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Review

Allorecognition in compound ascidians

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ABSTRACT In botryllids (colonial ascidians), there are two types of allorecognition: colony specificity and colony resorption. Colony specificity is manifested by fusion and rejection between two conspecific colonies. The genetic basis for this colony specificity resides in a single highly polymorphic gene locus (fusibility locus) with codominantly expressed alleles. Two colonies with no alleles in common at this locus reject each other, whereas colonies sharing at least one allele at the fusibility locus fuse and form a chimera. That is, in colony specificity, self components are distinguished from nonself components, and failure to recognize self induces rapid rejection reactions. The process of rejection in colony specificity is not uniform among all botryllid ascidians. Colony resorption can occur after the establishment of fusion between two colonies. Zooids of one partner of a chimera are resorbed more than 1 week after fusion, or, by amputation of fused blood vessels, the chimera becomes separated into the two original colonies. Colony resorption is also controlled mainly by the fusibility locus. It usually occurs in a chimera between two colonies sharing only one allele at this locus. In colony resorption, nonself determinants are recognized and chronic rejection reactions are induced resembling MHC-dependent graft rejection. Based on these findings, the fusibility locus of botryllids seems to be very similar to the MHC of vertebrates. Considering that vertebrates evolved directly from ascidians, it is likely that the fusibility locus is an ancestral form of the vertebrate MHC.

KEY WORDS: *ascidian, allorecognition, colony specificity, colony resorption*

Introduction

The self-nonself recognition system is one of the most important systems used by animals to maintain their individuality. It is well known that vertebrates, especially mammals, have a sophisticated recognition system known as the immune system. Using this immune system, animals can eliminate invasive micro-organisms such as viruses, bacteria, and parasites, and can also clear denatured cells and metabolic wastes from their bodies. Furthermore, they can recognize allogeneic tissues and organs transplanted from other individuals and reject them as nonself. This transplantation immunity, or allorecognition, is governed by the major histocompatibility gene complex (MHC). Humoral factors (antibodies, complement, lectins, and naturally occurring hemagglutinins) and cellular components (lymphocytes, macrophages, and natural killer cells) are involved in the complex responses of the immune system. Because this complicated self-nonself recognition system of vertebrates might have evolved from invertebrates, phylogenetic study of immune system is important for understanding the vertebrate immune system.

Ascidians, a group of protochordates, occupy a key evolutionary position on the phylogenetic line progressing toward vertebrates. Vertebrates are generally considered to have evolved directly from these animals (Berrill, 1955). Hence, ascidians are likely to share immunological characteristics with both vertebrates and invertebrates (Burnet, 1971). In this review, we discuss self-nonself recognition in ascidians and focus mainly on allorecognition in compound ascidians, which is considered to be analogous to transplantation immunity in vertebrates. The capacity for allorecognition is a bit mysterious for humans, because we are not naturally transplanted with organs or tissues from other individuals. On the other hand, compound ascidians do undergo a kind of transplantation reaction in nature and thus might give us some insight about the biological significance of allorecognition in other animals.

Self and nonself recognition in ascidians

It is thought that invertebrates, including ascidians, are not able to synthesize antigen-specific antibodies. However, naturally occurring hemagglutinins, lectins, and anti-microbial factors have

Abbreviations used in this paper: NFR, nonfusion reaction; SCR, sub-cuticular rejection; MHC, major histocompatibility gene complex.

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been found in the blood of ascidians (Table 1). Some of them are produced in blood cells (Parrinello and Arizza, 1989; Azumi *et al.*, 1990,1991; Cammarata *et al.*, 1993; Kelly *et al.*, 1993), though it is still not clear which tissues or cells produce these factors. In a colonial ascidian, *Polyandrocarpa misakiensis,* epithelial cells produce a kind of lectin that agglutinates stem cells to induce morphogenesis (Kawamura *et al.*, 1991). Most hemagglutinins and lectins are thought to have a role as opsonins in the ascidian body, but there is not enough information about their biological role. The major immune response of ascidians is phagocytosis and cytotoxicity by hemocytes which have been reported for other invertebrates as well (Smith and Peddie, 1992; Kelly *et al.*,1993; Parrinello *et al.*, 1993).

In 1903, Bancroft published an important and informative report of research on a phenomenon resembling the transplantation immunity of vertebrates in the compound ascidian *Botryllus schlosseri.* According to his report, when two pieces of a single colony came into contact with each other, they easily fused to form a single colony mass, but two pieces of different origin did not fuse together after grafting, regardless of conditions. On the other hand, among sibling colonies from the same mother colony, cases of fusion and nonfusion were seen. His work had not been evaluated until Oka and Watanabe found the same phenomenon in the Japanese botryllid ascidian *Botryllus primigenus* in 1957. They showed that this phenomenon was a type of self-nonself recognition under genetic control, and the recognition manifested by a complete fusion or a complete rejection among colonies of the same species was named "colony specificity".

Since then, colony specificity has been investigated in many species of compound ascidians (Table 2) and is found not only in botryllid ascidians, but also in several compound ascidians of other family. In solitary ascidians (particularly *Styela plicata*) transplantation experiments of allogeneic tissue have been carried out (Reddy *et al.*,1975; Raftos, 1990, 1991; Raftos and Briscoe, 1990; Raftos *et al.*,1987a,b, 1988). In *S. plicata,* each individual can recognize allogeneic tunic grafts as nonself and reject them. In that allorejection reaction, lymphocyte-like cells might detect nonself determinants on allogeneic cells (Raftos *et al.*, 1987b). It was also suggested that specific immune memory is present in that species (Raftos *et al.*, 1987a). Thus, analysis of histocompatibility in

