STUDIES ON THE COELOMIC CELLS OF SOME JAPANESE ASCIDIANS

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STUDIES ON THE COELOMIC CELLS OF SOME JAPANESE ASCIDIANS[1)]

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Coelomic cells of five solitary ascidians were examined. Their movement and behavior to foreign particles were observed under phase contrast microscopy. They were fixed and stained by various dyes, such as vital dyes and Giemsa. PAS-reaction, Hg-BPB reaction and OsO_4 fixation were performed on the coelomic cells. The following cell types were recognized: vesicular cells, large granular amoeboid cells, small granular amoeboid cells, fine granular amoeboid cells, minute granular cells, small vacuolated cells, large vacuolated cells, large basophilic cells, asidophilic granular cells, orange cells, brown cells and lymphocytes. The results are compared to those of other investigators and discussed.

In vertebrates, adaptive immunity has long been studied by many investigators. If the animal body is invaded by foreign materials, it discriminates 'not self' from 'self' and rejects the former from the body by immunological response. Recent studies on the immunological response revealed that early indication of the appearance of adaptive immunity was found in one of the oldest types of vertebrates, the hagfish, a cyclostome (Thoenes and Hildeman, 1970).

In the invertebrate kingdom, the existence of specific adaptive immunity is not known. However, 'immune-like' phenomena have been reported (Huff, 1940; Cooper, 1969; Theodor, 1970; Scott, 1971, Tanaka and Watanabe, 1973). It seems that the immunological response in vertebrates probably has evolved from such a physiological process in invertebrates. However, the question of whether immune systems of vertebrates developed from one of the 'immune-like' phenomena of invertebrates has not yet been solved (Burnet, 1968; Hildeman, 1974). Since ascidians occupy a unique position linking invertebrates and vertebrates, studies on these animals are expected to solve the question.

The naturally-occurring hemagglutinin in the coelomic fluid of an ascidian was investigated by Fuke and Sugai (1972). The data suggested that the hemagglutinin was polysaccharide or mucopolysaccharide, not considered a forerunner of vertebrate immunogloblin. Studies on the cellular 'immune-like' response of ascidians is expected to find the origin of the immune response. As one step in the study, the blood (coelomic fluid) cells of solitary ascidians were investigated. The

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blood cells of ascidians have been studied by numerous investigators (ENDEAN, 1960; GEORGE, 1939; OHUYE, 1936). A great diversity of blood cells have been described, but little is known about their biochemical, physiological and developmental roles.

In this paper, the structure of the coelomic cells, their histochemical properties are reported. The data are compared to the results of previous investigators and discussed.

MATERIALS AND METHODS

Five species of Japanese ascidians were used. Three of them, *Halocynthia roretzi* (DRASCHE), *Styela clava* (HERDMAN) and *Pyura mirabilis* (DRASCHE), were harvested at the Marine Biological Station of Asamushi in Aomori. *Halocynthia aurantium* (PALLAS) was collected at Otaru in Hokkaido and kept in aquaria at the Marine Biological Station of Asamushi. Specimens of *Styela clava* (HERDMAN) and *Styela plicata* (LEUSUER) were harvested at the Noto Marine Laboratory in Ishikawa.

To collect the colelomic cells of *Halocynthia roretzi*, *Halocynthia aurantium* and *Pyura mirabilis*, five to ten volumes of sea water were taken in a hypodermic syringe and then one volume of coelomic fluid was withdrawn from the space between the test and mantle of the ascidian. The coelomic cells of *Styela plicata* and *Styela clava* were collected by cutting the test and mantle without injuring the internal organs.

When the living cells were examined by phase contrast microscopy, the coelomic fluid was poured out onto a glass dish which was made as follows: a glass ring (20mm in diameter, 8 mm in height) was stuck onto the cover glass by adhesive agent. In some cases, they were dyed with vital dyes (0.005% neutral red; 0.001% toluidine blue; 0.001% Nile blue; all in sea water). For the observation of phagocytosis, the supernatant was discarded after ten minutes standing and then the living sperm of sea urchin (*Temnopleurus hardwichi* (GRAY)) or rabbit erythrocytes fixed with 1% glutaraldehyde were added.

Fixed cells for cytological examination were prepared as follows. The coelomic fluid was poured on the cover glass. After ten minutes, the supernatant was discarded and coelomic cells adhering to the glass were fixed with 1% glutaraldehyde (G.A) in sea water. After fixation had been carried out for one hour or more, the preparation was washed several times with distilled water and then stained with diluted ($\times 10$) Giemsa solution for 20 minutes. To make the samples for PAS-reaction and Hg-bromphenol blue staining, the coelomic cells were fixed according to ENDEAN (1960). A drop of coelomic fluid was put on the cover glass. After five minutes, excess fluid was drained off, and 5% formalin in sea water was added to each cover glass. After fifteen minutes, the fixative was drained off and the cover glass was flooded with distilled water. Polysaccharides

or mucoprotein were sought by utilizing the PAS-reaction (McMANUS, 1948). Proteins were examined by using Hg-bromphenol blue (BPB) (MAZIA, BREWER and ALFERT, 1953). In the last step of the staining, the cells were flooded with 0.02 M phosphate buffer (pH 6.0), instead of distilled water. For fixation of OsO_4, which is known to blacken the lipids, the cells were immersed in the 0.02‰ OsO_4 in sea water for fifteen minutes.

