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Ultrastructures and Classification of Circulating Hemocytes in 9 Botryllid Ascidians (Chordata: Ascidiacea)

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ABSTRACT—Ultrastructures of circulating hemocytes were studied in 9 botryllid ascidians. The hemocytes are classified into five types: hemoblasts, phagocytes, granulocytes, morula cells, and pigment cells. These five types are always found in the 9 species. They should represent the major hemocyte types of the circulating cells in the blood. Hemoblasts are small hemocytes having a high nucleus/cytoplasm ratio. There are few granular or vacuolar inclusions in the cytoplasm. Phagocytes have phagocytic activity and their shape is variable depending on the amount of engulfed materials. In granulocytes, shape and size of granules are different among the species. Morula cells are characterized by several vacuoles filled with electron dense materials. In pigment cells, the bulk of the cytoplasm is occupied by one or a few vacuoles containing pigment granules. We also described some other hemocyte types found in particular species. Furthermore, we encountered free oocytes circulating in the blood in two species, *Botryllus primigenus* and *Botrylloides lentus*.

Key words: colonial ascidian, blood cell, free oocyte, TEM, Tunicata

INTRODUCTION

As primitive chordates, much attention has been paid to the function of hemocytes in ascidian, particularly about immunology, sexual/asexual reproduction, and heavy metal accumulation (Cf. Wright, 1981; Burighel and Cloney, 1997). Whereas ascidian blood always contains 5-9 cell types that are morphologically distinguishable with one another, there are considerable variations among the species. Therefore, we do not have a common standard for hemocyte classification of ascidians to date, and there are still some controversy about the classification of hemocytes even in the same species, e.g., *Halocynthia roretzi* (Cf. appendix in Dan-Sohkawa *et al*., 1995). The difficulty of hemocyte identification may be due to the differences in 1) methods of observation, 2) interpretation of the intermediate stages of the hemocyte differentiation, and 3) physiological phenomena that the authors are concerned. Therefore, we need much more information about hemocyte ultrastructure, differentiation, and functions to discuss the homology of hemocyte types among different species.

The family Botryllidae is a member of the suborder Stolidobranchia and the monophyly of this taxon is supported by not only morphology but also molecular phylogeny (Cohen *et al*., 1998). The members of this group are all colonial species and some of them have been used in the studies of colonial allorecognition (Cf. Hirose, 2003) and sexual/asexual reproduction (Oka and Watanabe, 1957; Mukai and Watanabe, 1976; Manni *et al*., 1993; 1994) in which several types of hemocytes play important roles. Although morphological and/or histochemical studies of hemocytes were carried out in some botryllids (Milanesi and Burighel, 1978; Burighel *et al*., 1983; Schlumpberger *et al*., 1984; Ballarin *et al*., 1993; Shirae and Saito, 2000; Cima *et al*., 2001), comparative surveys were rarely carried out to discuss the homology of the hemocyte types among the species. Since phylogenetically related species would have similar types of hemocytes, comparative morphological studies of hemocytes would show the morphological variations among species and provide useful information for hemocyte classification in botryllid ascidians. In this study, we examined the ultrastructures of circulating hemocytes in 9 species of botryllid ascidians, and described five-hemocyte types in the all species; hemoblast, phagocyte, granulocyte, morula cell, and pigment cell. Moreover, circulating young oocytes were ultrastructurally described in two species.

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MATERIALS AND METHODS

Animals

The botryllid ascidians forms a sheet-like colony in which zooids are buried in a gelatinous tunic, and they grow on hard surfaces in intertidal or subtidal zone. The colonies were collected from the vicinity of Shimoda (Shizuoka Prefecture, Japan). The colonies were attached to glass plates and reared in culture boxes immersed in Nabeta Bay near the Shimoda Marine Research Center, University of Tsukuba.

Hemocyte morphology was examined in 5 *Botryllus* (*Botryllus primigenus, B. scalaris, B. schlosseri, B. delicatus,* and *B. sexiens*) and 4 *Botrylloides* (*Botrylloides simodensis, B. lentus, B. fuscus,* and *B. violaceus*) by means of electron microscopy. As for *B. simodensis*, the fixed hemocytes were also observed under a light microscope.

Light Microscopy

The colony pieces of *Botrylloides simodensis* were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate-0.45 M sucrose (pH 7.5) for 1 hr at 4°C. The vascular ampullae were cut with a razor blade and hemocytes were collected with a capillary. The hemocytes were mounted with the same buffer and observed under a light microscope equipped with Nomarski differential interference contrast optics (DCI).

Electron microscopy

Peripheral parts of the colonies were cut with a razor blade and they were fixed in 2.5% glutaraldehyde-0.1 M sodium cacodylate-0.45 M sucrose (pH 7.5) for 2 hr at 4°C and briefly washed with the same buffer. The specimens were postfixed with 1% osmium tetroxide-0.1 M sodium cacodylate (pH 7.5) for 1.5 hr at 4°C, dehydrated through an ethanol series or an acetone series, cleared with *n*-butyl glycidyl ether, and embedded in low-viscosity epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined in Hitachi HS-9 or JEOL JEM-1010 transmission electron microscope.

