# Morula-Like Cells in Photo-Symbiotic Clams Harboring Zooxanthellae

Authors: Nakayama, Koji, Nishijima, Miyuki, and Maruyama, Tadashi

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# Morula-Like Cells in Photo-Symbiotic Clams Harboring Zooxanthellae

Koji Nakayama[1]*, Miyuki Nishijima[2] and Tadashi Maruyama[1]

[1]*Marine Biotechnology Institute, Kamaishi Laboratories, 3-75-1 Heita, Kamaishi, Iwate 026, Japan*
[2]*Shimizu Laboratories, 1900 Sodeshi, Shimizu, Shizuoka 424, Japan*

**ABSTRACT**—Symbiosis is observed between zooxanthellae, symbiotic dinoflagellates, and giant clams and related clams which belong to the families Tridacnidae and Cardiidae. We have previously shown that a photo-symbiotic clam *Tridacna crocea* has three types of hemocytes, the eosinophilic granular hemocyte with phagocytic activity, the agranular cell with electron lucent granules, and the morula-like cell with large (ca. 2 μm in diameter) colorless granules. The function of the morula-like cell is not clear, but it has not been reported in any other bivalves except photo-symbiotic clams *T. crocea* and *Tridacna maxima*. In order to clarify whether it is specific to photo-symbiotic clams or not, we studied hemocytes in the photo-symbiotic clams *Tridacna derasa* (Tridacnidae), *Hippopus hippopus* (Tridacnidae) and *Corculum cardissa* (Cardiidae), and a closely related non-symbiotic clam *Fulvia mutica* (Cardiidae). The eosinophilic granular hemocytes and the agranular cells were found in all of the clams examined. However, the morula-like cells which were packed with many large electron dense granules (ca. 2 μm in diameter), were observed only in the photo-symbiotic clams. In *F. mutica*, a closely related non-symbiotic clam, this type of hemocyte was not found. Instead a hemocyte with vacuoles and a few large granules containing peroxidase activity was observed. The large granules of *F. mutica* varied in size from ca. 1–9 μm in diameter. Present data suggests that the presence of morula-like cells is restricted to photo-symbiotic clams and that the hemocytes associated with the morula-like cells may have some functional relationship to symbiosis with zooxanthellae.

## INTRODUCTION

Symbiosis is observed between microalgae and some marine bivalve molluscs from the families Tridacnidae and Cardiidae which are known to harbor extracellular symbiotic dinoflagellates, i.e. zooxanthellae. Carbon photosynthetically fixed by zooxanthellae was reported to be translocated to the tissues of giant clams (Goreau *et al*., 1973; Streamer *et al*., 1988). In the presence of a homogenate of Tridacnid clam mantle tissue, freshly isolated zooxanthellae released photosynthetically fixed carbon *in vitro* (Muscatine, 1967; Masuda *et al*., 1994). Minerals and bicarbonates are collaterally supplied to zooxanthellae for photosynthesis from giant clams (Trench, 1987). Some of the zooxanthellae in stomach of giant clams may be digested for nourishment (Morton, 1978; Maruyama and Heslinga, 1997).

In order to understand the mechanisms underlying the maintenance of the mutual association between the giant clam and zooxanthellae without impairment to the clam's defense system, we have characterized hemocytes in the giant clam, *Tridacna crocea* (Tridacnidae) (Nakayama *et al*., 1997a). Three types of hemocytes, eosinophilic granular hemocytes, agranular cells and morula-like cells, have been identified in *T. crocea*. The eosinophilic granular hemocyte is phagocytic and resembles the granulocytes of other bivalves (Cheng, 1981, 1984). The agranular cell is similar to other molluscan hyalinocytes. The morula-like cell which is packed with many colorless large granules, is apparently unique. The morula-like cell is clearly distinct from the serous or brown cells in other bivalve molluscs, which are yellowish or brownish and have acid-phosphatase activity (Zaroogian and Yevich, 1994). Hemocytes containing a single large vacuole with peroxidase activity are found in the closely related non-symbiotic clam, *Cerastoderma edule* (Cardiidae), but their role is obscure (Russell-Pinto *et al*., 1994).

