

— Report —

Monoclonal antibodies to hemocytes of the deep-sea symbiotic mussel, *Bathymodiolus japonicus*

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The deep-sea mytilid mussel, *Bathymodiolus japonicus*, harbors methane-oxidizing bacteria in the gill epithelial cells that are transmitted horizontally or environmentally to the next generation of the mussel. It remains to be elucidated how the symbiotic bacteria are maintained under the immune defense system of the host mussels. As hemocytes generally play a major role in the immune system, their characterization is important in understanding the immune defense system of the symbiotic mussel. In hemocytes of *B. japonicus*, two types of granulocytes and one type of agranulocyte have been reported. To develop biomarkers to identify these hemocyte subpopulations, we generated a monoclonal antibody (mAb) library against mussel hemocytes. We obtained 16 hybridoma clones producing mAbs against the hemocytes. These were divided into six categories based on reactivity to the hemocyte subpopulations. Ten out of 16 mAbs reacted to all of the hemocytes. Four of the remaining six mAbs respectively and exclusively reacted to granulocytes, agranulocytes, a subset of granulocytes, and a subset of agranulocytes. Two of them recognized both granulocytes and agranulocytes but only their subsets. These suggested that granulocytes and agranulocytes are respectively composed of heterogeneous subsets. The present mAb library may be useful not only for the classification of the hemocytes, but also for investigating their functions, differentiation and localizations in the body. The information obtained from the mAbs will be a baseline for understanding the relationship between the immunological defense system and stable maintenance of symbiotic bacteria in the deep-sea symbiotic bivalves.

Keywords : hemocyte, monoclonal antibody, *Bathymodiolus*, mussel, deep-sea, symbiotic bacteria

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1. Introduction

In deep-sea hydrothermal vents and seeps, unique symbiotic communities based on chemosynthesis are found (Cavanaugh et al., 1981; Felbeck et al., 1981). A deep-sea mytilid mussel, *Bathymodiolus japonicus* belonging to the family Mytilidae, harbors methane-oxidizing bacteria in the gill epithelial cells that are transmitted horizontally or environmentally (Fujiwara et al., 2000). The immune system is essential for protection against pathogenic bacteria and survival. However, it is unknown how the endosymbiotic bacteria are maintained by escaping from the immune defense system of host animals. Hemocytes generally play a major role in an immune defense system, and in bivalves, circulating hemocytes are responsible for both cell-mediated and humoral immunities (Bayne, 1983; Hine, 1999; Muroga and Takahashi, 2007; Takahashi and Muroga, 2008; Donaghy et al., 2009). Phagocytosis, one of the most important cellular immune responses, has been shown in the hemocytes of *Bathymodiolus* mussels (Bettencourt et al., 2009; Tame et al., 2015). In asymbiotic *Mytilus* mussels (Mytilidae), antimicrobial humoral immune factors from the hemocytes, such as defensin, mytilin and myticin, have been reported (Mitta et al., 1999a, 1999b, 2000), in addition to their phagocytic activities (Pipe, 1990; Carballal et al., 1997). Accumulation of immunological information seems to be a promising strategy in reaching an understanding of the symbiont maintaining mechanism in deep-sea symbiotic bivalves. We have recently reported that hemocytes of *B. japonicus* contain two types of granulocytes, basophilic granulocytes and eosinophilic granulocytes, in addition to an agranulocyte (Tame et al., 2015). They are recognized by observation under light microscopy with conventional hemocyte staining, and by electron microscopy (Tame et al., 2015). Only the granulocytes showed phagocytic activity, and bound a lectin, wheat germ agglutinin (WGA) (Tame et al., 2015). To understand the function and tissue distribution of mussel hemocytes, biological markers recognizing functional subpopulations of the hemocytes are mandatory. Monoclonal antibodies (mAbs) are highly specific molecular probes used in characterization of hemocyte subpopulations. In *Mytilus edulis*, some mAbs binding to granulocytes have been generated (Noel et al., 1994; Dzynda et al., 1997). However, this approach has not been developed further, nor is it widely used in invertebrate immunology or symbiosis biology. In the present study, we have generated a mAb

library against the hemocytes of *B. japonicus*, and report mAbs distinguishing particular hemocyte populations.

