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Author: Saito, Yasunori

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Xenogeneic Rejection among Three Botryllids (Compound Ascidians)

Yasunori Saito*

Shimoda Marine Research Center, University of Tsukuba, Shimoda 5-10-1, Shizuoka 415-0025, Japan

ABSTRACT—Xenogeneic rejection was observed among colonies of three botryllids, *Botryllus scalaris*, *Botryllus primigenus*, and *Botrylloides simodensis*. Allogeneic recognition occurs in each of these species, but the manner of allogeneic rejection differs among them. We studied xenogeneic rejection reactions among these species under the following conditions: colony contact at natural growing edges, colony contact at artificially cut surfaces, and injection of xenogeneic blood plasma into a vascular vessel. In the first two cases, xenogeneic rejection occurred only in *Botryllus primigenus* and *Botrylloides simodensis*. The features of that xenogeneic rejection were similar to those of allogeneic rejection in each of these two botryllids. Injection of xenogeneic blood plasma induced responses similar to those of allogeneic rejection in all three botryllids. It is interesting to note that colonies of *Botryllus scalaris* never showed any response against injected blood plasma from allogeneic incompatible colonies, unlike the responses seen in colonies of the other two botryllids under the same conditions. On the basis of these results, the relationship between allogeneic and xenogeneic rejection in botryllids is discussed.

Key words: ascidian, botryllid, colony specificity, xenogeneic rejection, allogeneic rejection

INTRODUCTION

Ascidians belong to the phylum Chordata, as do vertebrates, and it is generally believed that vertebrates evolved directly from this group (Berrill, 1955). Therefore, the study of self-nonself recognition in this group will offer some interesting insights about the origin of the vertebrate immune response. Certain species of compound ascidians have a capacity for allogeneic recognition called *colony specificity* (Bancroft, 1903; Oka and Watanabe, 1957; Mukai and Watanabe, 1974; Koyama and Watanabe, 1981; 1982; Saito and Watanabe, 1982; Hirose *et al*., 1988, 1997; Boyd *et al*., 1990; Shirae *et al*., 1999; Okuyama and Saito, 2001, 2002; Okuyama *et al*., 2002). Colony specificity is manifested by fusion incompatibility between two allogeneic colonies and is controlled by a single, multiallelic, Mendelian locus (Oka and Watanabe, 1960; Sabbadin, 1962) that resembles loci within the vertebrate major histocompatibility complex (MHC; Scofield *et al*., 1982). It is known that blood components participate in colony specificity (Mukai, 1967; Tanaka, 1973; Taneda and Watanabe, 1982b, 1982c; Saito and Watanabe 1984; Ballarin *et al*., 1995; Rinkevich *et al*., 1998). However, at present, research is ongoing to determine the details of the mechanism of colony specificity.

In contrast to the many studies of allogeneic rejection, there have been only a few reports about xenogeneic rejection in compound ascidians (Mukai and Watanabe, 1974; Rinkevich *et al*., 1994; Hirose *et al*., 2002). It has commonly been believed that most compound ascidians do not show any rejection against xenogeneic colonies, similar to the lack of response when they contact various substrata during their growth, such as stones, rocks, or seaweed (Mukai and Watanabe, 1974). However, the recent study showed the occurrence of xenogeneic rejection between closely related species (Hirose *et al*., 2002). In the present study, we also observed xenogeneic rejection among three Japanese botryllids, *Botryllus scalaris*, *Botryllus primigenus*, and *Botrylloides simodensis*. They are not so closely related to one another (Saito *et al*., 2001), but they live in the same habitat and probably come into contact with each other frequently under natural conditions. In these three botryllids, the occurrence of colony specificity (allogeneic recognition) has been studied. In fact, their mechanisms of allogeneic recognition are considered to be different from each other because their observed manners of allogeneic rejection are different from each other (Figs. 1 and 2; Saito *et al*., 1994). In the case where two colonies are brought into contact at their natural growing edges, the process of fusion is the same in all botryllids, and the allogeneic rejection appears to interfere with

* Corresponding author: Tel. +81-558-23-6358;
FAX. +81-558-22-0346.
E-mail: saito@kurofune.shimoda.tsukuba.ac.jp

the progress of fusion process. In *Botrylloides simodensis* the interference occurs at Stage 1–2 of the fusion process, in *Botryllus primigenus* at Stage 3–4, and in *Botryllus scalaris* at Stage 4-5 (Fig. 1). In the case of artificial cut surface contact, *Botrylloides simodensis* shows a very intensive rejection reaction differently from the rejection reaction in the case of growing-edge contact, though in the other two species the rejection reactions are almost the same between those two cases (Fig. 2). If such differences are reflected in their xenogeneic rejection reactions or if there is some relationship between allogeneic and xenogeneic rejection, research on xenogeneic rejection will provide valu-

