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A Comparison of Hemocytes and Their Phenoloxidase Activity among Botryllid Ascidians

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ABSTRACT—The colony specificity of colonial animals involves allorejection reactions, which are the defense reactions for allogeneic tissues that occur naturally. In colonial botryllid ascidians, all of the species already studied have colony specificity, and their allorejection modes differ from one another. However, in most of these botryllids, morula cells (MCs) always participate in the allorejection reactions, and the prophenoloxidase (proPO) system of MCs is considered to contribute to the allorejection reaction. The present study was performed using five botryllids and *Symplegma reptans*, which is closely related to botryllids, in an effort to clarify the relationship between the modes of allorejection and the characteristics of MCs, such as the ratio of MCs to total hemocytes and the phenoloxidase (PO) activity levels in the MCs. The MCs of these six ascidians resembled one another morphologically and the MCs of all species showed PO activity. In *Botryllus scalaris*, PO activity was also found in granular leukocytes, but the level of activity was much lower than that in MCs. The PO of these species resembles one another, at least in terms of their sensitivity to inhibition by common inhibitors of the proPO system. The PO activity per fixed number of hemocytes varied among these ascidians. This variation was due to a difference in the ratio of MCs to total hemocytes and/or a difference in PO activity per MC. In most ascidians, except for *B. scalaris*, the rejection reaction area showed a higher level of PO activity than the fusion area of the syngeneic colonies. These results suggest that the characteristics of MCs including their PO activity are closely correlated with the mode of the allorejection reaction.

INTRODUCTION

Colony specificity, which is a phenomenon observed in colonial animals from sponges to ascidians, is regarded as a type of allogeneic recognition in multicellular animals. When colonies touch at their growing edges, compatible colonies fuse to become a single mass, but incompatible colonies do not fuse and usually reject each other. In botryllids, a group of colonial ascidians, all of the species already examined show colony specificity (Taneda *et al.*, 1985; Rinkevich, 1992; Saito *et al.*, 1994). In botryllids, the fusion reaction occurs in the same way, but their allorejection modes differ even though they belong to the same family (Taneda *et al.*, 1985; Rinkevich, 1992; Saito *et al.*, 1994). Therefore, allorejection reactions in botryllids are very useful objects of study to elucidate the organization and diversity of allorejection systems in colonial animals.

In botryllids, allorejection reactions are initiated at different stages on the way to fusion, and the variation in allorejection reactions has been interpreted as reflecting a difference in nonself-recognition sites in their allogeneic recognition systems (Taneda *et al.*, 1985; Rinkevich, 1992; Saito *et al.* 1994). However, we recently pointed out that a difference in hemocyte behavior, especially the behavior of morula cells (MCs), induced by nonself-recognition also contributes to the variation in allorejection reactions (Shirae *et al.*, 1999). In *Botryllus scalaris* (Shirae *et al.*, 1999) and an unidentified species of *Botrylloides* from Israel (Rinkevich *et al.*, 1994), MCs do not have any role in the rejection reactions although they exist in the blood. However, in most other botryllid ascidians, MCs participate actively in their allorejection reactions. From blood vessels, they infiltrate into the tunic and degenerate there to form a necrotic area. Nevertheless, the degrees of their participation are different among botryllid species. In the allorejection of *Botrylloides*, called "subcuticular rejection"(Hirose *et al.*, 1997), the number of MCs infiltrating into the tunic is much fewer than that in the case of allorejection in *Botryllus schlosseri* or *Botryllus. primigenus*.

The MCs in some ascidians have phenoloxidase (PO) activity, which is one of the most common enzymatic activities in invertebrate immune responses. In solitary ascidians, it contributes to a cellular defense reaction (Chaga, 1980; Smith and Söderhäll, 1991; Jackson *et al.*, 1993; Akita and Hoshi, 1995; Arizza *et al.*, 1995; Parrinello, 1995; Hata *et al.*, 1998).

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Table 1. Characteristics of the allorejection reactions in the six colonial ascidians.

	Stages of allorecognition	Hemocyte types which mainly participate in the rejection reaction	Cut surface contact	Reference
S. reptans	partial dissolution of cuticle	morula cells	rejection	Mukai and Watanabe (1974) Shirae *et al.* (1999)
B. scalaris	ampullar fusion	phagocytes	rejection	Saito and Watababe (1982) Saito *et al.* (1994) Shirae *et al.* (1999)
B. primigenus	ampullar penetration	morula cells	rejection	Oka and Watanabe (1975, 1960) Tanaka and Watanabe (1973) Mukai and Watanabe (1974) Taneda and Watanabe (1982a, 1982b)
B. schlosseri	partial dissolution of cuticle	morula cells	rejection	Bancroft (1903) Sabbadin (1962) Mukai and Watanabe (1974) Rinkevich (1992) Rinkevich *et al.*(1994) Saito *et al.* (1994)
B. simodensis	partial dissolution of cuticle (subcuticular rejection)	morula cells	rejection	Hirose *et al.* (1990)
B. fuscus	partial dissolution of cuticle (subcuticular rejection)	morula cells	fusion (surgical fusion)	Hirose *et al.* (1994) Hirose *et al.* (1997)

Cammarata *et al.* (1997) showed that PO activity leads to the production of superoxide which contributes to cytotoxicity in the solitary ascidian *Styela plicata*. Furthermore, Ballarin *et al.* (1993, 1994, 1995, 1998) demonstrated that, in *B. schlosseri*, PO of MCs contributes to cytotoxicity and its activity increases both in the allorejection area between incompatible colonies and in hemocytes cultured with incompatible blood plasma. Therefore, PO of MCs seems to be a key enzyme in the allorejection reactions of botryllids.

In the present study, we tried to demonstrate that differences in the ratio of MCs to total hemocytes and in the levels of PO activity in the MCs are related to the variation in allorejection reactions among botryllids. For this study, six colonial ascidians were used: *B. scalaris*, *B. primigenus*, *B. schlosseri*, *Botrylloides simodensis*, *Botrylloides fuscus*, and *Symplegma reptans*. These five botryllids show allorejection reactions different from one another (Table 1). *S. reptans* of the family Styelidae, a sister family of Botryllidae (Berrill, 1936), is the species most similar morphologically to botryllids among the styelids. This species shows an allorejection reaction similar to that of *B. schlosseri* (Shirae *et al.*, 1999).

