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In Vitro Reactions of Coelomocytes against Sheep Red Blood Cells in the Solitary Ascidian *Halocynthia roretzi*

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ABSTRACT—Coelomocytes (blood cells) of the solitary ascidian *Halocynthia roretzi* were placed in culture to which sheep red blood cells (SRBCs) were introduced. Their reactions against SRBCs were observed by phase-contrast, time-lapse video and scanning electron microscopy. Three cell types, the phago-amoebocyte, the vacuolated cell and the fusogenic phagocyte, reacted to the SRBCs by phagocytosis. The vacuolated cell occasionally discharged the material of their vacuoles at the time of or shortly after ingesting the SRBC. The lymphoid cell captured SRBCs, but did not ingest them. Three other cell types, the fibroblastic cell, the giant cell and the multipolar cell, showed no reaction to the SRBC.

The SRBCs captured by the lymphoid cells were transferred either to phago-amoebocytes or fusogenic phagocytes, probably to be ingested. Such collaboration between multifunctional cells is considered to characterize the cellular defense mechanism of *H. roretzi*.

INTRODUCTION

Coelomocytes of the solitary ascidian *H. roretzi* have attracted the attention of many cell biologists interested in the defense mechanism of invertebrates. The coelomic fluid of this ascidian is inhabited by some ten cell types [1, 4, 6, 8–10]. A table of correlation between different nomenclatures of these cells used in the literature is given in the Appendix of Dan-Sohkawa *et al.* [4].

These cell types are reported to take different roles in the defense reaction of the ascidian body. The most abundant cell type, the vacuolated cell, releases lipopolysaccharide (LPS)-binding hemagglutinin and halocyamines [1] on encountering coelomocytes of "non-self" origin [7]. Cells of another type, the fibroblastic cell, form aggregates in reaction to a foreign environment, indicating a probable role in preventing the loss of body fluid [9]. The extracellar matrix (ECM)-secreting activity of these cells also suggests their role in wound healing [4]. Cells of the third type, the fusogenic phagocyte, form syncytia [3, 4, 10] which implies an encapsulating activity against large foreign bodies [5]. Phago-amoebocytes, the fourth cell type, are also shown to encapsulate aggregates of fibroblastic cells and fragments of tunic [9] and aggregates of starfish phagocytes [2].

Phagocytosis is one of the most fundamental means of self defense in invertebrates against particulate invaders. Phagocytosis against mammalian red blood cells has been reported with phago-amoebocytes [3, 6, 9, 10], fusogenic phagocytes [3, 10], vacuolated cells [3, 6, 10], fibroblastic cells [6] and giant cells [6]. Phago-amoebocytes also ingest *E. coli* cells [9]. In addition to such organic particles, phago-amoebocytes and fusogenic phagocytes were shown to take in latex beads of relatively small diameter (i.e., between ca 1.0

to 4.5 μm for the former and about 1 μm for the latter) [9].

There are, however, discrepancies of opinion among researchers concerning the phagocytotic ability of the coelomocyte types. While that of the vacuolated cell is accepted by Fuke [6], Dan-Sohkawa and Morimoto [3], and Sawada *et al.* [10], it is denied by Ohtake *et al.* [9]. Also, the phagocytotic ability of the fibroblastic cell and the giant cell claimed by Fuke [6] has not been confirmed by any of the other researchers.

In this study, we have reexamined *in vitro* the phagocytotic activities of the coelomocytes of *H. roretzi* against SRBCs by scanning electron microscopy (SEM) with an aim to facilitating more precise understanding of the phagocytotic abilities of these cells. The behavior of the cells on encountering SRBCs was also recorded by time-lapse video microscopy (VM) with the expectation that it would reveal the roles played by each cell type in defending the ascidian body from organic, particulate invaders. We show here for the first time a collaboration between lymphoid cells in their amoeboid form [4] and phago-amoebocytes or fusogenic phagocytes in clearing away the SRBCs.

MATERIALS AND METHODS

Materials

H. roretzi was a kind gift from the fisheries people of Mutsu Bay and from Asamushi Marine Biological Station of Tohoku University. Individual ascidians were transported and maintained in the laboratory aquarium (14°C) as described elsewhere [4]. They were used within a month.

SRBCs were purchased from Nippon Kohtai Kenkyujo, K. K. (Takasaki).