RESULTS

Many coelomic cells were found in the fluid obtained from five solitary ascidians. The cell number varied between different animals and also between the portion of the body. In the vicinity of branchial aperture and atrial aperture, there were more cells than for the other portions. Ordinarily, samples were collected from the coelom on the lateral side of animals. The mean number of cells per ml of ascidian fluid estimated by hemocytometer, were as follows: *Halocynthia roretzi*, $(1.4\pm0.3)\times10^7$; *Halocynthia aurantium*, $(1.2\pm0.2)\times10^7$; *Styela clava*, $(3.8\pm1.2)\times10^6$; *Styela plicata*, $(7.5\pm2)\times10^6$; and *Pyura mirabilis* $(1.2\pm0.2)\times10^7$.

It is difficult to classify coelomic cells of several species of ascidians absolutely, because, first, the external appearance of coelomic cells which are obtained from a species are usually very different from those of the other ascidians. Secondly, the physiological and developmental roles of the cells have not yet been studied sufficiently. Therefore, it is difficult to determine which types of the cells are homologous among the coelomic cells of several species of ascidians.

On the basis of the differences in structure and behavior toward various dyes and foreign particles, the following cell types were recognized: vesicular cells, large granular amoeboid cells, small granular amoeboid cells, fine granular amoeboid cells, minute granular cells, small vacuolated cells, large vacuolated cells, acidophilic granular cells, large basophilic cells, orange cells, brown cells and lymphocytes. The size and percentage of these cells are summarized in Table 1.

I. Coelomic cells of five ascidians

1) *Vesicular cells*

Vesicular cells are the most conspicuous and abundant type among coelomic cells of *Halocynthia roretzi* and *Pyura mirabilis* (Table 1). About 60% of the coelomic cells are in this type. They are characterized by possession of a large fluid-filled vacuole, and some have several vesicles besides a large one. The vesicles stained weakly, but clearly, by dyes for vital staining, Nile blue, toluidine blue and neutral red (Table 2).

The vesicles were not so strongly stained by Giemsa. The vesicles of *Halocynthia roretzi* colored faint red or green (Table 3). These stainability show that the fluid of vesicles is not basic but slightly acidic or neutral. The vesicles reticulated after the cells were fixed by glutaraldehyde (G.A.) and stained by

Table 1.
Coelomic cells of solitary ascidians

	Pyura mirabilis	*Halocynthia roretzi*	*Halocynthia aurantium*	*Styela clava*	*Styela plicata*
vesicular cell (compartment or signet ring cell*)	60–65% d; 8–12μ	50–65% l; 10–13μ w; 5–7μ	10–20% l; 10–16μ w; 6μ	—	—
large granular amoeboid cell (green cell or vanadocyte*)	15–20% l; 14–18μ w; 10μ	4–12% l; 10–13μ w; 7μ	30–50% l; 14–16μ w; 6μ	40–50% l; 16–18μ w; 6–10μ	15% l; 15–20μ w; 5μ
small granular amoeboid cell (phagocyte*)	10–15% l; 8–9μ w; 6μ	—	—	40–50% d; 6–10μ	80% d; 5–10μ
fine granular amoeboid cell (phagocyte*)	—	15–20% d; 10–14μ	40–50% d; 9–14–μ	—	—
minute granular cell	<5% l; 9–12μ w; 8μ	<5% d; 10μ	—	2% d; 6–12μ	N.D.
vacuolated cell (macrophage*)	2% d; 20–25μ	small v.c. 2%, d; 5–7μ large v.c. 2%, d; 20–25μ	<2% d; 20–25μ	<2% d; 25–30μ	<2% d; 20–30μ
large basophylic cell	—	2% d; 13–17μ	1–2% d; 12–15μ	—	1–2% d; 10–15μ
acidophylic granular cell	—	—	1–2% d; 12–15μ	—	—
brown or orange cell	<1% d; 6–8μ	<1% d; 6–8μ	—	<1% d; 5–7μ	—
lymphocyte	<1% d; 5–7μ	<1% d; 5–7μ	<1% d; 5–6μ	<1% d; 5–7μ	N.D.

The size and percentage of coleomic cells obtained from five solitary ascidians. The following abbreviations are used: l. length, w, width, d, diameter. N.D., 'not determined'. * see ENDEAN (1960) and GEORGE, 1939.

GIEMSA solution (Fig. 2-A). The fluid-filled vacuole of *Pyura mirabilis* does not exibit a fibrous structure like that of *Halocynthia roretzi*.

The vesicular cells of *Halocynthia roretzi* show an active amoeboid movement (Fig. 1-A). When they are put into contact with fixed reticulocyte or sea urchin's sperm, they stick to them and move with them. In some cases, they again set free the foreign material but frequently they demonstrate phagocytosis (Fig. 2-E).

The vesicular cells of *Pyura mirabilis* are approximately spherical (Fig. 1-D). They contain a large fluid vacuole and a nucleus which is peripheral in position (Fig. 2-B). One or two refractile granules are seen near the nucleus. On standing the vesicular cells were observed to elongate, occasionally to produce blunt pseudopods, to move slowly. They do not show active amoeboid movement as in *Halocynthia roretzi*.