MATERIALS AND METHODS

Animals

Colonies of *B. scalaris*, *B. primigenus*, *B. schlosseri*, *B. simodensis*, *B. fuscus* and *S. reptans* were collected in the vicinity of Shimoda (Shizuoka Prefecture, Japan) and in Uranouchi Inlet near the Usa Marine Biological Institute of Kochi University. Attached to glass slides, they were reared in culture boxes immersed in Nabeta Bay near the Shimoda Marine Research Center, University of Tsukuba. Colonies which grew well were used for the experiments.

Histochemical analysis of PO activity in hemocytes

Histochemical analysis of PO activity was performed using the method developed by Ballarin *et al.* (1995) with some modifications. The ascidian colonies were washed with filtered seawater (FSW) and immersed in FSW containing 10 mM L-cysteine for 5 min to prevent hemocytes from clotting during the collection of blood. After the FSW containing L-cysteine had been wiped up gently from the surfaces of the colonies, their vascular vessels were pricked with tungsten needles and the blood oozing through the prick-holes was collected. The collected blood was mixed with FSW, and hemocytes in the suspension were mounted on a glass slide coated with poly-L-lysine. This sample was then incubated in a moist chamber for 5 min to allow the hemocytes to adhere to the slide. The hemocytes on the slide were fixed with 1% glutaraldehyde in FSW containing 1% sucrose for 30 min at 4°C, and washed with FSW containing 1% sucrose. The fixed hemocytes were pre-incubated for 5 min at room temperature in 1 mM Na-cacodylate buffer (pH 8.5) containing 5 mM $CaCl_2$ (CAB-Ca), and incubated for 10 min at room temperature in a reaction mixture containing CAB-Ca plus 4% N, N'-dimethylformamide, dihydroxyphenyl-L-alanine (L-DOPA)-saturated CAB-Ca and 20.7 mM 3-methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH) in the ratio of 5: 2: 3. Then, these hemocytes were washed with CAB-Ca and examined under a Nikon optiphoto light microscope.

Assay of PO activity in hemocytes

Colonies were stripped from the glass slides on which they were cultured. After removal of detritus from the colony surface, the colonies were immersed in FSW containing 10 mM L-cysteine for 5 min to prevent aggregation of hemocytes and were cut into strips. Blood oozing from the cut surfaces of these strips was collected and stored in a microcentrifuge tube on ice. The blood was then centrifuged at 780×g for 15 min. The pellet was resuspended in FSW to obtain a hemocyte suspension at a cell concentration of 10^9 cells/ml. A 20 μl portion of the hemocyte suspension was mixed with 20 μl of 0.1% Tween 20 in phosphate buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, pH 7.2) and 160 μl of PBS on ice. This specimen was used as a "hemolysate" in the following assays.

To measure the PO activity in the hemolysate, the method used by Winder and Harris (1991) and Ballarin *et al.* (1998) was employed with some modifications. A 20 μl portion of the hemolysate was mixed with a reaction mixture consisting of 490 μl of PBS, 200 μl of L-DOPA-saturated PBS, and 290 μl of 20.7 mM MBTH in PBS containing 4%

of N, N'-dimethylfolmamide at 25°C, and, one minute after mixing, the absorbance at 505 nm was read by means of a Shimadzu UV-1200 spectrophotometer. PO activity in the hemocyte sample from each colony was measured in triplicate. The average was taken to be representative of the hemocyte PO activity in each colony. For each species, six colonies were examined. Data are expressed as means ± standard error (SE). Differences among the data for these species were analyzed statistically by the Kruskal- Wallis test.

Experiments on inhibition of PO activity

The effects of the following reagents on the PO activity of the hemolysate were investigated: the PO inhibitors Na-benzoate (20 mM) and tropolone (2 mM), the antioxidants L-cysteine (2 mM) and ascorbic acid (2 mM), and the scavenger enzymes superoxide dismutase (SOD) (120 U/ml) and catalase (140 U/ml). In order to evaluate the inhibitory effects on PO activity, these reagents were added to the reaction mixture described above. Then, the mixture was incubated for 10 min at 25°C, and its absorbance at 505 nm was measured. As a control, a mixture without any of these reagents added was used. In this assay, for each species, from three to six colonies were examined. The decrease in absorbance was calculated for each colony, and the average of the data for three colonies was taken to be representative of the inhibitory effect of a particular reagent on the PO activity in each species. Data are expressed as means ± standard deviation (SD). Significance was determined using the Wilcoxon Matched-Pairs Signed-Ranks Test.

Ratio of cell types among total hemocytes

Ascidian colonies were washed with FSW and fixed with 10% formaldehyde in FSW, pH 7.4, at 4°C overnight. After that, they were rinsed with FSW. Vascular vessels of the colony were then cut with a razor blade, and the blood oozing through the cut surface was collected with a micropipette and smeared on glass slides. After rinsing with FSW, the hemocytes were counted under a light microscope to obtain the ratio of each hemocyte type to total hemocytes (at least 200 hemocytes were counted per slide). Five colonies were examined for each species.

PO activity in the allorejection area

Colonies undergoing rejection were washed with FSW and fixed with 2% glutaraldehyde in FSW containing 1% sucrose for 1 hr at 4°C. Then, they were washed again with FSW containing 1% sucrose. These fixed colonies were incubated for 2-3 min at room temperature in a mixture consisting of CAB-Ca plus 4% N, N'-dimethylformamide, L-DOPA-saturated CAB-Ca and 20.7 mM MBTH in the same ratio as that used in the histochemical study of hemocytes. The colony parts, stained dark pink due to accumulation of dopaquinones, were examined under a Nikon optiphoto light microscope and an Olympus IX-70 inverted microscope. As a control, colonies undergoing fusion were also examined.

RESULTS

Morphology and PO activity of the hemocytes in the colonial ascidians

We classified the hemocytes of the six ascidians examined here into seven types principally, on the basis of the previous studies (Goodbody, 1974; Wright, 1981; Rowley *et al.*, 1982; Ballarin *et al.*, 1994), 1) hemoblasts, 2) hyaline amebocytes, 3) signet ring cells, 4) macrophages, 5) granular leukocytes, 6) pigment cells, and 7) MCs (Fig. 1).

