# Self and Nonself Recognition in a Marine Sponge, Halichondria japonica (Demospongiae)

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# Self and Nonself Recognition in a Marine Sponge, *Halichondria japonica* (Demospongiae)

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**The function of allogeneic recognition in a marine sponge, *Halichondria japonica*, was examined by use of cut pieces contact assay. Individuals of this species were able to distinguish an allogeneic individual from an autogeneic one, and showed rejection reactions against allogeneic individuals. There were two types of allogeneic rejection reaction: barrier formation at the contact area to separate from allogeneic individuals and necrosis with cytotoxic reactions at the contact area. In both types of rejection reactions, mesohyl cells accumluate at the contact area at the early stages of the rejection reaction. Fusion between two pieces of allogeneic individuals was very rare, and in most of combinations of allogeneic individuals rejection reactions appeared at the contact area. Xenogeneic rejections were also observed. *Halichondria japonica* showed rejection reaction against individuals of *Halichondria okadai*, but the intensity of rejection was less than that of allogeneic rejection.**

**Key words:** allogeneic recognition, rejection, sponge, *Halichondria japonica*, mesohyl cell

## INTRODUCTION

The self-nonself recognition system is one of the most important systems used by animals to maintain their individuality. Vertebrates, especially mammals, have a sophisticated recognition system called the "immune system." Using this system, animals can eliminate invasive organisms, such as viruses, bacteria, and parasites, from their bodies. Furthermore, they can recognize allogeneic tissues and organs transplanted from other individuals and reject them as nonself. This allogeneic recognition in vertebrates is called "transplantation immunity," and is mainly governed by the major histocompatibility gene complex (MHC). The self-nonself recognition system in vertebrates may have evolved from the recognition system of invertebrates, and as such the phylogenetic study of this system is important for understanding the vertebrate immune system.

The phenomenon of self-nonself recognition has been observed in many invertebrates and is especially well studied in colonial sessile animals, such as sponges (Van de Vyver, 1970; Hildemann and Johnson, 1979; Mukai and Shimoda, 1986), hydrozoans (Hauenschild, 1954; Buss et al., 1984), corals (Rinkevich and Loya, 1983a, b), nemerteans (Bierne, 1985), earthworms (Cooper, 1968), terrestrial slugs (Yamaguchi et al., 1999), cockroaches (Hartman and Karp, 1989), bryozoans (Chaney, 1983; Shapiro, 1992; Ishii and Saito, 1995), sea stars (Karp and Hildemann, 1976), and compound ascidians (Bancroft, 1903; Oka and Watanabe, 1957; Mukai and Watanabe, 1974). As the sponge is situated basally in metazoan phylogeny (Borchiellini et al. 2001), the ability to distinguish self from nonself in these animals is critical to developing an understanding of the evolution of the immune system in vertebrates. Using the cell aggregation method developed by Wilson (1907), Galtsoff first showed the xenogeneic recognition of sponge cells between two species with different color morphs, *Microciona* sp. and *Haliclona* sp. (1925). Subsequently, allogeneic recognition was reported in several species belonging to the two classes, Demospongiae and Calcarea (Hildemann et al., 1979; Bigger et al., 1981, 1983; Neigel and Avise, 1983; Buscema and Van de Vyver, 1984a, b, c; Zea et al., 1986; Amano, 1990). However, detailed information about the process of allogeneic rejection and the genetics of allogeneic recognition in sponges is limited.

The marine sponge *Halichondoria japonica* is very common in the inter-tidal zone of the rocky reefs on the island of Honshu in Japan. Individuals of this species spread on rock surfaces in tide pools or in grooves on the rocky shore, and form an irregular mass. Population densities are often very high, and contacts between individuals at their growing edges may occur very often under natural conditions. Additionally, if an individual can recognize allogeneic individuals as nonself, allogeneic recognition in this species would be common in nature. In the present study, the occurrence of allogeneic recognition in this species was examined and the manner of allogeneic recognition was observed. Furthermore, the mode of xenogeneic recognition against tissue of *Halichondria okadai*, which is also a very common species in Japanese coastal areas, was observed. As *H. okadai* occurs in the same habitat as *H. japonica*, individuals of *H. japonica* often come into contact with individuals of *H. okadai* as well as allogeneic individuals.