Reade and Reade (1976) reported that a hemocyte (type II cell) from the symbiotic clam *Tridacna maxima* (Tridacnidae), corresponding to the morula-like cell in *T. crocea*, initiated coagulation of hemocytes to make large clots when exposed to seawater. In the hemolymph of *T. crocea*, the morula-like cells surround such clots which are composed mainly of agranular cells (Nakayama *et al*., 1997a). This observation suggests that this type of hemocyte is involved in wound healing or exclusion of non-self materials by aggregation. Recently, another symbiotic function of the morula-like cell was sug-

* Corresponding author: Tel. +81-193-26-5814;
FAX. +81-193-26-6584.

gested. A protein with a molecular weight of 7.4-kDa was found to be specific to morula-like cells (Nakayama *et al.*, 1997b). Western blot analysis revealed that a protein from zooxanthellae was immunoreactive to the antibody against the 7.4-kDa protein from morula-like cells. We therefore investigated whether the morula-like cells are present in other marine symbiotic clams.

## MATERIALS AND METHODS

### Animals

Photo-symbiotic clams, *Tridacna derasa*, *Hippopus hippopus* and *Corculum cardissa* were purchased from Palau Mariculture Demonstration Center (Republic of Palau). A non-symbiotic Cardiid clam *Fulvia mutica* was purchased from a local supplier in Japan.

### Hemolymph collection

Hemocytes were extracted from the symbiotic clams, *T. derasa*, *H. hippopus* and *C. cardissa*, in the laboratory in Palau. The clams were chilled on ice for 30 min before use in order to prevent rapid aggregation of hemocytes. Hemolymph was withdrawn from the pericardial chamber with a syringe.

### Cytochemical staining methods

The hemocytes were collected by adhesion to a glass surface via incubation in a 2-well type Chamber Slide (Nunc Co., Illinois, USA) for 1 hr at 25°C, or by filtration (Cyto-shuttle, Cancer Diagnostics Inc., USA), and fixed and stained by the May-Grünwald-Giemsa method (Lewis and Dacie, 1990). Peroxidase activity was demonstrated in live hemocytes with 0.5 mg/ml diaminobenzidine (DAB) and 0.01% $H_2O_2$ in artificial Pacific seawater, APSW (Borowitzka and Larkum, 1976). After fixation in 60% acetone: 10% methanol: 30% water, acid phosphatase activity was visualized by the method of Burstone (1958) using naphthol AS-BI phosphate (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) as a substrate. Lipids were visualized by staining with Sudan Black B (Lewis and Dacie, 1990).

### Cell counts and size measurements

One ml of hemolymph was mixed with 10 μl of 0.3% May-Grünwald eosin methylene blue solution (Wako Pure Chemicals Industries, Ltd., Osaka, Japan), applied to a hematocytometer, and incubated for 30 min at room temperature. Each type of hemocyte was counted. For measurements of cell size, hemolymph was fixed with 2.5% glutaraldehyde for 2 hr at room temperature prior to addition of the May-Grünwald eosin methylene blue solution.

### Transmission electron microscopy

The hemocytes were prefixed overnight at 4°C with a mixture of 2% glutaraldehyde and 0.5% formaldehyde in APSW and then postfixed with 1% $OsO_4$ in 50% APSW, dehydrated through an ethanol series and embedded in Epon 812 resin (TAAB Co., Berkshire, UK). Thin sections were cut with an ultramicrotome (Ultracut N, Reichert-Nissei, Tokyo, Japan) using a diamond knife. Silver sections were counterstained with saturated aqueous uranyl acetate and Reynold's lead citrate and then observed in a Hitachi H-7000 electron microscope operated at 75 kV.

