Characterization of Coelomocytes of the Ascidian Halocynthia roretzi Based on Phase-contrast, Time-lapse Video and Scanning Electron Microscopic Observations

Authors: Dan-Sohkawa, Marina, Morimoto, Mayumi, Mishima, Hideki, and Kaneko, Hiroyuki

Source: Zoological Science, 12(3) : 289-301

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.12.289

ZOOLOGICAL SCIENCE 12: 289–301 (1995)

Characterization of Coelomocytes of the Ascidian *Halocynthia roretzi* Based on Phase-contrast, Time-lapse Video and Scanning Electron Microscopic Observations

MARINA DAN-SOHKAWA[1], MAYUMI MORIMOTO[2], HIDEKI MISHIMA[1] and HIROYUKI KANEKO[1]

[1]*Department of Biology, Faculty of Science, Osaka City University, Sughimoto-cho, Sumiyoshi-ku, Osaka 558, and* [2]*Biohistory Research Hall, Murasaki-cho, Takatsuki 569, Osaka, Japan*

ABSTRACT—Coelomocytes (blood cells) of the solitary ascidian *Halocynthia roretzi* were observed in culture by phase-contrast, time-lapse video and scanning electron microscopy. Seven types and one sub-type of cells were distinguished with respect to their morphology and behavior. These cell types are discussed in relation to those appearing in the literature. A table of correlation between cells reported under various names in the literature is presented in the Appendix.

INTRODUCTION

The Japanese solitary ascidian *Halocynthia roretzi* has become one of the most popular materials for studying invertebrate defense mechanisms. Its large body size (height and width of about 12 and 7 cm, respectively) and ease of availability (see Materials and Methods) have made it an excellent and handy material for investigation.

Coelomocytes, or blood cells, of *H. roretzi* were first characterized and classified by Fuke [7]. Along with reports that followed, some ten cell types have been recognized in the coelomic fluid of *H. roretzi* based on phase-contrast and staining properties [7, 10] and ultrastructural features [1, 9, 10].

Behaviorally speaking, some of these cells are reported to show active amoeboid movement [7, 11] and/or phagocytotic activity [7, 11] and/or to form syncytia [5, 11]. The most abundant cell type, the vacuolated cell, can recognize coelomocytes of "non-self" origin and discharge the inclusions of their vacuoles [8]. Substances released during this "contact reaction" are considered to include LPS-binding hemagglutinin and antimicrobial halocyamines [1, 2].

Another cell type, the phago-amoebocyte, is reported to encapsulate foreign living objects, such as fragments of tunic [10] and aggregates of starfish phagocytes [4]. Furthermore, some chemoattractant(s) may be released from phago-amoebocytes not only to attract its own cell type but also vacuolated cells and giant cells [4].

This study was conducted to further characterize the morphological and behavioral features of the coelomocytes of *H. roretzi*. Coelomocytes in culture were observed by phase-contrast, time-lapse video microscopy (VM) and scanning electron microscopy (SEM).

Accepted March 13, 1995
Received January 17, 1995

MATERIALS AND METHODS

Material

H. roretzi was a kind gift from the fisheries people of Mutsu Bay and from the Asamushi Marine Biological Station of Tohoku University. The individual tunicates were packed in a plastic bag with sea water and oxygen at the Asamushi Marine Station, sent by air transport in a cooling box with ice, kept in a laboratory aquarium (14°C) and used within a month.

Harvesting coelomocytes

Coelomocytes were obtained directly from the coelomic cavity of the papilla with a syringe and a needle. Coelomic fluid, 0.5 ml, was drawn into a syringe which contained 0.5 ml of Ca^{2+}, Mg^{2+}-free Jamarin (CMfJ: Jamarin Laboratory, Osaka) supplemented with 1 mM of ethylenediamine-tetraacetic acid (EDTA).

Coelomocyte culturing

Specially devised culture dishes [6] were used to raise the resolution of phase-contrast and VM observations. In brief, a square opening of 17×17 mm^2 was made on the bottom of a 3.5 cm Falcon culture dish. Vaseline grease was placed along the rims of this opening and a coverslip sized 24×24 mm^2 was placed over it. 1.5 ml of millipore-filtered (pore size 0.45 mm) Jamarin U supplement with 50 U/ml of penicillin and 50 mg/ml of streptomycin was placed on the surface of the coverslip, into which harvested coelomic fluid was dropped. Cell density was adjusted by the number of drops to be placed in the medium. The medium was changed once after 20 min of incubation to remove unattached cells. Cells were cultured at 20°C for 1.5 to 4 hr, if not otherwise stated, before being observed.

For SEM observations, coelomocytes were inoculated on Thermanox coverslips (13 mm in diameter, Sanko Junyaku, Tokyo) which were placed on the bottom of the culture dish.

Time-lapse VM

Coelomocyte movement was recorded at one shot per 2 sec, using a time-lapse video cassette recorder (Victor BR-9000) connected to an inverted phase-contrast microscope (Nikon Diaphot-

TMD) with a microscopic video camera (Nikon DK-3001).

Preparation for SEM observations

Coelomocytes were cultured on Thermanox cover slips for 1.5 to 4 hrs, washed once with Jamarin U, fixed serially with 2% OsO_4 in Jamarin U, 2% tannic acid in distilled water (D.W.) and 2% OsO_4 in D.W. for 10, 15 and 15 min, respectively. They were run through the alcohol series and treated for 20 sec with 100% isoamyl acetate. They were quickly transferred to a Hitachi HCP-2 critical point dryer to dry and coated with gold-palladium for 500 nm with Polaron SEM coating system. They were observed with a Hitachi S-800H scanning electron microscope.

RESULTS

Vacuolated cell

This is the cell type most abundant in the coelomic fluid of *H. roretzi* [7]. When placed on the cultural substratum and observed by phase-contrast optics, vacuolated cells are bright, phase-refringent bodies bearing an irregular shape (Figs. 1 and 7 a, v).

When observed by SEM, vacuolated cells are usually composed of three portions, "head", "body" and "tail". The head (Fig. 2, H) is a mass of cytoplasm bulging out from the central, vacuolated portion (body: Fig. 2, B). The leading front of the head is often formed into a small lamellipodium, by which the cell adheres to the substratum (Fig. 2 a, b, c: arrowheads). The cell surface of the head is rougher than the body with relatively even-pitched undulations. The body has a smooth, bulbous cell surface reflecting the presence of large cytoplasmic vacuoles within. The tail (Fig. 2, T) is a smaller mass of cytoplasm than the head usually bearing a small, trailing pseudopodium (Fig. 2, c, d: arrows). The surface features of the tail resemble those of the head.

Vacuolated cells look as if they are attached to the substratum only by their head and tail (Fig. 2 a, c). They often lift their body up from the substratum (Fig. 2 b, d). Fibrous material of unknown nature is sometimes seen to stretch between various parts of the cell surface and the substratum (Fig. 2 a, b: arrows).

Vacuolated cells move constantly about the cultural substratum in an inchworm fashion (Fig. 3). They project and withdraw their heads freely. They draw it back into the body whenever they wish to turn and form it anew in the direction they will migrate (Fig. 3, frames 2–3, 10–11). The average speed of migration is 17.4 μm/min.

Fibroblastic cell

Fibroblastic cells are rod-shaped cells, tightly adhering to the substratum when observed under phase-contrast optics (Fig. 1, F and inset). The only type of movement they show is to stretch on the substratum. In a longer time span, however, they migrate slowly to form small clusters of cells adhering laterally with one another (Fig. 1, inset).