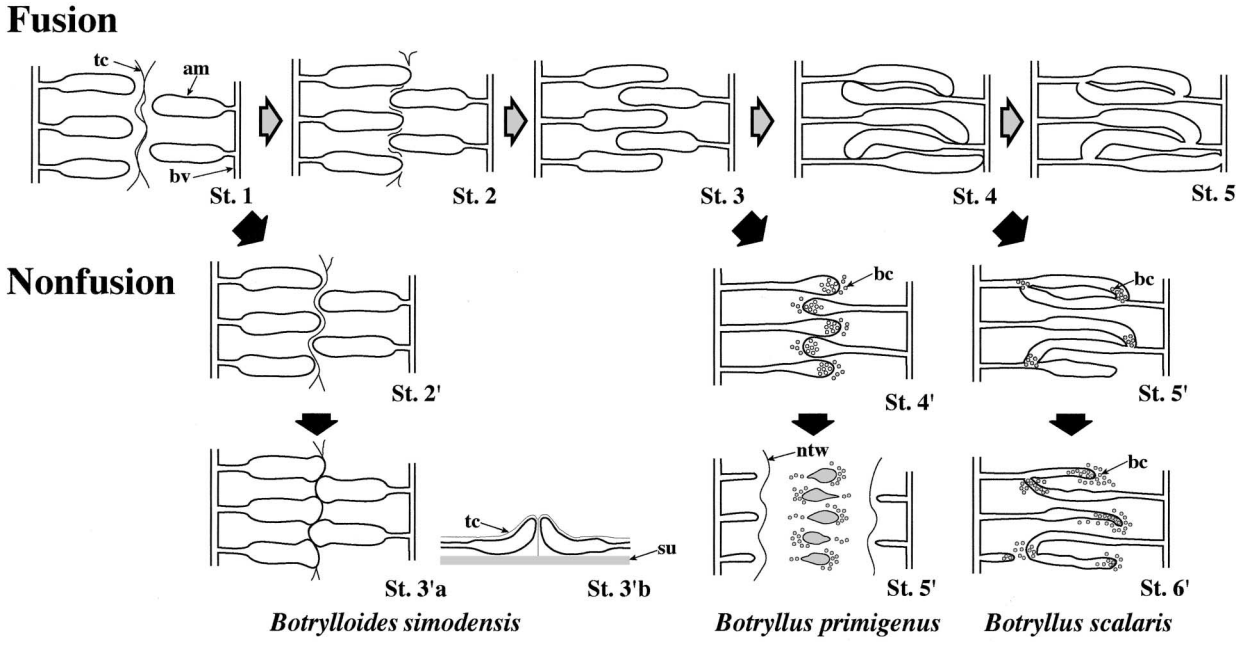

Fig. 1. Scheme showing the processes of fusion and nonfusion (allogeneic rejection) between allogeneic colonies in the case of growing-edge contact. The process of fusion (from Stage [St.] 1 to St. 5) is the same in all botryllids. The nonfusion process of each species is different among these three botryllids. am, ampulla; bc, blood cell; bv, blood vessel; ntw, new tunic wall; tc, tunic cuticle; su, substratum.

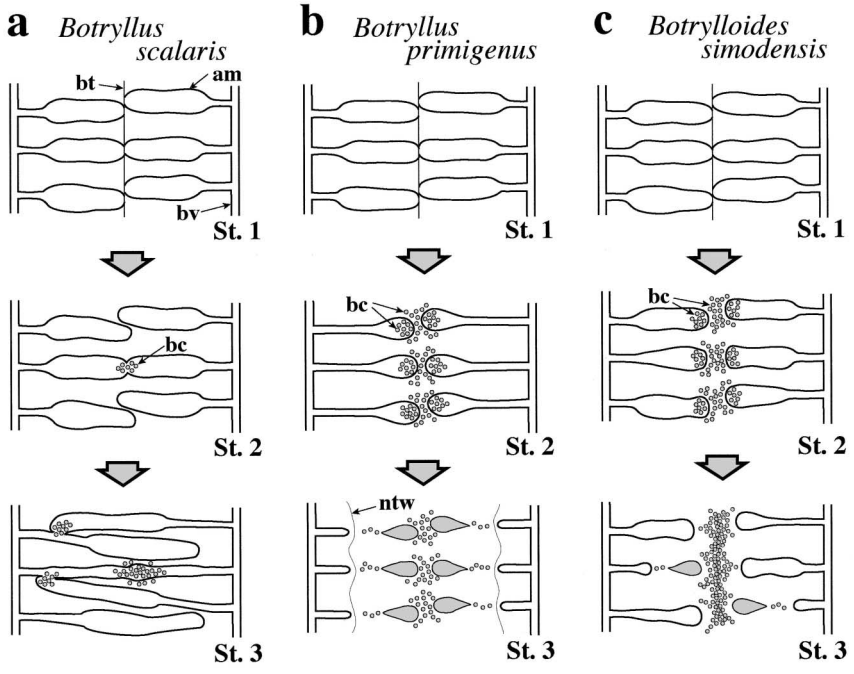

Fig. 2. Scheme showing the processes of allogeneic rejection (nonfusion) in the case of cut surface contact in *Botryllus scalaris* (a), in *Botryllus primigenus* (b), and in *Botrylloides simodensis* (c). am, ampulla; bc, blood cell; bt, boundary of tunic; bv, blood vessel; ntw, new tunic wall; St., Stage.

able information to add to our understanding of the self-nonself recognition systems in compound ascidians. In the present study, the processes and features of xenogeneic rejection reactions among *Botryllus scalaris*, *Botryllus primigenus*, and *Botrylloides simodensis* are reported, and the relationship between allogeneic rejection and xenogeneic rejection in botryllids is discussed.