2. Materials and Methods

2.1 Construction of the monoclonal antibody library

Bathymodiolus japonicus mussels were collected at a seep site off Hatsushima Island in Sagami Bay (35°0.948'N, 139°13.332'E, depth of 861 m), using the remotely operated vehicle (ROV) *Hyper Dolphin* during cruise aboard the R/V Natsushima (NT10-08, May 11-17, 2010) (Inoue, 2010). The hemolymph was withdrawn from the posterior adductor muscle using a 5 ml syringe with a 25-gauge needle. After determining cell density with a cell counter, the hemocytes were collected by centrifugation of 5 ml hemolymph at $180 \times g$ for 5 min at 4°C, and stored at -80 °C until used. The mAbs were generated by a conventional polyethylene glycol (PEG) method (Galfré and Milstein, 1981), as described previously (Nakamura et al., 2013). In brief, the hemocytes (6×10^5 cells) were injected into BALB/c mice three times at 2-week intervals. On day 4, after the third injection, the mouse splenic lymphocytes were collected and fused with cells of a murine myeloma cell line, NS-1. The fused hybridoma cells were incubated in hypoxanthine-aminopterin-thymidine selective medium (GIT medium containing 100 μ M Hypoxanthine, 0.4 μ M aminopterin, and 16 μ M thymidine; Wako Pure Chemical Industries, Osaka, Japan) for 10 days. Supernatants of the hybridoma cell cultures were stored at 4°C until used for dot blot indirect immunofluorescence staining or immunofluorescence microscopy. Cloning was performed for the immunofluorescence-positive hybridoma cells using the limited dilution method, which was repeated three times.

2.2 Detection of antibody in supernatant from hybridoma

Supernatant (5 μ l) from each hybridoma culture was transferred to nitrocellulose membrane (Merck Millipore Co. Ltd., Billerica, MA), and incubated with 1:2000 diluted goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Thermo Fisher Scientific Inc., Waltham, MA) for 2 hr at room temperature. After washing with PBS three times, the membrane was reacted with color development solution (1 tablet of 3,3'-Diaminobenzidine, tetrahydrochloride [Wako Pure Chemical Industries, Osaka, Japan] in 50 mM Tris-HCl [pH 8.0]) added with 500 μ l of

30% H₂O₂.

2.3 Immunofluorescence staining on the hemocytes with mAbs

Hemocytes of *B. japonicus* collected using the ROV *Hyper Dolphin* during the three research cruises aboard the R/V Natsushima (NT10-08, NT11-09: June 15–26, 2011, and NT13-07: April 2–10, 2013) (Inoue, 2010, 2011; Yoshida, 2013), were fixed on glass slides with equal volumes of 4% paraformaldehyde in seawater for 1 hr at 4°C. Supernatants of the hybridoma cell cultures were incubated with the hemocytes at 4°C for 2 hr. After washing with PBS three times, the glass slides were incubated with anti-mouse IgG goat antibody conjugated to Alexa Fluor 488 (Life Technologies Co. Ltd., Wyman, MA) and WGA conjugated to Alexa Fluor 594 (Life Technologies Co. Ltd., Wyman, MA) at 4°C for 30 min. The nuclear DNA of the hemocytes was stained with DAPI (4', 6-diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich Co. Ltd, St. Louis, MO). A Keyence fluorescence microscope (BZ-9000, Keyence Co. Ltd., Osaka, Japan) and a Nikon fluorescence microscope (Optiphot, Nikon Co. Ltd., Tokyo, Japan) were used for observation.

3. Results

We obtained 234 hybridoma clones in the selection medium, and confirmed the secretion of IgG in the supernatant of 127 of the 234 clones by dot blot method using goat anti-mouse IgG antibody conjugated to horseradish peroxidase. After screening through immunofluorescence staining and cloning three times, we obtained 16 hybridoma clones that demonstrated growth stability and production of hemocyte binding mAb. The list of obtained mAbs is shown in Table 1. The 16 mAbs were divided into six categories based on the reactive hemocyte subpopulations. Ten mAbs bound to all types of the hemocytes (category i, Table 1). The remaining six mAbs, on the other hand, reacted with a subset of the hemocyte population, suggesting the population was composed of multiple subsets. Because the lectin, WGA, has been shown to specifically bind to the granulocytes of *B. japonicus* (Tame et al., 2015), the reactivity of the six mAb clones to the hemocytes was examined by fluorescence staining with the respective mAb clones and WGA. Although two types of granulocytes were reported (Tame et al., 2015), we could not simultaneously identify them in the present study. As shown in Table 1, the six mAbs were divided into five categories: mAb in category ii specifically bound to WGA-positive [WGA(+)] hemocytes, that in category iii

