis and used one of them to investigate granulocyte distribution and ontogenesis in European flat oyster using immunohistochemistry. Xing and Zhan [20,21] produced four mAbs against haemocytes of scallop Chlamys Farreri, and analyzed haemocyte antigenicity of seven bivalve species by using these mAbs. Lin et al. [10] developed several mAbs against coelomocytes of the purple sea urchin Arbacia punctulata and demonstrated these mAbs could inhibit coelomocyte cytotoxicity against vertebrate target cells in a functional assay. Several authors [15–17] developed mAbs against haemocytes in Penaeus monodon and studied the differentiation, behavior and function of haemocytes by using the mAbs.

In this study, one mAb specific for coelomic spherulocytes of A. japonicus was developed and characterized by using indirect immunofluorescence assay test (IIFAT), immunocytochemical assay (ICA), Western blotting, fluorescence-activated cell sorter (FACS) and immunohistochemistry. The reaction of cells free in polian vesicle or hemal vessel of A. japonicus was analyzed with this mAb. Furthermore, the antigenicity of haemocytes or coelomocytes was compared among seven different species by using this mAb.

2. Materials and methods

2.1. Antigen preparation

Healthy sea cucumbers, 125 ± 25 g in weight, were collected from a local aquatic farm (Dalian, China) and kept in the aquaria with aerated seawater in the laboratory. Coelomic fluid was drawn from the right lateral side of the body and immediately diluted (1:3) in antiaggregant modified Alsever’s solution (MAS, 27 mM sodium citrate, 336 mM sodium chloride, 115 mM glucose, 9 mM EDTA, pH 5.6). The coelomocytes were washed twice with 0.01 M phosphate buffered saline (PBS, 0.13 M NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, pH 7.4), centrifuged at 800 × g, and then resuspended in PBS.

2.2. Coelomocyte monolayer preparation

Monolayers of coelomocytes were prepared by allowing cells to settle onto glass slides and incubated at room temperature in a wet chamber for 45 min. The slides were then air dried, fixed in acetone for 15 min and stored at −20 °C for later use.

2.3. Production of monoclonal antibodies

Three Balb/C mice were immunized by intraperitoneal injection with 100 μl coelomocytes suspension (4 × 107 cells ml−1). Two weeks later, a similar injection was administrated. Then another 2 booster injections were given by tail vein at 1-week intervals. Three days after the last injection, the mice were sacrificed. Spleen cells were collected from the immunized mouse and fused with myeloma cells, Sp2/0 using 45% polyethylene glycol 4000. The cells were distributed into 96-well culture plates (Costar) in RPMI-1640 medium (Hyclone) supplemented with 20% newborn calf serum (HyClone) and 1% HAT (Gibco), and the culture medium was changed every 3–5 days. After 12–14 days, the supernatants from those wells growing hybridomas were screened using IIFAT. Hybridomas giving positive results were cloned by limiting dilution three times, and the monoclonal antibodies (mAbs) were characterized by IIFAT, ICA, Western blotting, FACS and immunohistochemistry.

2.4. Indirect immunofluorescence assay test (IIFAT)

Monolayers were overlaid with 50 μl hybridoma culture fluid as primary antibody and incubated at 37 °C for 45 min in a wet chamber. After three washes in PBS with 5 min intervals, the cells were incubated for 45 min at 37 °C in darkness with goat-anti-mouse Ig conjugated with fluorescein isothiocyanate (GAM-FITC, Sigma) at the dilution of 1:256. The Evans blue dye (EBD, Fluka) was included at 1 μg/ml as the counterstain. After three washes, the slides were mounted in buffered glycerin and viewed under fluorescence microscope (Nikon). Myeloma culture supernatant was used as negative control.

2.5. Immunocytochemical assay (ICA)

Endogenous peroxidase was eliminated by incubation with 3% hydrogen peroxide in methanol for 20 min. After three washes in PBS containing 0.05% Tween-20 (PBS-T) with 5 min intervals, the cell slides were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. Then two incubations were successively performed with mAb against coelomocytes and horseradish peroxidase conjugated goat-anti-mouse IgG diluted 1:100 (GAM-HRP, Sigma). After antibody incubations, three washes in PBS-T were carried out. The reaction was developed with freshly prepared substrate solution, 0.03% diamino benzidine (DAB) and 0.03% H2O2 in PBS for 30 min, and then washed with tap water. Finally, the cell slides were slightly counterstained with haematoxylin and mounted in buffered glycerin. Myeloma culture supernatant was used as negative control.