MATERIALS AND METHODS

Animals

Live colonies of three Japanese botryllids, *Botryllus scalaris*, *Botryllus primigenus,* and *Botrylloides simodensis*, were used. In a colony of any of these three botryllids, the individual blastozooids are grouped into ladder- or star-shaped systems and are connected to one another by a ramifying network of vascular vessels, which terminate in sausage-shaped vascular ampullae at the periphery of the colony. Many colonies of these three species were collected from the rocky shore in the vicinity of Shimoda Marine Research Center, University of Tsukuba, Shizuoka Prefecture, in central Japan. The collected colonies were fastened to glass microscope slides with cotton thread. They were cultured in boxes immersed in seawater near the center of the cove where the environment was most natural and undisturbed. After culturing for 2 weeks, colonies that grew well on the glass slides were selected, and each colony was cut into several pieces to make subcolonies. Each colony piece was fastened to a glass slide or glass plate (82×107 mm) with cotton thread and then cultured again in the culture box in the cove.

Observations on reactions between two xenogeneic colonies

The cut colony assay was used in these experiments. A colony piece about 1 square centimeter in size was cut from each colony of two species with a razor blade. The two pieces were placed in contact with each other on a glass slide. In this experiment, two conditions were used to examine the effect of the tunic surfaces: In the first condition, contact occurred between the natural, growing edges of the two colony pieces. In the second condition, contact occurred between the cut surfaces of the two colony pieces. The cut surface was prepared by cutting off a very narrow part from the growing edge of a colony piece in order to remove the tunic surface layer. As *Botrylloides simodensis* shows quite different allogeneic rejection reactions between these two conditions (Figs. 1 and 2), these two conditions were used here. The two pieces placed in juxtaposition on a glass slide were kept in a moisture chamber. After 1 or 2 hr, the two pieces became attached to the glass slide, and this slide was kept in the laboratory aquarium with continuously renewed, running seawater. Observations were carried out under a binocular stereomicroscope (Nikon SMZ-10). Six or seven strains in each species were used for these experiments. In the case of growing-edge contact, about 30 pairs of colony pieces were observed in each combination among the three botryllids, while in the case of cut surface contact about 20 pairs were done in each combination.

Preparation of blood plasma for the microinjection assay

Large colonies that had grown well on the glass plates were used for these experiments. A colony was stripped off the glass plate using a razor blade. After removing the debris and hydrozoans adhering to the colony surface, the colony was washed with filtered seawater (FSW). The FSW remaining on the colony surface and in the branchial sacs of the zooids was soaked up with filter paper. Then, the colony was cut into strips about 3 to 5 mm wide, and blood droplets that exuded from the cut surfaces of the strips were collected in the cold (about 4°C). The collected blood was centrifuged at 12,000 rpm at 4°C for 20 min to remove cellular debris. The resultant clear, cell-free supernatant was used as intact blood plasma. About one milliliter of blood plasma was gained from a big colony, about 80×100 mm in size, of *Botrylloides simodensis*, but in both *Botryllus scalaris* and *Botryllus primigenus* the volume of blood plasma gained from the same size colony was less than 0.5 ml.

Preparation of recipient colonies for the microinjection assay

A large colony grown on a glass slide was cut into several small colonies, each of which was a square of about 5×5 mm, consisting of about 10 zooids. The small, square colonies on the glass slides were returned to the culture box in the cove. About 3 days later, the margins of most of these small colonies were fringed with newly formed ampullae. The small colonies fringed with good ampullae were used as recipients for the microinjection assay.

Microinjection assay for rejection reaction against blood plasma

The *in vivo* bioassay system developed by Taneda and Watanabe (1982b) was used. For each sample of blood plasma, about 7 μl was injected into each recipient colony through an ampulla with a micropipette (approximately 50 μm in tip diameter). As a control, the same volume of FSW was injected into a recipient colony. After the injection, the recipients were kept in the laboratory aquarium with continuously renewed, running seawater. Four hr after injection, they were observed under a binocular stereomicroscope to assess the intensity of the rejection reaction against injected blood plasma.

To determine the effects of blood plasma injection, the following four criteria, defined in the former experiments on allogeneic rejection (Taneda and Watanabe, 1982b; Saito and Watanabe, 1984), were applied:

(–): No harmful effect was induced except that the ampulla was injured by the injection.

(+): Slightly harmful effects, such as weak contraction of ampullae and a slight increase in opacity of ampullae, occurred.

(++): Contraction of ampullae and an increase in opacity of ampullae were distinctly observed.

(+++): Withdrawal of ampullae from the fringe of the colony, amputation of blood vessels, disintegration of ampullae, and/or degeneration of zooids were observed.

RESULTS

Xenogeneic rejection between *Botryllus scalaris* and *Botryllus primigenus*

In the allogeneic recognition at the growing edge of each species, following fusion of tunics, penetrating of ampullae into the facing colony usually occur between two incompatible colonies. Furthermore, in *Botryllus scalaris*, fusion of blood vessels also occurred between two incompatible colonies (Figs. 1and 2). However, fusion of tunic cuticles and matrices, and penetrating of ampullae into the facing colony never occurred between xenogeneic two colonies of those two species, not to mention fusion of vascular vessels.