TABLE 1

HUMORAL FACTORS IN SOME ASCIDIANS

Species	Form*	Factor**	Response to	Reference
Didemnum patulum	C	AG	mammalian erythrocytes	Coombe *et al.* (1984)
Aplidium australiensis	C	AG	vertebrate erythrocytes	Coombe *et al.* (1984)
Atapozoa fantasiana	C	AG	vertebrate erythrocytes	Coombe *et al.* (1984)
Phallusia mamillata	S	AG	vertebrate erythrocytes	Parrinello and Patricolo (1975), Parrinello and Canicatti (1983), Parrinello and Arizza (1989), Cammarata *et al.* (1993)
P. despressiuscula	S	AG	mammalian erythrocytes	Coombe *et al.* (1984)
Ascidia ceratodes	S	AG	invertebrate spermatozoa	Tyler (1946)
A. malaca	S	AG	mammalian erythrocytes	Parrinello and Patricolo (1975), Parrinello and Canicatti (1982)
A. thompsoni	S	AG	vertebrate erythrocytes	Coombe *et al.* (1984)
Ciona intestinalis	S	AG	vertebrate erythrocytes	Wright and Cooper (1975), Wright (1974) Parrinello and Patricolo (1975)
Microcosmos sulcatus	S	AG	human erythrocytes	Bretting and Renwrantz (1973)
M. nichollsi	S	AG	vertebrate erythrocytes	Coombe *et al.* (1984)
Pyura praeputialis	S	AG	mammalian erythrocytes	Coombe *et al.* (1984)
P. irregularis	S	AG	mammalian erythrocytes	Coombe *et al.* (1984)
Halocynthia hilgendorfi	S	AG	mammalian erythrocytes	Fuke and Sugai (1972)
H. pyriformis	S	AG	vertebrate erythrocytes	Anderson and Good (1975)
H. papillosa	S	AG	human erythrocytes	Bretting and Renwrantz (1973)
H. roretzi	S	AMB	viruses and bacteria	Azumi *et al.* (1990)
		AG	horse erythrocyte, bacteria	Azumi *et al.* (1991)
H. hispida	S	AG	mammalian erythrocytes	Coombe *et al.* (1984)
Herdmania momus	S	AG	mammalian erythrocytes	Coombe *et al.* (1984)
Botrylloides leachii	C	AG	vertebrate erythrocytes	Coombe *et al.* (1982, 1984)
B. mabnicoecus	C	AG	mammalian erythrocytes	Coombe *et al.* (1984)
Cemidocarpa etheridgii	S	AG	vertebrate erythrocytes	Coombe *et al.* (1984)
Polycarpa obtecta	S	AG	mammalian erythrocytes	Coombe *et al.* (1984)
P. papillata	S	AG	vertebrate erythrocytes	Coombe *et al.* (1984)
Polyandrocarpa misakiensis	C	AG	stem cells in its blood	Kawamura *et al.* (1991)
Styella plicata	S	AG	mammalian erythrocytes	Fuke and Sugai (1972)
S. clava	S	AG	vertebrate erythrocytes	Wright and Cooper (1984)
		OPS	yeast	Kelly *et al.* (1993)
Stolonica australis	C	AG	mammalian erythrocytes	Coombe *et al.* (1984)

*S, solitary ascidian; C, colonial ascidian; **AG, agglutinin or lectin; AMB, antimicrobial substance; OPS, opsonin

TABLE 2

THE OCCURRENCE OF COLONY SPECIFICITY IN SOME COMPOUND ASCIDIANS

Species	Contact with Growing edges	Cut surfaces	Presence of Colony specificity	Reference
Aplidium yamazii	F or R	F or R	present	Watanabe and Taneda (1982)
Aplidium constellatum	F or R	ND	present	Freeman (1970)
Polycitor proliferus	I	F	absent	Oka and Usui (1944)
Didemnum moseleyi	F or R	F or R	present	Mukai and Watanabe (1974)
Perophora viridis	F or R	ND	present	Freeman (1970)
P. japonica	F or R	ND	present	Koyama and Watanabe (1981)
P. orientalis	I	F	absent	Mukai and Watanabe (1974)
P. sagamiensis	F or R	ND	present	Koyama and Watanabe (1982)
P. bermudensis	F or R	ND	present	Freeman (1970)
Ecteinascidia tortugensis	F or R	ND	present	Freeman (1970)
Botryllus schlosseri	F or R	F or R	present	Bancroft (1903)
B. primigenus	F or R	F or R	present	Mukai and Watanabe (1974)
B. scalaris	F or R	F or R	present	Saito and Watanabe (1982)
Botrylloides simodensis	F or R	F or R	present	Mukai and Watanabe (1974)
B. fuscus	F or R	F	present	Authors (in preparation)
B. violaceus	F or R	F	present	Hirose *et al.* (1988)
*B. diegense**	F or R	ND	present	Yund and Feldgarden (1992)

F, fusion; R, rejection; I, indifference; ND, not done; *, more taxonomical study may be needed to identify this species.

solitary ascidians has identified a cell-mediated immune system that possesses functional characteristics similar to those of vertebrates (Kelly *et al.*, 1993; Raftos *et al.*, 1987b). In another solitary ascidian, *Halocynthia roretzi*, an interesting phenomenon called "contact reaction" was reported by Fuke (1980). When a blood cell contacts an allogeneic blood cell, both blood cells undergo rapid lysis after contact. Most types of blood cells are involved in this reaction. Humoral components are not involved in this reaction. It is also implied that the genes controlling the contact reaction are similar to those of colony specificity in botryllids (Fuke and Numakunai, 1982; Fuke and Nakamura, 1985). Raftos *et al.* (1987b) pointed out that tunicates might mount both rapid nonadaptive responses involving many cells and slower proliferative reactions requiring specific immunocytes.

Genetic control of colony specificity (fusibility) in *Botryllus primigenus*

Oka and Watanabe studied fusibility between colonies of *B. primigenus* taken from nature and among F_1 and F_2 progenies obtained by crossing of two nonfusible colonies (Oka and Watanabe, 1957, 1960, 1967; Oka, 1970; Watanabe and Taneda, 1990).

Fusion of two fragments derived from a single colony

When single colonies were divided into two pieces and the pieces reared separately, it was found that, upon contact, fusion occurred after various periods of rearing (2,3,5,7, and 30 days). Furthermore, fusion was possible even after separations of about 1 year.

Fusion between P and F_1 generations

When two different colonies were obtained from different locations (and did not fuse with each other while being in the same sexual phase), Oka and Watanabe designated them P and P'. If the two colonies were reared in the same aquarium for 3 days, including passage through the fertilization phase (asexual phase A), fertilization occurred between them. From such colonies (which were returned to the sea for further rearing), many larvae were released at about the 8th day after fertilization. The larvae underwent metamorphosis, attached themselves to glass slides, and formed colonies by asexual reproduction (Watanabe, 1953, 1975). These F_1 colonies were able to fuse with either parental colony, P or P'.