Fibroblastic cells lie on a thick, fibrous extracellular matrix (ECM) when observed by SEM (Fig. 4 a, arrow). They seem to secrete ECM component(s) from their proces-

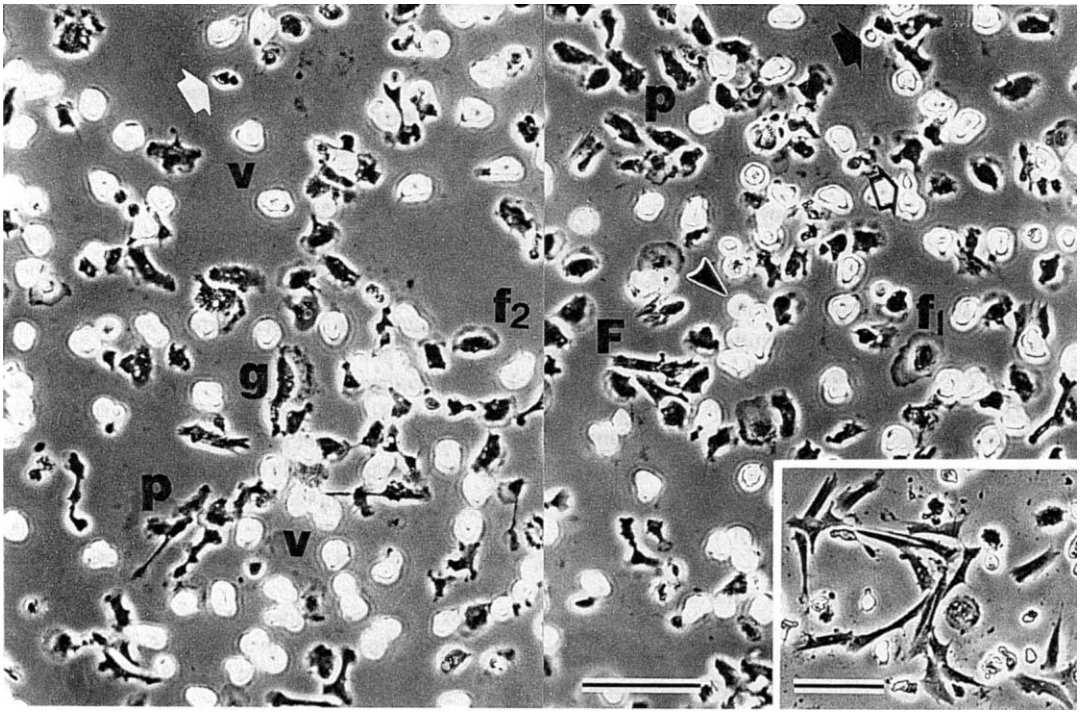

FIG. 1. Phase-contrast micrographs of a mixed population of the coelomocytes of *H. roretzi* 1.5 h in culture. Inset: a cluster of laterally adhering fibroblastic cells, 21 h in culture. F: fibroblastic cell, f1: fusogenic phagocyte in a steady state, f2: migrating fusogenic phagocyte, g: giant cell, p: phago-amoebocyte, v: vacuolated cell, filled black arrow: spherical, motionless lymphoid cell, open arrow: mono- or bipolar-lymphoid cell, white arrow: actively migrating lymphoid cell, arrowhead: macrogranular cell. Bars, 50 μm.

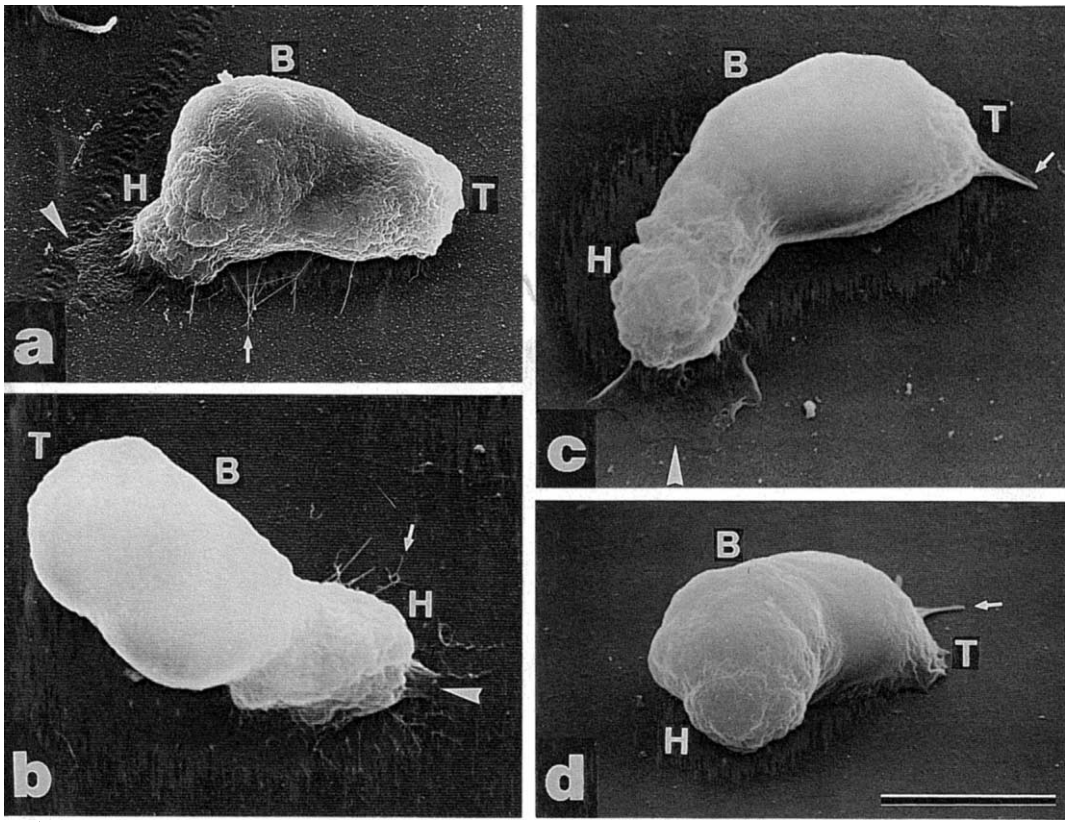

FIG. 2. Scanning electron micrographs of vacuolated cells. Cells of this type are composed of three portions, head (H), body (B) and tail (T). The head and the tail are freely projected and withdrawn for cell migration. Arrowheads: leading lamellipodia, arrows: (a, b) fibrous material of unknown nature, (c, d) trailing cytoplasm of the tail. Bar, 5 μm.

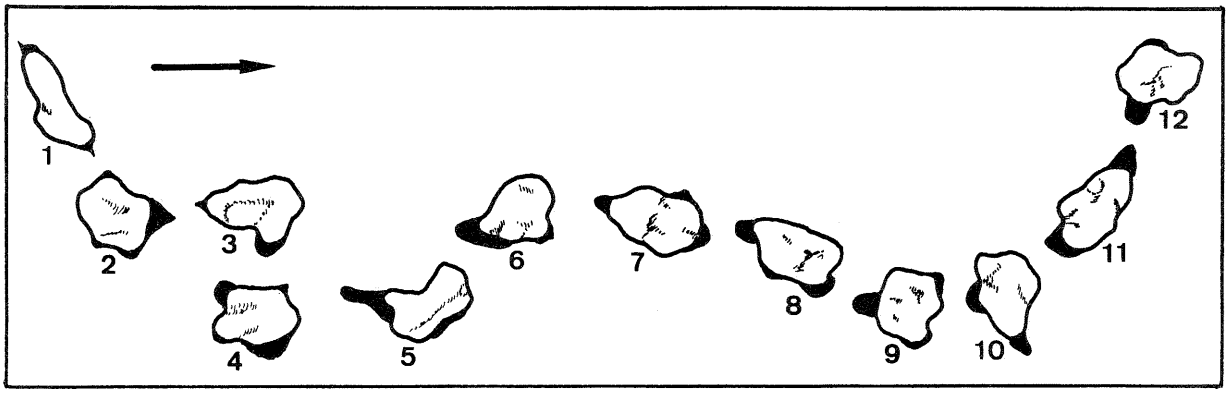

FIG. 3. Inchworm walk of a vacuolated cell. A serial tracing of a migrating cell under VM at 1-min intervals. The distance between each frame is arbitrary. Arrow : the direction of migration.

ses (Fig. 4 b: arrow). The parallel arrangement of the fibrous ECM along the long axes of the cells suggests that, while stretching gradually, the cells move back and forth on the newly laid ECM, adding more to the ends as they reach there, and eventually stretch fully as shown in Figure 1.