Harvesting and culturing coelomocytes

Coelomocytes were harvested and cultured as reported elsewhere [4]. In brief, coelomic fluid was obtained directly from the coelomic cavity of papilla with a syringe containing Ca^{++}, Mg^{++}-free Jamarin (CMfJ: Jamarin Laboratory, Osaka) supplemented

with 1 mM of ethylenediamine tetraacetic acid (EDTA). Harvested coelomic fluid was dropped into 1.5 ml of the culture medium (millipore filtered Jamarin U supplemented with 50 U/ml of penicillin and 50 mg/ml of streptomycin) loaded on the surface of a 24×24 mm^2 coverslip placed over an opening made on the bottom of a 3.5 cm Falcon culture dish. Cells were cultured at 20°C for 1.5 to 3 hr before adding SRBCs.

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.

Observation of phagocytosis

SRBCs were washed twice with CMfJ and added to the coelomocytes. SRBCs were also fixed with 2.5% glutaraldehyde for 2 hr at room temperature, washed six times with Jamarin U and stored. They were washed twice with CMfJ immediately before being added to the coelomocytes. The density of the SRBCs was adjusted in accordance with the observation by the number of drops added to the culture. The reaction of coelomocytes against SRBCs was observed by time-lapse VM and SEM.

Time-lapse video-microscopy

The phagocytotic behavior of the coelomocytes was recorded at one shot per 2 sec by time-lapse video recorder (Victor BR-9000) connected to an inverted phase-contrast microscope (Nikon Diaphot-TMD) with a microscopic video camera (Nikon DK-3001).

Scanning electron microscopy

SRBCs were given to the coelomocytes in a high density, i. e., enough to cover the entire dish surface, in order to maximize the phagocytotic incidence. Cells were allowed to take in SRBCs for 30 min, after which they were 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 a Polaron SEM coating system. They were observed with a Hitachi S-800H scanning electron microscope or ALPHA-25A scanning electron microscope (K. K. Topkon, Tokyo).

RESULTS

Three types of coelomocytes, the phago-amoebocyte, the vacuolated cell and the fusogenic phagocyte, reacted to SRBCs by phagocytosis. On the other hand, the amoeboid-form lymphoid cells captured the SRBCs and held them close to the surface of their elastic body. No difference in behavior against living or fixed SRBC was observed with any of these cell types. These reactions will be described below for the respective cell types.

Phago-amoebocytes

Phago-amoebocytes are the most active among the coelomocytes in ingesting SRBCs. They take in most of the SRBCs they encounter, so that they may have several of them in the cytoplasm at one time. Figure 1 shows phago-amoebocytes cultured in the presence of SRBCs. Though the cell shown in Figure 1 a is not in contact with an SRBC, it is considered to be in an activated state as judged by the double lamellipodia raised above the substratum (compare this with Fig. 6 of [4]). Phago-amoebocytes usually take in SRBCs from the cell surface facing the substratum (Fig. 1 b, c left and Fig. 2). However, they can also take in SRBCs from their upper surface, particularly when the concentration of the SRBC is high (see Materials and Methods) (Fig. 1 c right, d: arrowheads). Figure 2 a shows a serial tracing of a phago-amoebocyte encountering and engulfing an SRBC. On encountering the SRBC, the phago-amoebocyte simply ran over it without changing its speed of migration (from the first to the third frame) and then rounded up for a moment (the fourth frame). When the SRBC reappeared (the fifth frame), it moved with the phago-amoebocyte in a peculiar motion characteristic of cytoplasmic vesicles. The process of phagocytosis took only several seconds (the fourth frame). Virtually all phago-amoebocytes took in SRBCs when there were enough SRBCs around.

Vacuolated cells

Vacuolated cells recognize and ingest SRBCs by their "head" portions, i. e., the leading mass of the cytoplasm [4] (Fig. 3 a, b). Under VM, they look as if they were climbing over the SRBC before taking it in. It is only by the disappearance of the SRBC from the site after the vacuolated cell has moved away that we know the SRBC was ingested. Vacuolated cells sometimes circle around an SRBC for a while before taking it in (Fig. 4 a). In still other occasions, they linger around an SRBC for a while and move away leaving it alone.

Vacuolated cells were also observed to discharge the contents of their fluid-filled vacuoles at the time of or shortly after ingesting an SRBC. The cell shown in Figure 3 c is considered to represent the latter case. One of the bulging areas of the cell surface (arrowhead) is considered to reflect the presence of an ingested SRBC in the cytoplasm as judged from its shape and size. This notion is based on the fact that such bulge is only sometimes found to accompany the vacuolated cells which have burst, while a larger, more irregularly shaped bulge (arrow) is always found. The burst cells can continue to migrate with the ingested SRBC in their cytoplasm (Fig. 4 b).