2.6. Western blotting

Coelomocytes (1 × 107 cells ml−1) were resuspended in RIPA lysis buffer I (P005, Shanghai Sangon). After 10 min of incubation at 4 °C, cell debris was removed by centrifugation (12,000 g, 5 min) and the supernatant kept at −20 °C until used. The coelomocytes lysate was denatured and solubilized by 5 min boiling in buffer (0.5 M Tris–HCl, pH 6.8, containing 5% SDS, 50% glycerol, 5% 2-mercaptoethanol and 0.05% bromophenol blue), and then separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli [8]. Samples were electrophoresed at 150 V for 1 h and part of gel was stained with Coomassie brilliant blue R-250.

For Western blotting analysis, the samples separated by SDS-PAGE were electrophoretically transferred onto a 0.2 μm pore nitrocellulose membrane (Pall) at 200 mA for 5 h. The membrane was blocked with 3% BSA in PBS overnight at 4 °C, and incubated with mAb diluted 1:10 for 1 h at 37 °C, then washed three times with PBS-T. Antibody binding was detected with alkaline phosphatase conjugated goat-anti-mouse Ig (GAM-AP, Sigma) diluted 1:4000 in PBS for 1 h at 37 °C, and washed three times with PBS-T. Positive bands were stained with freshly prepared substrate solution (100 mM NaCl, 100 mM Tris and 5 mM MgCl2, pH 9.5) containing nitroblue tetrazolium (NBT, Sigma) and 5-bromo-4-chloro-3-indolyphosphate (BCIP, Sigma) for 5 min and stopped by washing with distilled water. Myeloma culture supernatant was used as negative control.

2.7. FACS analysis

Coelomocytes were drawn from healthy A. japonicus and was immediately diluted (1:3) in MAS. The coelomocytes were washed twice with PBS, centrifuged at 800 × g, and then resuspended in PBS. The anti-coelomocytes mAb was added to coelomocytes at the dilution of 1:10 and incubated for 1 h at 37 °C. Subsequently, the cells were washed three times with PBS and incubated with GAM-FITC diluted 1:256 in PBS for 1 h at 37 °C. After washes, the coelomocytes were analyzed with an FACScan (Becton Dickinson, FACScalibur™). Myeloma culture supernatant was used as negative control.
2.8. Comparison of antigenicity among haemocytes or coelomocytes of seven species

Seven species sea urchin Hemicentrotus pulcherrimus, ark shell Scapharca subcrenata, starfish Asterina pectinifera, starfish Asterias rollestoni, Japanese carpet shell Ruditapes philipinarum and blue mussel Mytilus edulis were purchased from market in Dalian (China) and kept in the aquaria with aerated seawater in the laboratory until next day experiment. Haemolymph of three bivalve species were obtained from the adductor muscle sinus and coelomic fluid of three echinoderm species were drawn from the coelom. They were withdrawn and immediately diluted (1:3) in MAS, monolayers of haemocytes or coelomocytes were prepared as above. The comparison of antigenicity among haemocytes or coelomocytes of above seven species was analyzed using mAb against coelomocytes of A. japonicus, employed method of IIFAT described above.

2.9. Reaction with cells free in polian vesicle or hemal vessel of A. japonicus

The cells free in polian vesicle or hemal vessel above the intestine were obtained using syringe pre-treated with cold MAS, and the monolayers of those cells were prepared according to the method mentioned above. The reactions of mAb against coelomocytes of A. japonicus with the cells free in polian vesicle or hemal vessel were analyzed using method of IIFAT mentioned above.

2.10. Immunohistochemistry

Several tissues of A. japonicus, including tentacle, body wall, respiratory tree, the structure of rete mirabile were fixed in Bouin’s fluid for 24 h. The fixed tissues were rinsed, dehydrated and embedded in paraffin wax. Longitudinal sections (5 μm) were cut and mounted on slides. After deparaffination in xylene and rehydration in diluted ethanol series, the sections were incubated with 3% hydrogen peroxide in methanol for 20 min to eliminate endogenous peroxidase. After three washes in PBS-T, antigen retrieval was performed by heating in water bath. Then the remaining procedures for antibody incubation, DAB substrate reaction and counterstain were identical to immunocytochemical assay mentioned above. As a negative control, myeloma culture supernatant instead of mAb was as primary antibody.

3. Results

3.1. Production and selection of mAbs

After the fusion, approximately 75% (144 hybridoma cultures) wells were found to yield hybridomas. Eleven hybridomas (approximately 8%) secreted antibodies were selected by IIFAT and ICA. Finally, one hybridoma secreted mAb reacting specifically with cytoplasm of spherulocytes (a subpopulation of coelomocytes of A. japonicus) was selected, cloned and subcloned by limiting dilution. The coelomocytes identified by the mAb showed uniform fluorescence in the cytoplasm by IIFAT and brown color by ICA (Fig. 1). The positive coelomocytes are spherical in shape, 8–10 μm in diameter with lower nucleo-plasmic ratio and some granules in the cytoplasm. This mAb was designated as 3F6 and isotyped as IgG.