Phago-amoebocyte

Under phase-contrast optics, phago-amoebocytes are recognized as a group of dark cells highly variable in shape (Fig. 1, p). This variability can be well explained when they are observed under VM. As shown in Figure 5, they change their shape vigorously as they swiftly migrate over the cultural substratum. The average speed of movement is 33.0 μm/min.

SEM reveals these cells as having a bulky mass of cytoplasm with one or more leading lamellipodia (Fig. 6: arrowheads; also see Fig. 10, d). Small portions of the cytoplasm are usually seen to trail on the opposite side (Fig.

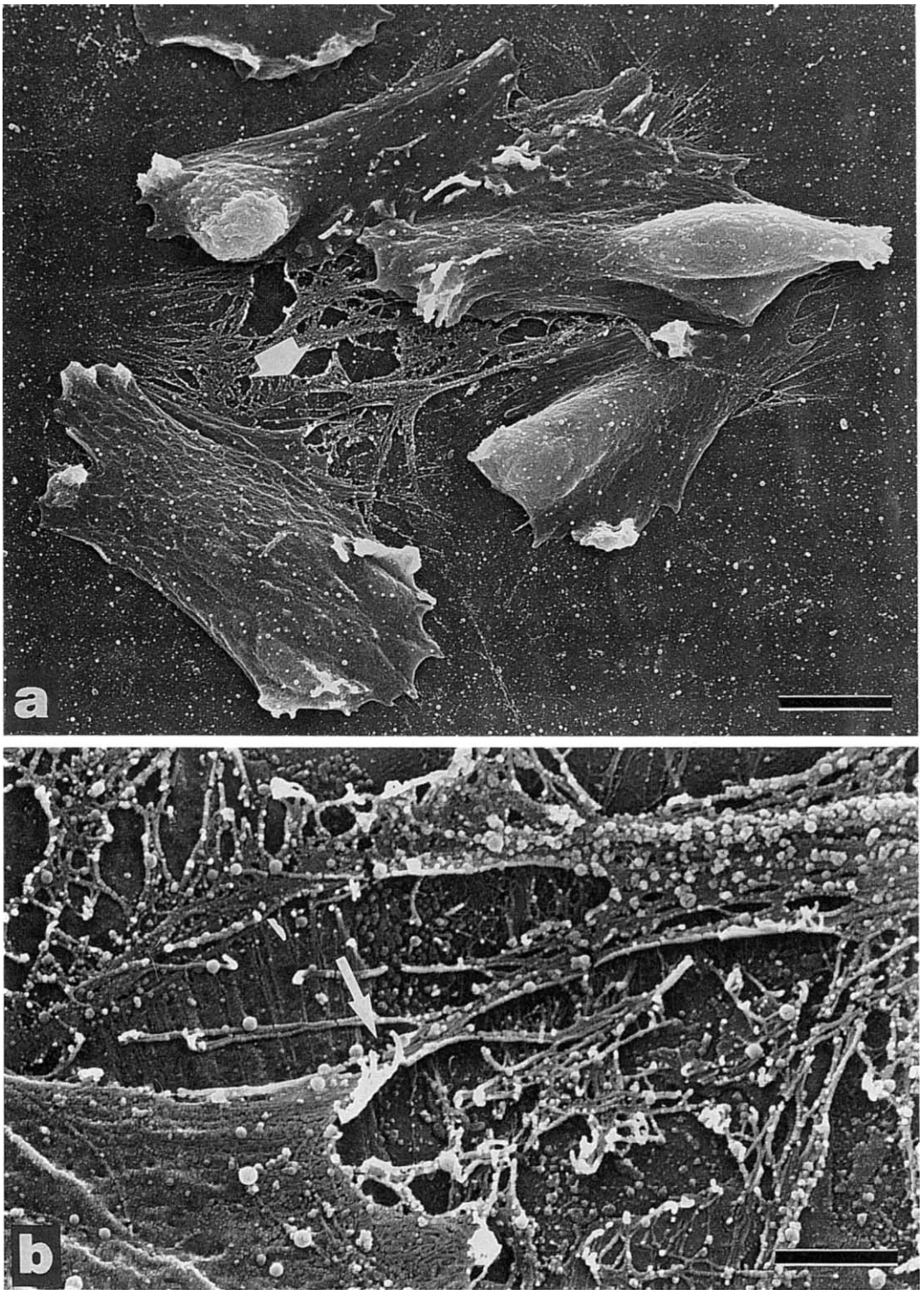

FIG. 4. Scanning electron micrographs of a cluster of fibroblastic cells. a: Four cells are in the process of spreading over the substratum. b: Higher magnification of the part of (a) indicated by the arrow. Fibrous component(s) of the ECM is secreted from a cell process (arrow). Bars, a: 5 μm, b: 1 μm.

6a: arrow) or on the portions of cell surfaces which seem to be in the process of withdrawing (Fig. 6b-d: arrows). The cell surface is slightly smoother than that of the head and tail portions of the vacuolated cell (Fig. 2).

Fusogenic phagocyte

Fusogenic phagocytes spread out flatly over the substratum with a broad, circumferential lamellpodium surrounding the bulk cytoplasm containing a nucleus and numerous vesi-

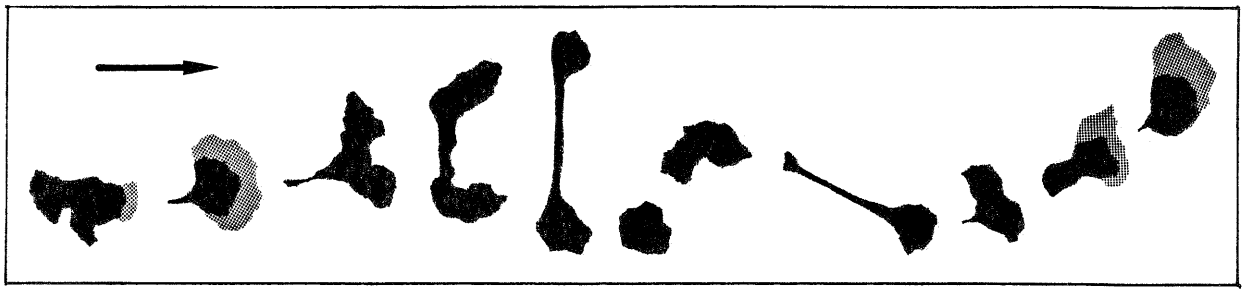

FIG. 5. Vigorous amoeboid movement of a migrating phago-amoebocyte. A serial tracing of a cell under VM. Other conditions, same as Fig. 3.