The hemoblasts (Fig. 1a) were small spherical cells, 4–5

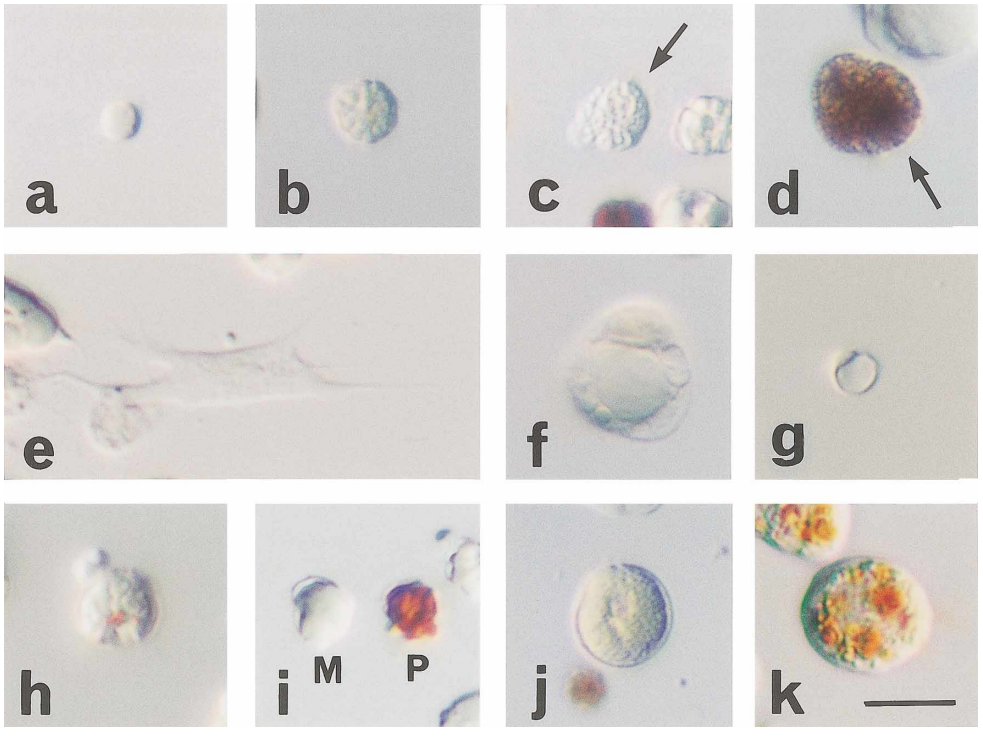

Fig. 1. Morphology of living hemocytes incubated in FSW.
a, hemoblast in *B. simodensis*; b, morula cell in *B. scalaris*; c, granular leukocytes (arrow) in *B. primigenus*; d, pigment cell in *B. fuscus*; e, hyaline amebocyte in *B. simodensis*; f, macrophage in *B. simodensis*; g, signet ring cell (arrow) in *B. fuscus*; h, morula cell in *S. reptans*; i, morula cell and pigment cell in *B. primigenus*; j, granular leukocyte in *B. fuscus*; k, granular leukocyte in *B. scalaris*. M, morula cell; P, pigment cell. Scale bar=10 μm.

μm in diameter, with a large nucleus. The hyaline amebocytes (Fig. 1e) were flat and variable in shape, about 6–20 μm in diameter, and they contained small granules. The macrophages (Fig. 1f) were large spherical cells, 12–15 μm in diameter, with a few large vacuoles filled with ingested materials. The signet ring cells (Fig. 1g) were spherical cells, about 5–10 μm in diameter, and they contained a vacuole which pushed the cytoplasm and the nucleus to the periphery. The granular leukocytes (Fig. 1c) were principally spherical in shape, about 6–8 μm in diameter, and they were filled with many small granules, about 1μm or less in diameter. In addition to this common type of granular leukocyte, in the two *Botrylloides* species, *B. simodensis* and *B. fuscus*, a specific type of granular leukocyte, which is larger than the common type in the six ascidians (about 10μm in diameter) was often seen among their hemocytes (Fig. 1j). In *B. scalaris*, another species-specific type of granular leukocyte was found (Fig. 1k). They were about 12–20 μm in diameter and had small red granules (about 1–2 μm in diameter) and white granules that appeared to be thick and short sticks and these were 5–8 μm long in the major axis in both fixed and fresh samples (data not shown). The pigment cells (Fig. 1d) were large vacuolated cells, 12–

