[REVIEW]

Colonial Allorecognition, Hemolytic Rejection, and Viviparity in Botryllid Ascidians

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ABSTRACT—Allorecognition is a fundamental system that animals use to maintain individuality. Although embryos are usually semiallogeneic with their mother, viviparous animals are required to allow these embryos to develop inside the maternal body, but must also eliminate an "invasion" by nonself. In colonial ascidians of the family Botryllidae, when two colonies are brought into contact at their growing edges, a hemolytic rejection reaction occurs between allogeneic colonies. Morula cells, a type of hemocyte, are the major effector cells in the hemolytic rejection. Morula cells infiltrate and aggregate where the two colonies make contact, and then discharge their vacular contents, which contain phenoloxidase and quinones. In viviparous botryllids, colonial contact at artificially cut surfaces always results in colonial fusion and establishment of a common vascular network even between allogeneic colonies in which the growing-edge contact results in rejection. This colonial fusion between incompatible colonies (surgical fusion) suggests that the allorecognition sites are not distributed in the vascular system in which the embryos are brooded. It is supposed that a common ancestor of the viviparous species lost the capacity for allorecognition in their vascular system to protect its embryos from alloreactivity, when it changed from ovoviviparous to viviparous in the course of evolution. The limited distribution of allorecognition sites would be a solution to the embryo-parent histoincompatibility in viviparity.

Key words: colonial ascidian, embryo-parent histoincompatibility, allorecogniton site, tunic cell, hemocyte

INTRODUCTION

The self–nonself recognition system mediates one of the most fundamental interactions between individuals, and its occurrence has been observed in various taxa of organisms. In metazoans, although the occurrence of self–nonself recognition is often represented by the allograft rejection, that type of tissue transplantation is an artificial procedure. In nature, the cellular interaction between allogeneic partners may occur only in fertilization, colony specificity, and viviparous reproduction.

In fertilization, including in conjugation and pollination, some gametes recognize and do not fuse with self-gametes to avoid inbreeding. This property is known as self-sterility, and it is particularly important in hermaphroditic organisms. Colony specificity is a type of allograft recognition that occurs naturally in colonial organisms and is manifested by fusibility between colonies; when colonies come into contact with each other, they fuse to form a single mass with self-colonies (fusion), but do not fuse with nonself colonies (rejection). Becoming a larger size through colonial fusion would be favorable for survival. Recently, Nakaya et al. (in press) demonstrated that the respirations of each zooid decreases when the colony size increases. This means that a larger colony can conserve more nutrient resources than a smaller colony, and thus, colonial fusion would increase the fitness of the animal. On the other hand, there are some disadvantages of fusion among allogeneic colonies, as discussed elsewhere (e.g., Buss and Green, 1985). In chimeric colonies of an ascidian, genetically distinct germ cells compete for access to developing gonads and, therefore, allogeneic fusion may result in domination by the germ cells originating from one of the partners in a chimeric colony (Stoner et al., 1999). In this case, highly polymorphic colony specificity would be required to avoid a takeover of germ lines. In some colonial ascidians, fusibility is essentially controlled by a single-locus and multiple-alleles system (Oka.
and Watanabe, 1960; Sabbadin, 1982; Scofield et al., 1982). Moreover, it has been suggested that the fusibility gene is also involved in self-sterility (Oka and Watanabe, 1960).

In contrast to fertilization and colony specificity, viviparous reproduction would not benefit from the occurrence of an allogeic response. In mammals, a mother is capable of rejecting her offspring's tissue that bears the paternally derived antigens, but fetal rejection by the maternal immune response is prevented by mechanisms as yet undefined. During pregnancy, although the maternal adoptive immunity is suppressed, suggesting a transient state of tolerance with respect to the fetus, the innate immune system is activated to cover the loss of maternal immune defense caused by this suppression (cf. Sacks, 1999). Viviparous reproduction has evolved in several lineages of metazoans independently, and nonmammalian-embryos would also need to avoid the alloreactivity of their mother in viviparous development.

