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Cell Types Involved in Allogeneic Contact Reactions of the Solitary Ascidian, *Halocynthia roretzi*

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ABSTRACT—The blood cells of a solitary ascidian, *Halocynthia roretzi* exhibit the allogeneic cellular reaction *in vitro* denoted as the "contact reaction"(Fuke, 1980). Nine cell types have been recognized in the blood and body fluid of *H. roretzi* (Fuke and Fukumoto, 1993). In the present study, the precise role of each cell type in allogeneic reactions is investigated *in vitro*. The vacuolated cells show devacuolation after contact with almost all other cell types from different reactive animals. These cells include hyaline amoebocytes, granular amoebocytes, macrogranular cells, small amoebocytes and giant cells (large basophilic cells), as well as vacuolated cells. The hyaline amoebocytes and small amoebocytes, which belong to the phagocyte series exhibit contact reactions with the cells of phagocyte series from other specimens of *H. roretzi*. They also show a contact reaction when in contact with granular amoebocytes. Therefore, almost all cell types have the materials responsible for individuality on their cell surface and can directly show the allogeneic cellular reactivity when they contact each other. To determine whether the contact reaction is involved in cell death, the loss of plasma membrane integrity was examined using fluorescent dyes. The number of cells showing uptake of ethidium bromide increased immediately after mixing of allogeneic cells. Almost the same cell types as those showing allogeneic behavior by light microscopy, as described above, exhibited loss of cell membrane integrity.

These results are discussed in the context of immune systems in invertebrates and vertebrates.

INTRODUCTION

The ability to distinguish self and non-self components seems to be one of the intrinsic characteristics of living organisms. It is not only found in higher vertebrates such as mammals but is widespread throughout the animal kingdom (Amano, 1990; Buss *et al*., 1990; Jokiel and Bigger, 1994; Ishii and Saito, 1995; Yamaguchi *et al*., 1999; Rinkevich *et al*., 1993). It remains uncertain, however, whether or not there is a homologous relationship between different animals, especially between invertebrates and higher vertebrates (Smith and Davidson, 1992; Humphreys and Reinherz, 1994; Rinkevich, 1996).

Ascidians are often regarded as being related to immediate forerunners of the vertebrates. Studies of self-nonself recognition in ascidians are therefore particularly relevant, as such studies can reveal crucial evolutionary steps toward the sophisticated immune systems possessed by vertebrates. From this viewpoint, extensive studies of the colonial ascidians have been particularly rewarding (Saito *et al*., 1994).

In the solitary ascidian, *Halocynthia roretzi*, an allogeneic cellular reaction, known as the "contact reaction" has been reported (Fuke, 1980). The reaction triggered by direct contact between reactive cells, results in mutual cell devacuolation and prolonged incapacitation of the cells in contact. The allogeneic reaction seems to be triggered by an absence of common self-markers (Fuke and Nakamura, 1983; Fuke, 1990), as reported for natural killer cells of mammals (Ljunggren and Karre, 1990). A high level of polymorphism in *H. roretzi*, of similar magnitude to that in the mouse MHC class I, has been revealed.

In higher vertebrates, special cell types that can distinguish non-self components such as T-cells are well known. It is important to know whether cell types specialized to distinguish self and non-self, also exist in ascidians.

Contact reactions between the allogeneic vacuolated cells of *H. roretzi* can be easily detected and have been described in detail (Fuke, 1980). However, the reactions between vacuolated cells and other types of cells, and those between other types of cells, have been barely described. Contact reactions seemed to occur in almost all types of blood cells because all blood cells eventually stopped moving (Fuke, 1980). However, it cannot be excluded that a cell type such as the vacuolated cell first distinguishes self and non-self components and exhibits the reaction, and that other types of cells then contribute indirectly to the reaction. In mammals, many sophisticated types of cell cooperation in the immune system have been reported, for example in helper T-cells and other lymphocytes. In ascidians, cell cooperation in phagocytosis of

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bacteria has been reported (Smith and Peddie, 1992).

For each type of blood cell, other than the vacuolated cell, there are few chances to contact others because each occupies a small percentage of the blood cell total. To obtain relatively pure cell populations and increase the chances of contact, cell separations were carried out. The cellular events between the separated cell groups were observed extensively *in vitro* to examine whether each cell type is able to distinguish self and non-self.

To fully understand the allogeneic contact reaction, it is important to clarify whether cell death occurs in the reactions. One of the morphological characteristics associated with cell death is loss of membrane integrity with concomitant inability to exclude dyes, such as ethidium bromide. The stainability of the nucleus by fluorescent dye after contact reactions was therefore examined.

MATERIALS AND METHODS

Animals

Specimens of the solitary ascidian, *H. roretzi*, were collected from Mutsu Bay, Aomori prefecture, and kept in running seawater (SW) at the aquarium facilities of Tohoku University Marine Biological Station, Asamushi. The animals were also cultured in artificial sea water (ASW) in the laboratory at 15°C at Kanazawa university, Ishikawa prefecture. From the three intra-specific variants available (Numakunai and Hoshino, 1973, 1974), Type C ascidians were used.

Fractionation of blood cells

Blood cells were collected from the sub-epidermal sinus by withdrawing body fluid into a hypodermic syringe containing a two-fold excess of marine anticoagulant (MAC) (0.1 M glucose; 15 mM trisodium citrate; 13 mM citric acid; 10 mM EDTA; 0.45 M NaCl; pH 7.0) according to Smith and Peddie (1992). After standing at 0°C for several hr, the supernatant was removed so that the concentration of blood cells was equivalent to that in ascidian blood ($(4.3\pm0.7)\times10^7$, n = 3). The blood cell populations of *H. roretzi* were separated by discontinuous density gradient centrifugation as follows. A stock 50% solution of Percoll (Pharmacia, Uppsala, Sweden) was prepared by mixing the Percoll with an equal volume of 10/9 M NaCl. Other solutions of Percoll at concentrations of 30% and 40% were prepared by dilution of the stock solution with MAC. The suspension of blood cells in a volume of 1.5 ml was layered over a discontinuous Percoll gradient that consisted of 2 ml each 30%, 40% and 50% Percoll. The gradient was centrifuged at 130 × g for 10 min at 5°C. Blood cells (groups L1, L2, L3) present at each interface between layers and those (P) in the pellet were collected (Fig. 1B). Each population of cells was washed with MAC by centrifugation at 300 × g for 10 min at 5°C. The cells in the pellet were suspended in the marine saline (MS)(12 mM $CaCl_2 \cdot 6H_2O$; 45 mM Tris; 38 mM HCl; 0.45 M NaCl; pH 7.4) described by Smith and Peddie (1992) and were laid immediately onto culture dishes to test their alloreactivity. To obtain a more purified fraction of vacuolated cells, a larger discontinuous gradient that consisted of 2 ml each of 20%, 30%, 40% and 50% Percoll was used. To minimize the contamination of vacuolated cells in L2 and L3, stronger centrifugal force of 350 × g was used.