The frequency of this "contact reaction" varied from experiment to experiment. That is, the proportion of the vacuolated cells to discharge their vacuoles varied from none to a great majority depending on the experiment. The condition that triggers the reaction is not known at present. More specifically planned experiments are necessary to determine whether the frequency of triggering the contact reaction differs between living and fixed SRBCs.

Virtually all vacuolated cells react to SRBCs sooner or later by phagocytosis, with or without the contact reaction.

Fusogenic phagocytes

On encountering an SRBC, fusogenic phagocytes slip their lamellipodium under it and let it slide over their surface
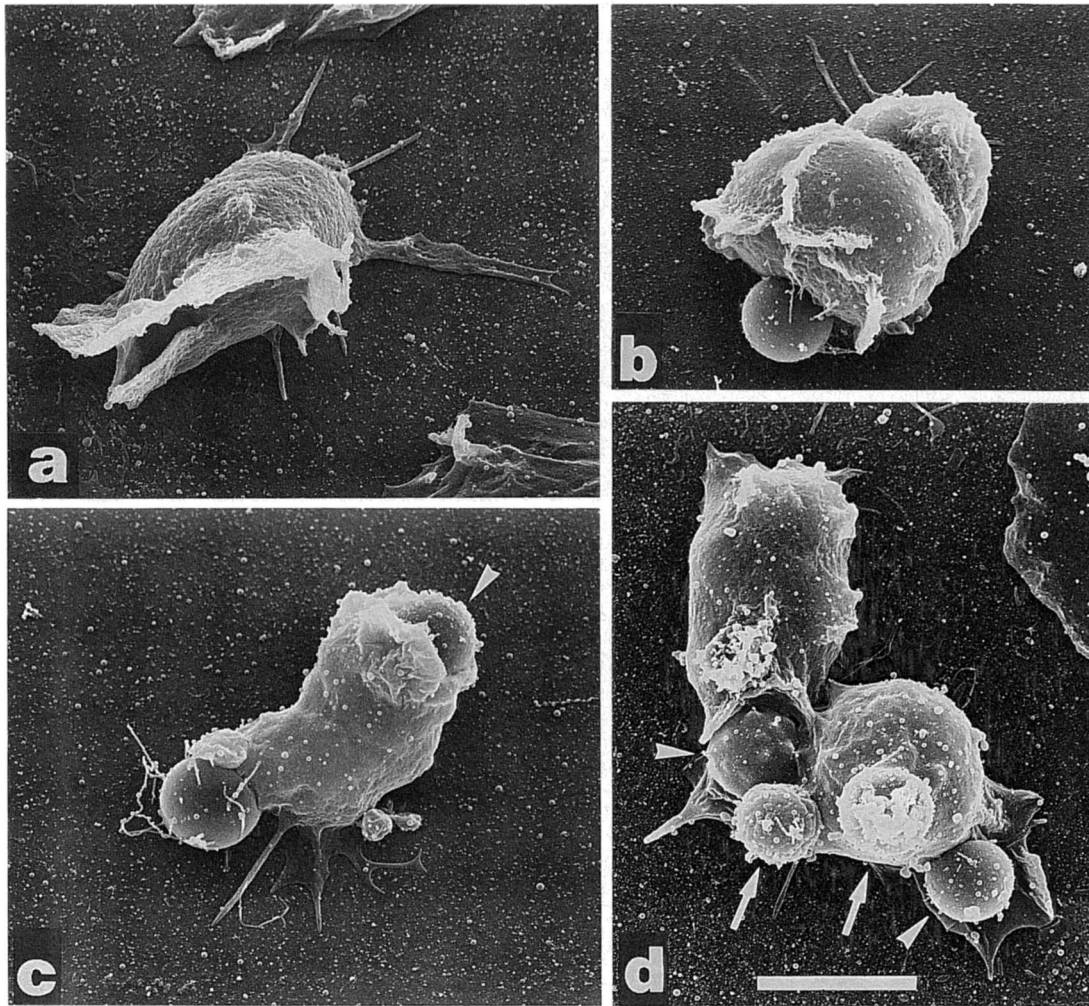
FIG. 1. Scanning electron micrographs of phago-amoebocytes cultured in the presence of SRBCs. See Fig. 6 of [4] for unstimulated states of these cells. Scale bar: 5 μm a: A cell probably in a stimulated state. Two lamellipodia are stretched out parallel to one another as if to enwrap an SRBC. b: A cell about to climb over and engulf an SRBC. c: A cell in the process of ingesting two SRBCs, the left one from its lower surface and the right one (arrowhead) from its upper surface. d: Two phago-amoebocytes in the process of engulfing SRBCs. The lower cell has already taken in two SRBCs (arrows) while holding two more (arrowheads) on its upper surface.
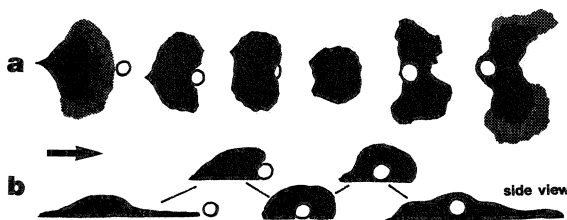