RESULTS

Light microscopic observation of whole-mount specimens (Fig. 1)

Fig. 1 shows light micrographs of the five-hemocyte types of *Botrylloides simodensis*. These five types are always found in the 9 species examined here. Hemoblast is a small, spherical hemocyte with a prominent nucleus and nucleolus (Fig. 1A). Phagocytes are irregularly shaped and often contain engulfed materials in their phagosomes. When the phagocyte engulfs large materials, the cell is a large sphere containing a large phagosome (Fig. 1B). The cytoplasm of granulocytes is full of granules of about 1 μm in major axis (Fig. 1C). Morula cells are characterized by several round, refractile vacuoles that occupy the bulk of the cytoplasm (Fig. 1D). Pigment cells are large spherical cells and have one or a few large vacuoles that contain pigment granules. One subtype of pigment cells has vacuoles of pale brown and it is often classified as nephrocyte (Fig. 1E). The other subtypes of pigment cells are yellow, orange, red, brown or purple due to the colorations of the pigment granule (Fig. 1F).

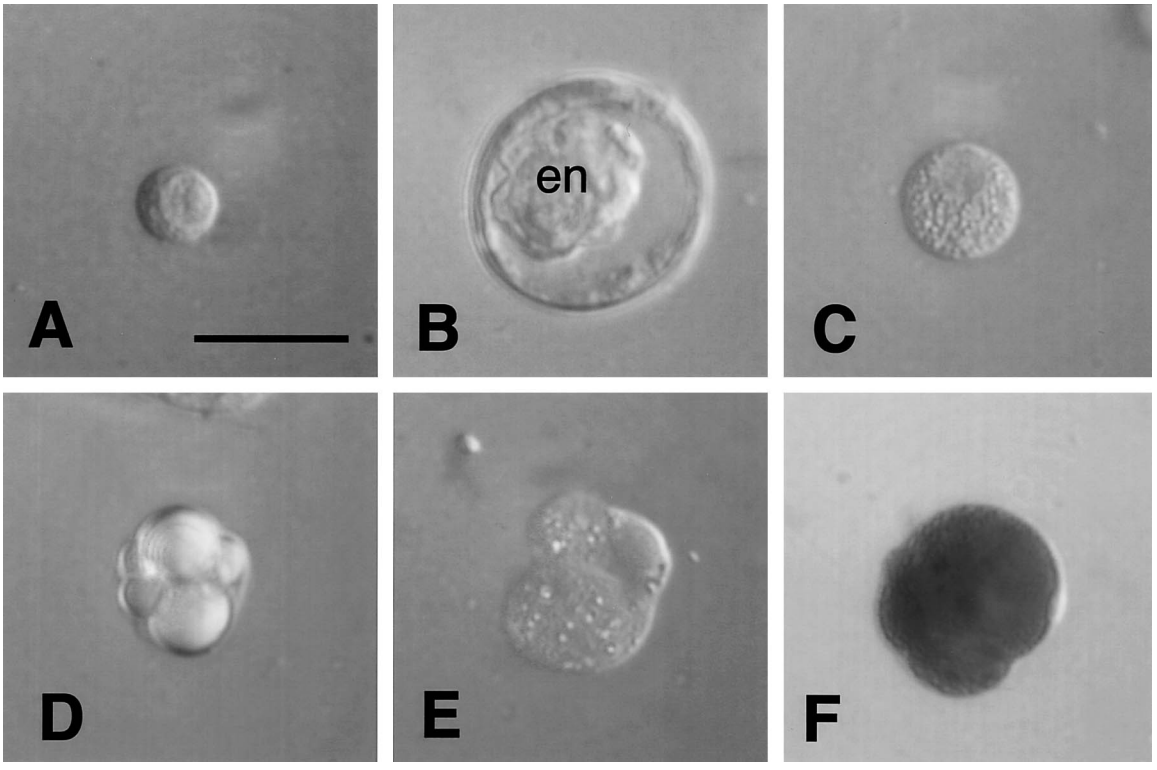


Fig. 1. Light micrographs of fixed hemocytes in *Botrylloides simodensis*. Hemocytes are classified into five types based on morphology. In pigment cells, there are several color-types. Hemoblast (**A**), phagocyte (**B**), granulocyte (**C**), morula cell (**D**), pigment cell (nephrocyte-type) (**E**), and pigment cell (**F**). en, engulfed material in the phagosome. Scale bar, 10 μm.

***Hemoblast* (Fig. 2)**

Hemoblasts are small hemocytes having a high nucleus/cytoplasm ratio. Round nucleus of about 3 μm in diameter usually has a large nucleolus. The cytoplasm contains some mitochondria, Golgi bodies, rough endoplasmic reticulum (rER), and many ribosomes suggesting active biosynthesis. There are few vacuoles, while some hemoblasts contain some small granules. Variations in hemoblast ultrastructures could not be found among the species examined here.

***Phagocyte* (Fig. 3)**

Phagocytes are characterized by phagosomes and pseudopodia. The numbers and sizes of phagosomes are variable. Phagocytes often contain other hemocytes in their phagosomes. There are sometimes two or more engulfed hemocytes in a phagocyte (Fig. 3C, D). Round granules are often found and they appear to be remnants of phagocytized materials. When the phagosome(s) is small and its total volume occupies small parts of the cell, the phagocyte usually has many, long pseudopodia. When the phagosome(s) is large and occupies the most part of the cell, the cytoplasm is almost a thin layer surrounding the engulfed materials and there are few pseudopodia. Fig. 3E and F show the typical two forms of phagocytes in *B. fuscus.*

***Granulocyte* (Fig. 4)**

Granulocytes contain many round or elliptical granules. The size and shape of the granules have some differences among the species. Granulocytes of *B. primigenus* have round granules, about 0.4 μm in diameter (Fig 4A); those of *B. scalaris* and *B. schlosseri* have round granules, about 0.7 μm in diameter (Fig. 4B); some elliptical granules are also found in *B. schlosseri*. In the other species, many of the granules are elliptical and their size (length of the major axis) is different from species to species; about 0.5 μm in *B. violaceus* (Fig. 4F), about 1 μm in *B. sexiens* and *B. fuscus* (Fig. 4E), about 1.5 μm in *B. delicatus* and *B. simodensis* (Fig. 4D), 1.8 μm in *B. lentus*. Round granules (up to 1.5 μm in diameter) are occasionally found in *B. lentus*.