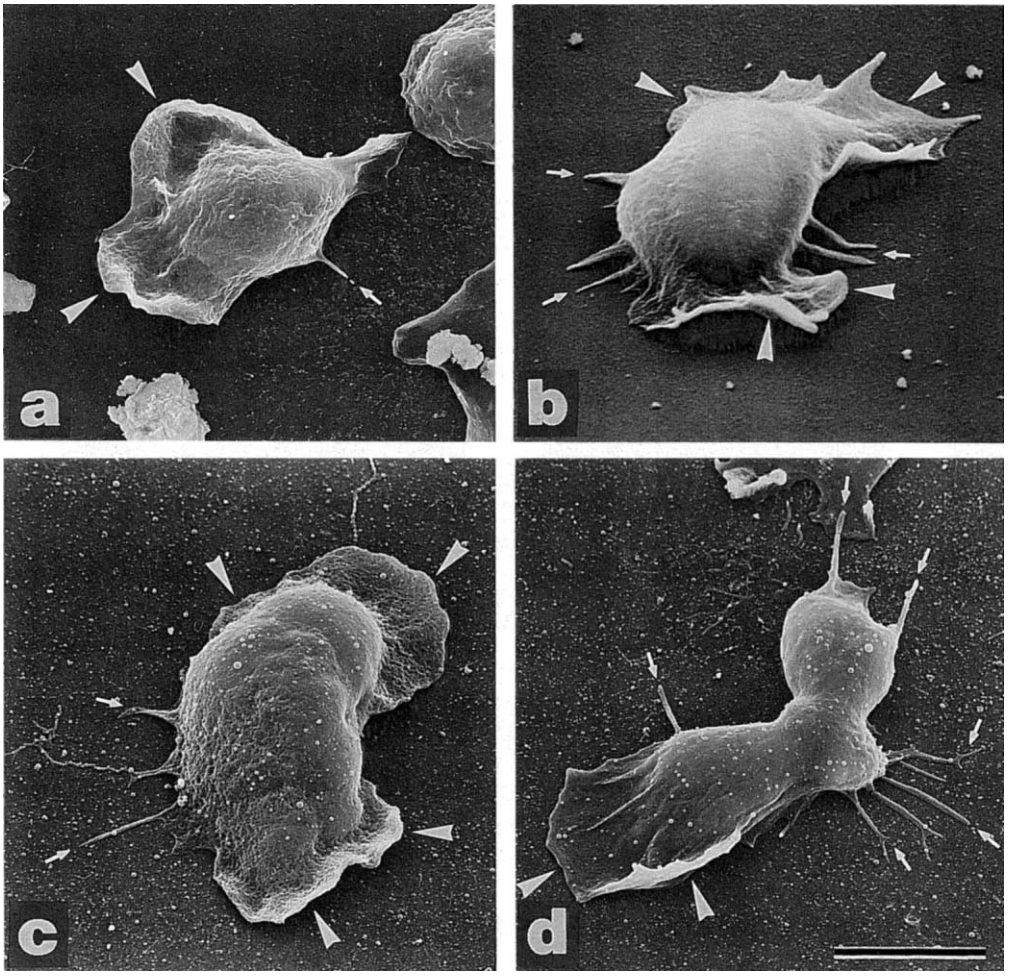

FIG. 6. Scanning electron micrographs of phago-amoebocytes. Arrowheads: lamellipodia formed in the direction of migration, arrows: trailing cytoplasm. Cells shown in b and c are considered to be in a state immediately before elongating in the perpendicular direction of the photograph. Bar, 5 μm.

cles (Fig. 1, f1, f2). They are disc-shaped when they are not migrating as observed under both phase-contrast optics and SEM (Figs. 1 and 11, f1 and Fig. 7c). They have a relatively smooth cell surface. They become crescent-shaped with a broad, leading lamellipodium when they are migrating (Figs. 1 and 7a, f2 and Fig. 7b).

Under certain conditions, fusogenic phagocytes form syncytia of various sizes (Fig. 7a: arrow). Although the exact condition of their formation is not known, they seem to arise from within large aggregates in the later phase of culture.

When observed under VM, fusogenic phagocytes glide over the substratum with an even pace and with a slight widening and narrowing motion (Fig. 8). The average speed of their migration is 10.8 μm/min.

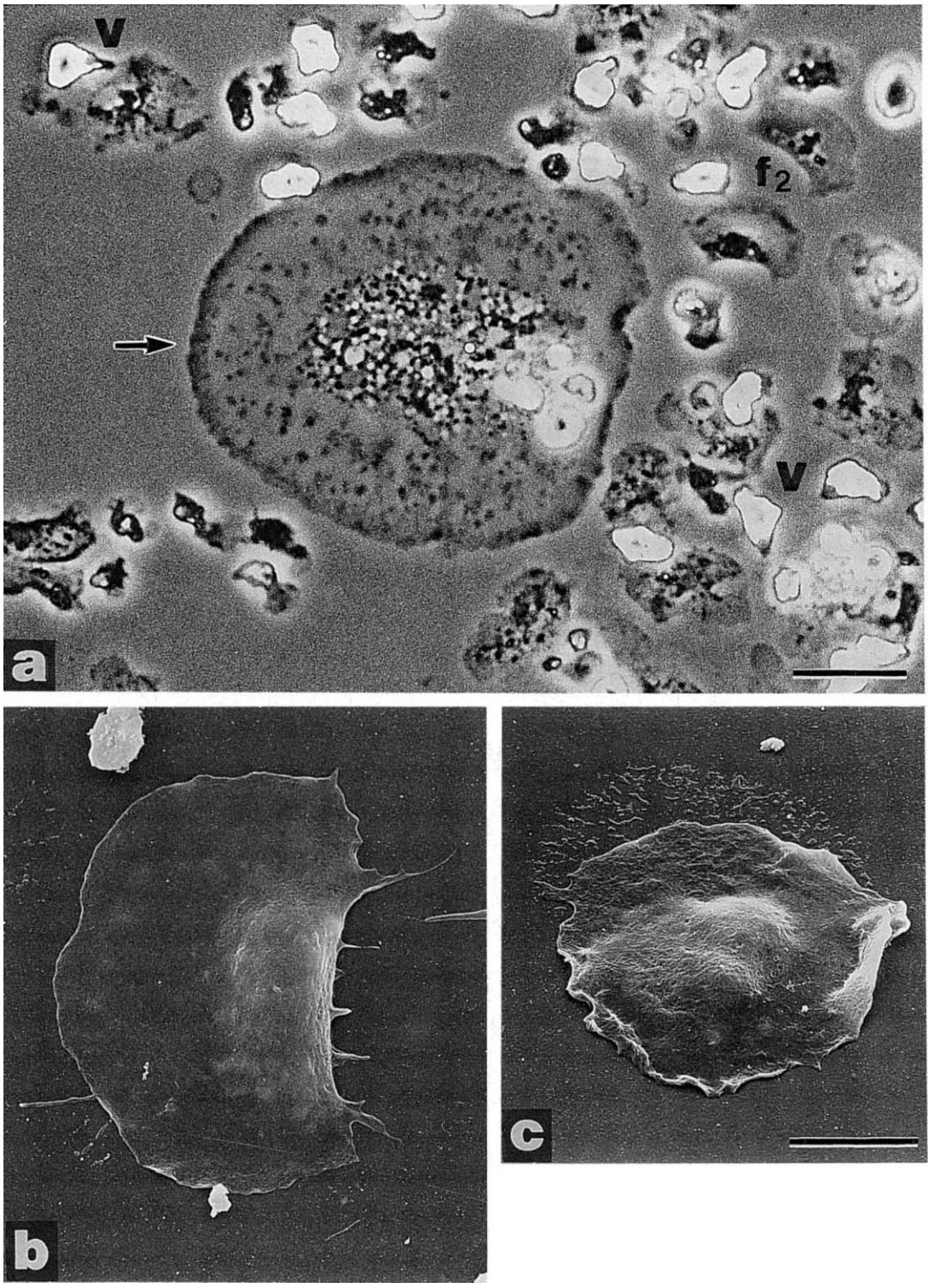

FIG. 7. Phase-contrast (a) and scanning electron micrographs (b, c) of fusogenic phagocytes. a: A large syncytium of fusogenic phagocytes (arrow) at 50 hr of culture. For symbols, see Fig. 1. b: A migrating fusogenic phagocyte. The cell is moving toward the left of the photograph. c: The same cell type in the steady state. Bars, a: 20 μm, b, c: 5 μm.

Giant cell

Giant cells are the largest among the coelomocytes of *H. roretzi* besides the problematical viriform cells (see Discussion). Giant cells usually expand to a width of 20~30 μm or more when they are gliding over the substratum (Fig. 1, g). They resemble fusogenic phagocytes in taking a crescent shape when they are migrating (Fig. 1, f2), but they are much larger and possess rougher lamellipodia. The cytoplasm

usually contains a few light and numerous dark vesicles.

The difference between giant cells and fusogenic phagocytes is unquestionable when they are compared under SEM. In contrast with the smooth cell surface of the fusogenic phagocytes (Fig. 7b, c), that of giant cells is much coarser with microvilli protruding from the cytoplasmic portion (Fig.