Contact reactions

A pair of animals that exhibited allogeneic contact reactions with each other was selected, because about 15% of animal pairs do not show such reactivity (Fuke, 1980). The contact reactions were tested according to Fuke (1980) with a slight modification. Briefly, freshly obtained blood cells in SW or fractionated cells in MS, from one animal, were cultured in glass chambers for 15 min. The concentration of cells was roughly the same as described previously (Fuke, 1980). After the cells had been vitally stained with 0.001% Nile Blue, a suspension of the blood cells, fractionated or non-fractionated, from a second animal, was introduced into the chamber. The contact reactions were observed under a phase-contrast microscope at a magnification of ×1000.

Test of plasma membrane integrity after staining with fluorescent dyes

Two fluorescent dyes, Hoechst 33342 (H-33342) and ethidium bromide (EB), were used for the test of plasma membrane integrity. H-33342 can cross the plasma membrane and stain all cells, dead or alive, whereas EB is excluded from viable cells which have integral cell membranes (Gorman, A. *et al*., 1996). The cultured cells after the contact reaction were stained with a dye cocktail consisting of H-33342 (1 μg/ml) and EB (4 μg/ml) in ASW. The nuclei stained with EB were examined under B illumination of a fluorescence microscope (BX-FLA: Olympus Co, Ltd). Soon after, the cells stained with H-33342 were counted under UV illumination. The proportion of cells showing cell membrane disruption was expressed as the percentage of the cells stained with EB to the total number of cells stained with H-33342.

RESULTS

Blood cell types and cell separation

In *H. roretzi*, nine blood cells have been recognized from correlative fine structural, behavioral, and histochemical analysis (Fuke and Fukumoto, 1993). While there is little disagreement about the classification of blood cell types in *H. rotretzi*, there is some confusion over their nomencleature (Sawada *et al*., 1991; Zhang *et al*., 1992; Ohtake *et al*., 1994; Dan-Sohkawa *et al*., 1995). In this paper, I adopt the names used in our previous paper (Fuke and Fukumoto, 1993) except for two cell types. The nine cell types are as follows: vacuolated cells (V), hyaline amoebocytes (H), granular amoebocytes (G), macrogranular cells (M), globular cells (GL), small amoebocytes (S), lymphocyte-like cells (L), giant cells (GI) and viriform cells (VI). The last two names, giant cells (large basophilic cells) and viriform cells (large granular cells), were changed according to the suggestion of other investigators, and the names in parentheses are the old names of these cells used in our previous paper (1993). The cell types and their differential cell counts reported in the previous paper are illustrated in Fig. 1A.

The blood cells were separated, and a typical result is shown in Fig. 1B. An appropriate cell fraction (L1, L2, L3, P) was used to test the contact reaction between some of the cell types. For example, to examine the alloreactivity between the hyaline amoebocytes, L2 fractions from different individuals were used. To test the contact reaction between hyaline amoebocytes and granular amoebocytes, fractions L2 and L3 were used for mixed cell culture. L1 fraction and P fraction or non-separated blood cells were used for testing the alloreactivity between small amoebocytes and vacuolated cells.

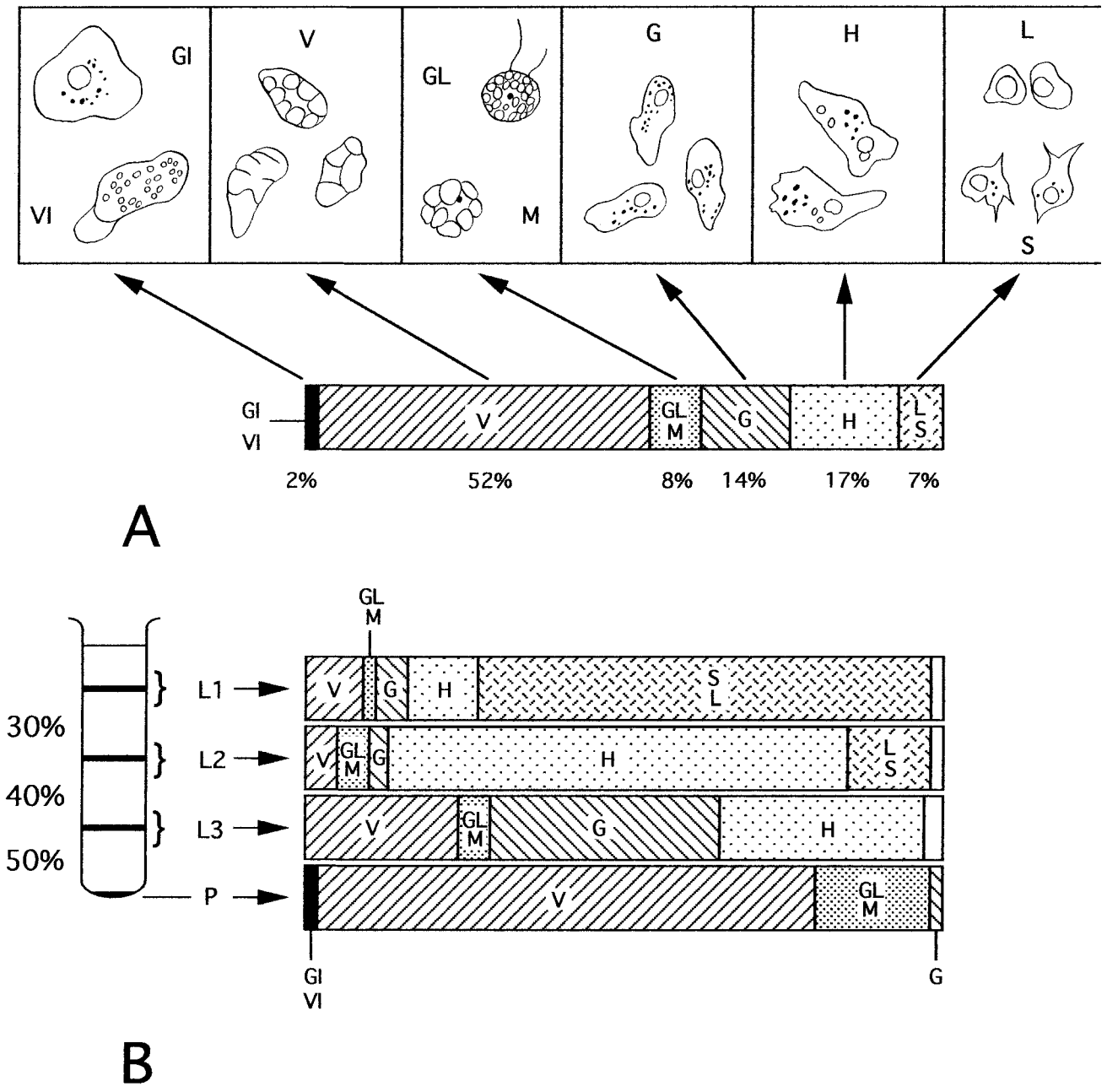

Fig. 1. Blood cell types and cell separation. A: Illustration of nine cell types and of differential cell counts reported in Fuke and Fukumoto (1993). V: vacuolated cell. H: hyaline amoebocyte. G: granular amoebocyte. M: macrogranular cell. GL: globular cell. S: small amoebocyte; L lymphocyte-like cell. GI: giant cell. VI: viriform cell. B: Fractionation of blood cells by discontinuous density gradient centrifugation in a solution of Percoll. The differential cell counts of each fraction are shown.