## MATERIALS AND METHODS

### Animals

Individuals of *H. japonica* and *H. okadai* were mainly collected in an area, about 3 m wide × 30 m long, of the rocky shore in

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Nabeta Bay near the Shimoda Marine Research Center, University of Tsukuba (34°39′53″N, 138°56′15″E). This bay faces the Pacific Ocean and is situated near the top of Izu Peninsula, Shizuoka Prefecture. Several individuals of *H. japonica* were also collected from the rocky shore of Shimoda Bay near Nabeta Bay. The distance between collected individuals was at least 1 m. Collected individuals were brought to the laboratory and kept in a running seawater tank. Other organisms, such as algae, barnacles, annelids and small arthropods, were removed from collected individuals. Each individual was cut into small pieces (about 7 mm wide × 20 mm long × 5 mm thick) for allogeneic and xenogeneic recognition assays (Fig. 1).

**Assays for allogeneic and xenogeneic reactions between two individuals**

Two pieces from different individuals were placed in juxtaposition on glass slides, and were brought into contact with each other either at their growing edges or at cut surfaces. Pieces were fastened to glass slides by cotton thread and maintained in the laboratory with running seawater between 17–20°C (Fig. 1). For growing edge assays, the layers of pinacocytes called "ectopinacoderm," which are epidermis-like, came into contact first. Alternatively, for cut surface assays, the mesohyl of the individuals came into contact first (Fig. 2). In order to examine the ability of self-nonself recognition of pinacocytes, two types of assays were carried out for each combination of individuals. Also for each combination, duplicated sets of a pair were always examined. Observations of allogeneic and xenogeneic reactions were made using a binocular stereomicroscope.

For the allogeneic assay, two experiments were done. Six individuals in the one experiment, and 12 individuals in the other experiment were used respectively.

**Light microscopy**

Animals used for the allogeneic and xenogeneic reaction assays were fixed in 10% formalin in seawater. Thereafter, they were rinsed, dehydrated with alcohol, and embedded in paraffin. All specimens were cut into sections of 7 μm thick and stained with Delafield's hematoxylin and eosin yellow (Merck). Sections were observed under a light microscope (Nikon Optiphoto).

**Scanning electron microscopy**

To observe the details of internal structure using scanning electron microscopy (SEM), paraffin-embedded specimens of the sponges demonstrating allogeneic recognition were cut with a microtome blade to expose the surfaces that were to be examined. The specimens were washed in xylene (1 h, three changes) to remove the paraffin. Next, they were dehydrated with acetone, and the acetone in the specimens was replaced with isoamyl acetate. After being dried at a critical point and sputter-coated with gold-palladium, the specimens were examined in a Hitachi S-570 scanning electron microscopy at 20 kV.

## RESULTS

### Allogeneic recognition in *Halichondria japonica*

When a piece of an individual was placed in contact with another piece of the same individual at the growing edge, the ectopinacoderm at the contact surfaces disintegrated, and the ectopinacoderm at the outer margin of the contact surfaces fused with the opposed ectopinacoderm a few hours after contact. The fusion of mesohyl followed after the disintegration of ectopinacoderm, and the fused area widened gradually. Approximately six hours after contact, the two pieces could be separated simple. About 24 hours after contact, the autogeneic pieces had fused completely with no visual boundary between two individuals (Fig. 3A–C). When two pieces of an individual were placed in contact with each other at cut surfaces, the fusion process was almost the same as for growing edge contact. However, since there was no ectopinacoderm at the contact area, the fusion of mesohyl progressed more quickly than that in the case of growing edge contact.

When two allogeneic pieces were used, rejection reactions were observed. When a piece of an individual was brought into contact with another piece of a different individual at the growing edge, the disintegration of ectopinacoderm at the contact area and fusion of ectopinacoderm at the outer margin of contact surfaces occurred within a few hours, and the fusion