In both the case of growing-edge contact and the case of cut surface contact, the *Botryllus scalaris* colony did not show any rejection reaction against the *Botryllus primigenus* colony. In contrast, the *Botryllus primigenus* colony always showed a rejection reaction against the *Botryllus scalaris* colony in both cases. The features and processes of the rejection reactions in both cases were similar to each other, except for the delay of the beginning of rejection in the case

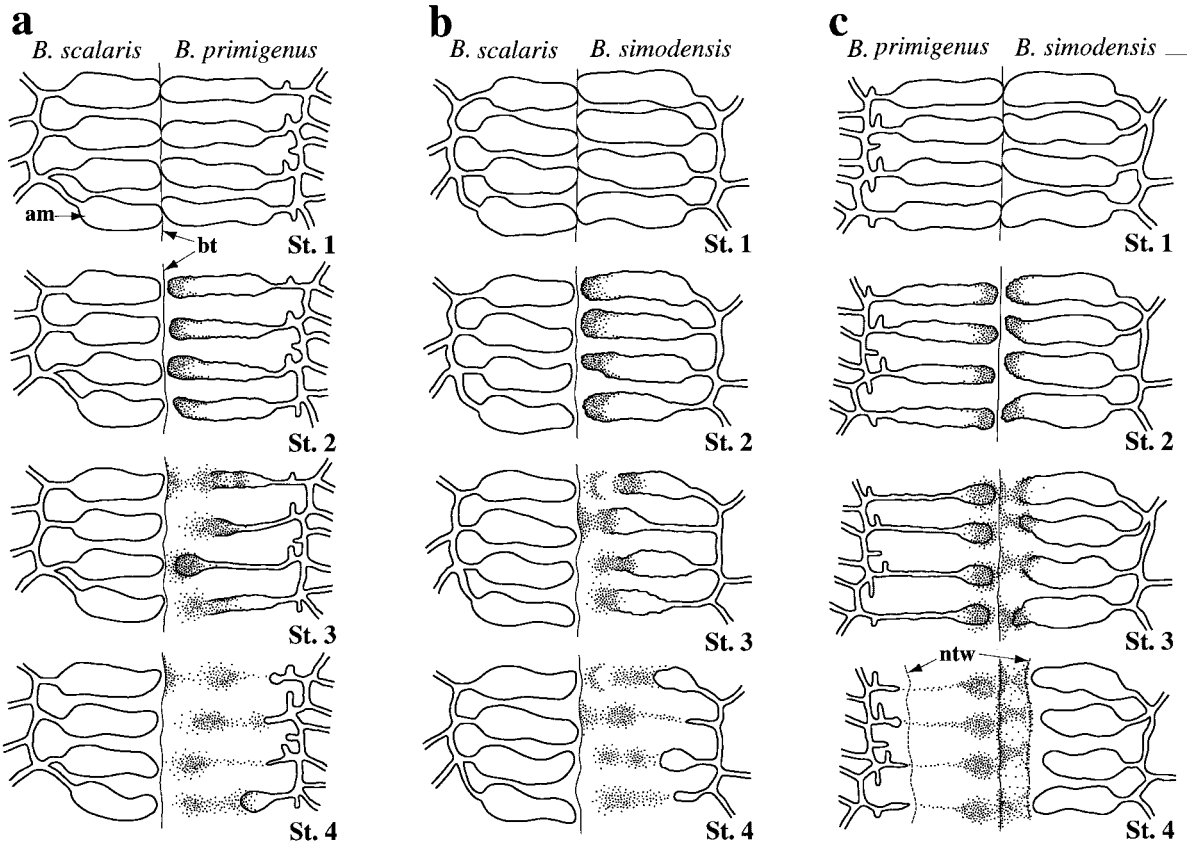

Fig. 3. Scheme showing the processes of xenogeneic rejection in the case of cut surface contact between *Botryllus scalaris* and *Botryllus primigenus* (a), between *Botryllus scalaris* and *Botrylloides simodensis* (b), and between *Botryllus primigenus* and *Botrylloides simodensis* (c). Details are explained in the text. am, ampulla; bt, boundary of tunic; ntw, new tunic wall; St., Stage.

of growing-edge contact. The rejection process in the case of cut surface contact is illustrated in Fig. 3a. The colonies of the two species were brought into contact with each other at their growing edges or cut surfaces (Stage 1). About 20 hr after contact in the case of growing-edge contact or within 2 hr in the case of cut surface contact, the first sign of rejection appeared in the ampullae of the colony of *Botryllus primigenus* at the contact area. First, adhesion and/or cluster formation of blood cells occurred in the distal part of the ampullae; then these ampullae began to contract or shrivel (Stage 2). Soon after that, morula cells (bright green blood cells) began to infiltrate the tunic matrix from the distal parts of the ampullae. While the atrophied ampullae were gradually withdrawing from the contact area, their tips disintegrated and many blood cells dispersed from these tips into the tunic matrix (Fig. 4a). The proximal portion of the ampulla often contracted tightly (Fig. 4b, Stage 3). Then, in most cases, these ampullae were amputated at their bases, but in some cases the ampullae withdrew from the contact area to the vicinity of the vascular network without amputation. Finally, the blood cells and epidermal cells of the disintegrated ampullae died, but the tunic matrix at the contact area remained relatively healthy (Stage 4). Therefore, separation of the contacted colonies was not observed for a few days after the rejection. In the case of cut surface contact, the rejection reaction progressed rapidly—only 5 to 6 hr from Stage 1 to Stage 4. In contrast, in the case of growing-edge contact, the time required for rejection was very variable.