Contact reactions between vacuolated cells and cells of other types

When vacuolated cells from incompatible individuals were mixed *in vitro*, a series of cellular events occurred, as described previously (Fuke, 1980) (Fig. 2a–2f). After intimate contact (Fig. 2b, 2c), the vacuoles of one cell released their contents (Fig. 2d) into the surrounding medium, and this was followed by the devacuolation of another cell (Fig. 2f). In the previous paper (Fuke, 1980), we mistakenly described this event as "cell lysis". It is more correctly termed "devacuolation". After devacuolation, the cells sometimes extended their pseudopodia and even moved around their partners (Fig. 2e, 2f). After devacuolation, the cell conjugates remained totally immobilized for several hr.

When vacuolated cells meet other types of cells, they show an allogeneic cellular reaction. Hyaline amoebocytes and small amoebocytes seem to be specialized for phagocytosis (Fuke and Fukumoto, 1993, Ohtake *et al.*, 1994). An allogeneic contact reaction between vacuolated cells and hyaline amoebocyte is recorded in Fig. 2g–2l. After the vacu-

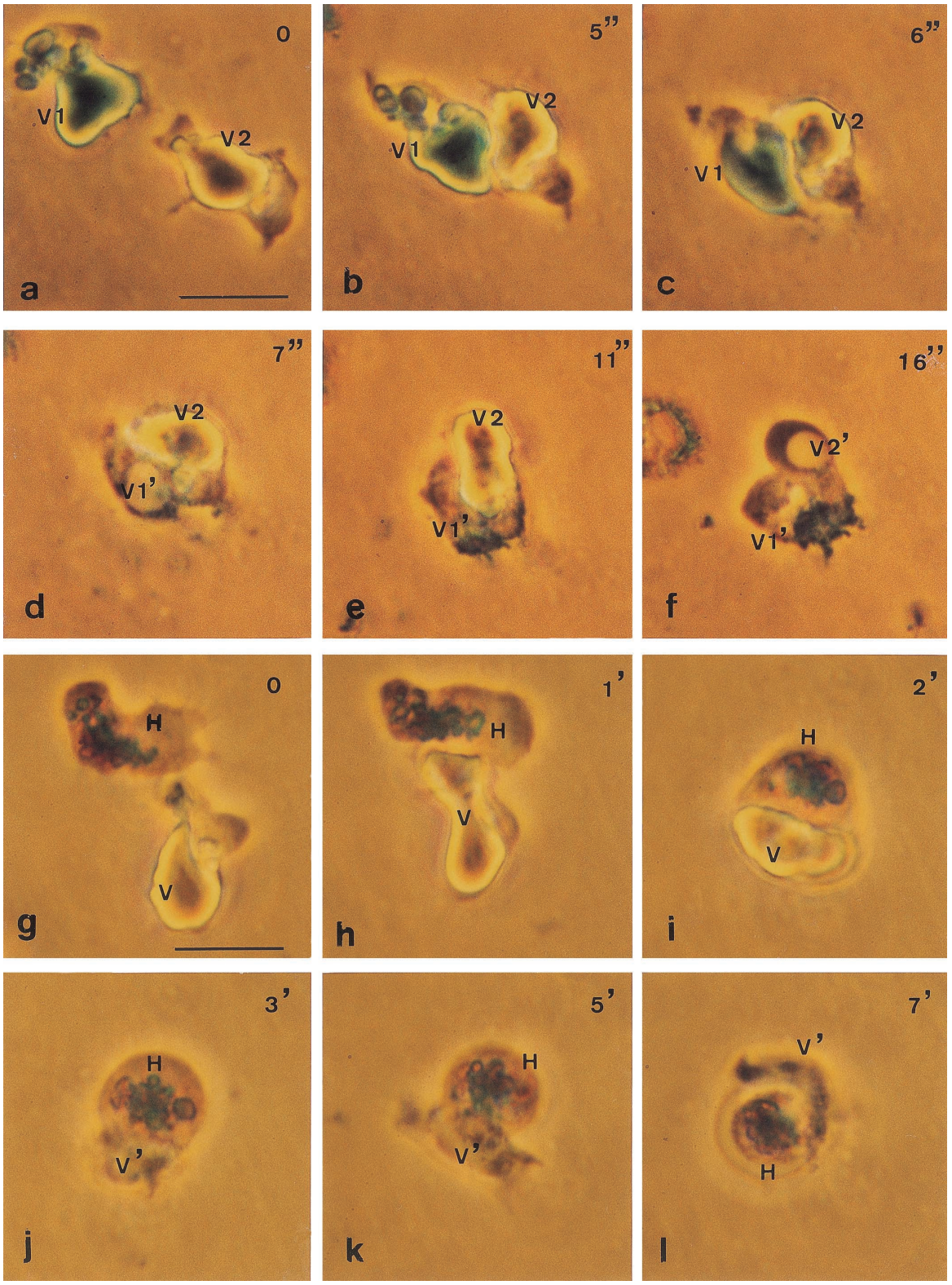

Fig. 2. Contact reactions between vacuolated cells and the cells of other types. a–f: The reactions between vacuolated cells from two individuals (V1, V2). V1 was stained with Nile Blue. After contact (b, c), the V1 cell discharged the contents of its vacuole (d, V1') and thenV2 (f, V2'). Afterthe devacuolation, V1' moves around V2. g–l: The allogeneic reaction between vacuolated cell (V) and a hyaline amoebocyte (H). The hyaline amoebocyte is stained blue. Following intimate contact (i), the vacuolated cell releases the contents of vacuole (j, V') and moves around the hyaline amoebocyte (k, l). The hyaline amoebocyte stops moving and swells abnormally (k, l). Scale bar = 10 μm. Time lapsed after the first frame of each reaction is indicated in seconds (") or minutes (') at the upper right corner of each frame.

