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Characterization of Abalone (*Haliotis diversicolor*) Hemocytes *In Vitro*

J. H. Chen*, H. Y. Yang, S. W. Peng, Y. J. Chen, and K. Y. Tsai
Department of Zoology, National Taiwan University
Taipei 107,Taiwan, Republic of China

ABSTRACT

The bled abalone (*Haliotis diversicolor*) hemocytes protruded their pseudopodia and became flatten within one minute at 25 ℃ *In Vitro*. When the hemocytes contacted each other before attached matrix like polystyrene or glass, the cell aggregation reaction was happened first. After touching matrix, cells adhered, extent their pseudopodia, and then radically migrated outward the cell clump. Three types of abalone hemocytes were morphologically distinguished: monocyte-like cells, lymphocyte-like cells (nuclear to cytoplasm ratio close to 1), and spherical cells. By using Percoll gradient centrifugation, only one band of hemocytes was seen from caffeine-treated hemocyte samples. All cells in the cell population were monocyte-like cells, which was the only subpopulation deal with phagocytosis in this study. Both the hemocyte aggregation and adhesion behaviors was affected by low temperature (4℃), caffeine (20 mM), EDTA (50 mM), but not by hypertonic (1100 or 1200 mOsm) or hypotonic (800 or 900 mOsm) conditions.

Key words: abalone hemocyte, hemocyte adhesion, hemocyte aggregation, phagocytosis

Introduction

Most bivalves and gastropods have shells to protect them from mechanical damage and environmental stress. However, shells cannot prevent pathogen invasion. When the external barriers are broken, internal defense systems are needed to reduce contamination. These internal defense systems utilize hemocytes (blood cells) and humoral mechanisms to eliminate invading particles (Sminia & van der Knaap, 1987). Such reactions resemble some elements of natural immunity in vertebrates. Since invertebtates have no antibody or antibody-like molecules in their hemolymph or coelomic fluid (blood and body fluid) to form powerful humoral immunity, the behaviors of hemocytes or coelomocytes including aggregation, adhesion, and phagocytosis are important as the first line of their immuno-defense (Ratcliffe *et al.*, 1985; Sminia and van der Knaap, 1987).

Hemocyte aggregation, adhesion, and phagocytosis in molluscs have been known for a long time (Geddes, 1880, cited by Narain, 1973; Drew, 1910, cited by Narain, 1973; Dundee, 1953; Bang, 1961; Sparks, 1972; Sminia, 1981; Chen and Bayne, 1995a). In molluscs, it has been presumed that cell aggregation and adhesion are involved in wound healing (Bang, 1961; Sparks, 1972; Sminia, 1981). However, wound healing in molluscs differs from that in vertebrates for two reasons. First, no extracellular fibers are formed in molluscs. Second, the hemocyte aggregation in molluscs is reversible and most aggregated cells later disperse, re-entering the circulatory system after wound repaired (Feng & Feng, 1974). Phagocytosis is also important on cellular immune response in molluscs (Fryer and Bayne, 1989).

Abalone (*Haliotis diversicolor*) is one of the important aquacultural molluscs in Taiwan, Japan, and Mainland China. However, their immunity are much less studied (Chen, 1996). In order to realize their cellular defense mechanism, it is necessary to know about their hemocyte types and hemocyte behaviors such as hemocyte aggregation, adhesion, or phagocytosis. In addition, the possible influence factors like low temperature (4℃), osmolarity, caffeine, or EDTA on cell adhesion and aggregation were also tested in this study.

* Corresponding author: J. H. Chen, FAX: 886-2-3636837, E-mail: chenjh @ ccms. ntu. edu. tw

Material and Methods

Chemicals

BCA protein assay kit was obtained from Pierce. Percoll solution, Sigmacote, and other chemicals were purchased from Sigma Co. USA.