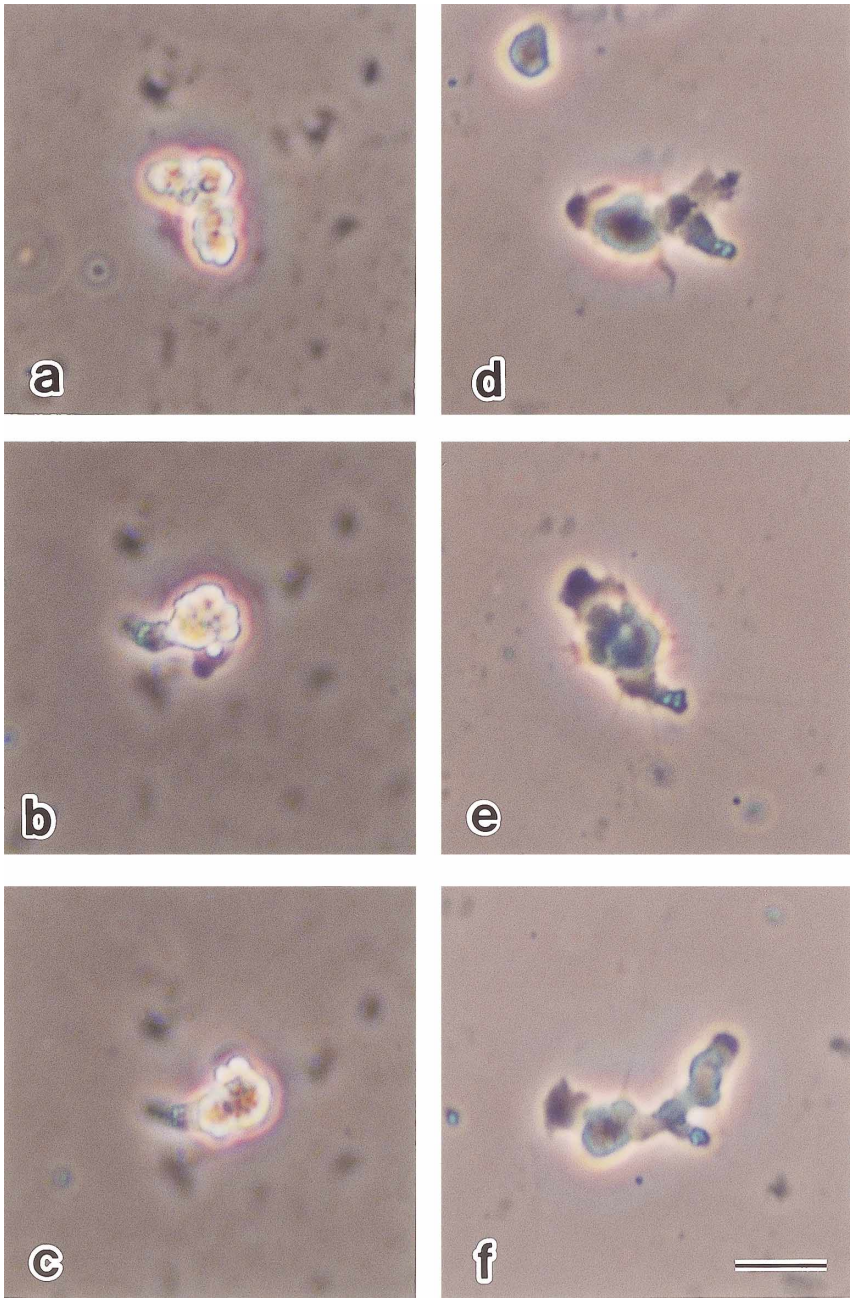

Fig. 2. Moving morula cells of two *Botrylloides*. Living morula cells changed their shapes during incubation in FSW, as seen by phase-contrast microscopy. a, morula cell in *B. simodensis*; b, after 20 sec; c, after 40 sec; d, morula cell in *B. fuscus*; e, after 20 sec; f, after 40 sec . Scale bar=10 μm.

15 μm in diameter, with one or a few large vacuoles (6–12μm in diameter) containing many small granules about 0.5 μm in diameter. The color of these granules determines the color of the colony (Hirose *et al*., 1998). Only in *B. primigenus*, there was a type of the pigment cells resembling the MC in size and shape, and the vacuoles in these cells were filled with a purple-colored material (Fig. 1i).

The MCs of the three botryllids, *B. schlosseri*, *B. primigenus*, and *B. scalaris*, were morphologically very similar to one another. Generally, the MCs are 6–10 μm in diameter and they were filled with many vacuoles about 2 μm in diameter (Fig. 1b). In *B. simodensis* and *B. fuscus*, the vacuoles of the MCs were irregular in size (2–8μm in diameter). Especially, the MCs of *B. fuscus* formed many long pseudopodia and showed various shapes during incubation (Fig. 2). The MCs of *S. reptans* contained several red granules, which were less than 0.5 μm in diameter (Fig. 1h).

In all of the ascidians, most MCs showed dark pink coloration in the L-DOPA-MBTH assay (Fig. 3). The degree of coloration dependent on PO activity varied among the MCs in each species. In *B. scalaris*, the species-specific granular leukocytes also showed weak PO activity (Fig.3b). In *B.*