Fusibility among F_1 colonies

Fusion experiments were carried out on the two groups of F_1 colonies, that is, those formed from larvae released by P and those formed from larvae released by P' (Fig. 1). The results revealed that F_1 colonies derived from P could be divided into four different classes, which occurred at approximately equal frequencies. Colonies belonging to any one class fused not only with each other but also with colonies from two of the remaining three classes, but not with those from the fourth class. F_1 colonies derived from P' were also separable into four different classes on the basis of comparable fusion behavior. If the four classes of F_1 colonies derived from P are compared with those of P', it is found that they correspond one to one, i.e., a class of F_1 colonies of P is identical with a class of those of P'. The whole of the colonies of F_1 generation is thus sorted into four groups. The essential features of these relationships are illustrated in Fig. 1.

Fusibility among F_2 colonies

Experiments were undertaken to obtain F_2 colonies by crossing pairs of F_1 colonies and those crossing experiments were successful for all combinations between the different classes.

In the case where F_2 colonies developed from larvae obtained by crossing nonfusible F_1 colonies of different classes (such as F1(I) and F_1(IV)), the resulting colonies could be separated into four

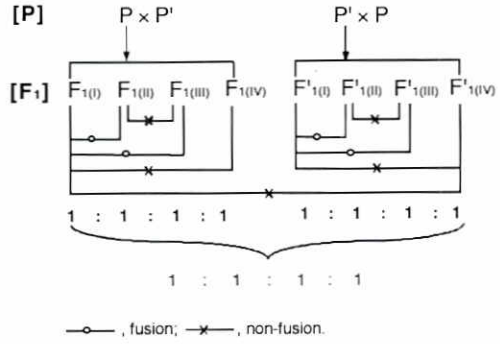

Fig. 1. Fusibility among F_1 colonies derived from P and P' (modified from Oka and Watanabe, 1967).

classes (F_2(I), F_2(II), F_2(III), and F_2(IV)) on the basis of their relative fusibility. The nature of this relationship was identical in form to that for F_1 colonies derived from P and P'.

When F_2 colonies were obtained by crossing two mutually fusible F_1 colonies of different classes (such as F_1(I) and F_1(II)), the resulting colonies were mutually fusible (Fig. 2). That is, fusion occurred between any two colonies derived from F_1(I) and F_1 (II). Further studies on fusibility between such F_2 and F_1 colonies revealed the existence of two types among colonies derived from larvae of these mutually fusible F_1 colonies. Among F_2 colonies derived from F_1(I) colonies by crossing with F_1(II) colonies, F_2(I') colonies were fusible with F_1(IV) but not with F_1(III), whereas F_2(II') colonies were fusible with both F_1(III) and F_1(IV). On the other hand, in the case of F_2 colonies derived from F_1(II) colonies by crossing with F_1(I) colonies, F_2(I'') colonies were fusible with both F1(III) and F_1(IV), whereas F_2(II'') colonies were fusible with F_1(III), but not with F_1(IV).

Fusibility among F_1 and F_2 colonies

Experiments were undertaken to determine the fusibility of the F_2(I), F_2(II), F_2(III), and F_2(IV) colonies with each of the four classes of F_1 colonies. Colonies belonging to F_2(II) and colonies belonging to F_2(III) were not fusible with colonies of F_1(III) and F_1(II), respectively.

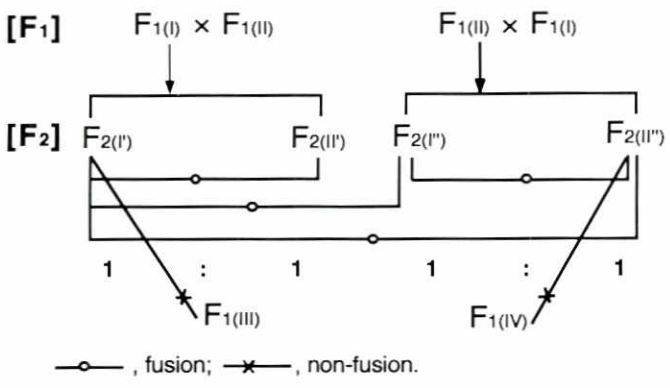

Fig. 2. Fusibility among F_2 colonies derived from fusible colonies of two different classes (modified from Oka and Watanabe, 1967).

Fusibility among P and F_2 colonies

Experiments were undertaken to determine the fusibility of four F_2 classes with the parental colonies P and P'. Colonies belonging to the mutually nonfusible classes F_2(II) and F_2(III) were fusible with colonies of either P or P'. However, colonies belonging to F_2(I) were fusible only with P, and colonies belonging to F_2(IV) were fusible only with P'. The colonies F_2(I) and F_2(IV) are thus fusible with all four classes of F_1 colonies and with one of the parent colonies (not with P' and P, respectively). They are classified together as group I (see Fig. 3). The colonies F_2(II) and F_2(III) are fusible with three of the four classes of F_1 colonies and with both kinds of parent colonies. They are classified together as group II (see Fig. 3).

The basis of fusibility

In order to explain the apparently complicated fusion and nonfusion phenomena outlined above, it is necessary to introduce some hypotheses as described by Oka and Watanabe.

(1) Under natural conditions, colonies are heterozygous for the genes controlling fusibility.

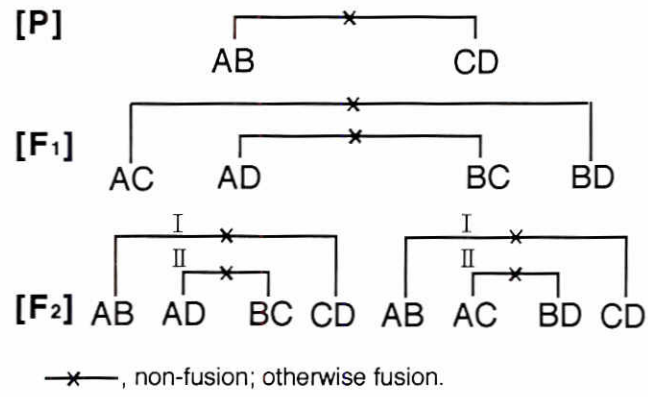

Fig. 3. Diagram of fusibility in P, F_1, and F_2 colonies (modified from Oka and Watanabe, 1967). *I, group I (AB and CD in F_2); II, group II (AD and BC or AC and BD in F_2).*

(2) These genes are similar to a series of multiple alleles of the type that control self-incompatibility in higher plants (S genes).