Besides the round granules, granulocytes of *B. scalaris* usually contain a rod-shaped, large granule that has tesselated substructures (Fig 4B: arrow and inset). In this species, granules having similar substructures are also found in large-granule tunic cells, a type of free cell distributed in the tunic (Fig. 4C).

***Morula cell* (Fig. 5)**

Morula cells are round and contain several vacuoles that are filled with electron dense materials. The size and numbers of vacuoles are variable even in the same species. In *B. primigenus,* vacuolar contents of some morula-shaped hemocytes are much more electron dense than usual morula cells (Fig. 5B). They are abundantly found in the mantle of upper part of the zooids. In the live specimens, they are dark purple contributing to the colony color of this species, and are referred as pigmentary morula cells in this report. In *B. schlosseri*, each vacuole contains an electron dense sphere, and there is a clear space around the sphere (Fig. 5D). Whereas this type of morula cells is sometimes found in the other botryllid species, most of morula cells are this type in *B. schlosseri.* Some morula cells are univacuolar in *B. fuscus* (Fig. 5H).

***Pigment cell* (Fig. 6)**

Pigment cells are large, roundish cells without pseudopodia. They have one or a few large vacuoles thrusting their nuclei and the cytoplasm at the periphery. In the vacuoles there are many pigment granules that do not have an individual limiting membrane. The granules vary in size and shape, and each pigment cell usually has single type of the granules. There may be some interspecific differences as for the repertory of the granules types. The ultrastructures of nephrocytes are basically indistinguishable from the other pigment cells, although the granule may have some different structures from the other pigment granules.

***Other cell types* (Figs. 7–10)**

Pigment cell-like hemocytes are often found in *B. sexiens*. They have a large vacuole that does not contain granules (Fig. 7). Although they may be precursors of pigment cells, hemocytes of similar morphology are rarely found in the other species examined here. Therefore, this hemocyte type is temporally referred as large-vacuole cell in this report.

In *B. lentus,* there are some vacuolated hemocytes that contain moderately electron dense materials (Fig. 8). They are similar in morphology to morula cells, but have distinct characteristics: the electron density of the vacuolar contents are much lower than those of morula cells and the vacuolar membranes are often obscure. The latter characteristic may suggest that these hemocytes are disintegrative stage. This hemocyte type is not found in the other species so far studied and temporally referred as multi-vacuole cells in this report.

In botryllid ascidians, young oocytes are known to circulate with blood in the common vascular system of the colony (Mukai and Watanabe, 1976). In the present study, we encountered young oocytes as free components of blood in *B. primigenus* (Fig. 9) and *B. lentus* (Fig. 10). The oocytes are round and much larger than the other hemocytes. Round nucleus is present at the center of the oocytes and it has a large prominent nucleolus. The oocytes are completely surrounded by the primary follicle cells. In Fig. 9, the follicle cells have a nucleolus and tightly attached to the oocytes, while they are loosely attached in Fig. 10. These observations were rare chances, because we had found the free oocytes once in each of the two species and never found in the other species so far.

