Alloimmune maturation in the coral *Stylophora pistillata* is achieved through three distinctive stages, 4 months post-metamorphosis

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SUMMARY

Adult colonies of the reef-building coral *Stylophora pistillata* discriminate precisely between 'self' and 'non-self' attributes, and respond selectively against specific allogeneic challenges. We studied the ontogeny of these allospecific responses on newly settled polyps by establishing allogeneic contacts within groups of 2–6 siblings or non-related offspring. Interactions were observed for up to 8 months. Three types of response, depending on the age of the interacting partners, were documented. The first was tissue fusion and the formation of a stable chimera, observed in partners less than 2 months old. The second was observed in contacts of partners 2–4 months old. It started with tissue fusion and transitory chimera since separation of the chimera partners or polyp death resulted when the oldest partner in the chimera reached the age of 4 months. The third type was the regular histoincompatibility response, as documented in allogeneic interactions of adult colonies, recorded here in all encounters with *S. pistillata* partners over 4 months old. Maturation of allorecognition in this species was therefore achieved through three time-dependent stages, 4 months following metamorphosis. Combinations of siblings or genetically unrelated partners did not affect the results. We propose that the coral alloimmune maturation system may be used as a new evolutionary model scheme for studying tissue transplantation and tolerance.

1. INTRODUCTION

One of the fundamental issues in mainstream immunology is the ontogeny of immune responses. The maturation of the vertebrate immune system is related to a series of complex biochemical and molecular processes, and is manifested by the lymphocytes' acquisition of a variety of humoral and cellular responses. The use of experimental manipulations during the ontogeny of vertebrate immune systems further revealed that immunological maturation varies greatly among different vertebrate taxa (Good & Papermaster 1964; Du Pasquier 1974; Ohki *et al.* 1988). These studies and others highlighted the paradox that rejection of transplanted tissues – a hallmark characteristic of vertebrate immune systems – is a phenomenon which never occurs naturally. In contrast to the vertebrates, many colonial invertebrates, especially those belonging to the Porifera, Cnidaria and Urochordata, manifest *in situ* fusion and rejection outcomes as a response to allogeneic contacts.

Reef-building corals produce sexually derived planktonic larvae (planulae) that settle on hard substrate, metamorphose and develop into new coral colonies. Several reports, dating back for more than a century, have documented that allogeneic planulae larvae which settle close to each other may fuse, producing chimeric coral colonies (cited by Rinkevich 1996). Hidaka (1985) was the first to show that while parent coral colonies never fused in allogeneic assays, and responded by rejection, their metamorphosed larvae fused with each other upon contact with no sign of tissue incompatibility. No further study has been done to reveal at which age or developmental stage corals reach alloimmune maturation, and what the fate of coral chimeras is after, and if, the fused genotypes become immunocompetent. The ontogeny of allorecognition responses in these organisms is therefore not only interesting as a fundamental characteristic of invertebrate immune systems, but is also of ecological and evolutionary significance regarding the question of the existence of natural chimerism, the evolution of 'self' and 'non-self' recognition, and other fundamental immunological issues such as tolerance (Rinkevich 1996).

The branching coral *Stylophora pistillata*, like other reef cnidarians, expresses highly complex and specific allorecognition responses (Rinkevich & Loya 1985*a*, *b*; Müller *et al.* 1984; Resing & Ayre 1985; Chadwick-Furman & Rinkevich 1994). While isografts are always

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accepted, allograft fusion has never been recorded in more than 500 assays done on adult colonies *in situ*. Several levels of intensity in histoincompatible responses, as well as delayed responses, reversals and non-transitive hierarchies, have been documented in this species. Effector mechanisms for transplant incompatibility in *S. pistillata* include unilateral cytotoxicity, formation of skeletal barriers, tissue and skeletal overgrowths, production of caustic enzymes for dissolving skeletons, allelopathy, translocation of metabolites, nematocyte discharge, retreat growth, and more (Rinkevich & Loya 1985*a*, *b*; Müller *et al*. 1984; Resing & Ayre 1985; Chadwick-Furman & Rinkevich 1994).