FIG. 2. Phagocytosis of an SRBC by a phago-amoebocyte. a: A serial tracing of a VM field. The interval between each frame is 1 min. The distances between the frames are arbitrary. b: The same process as seen from the side. Arrow: direction of migration.

to the central, cytoplasmic region (Figs. 5 a and 6) from where they ingest it (Fig. 5b). However, they are also able to take in SRBCs from their lamellipodium when the SRBC concentration is high (Fig. 5c). Fusogenic phagocytes can spend several minutes or longer to ingest one SRBC. They are often found to contain more than one SRBC under phase-contrast optics. Virtually all fusogenic phagocytes ingested SRBCs sooner or later.

Lymphoid cells in the amoeboid form

Lymphoid cells in their amoeboid state capture and enwrap the SRBCs with their elastic cell body and the filopodia (Fig. 7). However, they do not seem to ingest the SRBCs because they were never observed under VM to carry SRBCs in their cytoplasm. There is no possibility of over-looking the ingested SRBCs in phase-dark cell types even larger than lymphoid cells, such as phago-amoebocytes and vacuolated cells after bursting, as the SRBC will appear as clear and round areas in the dark cytoplasm (Figs. 2 a and 4b).

The probable fate of the SRBCs captured by lymphoid cells are shown in Figure 7 c, d. In these scenes, lymphoid cells carrying a cluster of SRBCs are approached by a
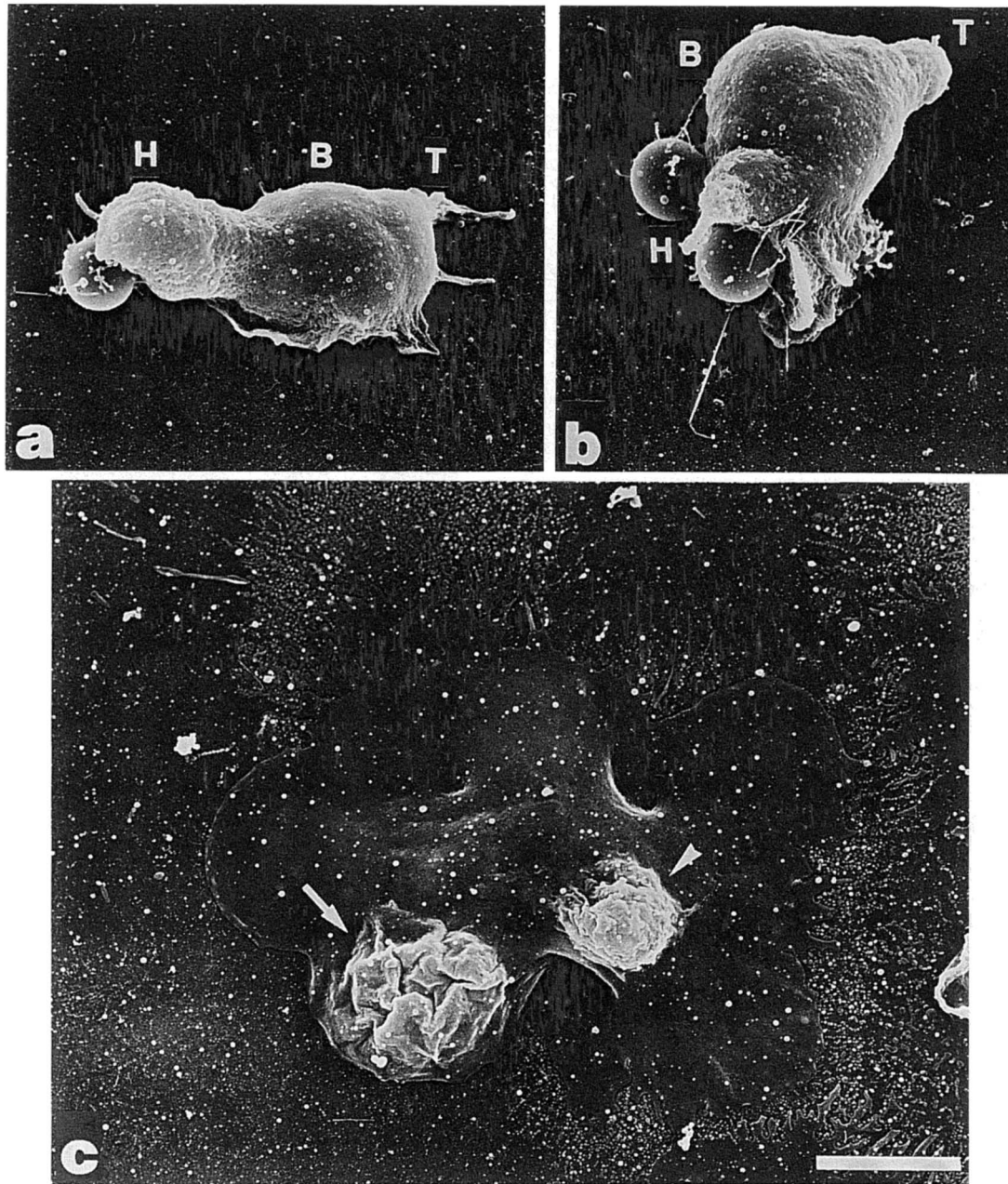
FIG. 3.   Scanning electron micrographs of vacuolated cells cultured in the presence of SRBCs.   See Fig. 2 of [4] for unstimulated states of these cells.   Scale bar: 5 μm.   a, b: Vacuolated cells in the process of ingesting SRBCs.   They always take in SRBCs from their "heads."   H: head, B: body, T: tail (c. f. Fig. 2 of [4])   c: A vacuolated cell immediately after discharging the contents of its vacuoles.   Arrowhead: a probable trace of an ingested SRBC, arrow : reflection of the bulk cytoplasm of the cell.