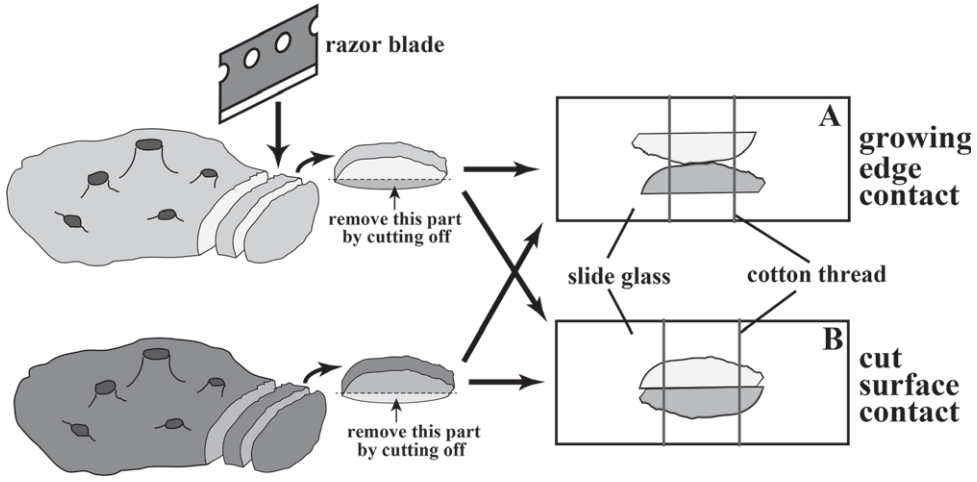


**Fig. 1.** Method of cut pieces contact assay for self-nonself recognition in sponges.

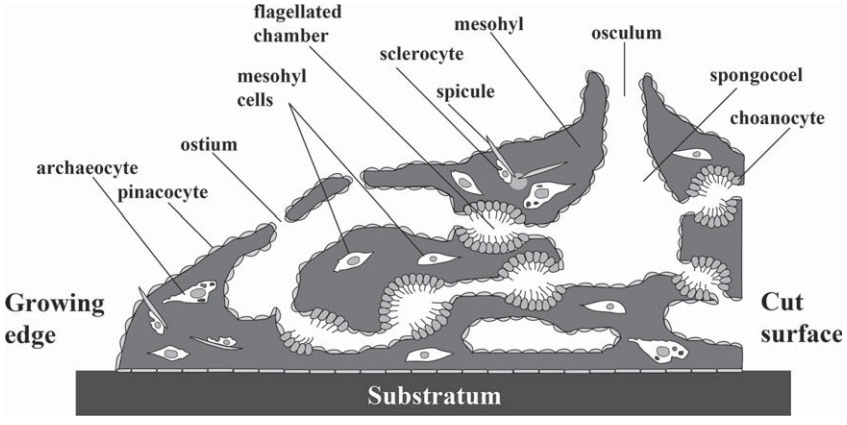


**Fig. 2.** Anatomical illustration of halichondrid sponges.

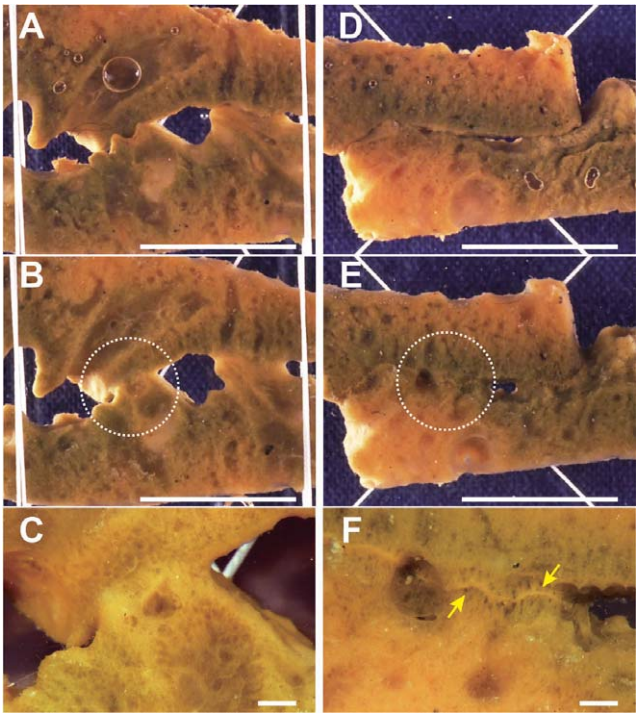


**Fig. 3.** Allorecogntion in *Halichondria japonica* at growing edge contact. The left column is a compatible case, and the right column is an incompatible case. **(A)** Just after contact between two autogeneic pieces (0 h). **(B)** The same, 24 h after the contact. Fusion occurred at the contact area. **(C)** Amplified image of the circled area in **(B)**. **(D)** Immediately following contact between two allogeneic pieces (0 h). **(E)** The same, 24 h after the contact. Clear line appeared at the boundary. **(F)** Amplified image of the circled area in **(E)** Arrows pointed the line formed at the boundary of two pieces. Scale bars in **(A, B, D, E)** represent 10 mm, and in **(C, F)** represent 1 mm.

of mesohyl followed. This process was almost same as that in autogeneic contact. However, 24 hours after contact, at the fused area a bright orange line with about 180 μm width began to appear as the boundary of the two individuals (Fig. 3D–F). On histological study, mesohyl cells began to gather around fused area in the early stage of the mesohyl fusion (Fig. 4A). The mesohyl cells from both individuals gradually increased in number at the fused area (Fig. 4B), and about 24 hours after contact the density of mesohyl cells became very high (Fig. 4C). By 48 hours after contact a barrier formed with fibrous materials in the center of the gathered mesohyl cells (Fig. 4D) and the number of mesohyl cells gathered in fused area were reduced.