Following some earlier evidence for the ontogeny of allorecognition responses in corals and other cnidarians (Hidaka 1985; Shenk & Buss 1991; Lange *et al*. 1992), we have looked for the possibility that young *Stylophora* polyps, immediately upon settlement, do not mount alloimmune responses as do mature individuals. This was studied by assaying allogeneic contacts of siblings, newly settled polyps released by the same mother colony, or by assaying combinations of non-related offspring taken from different colonies. Our results showed that allorecognition responses of this reef-building coral, belonging to one of the simplest invertebrate phyla, mature through three distinctive stages. These stages were time-dependent: Allogeneic contacts between colonies less than 2 m old led to stable chimeras, whereas fusion between 2–4 m old colonies was transitory, resulting in rejection. In colonies older than 4 m, no allogeneic fusions were recorded.

2. MATERIALS AND METHODS

Stylophora pistillata is a hermaphroditic species, reproducing sexually through internal fertilization. Brooded planula larvae are released from gravid colonies between January and June, usually during the night (Rinkevich & Loya 1979).

We collected planula larvae *in situ* from 11 gravid *S. pistillata* colonies (designated as A–K) near the Inter-university Institute for Marine Sciences (Eilat, Red Sea) at depths of 5–10 m. Plankton nets were placed before sunset over the colonies and the planulae were collected several hours later (as described in Rinkevich & Loya 1979). Groups of 30 planulae from each colony were shipped in 50 ml plastic tubes, containing filtered sea-water, to the laboratory in Haifa within 2 d of being collected. Larvae were transferred to 60 mm Petri dishes containing unfiltered sea-water (25 °C, 24 h constant light). Under these conditions, many larvae settled within 4–10 d upside down on the water surface layer. Others settled, in aggregates or at various distances from each other, on the bottom of the dishes. Upon settlement, primary polyps which metamorphosed on the water surface were collected on the barbs of fine paintbrushes. They were put on clean surfaces of plastic Petri dishes in aggregates of 2–6 polyps, either in direct tissue-to-tissue contact, or separately with gaps of several mm between them to delay allogeneic contact. Therefore, in these combinations, tissue-to-tissue contact was established at various polyp ages. We established combinations of siblings, polyps released by the same colony, and combinations of non-related offspring originating from different colonies. The dishes were placed in a humidified chamber for 20–30 min until polyps became firmly attached to the plastic. Petri dishes containing the attached polyps were held in running sea-water aquaria at 23–25 °C under a 12–12 h light–dark regimen (two 500 W halogen bulbs). The animals were fed daily with artificial plankton. They were observed once a week for up to 8 m under a stereo microscope. Dishes and animals were cleaned from fouling organisms using small pieces of razor blades and fine paintbrushes. Growth was measured in terms of additional polyps per unit of time.

3. RESULTS

Survival of metamorphosed larvae in the laboratory during the observational period of 8 m was high (> 90 %). Mortality in this period accidentally resulted from careless handling during the weekly cleaning and observational procedure. Primary polyps started to deposit calcareous skeletons 1–3 d following metamorphosis. Seven days post-settlement, additional polyps emerged extra-tentacularly from the peripheral tissue, or rarely intra-tentacularly. Growth rates in terms of new polyps over time were highly variable among the young colonies (data not shown). Polyp morphology and small colony structure appeared similar to field-growing young colonies. Polyps contained endosymbiotic algae (zooxanthellae), which were evenly distributed in their endodermal tissues.

Tissue contacts between allogeneic polyps caused one of three types of outcome, depending on their age when grafted. The first was, unexpectedly, a true tissue fusion, which was recorded in most encounters between young polyps (< 16 weeks old; table 1), usually 2–7 d following contact. Fusion was initially marked by a continuous layer of tissue across the contact area, and was followed by a perpetual skeletal deposition. Zooxanthellae were uniformly distributed in the fused zones where the original borderlines between the partners in the chimera gradually faded (figure 1*a*, *b*). Histological sections further confirmed that tissues between fused partners are continuous (data not shown). In several instances fusion did not occur (chimera 16, see table 1; and other cases, data not shown), or developed later due to the interference of turf algae that settled between the polyps before the establishment of tissue-to-tissue contacts. In these situations, upwalled tissues and skeletons usually developed from both interacting partners until allogeneic tissues eventually came into contact above the algae and fused. Neither cytotoxic responses nor overgrowths, characteristic of allogeneic interactions of adult *S. pistillata* colonies (Rinkevich & Loya 1985*a*, *b*; Müller *et al*. 1984; Resing & Ayre 1985; Chadwick-Furman & Rinkevich 1994), were recorded in the first 4 m of age. These outcomes were similarly recorded either between siblings or between polyps originating from different mother colonies (table 1).