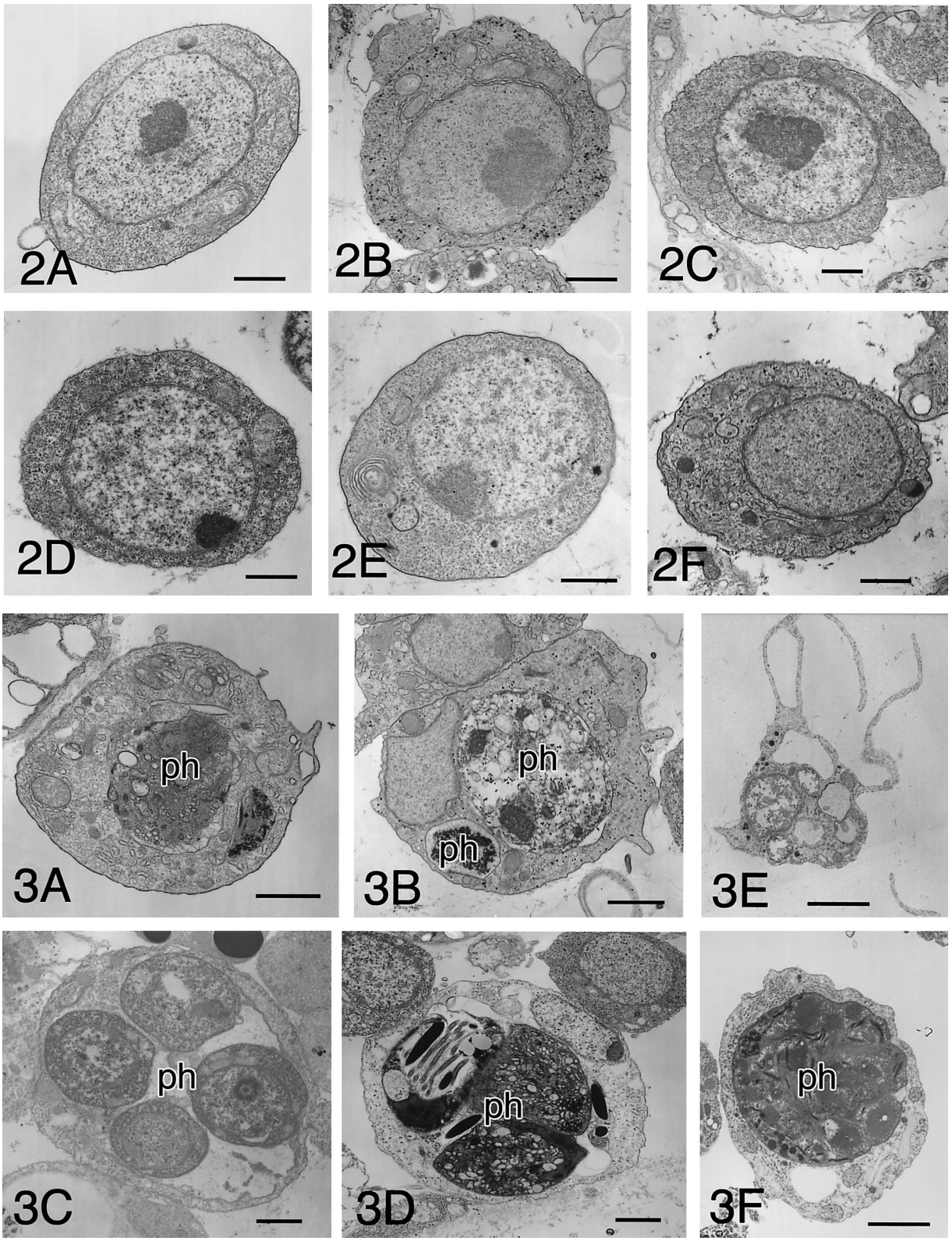


Fig. 2. Hemoblast of *B. primigenus* (**A**), *B. scalaris* (**B**), *B. schlosseri* (**C**), *B. sexiens* (**D**), *B. lentus* (**E**), and *B. violaceus* (**F**). Hemoblasts are small hemocytes having a high nucleus/cytoplasm ratio, and there are few variations in morphology among the species. Scale bars, 1 μm.