fusogenic phagocyte (Fig. 7c) or two phago-amoebocytes (Fig. 7d).   The activated state of the cell surfaces of these approaching cells (arrows) indicates that they are reacting to the SRBCs.   These scenes indicate that lymphoid cells hand over the SRBCs they have captured to phagocytotic cells for further treatment. The phago-amoebocyte and the fusogenic phagocyte are the only two cell types found so far in our SEM observations to react with the amoeboid-form lymphoid cell.

In these experiments, coelomocytes of *H. roretzi in vitro*

cleared away SRBCs at a density higher than 2 SRBCs / coelomocyte within about 20 min.

DISCUSSION

We have shown the phagocytotic behavior *in vitro* of three coelomocyte types of *H. roretzi* against SRBCs by means of time-lapse VM and SEM.   Cells which actively ingested SRBCs were the phago-amoebocyte (Figs. 1 and 2), the vacuolated cell (Figs. 3 and 4) and the fusogenic phago-cyte (Figs. 5 and 6).   The amoeboid-form lymphoid cell
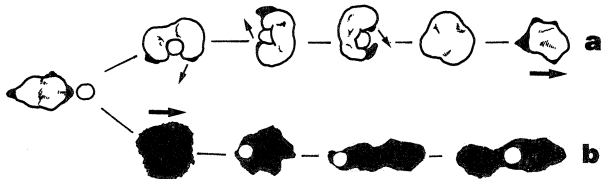
FIG. 4.   Phagocytotic behavior of vacuolated cells against SRBCs as traced from VM fields.   Conditions, same as Fig. 2 a.   a: A vacuolated cell circling around an SRBC before ingesting it.   b: A vacuolated cell migrating along after discharging its vacuoles at the time it ingested an SRBC.   Arrows: directions of migration.

trapped SRBCs until phago-amoebocytes or fusogenic phagocytes came along to take them in (Fig. 7).   Other cell types including the fibroblastic cell and the giant cell showed no apparent reaction to the added SRBCs.

The phago-amoebocyte seems to be the most active scavenger among the coelomocytes of *H. roretzi*.   They quickly take in most of the SRBCs they encounter and have the largest reported repertoire of phagocytic targets among

the *H. roretzi* coelomocytes.   These targets, both organic and inorganic, include native SRBCs [3, 9, 10], glutaraldehide-fixed rabbit [6] and sheep RBCs [9], *E. coil* [9] and latex beads [9].   As phago-amoebocytes are also responsible for encapsulating large foreign bodies [2, 9], it seems safe to conclude that this cell type plays a central role in the defense mechanism of *H. roretzi* against particulate invaders.