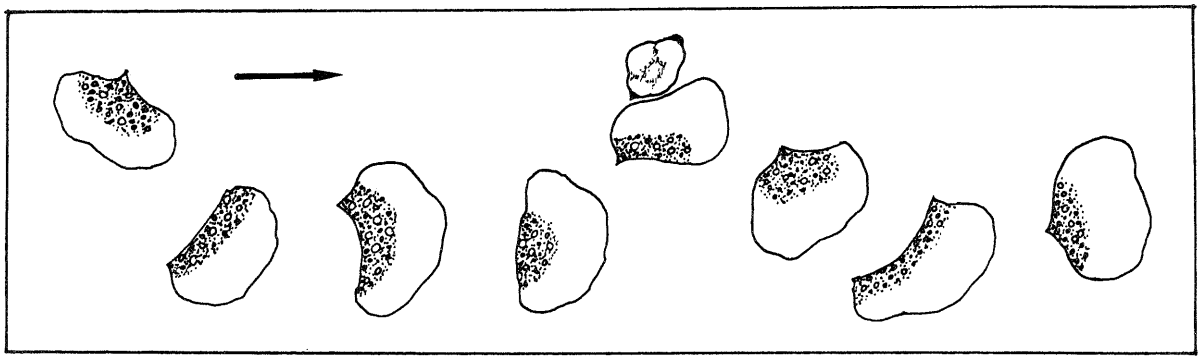

FIG. 8. Gliding movement of a fusogenic phagocyte. A serial tracing of a VM field. Conditions, same as Fig. 3. This particular cell encounters a vacuolated cell in the fifth frame and changes its direction of migration.

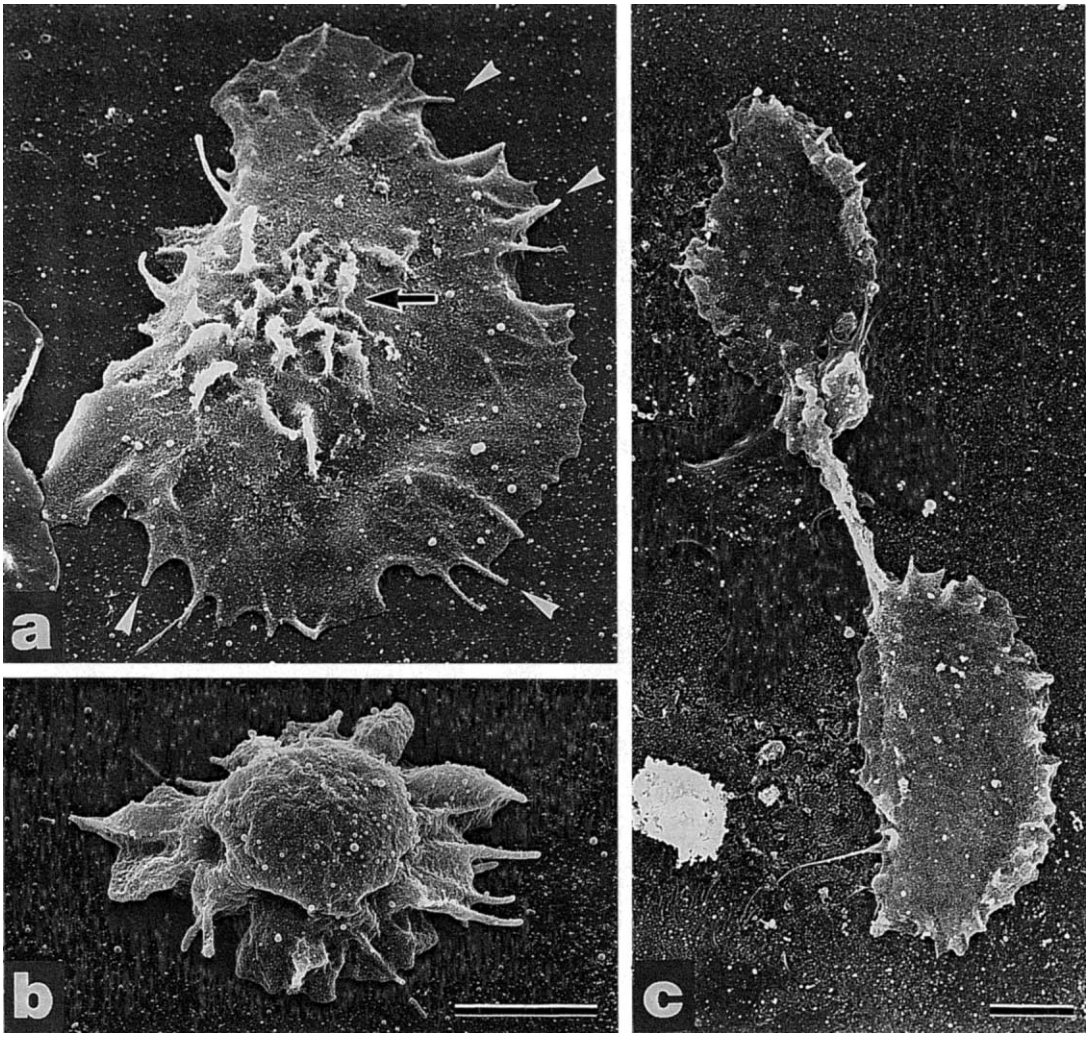

FIG. 9. Scanning electron micrographs of giant cells. a: A giant cell in migration. Arrow: cell processes, arrowheads: rod-like projections along which cytoplasm is considered to be carried. b: A giant cell immediately before transforming into the migrating form. c: A giant cell which has split its cytoplasm to "step over" a deformed vacuolated cell. This cell and that in (a) are migrating toward the right side of the photograph. Bars, 5 μm.

9a: arrow). The rim of the lamellipodium is also more irregular with numerous rod-shaped protrusions of what seem to be cytoskeletal bundles (Fig. 9a: arrowheads).

Giant cells stay rounded on the substratum for a longer period of time after inoculation as compared to phago-amoebocytes and fusogenic phagocytes. When they are ready to migrate, they start to project and withdraw their lamellipodium actively in all directions (Fig. 9b), then after a