Table 1. Reactivity of the mAbs to the hemocytes of *Bathymodiolus japonicus*

mAb ID No.	Cells reacted with mAb		Category	Data base ID JAMSTEC No.*	
	WGA-positive cells (Granulocytes)	WGA-negative cells (Agranulocytes)		Antibody (Supernatant)	Hybridoma cell
Bjh4-1A7	+	+	i	1100046928	1100046929
Bjh4-1A8	+	+	i	1100046930	1100046931
Bjh4-1B3	+	+	i	1100046932	1100046933
Bjh4-1D7	+	+	i	1100046934	1100046935
Bjh4-1E9**	+	+	i	1100046936	–
Bjh4-1G11	+	+	i	1100046937	1100046938
Bjh4-2C1	+	+	i	1100046939	1100046940
Bjh4-2C2	+	+	i	1100046941	1100046942
Bjh4-2C6	+	+	i	1100046943	1100046944
Bjh4-2G8	+	+	i	1100046945	1100046946
Bjh4-3G2	+	–	ii	1100046947	1100046948
Bjh4-2D11**	–	+	iii	1100046949	–
Bjh4-1F4**	H	–	iv	1100046950	–
Bjh4-2D6**	–	H	v	1100046951	–
Bjh4-3B12	H	H	vi	1100046952	1100046953
Bjh4-3B7	H	H	vi	1100046954	1100046955

+ or – was judged by immunofluorescence staining, using the hemocytes as antigens.

H, indicates heterogeneity, i.e., mAb reacted with a set of the cells of the category.

*, mAb and hybridoma cell line are available. Please see the JAMSTEC Data base (http://www.godac.jamstec.go.jp/bio-sample/index_e.html)

**, the hybridoma cell line was lost, but the supernatant is available.

specifically to WGA-negative [WGA(-)] hemocytes, that in category iv to a subset of WGA(+) hemocytes, that in category v to a subset of WGA(-) hemocytes, and that in category vi to a subset of WGA(+) hemocytes and a subset of WGA(-) hemocytes (Table 1). The immunofluorescence images of the hemocytes stained with mAb Bjh4-3G2 (category ii) are shown in Fig. 1. The mAb signal appeared only on the WGA(+) hemocytes, and it was clearly observed on the granules (Fig. 1). Conversely, the mAb Bjh4-2D11 (category iii) signal was observed only in the cytoplasm of WGA(-) hemocytes. The Bjh4-2D11-positive [Bjh4-2D11 (+)] dot-like signals were observed beneath the cell membranes (Fig. 2). Other four mAbs seemed to recognize a subset of WGA(+) and/or WGA(-) hemocytes. The mAb Bjh4-1F4 signal was found on the membrane in a subset of WGA(+) hemocytes (category iv, Table 1). Fig. 3 (a-c) shows images of a Bjh4-1F4 (+)/WGA(+) hemocyte (Fig. 3a), a Bjh4-1F4 (-)/WGA(+)/hemocyte (Fig. 3b), and a Bjh4-1F4 (-)/WGA(-) hemocyte (Fig. 3c). The mAb Bjh4-2D6 signal was observed in the cytoplasm only in a subset of WGA(-) hemocytes (category v, Table 1). Fig. 3 (d-f) shows images of a Bjh4-2D6 (+)/WGA(-) hemocyte

(Fig. 3d), a Bjh4-2D6 (-) WGA(+) hemocyte (Fig. 3e), and a Bjh4-2D6 (-)/WGA(-) hemocyte (Fig. 3f). Both mAbs Bjh4-3B7 and Bjh4-3B12, were bound to a subset of WGA-positive and a subset of WGA-negative hemocytes (category vi, Table 1). Fig. 4 shows images of mAb(+)/WGA(+) hemocytes (Fig. 4a, e), mAb(-)/WGA(+) hemocytes (Fig. 4b, f), mAb(+)/WGA(-) hemocytes (Fig. 4c, g), and mAb(-)/WGA(-) hemocytes (Fig. 4d, h). The signals of mAb Bjh4-3B7 were observed beneath the cell membrane and cytoplasm (Fig. 4a, c), but those of mAb Bjh4-3B12 were observed in the cytoplasm (Fig. 4e, g).