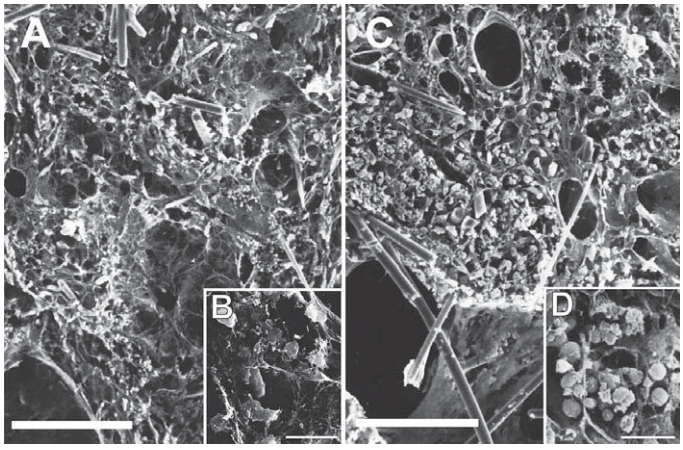


**Fig. 5.** SE-photographs of Allorejection in *H. japonica*. **(A)** Normal mesohyl. **(B)** Magnified image of normal mesohyl. **(C)** Mesohyl in fused area, 24 hours after the cut surface contact between two incompatible allogeneic individuals. **(D)** Magnified image of fusion area between two allogeneic individuals. Many cells gathered in mesohyl around allorejection area. Scale bars represent 100 μm in **(A, C)**, and 10 μm in **(B, D)**.

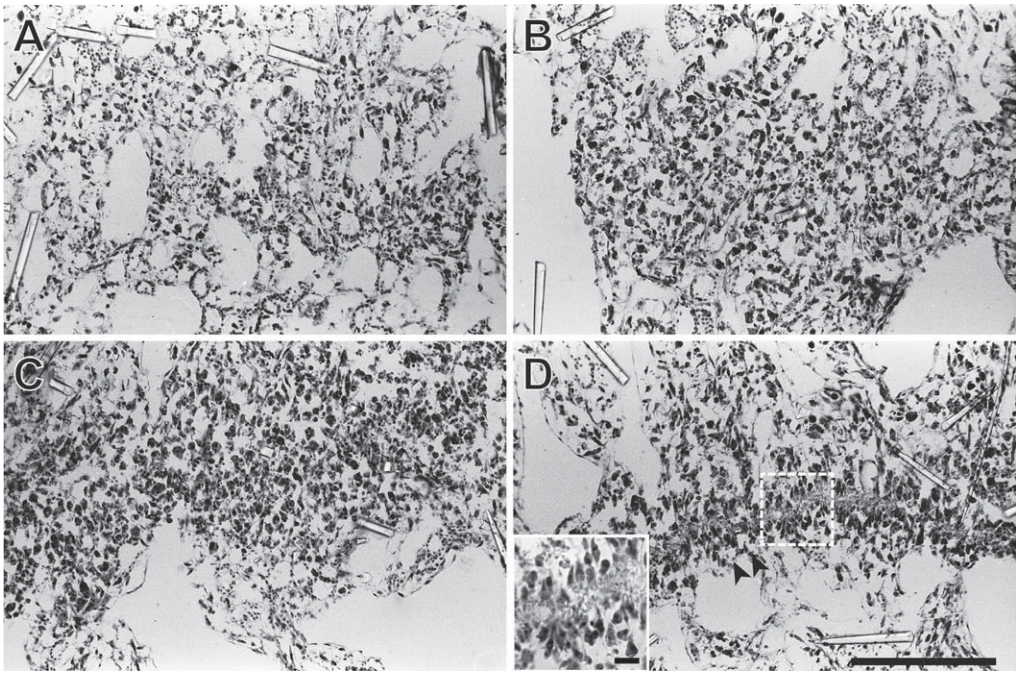


**Fig. 4.** Histological data of allorejection in *H. japonica* at growing edge contact. **(A)** Fused area of mesohyl between two incompatible individuals, 6 h after contact. **(B)** The same, 12 h after the contact. **(C)** The same, 24 h after the contact. **(D)** The same, 48 h after the contact. The area of high density of mesohyl cells became a narrow band (arrowheads) and fibrous materials accumulated to form a barrier between two individuals (see inset). Inset is a magnified photo of the area enclosed with broken line. Scale bar represents 100 μm and 10 μm in inset.