In some ascidians of the family Botryllidae, we find all three phenomena, namely, fertilization, colony specificity, and viviparous reproduction. All ascidians are hermaphroditic; all botryllid ascidians are colonial, and some botryllids are viviparous. In botryllid ascidians, the manner of reproduction is thought to have evolved from ovoviviparity to viviparity (Saito and Watanabe, 1985), and comparative studies on colony specificity suggest that viviparity affects the distribution of alloreactivity in the colonies (Hirose et al., 1988, 1994; Okuyama et al., 2002). In this review, I discuss the alteration of the allore cognition system linked with the evolution of viviparity based on findings in the viviparous botryllid colony's specificity.

**COLONY SPECIFICITY IN BOTRYLLID ASCIDIANS**

Diversity among rejection reactions

Colonial allore cognition occurs naturally when conspecific colonies growing on a substrate make contact at their edges. As a beginning, it is necessary to describe the general morphology of a botryllid colony (Fig. 1). Botryllid ascidians form a gelatinous, sheetlike colony in which the zooids are embedded in a transparent tunic, an integumentary tissue of tunicates. The zooids are interconnected via a common vascular network that extends throughout the colony. At the colony periphery, blood vessels form swollen termini called ampullae. In the vascular lumen, hemocytes are circulating in the blood, which is pumped by the synchronous heartbeats of the zooids. Hemocytes are morphologically discriminable into several types—hemoblasts, phagocytes, granulocytes, morula cells, and pigment cells—but detailed classification and nomenclature vary, depending on the species and the researchers (cf. Milanesi et al., 1978; Burighel et al., 1983; Shirae and Saito, 2000; Cima et al., 2001). The morula cell is the most abundant cell type of the hemocytes and has the leading role in the allorejection reaction (see below). The tunic is an integumentary tissue that covers the entire colony. It consists of tunic matrix, a tunic cuticle that overlies the tunic matrix, and tunic cells, which are “free” cells distributed in the tunic matrix. There are two to three types of tunic cells in the botryllid ascidians (Zaniolo, 1981; Hirose et al., 1991).

When isogeneic colonies are brought into contact, fusion proceeds as follows: (1) The first event is dissolution of the tunic cuticles at the contact point, and the cuticular layers of both colonies are fused into a continuous layer, resulting in fusion of the tunic matrices. (2) At the contact point, the ampullae of each colony extend into the tunic of the other colony. (3) The tips of the ampullae make contact with the basal sides of the opposite ampullae. (4) The epithelia of both colonies fuse with one another at the contact point, which means the blood vessels of the two colonies are interconnected, and the blood (i.e., hemocytes and blood plasma) circulates through the fused colonies. The colonial fusion process is completed in about 1 to 2 days. This fusion process is common in all botryllid ascidians studied thus far.

When incompatible colonies are brought into contact, fusion is interrupted by the rejection reaction. This rejection is an acute response as compared to the tissue transplantation in other organisms, and the rejection process is com-

![Fig. 1](https://example.com/fig1.png)  
**Fig. 1.** Schematic drawing of a botryllid colony: upper surface (left) and cross section (right). am, ampulla; ba, branchial aperture; bd, bud; bv, blood vessel; ca, cloacal aperture; cu, tunic cuticle; hm, hemocyte; ic, tunic cell; tu, tunic; zo, zooid.
pleted within a days. The timing of the initiation of rejection differs from species to species (reviewed in Saito et al., 1994). For instance, in Botrylloides species, the rejection reaction begins immediately after fusion of the tunic between the two colonies; infiltrating hemocytes are aggregated and disintegrated at the area where the tunics are partially fused. This hemolytic rejection reaction occurs in a very limited area in the tunic and is called subcuticular rejection (SCR) (Hirose et al., 1997). In contrast, in Botryllus primigenus rejection begins after the tip-to-side contact of the ampullae, and in Botryllus scalaris rejection begins after vascular fusion. In these species, the alloreaction reaction involves disintegration of the ampullae that have interacted with those of the opposite colony. These variations are probably caused by differences in the timing of allorecognition in the process of the fusion reaction. In botryllid ascidians, SCR is thought to be the most advanced and adaptive mode of the alloreaction reaction because the loss of tissue in SCR is less than that in the other variations of the rejection reaction studied thus far (Hirose et al., 1988, 1997). This view is consistent with the phylogeny of botryllid ascidians inferred from the modes of sexual reproduction, which proposes that the botryllids evolved from ovoviviparous species to viviparous ones and from having a shorter brooding period to having a longer one (Saito and Watanabe, 1985; Saito et al., 2001). The molecular phylogeny based on 18S rDNA sequences also supports the evolutionary course of allorecognition behavior from the internal (rejection after vascular fusion) to external (occurrence of SCR) (Cohen et al., 1998).

