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Allogeneic Responses between Three Remote Populations of the Cosmopolitan Ascidian *Botryllus schlosseri*[1]

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ABSTRACT—Colony allorecognition assays (CAAs) were performed between colonies of the world-wide distributed tunicate *Botryllus schlosseri*, sampled from the Mediterranean coast of Israel (Is), from Monterey, California (Mon) and from Mutsu Bay, Japan (Ja). While all 48 Is vs Ja CAAs resulted in nonfusion responses, unexpectedly, 4.4% of the 45 Is vs Mon pairs and 12.0% of the 25 Ja vs Mon assays ended in colony fusions. Allogeneic effector mechanisms in all 3 populations were similar, except for the Ja population which was characterized additionally by the appearance of masses of bright yellow blood cells gathered in the tips of interacting ampullae. A total of 201 multiple CAAs on 24 Is vs Mon, 22 Is vs Ja and 21 Ja vs Mon rejecting pairs did not show an allospecific memory in the rejection phenomenon. Results are discussed in view of the accumulated data on allogeneic responses in 5 remote populations of *B. schlosseri*.

INTRODUCTION

Botryllus schlosseri, a world-wide distributed colonial ascidian, is a common member of shallow water hard bottom communities. This species is found in European waters, Japan, the eastern and western coasts of North America, Australia, Hong Kong, and in many other localities [1–4]. This species, most likely of Mediterranean Sea origin, became a cosmopolitan species probably by ancestral colonies which attached to ship bottoms and were introduced into new localities.

Like other botryllid ascidians [5, 6], *B. schlosseri* colonies show colony specificity resulting either in vascular anastomosis (fusion) between separate parts of the same colony or between two compatible colonies, or in rejection between non-compatible colonies [7–10]. This histocompatibility discrimination is controlled by a single gene haplotype (termed the tunicate's fusibility-histocompatibility locus, Fu/HC [11]) with multiple codominantly expressed alleles [6–8]. Two colonies sharing no alleles at the Fu/HC locus will reject each other, whereas colonies sharing in common at least one allele on this locus will fuse upon direct contact [5–8].

When studying allogeneic reactions between *B. schlosseri* colonies collected from Monterey, California (Pacific Ocean) and from Woods Hole, Massachusetts (Atlantic Ocean), Boyd et al. [3] pointed to an interesting result that rejections were usually confined within the tunic and the peripheral ampullae (sausage-like termini of blood vessels) of only Woods Hole (WH) colonies. Four different types of rejections were developed by only WH ampullae which included blood cell infiltration, haemorrhage formation, retraction and ampullae amputation [4]. More intriguing is the result that rejection patterns are somehow different in each one of the two tested North American *B. schlosseri* populations [3, 4].

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Here we further analyze allorecognition responses between *B. schlosseri* colonies from 3 extremely separated populations: an eastern Mediterranean population (from along the coast of Israel), an eastern Pacific population (from Monterey, CA) and a western Pacific/Japan Sea population (from Mutsu Bay, Japan). Previous intrapopulational allogeneic assays revealed that rejection patterns are similar in these 3 populations [3, 4, 10, 12, 13]; in short, immediately after the first tunic-tunic contact was established between interacting colonies, tips of the marginal ampullae actively extended in a tip-tip orientation. Rejections were documented without ampullae penetration into the tunic matrix of the opposite colony, and without true matrix fusion.

MATERIALS AND METHODS

We used laboratory reared *B. schlosseri* colonies from Israel (Is) and Monterey, CA (Mon), and wild colonies from Mutsu Bay, Japan (Ja). Originally wild Mon colonies were collected from Monterey marina and were shipped to the National Institute of Oceanography, Haifa, Israel, in cool condition, where they were assayed against colonies of the Israeli population. Wild Mediterranean colonies were collected from 3 locations along the Israeli coast (Tel-Shikmona, Caesarea, Michmoret). Is and Mon colonies were kept in the laboratory [3, 12, 14], and offspring were collected and reared as described [3, 14, 15]. Assays were performed mainly on reared offspring. Ja colonies were collected in Mutsu Bay (Aomori, Aomori Prefecture) and shipped to Shimoda Marine Research Center where they were reared on glass slides in a wooden culture box immersed in Nabeta Bay, Shimoda. Is and Mon cultures were shipped to Shimoda where they were maintained in 17-liter standing seawater tanks until assayed against Ja colonies. Colony allorecognition assays (CAAs) and observations were performed as described [4, 10], and secondary and tertiary tests of CAAs were performed as previously [4, 16].