## RESULTS

### Hemocytes in photo-symbiotic and non-symbiotic clams

Three types of hemocytes were observed in photo-symbiotic clams *Tridacna derasa*, *Hippopus hippopus* and *Corculum cardissa*, and in a non-symbiotic clam *Fulvia mutica* (Fig. 1A–D). Granular hemocytes containing many small granules (ca. 0.6 μm in diameter) stained with eosin adhered to a glass or plastic surface with veil-like pseudopodia. Incubation of living cells with 0.8 μm latex particles showed that this type of granular hemocytes in *T. derasa*, *H. hippopus* and *F. mutica* (*C. cardissa* was not tested) had phagocytic activity indicating that they were eosinophilic granular hemocytes. Agranular cells were characterized by a high nucleus/cytoplasm ratio and by the fact that the cytoplasm remained unstained after treatment with the May-Grünwald-Giemsa stain (Nakayama *et al.*, 1997a). The agranular cells spread by extending pseudopodia on a glass surface. This type of hemocyte was observed in all of the clams examined. Morula-like cells are hemocytes packed with many large granules (Nakayama *et al.*, 1997a) and were observed in photo-symbiotic clams *T. derasa*, *H. hippopus* and *C. cardissa*, belonging to the families Tridacnidae and Cardiidae. However, hemolymph of *F. mutica* (Cardiidae), which does not harbor symbionts, contained no morula-like cells. Instead, some compartment cells with vacuoles and a few large granules were found (Fig. 1D). The morula-like cells in Tridacnid clams and the comparment cells in *F. mutica* were less abundant than the other two types of hemocytes (Table 1), whereas in *C. cardissa* the three types of hemocytes were almost equally abundant.

### Morula-like cells in photo-symbiotic clams

(1) *Tridacna derasa*

The morula-like cells in *T. derasa* were 9.9 ± 1.6 (mean ± SD) μm (n = 11) in diameter (Fig. 1A). The nucleus was oval and 3.8 ± 0.2 μm (n = 6) in diameter. The large granules in the morula-like cells were 1.9 ± 0.4 μm (n = 30) in diameter and some parts were electron dense (Fig. 2), as previously observed in *T. crocea* (Nakayama *et al.*, 1997a).

(2) *Hippopus hippopus*

The morula-like cells in *H. hippopus* were 8.9 ± 0.6 μm (n = 10) in diameter (Fig. 1B). The nuclei were 3.8 ± 0.2 μm (n = 5) in diameter. The large granules in the morula-like cell, 2.0 ± 0.4 μm (n = 30) in diameter, sometimes contained lower electron dense speckles in their central region (Fig. 3).

(3) *Corculum cardissa*

The morula-like cells in *C. cardissa* were 12.4 ± 0.9 μm (n = 12) in diameter (Fig. 1C) and contained large granules, 2.3 ± 0.6 μm (n = 42) in diameter. The nuclei were 5.2 ± 0.7 μm (n = 12) in diameter. The electron density of these granules varied (Fig. 4). A reticular structure was observed only in the large granules with low electron density.

### Compartment cell in a non-symbiotic clam *Fulvia mutica*

The hemocytes in *F. mutica* contained vacuoles and a few large granules and were 9.6 ± 0.8 μm (n = 14) in diameter (Fig. 1D). The nuclei, 4.8 ± 0.5 μm (n = 12) in diameter, were peripherally located. The large granules were 4.1 ± 2.4 μm (n = 51) in diameter and varied in size from ca. 1-9 μm in diameter. The vacuoles were electron-lucent and the large granules were electron-dense (Fig. 5). Peroxidase, detected only