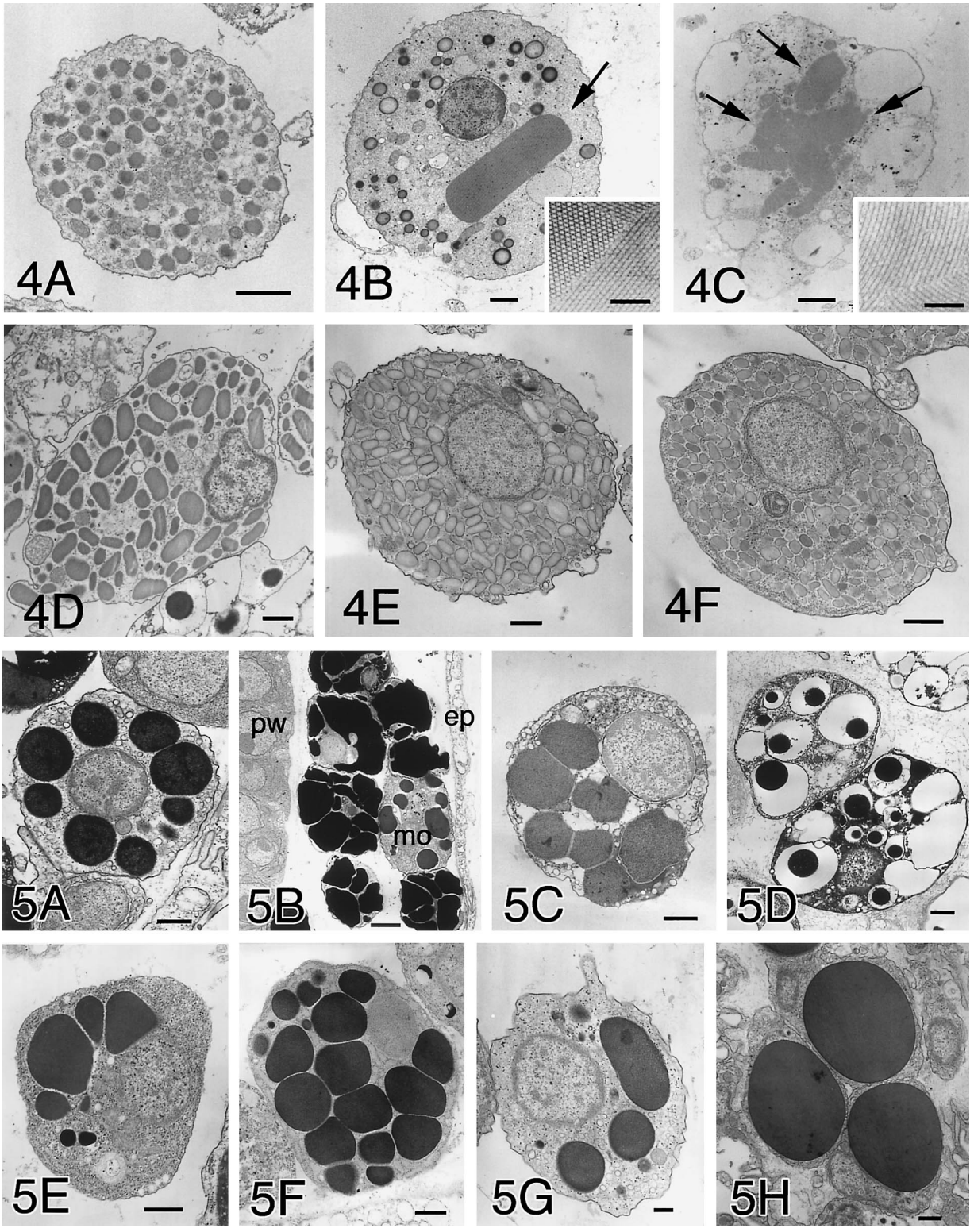
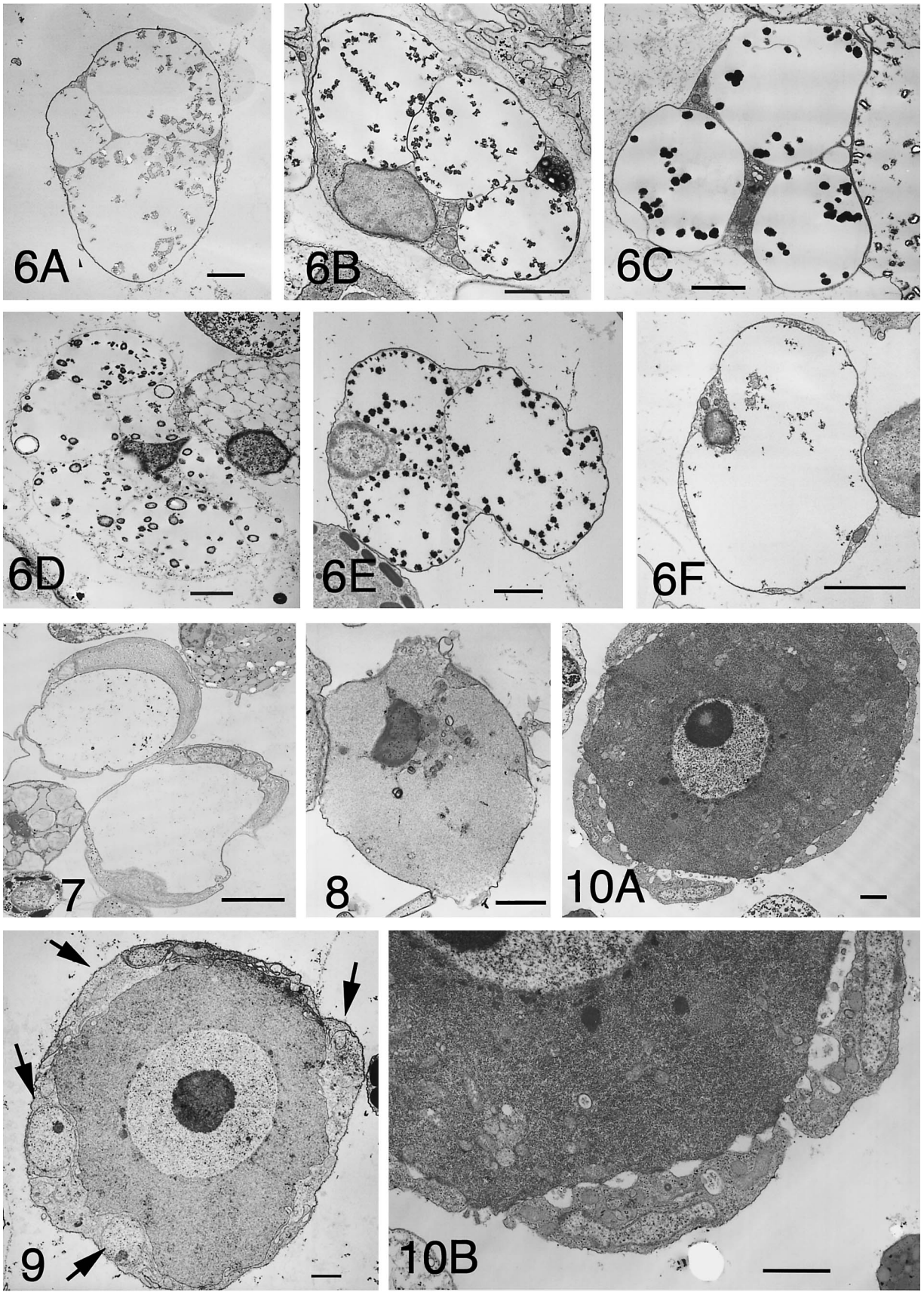


Fig. 3. Phagocyte of *B. primigenus* (**A**), *B. scalaris* (**B**), *B. delicatus* (**C**), *B. sexiens* (**D**), *B. fuscus* (**E** and **F**). Phagocytes contain phagosomes (ph) in the cytoplasm. They usually have several pseudopodia (A, B, and E), while those with large phagosomes often have few pseudopodia (C, D, and F). Scale bars, 2 μm.