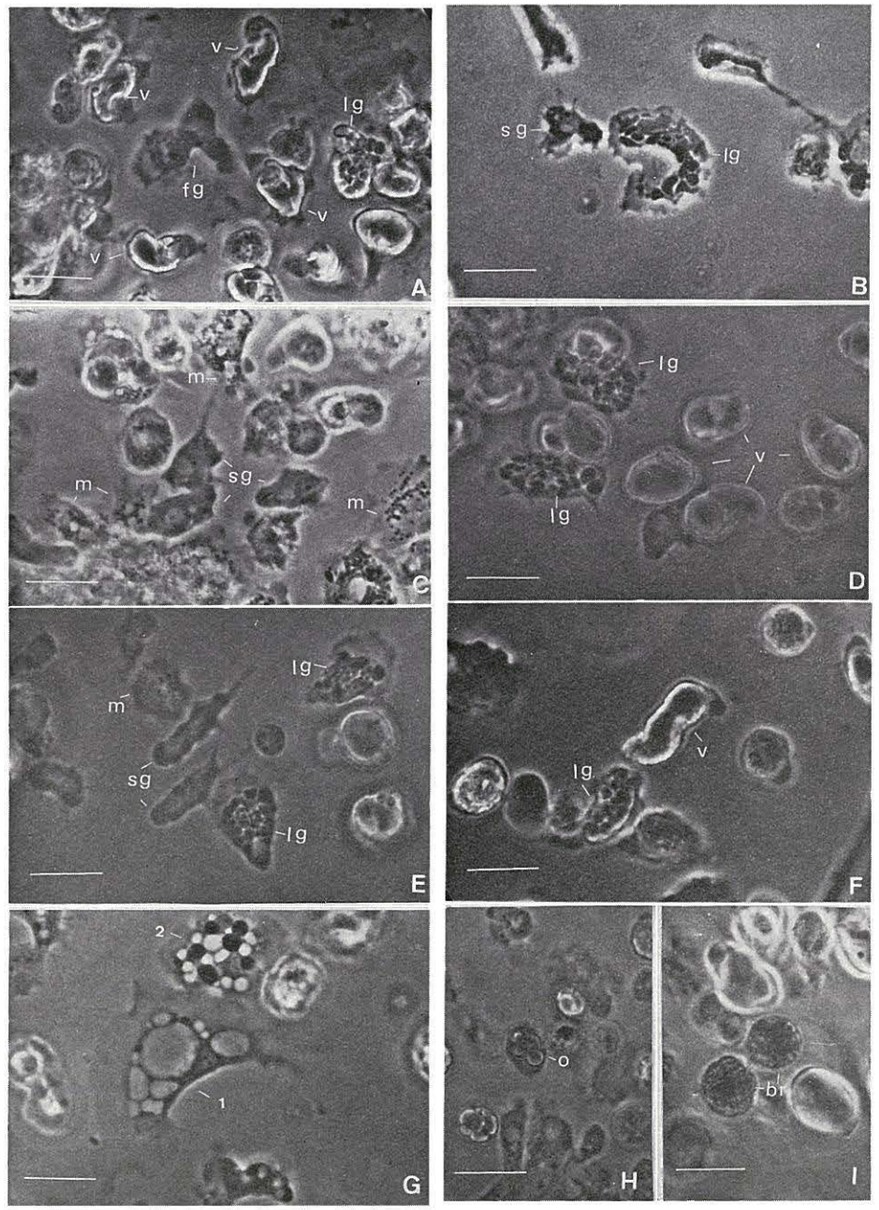

Figure 1. Coelomic cells of Japanese ascidians under phase contrast microscopy: A. *Halocynthia roretzi*. B.C. *Styela clava*, D.E. *Pyura mirabilis*. F. *Halocynthia aurantium*. G. The change of large granular amoeboid cell to vacualated cell (*Styela plicata*). H. Orange cell of *Halocynthia roretzi*. I. Brown cell of *Pyura mirabilis*. fg represents fine granular amoeboid cell; v, vesicular cell; lg, large granular amoeboid cell; sg, small granular amoeboid cell; m, minute granular cell; o, orange cell; b, brown cell. 1. represent the vacuolated cell originating from large granular amoeboid cell owing to the rupture of the large granules; 2 represents the process. The scale line indicates 10 μm.

In the coelomic fluid of *Halocynthia aurantium*, two types of globular cells were distinguished. One kind possesses a large vesicle which was packed full with bright small granules (Fig. 1-F). Tentatively, the cells are named vesicular cells. This type of cells is not so abundant as in *Halocynthia roretzi*. The granules which consist of a vesicle were not strongly stained by Giemsa. They were coloured faint pink or yellow. They show active amoeboid movement, and phagocytosis for reticulocytes were observed.

No vesicular cells were observed in *Styela plicata* and *Styela clava*.