(3) Colonies sharing at least one common gene are mutually fusible. Otherwise, they exhibit rejection. On the basis of these postulates, it is possible to explain the experimental results obtained.

Let us first consider the case where four classes of colonies are produced from two nonfusible colonies which have cross-fertilized. The majority of colonies formed in nature can be represented according to a series of letters AB, CD, EF,...., because they carry no common gene and are not fusible with each other. The crossing of AB with CD produces four classes of F1 colonies, which can be designated AC, AD, BC, and BD. All such individuals share common alleles with the parental colonies AB and CD and are therefore fusible with them. Members of each of the four classes share common alleles with two of the remaining three classes and are therefore fusible with them (Fig. 3).

Any cross between different, nonfusible classes of the F_1 generation, such as AC and BD, produces four classes in the F_2

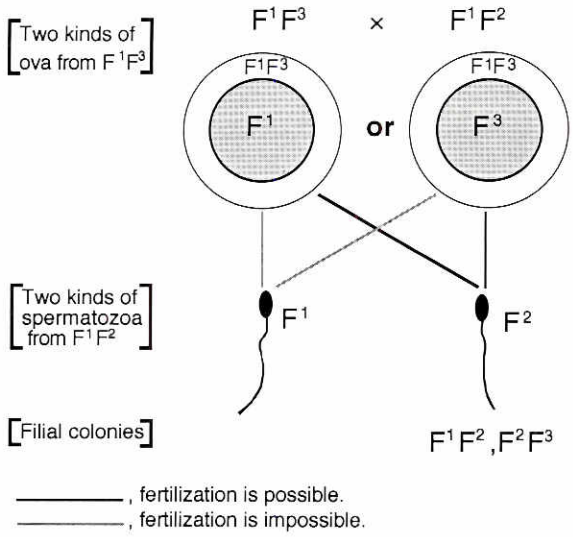

Fig. 4. Haploid-diploid incompatibility in *Botryllus primigenus* (modified from Oka and Watanabe, 1967).

generation with fusibility of the same kind as that described above for F_1 colonies. However, because of self-incompatibility (to be discussed in more detail below), the crossing of fusible colonies of different classes, such as AC and AD, produces only 2 classes in each case (AD and CD from AC, and AC and CD from AD). All pairs of different colonies selected from the three classes AC, AD, and CD have a single common allele, and so are fusible with each other. However, the absence of a common allele between AC and BD or AD and BC prevents their fusion. CD shares a common allele with both BC and BD and is thus fusible with them. To distinguish between the two classes of the F_2 generation produced by fusible F_1 colonies, it is thus necessary to attempt fusion with members of other generations.

In order to examine both the validity of these ideas and the actual distribution pattern of alleles controlling fusibility in *B. primigenus*, fusion experiments were carried out on a large number of colonies collected from three areas of the Izu Peninsula (Mukai and Watanabe, 1975a). No exceptions were found to the rules described above, thus providing strong evidence that fusibility among colonies of this species is controlled by a series of multiple alleles at a single locus. On the basis of these results, it appeared that a relatively large number of multiple alleles were involved in the fusion of naturally occurring colonies, perhaps several scores. These genes were designated F^1, F^2, F^3, ..., following the nomenclature of S genes of higher plants.

It is interesting to note how many other organisms display a fusibility of the type seen in *Botryllus*. In 1903, Bancroft could not find the evidence of colony specificity in all colonies of *Botrylloides gascoi* and *B. leachii*, but experiments on colonies of the Japanese species *Botrylloides simodensis* and the American species *Botrylloides diegense* have revealed a relationship similar to that seen among colonies of *B. primigenus* (Mukai and Watanabe, 1975b; Yund and Feldgarden, 1992). Furthermore, Hauenschild (1956) studied fusibility in the hydrozoan *Hydractinia echinata* and described that colony specificity (or, in his words, "tissue specificity") as being controlled by multiple alleles at a single locus. Although the results were not as clear as those with *Botryllus* and showed some differences, it is significant that even in such phylogenetically distant animals as ascidians and hydrozoans similar recognition systems may be operating.

Self-incompatibility

In compound ascidians, whose colonies are composed of hermaphroditic individuals, there are, theoretically, three ways in which fertilization could occur: fertilization within an individual, fertilization between individuals of a single colony, and fertilization between individuals of different colonies. It has been confirmed, however, that in *B. primigenus* neither self-fertilization nor fertilization between individuals of the same colony occurs. Fertilization is conducted only between different colonies, but does not necessarily occur between any two random colonies.

The result of crossing two nonfusible colonies is an F_1 generation consisting of four classes of colonies, among which two selected pairs are nonfusible with each other. Cross-fertilization within these nonfusible pairs yields, in each case, four different classes in the F_2 generation, as outlined above. Cross-fertilization between fusible F_1 colonies yields only two classes in the F_2 generation. Cross-fertilization between individuals of the same class is impossible. The latter case might be considered to represent a haploid-diploid incompatibility. (Diploid refers to the chromo-

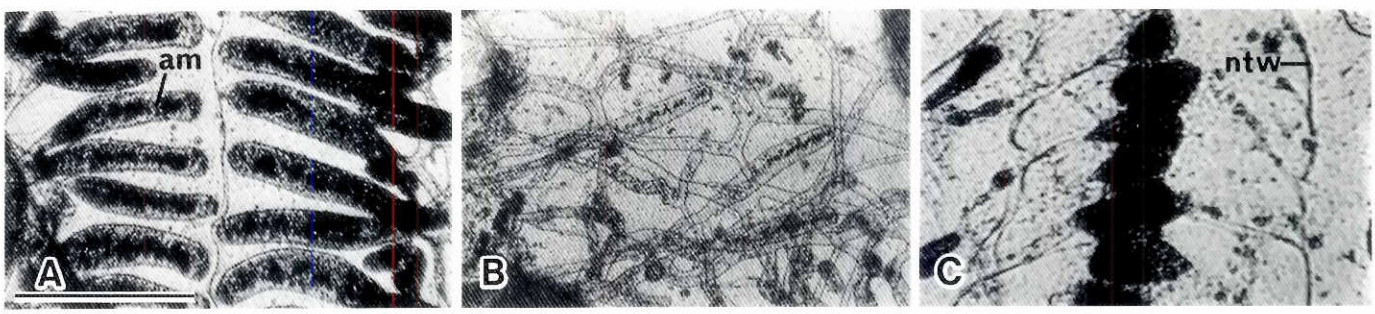

Fig. 5. Photographs of the outcome of contact between two colonies (left and right) of *B. primigenus*. (A) *Contact between two colonies at their tunic surfaces (Scale bar, 1 mm).* **(B)** *Fusion between two colonies. A common vascular network was formed.* **(C)** *Late stage of rejection between another pair of colonies. Amputation of ampullae of both colonies and clotting of blood cells in those ampullae were observed. A new portion of tunic wall was formed to separate the damaged part from the healthy part. am, ampulla; ntw, new tunic wall.*