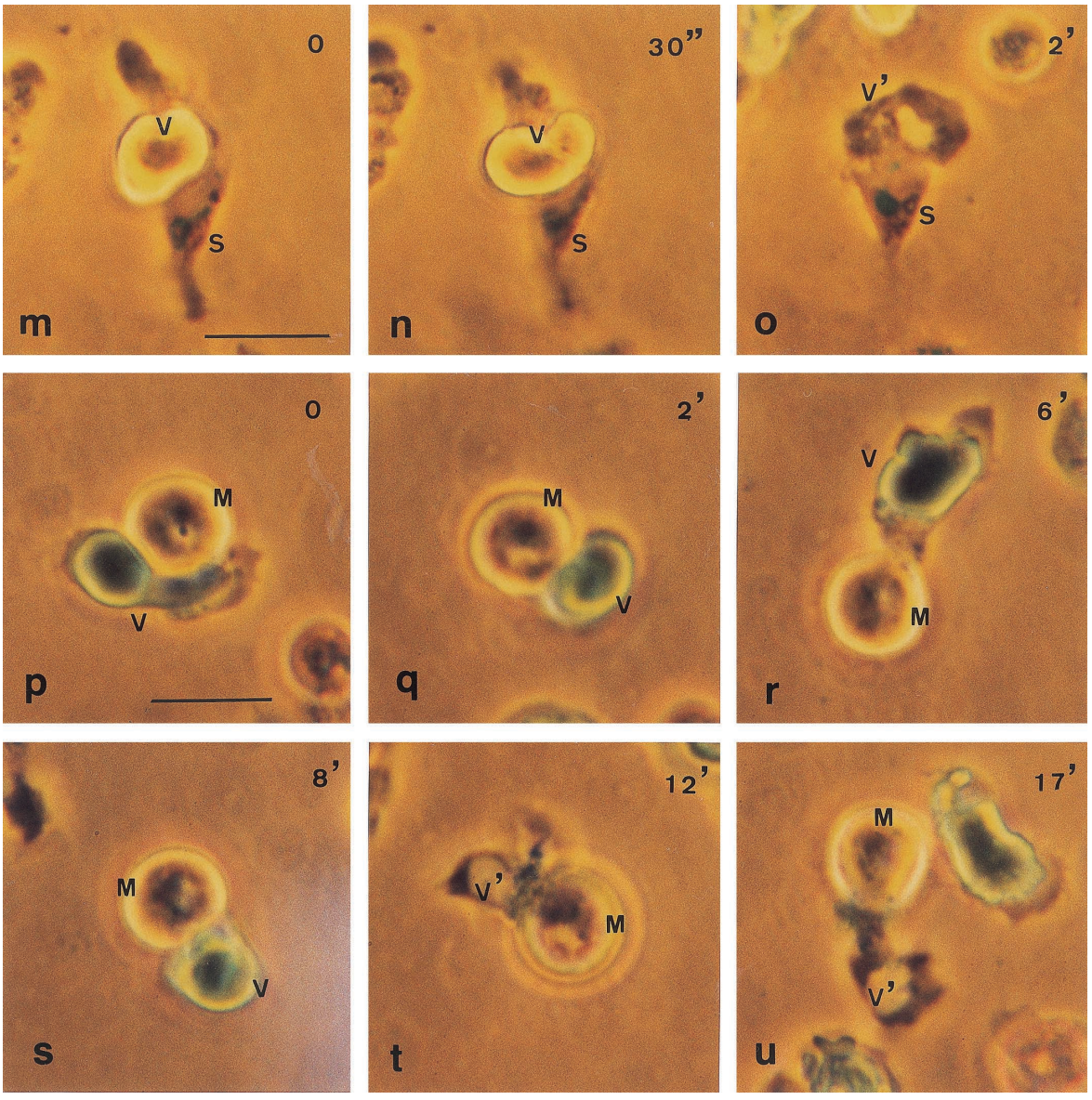

Fig. 2. (continued). Contact reactions between vacuolated cells and the cells of other types. m–o: The contact reaction between a vacuolated cell (V) and small amoebocyte (S). After intimate contact (m, n), the vacuolated cell releases its vacuole contents (V’). p–u: The allogeneic reaction between a vacuolated cell (V) and a macrogranular cell (M). The vacuolated cell is vitally stained blue. After contact between the allogeneic cells (p, q), the vacuolated cell moves and sticks to the macrogranular cell (r, M). Soon after, both cells stop moving and begin to cling to each other (s). Finally, the vacuolated cell discharges its vacuole content (t, V’) and continues to move around the macrogranular cell (u, M). Scale bar = 10 μm. The time after initiation is shown in seconds (”) or minutes (’) in each frame.

olated cell contacted the hyaline amoebocyte and appeared to recognize it as “non-self”, the former pressed itself tightly to the latter (Fig. 2h, 2i), and discharged the contents of its vacuoles (Fig. 2j). The devacuolated cells did not “die” immediately and moved around the hyaline amoebocyte (Fig. 2j–2l). The latter also exhibited a kind of allogeneic reaction. They stopped amoeboid movement, swelled slightly, and their granules tended to aggregate (Fig. 2j–2l). When vacuolated cells met small amoebocytes that belonged to the phagocyte group, a contact reaction occurred in the same way as between hyaline amoebocytes and vacuolated cells. After contact, the vacuolated cells discharged the contents of their vacuoles (Fig. 2m–2o). The small amoebocytes remained stationary and changed the appearance of their granules (Fig. 2o).

Granular amoebocytes, which have characteristic granules of uniform size, seem to play a role in hemostasis. When the vacuolated cells contact granular amoebocytes from different individuals, both cell types show an allogeneic contact reaction (Fig. 3a–3d). The vacuolated cells become devacuolated and remain in intimate contact with the granular cells. The changes in granular amoebocytes after the contact reaction are not clear, but sometimes the cells swell slightly.

Vacuolated cells were also able to induce the contact reaction in macrogranular cells (Fig. 2p–2u). Sometimes, it

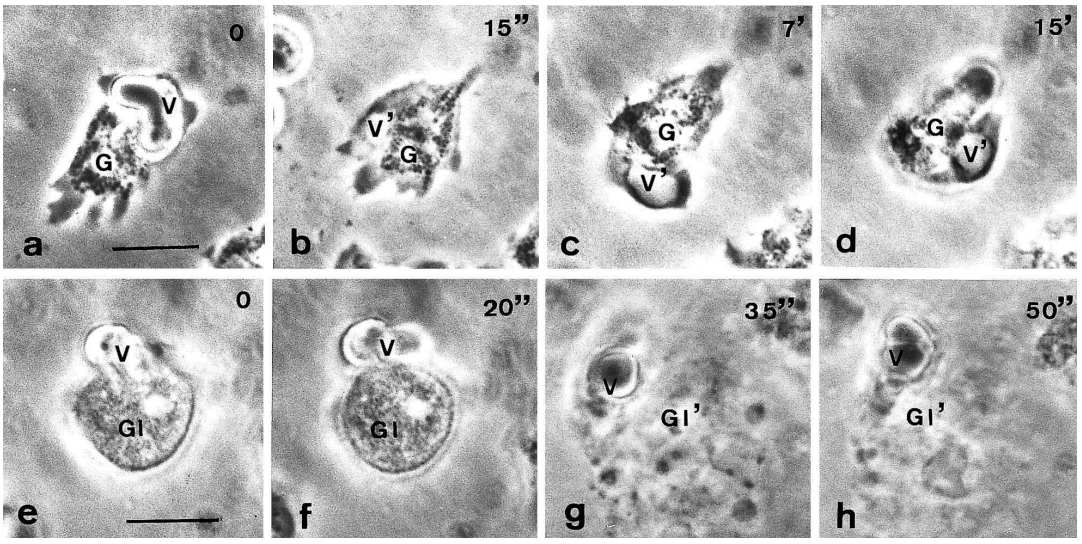

Fig. 3. Contact reactions of vacuolated cells and a granular amoebocyte and a giant cell. a-d: The reactions between a vacuolated cell (V) and a granular amoebocyte (G). Following contact, the vacuolated cell (V) shows devacuolation (b, V'). The cell (V') still moves around the granular amoebocyte (c, d). The granular cell draws in pseudopodia and develops a somewhat abnormal round shape and its granules tend to aggregate (d). e–f: Allogeneic reaction between a vacuolated cell (V) and a giant cell (GI). After contact, vacuolated cell (V) moves around the giant cell (e–f). The giant cell discharged its vacuoles (g, h, GI'). Scale bar = 10 μm. The time lapse after contact is indicated in seconds (") or minutes (').