2) *Large granular amoeboid cells*

Large granular amoeboid cells are very similar in all five ascidians. They possess about 20 large granules (Fig. 1-A, B). At some times, the cells of *Halocynthia roretzi* have a smaller number of these granules. It is difficult to distinguish these cells from vesicular cells. It seems that transitional stages between them exist. Large granular amoeboid cells of *Pyura mirabilis* are larger than other blood cells, being 16 μ in length and 10 μ in width (Fig. 1-D). These cells were usually observed to elongate and move very actively. Very elongated forms were frequently observed in *Styela clava* and *Pyura mirabilis* after standing for long time in sea water. (Fig. 1-B). The clear cytoplasm flew outward usually in one direction. One end of the cell was fixed on the surface of the slide glass and the free end was sent forth as pseudopodium. In an extreme case, the length of the cell reached 100 μ or more, while its breadth was only 2–3 μ. Their granules were sent in this narrow cytoplasm changing the shape into treads from one part to another. Large granular amoeboid cells of *Styela clava* were observed to phagocyte reticulocytes and sperm of sea urchin (Fig. 2-D). In 20–30 minutes after giving reticulocytes of *Pyura mirabilis*, almost all were seen to have two or more red cells in the cytoplasm (Fig. 2-D).

Dyes for vital staining colored the granules more strongly than those of vesicular cells. Dilute Nile blue and toluidine blue stained them blue. Neutral red colored them red. It is considered that there are more acidic substances in the granules than vesicles of vesicular cells. When the cells were fixed by G.A. and stained by Giemsa, no dyeing was observed (Table 3). For the coelomic cells of *Halocynthia aurantium*, the cells which have larger granules than those of vesicular cells were named large granular amoeboid cells (Fig. 1-F). When the cells were fixed by G.A. and stained by GIEMSA solution, it was difficult to distinguish the large granular amoeboid cells from vesicular cells. It seems that transitional stages between them exist.

The coelomic cells of *Styela plicata* were mentioned in a previous paper (FUKE and SUGAI, 1972). The large granular amoeboid cells are considered the same as those named "granular amoeboid cells" in that paper. The properties of these cells are very similar to those of *Halocynthia roretzi*, *Pyura mirabilis* and *Styela clava*.

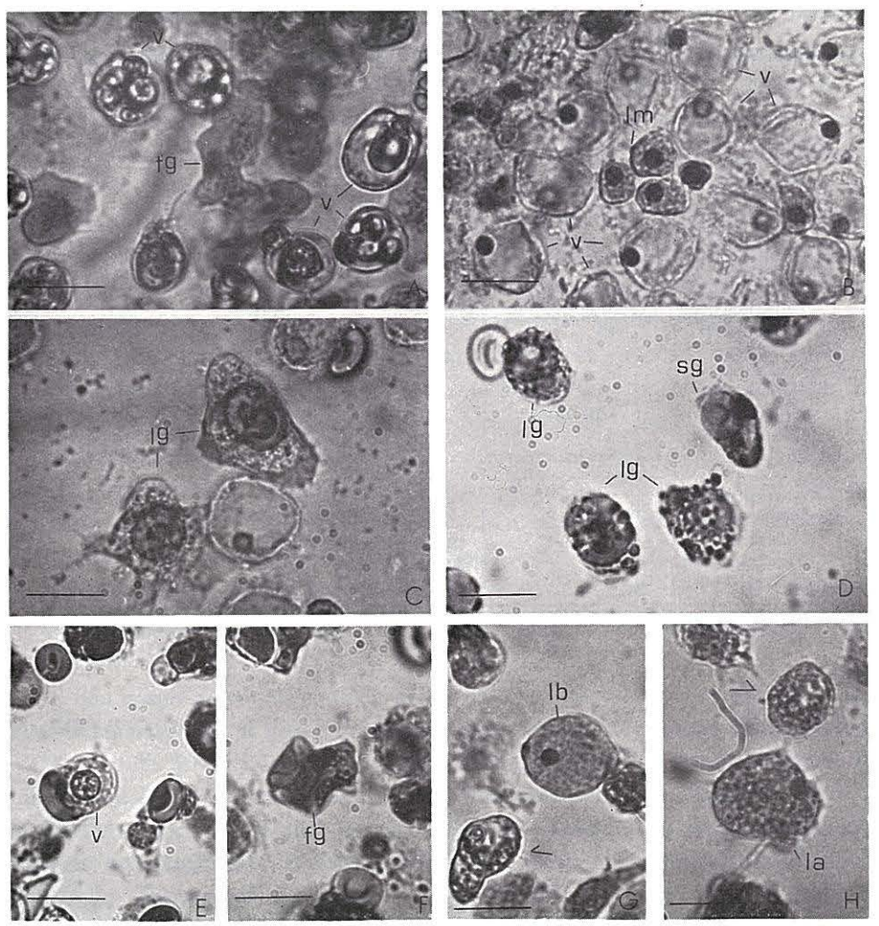

Figure 2. Coelomic cells of Japanese ascidians after fixation and staining by Giemsa: A. *Halocynthia roretzi*. B. *Pyura mirabilis*. C. Phagocytosis by large granular amoeboid cells of *Pyura mirabilis*. D. Phagocytosis by large granular amoeboid cells and small granular amoeboid cell of *Styela clava*. E. Phagocytosis by vesicular cell of *Halocynhtia roretzi*. F. Phagocytosis by fine granular amoeboid cell of *Halocynhtia roretzi*. G. H. Coelomic cells of *Halocynthia aurantium*. v. represents vesicular cell; fg, fine granular amoeboid cell; lm, lymphocyte; lg, large granular amoeboid cell; sg, small granular amoeboid cell; lb, large basophilic cell; la, large acidophilic cell. Arrow represents vesicular cell or large granular amoeboid cell. The scale line indicates 10 μm.