RESULTS

Interpopulational allogeneic reactions

We performed 45 CAA pairs of Is vs Mon colonies, 48 CAAs of Is vs Ja pairs, and 25 CAAs of Ja vs Mon. While all allorecognition assays of Is vs Ja resulted in nonfusion reactions (either in rejections or in indifference [16]), unexpectedly we observed 2 cases of vascular fusions in Is vs Mon pairs (4.4%) and 3 fusible assays in interacting Ja vs Mon pairs (12.0%).

Necrotic lesions (POR=points of rejection) were developed in assays of the three interpopulational combinations (Fig. 1a-i) in a similar way as they were established in intrapopulational allogeneic assays [3, 4, 10, 12, 13, 16]. The only significant difference in the allorecognition morphology between colonies of the 3 tested populations was the accumulation of bright yellow blood cells (morula cells; Tertakover and Rinkevich, unpublished) which usually gathered in masses within the tips of interacting ampullae of Ja colonies (this was recorded as well in Ja vs Ja combinations [13]). These cells disappeared after the formation of a full set of PORs. PORs were produced by colonies belonging to the 3 populations; however, in Ja vs Mon and Ja vs Is combinations, the peripheral ampullae of Ja genotypes were extended more actively towards those of their confrere colonies, and more cases were recorded where PORs were developed by Ja ampullae alone (in 50.5% of nonfusible Ja vs Mon cases; Fig. 1b-d, and in 60.0% in Is vs Ja pairs; Fig. 1a). Cases where PORs developed by only Mon or Is when confronted with Ja colonies were much lower (18.2% and 20.8%, respectively). We observed up to 11 PORs per assay although up to several tens of ampullae interacted reciprocally.

Three out of the 4 different characteristic types of PORs [4], including haemorrhages formation (Fig. 1a-f), ampullae amputation (Fig. 1d, f-h) and formation of an "ampulla POR" were recorded. Withdrawal of interacting ampullae from contact areas was another characteristic outcome of interpopulational allogeneic interactions (Fig. 1b-d). Ampullae regression appeared in colonies of the 3 tested populations, and usually started within 24 hr