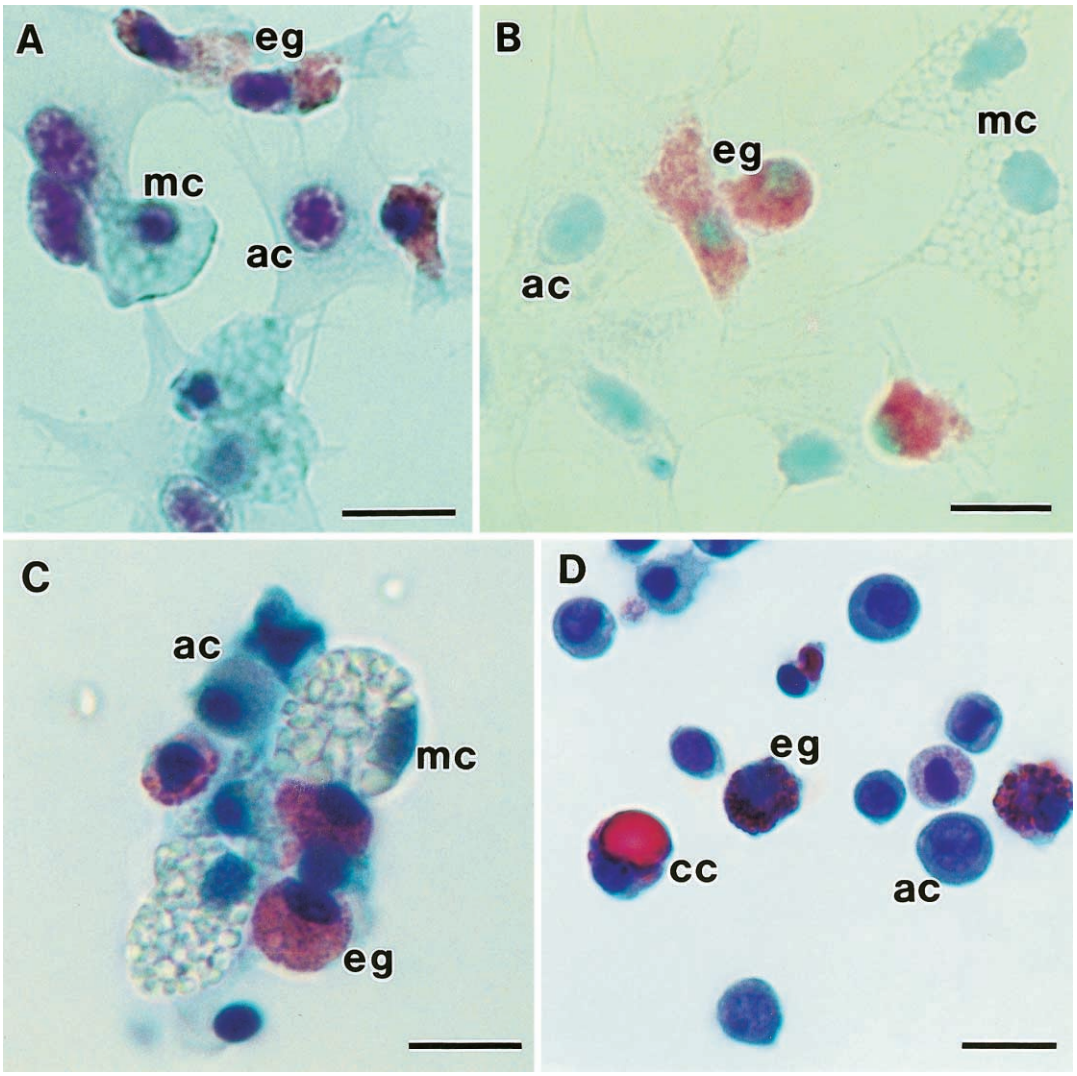


**Fig. 1.** Light micrographs of the hemocytes stained by May-Grünwald-Giemsa method. (**A**) Hemocytes in *Tridacna derasa*: eg, eosinophilic granular hemocyte; ac, agranular cell; mc, morula-like cell. (**B**) Hemocytes in *Hippopus hippopus*. (**C**) Hemocytes in *Corculum cardissa*. (**D**) Hemocytes in *Fulvia mutica*: cc, compartment cell. Scale bar = 10 μm.

