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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).
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, Botryllus simodensis, Botryllus 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 CaCl₂ (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-benzothiazolinonehydroxylcarbaldheyde (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 10⁶ 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% KH₂PO₄, 0.115% Na₂HPO₄, 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%
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of N, N'-dimethylformamide 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 trofolone (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 dopa-quinones, were examined under a Nikon optiphot 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
um in diameter, with a large nucleus. The hyaline amebocytes (Fig. 1e) were flat and variable in shape, about 6–20 um in diameter, and they contained small granules. The macrophages (Fig. 1f) were large spherical cells, 12–15 um in diameter, with a few large vacuoles filled with ingested materials. The signet ring cells (Fig. 1g) were spherical cells, about 5–10 um 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 um in diameter, and they were filled with many small granules, about 1um 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 10um 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 um in diameter and had small red granules (about 1–2 um in diameter) and white granules that appeared to be thick and short sticks and these were 5–8 um 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 um.](https://bioone.org/journals/Zoological-Science/0303-7865/article.aspx?doi=10.1093/zsc/42.12.884)
15 µm in diameter, with one or a few large vacuoles (6–12 µm 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 (2–8 µm 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.

![Image](https://bioone.org/journals/Zoological-Science/10.7894/0024-2489-23-10-925/fig3.jpg)

**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>
<thead>
<tr>
<th></th>
<th>Na-benzoate 20 mM</th>
<th>tropolone 2 mM</th>
<th>ascorbid acid 2 mM</th>
<th>cysteine 2 mM</th>
<th>SOD 120U/ml</th>
<th>catalase 140U/ml</th>
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<tr>
<td><strong>Inhibition (%)</strong></td>
<td></td>
<td></td>
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<tr>
<td>S. reptans</td>
<td>97.5±0.8%</td>
<td>99.3±0.8%</td>
<td>98.9±0.8%</td>
<td>99.1±0.4%</td>
<td>20.9±8.6%</td>
<td>21.1±5.4%</td>
</tr>
<tr>
<td>B. fuscus</td>
<td>95.0±3.8%</td>
<td>96.4±3.7%</td>
<td>93.6±5.8%</td>
<td>98.0±2.0%</td>
<td>8.3±5.1%</td>
<td>–</td>
</tr>
<tr>
<td>B. simodensis</td>
<td>97.2±3.1%</td>
<td>98.9±0.6%</td>
<td>97.2±2.5%</td>
<td>98.4±0.5%</td>
<td>36.3±14.6%</td>
<td>–</td>
</tr>
<tr>
<td>B. schlosseri</td>
<td>98.3±0.0%</td>
<td>99.1±0.1%</td>
<td>98.4±0.8%</td>
<td>98.7±0.5%</td>
<td>24.3±24.0%</td>
<td>–</td>
</tr>
<tr>
<td>B. primigenus</td>
<td>96.8±2.2%</td>
<td>97.3±3.6%</td>
<td>98.4±1.5%</td>
<td>97.3±2.6%</td>
<td>29.7±14.7%</td>
<td>–</td>
</tr>
<tr>
<td>B. scalaris</td>
<td>88.9±4.6%</td>
<td>91.7±8.1%</td>
<td>81.5±23.6%</td>
<td>97.9±4.3%</td>
<td>35.7±11.5%</td>
<td>–</td>
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Data are expressed as means ± SD. Bars mean no reduction of absorbance in the experiments.

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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. Allojection 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 (ΔA₅₀₅ / min ×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 troponine (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 pig-
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 Botryllioidea (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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