Animals

The cultured abalones (4 to 7 cm long) were maintained in a filtered, aerated, recirculating, and photoperiod-controlled sea water system and generally fed with kelp three times a week, as described by Chen (1996).

Buffered saline preparation

The Ca^{++}, Mg^{++} Tris-bufferred saline (CMTBS; 10 mM $CaCl_2$, 60 mM $MgCl_2$, 50 mM Tris base) was prepared according to Chen (1996). The pH was adjusted to 8.0 by using concentrated HCl. NaCl was added to reach the normal osmolarity, 980 mOsm, of sea water.

Yeast solution preparation

Commercial baker's yeast (*Saccharomyces cerevisiae*), purchased from grocery, was suspended in 10 % formalin for 60 min at room temperature. After three washes by centrifugation in CMTBS, the yeast particles were adjusted to the density of 1×10^7/ml, and stored at -20 ℃. For phagocytic assay, the yeast density was adjusted to 1×10^6/ml in cell-free plasma/CMTBS (1:1) before using.

Hemolymph collection

The method for abalone hemolymph collection was as follows: each abalone was weighted and measured its length before bleeding. The maximum volume of hemolymph collected from each abalone was limited (50μl per gram). The entire bleeding procedure were performed at 4 ℃. Hemolymph was collected from the hemosinus of the foot muscle (gastropod) by using a pre-cooled sterile syringe with 27G needle, and was then transferred into a sterile test tube for further treatment (Chen, 1996).

Density separation of hemocyte subpopulations

Continuous gradients of 65% Percoll in CMTBS (100% Percoll : 2.8X CMTBS = 65 : 35) were formed in round bottomed centrifuge tubes (Corex, No. 8441, USA) by centrifugation in an angle rotor at 10,000g, 4 ℃ for 30 min. The centrifuge tubes were cleaned by acid methanol and coated by Sigmacote to reduce hemocyte attachment. To prevent the hemocytes from forming aggregates *in vitro* during centrifugation, caffeine was used to treat hemocytes. Caffeine was dissolved in 2.8X CMTBS, and its final concentration in 65% Percoll was 20 mM. The mixed hemolymph was carefully loaded onto the top of the Percoll gradients, and the centrifuge tubes were centrifuged in a swing rotor at 300 g for 15 min at 4℃.

Observation of hemocyte adhesion *in vitro*

After hemolymph had been mixed with CMTBS, the diluted hemolymph was loaded (50 μl/well) into wells of pre-cooled 8-well slides (Cel-Line, NJ). A coverslip (24 × 50 mm), which was held by two 1 mm thick spacers, was mounted. The extent of cell adhesion/spreading observed under inverted phase contrast microscopy was classified at five different stages (Chen and Bayne, 1995a).

Hemocyte aggregation

The harvested hemolymph was aliquoted into microtubes (Sorenson BioScience, Utah), mixed (1:1) with CMTBS or test solutions, and then vortexed. The well-mixed hemolymph was loaded up-side-down onto the well of cooled 8-well slide (50μl/well). These were maintained as hanging-drops during gyration at 100 rpm performed by Lab Rotator (model 2800A; Digisystem Laboratory Instrument Inc.) at 25 ℃. Following 15 minutes gyration, the slide was carefully turned right-side-up, and added 100 % formalin (2 μl/well) to fix the cells. Cell aggregation were observed under inverted phase contrast microscopy. The extend of cell aggregation was distinguished: no aggregation, weak aggregation, or cohesive aggregation (Chen and Bayne, 1995a).

Hemocyte phagocytosis

Phagocytosis of formalin-killed yeast was carried out *in vitro* using monolayer of hemocytes, as described in Fryer and Bayne (1989). The hemocyte monolayer was washed three times with CMTBS to remove unadhered hemocytes and plasma, and were incubated with the formalin-killed yeast (10^6/ml). After the phagocytosis period, free yeast particles were removed with 3 washes of CMTBS. The remaining hemocytes and phagocytozed yeast cells were then fixed in 100 % methanol for 10 min, air dried and stained with Giemsa's stain (10 min), washed and destained in acetone, cleared with Histo-clear, and mounted in Histomount.