In an additional set of experiments, four newly settled polyps of *S. pistillata* were grafted onto four older colonies at the age of 3 m: two were non-chimeric colonies of nine and seven polyps respectively, and two

Table 1. *Fate of chimeras composed of non-related genotypes (nos. 1–20), or of siblings (nos. 21–25)*

(When not specified, chimeras have lasted without any sign of separation/death until the end of the observations (8 m post-settlement).)

chimera	mother's colony origin	major steps in chimera history in weeks (w)
1	A, B, C, H, J, K	A multichimera of five partners within 1 w. The sixth partner fused at the age of 10 w, following suture formation, and separated at the age of 16 w.
2	A, B, C, H	A multichimera of three genotypes. The fourth partner fused at the age of 14 w, and died at the age of 15 w.
3	A, B	Chimera established at the age of 1 w.
4	A, C	Chimera established at the age of 1 w.
5	A, C	Chimera established at the age of 1 w.
6	A, C	Chimera established at the age of 9 w, and separated at the age of 17 w.
7	A, C	Chimera established at the age of 1 w.
8	A, C	Chimera established at the age of 1 w.
9	A, C	Chimera established at the age of 8 w, and separated at the age of 15 w.
10	A, C	Chimera established at the age of 1 w.
11	A, C	Chimera established at the age of 1 w.
12	A, C	Chimera established at the age of 10 w, and separated at the age of 18 w.
13	A, B, C, F, L	A multichimera established at the age of 2 w.
14	A, E, H, I	A bichimera was established at the age of 2 w. The others fused at 8 and 10 w respectively. The middle partner in the chimera died at the age of 16 w.
15	B, D	Chimera established at the age of 3 w.
16	B, D, D, F	A bichimera was established at the age of 2 w. The others did not fuse as a result of algae settlement.
17	A, B, D	A multichimera was established at the age of 3 w.
18	A, B, D	A bichimera was established at the age of 1 w. The third partner fused at the age of 10 w and separated at the age of 17 w.
19	A, D, F	A bichimera was established at the age of 2 w. The third partner fused at the age of 11 w and died at the age of 17 w.
20	D, F, G, X	A multichimera was formed at the age of 3 w. An additional polyp from a different mother colony (X) was grafted onto the chimera at the age of 12 w. Fusion occurred within a week, and the polyp died at the age of 4 w.
21	A, A, A, A, A	Three polyps fused at the age of 2 w. The other two fused at the age of 8 and 10 w respectively, and separated at the age of 17 w following the death of the middle genotype in the chimera.
22	C, C	Chimera formed at the age of 10 w and separated at the age of 16 w.
23	G, G, G	The polyps fused at the age of 1 and 2 w respectively.
24	H, H	Chimera was formed at 1 w.
25	I, I	Chimera established at the age of 3 w.

were 36- and 15-polyp chimeras, originating from three and two primary polyps respectively. As before (table 1) fusion resulted in all four cases.

After fusion, the chimeras grew and new polyps emerged (figure 1*b*). They developed on all parts including the fusion area, where it was not clear morphologically to which partner of the chimera these new polyps belonged. Chimeras that fused at an earlier stage than 2 m remained stable throughout the 8 m of observations. However, all those that fused at 2–4 m of age demonstrated a second outcome, separation between the fused partners and/or death of one of them. This occurred at the age of 15–16 weeks (table 1). We have termed this type of fusion 'transitory fusion'. The first sign of separation in transitory fusions was the disappearance of zooxanthellae from the original fusion area. A bleached line emerged, demarcating the partners in the chimera at the site of fusion (figure 1*c*). The width of the bleached line corresponded to the age at which fusion was established. In chimeras where fusion occurred shortly before separation, about 2.5–4 m post-metamorphosis, the line was thin (0.1 mm). In partners that fused earlier, at ages of 2–2.5 m, the line was wider, up to 1 mm. The bleaching was followed by tissue death, which separated the partners. Young polyps that had fused with older colonies exhibited a more extensive response. They died when the older partners in the chimera reached an age of 4 m. In two cases, the bleached lines within the chimeras bisected polyps that emerged in the fusion areas (figure 1*c*). Half of these two polyps bleached completely and subsequently died, while half survived and regenerated complete polyps.