**Table 1.** Percentages of hemocyte types in marine molluscs

| | n | Eosinophilic granular hemocyte | Agranular cell | Morula-like cell | Compartment cell |
|---|---|---|---|---|---|
| *Tridacna crocea*[1)] | 6 | 48.2 ± 7.6[3)] | 44.9 ± 8.8 | 6.9 ± 3.0 | n.d. |
| *Tridacna derasa* | 3 | 42.4 ± 0.4 | 41.5 ± 1.5 | 16.1 ± 1.4 | n.d. |
| *Hippopus hippopus* | 1 | 33.1 | 58.5 | 8.5 | n.d. |
| *Corculum cardissa* | 2 | 36.1 ± 1.2 | 31.7 ± 9.6 | 32.2 ± 8.4 | n.d. |
| *Fulvia mutica* | 5 | 45.3 ± 8.1 | 38.4 ± 13.3 | n.d. | 16.3 ± 7.7 |
| *Cerastoderma edule*[2)] | 43 | 64[4)] | 22[5)] | n.d. | 14[6)] |

[1)] Nakayama *et al.*, 1997a.
[2)] Russell-Pinto *et al.*, 1994.
[3)] Mean ± S.D.
[4)] Type Ia + Ib cells in 2).
[5)] Type II cell in 2).
[6)] Type III cell in 2).
n.d., not detected.

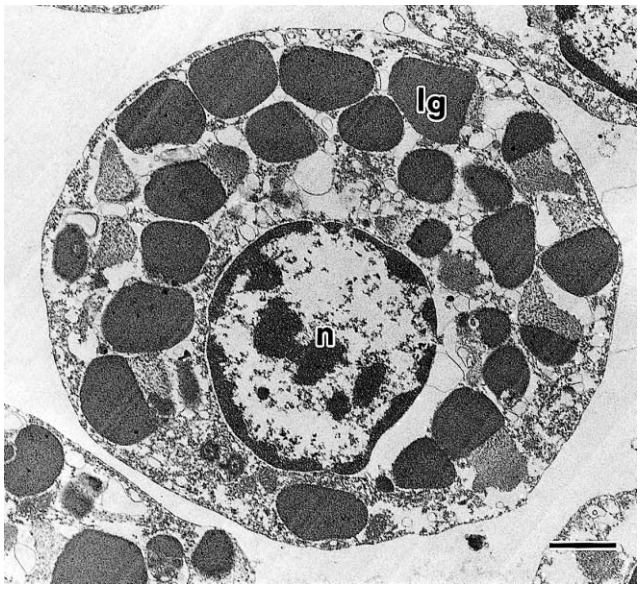


**Fig. 2.** Transmission electron micrograph of morula-like cell in *Tridacna derasa*: n, nucleus; lg, large granule. Scale bar = 1 μm.

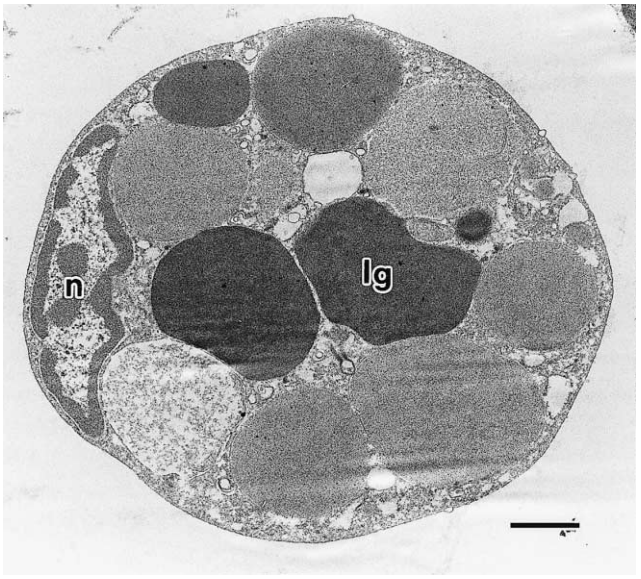


**Fig. 4.** Transmission electron micrograph of morula-like cell in *Corculum cardissa*: n, nucleus; lg, large granule. Scale bar = 1 μm.