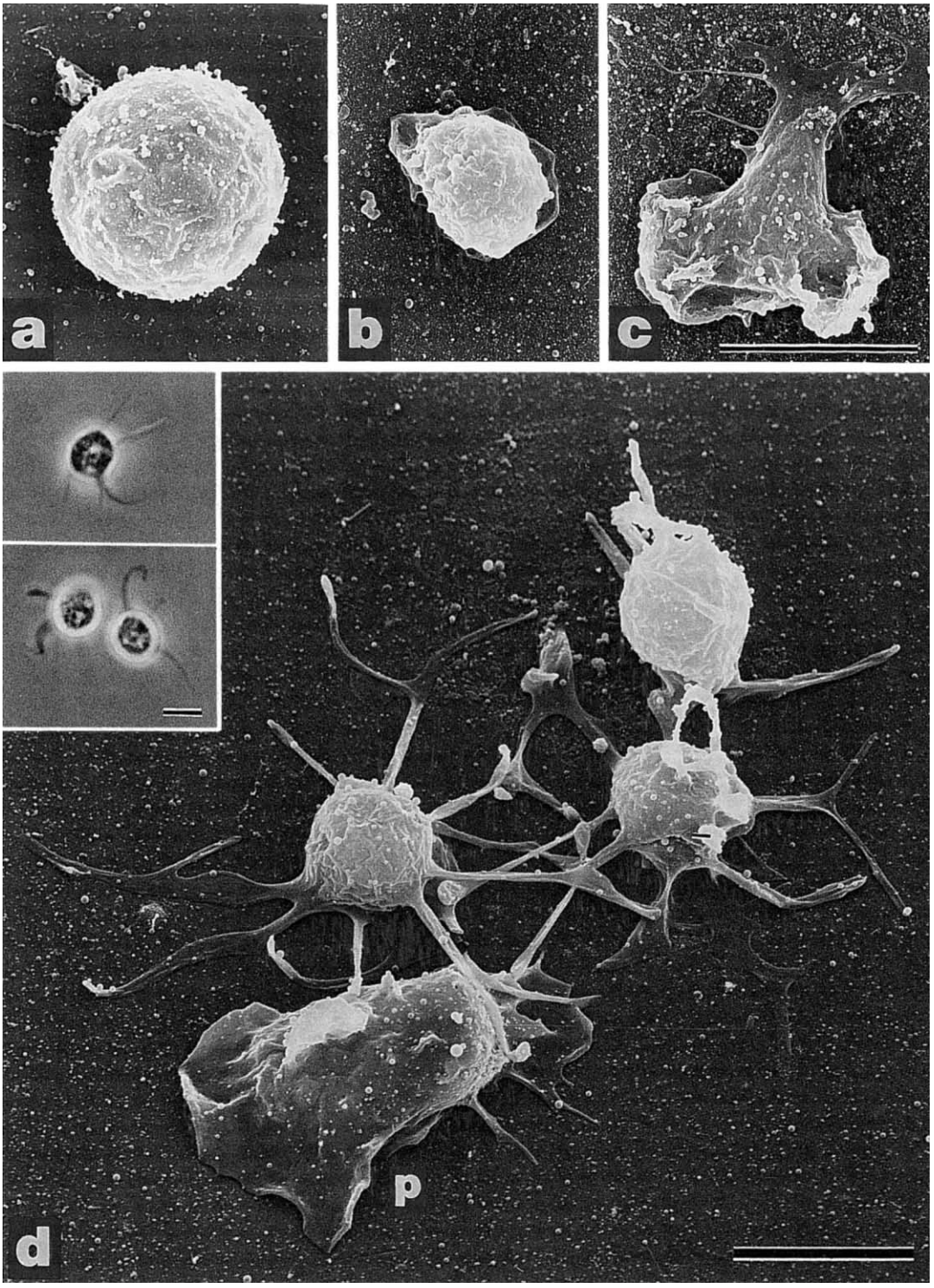

FIG. 10. Phase-contrast and scanning electron micrographs of lymphoid cells. a: A spherical and motionless lymphoid cell. b: A lymphoid cell probably in the process of transforming into the migrating form. c: A lymphoid cell in the actively migrating state. This cell is migrating toward the bottom of the photograph. d: Three lymphoid cells forming a cluster in company with a phago-amoebocyte (p). Cells in a similar state as seen under phase-contrast microscopy are shown in the inset. Bars, 5 μm.

short while, they assume the crescent shape within a few seconds (Fig. 9b→a). The same rough microvilli shown in Figure 9 a (arrow) are found sometimes on the surface of the giant cells in this state. Migrating giant cells often split their body in two to "step over" other cells (Fig. 9 c). They glide with an even pace as do fusogenic phagocytes although more vigorously, with a similar widening and narrowing motion. The speed of migration, however, is much greater than that of fusogenic phagocytes. It reaches the speed of the phago-amoebocyte, i.e. an average of 33.0 μm/min.

Lymphoid cell

Lymphoid cells are the smallest of the coelomocytes of *H. roretzi*. They take three different states under culture when observed by phase-contrast optics. They can be spherical and motionless (Fig. 1, filled black arrow) or migrate slowly in mono- or bipolar shapes (Fig. 1, open arrow). When they are migrating swiftly, they change their shape constantly as the result of vigorous amoeboid movement (Fig. 1, white arrow). The average speed of migration in this state is 8.0 μm/min.

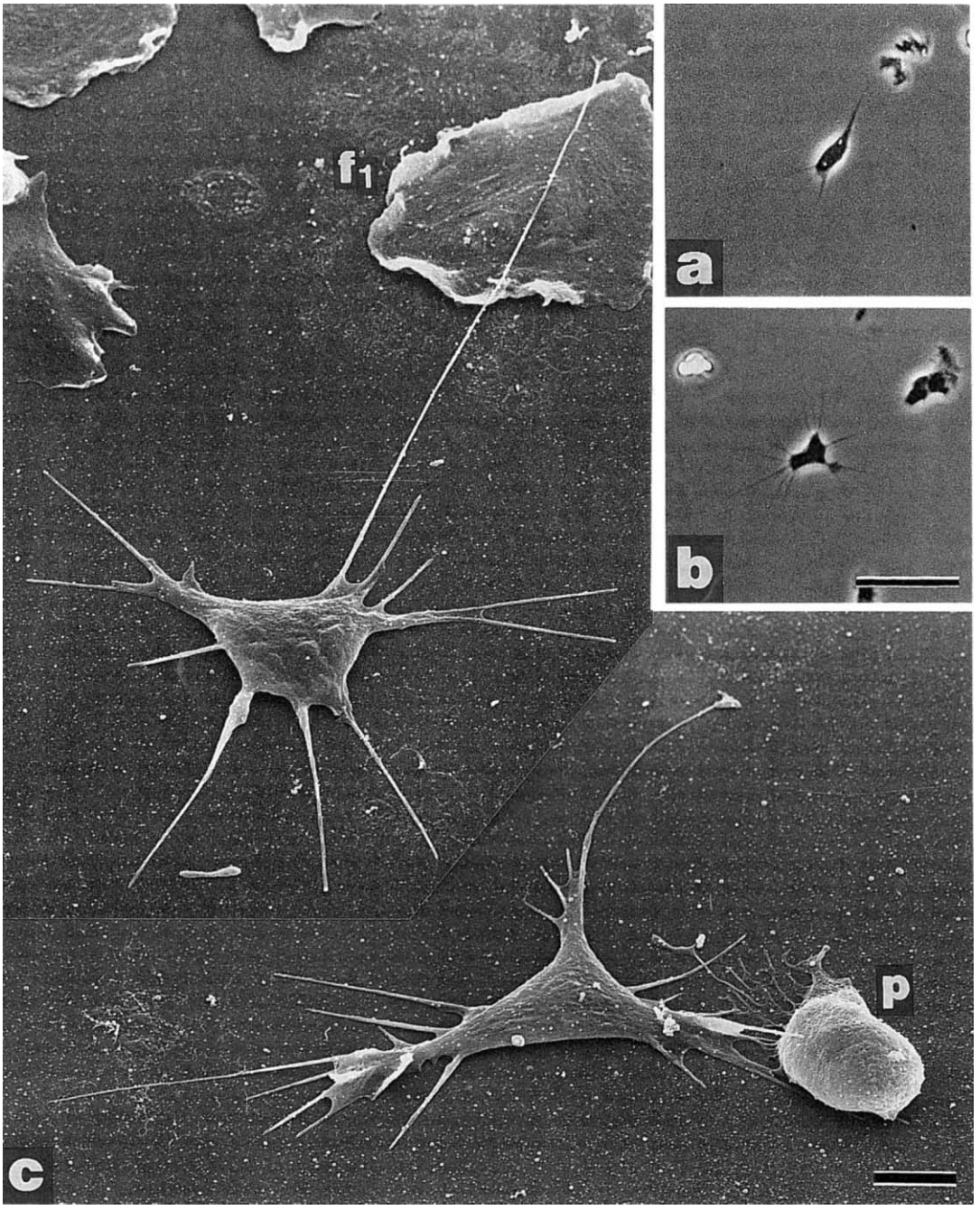

FIG. 11. Phase-contrast (a, b) and scanning electron micrographs (c) of multipolar cells. Symbols, same as Fig. 1. Bars: a, b 50 μm, c 5 μm.

SEM reveals various forms and numbers of pseudopodia accompanying the spherical and polar states of lymphoid cells (Fig. 10b, d). Such pseudopodia are also visible under high magnification with phase contrast (Fig. 10d, inset). Although lymphoid cells are usually scattered singly among other cell types (Fig. 1), small networks of lymphoid cells are often found at sites where small aggregates have dispersed (Fig. 10d). In the actively migrating state, lymphoid cells show a highly elastic feature, so that the cell body is indistinguishable from the broad ruffling membranes (Fig. 10c).