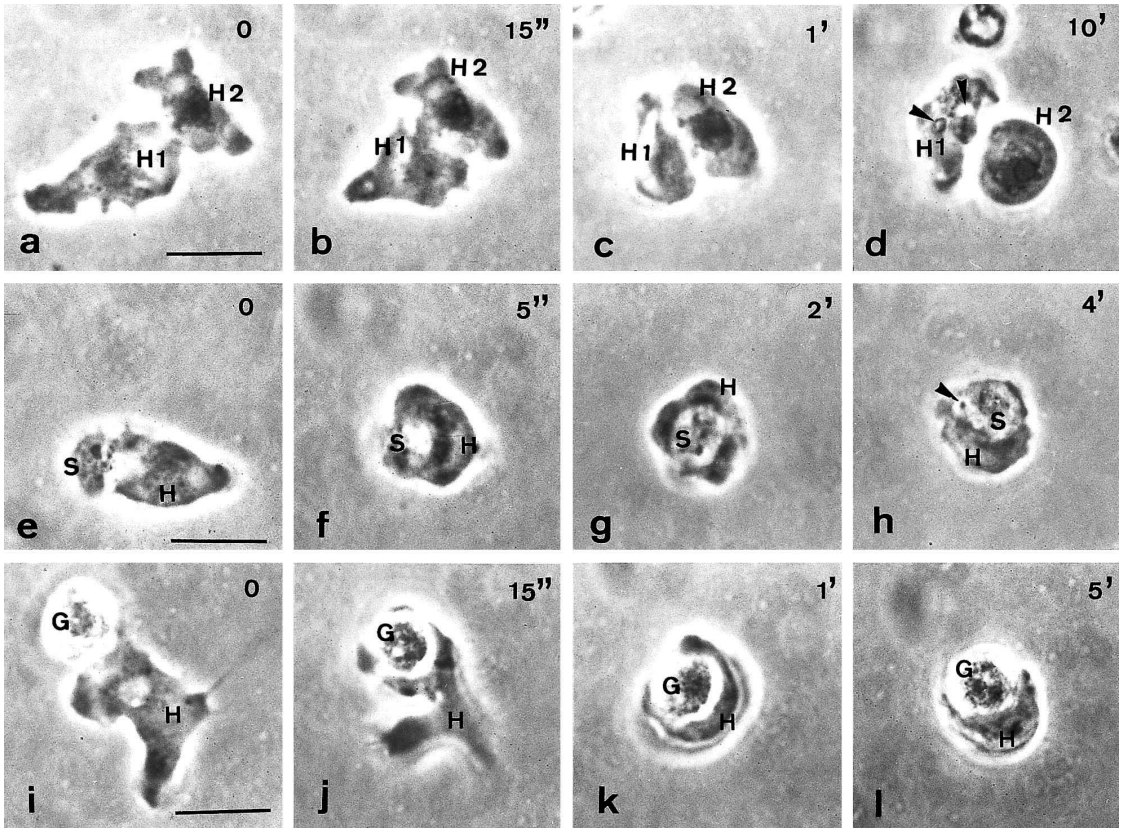

Fig. 4. Allogeneic cellular reactions not involved in vacuolated cells. a–d: Contact reaction between allogeneic hyaline amoebocytes (H1, H2). The H2 cell is stained vitally with Nile Blue. The darker granules of H2 can be distinguished from those of the unstained H1. After contact, the cells stop their amoeboid movement (b) and swelled abnormally (d). The granules tend to aggregate and empty their contents (d, arrowheads). e-h: Contact reaction between a hyaline amoebocyte (H) and a small amoebocyte (S). After contact (e), the hyaline amoebocyte (H) surrounds the small amoebocyte (S) as if the former is attempting to phagocytose the latter (f, g). The small amoebocyte releases the contents of its granules (h, arrowhead). i-l: Contact reaction between a hyaline amoebocyte (H) and a granular amoebocyte (G). When the hyaline amoebocyte meets an allogeneic granular cell (i), the former encloses the latter (j, k). Thereafter they remain for a long period and become rounded without protruding their pseudopodia (l). The scale bar indicates 10 μm. The time after initiation is shown in seconds ("), or minutes (') in the upper right corners of each frame.

was observed that the vacuolated cells moved and adhered to the macrogranular cells (Fig. 2r). After discharging the contents of their vacuoles, the vacuolated cell moved around the macrogranular cells (Fig. 2t–2u). Vacuolated cells also induced the allogeneic reaction in giant cells, whose cytoplasm was occupied by numerous vacuoles, as shown in Fig. 3e–3h. In these reactions, devacuolation of the giant cell occurred first. In another case, the vacuolated cell discharged the contents of its vacuoles, followed by the devacuolation of giant cells (data not shown).

We have not yet been able to describe the contact reactions between vacuolated cells and viriform cells, globular cells and lymphocyte-like cells, because of the infrequency of contact between them.

Contact reactions between non-vacuolated cells

The following experiments were designed to see if cells other than vacuolated ones could interact to exhibit a contact reaction.

The allogeneic cellular events between incompatible hyaline amoebocytes are shown in Fig. 4a–4d. After being in intimate contact (Fig. 4b, 4c), both cells swelled and became rounded. The cell granules tended to aggregate and empty their contents (Fig. 4d, arrowheads). Thereafter, the cells usually did not conjugate tightly with each other and stayed stationary, not extruding pseudopodia (Fig. 4d). Similar contact reactions were observed between hyaline and small amoebocytes (Fig. 4e–4h). When they contacted each other, the hyaline type rapidly surrounded the small type as if it was going to phagocytose it (Fig. 4f–4g). The small amoebocytes then discharged their granules (Fig. 4h, arrowhead). Allogeneic contact reactions between small amoebocytes were also observed and these were similar to those between hyaline amoebocytes (data not shown).

The blood cells specialized in phagocytosis, hyaline amoebocytes and small amoebocytes, also exhibited allogeneic contact reactions when touching non-phagocytic-type cells. When hyaline amoeboid cells contacted granular amoebocytes (Fig. 4i), the former enclosed the latter very rapidly (Fig. 4j, 4k) and remained stationary for an extended period (Fig 4l).