Xenogeneic rejection between *Botryllus scalaris* and *Botrylloides simodensis*

The *Botryllus scalaris* colony also did not show any rejection reaction against the *Botrylloides simodensis* colony in either the case of growing-edge contact or the case of cut surface contact. However, the *Botrylloides simodensis* colony always showed a rejection reaction against the *Botryllus scalaris* colony in both types of contact. The features and processes of the rejection reactions in both cases were similar to each other. The rejection process in the case of cut surface contact is illustrated in Fig. 3b. The colonies of the two species were brought into contact with each other (Fig. 5a; Stage 1). About 2 to 3 days after the growing-edge contact or within 2 hr after the cut surface contact, the adhesion of blood cells to the ampullar wall and/or the cluster formation of blood cells began at the distal parts of the ampullae of the colony of *Botrylloides simodensis* at the contact area; then these ampullae began to contract or shrivel (Stage 2). After a little while, morula cells began to infiltrate the tunic matrix from the distal end of the ampullae. Then, one or both

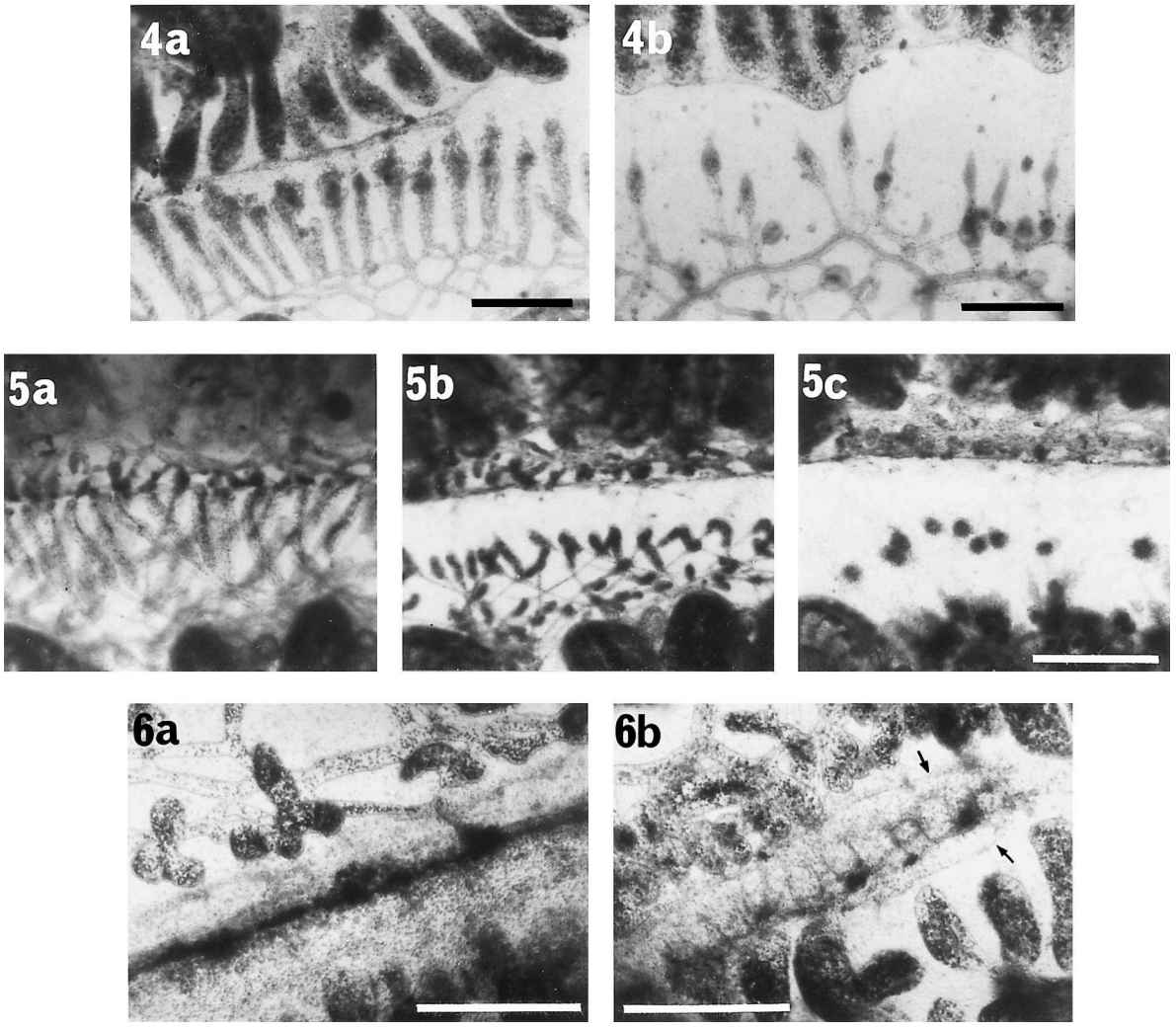

Fig. 4. Xenogeneic responses of *Botryllus primigenus* against *Botryllus scalaris*. (a) the case of cut surface contact, about 3 hr after the contact. A rejection reaction is observed only in the *Botryllus primigenus* colony. Most of the ampullae show disintegration at their distal parts. (b)growing-edge contact, 1day after the contact. A rejection reaction is observed only in the *Botryllus primigenus* colony. The ampullae of *Botryllus primigenus* make contact and then withdraw from the contact area. Upper side, colony of *Botryllus scalaris*; lower side, colony of *Botryllus primigenus*. Scale bar is 500 μm.