The third type of outcome was characterized by the formation of sutures in the contact areas and by overgrowth of one partner above the tissue and skeleton of its confrère, as previously recorded in allogeneic assays done on adult colonies. This response occurred between all partners that first fused when older than 4 m, either between single colonies or in cases where chimeras confronted other chimeras or individual polyps (table 1).

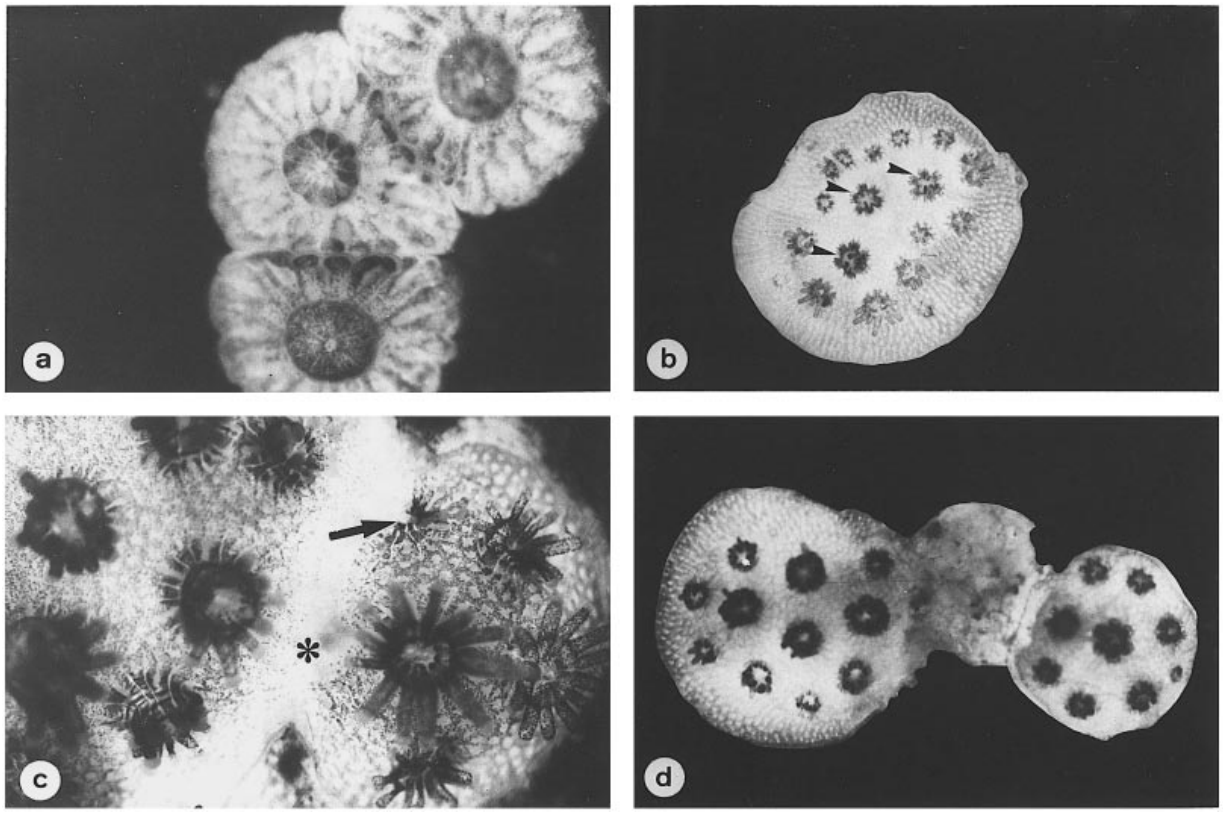

Figure 1. Chimerism and alloimmune maturation in *S. pistillata*. Polyp size is *ca.* 2 mm in diameter. (a) Chimera no. 2 (table 1). Three allogeneic polyps immediately after metamorphosis in tissue-to-tissue contacts. Fusion resulted within 48 h. (b) The same, 5 weeks later. Tissue and skeleton fusions are evident. Arrowheads point to the three original polyps. (c) Chimera no. 18 (table 1), 17 weeks after metamorphosis. A bleached line (asterisk) emerged between the third partner and the bichimera. One polyp on the edge of this line has bleached in half. The bleached tissue disintegrated, separating the two partners. (d) Chimera no. 14 (table 1), 16 weeks after settlement. The middle partner died.

4. DISCUSSION

Maturation of allorecognition in *S. pistillata* was time-dependent, and was achieved through three distinctive stages, all culminating within the first 4 m after settlement and metamorphosis (figure 2*a–c*). Colonies younger than 2 m fused to form morphologically stable chimeras (figure 2*a*). Contacts that occurred between 2–4 m old colonies resulted in transitory fusion, which terminated at the age of 4 m in tissue separation or death of the smaller partner (figures 1*c*, *d*, 2*b*). After the age of 4 m, full alloimmune competence was acquired, and no fusions with allografts were recorded (figure 2*c*).