The phagocytotic activity of the vacuolated cell seems to depend on its physical state, i. e., whether it is "floating" or "attached".   In contrast to the active scavenger behavior shown in this report and in the literature [6, 10], vacuolated cells are unreactive to SRBCs when applied in suspension [9]. This difference indicates that vacuolated cells require a substratum in order to convert their rounded body of the floating state [8, 9] to the tri-partite configuration of the attached state (Fig. 3 a, b) [4].   This tri-partite configuration, in turn, is considered to give cytological basis for the phagocytotic machinery in which the head portion seems to be indispensable (Fig. 3 a, b).

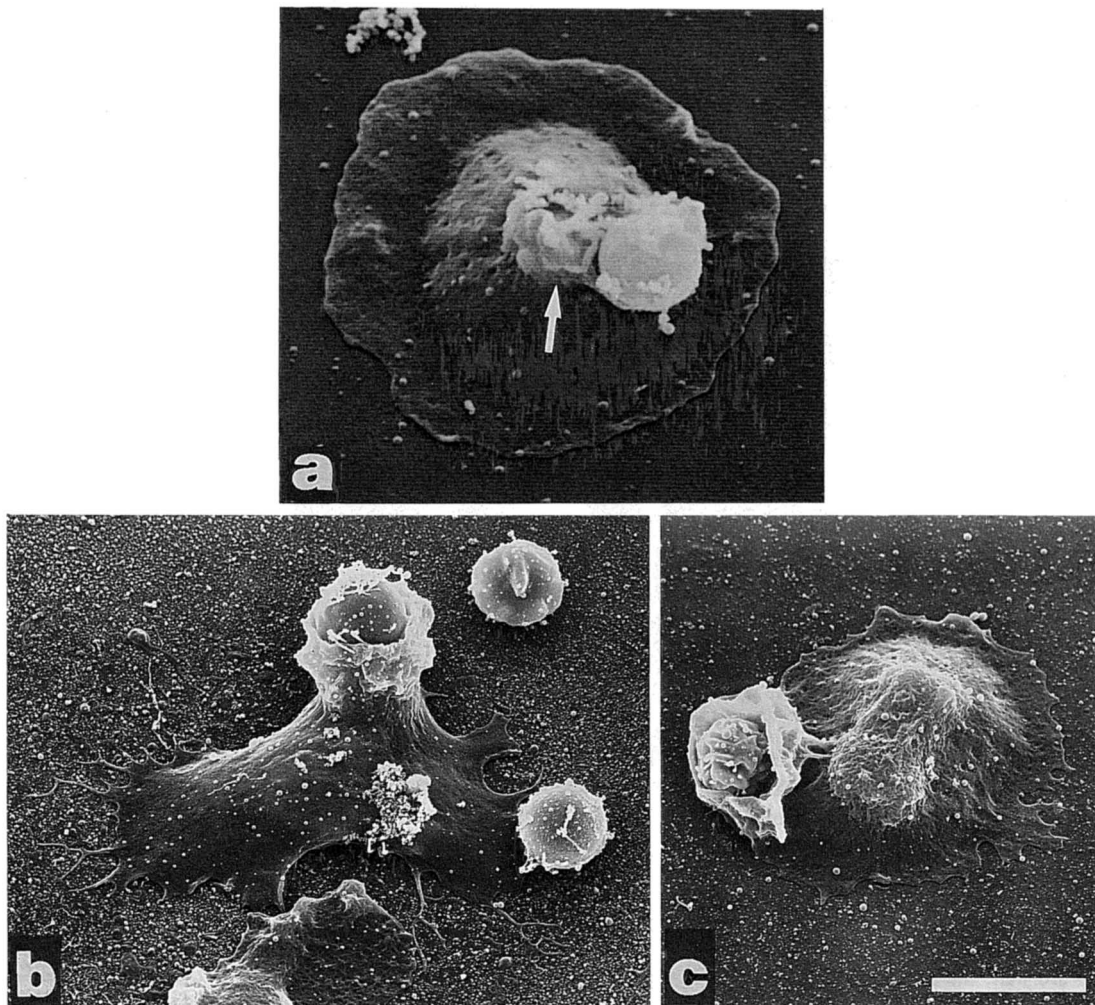Vacuolated cells attack SRBCs in two different ways,