Histological and ultrastructural investigations showed that morula cells are the major effector hemocytes in the alloreaction reaction in most botryllids (Taneda and Watanabe, 1982a; Hirose et al., 1990, 1997). Responding to the allogeneic challenge, morula cells infiltrate the tunic and disintegrate there, discharging their vascular contents (M-type rejection). In contrast, in Botryllus scalaris, phagocytes mediate the aggregation of hemocytes in the vascular lumen, resulting in interruption of blood flow after vascular fusion (P-type rejection), and morula cells do not participate in this alloreaction reaction (Shirae et al., 1999). To date, P-type rejection has only been described in B. scalaris.

In botryllid ascidians, the fusibility among conspecifics is controlled by a single-locus and multiple-alleles system (Oka and Watanabe, 1960; Sabbadin, 1982; Scofield et al., 1982); the colonies sharing one or both alleles results in fusion, and the colonies sharing no alleles results in rejection (see Saito et al., 1994 for review). Accordingly, the colonies sharing one allele, i.e., semi-allogeneic colonies, fuse to form a chimera colony. The occurrence of chronic alloreaction has been reported in the chimera colonies; the chimera separated into two original colonies, or blastozooids of either one or both of partners in the chimera become resorbed in several weeks or more (Saito and Watanabe, 1982; Scofield et al., 1982; Rinkevich and Weissman, 1987; 1989, 1992). I may leave the details on the chronic rejection to some reviews (Weissman et al., 1990; Rinkevich, 2002).

**Cellular process of subcuticular rejection (SCR) in Botrylloides**

The family Botryllidae consists of two genera, Botryllus and Botrylloides. The morphological process of alloreaction reaction has been reported in several Botryllus and Botrylloides (see Saito et al., 2001), while viviparous species have been described in Botrylloides but not Botryllus. I describe here the detailed process of SCR in Botrylloides species, because this review is aiming to discuss the allorecognition system in relation to the evolution of viviparity from ovoviviparity. In the genus Botrylloides, the process of the alloreaction reaction has been reported in five species: B. simodensis (Hirose et al., 1990, 1997), B. leachi (Zaniolo and Ballarin, 2001), B. lentus (Okuyama et al., 2002), B. fuscus (Hirose et al., 1994), and B. violaceus (Hirose et al., 1998). Among them, B. simodensis and B. leachi are ovoviviparous, and the others are viviparous. All of these five species exhibit SCR when responding to contact with an allogeneic colony. It is thought that the process of SCR occurs according to the steps shown in Fig. 2, mainly on ultrastructural investigation (Hirose et al., 1997).

**Dissolution of tunic cuticle**

Culciferous cuticle always occurs in any allogeneic combination, indicating that the tunic cuticle is not a barrier to allogeneic fusion. The fusion of tunic cuticles occurs and SCR is induced even in some xenogeneic combinations, such as B. simodensis–B. lentus, B. lentus–B. fuscus, and B. fuscus–B. violaceus (Hirose et al., 2002). With respect to phylogeny, the species in these xenogeneic combinations are thought to be closely related to one another (cf. Saito and Watanabe, 1985). Therefore, the tunic cuticle seems to be a barrier to avoid interspecific fusion of colonies, but it cannot distinguish some closely related congeneres from conspecifics. If culciferous fusion occurs, SCR is induced in allogeneic and xenogeneic combinations, resulting in the loss of tissues as a result of the hemolytic rejection reaction. Xenogeneic discrimination at the tunic cuticle does not require the hemolytic reaction and it would profit the animal to save the loss of tissue. It is possible that some enzymes are involved in the dissolution of the cuticle and that the substrate specificity of the enzymes prevents the xenogeneic fusion of the tunics.