Fig. 5. Xenogeneic rejection of *Botrylloides simodensis* against *Botryllus scalaris*. (a) The two colonies coming into contact with each other at their cut surfaces. (b) Three hr after contact. Contraction and withdrawal of ampullae are shown in the *Botrylloides simodensis* colony. (c) Nine hours after contact. Disintegration of amputated pieces of ampullae is seen in the *Botrylloides simodensis* colony, and disintegration is also seen at the withdrawn ampullae. Upper side, colony of *Botryllus scalaris*; lower side, colony of *Botrylloides simodensis*. Scale bar is 1.0 mm.

Fig. 6. Examples of xenogeneic rejection between *Botryllus primigenus* and *Botrylloides simodensis* in the case of cut surface contact. (a)Many blood cells are seen at both sides of the boundary between the two colonies. The boundary that appears as a black line is composed of dead blood cells. (b)New tunic walls (arrows) are seen at the contact area of both colonies. The area surrounded by new tunic walls is filled with many blood cells. Upper side, colony of *Botryllus primigenus*; lower side, colony of *Botrylloides simodensis*. Scale bar is 500 μm.

of the following reactions occurred in each ampulla: (1) The atrophied ampulla gradually withdrew from the contact area; in this case, the tip of the ampulla usually disintegrated and many blood cells dispersed from the tip into the tunic matrix, or sometimes a constriction occurred near the distal end of the ampulla and amputation followed. (2) Tight contraction of the proximal portion of the ampulla occurred (Fig. 5b, Stage 3); in this case, the ampulla was always amputated at its base, whereas in the other case, the atrophied ampulla withdrew to the vicinity of the vascular network. The pieces of the amputated ampullae disintegrated and the blood cells within them dispersed into the tunic matrix (Fig. 5c, Stage 4). Finally, the blood cells and the epidermal cells of the disintegrated ampullae died, but visible disturbance of the tunic matrix at the contact area did not occur for some days. In the case of cut surface contact, the rejection reaction in *Botrylloides simodensis* progressed rapidly—only about 5 hr from Stage 1 to Stage 4. However, in the case of growing-edge contact, the time required for rejection was long and variable.

Xenogeneic rejection between *Botryllus primigenus* and *Botrylloides simodensis*

When a *Botryllus primigenus* colony and a *Botrylloides simodensis* colony came to contact with each other at their growing edges, no rejection reaction occurred in either colony. About 1 week after contact, the *Botrylloides simodensis* colony began to grow over the *Botryllus primigenus* colony, but still no rejection reaction was observed. However, when two such colonies were brought into contact with each other at their cut surfaces, remarkable rejection reactions occurred in both colonies. The processes of these reactions are illustrated in Fig. 3c. First, the two colonies were brought into contact at their cut surfaces (Stage 1). About 6 hr later, blood cells began to adhere to the ampullar wall and/or they began to cluster together at the distal parts of the ampullae of both colonies in the contact area, and then these ampullae began to contract or shrivel (Stage 2). Subsequently, morula cells began to infiltrate the tunic matrix from the tips of the ampullae. In the *Botryllus primigenus* colony, contraction of the ampullae proceeded further, especially at the proximal portions of these ampullae, and the bloodstream in the ampullae stopped completely. In the *Botrylloides simodensis* colony, the distal ends of the ampullae atrophied remarkably, and these ampullae began to withdraw from the contact area (Stage 3). Next, the contracted ampullae in the *Botryllus primigenus* colony were cut off from the healthy parts of the proximal vascular network, and the pieces of the ampullae began to disintegrate. About 14 to 15 hr after the initial contact, the infiltrated blood cells turned brown in both colonies (Fig. 6a), and new tunic walls were formed to separate the contact zone from the healthy parts of the colonies (Fig. 6b; Stage 4).

Responses against injected allogeneic blood plasma in *Botryllus scalaris*

It is known that colonies of both *Botryllus primigenus* and *Botrylloides simodensis* exhibit rejection reactions against injected allogeneic blood plasma of incompatible colonies (Table 1; Taneda and Watanabe, 1982b; Saito and Watanabe, 1984)), but there has been no report on the response of *Botryllus scalaris* against injected allogeneic blood plasma. Therefore, a microinjection experiment using allogeneic blood plasma was performed in *Botryllus scalaris* before examining this species' reactivity against injected xenogeneic blood plasma. As allogeneic rejection occurs after the fusion of blood vessels between two incompatible colonies in *Botryllus scalaris* (Figs. 1 and 2a), the allogeneic recognition in this species appears to depend on a direct contact of blood components of two colonies within the blood vessel. Accordingly, this injection experiment was necessary in order to know which component(s) of blood participated in the allogeneic recognition in this species.

Injection of FSW induced no harmful effect except that the ampulla was injured by the injection. Almost all of the recipient colonies of *Botryllus scalaris* did not show any reaction against syngeneic blood plasma from their subcolonies, and none of them showed any rejection against allogeneic blood plasma from compatible (fusible) colonies (Table 1). Moreover, nearly all of the recipient colonies did not show any rejection reaction against allogeneic blood plasma from incompatible (nonfusible) colonies.