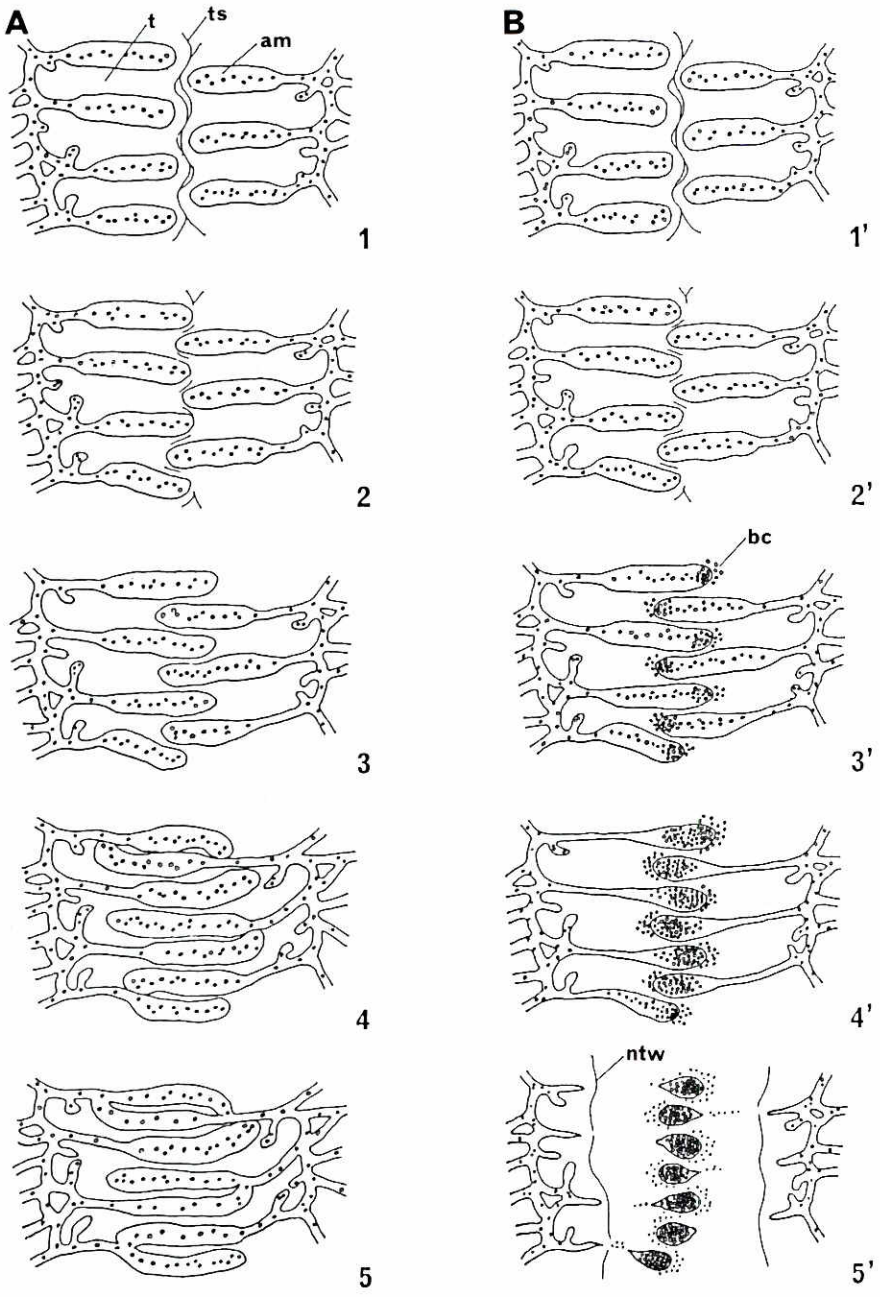

Fig. 6. Diagram summarizing the processes of fusion and nonfusion in *B. primigenus*. *Numerals represent the stages of fusion* **(A)** *and rejection* **(B)**. *am, ampulla; bc, blood cell; t, tunic; ts, tunic surface; ntw, new tunic wall.*

some number characteristic of the somatic cells, and haploid to half that number). That is, in terms of the F alleles controlling fusion, fertilization would occur only in cases where the alleles of the spermatozoa and the alleles of the colony that produces ova were different. In crossing F^1F^2 with F^3F^4, the F^1 and F^2 spermatozoa produced by the former are able to fertilize the F^3 and F^4 ova of the latter. In this way, four classes, F^1F^3, F^1F^4, F^2F^3, and F^2F^4, are produced. When F^1F^3 is crossed with F^1F^2, spermatozoa F^1 are unable to fertilize the ova because F^1 is shared between F^1F^3 and F^1F^2, but spermatozoa F^2 from F^1F^2 are able to fertilize the ova F^1 and F^3 of F^1F^3 to produce the two classes of progeny, F^1F^2 and F^2F^3 (Fig. 4).

The above situation corresponds exactly to the homomorphic self-incompatibility prevailing among angiosperms. In that case, the self-incompatibility is controlled by the S alleles, S^1, S^2, S^3, ..., and fertilization occurs only when there is a difference between pollen and stylar tissue alleles.