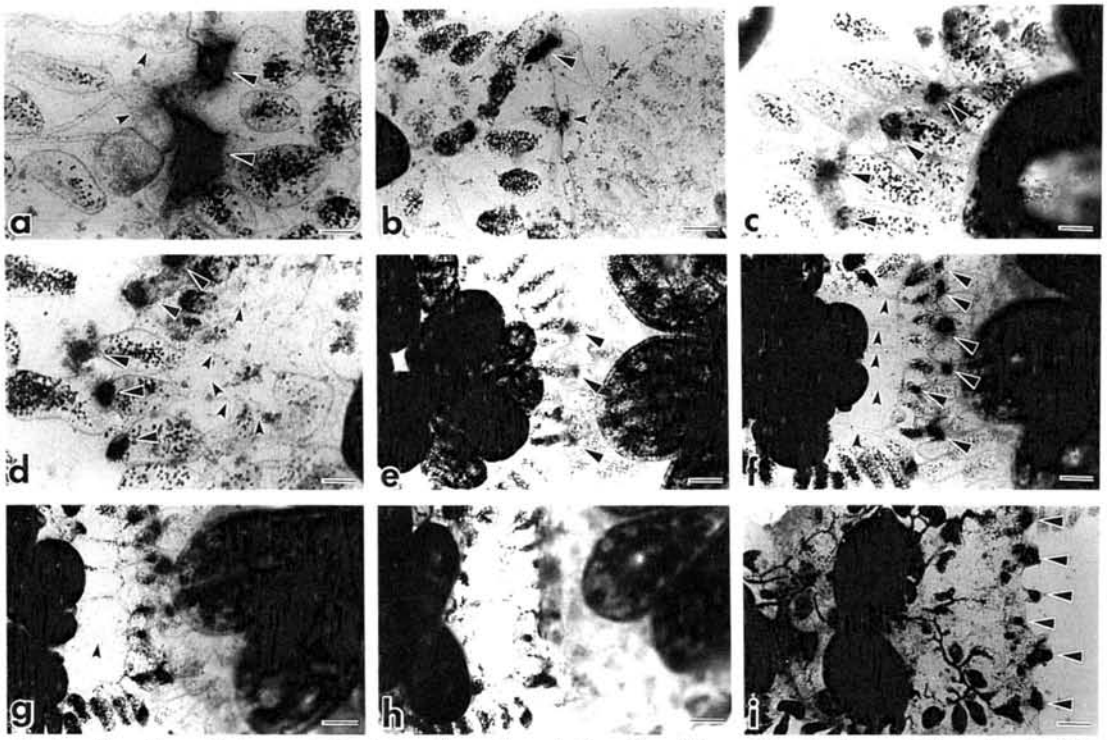

FIG. 1. *B. schlosseri* interpopulation interactions, Ja vs Is (a, e-i) and Ja vs Mon (b-d). a: 5 days after Ja (left) ampullae produced 2 large and diffused PORs, which resulted through ampullar bleeding (arrowheads). Both previous bleeding ampullae remained intact near PORs (small arrowheads). b: 48 hr after first ampulla-ampulla contacts. One of the Ja (left) ampullae formed an "ampullar POR" [4] and was amputated and formed a large POR (arrowhead). Another ampulla formed a typical POR through bleeding (small arrowhead). Zooids of both partners retreated up to 2 mm from the previously interacting site. c-d: 24 and 48 hr, respectively, after first ampulla-ampulla contact was established. Initial Ja (right) ampullar bleeding, which started with 4 PORs (c, arrowheads), progressed by the formation of the 5th POR (d, arrowheads) and by ampullae amputation (small arrowheads). Zooids of both colonies and some of the Mon ampullae retreated from the contact zone. e-i: A case where Is (left) ampullae were amputated after developing PORs. e: 24 hr after CAA, 3 PORs (arrowheads). f: 24 hr later, 6 PORs (arrowheads), amputation of Is blood vessels leaving only 5 connecting vessels (small arrowheads) to peripheral ampullae. g: 24 hr thereafter, no more PORs but an additional blood vessel was amputated (small arrowhead). Ja ampullae started overgrowing the contact area. h: 4 days later, all connecting vessels were amputated, Ja colony progressed, overgrowing Is ampullae and tunic matrix. i: 6 days later, Ja colony was removed to show 6 old PORs (arrowheads) and degenerated tunic of the Is colony. Scale bars: a, c, d =0.25 mm; b, e-i=0.5 mm.

after the first POR was developed. After ampullar retreat, the remaining bare tunic gradually degenerated, forming an empty space between the interacting colonies.

Multiple colony allogeneic interactions

A total of 201 CAAs were carried out in primary, secondary and tertiary interaction with 24 Is vs Mon, 22 Is vs Ja and 21 Ja vs Mon rejecting pairs (Table 1). Secondary and tertiary interpopulational allogeneic interactions resulted in similar outcomes as primary interaction, that is, all colonies continued to express their former mode of allorecognition responses where again, Ja colonies, when confronting either Is or Mon colonies, developed the yellow color in the tips of interacting ampullae, and were more active in producing PORs than the colonies of the other populations.

TABLE 1. Morphological analyses of colony allorecognition assays carried out in primary, secondary and tertiary *Botryllus schlosseri* repeated interpopulational allogeneic reactions