Hemocyte adhesion

The procedure for hemocyte adhesion assay was described in Chen and Bayne (1995a). The harvested hemolymph was mixed with CMTBS or test solution,

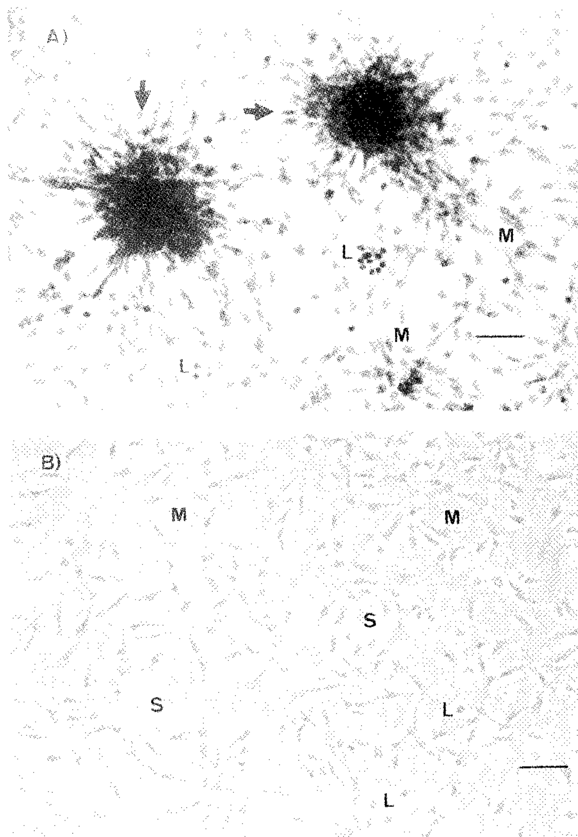

Figure 1. the abalone (*Haliotis diversicolor*) hemocytes. The hemocytes were loaded on the well slide, and incubated at 25 ℃ for 30 minutes. A) The hemocytes protruded their pseadopodia like fry-eggs (F). The aggregated cells (arrow) radically migrated from aggregates. Two types of hemocytes were distinguished, monocyte-like cells (M) and lymphocyte-like cells (L) with darker-stained nucleus and less cytoplasm. Giemsa stain. B) The spherical cells (S) did not spread in 30 min incubation. Observation in inverted phase contrast microscope. The scale bar in each micrograph represents 0.1 mm

loaded 50μl/well into wells of a pre-cooled (4℃), flat-bottom 96-well tissue culture plate (Corning, New York), and incubated at 25 ℃ for 15 min. The unattached cells and plasma were removed and each well was washed 3 times with CMTBS (200μl/well). As an estimation of the number of adherent cells, protein concentrations of each well were measured by means of the BCA protein assay (Pierce). The absorbance of each well was read at 550 nm using a microtiter plate reader (Molecular Devices, THERMO-max).

Data analysis

Each experiment was repeated at least three times, using fresh hemolymph samples. Data are presented as mean ± S.D.. Statistical analysis of data was performed using paired or unpaired Student's t-test as appropriate. Differences were considered significant when $p<0.05$.

Results

The subpopulations of abalone hemocytes and their behaviors *in vitro*

As soon as hemolymph was removed from the hemo-sinus in abalone foot, most hemocytes protruded their pseudopodia within one minute at 25 ℃. When the cells attached matrix either glass or plastics (polystyrene), the adhesion reaction was initiated. The cells became flatten and spread like fry-egg (Fig. 1a), and might migrate around glass or plastics. Those cells might form aggregates, when they contacted each other before attached a matrix. The aggregates still remained the competence of adhesion, and the aggregated hemocytes radically migrated from the edge of the aggregates in few minutes (Fig. 1a).