In contrast to the vertebrate immune system, allorecognition in the Cnidaria is not dependent on the differentiation of specific stem cells acting within distinct morphological environments. 'Self'/'non-self' recognition in *S. pistillata* is expressed on all outer surface areas of the colony. After the settlement of the planula larva and the establishment of the primary polyp, there are no further profound physiological or morphological changes like those recorded in lower vertebrates, e.g. the anuran amphibians (Flajnik *et al.* 1987). Therefore, it may be proposed that ontogenic changes in the coral allorecognition system are not the result of replacement of tissues or organs, but reflect changes in the expression of 'self'/'non-self' attributes during the first 4 m after settlement.

The long-term acceptance of grafted tissue (that occurs at the age of < 2 m) is reminiscent of the phenomenon of vertebrate transplantation tolerance (Charlton *et al.* 1994; Matzinger 1994), although the mechanisms are not known. Moreover, allotolerance in *S. pistillata* was specific: when maturation of allorecognition in a chimera was achieved, early fusions, characterized by prolonged coexistence of the partners, continued in morphologically stable chimeras. These chimeras, however, rejected all other allogeneic grafted genotypes, or separated from transitory-fused partners. The delayed response of 2–4 m old fusions, however, resembles results obtained from vertebrates, including the outcomes of grafting quail embryonic tissue onto chicken embryos (Ohki *et al.* 1988), and the responses against transplanted tumours during amphibian alloimmune maturation (Robert *et al.* 1995). In the chicken system, fusions at early stages (4–5 d) lead to normal development of transplanted organs. A few weeks later, when the recipient chick becomes immunocompetent, it rejects the quail organs, as if it has not been 'educated' during embryonic life that the grafted tissue is regarded as 'self'. In the amphibian system, lymphoid tumour cells grow after their transplantation into tadpoles or young post-metamorphic animals, but do not grow in fully grown adults. The coral system may also be used as a new evolutionary model scheme for studying tissue transplantation.