FIG. 5.   Scanning electron micrographs of fusogenic phagocytes in the process of ingesting SRBCs.   Scale bar: 5 μm.   a: A fusogenic phagocyte carrying an SRBC over the cell surface to the central, cytoplasmic region.   Note the activated state of the cell surface of the central region (arrow).   b: A fusogenic phagocyte in the process of phagocytosis.   c: A fusogenic phagocyte ingesting an SRBC from the tip of the lamellipodium.
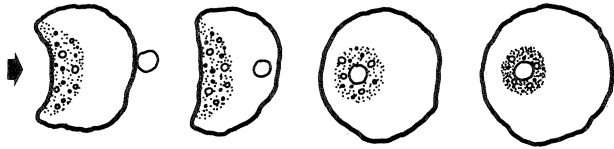
FIG. 6. Phagocytotic behavior of the fusogenic phagocyte against SRBC as traced from a VM field. A migrating fusogenic phagocyte is encountering and ingesting an SRBC. Conditions, same as Fig. 2 a. Arrow: direction of migration.

namely via phagocytosis and contact reaction. These two reactions are independent of each other as deduced from the fact that vacuolated cells react exclusively by the latter reaction when they encounter coelomocytes of non-self origin [7]. The usage of both of these machineries for attacking SRBCs is considered to imply, at one hand, that the size of the SRBC, i. e. about 3 μm in diameter, marks the borderline between the size of an object which vacuolated cells recognize as "small" to ingest or "large" to attack with cytotoxic halocyamins. On the other hand, additional factor(s) may
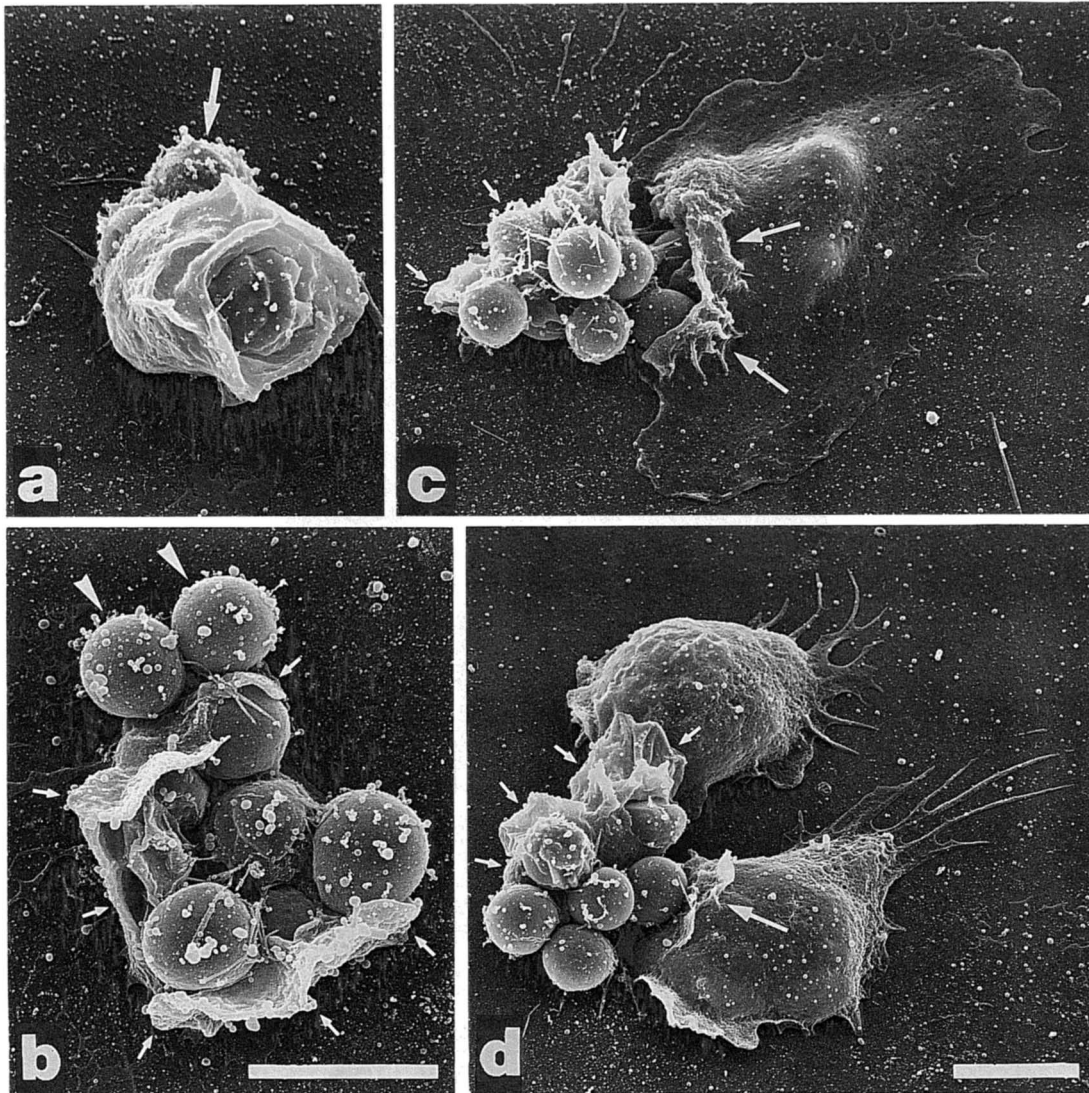