4. Discussion

In the present study, we obtained six categories of mAbs that bound to specific hemocyte populations (Table 1). These mAbs and hybridoma cell lines are available (JAMSTEC Marine Biological Samples Database, <http://www.godac.jamstec.go.jp/bio-sample/index_e.html>). The mAb Bjh4-3G2 (category ii) reacted with the granules in the granulocytes (Fig. 1). The granulocytes in *Bathymodiolus*

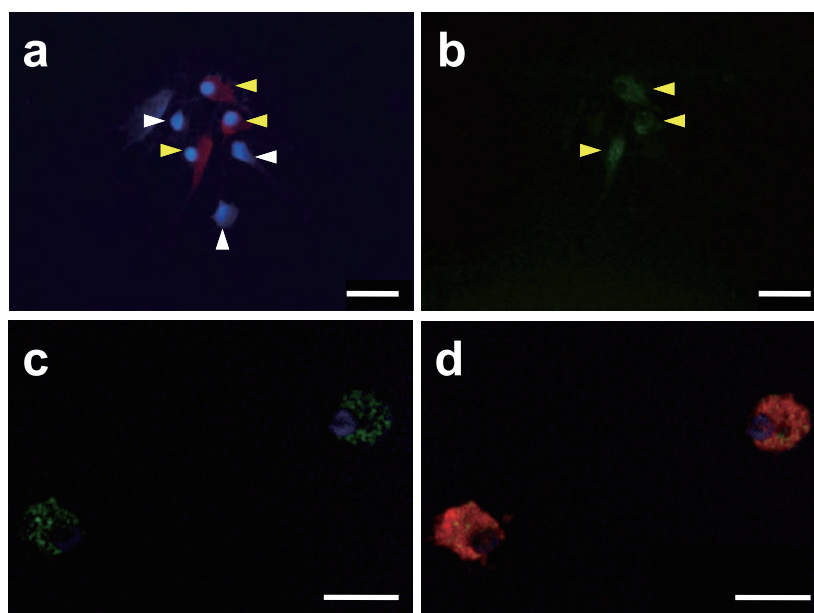


Fig. 1. Immunofluorescence micrographs of the monoclonal antibody (mAb) Bjh4-3G2 (category ii) bound *Bathymodiolus japonicus* hemocytes. **a and b**, The same seven hemocytes stained with wheat germ agglutinin (WGA), and 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI), and mAb Bjh4-3G2, were observed under two different fluorescence optics. **a**, Merged fluorescent images of WGA (red) and DAPI (blue) stains. Yellow arrowheads, WGA(+) hemocytes. White arrowheads, WGA(-) hemocytes. **b**, Fluorescent images showing only mAb Bjh4-3G2 (+) hemocytes (green). The signals were detected only in the three WGA(+) hemocytes (yellow arrowheads). **c and d**, The same two granulocytes stained with mAb Bjh4-3G2, WGA and DAPI, were observed at higher magnification under different fluorescence optics. **c**, Merged images of mAb Bjh4-3G2 (green) and DAPI (blue) stains. **d**, Triple merged images of mAb Bjh4-3G2 (green), WGA (red), and DAPI (blue) stains. mAb Bjh4-3G2 (green) bound to the granules in the WGA(+) granulocytes. Scale bars indicate 10 μ m. Nikon fluorescence microscope was used in (**a and b**), and a Keyence fluorescence microscope with the haze reduction function was used in (**c and d**) to eliminate fluorescence blurring.

mussels have phagocytic activity (Bettencourt et al., 2009; Tame et al., 2015). The granules are thought to be lysosomes (Tame et al., 2015). In oyster (*Crassostrea virginica*) and hard clam (*Mercenaria mercenaria*), the granules in the granulocytes have been shown to possess lysozyme activity, which is released into hemolymph (Cheng and Rodrick, 1975; Cheng et al., 1975; Cheng and Downs, 1988),