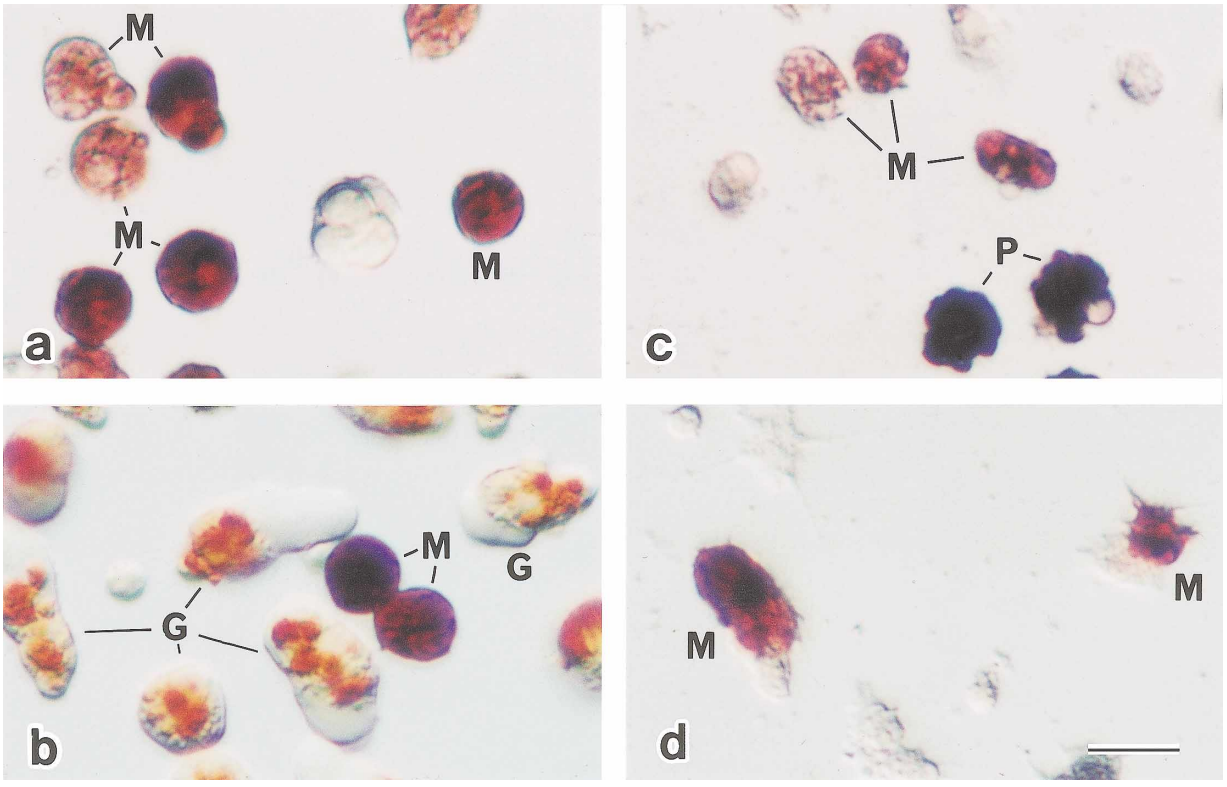

Fig. 3. Phenoloxidase activity in hemocytes as determined by the L-DOPA-MBTH assay. Morula cells of all botryllids showed dark-pink coloration. a, *S. reptans*; b,*B. scalaris*. The granular leukocytes of *B. scalaris* with pigmentation also showed such coloration in their cytoplasm. c, *B. primigenus*. In *B. primigenus*, the pigment cells resemble morula cells morphologically and their vacuoles include purple-colored components. In this assay, the pigment cells did not show any increase in dark pink coloration. d, *B. fuscus*. M, morula cell; G, granular leukocyte; P, pigment cell. Scale bar=10 μm.

Table 2. Effects various reagents on phenoloxidase activity

	Na-benzoate 20 mM	tropolone 2 mM	ascorbid acid 2 mM	cysteine 2 mM	SOD 120U/ml	catalase 140U/ml
			Inhibition(%)			
S. reptans	97.5±0.8%	99.3±0.8%	98.9± 0.8%	99.1±0.4%	20.9± 8.6%	21.1±5.4%
B. fuscus	95.0±3.8	96.4±3.7	93.6± 5.8	98.0±2.0	8.3± 5.1	–
B. simodensis	97.2±3.1	98.9±0.6	97.2± 2.5	98.4±0.5	36.3±14.6	–
B. schlosseri	98.3±0.0	99.1±0.1	98.4± 0.8	98.7±0.5	24.3±24.0	–
B. primigenus	96.8±2.2	97.3±3.6	98.4± 1.5	97.3±2.6	29.7±14.7	–
B. scalaris	88.9±4.6	91.7±8.1	81.5±23.6	97.9±4.3	35.7±11.5	–

Data are expressed as means ± SD. Bars mean no reduction of absorbance in the experiments.

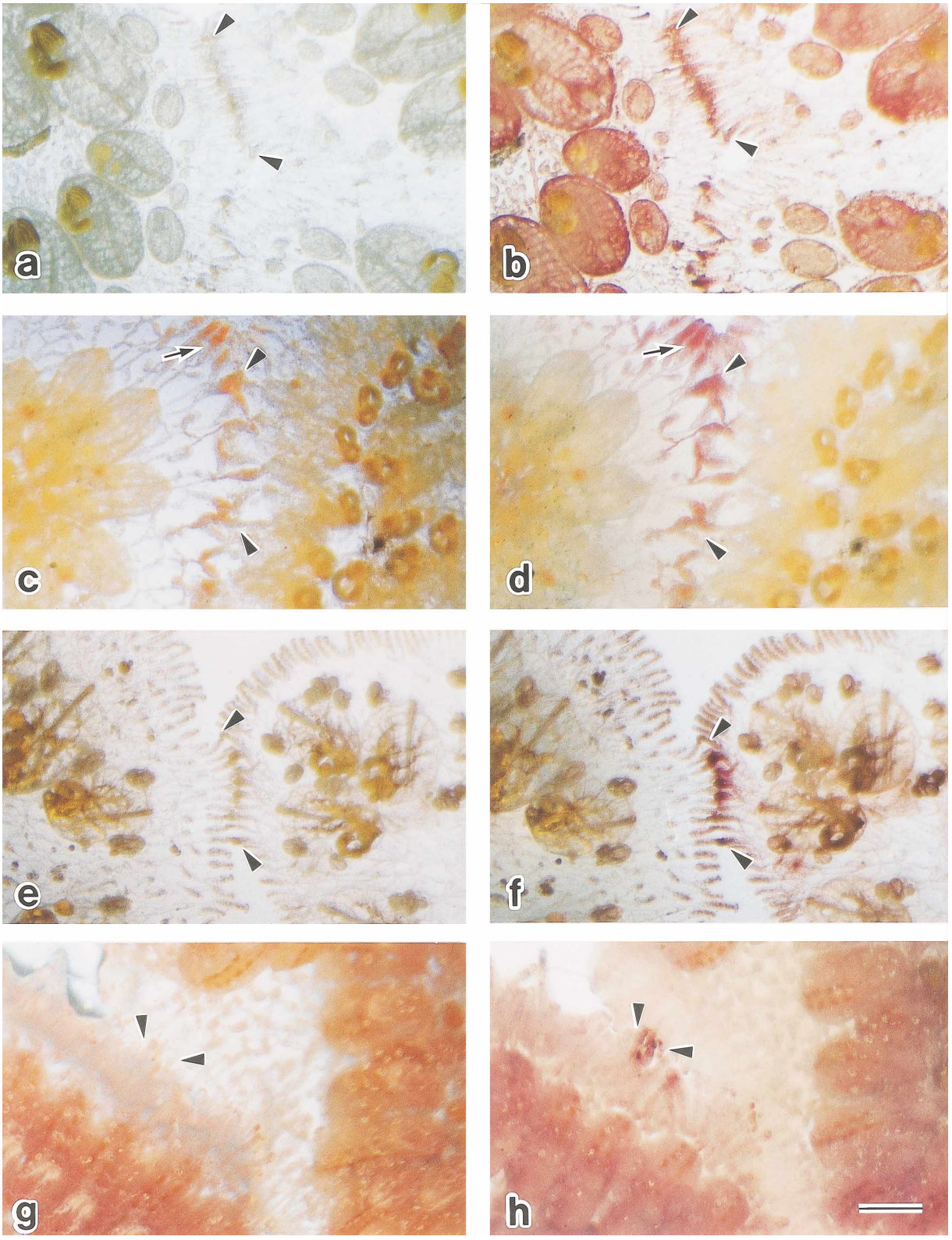

Fig. 4. PO activity in the allorejection reaction area in colonial ascidians as determined by the L-DOPA-MBTH assay after fixation in 2% glutaraldehyde. a, *S. reptans* colonies before treatment; b, *S. reptans* colonies after treatment; c, *B. scalaris* colonies before treatment; d, *B. scalaris* colonies after treatment; e, *B. primigenus* colonies before treatment; f, *B. primigenus* colonies after treatment; g, *B. fuscus* colonies before treatment; h, *B. fuscus* colonies after treatment. Arrowheads indicate the allorejection sites. Arrows indicate ampullae not undergoing rejection in *B. scalaris*. Scale bar=1mm.