Multipolar cell

Multipolar cells are relatively large cells possessing several to about a dozen filopodia spreading out in all directions (Fig. 11). They were never observed to migrate or to form lamellipodia. The cell body was always in a single mass and the surface was smooth.

DISCUSSION

We have described seven types and one subtype of coelomocytes of *H. roretzi* as observed under phase-contrast microscopy, SEM and time-lapse VM. The naming of these cell types differ among investigators and should be standardized to facilitate further understanding of their structural and functional aspects. The Appendix presents a table correlating the different names given in the literature as agreed by the major authors. The cell types described in this report are discussed below in reference to those shown in the table.

Vacuolated cell

This cell type has been called the vesicular cell and the large granular amoeboid cell by Fuke [7], the vacuolated cell by Fuke and Fukumoto [9], v1, v3 and v4-cells by Sawada *et al.* [11], V1, V3, V4, V6 and V7 by Ohtake *et al.* [10], Type F hemocyte by Azumi *et al.* [1] and the morula cell by Dan-Sohkawa and Morimoto [5].

The most prominent structural feature of this cell type is the fluid-filled vesicles of various sizes and numbers [1, 7, 9–11] carried in the "body" portion of the cell (Fig. 2, B). This portion corresponds to the main, phase-refringent part of the cell body of vacuolated cells under phase-contrast optics (Figs. 1 and 7a, v). The "head" and "tail" portions, on the other hand, are formed, projected and withdrawn freely at will. These portions are seen under phase-contrast optics as phase-dark "feet" peeping out from underneath the body (Figs. 1, 3 and 7a).

The tri-partite body structure of vacuolated cells explains their inchworm walk as seen in VM (Fig. 3). After a cell chooses the direction of migration, it migrates first with its head, stretching its body as a result (Fig. 3, frames 4–5, 6–8, 10–11). The cell then draws its tail toward its head and rounds up (Fig. 3, frames 5–6, 8–9, 11–12).

Functionally speaking, vacuolated cells are reported to discharge the inclusion of their vacuoles when they encounter coelomocytes of a "non-self" origin [8]. The vacuoles are reported to contain antimicrobial substances and hemagglutinins [1, 2]. These cells *in vitro* were also shown to phagocytize sheep red blood cells [4].

Fibroblastic cell

This cell type corresponds to the minute granular cell [7], the granular amoebocyte [9], g1-cell [11], the large granular amoebocyte [10], Type B hemocyte [1] and the fibroblastic cell [5]. This is the first report, to our knowledge, to describe the ECM secreting activity of this cell type (Fig. 4).

Fibroblastic cells spread over the substratum much more slowly than cell types possessing active spreading tendencies, such as the phago-amoebocyte, the fusogenic phagocyte and the giant cell. For this reason, they are harder to find in the early phases of culture. They hardly migrate over the substratum, displaying only a slow, expanding motion. Over a longer time span, however, they show a tendency to form clusters of laterally adhering cells (Fig. 1, inset). This reluctance to move is considered to reflect their requirement for an ECM carpet for expansion and migration.

Fibroblastic cells are reported to aggregate around small pieces of tunic or gill pouch (of self or non-self origin) added to the coelomocyte suspension. This behavior is interpreted as their role in preventing bleeding [10]. The ECM secreting ability of these cells, as shown in this report, is considered to suggest an additional role in wound healing.

Such quickly aggregating ability [10] and the reported amoeboid movement in the early phase of culture [9] contrast sharply with the static feature of fibroblastic cells described in this report. The difference suggests the possibility that fibroblastic cells behave quite differently in the floating and the attached states. Further study is required to correlate these two states and the two different roles which have been assigned to this cell type.

Phago-amoebocyte

This cell type corresponds to the fine granular amoeboid cell [7], the hyaline amoebocyte [5, 9], p2-cell [11], the small granular amoebocyte [10] and Type C hemocyte [1]. This is the cell type most active not only in amoeboid movement (Fig. 5), but also in phagocytotic activity among the coelomocytes of *H. roretzi* [5, 7, 10, 11].

Phago-amoebocytes are reported to encapsulate foreign bodies such as pieces of tunic and large latex beads (27.4 μm in diameter) as well as aggregates of "self" fibroblastic cells [10]. Phago-amoebocytes are also the putative candidate for secretion of chemoattractant(s) during such an encapsulation reaction [4].

Fusogenic phagocyte

This cell type has been described as a p1-cell [11], the small granulated amoebocyte [10], Type A hemocyte and the granulated amoebocyte [5]. Cells of this type are characterized by an ability to form syncytia [5, 11]. The phagocytotic activity of this cell type will be reported elsewhere.

Interestingly, the fusogenic phagocytes were not recognized by Fuke [7], and Fuke and Fukumoto [9] and were not distinguished from the phago-amoebocytes by Ohtake *et al.* [10]. Since these studies were based on TEM observations, the oversight may reflect the incidental similarity of cytoplasmic features between the fusogenic phagocytes and the phago-amoebocytes, in spite of their difference in SEM (Figs. 6 and 7) and behavioral features (Figs. 5 and 8). Conversely, the two cell types may be two different states of a single cell type. This last possibility, however, seems unlikely for the following three reasons. Firstly, the difference in SEM features between the two cell types (Figs. 6 and 7) is too great to assume that they belong to a single cell type. Secondly, no conversion was ever observed under VM between these two cell types. Thirdly, no fusion event was observed among the phago-amoebocytes. Hence, we consider these two cell types to be truly different.

Giant cell

This cell type corresponds to the large basophilic cell [7, 9, 10], g2-cell [11], V5 [10], Type E hemocyte [1] and the giant cell [5]. Giant cells are reported to phagocytose erythrocytes [7] and to release a metallo-protease in response to various chemical stimuli [1, 3].

The rod-like processes projecting from the rim of the lamellipodium (Fig. 9a; arrowheads) of actively migrating giant cells are considered to be the leading cytoskeletal backbones, along which cytoplasmic components are quickly transported forward. Although such a mechanism of locomotion is purely speculative, the giant cells are likely to provide an excellent model system for investigating the relation between the cytoskeleton and cell locomotion. It is worth mentioning, from this line of consideration, that giant cells isolated by discontinuous density gradient centrifugation in a solution of bovine serum albumin [1] behave quite normally in culture.

The rough microvilli (Fig. 9a, arrow) on the other hand, are obviously of a different nature from the rod-like projections. They are expected to be related to the functional aspect of giant cells.

Lymphoid cell

This cell type has been called a lymphocyte [5, 7], the lymphocyte-like cell [9], Type D hemocyte [1] or the lymphoid cell [10, 11]. The name "lymphoid cell" was chosen after its small and spherical features. However, since there is no evidence indicating a homology between it and the mammalian lymphocyte, we feel that the name should be changed to a more adequate one as soon as its function has been elucidated.

Lymphoid cells are reported to release a metallo-protease under experimental conditions along with fusogenic phagocytes and giant cells [1, 3]. On the other hand, they are often found to form small networks in culture (Fig. 10d) in association with cell aggregates. Implications of these phenomena on their function *in vivo*, however, remain to be found in future studies.

We have classified small spherical cells (Fig. 1: filled black arrow and open arrow, Fig. 10a, b, d) and small amoeboid cells (Fig. 1: white arrow, Fig. 10c) as static and active forms, respectively, of a single cell type. Although there is no direct evidence to justify such classification, there are reasons to support this. Firstly, such a condition of the lymphoid cell as shown in Figure 10b is considered to suggest a transformation between the two forms. Secondly, the combined proportion of the two forms against other cell types is fairly constant throughout the culture, i. e., about a dozen in a field as shown in Figure 1. Furthermore, one or the other of the two forms often makes up the entire population under certain unidentified conditions.

The globular cell of Fuke and Fukumoto (see Fig. 5 of [9]) is considered to be identical with the spherical form of the lymphoid cell (cf. Fig. 10d, inset).