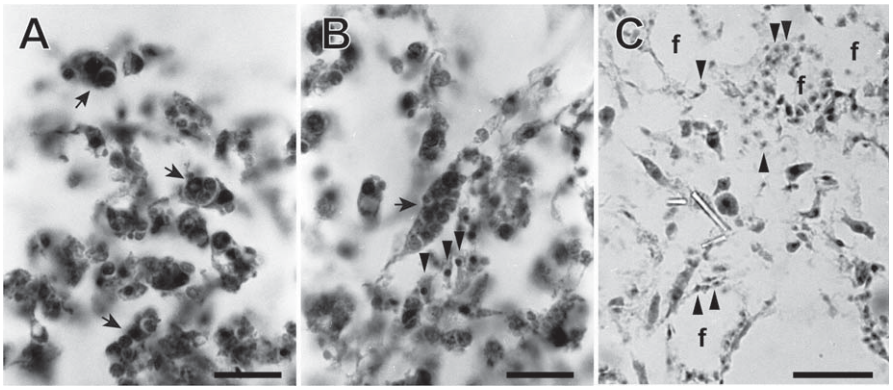


**Fig. 6.** Phagocytosis in fused area between allogeneic pieces. **(A)** and **(B)** Cells having phagosomes (arrows) in the mesohyl of fused area. **(C)** Intact mesohyl. The density of mesohyl cells is low and cells having phagosomes are rare. Arrowheads indicate choanocytes. f, flagellated chamber. Scale bar represents 10 μm in **(A, B)**, and 50 μm in **(C)**.

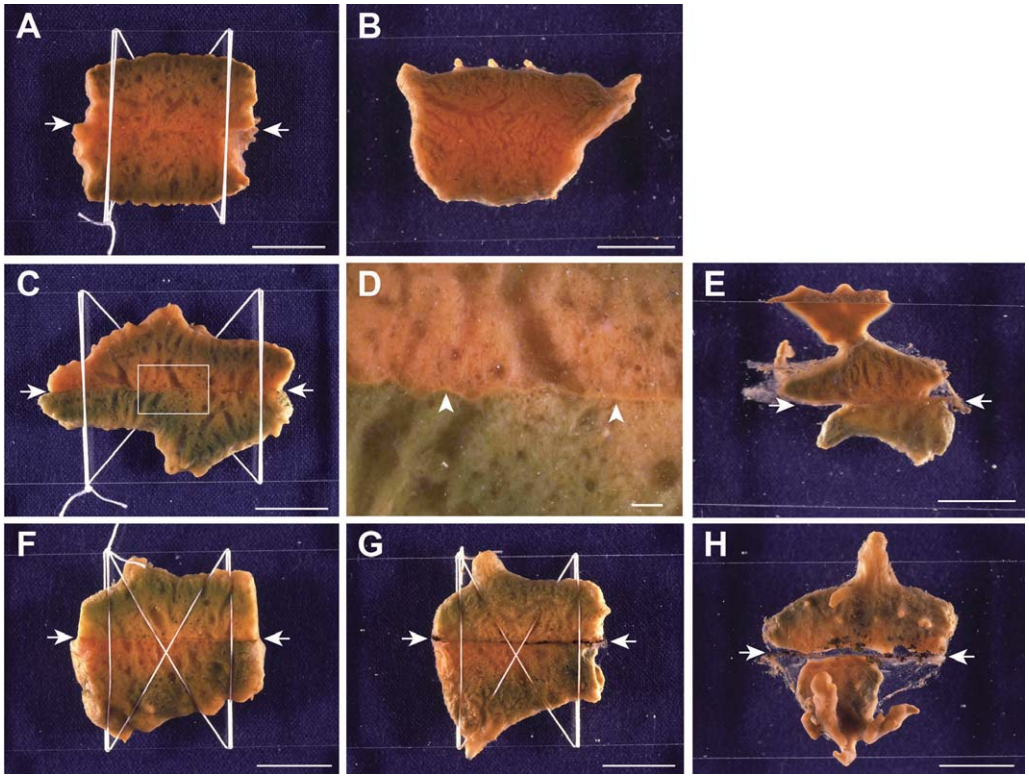


**Fig. 7.** Two manners of allorejection in *H. japonica* at cut surface contact. **(A)** Two autogeneic pieces 1 day after contact. **(B)** The same, 30 days after contact. They are completely fused into a single individual. **(C)** Two allogeneic pieces one day after contact. **(D)** Magnified image of the square in **(C)**. Clear line appeared at the contact area (arrowheads). **(E)** The same, 30 days after the contact. Nothing happened at the contact area. **(F)** Two allogeneic pieces one day after contact. **(G)** The same, three days after contact. A black line appeared at the contact area. **(H)** The same, 30 days after contact. Two pieces were separated by necrosis at the contact area. Arrows indicate the boundary of two pieces. Scale bars represent 1 cm, except in **(D)**, and 1 mm in **(D)**.