Responses against injected xenogeneic blood plasma

As the fusion of tunics, as well as the fusion of blood vessels, never occurs between two xenogeneic colonies,

Table 1. Responses to intraspecific injection of blood plasma in three botryllids

Injected Blood Plasma	Response of Recipient*				Total Number of Challenges	Reference
	+++	++	+	–		
Botryllus scalaris						Present study
Auto BP	0	0	4	44	48	
F-Allo BP	0	0	0	13	13	
NF-Allo BP	0	0	3	60	63	
Botryllus primigenus						Taneda and Watanabe (1982b)
Auto BP	0	0	3	39	42	
NF-Allo BP	0	5	44	7	56	
Botrylloides simodensis						Saito and Watanabe (1984)
Auto BP	0	3	24	70	97	
F-Allo BP	0	2	3	30	35	
NF-Allo BP	35	70	27	8	140	

Auto BP: blood plasma from autogeneic (syngeneic) colonies.
F-Allo BP: Blood plasma from fusible (compatible) allogeneic colonies.
NF-Allo BP: blood plasma from nonfusible (incompatible) allogeneic colonies.
* See MATERIALS AND METHODS for explanation of response scores.

Table 2. Responses to injection of xenogeneic blood plasma in three botryllids

Species of Recipient Colony	Donor of Injected Blood Plasma	Response of Recipient*				Total Number of Challenges
		+++	++	+	–	
Botryllus scalaris	*B. primigenus*	40	3	0	0	43
	B. simodensis	3	44	6	7	60
Botryllus primigenus	*B. scalaris*	38	0	0	1	39
	B. simodensis	9	40	5	21	75
Botrylloides simodensis	*B. scalaris*	42	1	0	0	43
	B. primigenus	32	5	0	1	38

* See MATERIALS AND METHODS for explanation of response scores.

only diffusible humoral factor(s) can move from one colony to the other colony. Thus, xenogeneic rejection might be caused by the recognition of xenogeneic humoral factor(s) in a recipient colony. Therefore, this experiment was performed to make sure the existence of recognition sites for xenogeneic humoral factor(s) in blood vessels and to observe the manner of the rejection reaction against xenogeneic factor(s).

Injection of FSW did not induce any harmful reactions in recipient colonies except that the ampulla was injured by the injection. As shown in Table 2, all recipient colonies of the three botryllids showed rejection reactions against injected blood plasma from xenogeneic colonies. The criteria for assessing allogeneic rejection (see MATERIALS AND METHODS) were applicable to xenogeneic rejection as well. The blood plasma of *Botrylloides simodensis* induced rejection reactions in 88.3% of recipient colonies of *Botryllus scalaris* and in 72.0% of *Botryllus primigenus* recipients, and the main rejection response was “2+” (++). The blood plasma of both *Botryllus scalaris* and *Botryllus primigenus* induced more extensive rejection reactions in *Botrylloides simodensis* recipients, mainly “3+” (+++), with more than 97% of recipients showing rejection responses. Thus, it appears that recipient colonies of *Botrylloides simodensis* were very sensitive to xenogeneic blood plasma.

DISCUSSION

In these cut colony assay experiments, two conditions—growing-edge contact and cut surface contact—were used. In the case of growing-edge contact, colonies of *Botryllus primigenus* and *Botrylloides simodensis* both showed rejection reactions against colonies of *Botryllus scalaris,* although it took a long time (1 to 3 days) for initiation of the rejection. In growing-edge contact assays between colonies of *Botryllus primigenus* and *Botrylloides simodensis*, rejection reactions did not appear in either colony for more than 1 week after contact. In contrast, in the case of cut surface contact, colonies of *Botryllus primigenus* and *Botrylloides simodensis* showed rejection reactions against colonies of *Botryllus scalaris* within 2 hr after contact. In cut surface contact assays between *Botryllus primigenus* and *Botrylloides simodensis*, rejection reactions were also seen in both colonies about 6 hr after contact. However, *Botryllus scalaris* colonies did not show any rejection reactions against colonies of the other two species in either the growing-edge contact assays or the cut surface contact assays.

Because fusion of tunic surfaces, tunic matrices, and vascular ampullae never occurred between two colonies of different species, it is supposed that one or more diffusible substances, such as a humoral factor(s), can move from one colony into the other colony. Therefore, the xenogeneic rejection seen in our experiments was probably caused by the recognition of some xenogeneic humoral factor(s). The delay or non-appearance of rejection in the case of growing-edge contact between *Botryllus primigenus* and *Botrylloides simodensis* may be due to the presence of tunic surfaces (cuticle layers). According to electron microscopic studies on the tunic of botryllids, the tunic surface is composed of a high-electron-dense layer (Katow and Watanabe, 1978; Milanesi *et al*., 1978). This structure seems to be obstructive to the diffusion of the humoral factor(s). Our results in the growing-edge contact assays suggest that the humoral factor(s) of *Botryllus scalaris* could barely pass through the layer. As stated above, in the cut colony assay, xenogeneic rejection appeared in only two species, *Botryllus primigenus* and *Botrylloides simodensis*. Hence, it seems that colonies of these species can recognize the xenogeneic humoral factors invading their tunic matrices. On the other hand, *Botryllus scalaris* colonies did not show any rejection reactions against colonies of the other two species. However, the results of the microinjection assays using xenogeneic blood plasma showed that all species might have the ability to recognize xenogeneic humoral factors that are introduced directly into their blood vessels. Therefore, *Botryllus scalaris* seems to be lacking the capacity to recognize xenogeneic humoral factors coming through the tunic matrix. These observations suggest that there are some differences between *Botryllus scalaris* and the other two botryllids in the distribution of recognition sites of xenogeneic factors.