Fusion and nonfusion reactions in Botryllid Ascidians

In botryllid ascidian colonies, zooids are covered with a common tunic that is gelatinous and translucent or transparent, and they are interconnected by a ramified vascular network. The margin of a colony is fringed by many vascular ampullae, which are the

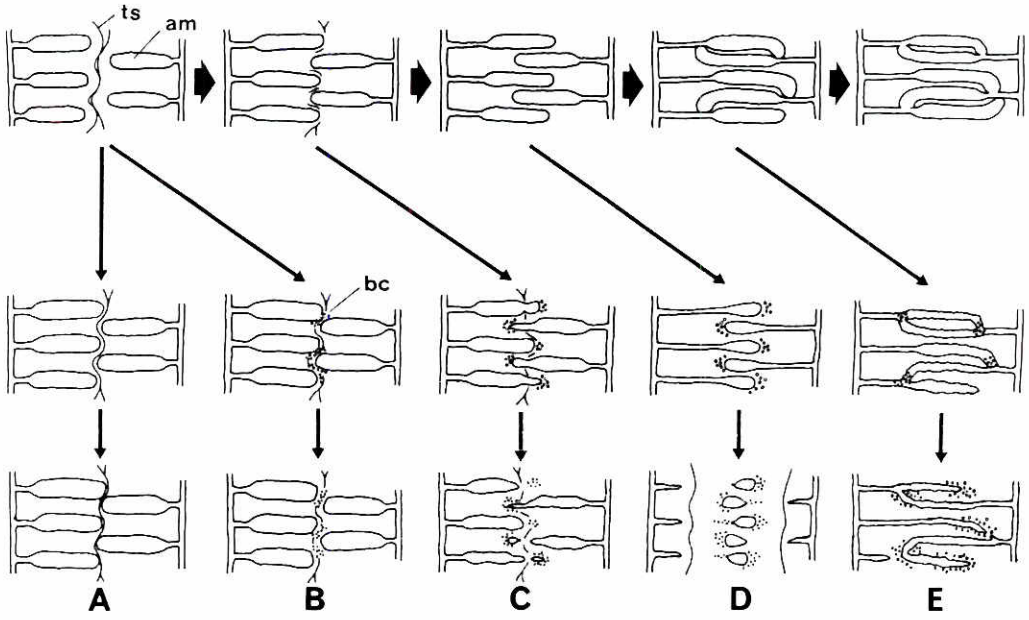

Fig. 7. Variations in the appearance of rejection for different botryllid species. (A) *Botrylloides simodensis, B. fuscus,* and *B. violaceus.* **(B)** Monterey *Botryllus schlosseri.* **(C)** Woods Hole *B. schlosseri.* **(D)** *B. primigenus.* **(E)** *B. scalaris. Upper row shows the fusion process. ts, tunic surface; am, ampulla; bc, blood cell.*

terminals of vascular vessels. Fusion and rejection occur mainly at the tunic covering the ampullae and at the ampullae themselves (Fig. 5; Tanaka and Watanabe, 1973; Katow and Watanabe, 1980). The processes of fusion and rejection in *B. primigenus* are summarized schematically in Fig. 6.

The process of fusion

First, two colonies make contact with each other at the surfaces of their tunic cuticle layer, which has an electron-dense structure (Stage 1), and then the cuticle begins to dissolve at the contact area (Stage 2). After disappearance of the tunic cuticle boundary, vascular ampullae of each colony expand into the facing colony (Stage 3). The penetration of ampullae continues until their tips touch the proximal parts of ampullae of the facing colony (Stage 4). Finally, fusion of vascular vessels occurs between the tips and proximal parts of ampullae, and two colonies become a single colony with a common tunic and a common vascular system (Stage 5). These processes are common in all botryllids that have been studied (Saito and Watanabe, 1982; Hirose *et al.,* 1988; Boyd *et al.,* 1990).

The process of nonfusion

If two colonies are incompatible, they can either simply not fuse or they can show rejection reaction at the contact area. The rejection reaction is called "non-fusion reaction (NFR)" (Tanaka and Watanabe, 1973). In *Botryllus primigenus*, the first sign of NFR appears when ampullae penetrate into the facing colony (Stage 3 of the fusion process). Three to five hours after contact, the blood cells begin to infiltrate from the ampullar tips into the tunic of the facing colony (Stage 3').

The infiltrating blood cells are mainly morula cells. Nine to ten hours after contact, blood current becomes slow, cell aggregation occurs at the ampullar tips, and the proximal parts of ampullae gradually become thin (Stage 4'). About 12 hours after contact, the proximal part of an ampulla shrinks and finally becomes amputated. At this time a new portion of tunic wall is formed to separate the healthy part from the colony part that was damaged by NFR (Stage 5'). This new tunic wall has the same structure of tunic cuticle layer. Soon, the amputated ampullar tips and the blood cells in that area begin to disintegrate. Then, this necrotic part is detached, and the two colonies separate completely. Thus, NFR occurs mainly at the vascular ampullae, and its characteristics are as follows: infiltration of blood cells, aggregation of blood cells, and shrinkage and amputation of blood vessels (Taneda and Watanabe, 1982b).

When two colonies are brought into contact with each other at their artificial cut surfaces, fusion or rejection occurs faster than in the case of contact at their natural growing edges. The tunic becomes fusible soon after contact, and the ampullae begin to penetrate into the facing colony to effect fusion (if the colonies are compatible) between their tips and the proximal parts of the opposite ampullae, or injured ampullar tips fuse with the tips of injured ampullae of the facing colony. When two colonies are incompatible, a series of NFR responses begins before the penetration of ampullae occurs.

Blood components are primarily involved in NFR. When a small AB colony fuses with a large BC colony, the blood of the AB colony is replaced by the blood of the BC colony. A few days after fusion, these two colonies are separated. When the small AB colony is brought into contact with a naive CD colony, it is now fusible with the CD colony — although prior to fusion with the BC colony, it would not fuse with a CD colony (Mukai, 1967). In another experiment, three colonies, AB, BC, and CD, are arranged in a line to allow contact between neighboring colonies; fusion between AB and BC and fusion between BC and CD are allowed to occur at the same time. Subsequently, NFR appears in the vascular network of the central BC colony (Tanaka, 1973). It has also been found that NFR is suppressed when two incompatible colonies have been irradiated with X rays (Taneda and Watanabe, 1982a). In an irradiated colony, the number of lymphocyte-like cells is remarkably reduced, therefore lymphocyte-like cells might play an important role in NFR. It is also thought that colony-specific humoral factors exist in the blood, because blood plasma can induce NFR in the blood vessels of an incompatible colony by microinjection