The details of serial cellular events between hyaline amoebocyte and macrogranular cell or globular cell were not evident. However, vestiges of contact reactions between them were often observed. Macrogranular cells were often surrounded tightly by hyaline amoebocytes (Fig. 5a). After the contact reaction, many empty vacuoles appeared in hyaline amoebocytes (Fig. 5b, arrowheads). On the other hand, changes in macrogranular cells were not clear. Allogeneic contact reactions between small amoebocytes and macrogranular cells also probably occurred (Fig. 5c).

As granular amoebocytes rarely exhibit active amoeboid movement, there is little chance of their contacting allogeneic cells. Also it isdifficult to differentiate the origin of cells because their granules can not be stained vitally. Therefore it was difficult to observe a series of allogeneic reactions between granular amoebocytes. However, changes in

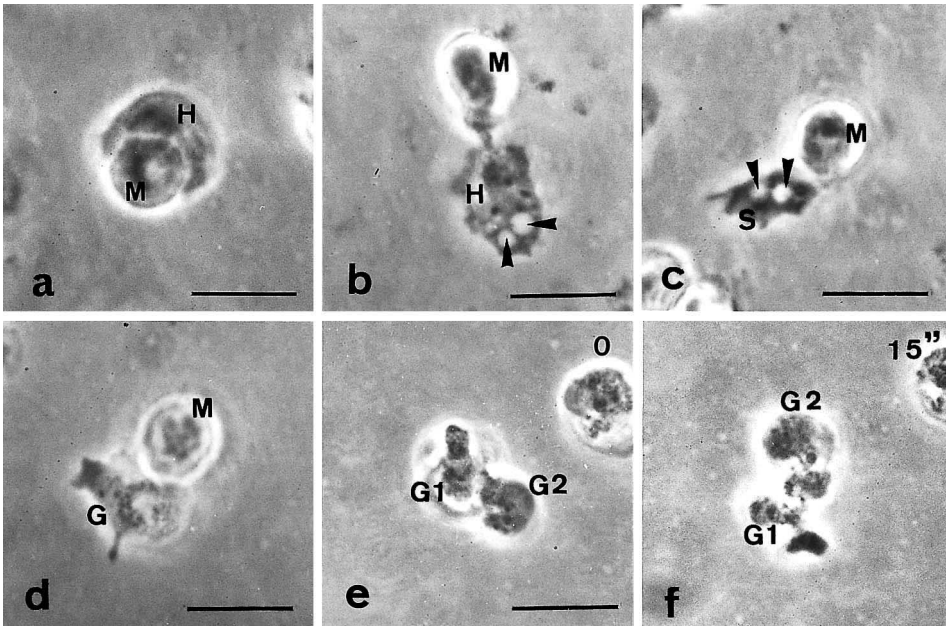

Fig. 5. The final appearance of the cells appears to indicate that allogeneic cellular reactions have occurred. a: Contact reaction between a macrogranular cell (M) and a hyaline amoebocyte (H). b: Contact reaction between a macrogranular cell (M) and a hyaline amoebocyte (H). Hyaline amoebocyte showed de-granulation (arrowheads). c: Contact reaction between a macrogranular cell (M) and a small amoebocyte (S). The small amoebocyte (S) releases the contents of its granules (arrowheads). d: Contact reaction between a macrogranular cell (M) and a granular amoebocyte (G). e–f: Two granular amoebocytes from different individuals (G1, G2) in close contact and appearing to show a contact reaction. They cling to each other. Note the position of G1 and G2 in e and f. Time elapsed after e is indicated in seconds (") at the upper right corner of f. Scale bar = 10 μm.