Using cell morphology and behaviors as the characters of abalone hemocyte classification, three groups of cells were distinguished: monocyte-like cells, lymphocyte-like cells, and spherical cells (Fig. 1b). Monocyte-like cells could spread and adhere very well on both glass and polystyrene. Lymphocyte-like cells could adhered, but showed pool-spreading competence. The spherical cells always remained rounded shape, and could not adhere during the incubation period. The monocyte-like cells, 15μm in diameter with no granules in their cytoplasms, are the major sub-populations in abalone hemolymph. The lymphocyte-like cells were rare in all harvested hemolymph. The percentage of sphere cells varied. They might be absent, but were more than 50% in one special sample (data not shown). The hemocyte population separated by Percoll gradient centrifugation showed only one band (density around 1.068), and all of the cells in the band are monocyte-like hemocyes (data not shown).

Phagocytosis of abalone hemocytes

Formalin-killed yeasts could be phagocytosed by monocyte-like hemocytes (Fig. 2). No yeast particle was observed in the cytoplasm of lymphocyte-like hemocytes in this study.

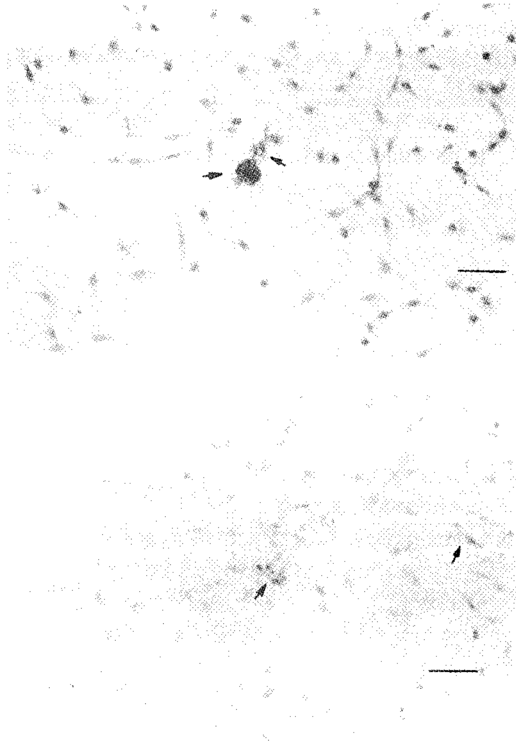

Figure 2 The phagocytosis of abalone (*Haliotis diversicolor*) hemocytes. Several formalin-fixed yeasts (arrow) were engulfed into monocyte-like hemocytes. Giemsa stain. The scale bar in each micrograph represents 0.1 mm.

Table 1. Abalone (*Haliotis diversicolor*) hemocyte aggregation in various conditions.

Treatment		Aggregation level
Osmolarity (mOsm)	800	CA
	900	CA
	980 *	CA
	1100	CA
	1200	CA
Temperature (℃)	4	WA
	15	CA
	25	CA
	4-25 ※	CA
Caffeine (mM)	1	CA
	5	CA or WA
	10	WA or NA
	20	NA
EDTA (mM)	0.05	CA
	0.5	CA
	5.0	CA
	50.0	NA

※ : Hemocytes were incubated at 4℃ for 10 min and were then transferred to 25℃ for additional 5 min incubation.
* :The normal osmolarity of sea water for abalone aquaculture in this study.
! : Hemocyte aggregation level: cohesive aggregation (CA), weak aggregation (WA), and no aggregation (NA).