As shown in Figures 4 and 5C–D, several types of mesohyl cells gather at sites of fusion between two allogeneic individuals. On the other hand, Figure 5A–B shows the density of mesohyl cells in normal tissue is very low. In the fused area between two allogeneic individuals, cells having phagosomes were frequently found (Fig. 6) in addition to the accumulation of mesohyl cells.

### Types of allogeneic rejection in *H. japonica*

In most pairs of individuals of *H. japonica*, the allogeneic rejection was as described above. The barrier formed between two individuals remained for more than one month, and each individual grew independently (Fig. 7C–E). However, a different type of allogeneic rejection was also observed in several pairs of individuals, in which a reaction resembling necrosis was observed. At the contact area, tissues became black and a black line was formed between two individuals instead of the formation of a barrier (Fig. 7F–G). Furthermore, the black line thickened and then peeled away and fell from the contact area. Finally the individuals grew apart from each other as result (Fig. 7H). Pair showing this type rejection did not always show the same type of rejection. As shown in Fig. 8, in eight cases among 66 allogeneic combinations, both types of rejection were respectively shown in two pairs of the same combination.

### Frequency of allorejection among a population of *H. japonica*

Fusibility was examined by cut piece assay between allogeneic individuals from populations from the rocky shore of Nabeta Bay. Among 12 individuals, all combinations were examined, and there was no fusion between allogeneic individuals (Fig. 8). Another experiment in which all combinations of six individuals were examined, 14 allogeneic pairs showed rejection and only one pair fused. Individuals from the population of Nabeta Bay did not fuse with any allogeneic individuals from the population of Shimoda bay.

### Xenogeneic rejection of *H. japonica* against *H. okadai*

Individuals of *H. japonica* never fused with individuals of *H. okadai* when they came into contact with each other at their growing edges and no rejection reaction was observed

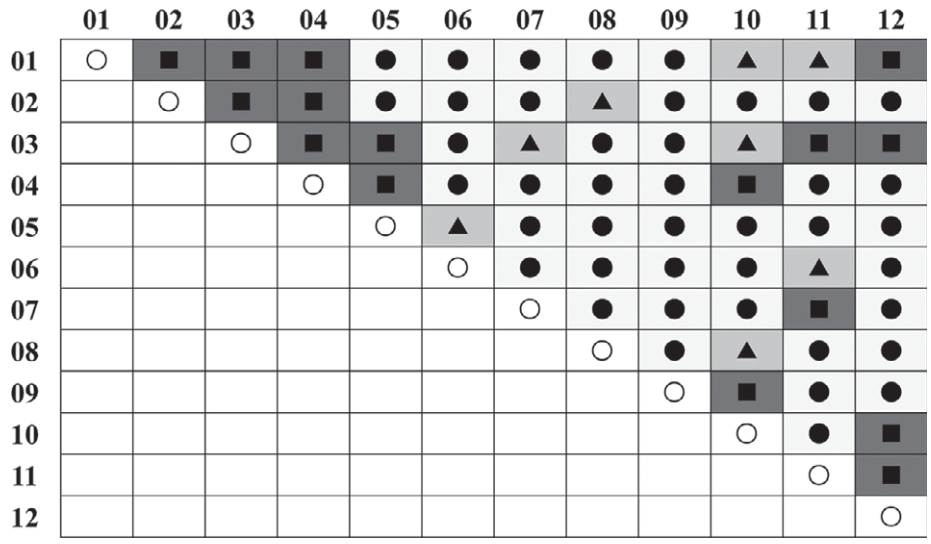


**Fig. 8.** Results of cut piece contact assay among 12 individuals of *Halichondria japonica*. Duplicated sets were examined for each combination. The observation was made 21 days after cut surface contact. Specimens were kept in a running seawater tank. Seawater temperature was 17.5°C. ○, Fusion; ■, Rejection, both sets showed necrosis; ▲, Rejection, one showed necrosis and the other showed formation of a barrier; ●, Rejection, both showed formation of a barrier.