**Diffusion of allogeneic humoral factor(s)**

Fusion of tunic cuticles results in direct contact of the tunic matrices of the colonies, and humoral components in the tunic diffuse into the tunic of the opposite colony via the partial fusion of the tunic. In some Botryllus species, blood plasma from a colony has been injected into the blood vessel of an allogeneic colony and has been shown to induce allospecific responses of hemocytes (Taneda and Watanabe, 1982b). The demonstration of allospecific responses of hemocytes by in vitro incubation of hemocytes in blood plasma (Ballarin et al., 1995, 1998) provides another indication that blood plasma may have the capacity to,
induce allogeneic rejection. In addition to being in the blood, the allogeneic factor(s) is probably also distributed in the tunic, where it could activate hemocytes to induce allorejection. In the early stage of SCR, some tunic cells break down, discharging their contents around the points of partial fusion (Hirose et al., 1997). The factor(s) could induce tunic cell breakdown and the material discharged from these cells may contain the activity to induce the subsequent rejection reaction. It is possible that components of the dissolved tunic cuticle are also involved in the induction of the rejection reaction.

Hemocyte infiltration and aggregation

After partial fusion of the tunic, hemocytes infiltrate the tunic. This infiltration is probably induced by the humoral factor(s) from the allogeneic colony or from the discharged material from the disintegrating tunic cells, or from both. Permeability of the ampullar epithelium increases at this stage (Taneda and Watanabe, 1982a), and hemocytes pass through the space between the epithelial cells of the ampullae. The infiltrated hemocytes aggregate where the tunic is partially fused with its counterpart. Diffusion of a chemotactic factor may be involved in the hemocyte aggregation.

The majority of the infiltrated hemocytes are morula cells that have eosinophilic vacuoles (Hirose et al., 1990, 1997). They are the major effector cells for the allorejection in botryllids (M-type rejection). Morula cells contain phenoloxidase (PO) and quinones (Frizzo et al., 2000; Shirae et al., 2002), and the PO activity and amount of quinones increase in response to allogeneic blood plasma (Ballarin et al., 1995, 1998) or allogeneic contact of colonies (Shirae et al., 2002). Furthermore, stimulation of morula cells with mannan or phorbol 12-mono-myristate induces a significant increase in the expression of interleukin-1-α- and tumor necrosis factor-α-like molecules, suggesting the immunomodulatory functions of morula cells (Ballarin et al., 2001).

Hemocyte breakdown

Morula cells in the aggregates disintegrate and form small rejection lesions at the partially fused area of the allogeneic tunics. Some infiltrated hemocytes also break down in the tunic, apart from the cell aggregates. The disintegrating morula cells discharge their vacuolar contents, for example, PO and quinones that produce oxidants in the tunic, reacting with allogeneic tissue. The oxidants derived from quinone oxidation might alter the tunic architecture, perhaps leading to formation of the rejection lesion. Because ascorbic acid (an antioxidant) inhibits the hemocyte infiltration and disintegration, the oxidants may also promote these hemolytic events (Ballarin et al., 1998; Shirae et al., 2002). It is possible that a positive feedback regulation exists between morula cell infiltration and breakdown.

Boundary formation

Electron-dense fibers appear around the disintegrating hemocytes and rejection lesion. The fibers seem to originate from fibrous components of the tunic matrix. The discharged fibers seem to originate from fibrous components of the tunic matrix. The discharged fibers seem to originate from fibrous components of the tunic matrix.
materials from the disintegrating hemocytes appear to react with the tunic matrix to form electron-dense fibers. Around the rejection lesion, these fibers aggregate to form a continuous boundary separating the lesion from the contacting colonies. The boundary might be essential to confine the hemolytic rejection to the limited area of the tunic that is fused with the allogeneic tunic.