With respect to allogeneic rejection, it is believed that colonies of *Botryllus primigenus* and *Botrylloides simodensis* can recognize allogeneic humoral factors diffusing to their ampullae through their tunic matrices and show rejection reactions (Mukai and Watanabe, 1974; Hirose *et al*., 1997). Furthermore, in these two species, colonies can recognize

allogeneic factors in injected blood plasma. That is, the recipient colony can distinguish between syngeneic or compatible allogeneic blood plasma and incompatible allogeneic blood plasma (Taneda and Watanabe, 1982b; Saito and Watanabe, 1984). In contrast, in *Botryllus scalaris* allogeneic rejection does not occur until the fusion of vascular vessels is established between two nonfusible colonies (Figs. 1 and 2; Saito and Watanabe, 1982; Shirae *et al*., 1999), and therefore the allogeneic rejection is not induced by a humoral factor(s) diffusing through the tunic matrix. That is, in this species, there might be no allogeneic humoral factor, or there might be no recognition sites for the allogeneic humoral factor(s) diffusing through the tunic matrix. In the present study of microinjection of blood plasma, it was shown that recipient colonies of *Botryllus scalaris* could not distinguish between syngeneic and allogeneic blood plasma, but the recipients could recognize xenogeneic blood plasma as nonself and showed rejection reactions. These facts point out two possibilities; one is that in the blood plasma of *Botryllus scalaris* there is no allogeneic humoral factor concerned with colony specificity, and the other is that there is no recognition sites for allogeneic humoral factors in the blood vessel. Therefore, in this species, the allogeneic rejection reaction between two incompatible colonies may be induced only by direct contact of allogeneic blood cells. The recent morphological study on the details of allogeneic rejection in this species has also shown that the first sign of allogeneic rejection is attachment of blood cells at the fused vessels (Shirae *et al*., 1999).

In xenogeneic rejection between *Botryllus primigenus* and *Botrylloides simodensis*, the rejection reaction in each species progressed in the same manner as that in their allogeneic rejection, respectively. The rejection reaction of *Botryllus primigenus* against *Botryllus scalaris* was similar to that of *Botrylloides simodensis*, and these reactions were more intense than those between *Botryllus primigenus* and *Botrylloides simodensis*. Thus, the reactions of xenogeneic rejection in both species, *Botryllus primigenus* and *Botrylloides simodensis*, progressed in almost the same manner as those of their allogeneic rejection and showed common features, as follows: (1) adhesion of blood cells to the vascular wall, (2) cluster formation of blood cells in vascular vessels, (3) contraction or atrophy of ampullae, (4) infiltration of blood cells (morula cells) into the tunic, and (5) amputation and withdrawal of ampullae. All of these features are also shown in their allogeneic rejection reactions (Tanaka and Watanabe, 1973; Mukai and Watanabe, 1974; Taneda and Watanabe, 1982a). Therefore, in these botryllids the same mechanism may be involved in both allogeneic and xenogeneic rejection caused by nonself recognition though there might be some differences between the mechanisms of allogeneic recognition and xenogeneic recognition, and morula cells may play a leading role in their rejection reactions (Ballarin *et al*., 1995; Rinkevich *et al*., 1998; Shirae and Saito, 2000). On the other hand, in *Botryllus scalaris*, it is known that phagocytes (another type of blood cell) play the leading role in allogeneic rejection (Shirae *et al*., 1999). However, the responses against injected xenogeneic blood plasma in this species were the same as those of the other two species. That is, *Botryllus scalaris* has recognition sites for xenogeneic humoral factor(s), and possibly has the similar mechanism of rejection reaction to those of the other two species. Thus, might be some differences between the mechanisms of allogeneic rejection and xenogeneic rejection in this species.

In some solitary ascidians, a recognition reaction exists that is known as *contact reaction*. When coelomic cells from a solitary ascidian are mixed in vitro with coelomic cells from a different species or from another individual of the same species, cell lysis is caused by direct contact between host- and donor-derived cells (Fuke, 1980). This observation clearly shows that coelomic cells of these solitary ascidians have the recognition sites of self-nonself on their surfaces. On the other hand, in the most case of xenogeneic or allogeneic recognition between two colonies of botryllids, the humoral factor(s) derived from the donor colony is surely recognized as nonself by the host colony, but the recognition loci of the host colony are not known at present. Blood components, especially blood cells, are considered to be participants in allogeneic recognition reactions of botryllid colonies (Mukai, 1967; Tanaka, 1973). It is known that morula cells of *Botryllus schlosseri* can recognize allogeneic factors (Ballarin *et al*., 1995; Rinkevich *et al*., 1998), and in *Botryllus primigenus* stem cells (hemoblasts) are considered as participants in allogeneic recognition (Taneda and Watanabe, 1982c). Furthermore, in *Botryllus scalaris* phagocytes might play the important role in the allogeneic recognition (Shirae *et al*., 1999). Therefore, it is likely that blood cells directly recognize nonself humoral factors or cell surface molecules, but more investigation is needed.

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