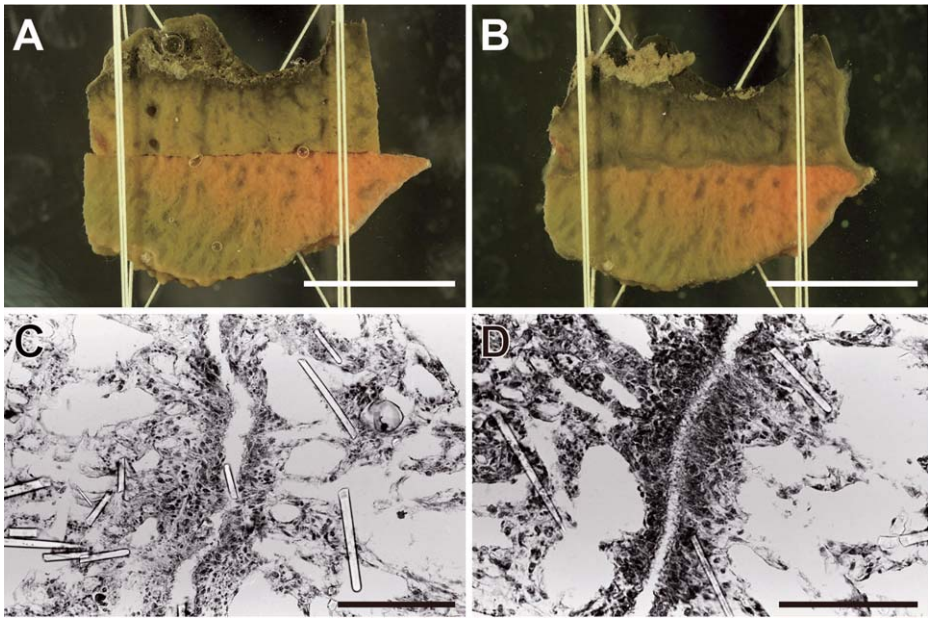


**Fig. 9.** Xenorejection between *H. japonica* and *H. okadai* at cut surface contact. **(A)** Two xenogeneic pieces 6 h after the contact. **(B)** The same, 96 h after the contact. **(C)** Histological section of contact area 24 h after the contact. **(D)** The same 96 h after the contact. In **(C, D)**, right side is *H. japonica*. Scale bars represent 1 cm in A and B, and 100 μm in **(C, D)**.

(Fig. 9A–B). When two individuals came to contact each other at their cut surfaces, mesohyl cells gathered at the contact area. However, the fusion of mesohyl between two individuals did not occur, and filamentous materials formed between the two individuals (Fig. 9C–D).

## DISCUSSION

All individuals of *H. japonica* showed self and nonself recognition. Each individual could accept autograft as self, and can reject allograft as nonself. From the experiments using two conditions, growing edge contact and cut surface contact, it became clear that the ectopinacoderm which is the outermost tissue of the sponge body did not participate in allogeneic recognition. The ectopinacoderm of two allogeneic individuals always fused with each other and formed a common ectopinacoderm as if autogeneic. Ectopinacoderm of *H. japonica* may have no role in allorecognition. Therefore, allogeneic rejection may be induced after the fusion of mesohyl between two incompatible individuals. Mesohyl cells gathered around fused areas of mesohyl, and several cell types were found, in contrast to previous reports in other species, *Microciona prolifera*, *Leucandra abratsbo*, and *Callispongia diffusa* (Zea et al., 1986; Amano, 1990; Humphreys, 1994; Yin and Humphreys, 1996).

From the first contact with an allogeneic individual, it took more than 20 hours for the rejection reaction to become visible. However, the histological sections showed that mesohyl cells began to gather around the contact area within six hours. It is unclear what attracted mesohyl cells to the contact area, but it can be assumed that some trigger is released during the fusion of mesohyl between two incompatible individuals. About 24 hours after contact, the cell density around fused area was highest (Fig. 4C), and at this time a line appeared at the fused area (Fig. 3E–F). Therefore, the gathering of the mesohyl cells around the fused area may form the line that demarcates the two individuals, after which the gathered cells form the barrier from a fibrous material. It is thought that this fibrous materials is collagen, but its identity has not been determined conclusively. Blackening of the fused area was also observed in several allogeneic combinations, as shown in Figs. 7 and 8. That is, there are two manners of allogeneic rejection in *H. japonica*; one is the formation of a barrier without remarkable cytotoxic reactions, and the other is the blackening of tissues with cytotoxic reactions. Both manners of allogeneic rejection, with and without cytotoxic reaction, were sometimes observed in the same allogeneic combination. From only the data shown in Fig. 8, the genetic rela-

tionship was not clear between the allogeneic combination and the type of allogeneic rejection. The ratio of allogeneic combination showing blackening of tissues in at least one of duplicated sets was not high, about 36% of combinations among 12 individuals and 0% in the other experiments using six individuals. The manner of allogeneic rejection with formation a fibrous barrier was common in *H. japonica* (cf. Fig. 8).