Because the process of boundary formation is ultrastructurally the same as that of regeneration of tunic cuticle, one might expect the boundary to eventually become tunic cuticle, and boundary formation and cuticle regeneration probably share the same molecular process. In cuticle regeneration, some proteases and protease inhibitors inhibit the electron-dense fiber formation, suggesting the involvement of proteolysis (Hirose et al., 1995). Boundary formation in allo rejection is also inhibited by antioxidants or protease inhibitors (Shirae et al., 2002). Therefore, some proteases and oxidation of the discharged materials (e.g., quinones) from hemocytes are involved in boundary formation.

**SURGICAL FUSION AND VIVIPARITY**

Surgical fusion indicates restricted distribution of allo recognition sites

In ovoviviparous botryllids, when conspecific colonies are brought into contact at their artificially cut surfaces, the hemolytic rejection reaction occurs immediately at the contact edges in the incompatible combinations in which allo rejection is induced by the growing-edge contact. The rejection is so intense that the rejection lesion forms a conspicuous black line between the colonies in some species, such as Botryllodes simodensis (Hirose et al., 1990).

Morula cells are the major effector cells for this rejection at the cut surface, as they are in the rejection reaction at the growing edges.

In contrast, in viviparous species, when the colonies are brought into contact at their cut surfaces, the colonies always fuse and form a chimera in any conspecific combination. This means that the colonies of the same combination undergo rejection when making contact at the growing edges but fuse at the cut surfaces (Fig. 3). This type of fusion between incompatible colonies is referred to as surgical fusion, and it occurs exclusively in viviparous species, for example, Botryllodes lentus (Okuyama et al., 2002), B. fuscus (Hirose et al., 1994), and B. violaceus (Hirose et al., 1988). Under laboratory conditions, surgical fusion can be maintained for several days, and no signs of hemolytic rejection are observed. The chimeric colony resulting from surgical fusion shares a common vascular network, and thus hemocytes are exchanged between the fused colonies. Because young oocytes circulate throughout the colony as hemocytes in botryllid ascidians (Mukai and Watanabe, 1976), the oocytes of the two colonies are probably mixed in the chimeric colony. Needless to say, the cut surface contact of colonies is an artificial treatment, and thus surgical fusion would never occur in nature.

The occurrence of surgical fusion indicates the absence of acute allo recognition in the blood vessels of these viviparous botryllids. On the other hand, the occurrence of SCR indicates not only the presence of the allo recognition system in the growing edge but also the presence of the effector system for the rejection reaction, in which morula cells play the leading role. Therefore, the allo recognition system and the effector system appear to be a discrete system, and the

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**Fig. 3.** Surgical fusion in viviparous Botryllodes species. Colonies that are brought into contact at cut surfaces always fuse into a single colony even in the incompatible combinations in which subcuticular rejection is induced by contact at the growing edges.
major effectors are hemocytes in all botryllids. In the viviparous botryllids, the capacity for allorecognition seems to be absent in the blood vessels but present in the growing edge, probably in the subcuticular area of the tunic, in which many tunic cells are distributed.

In most Botryllus species, when incompatible colonies come into contact at their growing edges, allorecognition is initiated after the fusion or contact of the vascular ampullae, and the rejection reaction results in the disintegration of the ampullae that have interacted with the allogenic tissue (cf. Saito et al., 2001). These species possess the capacity for allorecognition in the blood vessels—probably in a particular type of hemocyte—but not in the tunic. In the other botryllids studied thus far, allorecognition sites are distributed in the tunic, probably in the tunic cells, because allorecognition begins after fusion of the tunic. On the other hand, cut surface contact between incompatible colonies induces allogenic rejection in ovoviviparous botryllids, indicating that allorecognition sites are also present in the hemocytes. As discussed above, the occurrence of surgical fusion indicates the absence of allorecognition sites in the hemocytes in the viviparous botryllids. Accordingly, it is assumed that there are three distribution patterns of allorecognition sites for colony specificity in botryllid ascidians: hemocytes, hemocytes plus tunic cells, and tunic cells (Fig. 4).