3.2. Western blotting

The result of Western blotting was shown in Fig. 2. Many bands were observed after Coomassie staining of the total proteins from coelomocytes (lane 2). Probing these electro blots with mAb 3F6 recognized an antigen of coelomocytes and revealed a definite band at 136 kDa (lane 3).

3.3. FACS analysis

At least 20,000 cells were counted in the flow cytometry assay. The results were presented as cell cytograms using a dot plot combination of low angle forward scattered (FSC) and right angle scattered (SSC) laser light and FITC fluorescence histograms of immunostained cells (Fig. 3). A 51.24% of coelomocytes labeled with mAb 3F6 was counted by the cytometer. The result of flow cytometry assay in this study confirmed the microscopy findings and demonstrated coelomocytes positive to mAb 3F6.

3.4. Comparison of antigenicity among haemocytes or coelomocytes of seven species

Detected by IIFAT, none of the haemocytes or coelomocytes from sea urchin, starfish A. pectinifera, A. rollestoni, ark shell, Japanese carpet shell, Yesso scallop or blue mussel showed positive with mAb 3F6. Only coelomic spherulocytes from A. japonicus showed positive reaction with mAb 3F6.

Fig. 1. Detection of coelomocytes reacted with mAb 3F6 by indirect immunofluorescence assay test (A) and immunocytochemical assay (B). Bar = 10 μm.
3.5. Reaction with cells free in polian vesicle or hemal vessel of \textit{A. japonicus}

Most of cells free in polian vesicle were positive with mAb 3F6. The positive cells are in spherical shape, 5–7\( \mu \)m in diameter, smaller than coelomic spherulocytes of \textit{A. japonicus} (Fig. 4A, B). After immunofluorescent staining, some cells collected from hemal vessel showed strong positive signals on cell cytoplasm. These cells are in spherical shape with diameter of 7–8\( \mu \)m. The other cells with higher nucleo-plasmic ratio, about 5–6\( \mu \)m in diameter usually showed weak positive signals on membrane (Fig. 4C, D).

3.6. Immunohistochemistry

Analyzed by immunohistochemistry, the positive signals were mostly observed in the lumen structure of rete mirabile and haemocoel of the respiratory tree (Fig. 5A, C). In addition, the outer epidermis of body wall and tentacle also showed positive (Fig. 5E, G).

4. Discussion

In our previous study, coelomocytes of \textit{A. japonicus} were divided into six cell types: lymphoid cells (41 ± 1.47\%), spherulocytes (30 ± 0.89\%), amoeboid phagocytes (25 ± 0.98\%), hyaline cells, fusiform cells and crystal cells [9] based on morphological and ultrastructural features. The main objective in preparing mAbs against \textit{A. japonicus} coelomocytes was to develop specific markers for further identification of coelomocyte subpopulation on the basis of morphological and ultrastructural features, coelomocyte distribution in tissues, ontogenesis and regeneration of coelomocytes, etc. In this study, we attempted to separate different types of coelomocytes from \textit{A. japonicus} by isopycnic centrifugation in discontinuous density gradient of sucrose, Percoll or Ficoll, but none of the methods generated ideal results (data not shown). Therefore, we had to use all of coelomocytes as antigen to immunize mice.

Several antibodies against coelomocytes of \textit{A. japonicus} were screened in this study. However, most of them showed strong cross-reaction with coelomic fluid components of \textit{A. japonicus} (data not shown).

![Fig. 2. Analysis of mAb 3F6 by Western blotting. Lane 1, marker; lane 2, protein profile of coelomocytes of \textit{A. japonicus} by sodium dodecyl sulfate polyacrylamide gel electrophoresis; lane 3, Probing with mAb 3F6 recognized an antigen of coelomocytes and revealed a definite band at 136 kDa.](image)

![Fig. 3. Detection reaction of mAb 3F6 with coelomocytes by FACS. A: Flow cytometric immunofluorescence dot plots of \textit{A. japonicus} coelomocytes labeled with mAb 3F6 or C: labeled with myeloma culture supernatant (negative control). B: The distribution of fluorescence of coelomocytes labeled with mAb 3F6 gated in A and D: The distribution of negative control coelomocytes gated in C.](image)
shown). This indicates that there are common antigens between coelomocytes and coelomic fluid of *A. japonicus*. Among those hybridomas secreted antibodies, only 3F6 was raised since it produces specific antibody. After characterized by four methods, the mAb 3F6 was demonstrated specifically positive with spherulocyte. Unfortunately, different coelomocyte subpopulations of *A. japonicus* were not distinguishable according to cell size and granularity by FACS analysis. In a previous study, Xing et al. [18] reported *A. japonicus* coelomocytes could be distinguished to three subpopulations according to partial size as analyzed by flow cytometry (FCM). However, the result in dot plot was not yet significant in this study.