6A
6B
6C
6D
6E
6F
7
8
10A
9
10B

DISCUSSION

Circulating hemocytes are classified into five-hemocyte types in the 9 botryllid ascidians based on the ultrastructure: hemoblasts, phagocytes, granulocytes, morula cells, and pigment cells. These five types are always found in the 9 species examined here. The electron micrographs in this report show some typical form of each type. It should be noted that there are some transient or disintegrative forms of hemocyte differentiation. The five types do not include some other hemocyte types described here, e.g., large-vacuole cells, multi-vacuole cells, and young oocytes. Although there may be some minor types that are overlooked, the five-hemocyte types should represent the major hemocyte types of the circulating cells in the blood of botryllid ascidians. Several studies have dealt with the hemocyte classification in some botryllids, and they classify the hemocytes into seven or more types. Table 1 shows a possible correspondence of the hemocyte types reported for botryllid ascidians by several authors (Milanesi and Burighel, 1978; Burighel *et al*., 1983; Schlumpberger *et al*., 1984; Shirae and Saito, 2000; Cima *et al*., 2001).

Hemoblasts are often referred as stem cells or lymphocyte-like cells. They appear to be undifferentiated cells and are believed to differentiate into some other cell types. In vascular budding of *B. primigenus,* asexual buds are formed from the aggregations of hemoblasts and epithelial cells, and this indicates the extreme pluripotency of hemoblasts (Oka and Watanabe, 1957).

Phagocytes are characterized by their phagocytic activity. Since they often contain other hemocytes in the phagosomes, one of their functions should be a scavenger to keep free of discarded cells in the vascular system. Phagocytes are irregularly shaped when they do not engulf large materials, but their cell shape easily changes depending on the amount of phagosomes. In *B. schlosseri,* Cima *et al*. (1996)

Table 1. Comparison of major hemocyte types reported in botryllid ascidians

Present study (9 botryllids)	*Botryllus schlosseri*[1]	*Botryllus schlosseri*[2]	*Botrylloides leachi*[3]	5 botryllids[4]
Hemoblast	Hemoblast	Lymphocyte -like cell	Stem cell	Hemoblast
Phagocyte	Macrophage Macrogranular amoebocyte?	Macrophage Signet-ring cell?	Hyaline amoebocyte? Macrophage-like cell	Hyaline amoebocyte Macrophage Signet-ring cell?
Granulocyte	Microgranular amoebocyte	Microgranular amoebocyte	Granular cell Compartment amoebocyte Compartment cell	Granular leucocyte
Morula cell	Morula cell	Morula cell Compartment cell Macrogranular amoebocyte?	Morula cell Granular amoebocyte?	Morula cell
Pigment cell* Nephrocyte	Pigment cell Nephrocyte	Carotenoid pigment cell Granular pigment cell Nephrocyte	Pigment cell Nephrocyte	Pigment cell

[1] Milanesi and Burighel, 1978; Burighel *et al*., 1983 (ultrastructural study).
[2] Schlumpberger *et al*., 1984 (light microscopic study).
[3] Cima *et al*., 2001 (ultrastructural study).
[4] Shirae and Saito, 2000 (light microscopic study for *B. primigenus, B. scalaris, B. schlosseri, B. simodensis, B. fuscus*).
* Pigment cells and nephrocytes are indistinguishable in electron microscopic observation.

Fig. 4. Granulocytes (**A**, *B. primigenus*; **B**, *B. scalaris*; **D**, *B. simodensis*; **E**, *B. fuscus*; **F**, *B. violaceus*) and large-granule tunic cell of *B. scalaris* (**C**). They are characterized by round (A and B) or elliptical (D, E, and F) granules filled in the cytoplasm. Arrows in **B** and **C** indicate the large-granules. Insets in **B** and **C** are enlargement of the large granules. Scale bars, 1 μm. Scale bars in insets, 0.2 μm.

Fig. 5. Morula cells of *B. primigenus* (in blood vessel, **A**; in mantle, **B**), *B. scalaris* (**C**), *B. schlosseri* (**D**), *B. sexiens* (**E**), *B. delicatus* (**F**), *B. lentus* (**G**), and *B. fuscus* (single vacuole type, **H**). They contain several vacuoles filled with electron dense materials. In **B**, a morula cell (mo) is surrounded by pigmentary morula cells in which vacuolar contents are heavily electron dense. ep, epidermis; pw, peribranchial wall. Scale bars, 2 μm for **B** and 1 μm for the others.

Fig. 6. Pigment cells (or nephrocyte) of *B. primigenus* (**A**), *B. scalaris* (**B**), *B. schlosseri* (**C**), *B. sexiens* (**D**), *B. simodensis* (**E**), and *B. lentus* (**F**). The bulk of the cytoplasm in each pigment cell is occupied by one or a few vacuoles containing pigment granules. Scale bars, 2 μm.

Fig. 7. Large-vacuole cells in *B. sexiens*. Unlike the pigment cells (Fig. 6), a large vacuole does not contain granules. Scale bar, 2 μm.

Fig. 8. Multi-vacuole cell in *B. lentus*. They have several vacuoles containing moderately electron dense materials. Scale bar, 2 μm.

Fig. 9. A young oocyte of *B. primigenus*. A large prominent nucleolus is found in the round nucleus. The oocyte is wholly covered with primary follicle cells (arrows). Scale bar, 2 μm.

Fig. 10. A young oocyte of *B. lentus* (**A**) and enlargement (**B**). There is a large nucleolus in the nucleus. Primary follicle cells are loosely attached to the cell membrane of the oocyte. Scale bar, 2 μm.

described this morphological change of phagocytes in detail. After the digestion of the phagocytized materials, the remnants may turn into granules. To date, this cell type may be given several names, such as hyaline amoebocytes, macrophages, granular amoebocytes, and etc. According to the histochemical analysis in *B. schlosseri* (Ballarin *et al*., 1993) and *B. leachi* (Cima *et al*., 2001), acid phosphatase and some other hydrolytic enzymes are present in hyaline amoebocytes, macrophages, and signet-ring cells. This indicates that these hemocyte types are involved in the same function, i.e., phagocytosis, and thus they probably share the same cell lineages of phagocytes.