3) Fine granular amoeboid cells

The fine granular amoeboid cells are restricted to the genus *Halocynthia*. They are very different from the two types of cells mentioned above. The cytoplasm spread thinly on a glass surface, and there are many small granules and one or two larger ones (Fig. 1-A, Fig. 2-A). The size of the cells varies widely. Fully

Table 2.
Vital staining of granules or vesicles in coleomic cells

	Pyura mirabilis	*Halocynthia roretzi*	*Halocynthia aurantium*	*Stylea clava*
vesicular cell				
Nile blue	faint blue	blue	N.D.	—
toludine blue	faint purple	blue	faint blue	—
netural red	pink	pinkish purple	N.D.	—
large granular amoeboid cell				
Nile blue	blue	blue	N.D.	blue
toludine blue	blue	blue	blue	blue
netural red	red	red	N.D.	deep red
small granular amoeboid cell				
Nile blue	faint blue	—	—	purplish blue
toluidine blue	purple	—	—	purple
netural red	pinkish brown	—	—	yellow
fine granular amoboid cell				
Nile blue	—	purplish blue	N.D.	—
toludine blue	—	purple	not stain	—
netural red	—	pinkish brown	N.D.	—

Stainability by dyes for vital staining. N.D. represents 'not determined'.

developed cells have diameters between 10–14 μ for *Halocynthia roretzi* and 9–14 μ for *Halocynthia aurantium*. These cells are most abundant in *Halocynthia aurantium* (40–50%).

The cytoplasm was not stained by any supervital dyes. The granules of *Halocynthia roretzi* were stained purplish-blue by Nile blue, purple by toludine blue and pinkish brown by neutral red. When the cells were fixed with G.A. and stained by GIEMSA solution, the cytoplasm was clearly basophylic. A variable number of minute granules, colored red or purple by Giemsa staining were seen near the nucleus (Fig. 2-A). The properties of the granules are very different from those of large granular amoeboid cells and vesicular cells. They are acidophilic.

Observations made on fine granular amoeboid cells under phase contrast microscopy revealed that they moved more rapidly than vesicular cells and large granular amoeboid cells. They move more actively with granulated cytoplasm in the head. The granules are occasionally observed to elongate like a thread. Their phagocytosis was also more active than for vesicular cells or large granular amoeboid cells mentioned above. After the cells stuck to the reticulocyte, they did not set the foreign materials free. The cells phagocyted the reticulocyte within ten seconds after contact. It was often observed that numerous mammalian reticulocytes were engulfed within one cell (Fig. 2-F).

4) *Small granular amoeboid cells*

Small granular amoeboid cells of *Pyura mirabilis* constitute about 15% of the total number of cells. In *Styela clava*, they form about 40–50% (Table 1). In *Styela plicata*, they are most abunbant and 80% of coelomic cells are in this type. They are not found in the genus *Halocynthia*, in which many fine granular amoeboid cells are seen. In a previous paper (Fuke and Sugai, 1972), these cells were named "fine granular amneboid cells" in *Styela plicata*.

These cells in *Pyura mirabilis* are similar to large granular amoeboid cells except their size under phase contrast microscopy. They have a clear round nucleus in which one or two nucleoli are seen. Granules in the cytoplasm are smaller and more indistinct than those of large granular amoeboid cells and occasionally take the shape of a string, just like those of large granular amoeboid cells (Fig. 1-B, C, E). They are usually observed to produce projecting pseudopodia (Fig. 1-E). In *Pyura mirabilis*, the granules gave a positive reaction (red), but there was much variation after staining by Giemsa. In *Styela clava*, as well as *Styela plicata*, they were stained reddish. The cytoplasm was a faint purple after fixation and staining by Giemsa (Table 3). They occasionally had some vacuoles.

The small granular cells of *Styela clava* and *Styela plicata* show active amoeboid movement, but those of *Pyura mirabilis* were not so active. They show active phagocytosis (Fig. 2-D).

5) *Minute granular cells*

These cells are characterized by the possession of minute granules which show Brownian movement (Fig. 1-C). They are found in the coelomic fluid of *Halocynthia roretzi*, *Pyura mirabilis* and *Styela clava*. Although their numbers fluctuate somewhat from specimen to specimen, these cells constitute less than 5% (Table 1). The cytoplasm, as well as minute granules, was not stained by any vital dye. The minute granular cells are usually oval, but after standing for many hours, they change their shape to fibroblast-like cells. They do not move actively; rather they stay at one point protruding and withdrawing their cytoplasm.

When mammalian reticulocytes were given to the cells, they phagocyted them within 2 seconds and attached to the other reticulocytes subsequently.