The appearance of an ontogenic process in cnidarian

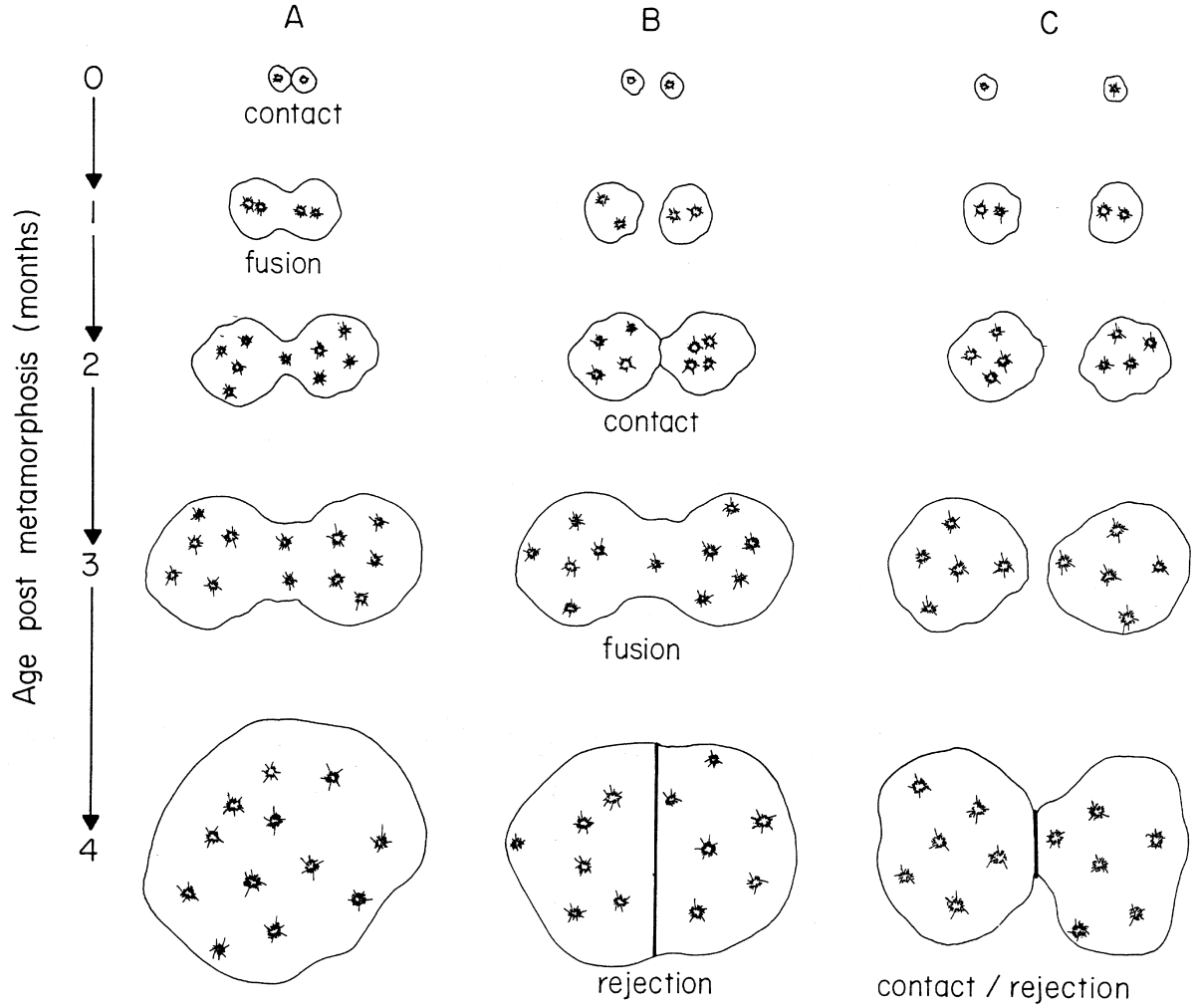

Figure 2. A schematic illustration of the three stages (A, B, C) in the maturation of allorecognition in *S. pistillata*. (A) First stage: tissue contacts immediately post-metamorphosis (time = 0), and up to 2 m in age, result in fusion and the establishment of a stable chimera. (B) Second stage: tissue contacts 2–4 m post-metamorphosis lead to fusion and transitory chimera; rejection causes their termination at the age of 4 m. (C). Third stage: tissue contacts established after 4 m post-metamorphosis result in histoincompatibility response, as observed in adult colonies.

alloimmunity has already been demonstrated in a few previous studies (Hidaka 1985; Shenk & Buss 1991; Lange *et al*. 1992). Our work confirmed these results and added two important points to this paradigm. First, since we followed the whole maturation process in detail, we were able, for the first time in invertebrates, not only to determine the age at which allorecognition matures, but also to observe the various stages leading to full allorecognition maturation. Second, in our study we assayed combinations of siblings, as well as genetically non-related individuals, demonstrating that relatedness was not a factor that may alter the ontogeny of immunocompetence.

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