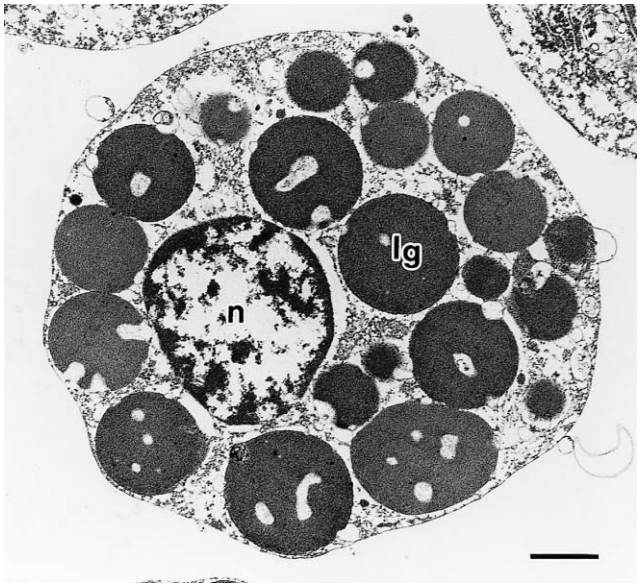


**Fig. 3.** Transmission electron micrograph of morula-like cell in *Hippopus hippopus*: n, nucleus; lg, large granule. Scale bar = 1 μm.

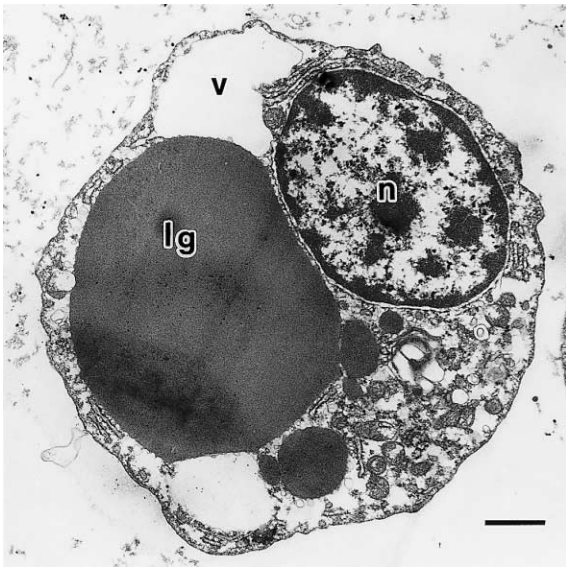


**Fig. 5.** Transmission electron micrograph of compartment cell in *Fulvia mutica*: n, nucleus; lg, large granule; v, vacuole. Scale bar = 1 μm.

in the compartment cells (Fig. 6), was inactivated by fixatives such as methanol, ethanol, formalin and glutaraldehyde. The compartment cell had neither acid phosphatase activity nor lipid droplets.

## DISCUSSION

Hemocytes packed with large granules or vacuoles are observed in some invertebrates. In the coelom of some ascidians, morula cells characterized by the presence of several yellow-green vacuoles approximatelly 2 μm in diameter occur (Goodbody, 1974; Fuke, 1979). In rejection reactions between incompatible colonies, morula cells of *Botryllus schlosseri* (Pleurogona, Ascidiacea) release the contents of their vacuoles, mainly oxidative enzymes (Ballarin *et al*., 1995). Although hemocytes packed with large granules are also observed in molluscs and named as brown or serous cell in *Crassostrea virginica* (Ruddell and Wellings, 1971; Cheng, 1981), type III cells in *Cerastoderma edule* (Russell-Pinto *et al*., 1994), type II cells in *Tridacna maxima* (Reade and Reade, 1976) and morula-like cells in *Tridacna crocea* (Nakayama *et al*., 1997a), their functions are not well known.