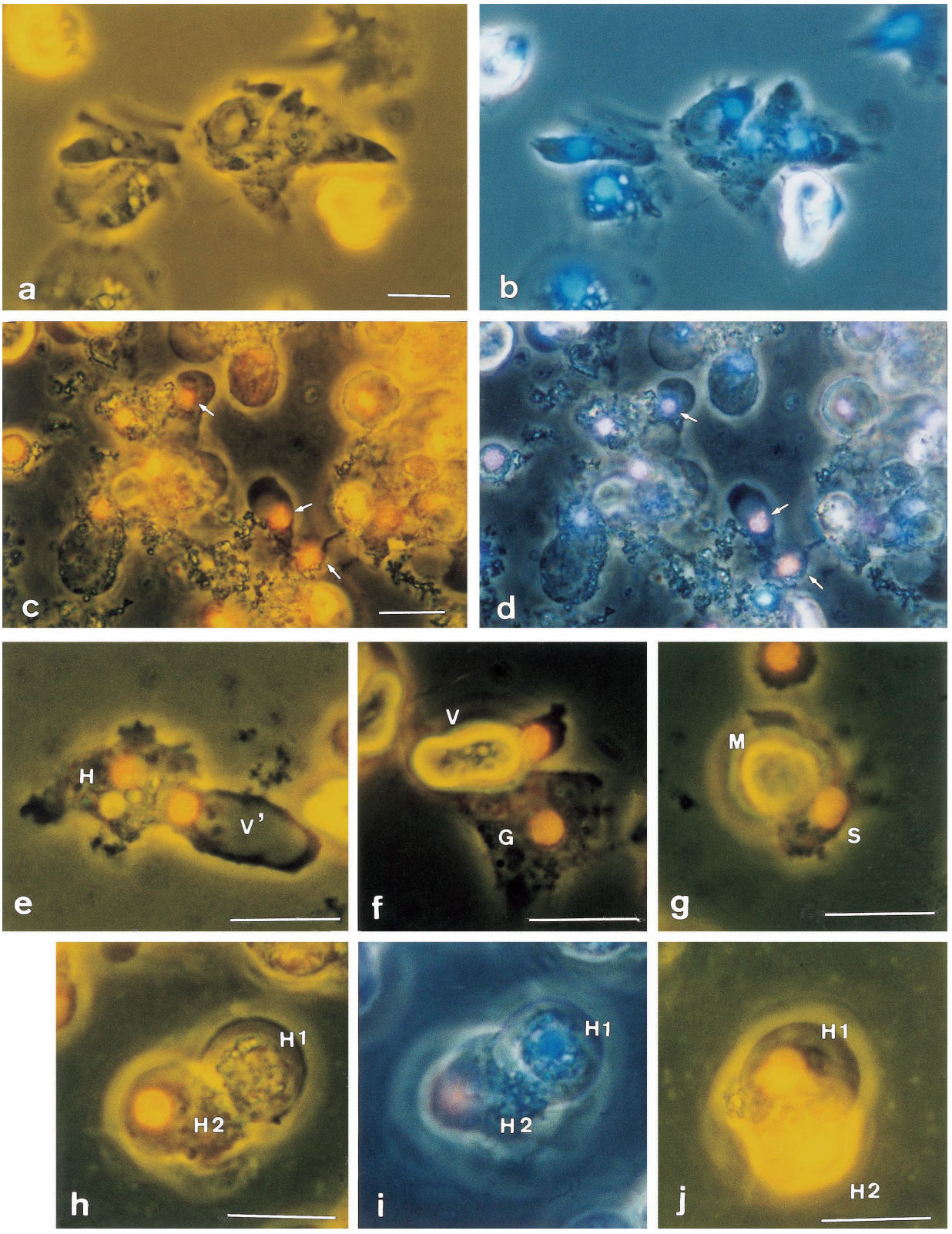

Fig. 6. Cell membrane integrity after the contact reaction examined using fluorescent dyes (a, b, c, d). After blood cells had been cultured with auto-cells (a, b) or allo-cells (c, d) for 1 hour, they were stained with a dye cocktail of EB and H-33342. The cells were first observed under B illumination (a, c) and then under UV illumination (b, d). After the allogeneic contact reaction, some nuclei were stained orange with EB (c, arrows) and pink with EB and H-33342 (d, arrows). Cell types showing membrane disruption after the contact reaction (e, f, g, h, i, j). e: Contact reaction between a vacuolated cell (V') and a hyaline amoebocyte (H), 1 hr after mixing of the cells. Both nuclei are stained orange by EB. The vacuolated cell shows devacuolation. f: Contact reaction between a vacuolated cell (V) and a granular amoebocyte (G) at 30 minutes after the contact reaction. The vacuolated cell with a stained nucleus is moving and has not yet become devacuolated. g: Contact reaction between a

cells indicative of a contact reaction between granular amoebocytes and globular or macrogranular cells were often found (Fig. 5d). We showed one example of an allogeneic reaction between granular cells which were clinging to each other (Fig. 5e, 5f). No contact reactions were observed between autologous granular cells.

Cooperation of cells in contact reactions

To obtain a more purified fraction of vacuolated cells, a longer discontinuous gradient of Percoll was used. After the cell fractionation, the P fraction that contained mainly vacuolated cells (about 90%) and small amounts of globular or macrogranular cells, giant cells and viriform cells, but a few or no granular amoebocytes, hyaline amoebocytes and small amoebocytes (below 3%), was obtained. When the cells of the P fraction were mixed with cells of the same fraction but from a different animal, a vigorous contact reaction between vacuolated cells immediately took place (data not shown). Therefore, the vacuolated cells seemed to be able to exhibit a contact reaction without the help of granular amoebocytes, hyaline amoebocytes or small amoebocytes. When the cells were fractionated by a slightly stronger centrifuged force (350 x g), we obtained the fractions L2 and L3 that had a few vacuolated cells (below 4%). As an example, the cell mixture of L2 and L3 contained hyaline amoebocytes (60%), granular amoebocytes (26%), small amoebocytes (10%) and vacuolated cells (4%). When the cells (L2 and L3) were introduced to these from an allogeneic animal, a contact reaction occurred without vacuolated cells. There were large aggregates consisting of hyaline amoebocytes, granular amoebocytes and small amoebocytes without vacuolated cells.

Change of cell membrane integrity with contact reaction

Dye exclusion tests were carried out to examine cell death after the contact reaction. The nuclei of blood cells were usually not stained by EB, but were vitally stained with H-33342 (Fig. 6a, Fig. 6b). After mixing the allogeneic cells, the cells which had nuclei stained orange with EB under B illumination (Fig. 6c. arrows). Under UV illumination, the nuclei were stained pink (Fig. 6d. arrows). After the contact reaction, the percentage of cells stained orange by EB relative to total cells increased rapidly, indicating that the permeability of the cell membrane had changed (Fig. 7).

The cell types stained with EB were then examined. Vacuolated cells, which showed devacuolation after the contact reaction, were usually stained by EB (Fig. 6e), as were vacuolated cells that had yet not been become devacuolated (Fig. 6f). The nuclei of small amoebocytes, which were in contact with macrogranular cells, were stained orange by EB (Fig. 6g). Hyaline amoebocytes in contact with allogeneic vacuolated cells (Fig. 6e) and hyaline amoebocytes (Fig. 6h, 6i, 6j)

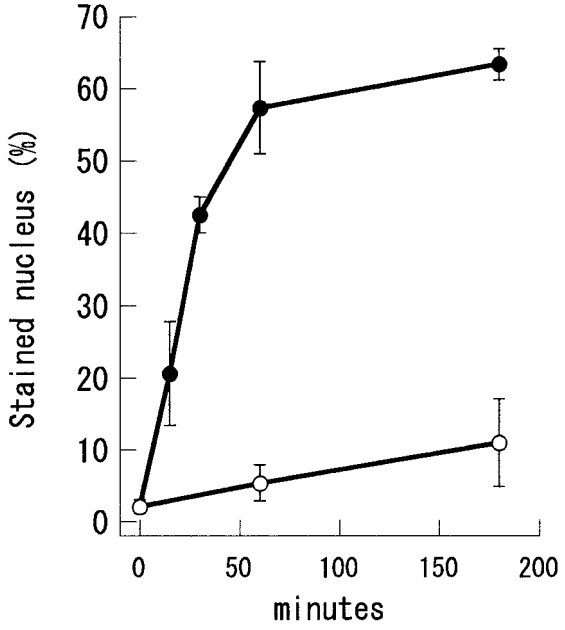

Fig. 7. Increases in the number of cells with disrupted cell membranes after the contact reaction. The rates of the cells are expressed as percentages of nuclei stained by EB, relative to all nuclei stained by H-33342. Error bars represent ±SD of three independent experiments.

showed cell membrane disruption. In other cell types, such as granular amoebocytes (Fig. 6f) and giant cells (data, not shown), the nuclei were stained with EB, indicating cell membrane disruption.

Contrary to expectation, the nuclei of moving (not "dead") vacuolated cells, which had devacuolated, were stained orange by EB, as were those that had not yet exhibited devacuolation (Fig. 6f).

DISCUSSION

The results of contact reactions between various cell types are summarized in Table 1. The allogeneic reactivities of lymphocytes and viriform cells were not clarified because they occupied small percentages and had little chance of encountering allogeneic cells. Almost all cells (above 95%) have an ability to distinguish self from nonself. Recently, Ohtake *et al* (1996) reported that viriform cells did not show any sign of contact reactions with the blood cells, as well as viriform cells from another individuals.

The characteristics of the contact reaction varied with cell type. Vacuolated cells clearly showed devacuolation after contact with all other cell types, as well as with other vacuolated cells. The giant cells were also induced to de-granulate by contacting incompatible vacuolated cells (Fig. 3e–3h). After contacting vacuolated cells, hyaline amoebocytes, small

small amoebocyte and a macrogrobular cell at 20 min after cell mixing. The small amoebocyte, in contact with a macrogranular cell, has an EB-stained nucleus. h-i: Contact reactions between allogeneic hyaline amoebocytes (H1, H2) at 90 min after allogeneic cell contact. A cell (H1) has an intact cell membrane but the other has not (H2). The cells were observed under B illumination (h) and the immediately observed under UV illumination (i). j: A pair of hyaline amoebocytes (H1, H2) showing the contact reaction at 2 hr after mixing with allogeneic cells. Both nuclei are stained by EB. Scale bars = 10 μm.