FIG. 7. Scanning electron micrographs of lymphoid cells cultured in the presence of SRBCs. Scale bars: 5 μm. a: A lymphoid cell enwrapping an SRBC with its elastic body. It probably will not ingest the SRBC (see text for explanation). This cell is accompanied by one spherical lymphoid cell (arrow). Scale same as b. b: An amoeboid-form lymphoid cell capturing four SRBCs with its expanded body (small arrows) and carrying two more on its outer surface (arrowheads). c: A lymphoid cell trapping several SRBCs is aided by a fusogenic phagocyte probably to take over the SRBCs. The fusogenic phagocyte is considered to have appoached from the upper right corner of the photograph. The thin and ragged appearance of the rightward half rim of its lamellipodium, as compared to the thicker and smoother one of the leftward half, indicates that the cytoplasm is moving from the upper right half of the cell to the lower left half. The lack of lamellipodium in the lymphoid cell indicates that it is not migrating. Large arrows : activated cell surface of the fusogenic phagocytes, small arrows same as b. Scale, same as d. d: A lymphoid cell holding several SRBCs is about to hand the SRBCs over to two approaching phago-amoebocytes. The trailing tails of the cytoplasm of the phago-amoebocytes, which stretch toward the upper right corner of the photograph, indicate that these cells have come from that direction. Large arrow : activated cell surface of the phago-amoebocyte, small arrows same as b. The activated cell surface of the upper phago-amoebocyte, if any, is not visible.

affect the judgment of the cells to trigger the contact reaction. This idea is based on the fact that while virtually all vacuolated cells take in SRBC sooner or later when there are enough around, the frequency with which the cells discharge their vacuoles varies greatly from experiment to experiment. Further study is necessary to reveal the nature of the factor(s).

The phagocytotic machinery of the fusogenic phagocyte seems also to be substratum-dependent as deduced from their phagocytotic behavior (Figs. 5 and 6). That is, they always take in SRBCs from the free cell surface on the opposite side of that facing the substratum. However, since we do not know at present whether they can take in SRBCs in the floating state or not, this issue has to await further study for verification.

No trace of the fusion event of fusogenic phagocytes was observed in the present study, in which the focus was on coelomocytes in a single state and in small clusters. This fact agrees with our previous observation that syncytia of fusogenic phagocytes arise from large aggregates [4].

In spite of the reported phagocytotic activity of the fibroblastic cell and the giant cell [6], these two cell types were indifferent to SRBCs under the conditions applied in this study. The situation did not change even when the SRBCs were given to the cells in suspension at the time of inoculation. These findings agree with the results of Ohtake et al. [9].

Direct collaboration in clearing the SRBCs was observed between the amoeboid-form lymphoid cell and two other cell types, the phago-amoebocyte and the fusogenic phagocyte (Fig. 7 c, d). A similar case of collaboration has been reported by Ohtake et al. [9] in which phago-amoebocytes enwrapped aggregates of fibroblastic cells in suspension. Furthermore, the same three phagocytic cell types described in this study have been shown in vitro to attack added starfish phagocytes in collaboration [2]. These findings indicate that the cellular aspect of the defense mechanism of H. roretzi consists of many complex cell interactions. Our experimental system should provide a useful tool for analyzing such interactions.

Our present results, along with those described in the literature, show that the characteristic cellular feature of the defense mechanism of H. roretzi consists of collaboration among multifunctional cells. Further cytological analyses should reveal other cellular functions and collaborative actions.

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