Morula-like cells in symbiotic clams (type II cells in *T. maxima*) are characterized by the presence of many large

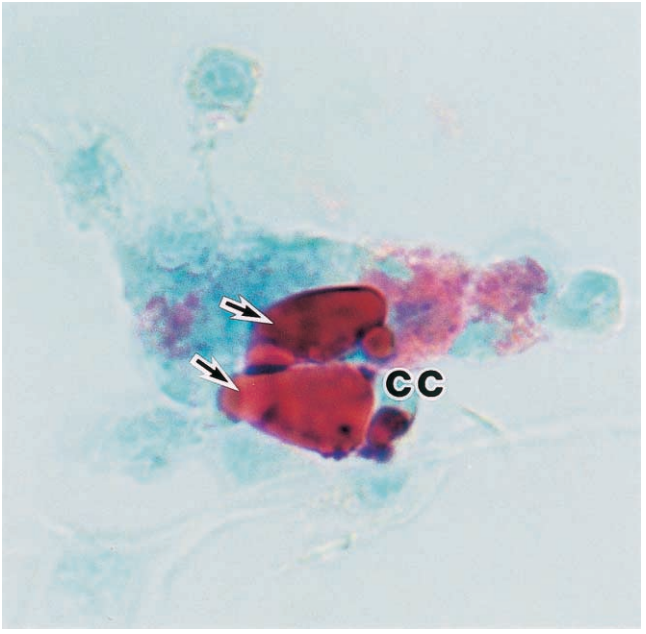


**Fig. 6.** Peroxidase activity in hemocytes of *Fulvia mutica*, visualized with DAB and $H_2O_2$ (arrows), and counterstained by Giemsa method: cc, compartment cell.

granules (ca. 2 μm in diameter) which are colorless under a light microscope and are electron-dense. In *T. crocea*, *Tridacna derasa* and *Hippopus hippopus* (Tridacnidae), some of these large granules in the morula-like cells contain patches of low electron density, whereas those of *C. cardissa* (Cardiidae) rarely contain these low-electron density patches although the electron density of the granules varied. Brown cells in the oyster *C. virginica* (Ostreidae) differ from morula-like cells in the symbiotic clam *T. crocea* (Tridacnidae) because the former has acid phosphatase activity and lipofuscins but the latter does not (Zaroogian and Yevich, 1994; Nakayama *et al*., 1997a). Brown cells were thought to be involved in the extraction of hippuric acid and other metabolic by-products from hemolymph, storage of fats, and secretion of shell-forming materials (White, 1942; Cheng, 1981, 1984). The present study indicates that a non-symbiotic clam *Fulvia mutica* (Cardiidae), closely related to Tridacnidae, lacks morula-like cells but contains compartment cells which are morphologically and enzymatically different from the morula-like cells in symbiotic clams. Because of the presence of vacuoles and granules with peroxidase activities, the compartment cells in the non-symbiotic clam *F. mutica* resemble type III cells from *C. edule* (Cardiidae), which is also a non-symbiotic clam. While the type III cells in *C. edule* have been compared with serous or brown cells (Russell-Pinto *et al*., 1994), the absence of acid phosphatase activity indicates that they are distinct from each other. The absence of peroxidase or acid phosphatase activity in the morula-like cell of the symbiotic clam *T. crocea* (Nakayama *et al*., 1997a) indicates that it is also different from serous or brown cells. These findings indicate that morula-like cells in symbiotic clams may be evolutionally related to compartment cells in non-symbiotic clams *F. mutica* and *C. edule*, but their characteristics are apparently different.

In Tridacnid clams, Fankboner (1971) described intracellular digestion of moribund zooxanthellae in the siphonal mantle. To establish symbiosis between host animals and microalgae, the host animals should be able to recognize the symbiont cells and accept them. We previously described that an antibody raised against a 7.4-kDa protein in the large granules of morula-like cells in the symbiotic clam *T. derasa* (Tridacnidae) crossreacted with a 7.4-kDa protein in the zooxanthellae of *T. derasa* (Nakayama *et al*., 1997b). Since morula-like cells are found only in symbiotic clams belonging to the families Tridacnidae and Cardiidae, we postulate they may have some recognition role or other in establishment and maintenance of symbiosis.

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