Table 1. Reciprocal contact reactivities among various types of cells.

	V	H	G	M, GL	S	GI
V	+	+	+	+	+	+
H		+	+	+*	+	n.d.
G			+*	+*	n.d.	n.d.
M, GL				n.d.	+*	n.d.
S					+	n.d.
GI						n.d.

+: A series of contact reactions could be observed. +*: Positive contact reaction indicated by morphological changes could be found. n.d.: not determined. V: vacuolated cell. H: hyaline amoebocyte. G: granular amoebocyte. M: macrogranular cell. GL: globular cell. S: small amoebocyte. GI: giant cell.

amoebocytes and granular amoebocytes stopped moving and swelled abnormally (Fig. 2j–2l, Fig. 2o, Fig. 3c–3d). However, macrogranular cells appeared to change relatively little after a contact reaction (Fig. 2s–2u).

Non-vacuolated blood cells were shown to exhibit allogeneic cellular reactions upon contact with each other. When allogeneic hyaline amoebocytes encountered each other, they attached to their partners as if they were going to carry out phagocytosis. Finally, however, they stopped their movement without phagocytosis and swelled abnormally (Fig. 4c–4d). A similar pattern of contact reaction was observed between hyaline amoebocytes and small amoebocytes (Fig. 4f–4h), as well as between hyaline amoebocytes and granular amoebocytes (Fig. 4j–4l). Certainly, macrogranular cells are targets of many types of allogeneic cells. However, it is uncertain whether the cells show active allogeneic behavior toward other types of cells.

In colonial ascidians, the cell type involved in self-nonself recognition seems to vary with the species. In *Botryllus pirimigenus*, morula cells (vacuolated cells) and lymphocyte-like cells play a central role in the rejection reaction (Tanaka and Watanabe, 1973; Taneda and Watanabe, 1982b). Phagocytes were recently reported to play an important role in self-nonself recognition in Botryllus scalaris (Shirae and Saito, 1999).

To fully understand the allogeneic contact reaction, it is important to know whether cell death is involved in the reaction. In *Styela clava* blood cells were reported to die after allogeneic cellular reactions *in vitro* (Kelly *et al*., 1992). Initially, in *H. roretzi*, we mistakably believed that the contact reaction was involved in immediate cell lysis and consequently in immediate cell death (Fuke, 1980). However, devacuolated cells did not “die” at least immediately, judging from their mobility, as shown in Fig. 2d–2f, Fig. 2j–2l, and Fig. 2t–2u.

One useful method for differentiating dead and viable cells is dye exclusion. Our test using EB showed that blood cells lost their cell membrane integrity soon after mixing of allogeneic blood cells (Fig. 6, Fig. 7). Almost all cell types, vacuolated cells, hyaline amoebocytes, granular amoebocytes, small amoebocytes, and giant cells, were confirmed to lose their membrane integrity (Fig. 6). Unexpectedly, in vacuolated cells, loss of membrane integrity did not occur as a result of devacuolation. Many vacuolated cells, which had not yet shown devacuolation and were moving actively, had nuclei that were stained orange by EB (Fig. 6). However, the blood cells did not “die” at least for some time, judging from their ability to move. All cells formed aggregates and eventually stopped moving after the contact reaction (Fuke, 1980). Therefore, it is natural to consider that cells which have a disrupted cell membrane will not live for a long time. The cause and mechanisms of cell membrane integrity are very important and remain to be clarified.

Cell cooperation during phagocytosis of bacteria in *Ciona intestinalis* has been reported (Smith and Peddie, 1992). Is there cooperation between different cell types in the contact reaction? As the vacuolated cells showed vigorous contact reactions immediately after contact with allogeneic vacuolated cells *in vitro*, it is clear that they can exhibit the allogeneic reaction without help of other cell types. Other types of cells, such as hyaline amoebocytes, granular amoebocytes and small amoebocytes, showed a kind of allogeneic reaction with a few vacuolated cells at below 4%. As described above, they were directly observed to show cellular behavior toward allogeneic partners. Therefore, it seems conceivable that each type of cell exhibits the allogeneic reaction without the cooperation of other cell types.

The conclusion that there is no cell cooperation between different cell types is supposed from the experiments using ‘conditioned medium’. The cell free ‘conditioned medium’ obtained after the contact reaction was examined to see if it induced the allogeneic contact reaction with fresh cells. Preliminary experiments using the medium obtained by mixing incompatible blood cells at low concentration (4×10^6), were reported previously (Fuke, 1980). The cell free medium, which contained about 4 times the amount of materials released from reactive cells (1.72×10^7), also did not induce any sign of a contact reaction in fresh blood cells. The possibility that the products of contact reactions between cells of one type, such as vacuolated cells, may affect other cell type is unlikely. However, it remains possible that the effective factor(s) released from the reacted cells is very labile, and therefore was not detectable in the experiment.

Vacuolated cells were reported to release phenoloxidase upon contact reaction in *H. roretzi* (Akita and Hoshi, 1995). In *Botryllus sholosseri*, morula cells (vacuolated cells) released phenoloxidase when they were incubated with blood plasma from non-fusible colonies *in vitro* (Ballarin *et al*., 1995). It was suggested that the phenoloxidase is responsible for localized cell death in the necrotic region. In *H. roretzi*, it is considered that the substrates obtained from vacuolated cells damage partner cells. However, cells other than vacuolated cells, which, in the absence of vacuoles are unlikely to contain phenoloxidase, also exhibited a kind of contact reaction. The cause(s) and effect(s) of the allogeneic reactions caused by these cells remain to be elucidated.

The contact reactions complete very rapid and are reciprocal (bilateral). The allogeneic reactions seem to be triggered by an absence of common self-markers (Fuke and Nakamura, 1985), as in mammalian natural killer cells (Ljunggren and

Karre, 1990).

Allograft rejections have been reported in solitary ascidians (Reddy *et al*., 1975, Raftos *et al*., 1987, 1988). Only one cell type, the lymphocyte-like cell, was responsible for allograft rejection. The transplantation immunity in *Styela plicata* required a much longer time, that is about 38 days for the first set of allografts, and was unilateral that is, one animal retained a reciprocal graft while the other rejected it. The reactions are triggered by the presence of non-self markers, as in classical transplantation immunity. In solitary ascidians, it seems that there are two types of allogeneic reactions: transplantation immunity and contact reaction. In colonial ascidians, two similar types of reactions, these are bilateral colony non-fusion reaction and unilateral colony resorption, have been reported (Rinkevich, *et al*., 1993).

Detailed comparative studies of two allogeneic reactions in ascidians were needed to understand more fully the phylogenic relationships of invertebrate immunity.

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