6) *Vacuolated cells*

These cells do not have any characteristic vesicles or granules in their cytoplasm but they have several empty vacuoles. Vacuolated cells can be classified into two types according to their size, small vacuolated cells and large vacuolated cells, in *Halocynthia roretzi*. The size of small vacuolated cells is about 6 μ. Their number varies depending on the specimens. The cells do not move actively. They phagocyte fixed reticulocytes and sperm of sea urchin. The cells are thought to be a transitional type between lymphocytes and other

Table 3.
Giemsa staining of coleomic cells after G.A. fixation

	Pyura mirabilis	*Halocynthia roretzi*	*Halocynthia aurantium*	*Styela clava*	*Styela plicata*
vesicular cell	not stain	vesicles; faint red or faint green	vesicles; faint pink or yellow	—	—
large granular amoeboid cell	not stain	not stain	granules; faint pink or yelow	cytoplasm; faint purple granules; not stain	not stain
small granular amoeboid cell	granules; red (variation)	—	—	cytoplasm; faint purple granules; red	cytoplasm; faint purple granules; red
fine granular amoeboid cell	—	cytoplasm; purple granules; red	granules; purple	—	—
minute granular cell	not stain	not stain	—	not stain	N.D.
vacuolated cell	cytoplasm; faint purple	cytoplasm; faint purple	cytoplasm; faint purple	cytoplasm; faint purple	cytoplasm; faint purple
large basophylic cell	—	cytoplasm; light blue granules; pink	cytoplasm; light blue granules; pink	—	cytoplasm; grayish blue granules; pink
acidophylic granular cell	—	—	cytoplasm; light blue granules; red purple	—	—
brown or orange cell	not stain	not stain	—	not stain	—
lymphocyte	faint purple	faint purple	faint purple	faint purple	faint purple

Giemsa staining of coleomic cells after glutarladehyde fixation. N.D. represents 'not determined'.

differentiated cells. An other possibility is that vacuolated cells might originate from vesicular cells or large granular cells.

Large vacuolated cells are thin when spread on the glass. They have ordinarily two or three large vacuoles near the nucleus which are located in the center of the cells. The size of these cells is about 20–25 μ. The number of the cells varies depending the physiological condition. There are about 2% when they are counted soon after they were harvested from the sea, but the number increases after they are long cultured under starvation. They are not amoeboid, but they show phagocytosis for rabbit reticulocytes and sperm of the sea urchin. It seems that the large vacuolated cells originate from the vesicular cells or large granular cells as do small vacuolated cells. It was actually observed that these cells originated from the large granular amneboid cells in *Styela plicata* (Fig. 1-G). The large granules

exibit autolysis when the ascidians were cultured under starvation for a long period of time.

7) *Large basophilic cells*

These cells are rare in the blood (about 2%), but they are conspicuous by their characteristic appearance. The nucleus is small, round and located eccentrically (Fig. 2-G). The cytoplasm is very granular. The cells are often observed to have bulged out lopopodia. They occasionary show active amoeboid movement. Some of the granules stain vitally with toluidine blue. After fixation and staining by Giemsa, the cytoplasm was colored light blue and minute granules were dyed pink. Phagocytosis for reticulocytes was observed.

8) *Acidophilic granular cells*

The acidophilic granular cells are found only in *Halocynthia aurantium* as far as investigated. These cells are rare in blood (about 1–2%), but they are easy to find because they have a characteristic appearance. The nucleus is small, round and located eccentrically just as in large basophilic cells, but the cells are a little larger than large basophilic cells. After fixation and staining by Giemsa, the cytoplasm was colored light blue, with red-purple granules scattered throughout (Fig. 2-H). Phagocytosis is not yet confirmed.

9) *Lymphocytes*

These small and spherical cells are like mammalian lymphocytes. They possess a large nucleus and small amount of cytoplasm. These cells are rare in the blood. They are often observed to produce pseudopodia. They do not show phagocytosis. Lymphocytes often seen to aggregate in the coelomic fluid (Fig. 2-B). Lymphocytes are believed to be the primitive blood cells of ascidians (Endean, 1960).

10) *Orange cells or brown cells*

These cells are rare in the coelomic fluid, but they are very conspicuous because they have a natural brown or orange color (Fig. 1-I, H). In *Pyura mirabilis*, so many brown granules fill the cytoplasm that it is difficult to find the nucleus. They were usually observed to have protruding pseudopodia. They did not show active amoeboid movement nor were they observed to phagocyte foreign bodies.

II. Histochemical properties of the coelomic cells

Some histochemical properties of coelomic cells obtained from three ascidians, *Halocynthia roretzi*, *Pyura mirabilis* and *Styela plicata*, were examined. PAS-reaction, Hg-BPB reaction and OsO_4 fixation were performed.

1) *PAS-reaction*

When the coelomic cells of *Halocynthia roretzi* were fixed with formaldehyde, it was difficult to discriminate vesicular cells from large granular amoeboid cells. The vesicles or granules of these cells were PAS-positive, but there was much variation in the intensity of the color (Table 4). In some large granules, the PAS-positive material was confined to the periphery of the granules; in others, whole granules were colored strongly. On rare occations, a large vesicle, in which minute granules were seen, was found PAS-negative but its cytoplasm, except for the granule, gave a positive reaction. In vesicular cells of *Pyura mirabilis*, the cytoplasm was scarcely stained red except for the granules near the nucleus. The large granules of the amoebocyte of same species were PAS-positive, but there was much variation in intensity of the color just as those of *Halocynthia roretzi*. The granules of large granular amoeboid cells of *Styela clava* were also colored red, but there was also much variation from granule to granule.

Table 4.
PAS-reaction of coelomic cells

	Pyura mirabilis	*Halocynthia roretzi*	*Styela clava*
vesicular cell	cytoplasm; weak red granules; strong red near the nucleus	*vesicles or granules; positive (variation)	—
large granuler amoeboid cell	granules; positive (variation)		cytoplasm; pink granules; positive (variation)
small granular amoeboid cell	cytoplasm; faint positive granules; strong positive near the nucleus	—	cytoplasm; faint positive granules; strong positive near the nucleus
fine granular amoeboid cell	—	cytoplasm; faint pink granules; strong red in the periphery of the cytoplasm	—
large basophilic cell	—	cytoplasm; positive	—

PAS-reaction of coelomic cells after formaldehyde fixation. *In *Halocynthia roretzi*, it is difficult to discriminate vesicular cells from large granular amoeboid cells.