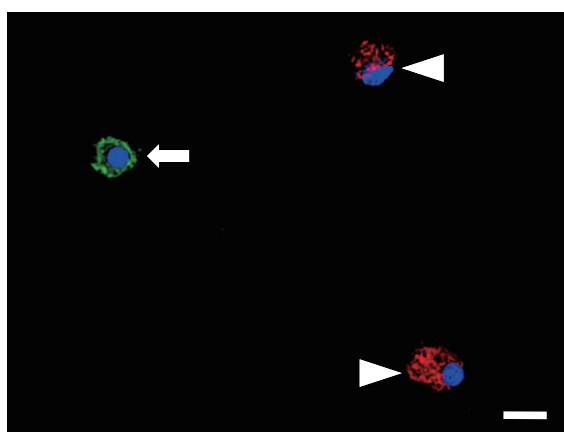


Fig. 2. Immunofluorescence micrograph of *Bathymodiolus japonicus* hemocytes bound by the monoclonal antibody (mAb) Bjh4-2D11 (category iii). The hemocytes were triple stained with mAb Bjh4-2D11 (green), wheat germ agglutinin (WGA) (red), and 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue). mAb Bjh4-2D11 signals (green) were observed in the WGA(−) hemocyte (white arrow) but not in WGA(+) (red) hemocytes (white arrowheads). Scale bar, 5 μ m. Keyence fluorescence microscope with the haze reduction function was used.

suggesting that the granules are also involved in humoral immunity. The Bjh4-3G2 mAb most likely reacted with lysosome and may be a good marker of lysosome in *B. japonicus* hemocytes. On the other hand, as the function of agranulocyte is unknown, mAb Bja4-2D11 (category iii, Fig. 2) may be useful in detecting agranulocyte in various tissues and, hence, in studying its function. In bivalve hemocytes, agranulocyte has often been called as blast-like cell suggesting a possibility of its differentiation to granulocytes (Hine, 1999). This mAb may also be useful to study hematopoiesis or hemocyte differentiation in *Bathymodiolus*. This is the first report of mAb reacting to agranulocytes in bivalves. The presence of the mAbs that reacted to a subset of WGA(+) and/or WGA(−) hemocytes may indicate that granulocytes and agranulocytes are composed of heterogeneous subsets, respectively (Fig. 3, 4). Hemocytes of *B. japonicus* have been reported to contain two types of granulocytes, basophilic and eosinophilic granulocytes (Tame et al., 2015). We observed that Bjh4-1F4 mAb (category iv) (Fig. 3a-c) bound to only a subset of the granulocytes. However, it was difficult to determine whether the mAb was reacting to the basophilic or eosinophilic granulocytes. Although both of two granulocytes have phagocytic activity, their functional difference remains to be studied (Tame et al. 2015). If this mAb, Bjh4-1F4, specifically recognizes one of the granulocytes, it would be useful to study their functional difference.