In *Axinella polypoides*, *Leucandra abrastsbo*, and *Ephydatia muelleri*, the accumulation of archaeocytes was found in the fused area of the two incompatible individuals (Buscema and Van de Vyver, 1984a; Amano, 1990; Mukai, 1992) and in *Microciona prolifera* and *Callyspongia diffusa* gray cells gathered at the fused area in the early stage of allogeneic rejection (Zea et al., 1986; Humphreys, 1994; Yin and Humphreys, 1996). In *Toxadocia violacea*, a striking mobilization of a specific cell, leukocyte-type cells was involved in allogeneic rejection reactions (Bigger et al., 1983). Several types of mesohyl cells, including archaeocytes and gray cells, move around within the sponge mesohyl, but the specific role of them has not yet to be determined. Archaeocytes are amoeboid, very mobile cells and capable of being the origin of any other type of cell. Gray cells are also very mobile cells, and contain large oval to spherical, basophilic granules and small glycogen-containing granules (Müller, 1982). In the above-mentioned six species, specific cells among mesohyl cells were involved in the allogeneic recognition, and, in the five species except *E. muelleri*, remarkable cytotoxic reactions occurred at the fused area. On the other hand, in this study of *H. japonica* several types of mesohyl cells were accumulated at the fused area (Fig. 4) as with the *Ephydatia fluvatilis* (Buscema and Van de Vyver, 1984c). And in those two species a fibrous barrier was usually formed at the fused area of two allogeneic individuals without remarkable cytotoxic reactions. The relationship between the accumulation of homogenous cells at the fused area and cytotoxic reactions is not clear. In *Axinella verrucosa* and *Axinella damicornis*, collencyte, which seems to secret collagen, mainly gathered at the fused area of two allogeneic individuals, but cytotoxic reaction was not shown. The barrier formation was shown in those two species, and it seemed that gathered collencytes formed a barrier of collagen fiber (Buscema and Van de Vyver, 1984b). However, biochemical work on the fibrous materials is still required.

As mentioned above *H. japonica* showed two types of allogeneic rejection. *Axinella verrucosa* also showed two manners of rejection reaction, which are genetically fixed (Buscema and Van de Vyver, 1984b). However, in *H. japonica*, it is not clear whether the manner of allogeneic rejection is genetically controlled or not. As shown in Fig. 8, there were several cases where both necrotic reaction and barrier formation were observed in the same combination of allogeneic individuals. Therefore, the physiological condition of the individuals might influence the allogeneic rejection as well as the genetic control. Figure 8 also shows that there was no fusible combination among allogeneic individuals. During this study, only one fused pair was found, and fusion never occurred between two individuals from the different populations. This suggests that for this species the genes related to the allorecognition have high polymorphism, similar to that of *C. diffusa* (Hildemann et al., 1980).

Furthermore, *H. japonica* shows rejection reaction against tissues of *H. okadai*. These two species occur in the same habitat in Japan, and therefore there are many opportunities for an individual of *H. japonica* to come into contact with individuals of *H. okadai* at its growing edge. In the rejection reaction, mesohyl cells gathered to the contact area like the case of allogeneic rejection, but the fusion of mesohyl between both individuals did not occur. Gathered cells were aligned along the contact surface in both individuals, and then filamentous material was secreted in the small gap of the contact area (Fig. 9C–D). However, the filamentous material did not become a tough barrier as shown in allogeneic rejection, and the small gap between individuals was maintained for several days. As the affinity between tissues of xenogeneic individuals might be lower than that of allogeneic individuals, the intensity of xenogeneic rejection would be less than that of allogeneic rejection. Similar phenomena were shown in xenogeneic rejection among 4 colonial ascidians of the genus *Botrylloides* (Hirose et al., 2002).

Many species of sponges can distinguish allogeneic tissues from autogeneic tissues, and they show rejection reactions against allogeneic tissues. Though the manner of rejection is different among species, those manners are basically divided into two types; one is necrotic reaction with cytotoxic reactions at the contact area and the other is formation of a barrier with fibrous material to separate allogeneic tissues. And, in most species, it is fixed genetically which type of rejection reaction is shown. Therefore, there is need to study how and why *H. japonica* shows both types of rejection reactions against allogeneic individuals. In the *H. japonica* population of Nabeta Bay, the rate of fusion between two individuals is very low (ca. 1.2 %) in comparison with the rate in the *Haliclona* sp. population of Vancouver Island (25 %; McGhee, 2006). Therefore, research on the high value of polymorphism in genes related to allogeneic recognition is also necessary. Furthermore, the trigger of accumulation of mesohyl cells and the cells secreting fibrous materials should also be clarified.

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