Detected by IIFAT, none of the haemocytes or coelomocytes of *H. pulcherrimus*, *A. pectinifera*, *A. rollestoni*, *S. subcrenata*, *R. philipinarum*, *P. yessoensis* or *M. edulis* was positive with mAb 3F6. The finding indicated that mAb 3F6 was species specific and did not recognize antigen exist in the haemocytes or coelomocytes of above seven species. However, the result could not exclude that there might be other common antigens exist between *A. japonicus* and above other species.

Water vascular system of sea cucumbers has no connection to the outside so it is filled with the internal coelomic fluid. Polian vesicles function as pumps to maintain pressure and circulation of fluid in the water vascular system [14]. Immunofluorescence results showed most of cells free in polian vesicle were positive with mAb 3F6. The positive cells in polian vesicle are different from coelomic spherulocytes, which possess higher nucleo-plasmatic ratio and are smaller in diameter. The result in this study shows that there are common antigens between cells free in coelom and polian vesicle. However, cells from both organs are not identical. Further research is needed to determine the origin of coelomocytes and understand the relationship between cells free in coelom and polian vesicle.

Xing et al. [19] reported four types of cells in hemal vessel of *A. japonicus*: lymphocytes, amoebocytes, morula cells and crystal cells based on light and scanning electron microscopical studies. In this study, cells with strong fluorescent signals on cytoplasm were morphologically like morula cells, and cells with weak positive signals on membrane were lymphocytes. These results demonstrated that common antigens exist between haemocytes and coelomocytes of *A. japonicus*. However, the relationship of haemocytes and coelomocytes was not yet clear.

A few previous studies described the relationship between coelomocytes and hemal vessel or respiratory tree of holothurians. Cuénot [1] and Hatanaka [5] reported that coelomocytes originated from the hemal ring and vessels. Prosser and Judson [12] noted the possibility that coelomocytes of *Sicthopus* might originate in the hemal vessel. Hetzel [7] suggested that at least some, if not all, lymphocytes possibly originated from mesenchymal cells in the hemal vessels of holothurians. Hérouard [6] indicated that the coelomocytes originated in the tissues of the respiratory trees. Endean [4] indicated that the homogeneous amoebocytes (lymphocytes) originated from the lining epithelium of the respiratory trees in *Holothuria leucospilota*, migrated into the coelomic fluid, and differentiated into morula cells and possibly other coelomocyte types. However, the cell distinctions were mainly based on morphological and microstructural observations. In this study, we demonstrated the correlation between spherulocytes and the structure of rete mirabile and respiratory tree of *A. japonicus* on the basis of antigen distribution.

![Fig. 4. Detection of cells free in polian vesicle or hemal vessel reacted with mAb 3F6 by IIFAT. Polian vesicle (A) or hemal vessel (C) above the intestine of *A. japonicus* (arrow indicated) B, D: IIFAT demonstrated cells free in polian vesicle (B) or hemal vessel (D) of *A. japonicus* after immunofluorescence stain with mAb 3F6.](image-url)
Fig. 5. Immunohistochemistry of the structure of rete mirabile (A, B), respiratory tree (C, D), body wall (E, F) and tentacle (G, H). The pictures on the left (A, C, E and G) were positive reaction using mAb 3F6 and the pictures on the right (B, D, F and H) were negative control using myeloma culture supernatant. Bar = 20 μm. Lu: lumen; M: muscle; Ca: center antrum; He: haemocoel; C: cuticula; E: epithelium; Ct: connective tissue.
In addition, the outer epidermis of body wall and tentacle show positive in immunohistochemistry assays, which indicated that there were spherulocytes or similar antigen distribution on the outer epidermis of body wall and tentacle. The spherulocytes or antigen may have certain immune defense functions.

In summary, one mAb 3F6 was developed and demonstrated to be specific to spherulocytes of A. japonicus. The spherulocytes can be identified and distributions of spherulocytes can be located in different tissues using mAb 3F6 by IIFAT, ICA and immunohistochemistry. Western blotting with mAb 3F6 revealed an antigen in different tissues using mAb 3F6 by IIFAT, ICA and immunohistochemistry. Western blotting with mAb 3F6 revealed an antigen of coelomocytes and showed a definite band of molecular weight 136 kDa. The mAb 3F6 will be a useful tool for future studies on the relationship between haemocytes and spherulocytes, spherulocytes ontogenesis and origin, and functions of spherulocytes.

Acknowledgements

This study was supported by National Natural Science Foundation of China (30800853), Educational Commission Project of Liaoning (2008144), National Key Projects, National Science & Technology Pillar Program during the eleventh Five-Year-Plan period (2006BAD09A01), National 908 Special Projects (908-ZH-03).

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