The inhibitory effect of temperature, osmolarity, caffeine, or EDTA on hemocyte aggregation

The aggregation competence of hemocytes was not be inhibited in different osmolarity solutions (from 800 to 1200 mOsm). Also, it was not affected at 15℃, but at 4℃. When the incubation temperature was raised from 4℃ to 25℃, the aggregation competence was completely recovered (Table 1). The hemocyte aggregation was completely inhibited in 20 mM caffeine. It showed various effects of caffeine in lower concentrations such as 10 mM or 5 mM. In most tests, hemocytes formed weak aggregates in either 10 mM or 5 mM caffeine treatments. However, they might form cohesive aggregates in 5 mM caffeine or form no aggregates in 10 mM caffeine solution. Hemocytes could cohesively aggregate in 1 mM caffeine treatment (Table 1). At 50 mM EDTA, hemocyte aggregation was inhibited. No significantly inhibitory effect on cell aggregation was showed, when 5 mM, 0.5 mM or 0.05 mM EDTA was present (Table 1).

The inhibitory effect of temperature, osmolarity, caffeine, or EDTA on hemocyte adhesion

The hemocytes could adhere well at both 15℃ and 25℃, but not at 4℃ (Fig. 3a). As same in aggregation test, Osmolarity (from 800 mOsm to 1200 mOsm) showed no effect on hemocyte adhesion (Fig. 3b). The hemocyte adhesion could be significantly inhibited in 20 mM caffeine treatment. However, the lower concentrations such as 10 mM or 5 mM caffeine showed various effect. No effect was seen in 1 mM caffeine treatment (Fig. 3c). At 0.05, 0.5 or 5.0 mM EDTA, hemocyte adhesion could not be block, but the cell behavior was apparently inhibited in 50 mM EDTA (Fig. 3d).

Discussion

The hemocyte behaviors of abalone(*H. diversicolor*) *in vitro* including pseudopodia protrusion, cell adhesion, aggregation, and phagocytosis are similar to those of other molluscs (Cheng, 1981; Sminia, 1981; Chen and Bayne, 1995a). However,