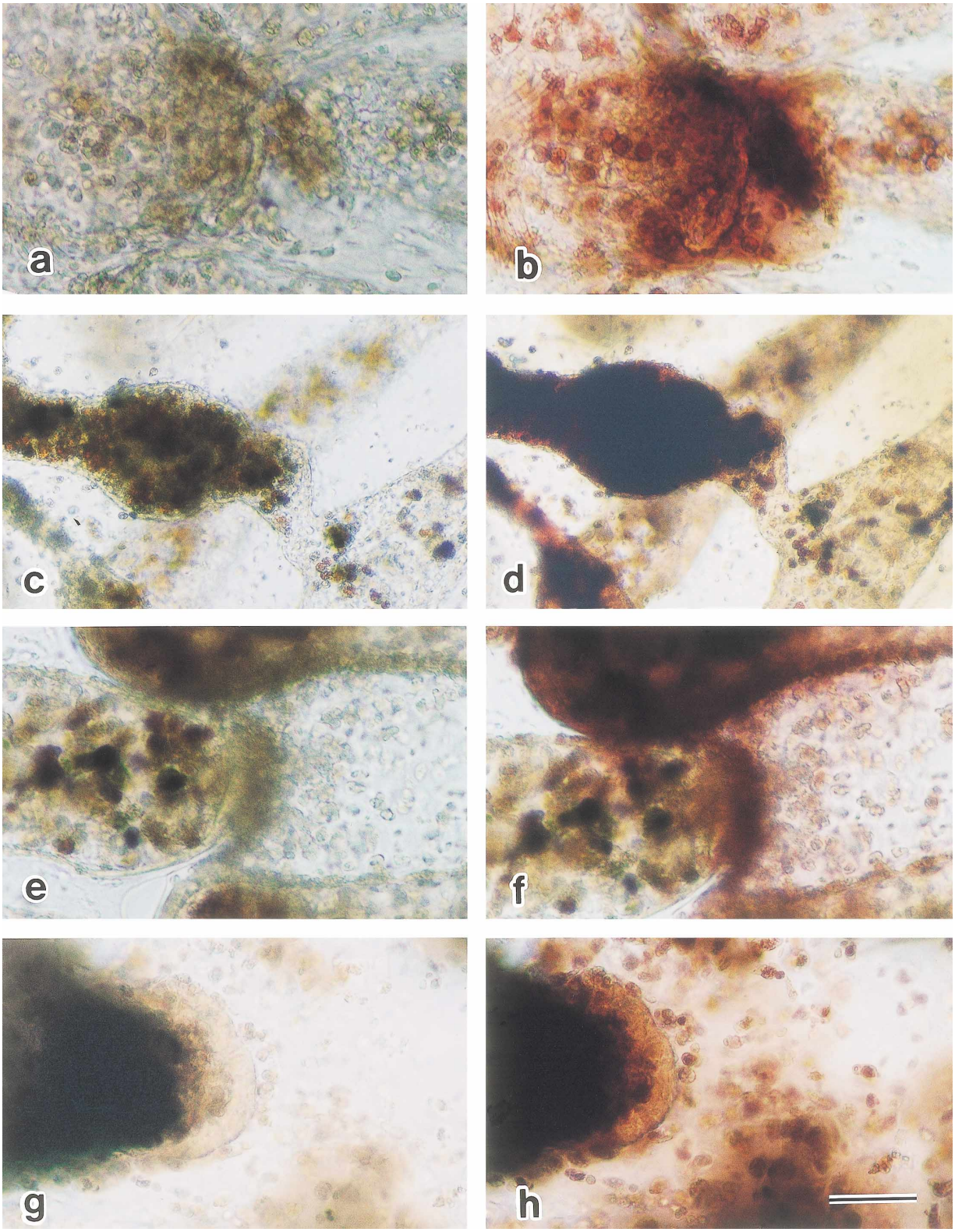

Fig. 5. Allorejection reaction area as determined by the L-DOPA-MBTH assay, at high magnification. a, *S. reptans* ampullae before treatment; b, *S. reptans* ampullae after treatment; c, *B. scalaris* ampullae before treatment; d, *B. scalaris* ampullae after treatment; e, *B. primigenus* ampullae before treatment; f, *B. primigenus* ampullae after treatment; g, *B. fuscus* ampulla before treatment; h, *B. fuscus* ampulla after treatment. Scale bar=50 μm.

primigenus, the MC-like pigment cells did not show further coloration by dopa-quinones (Fig. 3c).

Ratios of MCs to total hemocytes

The ratio of MCs to hemocytes differed among the six ascidians. *B. primigenus* showed the highest MC ratio (54.1± 8.1%), followed by *S. reptans* (46.8±10.0%), *B. simodensis* (39.9 ±11.4%), *B. schlosseri* (37.5±5.2%), and *B. scalaris* (14.6 ±8.3%) in that order, and the lowest MC ratio was that observed in the case of *B. fuscus* (8.5±2.2%). In *B. scalaris*, the ratio of granular leukocytes, which showed little PO activity, to hemocytes was 24.0±11.8%.

PO activity of hemocytes

In the six ascidians studied here, their hemolysates, prepared from the same number of hemocytes in each instance, showed significantly different levels of PO activity (ΔA_{505} / min $\times$100) in the first minute after mixing with the reaction mixture ($p<0.001$). *B. schlosseri* showed the highest level of activity (22.0±2.5) followed by *S. reptans* (17.7±2.3), *B. primigenus* (5.6±2.4), *B. simodensis* (3.2±0.7), and *B. scalaris* (0.8± 0.1) in that order. The lowest level of activity was that observed in the case of *B. fuscus* (0.3±0.1).