The granules of granulocytes show interspecific variations: some species have round granules and some have elliptical granules (Fig. 4). The cytoplasm of granulocytes is full of the granules that are roughly uniform in size and shape. While granular inclusions are found in some other types, they usually vary in size and are distributed sparsely or unequally. In live specimens, granulocytes often attach on the glass slides changing them into irregular shape, and thus this cell type may be classified as some granular amoebocytes (or leucocytes) or compartment amoebocytes in some studies. While the function of granulocytes is uncertain, Cima *et al*. (2001) suggest that they are involved in nutrient storage/supply or immunosurveillance of the alimentary tract. Tunic cells are free cells distributing in the integumentary tissue, tunic. In botryllids, there are several types of tunic cells that are free cells distributing in the integumentary matrix (tunic) (Hirose *et al*., 1991), and the tunic cells are thought to differentiate from some hemocytes. Since large granules with tessellate substructures are contained in both granulocyte and large-granule tunic cells of *B. scalaris* (Fig. 4B and C), the large-granule tunic cells seem to be originated from the granulocytes that migrate in the tunic from the blood. On the other hand, while the granules of granulocytes in *B. lentus* are larger than those in any other species studied here, the granules of vacuo-granular tunic cells in *B. lentus* are also the largest in 4 *Botryllus* and 4 *Botrylloides* species so far studied (Hirose *et al*., 1991). This may indicate that granulocytes are related with vacuo-granular tunic cells, although the granules of granulocytes are different from those of vacuo-granular tunic cells in shape and size in each species.

Morula cells are characterized by their roundish vacuoles containing electron dense materials. The vacuoles are demonstrated to contain phenoloxidase (PO) and quinones (Cf. Ballarin *et al*., 1993; Frizzo *et al*., 2000). Morula cells are known to be cytotoxic cells (Ballarin *et al*., 1998) and the major effector cells in the allorejection reaction between colonies (Hirose *et al*., 1997; Shirae *et al*., 2002). On the other hand, the PO activities of hemolysate are much different among the species; the activity is much higher in ovoviviparous species than that in viviparous species (Shirae and Saito 2000; Hirose *et al*., 2002). Moreover, in *B. schlosseri,* Ballarin *et al*. (2001) demonstrated that the antibody against IL-1-α and TNF-α specifically label phagocytes and morula cells, and they supposed that morula cells play an important immunomodulatory role in the blood. In live specimens, morula cells often attach on the glass slides having irregular shape, while they are spherical in the blood. Thus, they might be sometimes described as granular amoebocytes. In *B. schlosseri* and *B. leachi,* histochemical studies revealed the presence of peroxidase and PO activities in granular amoebocytes as well as morula cells (Ballarin *et al*., 1993; Cima *et al*., 2002), indicating these hemocytes may share the same cell lineage. The granular amoebocytes with PO activity do not belong to the granulocytes defined in this study, and they should be included in morula cell. The size and amount of the vacuoles vary even in the same specimens, and this variation probably reflects the stages of a cell differentiation pathway. Many univacuolar morula cells are found in *B. fuscus* (Fig. 5H). Similarly, in a solitary ascidian *Ciona intestinalis,* univacuolar refractile refringent granulocytes with high PO activity differentiate to morula cells with lower PO activity (Parrinello *et al*., 2001). In *B. primigenus*, pigmentary morula cells are distributed in the mantle wall (Fig. 5B). Except for the electron density and the coloration of their vacuolar contents, they are the same with morula cells in morphology and much different from pigment cells. Therefore, these pigmentary morula cells are probably derived from morula cell, and they are listed as subtype of morula cells here.

In *B. schlosseri,* the presence of iron was shown in the morula cell pellets by means of X-ray microanalysis (Milanesi and Burighel, 1978), and Scofield and Nagashima (1983) speculated that the iron may participate in the allorejection reaction by performing a catalytic function. Indeed some metals are known to be accumulated in ascidian hemocytes, and vanadium and irons had been believed to be concentrated in the vacuoles of the morula cells, however, lacking reliable evidences (reviewed in Wright, 1981). In solitary ascidians of the family Ascidiidae, scanning X-ray microscopy strictly confirmed that vanadium are accumulated in the vacuoles of signet-ring cells but not in the morula cells (Ueki *et al*., 2002). Therefore, it is necessary to reconsider the localization of the metals in botryllid hemocytes as well as their functions with reliable methods.

Although refractile granules distinguish nephrocytes from the other pigment cells,ultrastructures of nephrocytes and pigment cells are basically the same. Ultrastructures of the both cell types described here are consistent with the detailed description in *B. schlosseri* (Burighel *et al*., 1983) and *B. simodensis* (Hirose *et al*., 1998). Whereas the function of nephrocytes is thought to be storage and excretion of nitrogenous wastes (Cf. Wright, 1981), nitrogenous compounds are also isolated as pigment substances, e.g., pteridines and purines (Hirose et al, 1998). This means that the colored pigment cells may also have excretory function as well as nephrocytes, and the coloration of pigment granules is the only difference between nephrocytes and the other pigment cells. Therefore, nephrocytes are listed as a subtype ranking with other color types of pigment cells. This

would be a reasonable treatment, because refractile cells (e.g., leucophore and iridophore) are usually categorized in members of pigment cells (chromatophores) in vertebrates. There are several variations in the ultrastructures of pigment granules. In *B. schlosseri,* Burighel *et al*. (1983) showed the granule colors are related with the granule ultrastructures.