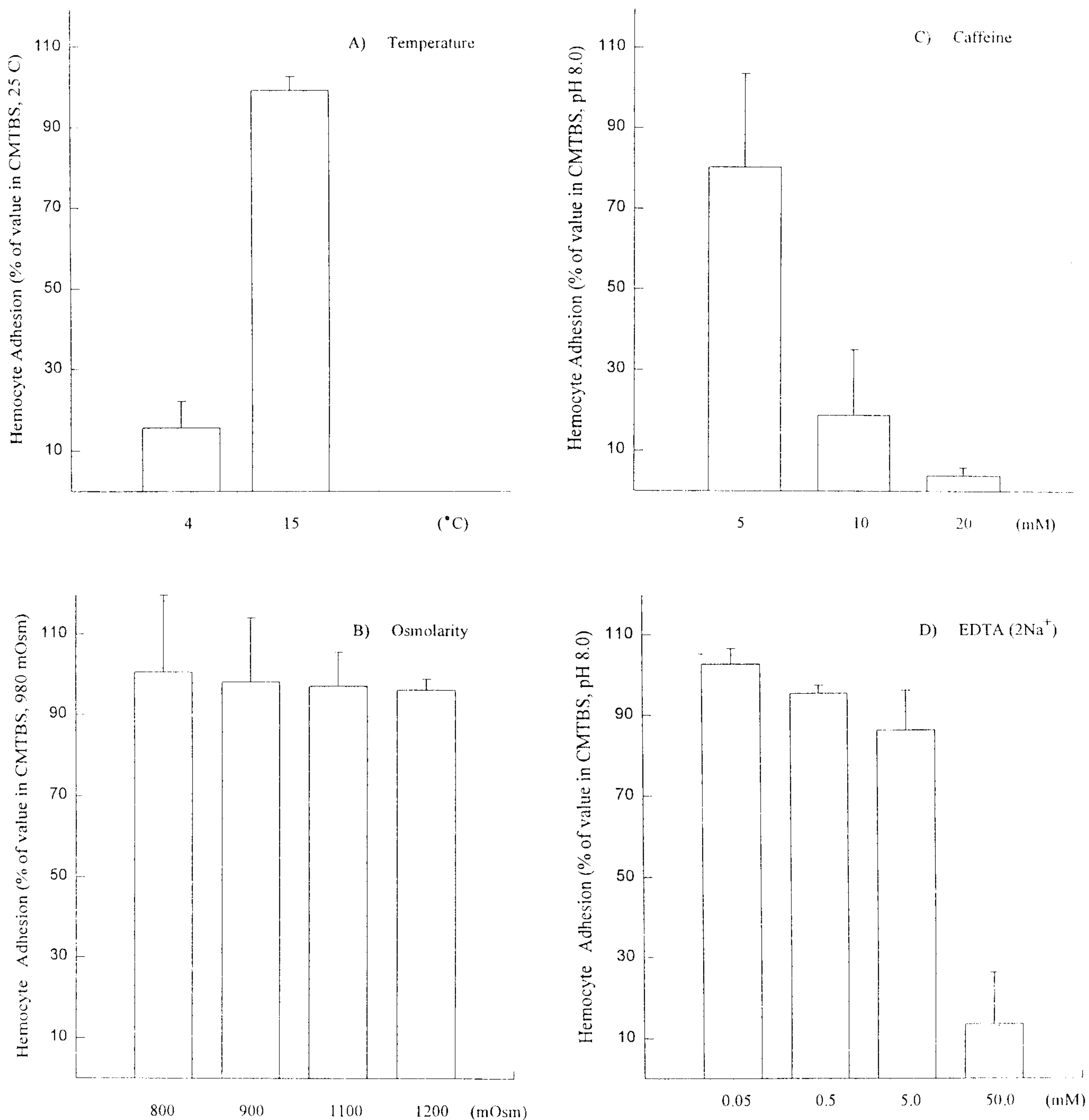

Figure 3. The influence of temperature, osmolarity, caffeine, and EDTA on abalone (*Haliotis diversicolor*) hemocyte adhesion *in vitro*. A) Hemocyte adhesion did not be affected at 15°C, but significantly inhibited at 4°C ($p<0.05$). B) Either hypotonic (800 and 900 mOsm) or hyper tonic (1100 or 1200 mOsm) solutions showed no effect on hemocyte adhesion. C) At 20 mM caffeine, hemocyte adhesion was significantly inhibited ($p<0.05$). However, the influence effect of 10 mM caffeine varied. Less inhibitory effect was seen in 5 mM caffeine treatment. D) The hemocyte adhesion was significantly inhibited in 50 mM EDTA ($p<0.05$). At 0.05, 0.5 or 5.0 mM EDTA, the inhibitory effect on hemocyte adhesion was not significant.

the morphology of hemocytes showed different among other molluscan species. Especially, the spherical, non-adherent cells were never described in other mouuscan species before. The hematology of more than 30 species of molluscs has been studied (Anderson, 1981; Cheng, 1981; Sminia, 1981; Rasmussen et al., 1985; Auffret, 1988). However, no single taxonomic system has been generally accepted for molluscan hemocyte classification. So far, two main schemes are broadly followed for molluscan hemocyte classification (Narain, 1973). Yet the hemocyte classification may vary between closely related species even according to that two schemes. Any comprehensive system for the classification of molluscan hemocytes must draw on more than morphological and behavioral criteria. Data obtained from ultrastructure studies (Cheng & Foley, 1975), cell surface marker labeling (Yoshino & Granath, 1983), specific gravity (Cheng *et al.*, 1980), as well as intracellular acid phosphotases and lysozyme levels (Cheng & Downs, 1988) collectively provide a basis for the classification of hemocyte subpopulations. It is now clear that hemocytes may be quite distinctive even between closely related molluscan species (Auffret, 1988). In this study, three types of cells could be distinguished by their morphology and cell behaviors. However, only one subpopulation which belongs to monocyte-like cells was separated by density. It might be due to low population density of the two minor populations, therefore they did not form the obvious bands which could be seen by naked eyes in Percoll gradient. In addition, the spherical, non-adherent cells were only found occasionally and the cell density varied. Since steroid hormones released from stressed vertebrates could suppress their immuno-responses (Su *et al*, 1988; Khansari *et al.*, 1990), was suspected that the spherical cells might be derived from monocyte-like hemocytes which cell behaviors were changed due to the stress of bleeding. However, immuno-suppression was little known in invertebrates (Ratcliffe *et al.*, 1985). We need more further studies to unravel what is the hemocyte activator(s) or inactivator(s) in abalones.