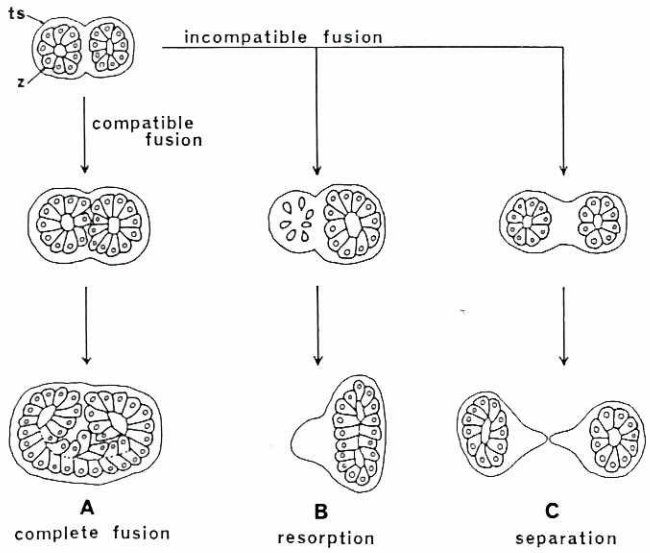

Fig. 8. Three types of responses after the establishment of fusion. (A) *Complete fusion of compatible colonies.* **(B and C)** *Incompatible fusion. Either resorption of zooids* **(B)** *or re-separation into two colonies* **(C)** *occurs about 2 weeks after fusion. z, zooid; ts, tunic surface.*

into a vessel (Taneda and Watanabe, 1982c; Saito and Watanabe, 1984). These factors seem to be present in the tunic matrix, because NFR begins before fusion of blood vessels in *B. primigenus* (Taneda, 1985). It may be the lymphocyte-like cells that recognize these factors, but the possibility cannot be excluded that the epithelial cells of the ampullae and the tunic cells can recognize them.

Variations of nonfusion reactions among botryllid species

In botryllid ascidians, five species — *Botryllus scalaris, Botryllus schlosseri, Botrylloides simodensis, Botrylloides fuscus*, and *Botrylloides violaceus* — have colony specificity, as does *B. primigenus* (Sabbadin, 1962; Mukai and Watanabe, 1974; Saito and Watanabe, 1982; Scofield and Nagashima, 1983; Hirose *et al.*, 1988). That is, fusion or nonfusion occurs between two colonies of the same species. The fusion reaction process is the same in all botryllids, but the nonfusion reaction process is not uniform among them. The nonfusion reaction is not merely the absence of fusion between two colonies, as in the case of contact between two xenogeneic colonies. The rejection reaction appears as a way to interfere with the fusion reaction process.

As mentioned above, in *B. primigenus* NFR begins between Stage 3 and Stage 4 of the fusion process (Fig. 7D), but in *B. scalaris,* the first sign of rejection appears after fusion of ampullae of both incompatible colonies, that is, just before Stage 5 of fusion process (Fig. 7E). The characteristics of rejection in this species are almost the same as those of NFR in *B. primigenus*. Soon after the exchange of blood begins through the fused ampullae, clotting of blood cells occurs in the fused vessels and the blood flow stops there. Then, the fused vessels shrink and many blood cells infiltrate from those vessels into the tunic matrix. Finally, the vessels become amputated there (Saito and Watanabe, 1982).

In B. *schlosseri* of Woods Hole, Massachusetts (USA), the rejection reaction appears between Stage 2 and Stage 3 (Fig. 7C). In this species, fusion of the tunic does not occur widely at the contact area, but only in small areas around the ampullar tips near the boundary of the facing colonies. Therefore, after penetration of the ampullae, the boundary between the two colonies remains intact except in the areas where ampullae are penetrating. Blood cells infiltrate around the tips of the penetrating ampullae. Then, the penetrating ampullae begin to shrink and withdraw or become amputated near the boundary (Boyd e*t al.*, 1990).

In Monterey *B. schlosseri* of California (USA), rejection occurs before Stage 2 (Fig. 7B). After contact occurs between two incompatible colonies, fusion of the tunics of these two colonies cannot be seen under the binocular stereomicroscope. The ampullae of both colonies push against each other through their tunic cuticle layers, and then blood cells begin to infiltrate into the tunic from the ampullar tips. These blood cells become necrotic and their color turns black. Thus, several black spots, which are visible to the naked eye, are formed between facing ampullae, and then the ampullae withdraw a little from the boundary (Scofield and Nagashima, 1983; Boyd et *al.*, 1990). Woods Hole and Monterey *B. schlosseri* have the same morphology and life history, and colonies from the two populations are capable of interbreeding to produce fertile progeny; nevertheless their manner of rejection is different. When a Woods Hole colony makes contact with a Monterey colony, tunic fusion does not occur between them, and extensive rejection appears in the Woods Hole colony, but little or no rejection appears in the Monterey colony. Therefore, perhaps species differentiation is progressing between these two geographically separated populations (Boyd *et al.*, 1990).

In three Japanese botryllids belonging to the genus *Botrylloides* —*B. simodensis, B. fuscus,* and *B. violaceus* — rejection also occurs before Stage 2 (Fig. 7A). In these species, as well as in Monterey *Botryllus*, fusion of the tunic is not seen under the binocular stereomicroscope. Furthermore, they do not show a remarkable infiltration of blood cells from the ampullae, and they behave as if they had made contact with colonies of a different species or with a substratum such as stones or seaweed. However, in histological studies of the contact area between the colonies, it was observed that the structure of the cuticle layers had disappeared at some tiny areas and tunic fusion was observed there. In these small fused areas, there are clusters of tunic cells which are normally found in the subcuticular zone of the tunic, and several morula cells come out of the ampullae near these areas. These tunic cells and morula cells might destroy the fused areas and prevent the expansion of fused areas. This type of limited rejection is called "sub-cuticular rejection" (SCR; Hirose e*t al.*, 1988).

When two nonfusible colonies of each species, *B. scalaris, B. schlosseri,* and *B. simodensis,* are brought into contact with each other at their cut surfaces, they show rejection reactions like the NFR of *B. primigenus,* although the intensity of the reactions is different among them. The most intense reaction is observed in *B. simodensis* (Hirose *et al.*, 1990). On the other hand, *Botrylloides fuscus* and *B. violaceus* do not show any rejection reactions — fusion occurs at cut surfaces of nonfusible colonies (Hirose *et al.*, 1988). These two species can show sub-cuticular rejection when nonfusible colonies make contact at their growing edges. It seems that the allorecognition of colony specificity is lacking inside the tunic cuticle layer in these two species.