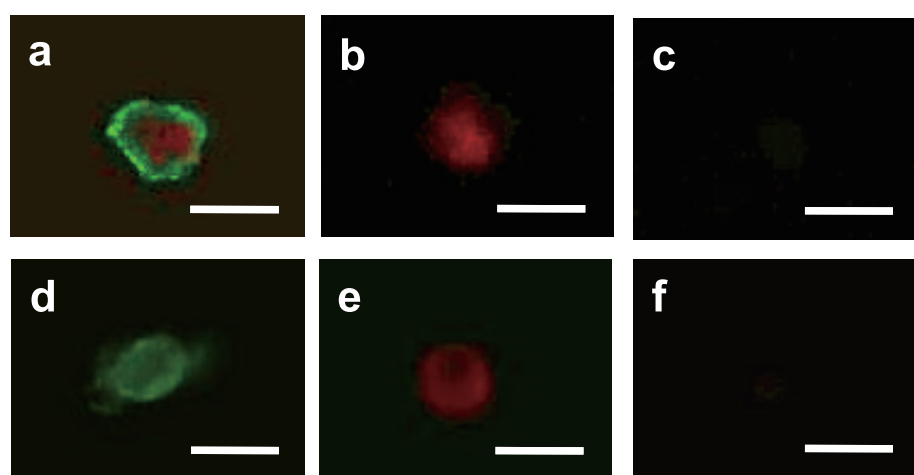


Fig. 3. Immunofluorescence micrographs of *Bathymodiolus japonicus* hemocytes bound by the monoclonal antibody (mAb) Bjh4-1F4 (category iv; a-c) and Bjh4-2D6 (category v; d-f). The hemocytes were double stained with wheat germ agglutinin (WGA) (red), and one of the mAbs (green). **a, b, and c**, Three different hemocytes showing different binding affinities to mAb Bjh4-1F4 and to WGA. **a**, A hemocyte showing signals of both of the Bjh4-1F4 and WGA [Bjh4-1F4(+)/WGA(+)]; **b**, a hemocyte showing WGA signal but no mAb signal [Bjh4-1F4(−)/WGA(+)]; **c**, a hemocyte showing neither signal of the mAb and WGA [Bjh4-1F4(−)/WGA(−)]. **d, e, and f**, Three different hemocytes showing different affinities to mAb Bjh4-2D6 and to WGA. **d**, a Bjh4-2D6 (+)/WGA(−) hemocyte, **e**, a Bjh4-2D6 (−)/WGA(+) hemocyte, **f**, a Bjh4-2D6 (−)/WGA(−) hemocyte. Scale bars, 10 μ m. Keyence fluorescence microscope was used for the observations.

In the present study, the antigens of the mAb recognizes were not determined, and thus, should also be further studied. Further studies including generation of mAbs against separated hemocytes by density gradient centrifugation, will be needed to for understanding the immunology of *B. japonicus* with a reference to the maintaining mechanism of the symbiont. We have reported three types of hemocytes in *B. japonicus* (Tame et al., 2015), however, we do not know their differentiation processes. The mAb library would be important markers not only for the immunological functions of hemocytes but also for their differentiation.

Classification of human leukocytes based on their surface molecules has been studied extensively. More than 350 cluster differentiation (CD) molecules on human leukocyte surfaces have been identified, and the mAbs against these molecules have greatly contributed to characterizing their immunological functions (Human leukocyte differentiation antigens workshop, <<http://www.hcdm.org/>>). Therefore, studying hemocytes by using mAbs is a promising approach in understanding immunological mechanisms in bivalves and may lead to a better understanding of the mechanisms that allow for the survival of symbiotic bacteria.

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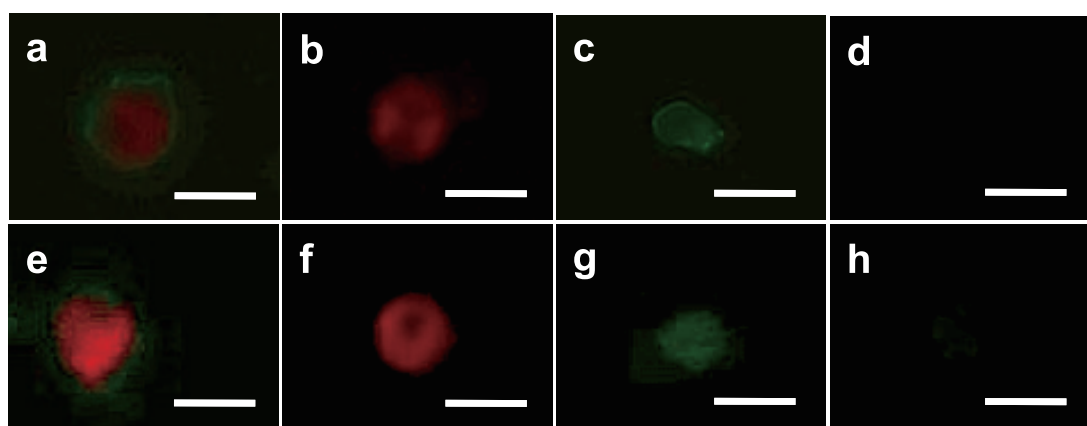


Fig. 4. Immunofluorescence micrographs of *Bathymodiolus japonicus* hemocytes bound by the monoclonal antibody (mAb) Bjh4-3B7 (category vi; **a-d**), or Bjh4-3B12 (category vi; **e-h**). Hemocytes were double stained with wheat germ agglutinin (WGA) (red), and one of the mAbs (green). **a**, Bjh4-3B7(+)/WGA(+) hemocyte; **b**, Bjh4-3B7(-)/WGA(+) hemocyte; **c**, Bjh4-3B7(+)/WGA(-) hemocyte; **d**, Bjh4-3B7(-)/WGA(-) hemocyte; **e**, Bjh4-3B12(+)/WGA(+) hemocyte; **f**, Bjh4-3B12 (-)/WGA(+) hemocytes; **g**, Bjh4-3B12(+)/WGA(-) hemocytes; **h**, Bjh4-3B12(-)/WGA(-) hemocytes. Scale bars, 10 μ m. Keyence fluorescence microscope was used.

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