In the fine granular amoebocyte of *Halocynthia roretzi*, the whole cytoplasm gave a faint PAS-positive reaction. Strong PAS-positive granular material was seen in periphery of the cytoplasm.

In the small granular amoeboid cells of *Pyura mirabilis* and *Styela clava*, the whole cytoplasm gave a faint PAS-positive reaction. Strong PAS-positive granular material was evident in the cytoplasm near the nucleus rather than at the periphery.

Large basophilic cells gave a positive reaction on whole cytoplasm. No other cells were observed PAS-positive.

2) *$HgCl_2$-bromphenol blue*

$HgCl_2$-bromphenol blue procedure was used according to Mazia *et al.* (1953). The cells were stained by $HgCl_2$-BPB and treated with acetic acid, then rinsed in 0.02 M phosphte buffer at pH 5.5, pH 7.0 and pH 8.5. At pH 8.5, almost all of dye was released from the cells. At pH 5.5 the cells were stained stronger than at pH 7.0. Then, the coelomic cells were observed in 10% glycerin, with 0.02 M phosphate buffer at pH 5.5

In *Halocynthia roretzi*, it is difficult to discriminate between vesicular cells and large granular amoeboid cells after formaldehyde fixation as mentioned above. The granules or vesicles of these cells showed variations in staining. Most vesicles became blue but a few were greenish or yellow. The vesicular cells of *Pyura mirabilis* was scarecely stained by $HgCl_2$-BPB except for its fibrous structure. The granules of the large granular amoeboid cells of *Pyura mirabilis* and *Styela clava* were also stained with variety. Most granules became deep blue, but a few were faint blue or yellow. In *Pyura mirabilis*, the granules of small granular amoeboid cells were stained just as those of large granular amoeboid cells. In *Styela clava*, the cytoplasm of small granular amoeboid cells was colored faint blue, but near the nucleus, it was strongly dyed purplish blue. In *Halocynthia roretzi*, the cytoplasm of fine granular amoeboid cells looked a faint blue. Near the nucleus the coloration of the cytoplasm was a little stronger than at the periphery. The cytoplasm of large basophylic cells became faint blue, and its granules were dyed reddish purple and blue purple. The data are summarized in Table 5.

The other cells such as lymphocytes, vacuolated cells and minute granular cells gave no detectable color by this method.

3) *Fixation with OsO_4*

Fixation with osmium tetroxide resulted in immediate blackening of large granular cells and vesicular cells in *Halocynthia roretzi* and *Styela clava*. The intensity of blackening of the granules varied from one granule to another. The minute granules colored strong black were often seen in the large granules or vesicles. The vesicular cells of *Pyura mirabilis* were not blacked by OsO_4 at all. In large granular amoeboid cells of same species, the granules were blacked strongly and did not show variation.

Fine granular amoeboid cells of *Halocynthia roretzi* did not became black, except that the minute granules near the nucleus often became black.

The small granular amoeboid cells were also blackened by OsO_4, but the intensity was not so strong as those of large granular amoeboid cells in *Pyura*

Table 5.
$HgCl_2$-BPB staining of coelomic cell

	Pyrua mirabilis	*Halocynthia roretzi*	*Styela clava*
vesicular cell	not stain		—
large granular amoeboid cell	granules; most, blue few, faint blue or yellow	*granules or vesicles, most, blue few, greenish or yellow	granules; faint blue or yellow
small granular amoeboid cell	granules; most, blue few, faint blue or yellow	—	cytoplasm; faint blue, purplish blue (near the nucleus)
fine granular amoeboid cell	—	cytoplasm; faint blue, (near the nucleus)	—
large basophylic cell	—	cytoplasm; faint blue, granules; reddish purple and blue purple	—

$HgCl_2$-BPB staining of coelomic cells after formaldehyde fixation. *In *Halocynthia roretzi*, it is difficult to discriminate vesicular cells from large granular amoeboid cells.

mirabilis. In some cases, minute black granules were found in the cytoplasm of the small granular amoeboid cells. In *Styela clava*, two cell types, as for smaller cells, were distinguished after fixation with OsO_4. One of them possessed small granules with less intense blackening than the large granules in large granular amoeboid cells. These cells are similar to the small granular amoeboid cells in *Pyura mirabilis*. The other cells did not became black except for one or two minute granules near the nucleus. These cells are similar to fine granular amoeboid cells in *Halocynthia roretzi*.

Discussion

There are ten kinds of coelomic cells observed in five Japanese ascidians. These cells are classified into five groups as shown in Table 1.

The first group contains vesicular cells and large granular amoeboid cells. These two types of cells are characterized by the possession of fluid-filled globules. The globules of both kind of cells are similar in character. They are vitally-stained by neutral red, toluidine blue and Nile blue, scarcely dyed by Giemsa after fixation, blackened by OsO_4, colored blue by $HgCl_2$-BPB and are PAS-positive.