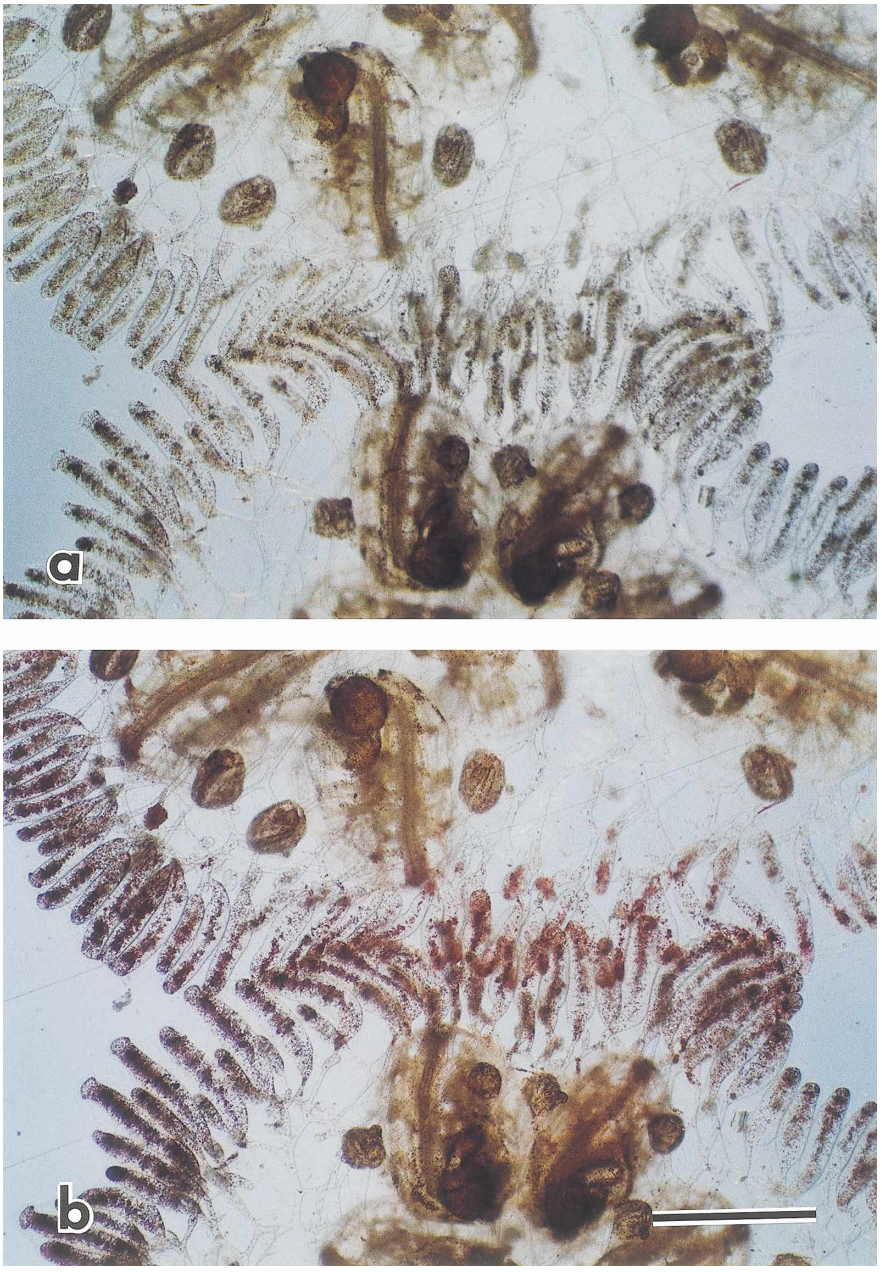

Fig. 6. Fusion reaction area as determined by the L-DOPA-MBTH assay in *B. primigenus*. a, colonies before treatment; b, colonies after treatment. Scale bar=1mm.

Effects of inhibitors on PO activity

The effects of six reagents on the PO activity in the six ascidians are summarized in Table 2. The PO activity in all six species was almost completely inhibited by Na-benzoate (20 mM) or tropolone (2 mM), and was fully inhibited by L-cysteine (2 mM) or ascorbic acid (2 mM; $p<0.05$). Upon incubation with SOD (120 U/ml), there was a significant decrease in absorbance at 505nm ($p<0.05$), whereas the other scavenger enzyme tested, catalase, had no effect on the PO activity in the botryllids even at a high concentration, 140 U/ ml. Only in the case of *S. reptans*, catalase (140 U/ml) induced a significant decrease in absorbance at 505nm ($p<0.05$), similar in degree to that observed with SOD (120 U/ml). The effect of each of these reagents on the PO activity in MCs was similar for all of the botryllids. Histochemical observations showed that the coloration by dopa-quinone in the MCs of all of the ascidians and in the granular leukocytes of *B. scalaris* was inhibited by these PO inhibitors and antioxidants. The coloration in the hemocytes was inhibited by the reagents at concentrations ten times as high as those required in the case of the hemolysate (data not shown).

PO activity in the allorejection areas

Even in the fixed normal colonies, MCs showed strong PO activity upon incubation with the reaction mixture containing L-DOPA and MBTH. Therefore, in this experiment, the incubation time was set at 2–3 min, at room temperature, to obtain better contrast between the rejection area and the non-rejection area (Fig. 4). In *S. reptans* and the four botryllids other than *B. scalaris*, a higher level of PO activity was observed in the allorejection area than in the non-rejection area or the fusion area between compatible colonies. The dark pink colored area was consistent with the range of the rejection area in each species, like the result obtained for *B. schlosseri* as shown by Ballarin *et al*. (1995). PO activity outside of MCs was also found in the tunic in the rejection area, especially around the MCs that infiltrated into the tunic (Figs. 5b and 5f). In *B. scalaris*, PO activity was observed only inside of the vascular vessels, and the change in coloration of the hemocyte aggregates in the ampullae undergoing rejection was not marked (Figs. 4d and 5d). In *B. fuscus*, although the ampullae undergoing rejection and the infiltrating MCs were dark pink in color, PO activity was not observed in the tunic around the infiltrating MCs (Figs. 4h and 5h). In the fusion reactions between syngeneic colonies in the case of all of the ascidians, an increase in PO activity was not evident in the fusion area (Fig. 6).