Multipolar cell

Multipolar cells have never been reported in literature to our knowledge. Although they are always found in culture, the frequency of their appearance is low ($<1\%$). They were never seen to migrate nor be seen in the process of expanding.

Viriform cell

This cell type has been called the large granular cell [9], g3-cell [11] and V8 [10]. This cell type requires a special comment, for we doubt its identity as a genuine *H. roretzi* cell. Viriform cells can be easily introduced into a culture by placing a piece of tunic in the culture dish containing the same medium used for culturing coelomocytes. When they are moving about, their body length and width reach those of migrating giant cells. However, viriform cells are much more massive than giant cells since they are never flattened as the latter. Viriform cells adhere to the substratum by a small organ on the tip of their body (see Fig. 9 b of [9]). They rush around the culture field with this organ at tremendous speed, paying no heed to the coelomocytes. In fact, we can easily see them moving around the substratum without the aid of time-lapse VM. From time to time, they abruptly stop moving, stand on their tips and let their bodies float. After remaining in this position for various intervals, they suddenly return to rushing about again.

These features of viriform cells suggest that they may be a protozoan symbiont which lives deep in the tunic of the ascidian, as mentioned by Fuke and Fukumoto [9]. However, further investigation is necessary before a conclusion is reached.

A comment on the macrogranular cell

We paid no particular attention to macrogranular cells (Fig. 1, arrowhead) in the present study, since we had an impression that they represent an unspread form of a known cell type. This impression comes from the fact that macrogranular cells are more abundant in the early phase of culture

and decrease with time. In order to verify this impression, we conducted a brief counting.

We chose fibroblastic cells as the most probable candidate for the spread counterpart of macrogranular cells. This is the cell type which not only appears relatively late in culture but also occupies a large enough proportion of the population to account for macrogranular cells, i. e., 8.3± 1.2% [9]. Round and elongated cells (Fig. 1 arrowhead and F, respectively) were counted at 20 min and 10 h of the same unwashed culture. The washing procedure at 20 min of culture (see Materials and Methods) was omitted for fear of disturbing the original proportion of cell types. The counting showed that at 20 min, macrogranular cells occupied about 10% of the population. No typically elongated fibroblastic cells were found. At 10 h, elongated fibroblastic cells occupied some 5% of the population while the proportion of macrogranular cells had decreased to about 5%. These values suggest that macrogranular cells are, indeed, the unspread form of fibroblastic cells. A more precise investigation, particularly at a fine structural level, should lead to a definite identification of macrogranular cells.

Although unattached cells were removed by washing after 20 min of incubation, hardly any cell type seems to be lost in our culture method. It is also worth mentioning that there were no great discrepancies in the proportion of cell types based on our method and that of Fuke [7]. These facts indicate that most types of coelomocytes of *H. roretzi* have equally strong power to adhere to the cultural substratum regardless whether they are in the spread or unspread state.

We did not compare coelomocytes of different ascidian species, because coelomocytes are so highly variable among different ascidian species that there is still confusion in the general understanding of them [9]. At present it is more important to better understand the individual cell types. Only then can there be a truly meaningful comparison of coelomocytes among different ascidian species.

ACKNOWLEDGMENTS

We are grateful to the fisheries people of Mutsu Bay for kindly offering the *H. roretzi*. Thanks are also due to the staff members of Asamushi Marine Biological Station for generously supplying, packing and sending the animals. This work was supported in part by a Grant-in-Aid of the Ministry of Education, Science and Culture of Japan (No. 04640668).

REFERENCES

1 Azumi K, Satoh N, Yokosawa H (1993) Functional and structural characterization of hemocytes of the solitary ascidian, *Halocynthia roretzi*. J Exp Zool 265: 309–316

2 Azumi K, Yokosawa H, Ishii S (1990) Halocyamins: Novel antimicrobial tetrapeptide-like substance isolated from the hemocytes of solitary ascidian, *Halocynthia roretzi*. Biochemistry 29: 159–165

3 Azumi K, Yokosawa H, Ishii S (1991) Lipopolysaccharide induces release of a metallo-protease from hemocyte of the ascidian, *Halocynthia roretzi*. Dev Comp Immunol 15: 1–7

4 Dan-Sohkawa M (1990) Video-microscopic analysis of the coelomocytes of *Halocynthia roretzi*. Zool Sci 7: 1163 (Abstract)

5 Dan-Sohkawa M, Morimoto M (1991) Coelomocytes of *Halocynthia roretzi* as observed by scanning electron microscopy. Zool Sci 8: 1161 (Abstract)

6 Dan-Sohkawa M, Suzuki J, Towa S, Kaneko H (1993) A comparative study on the fusogenic nature of echinoderm and nonechinoderm phagocytes in vitro. J Exp Zool 267: 67–75

7 Fuke TM (1979) Studies on the coelomic cells of some Japanese ascidians. Bull Mar Biol St Asamushi Tohoku Univ 16: 143–159

8 Fuke TM (1980) "Contact reactions" between xenogeneic or allogeneic coelomic cells of solitary ascidians. Biol Bull 158: 304–315

9 Fuke M, Fukumoto M (1993) Correlative fine structural, behavioral, and histochemical analysis of ascidian blood cells. Acta Zoologia (Stockholm) 74: 61–71

10 Ohtake S, Abe T, Shishikura F, Tanaka K (1994) The phagocytes in hemolymph of *Halocynthia roretzi* and their phagocytic activity. Zool Sci 11: 681–691

11 Sawada T, Fujikura Y, Tomonaga S, Fukumoto T (1991) Classification and characterization of ten hemocytes types in the tunicate *Halocynthia roretzi*. Zool Sci 8: 939–950

APPENDIX

A meeting was held at the Asamushi Marine Biological Station of Tohoku University in July, 1993, with the aim to standardize the nomenclature of different types of coelomocytes (blood cells, hemocytes) of *Halocynthia roretzi*. Participant investigators of the meeting were Masako Fuke, Tomoo Sawada, Shin-ichi Ohtake and Marina Dan-Sohkawa. Although a final conclusion on the nomenclature was not reached for all the cell types, correlation between cell types was reached relatively easily (see Table). The names of the cell types given in the leftmost column of the table were chosen by the present authors on the basis of the general agreement reached at this meeting that the names should follow, as much as possible, the functional features of the respective cell types. This table should facilitate further investigations on the coelomocytes of *H. roretzi* as the basis for understanding the defense mechanism of higher vertebrates including humans.

Literature / Cell Type (This report)	Fuke [7]	Fuke and Fukumoto [9]	Sawada *et al.* [11]	Ohtake *et al.* [10]	Azumi *et al.* [1]	Dan-Sohkawa and Morimoto [5]
Vacuolated cell*	vesicular cell / large granular amoeboid cell	vacuolated cell (T1, T2) (T3)	v1, v3, v4-cell	V1, V3, V4, V6, V7	Type F hemocyte	morula cell
Fibroblastic cell	minute granular cell	granular amoebocyte	gl-cell	large granular amoebocyte	Type B hemocyte	fibroblastic cell
Phago-amoebocyte	fine granular amoeboid cell	hyaline amoebocyte	p2-cell	small granular amoebocyte	Type C hemocyte	hyaline amoebocyte
Fusogenic phagocyte	—	—	p1-cell		Type A hemocyte	granulated amoebocyte
Giant cell*	large basophilic cell	large basophilic cell	g2-cell	V5: large basophilic cell	Type E hemocyte	giant cell
Lymphoid cell spherical	lymphocyte	lymphocyte-like cell	lymphoid cell	lymphoid cell	Type D hemocyte	lymphocyte
Lymphoid cell amoeboid	—	small (hyaline) amoebocyte				active form lymphocyte
Macrogranular cell	—	macrogranular cell	v2-cell	—	—	—
Globular cell	—	globular cell		V2	—	—
Viriform cell*	—	large granular cell	g3-cell	V8	—	—
Others	brown or orange cell, vacuolated cell (macrophage)			dense granular cell		multipolar cell

*: Names agreed upon by all participants.