The vesicular cell of *Pyura mirabilis* is exceptional because the glouble is not blackened by OsO_4 or colored by PAS-reagents. The vesicular cells are labile and have a tendency to burst. These exceptional properties are considered to be due to the failure in fixation of the globule.

Both types of the cells exibit active amoeboid movement. They show phagocytosis.

Small granular amoeboid cells and fine granular amoeboid cells are classified in the second group. They have typical properties of phagocyte. The fine granular amoeboid cells are peculiar to the genus *Halocynthia*. These cells have characteristic fine granules near the nucleus which are stained red by Giemsa. They move very rapidly and immediately attack foreign materials.

It is difficult to classify the small granular amoeboid cells. On the one hand, the properties of small granular amoeboid cells such as active amoeboid movement, strong phagocytosis, and possession of granules near the nucleus are similar to those of fine granular amoeboid cells. On the other hand, the small granules are rather similar to those of the large granular amoeboid cells rather than to those of fine granular amoeboid cells under phase contrast microscopy.

Judging from the stainability by $HgCl_2$-BPB reagents, small granular amoeboid cells of *Pyura mirabilis* are similar to large granular amoeboid cells. Those of *Styela clava* are stained weakly by $HgCl_2$-BPB just as fine granular amoeboid cells of *Halocynthia roretzi*.

Small granular amoeboid cells of *Styela clava* can be divided into two subgroups on the basis of OsO_4 fixation. One type are cells which have small black granules like fine granular amoeboid cells of *Halocynthia roretzi*. The other cells have granules which are similar to those of large granular amoeboid cells.

Considering the facts above mentioned, small granular amoeboid cells are heterogeneous. They are a mixture consisting of both the cells related to large granular amoeboid cells and cells related to fine granular amoeboid cells (phagocyte).

The ascidians which have small granular amoeboid cells, (that is *Pyura mirabilis*, *Styela clava* and *Styela plicata*) do not have fine granular amoeboid cells and *vice versa*. It is a wonder that the cell kind which is abundant and plays such an important role in one ascidian is not found in the other ascidians. So it is natural to say that the small granular amoeboid cells contain phagocyte-like fine granular amoeboid cells. The small granular amoeboid cells also contain intermediate cells which develop from stem cells to large granular amoeboid cells.

The third group contains minute granular cells and vacuolated cells. Both cells are thought to originate from other types of cells. The vacuolated cells of *Styela plicata* are observed to originate from large granular amoeboid cells through bursting of the granules when the animals are cultured under starvation. George (1939) suggested that the cells of the vanadocyte type act as nutritive cells for the tissue. The present investigation seems in accord with this suggestion, but under conditions of starvation, bacteria increase in the coelomic fluid. So the possibility is not excluded that the cells release an anti-bacterial substance.

Minute granular cells are frequently observed when the cells are cultured *in vitro*. But the problem remains to be solved about where the cells originate.

The fourth group contains large basophilic cells, acidophilic granular cells, and brown or orange cells. Although they occupy a small percentage among

coelomic cells, they are conspicuous from their characteristic appearance. The roles of these cells are unknown.

The fifth group is lymphocytes. The cells are considered as primitive cells by other investigators (GEORGE, 1939; ENDEAN, 1960), because they are similar to mammalian lymphocyte cells, but the evidence is insufficient.

OHUYE (1936) also investigated the blood cells of Japanese ascidians. He said that staining after fixation with Giemsa etc., usually resulted in failure because of the high concentration of salt in the blood. So it is difficult to compare directly the results of this paper to those of OHUYE. The large granular amoeboid cells of *Styela clava* are thought to be "finely granular amoeboid cell" named by Ohuye, but he said that there are "green cells" in addition to "finely granular amoeboid cells". On the other hand, GEORGE (1939) named the large granular amoeboid cells as "green cells", and he did not find naturally colored "green cells" in the coelomic cells of *Styela clava*.

The observation for *Halocynthia roretzi* are also different from those of Ohuye. He reported that the "colorless" morula cell" is abundant in the blood. The form of the cells are like those of vesicular cells after fixation. But the "colorless morula cell" was reported not to be stained by neutral red by Ohuye. The vesicular cells are stained by neutral red.

ENDEAN (1960) reported blood cells of *Phallusia mammillata* in detail. The author carried out the cell staining with $HgCl_2$-BPB, PAS-reagents and fixation with OsO_4 before the coelomic cells were compared to the results of ENDEAN. The large granular cells are thought to be vanadocytes stated by Endean because the cells are blackened by osmic acid, stained yellow or blue, and stained vitally by neutral red. GEORGE called these cells "green cells". ENDEAN said that the strong reducing properties of the chromogen would account for the blackening of the vanadocyte in the presence of OsO_4. He said also that the vanadocyte have PAS-positive materials which are considered mucopolysacchride and the role of the cells may be production of the precursor of the tunicin of the test. The large granular cells of Japanese ascidians have the same properties as "vanadocyte", but the physiological roles remain to be solved.

Vesicular cells are in accord with signet ring cells from their form and stainability. From their possession of acid granules near the nucleus and exibition of active phagocytosis, the fine granular amoeboid cells and small granular amoeboid cells are thought to be the phagocytes described by ENDEAN (1960). The classification of the coelomic cells is as yet an arbitrary one. It is still necessary to carry out studies on the development and physiological role of the coelomic cells of ascidians.

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