The phylogeny of the family Botryllidae that is based on the mode of sexual reproduction proposes that primitive botryllids were ovoviviparous. Viviparous botryllids emerged in the lineage of Botrylloides species that have a brooding organ derived from the peribranchial wall, and the brooding period increased in the advanced species (Saito and Watanabe, 1985; Saito et al., 2001). The molecular phylogeny that is based on 18S rDNA also supports the monophyly of the genus Botrylloides, although it does not support the monophyly of the viviparous Botrylloides (Cohen et al., 1998). According to this view, the distribution pattern of allorecognition sites is expected to have evolved according to the steps shown in Fig. 4: hemocytes were originally the only allorecognition sites in primitive Botryllus, then tunic cells acquired the capacity for allorecognition in advanced Botryllus and ovoviviparous Botrylloides, and finally, hemocytes lost the capacity for allorecognition in viviparous Botrylloides.

Does viviparity require the loss of allorecognition in the blood vessels?

Viviparity is probably associated with the loss of allorecognition sites in the blood vessels in botryllid ascidians, because surgical fusion occurs exclusively in viviparous species. In viviparous botryllids, each embryo is enveloped by the brood pouch, which is derived from the peribranchial wall, and it is brooded in the lumen of the blood vessels of the mother colony (Mukai et al., 1987; Zariolo et al., 1998). If the viviparous species had not lost the capacity for allorecognition in the blood vessels, the allorecognition effectors might attack the embryos in the mother colony. According to the single locus–multiple alleles model for the genetic control of colony specificity, colonies fuse if they share at least one allele at the fusibility locus (Oka and Watanabe, 1960), and thus the offspring are always fusible with their mother colony. However, subsequent separation or colony resorption occurs in this type of chimeric colony, indicating the presence of the semiallogeneic reaction (Saito and Watanabe, 1982; Rinkevich and Weissman, 1987, 1992). For example, colony resorption reported on Mediterranean Botrylloides species starts 1–26 days after fusion (Rinkevich, 1995). Because the viviparous embryos are brooded for 10 days or more, some rejection reactions could be induced against the brooded embryos. Therefore, the loss of the allorecognition sites in the blood vessels would be essential to acquire viviparity.

The PO activity in the hemolysate is much lower in viviparous species than in some ovoviviparous species. For instance, the PO activity in viviparous species is less than one eighth of the PO activity in ovoviviparous Botrylloides simodensis (Shirae and Saito, 2000; Hirose et al., 2002). PO
is known to be one of the key enzymes for biological defense in various organisms, as well as being a major effector in the allorejection reaction in botryllids. Therefore, because of the low PO activity in blood vessels, the embryos do not suffer much cytotoxic stress from the activation of PO. However, it should be noted that the effector system for allorejection in viviparous species is sufficient for the occurrence of SCR. Moreover, cut surface contact induces hemolytic rejection in some xenogeneic combinations of viviparous Botrylloides, for example, *Botrylloides lentus-* *B. fuscus* (Hirose et al., 2002). Thus, the low PO activity is not the primary reason for the occurrence of surgical fusion.

Viviparity is found in various taxa of animals, including mammals, and each animal needs to cope with both viviparity (acceptance of allogeneic individuals) and immunity (elimination of nonself). In the case of botryllid ascidians, when a common ancestor of the viviparous *Botrylloides* acquired viviparity in the course of evolution, it would need to lose the capacity for allorejection in the vascular system where the embryos are brooded. The loss of allore cognition sites would cause few disadvantages for survival, because these *Botrylloides* species can carry out allore cognition and subsequent allorejection in their integumentary tissue, the tunic. The limited distribution of allore cognition sites is a solution to the embryo-parent histoincompatibility in viviparity, and a similar mechanism might have been adopted in some other lineages of viviparous animals.

**PERSPECTIVES**

Undoubtedly, the most essential question in colony specificity is, How does a colony discriminate between self and nonself? To answer this question, it is necessary to discover, on a molecular level, what recognizes nonself and what is recognized as nonself. As shown in Fig. 4, the possible candidates for location(s) of allore cognition sites are hemocytes and tunic cells. However, the molecule(s) recognized as nonself is poorly known to date. On the other hand, results of recent studies have provided increased knowledge about the molecular bases of the effector system of the allorejection reaction. With future research we can go up the cascade of the allorejection reaction in order to reach the initial point of the cascade, namely, allogeneic recognition.

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