In botryllid ascidians, oocytes are thought to differentiate from hemoblasts in the loose cell mass of the buds of early stage 8, and then they leave the buds (Mukai and Watanabe, 1976). The young oocytes circulate in the blood until they are caught in the gonadal space of the buds of stage 9. The development of the free oocytes is subdivided into two phases: multinucleololar phase (early phase) and mononucleolar phase (late phase). Moreover, Manni *et al*. (1993; 1994) described the oogenesis and egg envelope cytodifferentiation in the blastzooids of *B. schlosseri*, and they divided the oogenesis into five stages. The oocytes observed here are all mononucleolar phase/stage 2 (Fig. 9 and 10). In this study, we found only two mononucleolar oocytes and no multinucleolar oocytes. It is possible that the free oocytes rarely come to colony periphery where we mainly examined. In *B. primigenus*, multinucleolar oocytes are 5 to 6 μm in diameter and usually found in groups (Mukai and Watanabe, 1976). Some of them might be regarded as hemoblasts in the present observation, because there are few morphological characteristics that discriminate multinucleolar oocytes from hemoblasts except for the number of nucleoli.

Hemoblasts probably differentiate into some other hemocyte types, and each type includes transient and disintegrative forms of the differentiation. The hemocyte types in the present classification represent the pathways of the hemocyte differentiation, and this is the reason why they are smaller in number than other classifications (Cf. Table 1). Although it might be possible that one hemocyte type defined here consists of some hemocyte types of discrete cell lineage, we think that the present classification is a starting point for more precise and comprehensive hemocyte classifications in botryllids. Ideally, the hemocytes should be classified based on their functions and the pathway of cytodifferentiation. There are some specific characteristics related to the functions of a particular hemocyte type: pluripotency for hemoblasts, phagocytic activity and hydrolytic enzymes for phagocytes and PO for morula cells. Pigment cells may be defined by the presence of pigment granules of pteridines and/or purines. Although granulocytes are characterized in morphology by the distinctive granules, their function is not well known to date. The prospective pathways of the cytodifferentiation are summarized in Fig. 11. Future studies are expected to subdivide the hemocyte types and disclose the functions of each subtype.

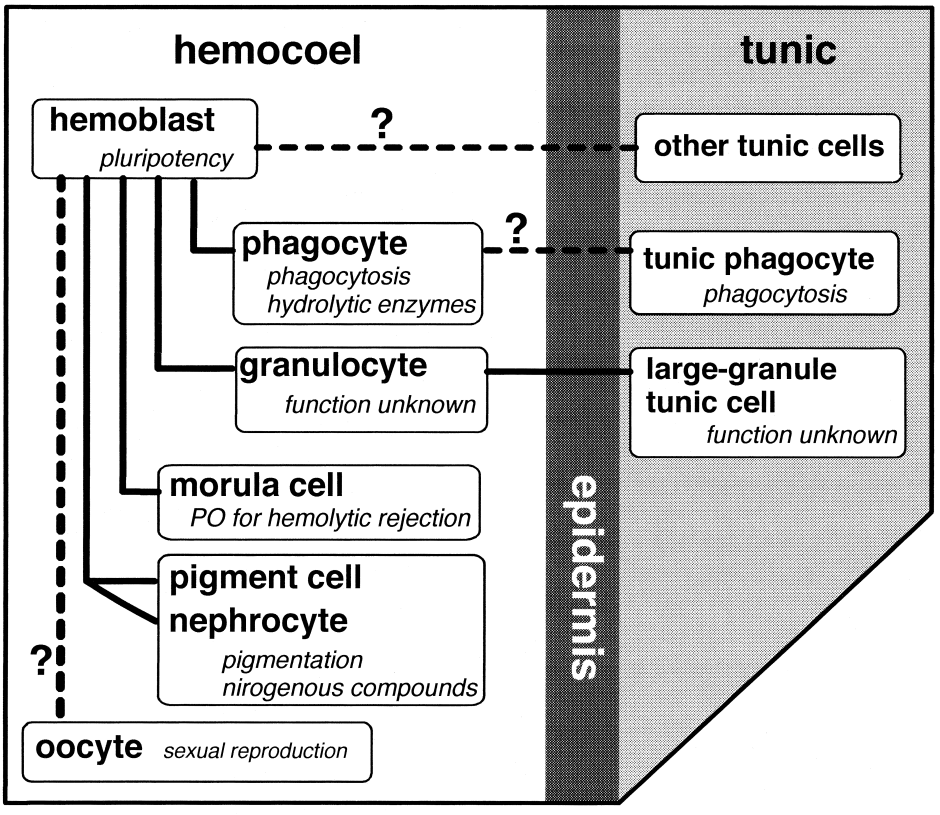


Fig. 11. Hypothetical pathways of the cytodifferentiation of hemocytes and tunic cells in botryllid ascidians. Pluripotent hemoblasts are thought to differentiate into the other types of hemocytes. Some hemocytes infiltrate in the tunic passing through epidermis, and they differentiate into tunic cells. Functional characteristics are appended in italic letters.

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