The inhibitory effect of caffeine could be resulted in the competition of caffeine with adenosine-like receptors on hemocyte surfaces (Chen and Bayne, 1995b). In order to figure out the varied effect of either 10 mM or 5 mM caffeine on hemocyte adhesion, we analyzed the difference of the size, weight, sex, or bleeding volume of the bled abalones. Nothing could be linked together. Therefore, it could be due to individual difference. EDTA is well known as an inhibitor on Ca^{++}, Mg^{++} dependent cell adhesion (Gumbiner and Yamada, 1992). In this study, the concentration of EDTA equal or less than 5 mM showed no effect on either cell adhesion or aggregation. This results are different with that in *Mytilus californianus* (Chen and Bayne, 1995a). Since the EDTA in solution may act as multi-protic molecule, the pH value of the buffered solution which EDTA was dissolved can be changed by its different dissociation. Therefore, some investigators suspected that the inhibitory effect of EDTA might come from acidic, not by divalent-cation chelating (Kenney *et al.*, 1972).

Only monocyte-like hemocytes performed phagocytosis in this study. However, according to this experimental design, it was hard to make the conclusion that the other two cell types can not perform phagocytosis. Since the population of lymphocyte-like hemocytes was low and the spherical hemocytes did not adhere on glass slide, this two reasons might cause the uncertain results.

Since the inhibitory effect of temperature (4°C), caffeine (20 mM), or EDTA (50 mM) on either hemocyte aggregation or hemocyte adhesion in abalones are similar to that in mussel, *M. californianus* (Chen and Bayne, 1995a), it was inferred that the cellular immuno-responses in those two molluscs should be regulated by same mechanism. Mussels belong to bivalves and abalones are gastropods. This two animals have been evolutionarily separated for more than one millions years (Clarkson, 1993). Therefore, it was implied that the inhibitory effect of those tested inhbitors on hemocyte behaviors might be common responses among all bivalves and gastropods.

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離體九孔螺血液細胞特性之研究

陳俊宏 楊欣雅 彭賢巍 陳盈君 蔡坤穎
國立台灣大學動物學系

摘 要

離體之九孔螺血液細胞會伸出偽足，當觸及適當的基質如玻璃或塑膠，就會進行附著反應；若觸及其他血液細胞，就會凝集形成細胞團，細胞團外圍細胞在觸及玻璃或塑膠後，亦會伸出偽足進行附著反應，接著細胞會行變形蟲運動，呈輻射狀向外遷移。九孔螺血液細胞經外形分類，至少可區分為三種：單核球(細胞核呈圓形、橢圓形或馬蹄形且染色較淡)、淋巴球(細胞核呈圓形、染色較深且核質比小於一)及一群完全不伸出偽足的細胞。依細胞比重分離實驗的結果，只形成一條細胞帶，其中的細胞依外形區分皆屬單核球。依本實驗結果，單核球是唯一具有吞噬能力的細胞。九孔螺血液細胞的附著及凝集能力會受到低溫(4℃)、咖啡因(20 mM)及EDTA(50 mM)的抑制，然而在高張液(1100 及1200 mOsm)或低張液(800 及900 mOsm)中卻不受影響。

關鍵詞：九孔螺血液細胞、細胞附著、細胞凝集、細胞吞噬