For the species that exhibit colony specificity, two compatible colonies must be able to fuse when they make contact at their

growing edges. For species that demonstrate allorecognition ability, the colonies first have to distinguish allogeneic colonies from xenogeneic colonies at their tunic surface. The rejection reactions in botryllids appear to prevent the fusion process, and the stage of appearance of rejection is not the same among them. In *B. simodensis, B. fuscus* and *B. violaceus,* soon after the beginning of fusion at tunic cuticle layer, each colony recognizes the incompatible partner as a nonfusible colony (the first step of recognition). In *B. primigenus,* colonies recognize each other as nonfusible after ampullar penetration (the second step of recognition). In *B. scalaris,* colonies recognize that they are nonfusible with each other only after the fusion of blood vessels (the third step of recognition).

Colony resorption

The genetic basis for colony specificity resides in a single highly polymorphic gene locus with codominantly expressed alleles, and these alleles seem to be involved in determining the compatibility of fertilization (Oka and Watanabe, 1960; Scofield *et al.,* 1982; Yund and Feldgarden, 1992). These characteristics are similar to those of the major histocompatibility gene complex (MHC), which controls vertebrate transplantation immunity and mating preference in rodents (Yamazaki *et al.,* 1976). However, there are two differences between the colony specificity of botryllids and the transplantation immunity of vertebrates. First, in botryllids, rejection appears about 1 day after contact between two nonfusible colonies, whereas in vertebrate transplantation immunity rejection takes much longer. The second, and major, difference is that in transplantation immunity of vertebrates, grafts are accepted only between animals that share both MHC haplotypes, whereas in colony specificity of botryllids, two colonies sharing at least one allele at the fusibility locus may fuse and rejection develops only between colonies that share no allelic determinants at this locus.

Recently, in *B. schlosseri* and *B. scalaris,* an interesting phenomenon was found that may help to explain the relationship between colony specificity and MHC-dependent graft rejection (Fig. 8; Saito and Watanabe, 1982; Scofield *et al.,* 1982). When fusion occurs between two pieces derived from the same colony, these two pieces always become a single colony. On the other hand, when fusion occurs between two pieces derived from different colonies, blastozooids of one or both partners in that chimera often become resorbed more than 1 week after the fusion, or, sometimes the two pieces become separated again by amputation of the fused vessels. A similar phenomenon has also been observed in the other four Japanese botryllids (authors' unpublished data). This observation suggests that initial fusion does not always indicate acceptance of the fused partner as self and that more critical self-nonself recognition is carried out after fusion.

From studies of this phenomenon, it has become clear that there are two types of fusion, "compatible fusion" and "incompatible fusion", and these two types of fusion are determined genetically (Rinkevich and Weissman, 1987, 1989). When fusion occurs between two colonies sharing only one allele at the fusibility gene locus (such as AB and AC colonies), rejection occurs resulting from incompatible fusion (Weissman *et al.,* 1988; Rinkevich and Weissman, 1989). Almost 70% of the cases of rejection owing to incompatible fusion are resorption of the blastozooids of one partner in the chimera (Rinkevich and Weissman, 1992), thus the recognition after fusion is called «colony resorption». The direction of resorption appears to be inherited, as multiple subclones of asexually derived individuals from colony #1 always resorb paired subclones from colony #2, independent of laboratory condition and colony age (Rinkevich and Weissman, 1987). In the resorption phenomenon, a linear hierarchy was shown within each generation of inbred strain of *B. schlosseri* (Rinkevich *et al.,* 1993). Moreover, colony resorption is also occasionally observed when two colonies that have the same set of alleles at the fusibility locus are fused. This could mean that minor histocompatibility genes are also present in *Botryllus* (Rinkevich, 1993; Rinkevich *et al.,* 1993).

From these findings, it has become clear that there are two types of allorecognition responses in botryllid ascidians: colony specificity and colony resorption. Regarding these two types of allorecognition, the authors propose a hypothesis that colony resorption is common in all botryllids and it might have been conserved for a long period of time. On the other hand, colony specificity might be a newer ability, obtained more recently in their evolutionary history. In sessile animals, such as botryllids, which grow horizontally on the substratum to increase their occupying space, two parts of the same colony often come into contact with each other at their growing edges. In this case, they have to compete with themselves at the growing edges if they cannot fuse with each other. We speculate that the colonies developed the ability to fuse with each other at their growing edges in order to avoid this competition. However, the ability to fuse was demonstrated even between conspecific incompatible colonies, and that fusion resulted in the occurrence of colony resorption, resulting in significant damage to the chimera. Therefore, colony specificity evolved to prevent fusion with incompatible colonies at as early a stage as possible, that is, from the third step of recognition to the first step of recognition (cf. section entitled "Variations of nonfusion reactions among botryllid species" in this text). This hypothesis may be supported by the following facts. As mentioned above, all of the six botryllids that we examined show colony resorption, and colony specificity appears to prevent the progression of the fusion process in those species. Furthermore, the allorejection process is different for two remote botryllid populations, Woods Hole and Monterey, in spite of the fact that they belong to the same species *(B. schlosseri).* This suggests that the manner of colony specificity might be able to change relatively quickly.

Concluding remarks

Colonial ascidians are unique in that they can experience grafting of allogeneic tissue in their natural environment. Therefore, allorecognition is indispensable for maintenance of their individuality. Botryllid ascidians have two types of allorecognition responses. One type is colony resorption, which may be homologous to the transplantation immunity of solitary ascidians and also similar to vertebrate allorecognition controlled by the MHC. The other type of allorecognition response is colony specificity, which occurs rapidly and may resemble the contact reaction in solitary ascidians. The former may recognize nonself determinants of allogeneic cells, whereas the latter may distinguish self components from nonself components, as described by Burnet (1971).

Many studies on allorecognition (colony specificity and colony resorption) in botryllid ascidians strongly suggest that the fusibility gene advocated by Oka and Watanabe 40 years ago is the histocompatibility gene in ascidians and that the fusibility gene may be an ancestral form of the MHC, or may share a common ancestor with the MHC. However, we have many questions about the nature of the fusibility gene itself; for example, which chromosome is the gene located on, is there a complex of genes, and what are the

products of these genes. Much work remains to be done in order to compare the fusibility gene with the MHC at the molecular level.

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