DISCUSSION

This comparative study of hemocyte morphology among botryllid ascidians and *S. reptans* has made clear that their hemocytes consist of the same cell types basically. However, in a few cases, hemocytes with genus- or species-specific characteristics were found and these might serve as marker hemocytes in particular species. In *B. primigenus*, the pigment cells resembled MCs morphologically except for the purple-coloration of their granules. However, these pigment cells did not display any PO activity. As observed under the light microscope, these cells were mostly deposited under the epithelium of the zooids and they contributed to the purple color of the zooids (data not shown). These cells were also seen in the ampullae, although they never infiltrated into the tunic in the allorejection reaction. The pigment cells and the MCs surely have a different function and a different origin in terms of cell lineage. Therefore, we should be careful not to confuse these two types of hemocytes in the sectional specimens of *B. primigenus*.

The MCs of *B. fuscus* do not have sufficient granules to fill up the cytoplasm, and there is more cytoplasmic matrix than in the case of the MCs of other species. The MCs of this species form philopodia actively and move much during incubation in FSW. The MCs of *S. reptans* were very distinctive in terms of having red granules. However, because of the common morphological characteristics, they can be classified into the same hemocyte type. The MCs of the botryllids and *S. reptans* were found to have PO activity in their vacuoles, as in the case of *B. schlosseri* and some solitary ascidians (Ballarin *et al*., 1993; Jackson *et al*., 1993; Akita and Hoshi, 1995; Arizza *et al*., 1995). Therefore, PO activity is regarded as one of the common features of MCs in these ascidians. In *B. scalaris*, the granular leukocytes also showed weak PO activity in their cytoplasm. As the PO inhibitors and antioxidants had the same inhibitory effect on the PO activity of the granular leukocytes as that observed in the case of MCs, the PO enzymes present in these two types of hemocytes may be highly similar. In this species, when a colony is injured and its blood oozes out, the granular leukocytes immediately form aggregates to stop the blood leak (data not shown). The role of these hemocytes, especially in relation to the PO activity, should be further studied in *B. scalaris*.

Both the ratio of MCs to total hemocytes and the PO activity of the hemocytes varied among the ascidians examined, and the differences in these two factors in these ascidians were not parallel. The variation in PO activity among species is more conspicuous when the PO activity per MC is estimated. The level of PO activity in the hemocytes was highest in *B. schlosseri* and *S. reptans* among the ascidians examined. *B. simodensis* and *B. primigenus* showed a lower level of PO activity than *B. schlosseri*, but the ratio of MCs to total hemocytes was almost the same for both, and even higher than that in the case of *B. schlosseri*. In these four species, PO activity was observed only in the MCs. These results suggest that in *B. simodensis* and *B. primigenus*, the PO activity per MC is lower than that in *B. schlosseri*. In contrast, in *B. scalaris* and *B. fuscus*, both the MC ratio and the PO activity per MC were markedly lower than those in *B. schlosseri*. In *B. scalaris*, the granular leukocytes also had PO activity, although the level of activity was lower than that in the MCs as described above. Therefore, without the granular leukocytes taken into account, the level of PO activity in the MCs would be estimated as being much lower. In the other experiment

concerning PO activity, the characteristics of the PO in the six ascidians were found to be similar in terms of the effects of inhibitors, except for the weak inhibition by catalase in the case of *S. reptans*. These results suggest that in the hemocytes of the six ascidians, the difference in PO activity is attributable to the two factors, the ratio of MCs to total hemocytes and the PO activity per MC.

The PO activity might be related to the allorejection modes in colony specificity. In the compound ascidians examined here, the morphological processes of fusion and allorejection have already been described in detail. Table 1 is a list of the characteristics of the allorejection reactions that have been reported in five botryllids and *S. reptans*. A close correlation between the characteristics of the MCs and the allorejection reaction is obvious as follows. 1) *B. schlosseri* and *S. reptans*, which have hemocytes with a high level of PO activity, show a restricted allorejection reaction. In both species, the allorejection reaction is initiated after their ampullae come into contact, and MC infiltration and disintegration of these cells leads to severe necrosis around the ampullae undergoing rejection. 2) In *B. scalaris*, the behavior of hemocytes in the allorejection reaction is different from that in the case of the other botryllids and *S. reptans*. The allorejection reaction is initiated in the latest stage of the fusion process, after vascular fusion and blood exchange. In the allorejection reaction in this case, the MCs do not perform any noteworthy role. The blood exchange between two incompatible colonies, however, does not involve necrosis with disintegration of hemocytes. The low level of PO activity might affect this mild rejection reaction in *B. scalaris*. 3) *B. fuscus* and *B. simodensis* belong to the same genus *Botrylloides* (Monniot, 1988). Recent molecular analysis has demonstrated that these two species are more closely related to each other than to the other botryllids (Cohen *et al*., 1998). However, *B. fuscus* has a much lower proportion of MCs and also has a much lower level of PO activity than *B. simodensis*. Whereas they show the same mode of allorejection in the case of growing edge contact, these two species show opposite reactions in the case of contact between the cut surfaces of two incompatible colonies (Table 1). In *B. fuscus*, two incompatible colonies become fused without infiltration or disintegration of MCs (Hirose *et al*., 1994). In contrast, in *B. simodensis*, severe necrosis is caused by MC disintegration in the contact area (Hirose *et al*., 1990). The lower level of PO activity and the lower ratio of MCs to total hemocytes in the case of *B. fuscus* than those in the case of *B. simodensis* might be related to the occurrence of artificial fusion between two incompatible colonies.

Previously we demonstrated that the difference in allorejection modes might be due to difference in MC behavior, and in this study we showed that there is a correlation between the allorejection mode and the ratio of MCs to total hemocytes and their PO activity among botryllids. To understand the mechanisms responsible for the variation in allorejection modes in botryllids, further comparative studies will be necessary, such as studies on various factors related to the proPO system, MC infiltration, MC degranulation, and the allogeneic recognition system.

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