Pair No.	Repeated Is vs Mon CAAs, in test no.*					Repeated Is vs Ja CAAs, in test no.*					Repeated Ja vs Mon CAAs, in test no.*				
	1st**	Int†	2nd**	Int†	3rd**	1st**	Int†	2nd**	Int†	3rd**	1st**	Int†	2nd**	Int†	3rd**
1	2 P, mi	I	4 P, Mon	39	3 P, Mon	2 P, rec	4	3 P, Ja	6	10 d, in	5 P, Ja	5	1 P, Ja	8	3 P, Ja
2	4 P, rec	13	3 P, Is	5	6 P, rec	2 P, Ja	5	2 P, Ja	4	15 d, in	8 d, in	I	8 d, in	I	3P, Ja
3	7 P, rec	14	17 d, in	25	5 P, Is	1 P, Is	3	10 d, in	I	5 P, Ja	7 P, Ja	5	10 d, in	I	5 P, Ja
4	2 P, Is	10	1 P, rec	5	6 P, mi	8 P, Ja	4	2 P, Is	9	3 P, Ja	3 P, Mon	5	4 P, Mon	8	2 P, mi
5	5 P, Is	I	2 P, Is	12	2 P, Is	3 P, Ja	3	7 P, Ja	8	1 P, Ja	4 P, Ja	5	3 P, Ja	3	1 P, Mon
6	8 d, in	13	1 P, Is	74	1 P, Mon	6 P, Ja	4	4 P, rec	8	3 P, Ja	5 P, Ja	5	2 P, Mon	3	2 P, Mon
7	5 P, rec	17	2 P, Mon	18	15 D, in	7 P, Is	4	10 d, in	I	15 d, in	2 P, Mon	4	10 d, in	I	4 P, Ja
8	4 P, Is	5	1 P, Mon	3	4 P, Mon	2 P, Ja	4	4 P, Ja	6	10 d, in	4 P, Ja	5	1 P, Ja	8	10 d, in
9	10 P, mi	4	2 P, Is	5	1 P, Mon	2 P, Ja	4	4 P, Ja	8	4 P, Ja	1 P, Ja	5	10 d, in	I	15 d, in
10	2 P, Is	5	3 P, Mon	12	2 P, mi	5 P, Is	4	10 d, in	I	7 P, Ja	5 P, Ja	7	10 d, in	I	3 P, Mon
11	2 P, Mon	6	1 P, Mon	3	4 P, Mon	1 P, Is	4	1 P, Ja	8	2 P, Ja	1 P, Mon	3	10 d, in	I	2 P, Mon
12	1 P, Is	5	1 P, Is	10	7 P, rec	2 P, Ja	3	2 P, Ja	8	3 P, Ja	8 P, Ja	7	4 P, Ja	4	2 P, rec
13	2 P, Is	3	5 P, rec	2	2 P, rec	2 P, rec	5	10 d, in	I	2 P, Ja	3 P, Ja	3	4 P, Mon	6	3 P, Ja
14	2 P, Is	5	2 P, Is	2	6 P, Mon	2 P, Ja	3	3 P, rec	6	10 d, in	2 P, Ja	3	3 P, mi	4	10 d, in
15	2 P, Is	5	3 P, Is	8	4 P, Mon	1 P, Ja	3	3 P, Ja	8	2 P, Ja	4 P, Ja	6	5 P, Mon	5	1 P, Mon
16	4 P, Mon	5	1 P, Mon	5	2 P, Mon	2 P, Ja	3	3 P, Ja	6	10 d, in	4 P, Ja	6	2 P, Ja	4	1 P, Mon
17	4 P, Mon	5	1 P, Is	11	5 P, Mon	8 d, in	I	10 d, in	I	1 P, Ja	6 P, Ja	4	3 P, Ja	4	1 P, Ja
18	1 P, Is	6	3 P, mi	1	3 P, Mon	3 P, mi	4	2 P, Is	6	2 P, rec	8 d, in	I	4 P, Ja	4	1 P, Ja
19	1 P, Mon	I	2 P, Is	10	4 P, Mon	5 P, Is	5	4 P, Ja	6	1 P, Ja	4 P, mi	4	2 P, Mon	4	5 P, Mon
20	3 P, mi	7	3 P, mi	3	5 P, Is	2 P, rec	3	10 d, in	I	3 P, rec	4 P, rec	5	2 P, Ja	4	1 P, Ja
21	2 P, Mon	11	2 P, rec	5	2 P, rec	8 d, in	I	3 P, Is	8	1 P, Ja	7 P, Mon	6	4 P, Ja	4	5 P, Ja
22	17 d, in	I	4 P, Is	5	5 P, Is	17 d, in	I	1 P, Ja	I	10 d, in					
23	10 P, Is	9	6 P, rec	1	2 P, Is										
24	4 P, Mon	9	1 P, Is	5	8 P, rec										

* Is, Mon and Ja refer to the Israeli, Monterey and Japanese colonies, respectively.

** Results in the first to third tests: P = number of PORs; area where PORs were observed: Is, Mon, Ja, rec (reciprocal), mi (middle, on the borderlines between the interacting colonies), in (indifference, no POR was recorded during the period in days [d] specified).

† Int = time interval (in days) from the day on which a full set of PORs was completed until the next assay was performed. I = a case where a preceding CAA was done immediately after the specified period of indifference or immediately after the last POR of the former CAA was observed.

Ja interactions with Is or Mon colonies resulted also in up to 2–3 times more cases of "indifference" within secondary and tertiary assays when compared to the primary assay of a specific set of colonies (Table 1). The number of PORs in each combination of colonies are developed irrespective of the number of the repeated assay, and their locations (reciprocally, in the middle between both colonies or within the tunic of only one partner in the CAA) were not confined to either one of the interacting colonies of each specific set of genotypes (Table 1). The results presented in Table 1 further indicate that secondary and tertiary interpopulational allogeneic assays done on the same pairs of colonies (immediately to 17 days after the last POR of the former CAA was observed; Table 1) do not reveal an allospecific memory in rejection, for example in an accelerated formation of PORs and/or augmentation in POR numbers. These results confirm previous conclusions on assays done with Mon vs Mon [16] and Mon vs WH [4] interactions, and further point to the high variability in directionality of responses/number of PORs/repertoire of allorecognition responses characteristic of this species.

DISCUSSION

Recently, 5 *B. schlosseri* populations were studied for intrapopulation morphology or the genetics of allorecognition: the population from the Venetian Lagoon, Italy [7, 17], from the Mediterranean coast of Israel [12], from Woods Hole, MA, Atlantic Ocean [3, 4, 8], from Monterey and Santa Barbara, CA, eastern Pacific Ocean [3, 4, 10, 16, 18], and from Mutsu Bay, western Pacific Ocean/Japan Sea [13]. The results of these studies elucidate the capacity for distinction of colony which is manifested by either vascular fusion or the formation of necrotic lesions when two different genotypes meet each other through their peripheral ampullae. In 4 of the above studied *B. schlosseri* populations (except the WH population [3, 4]), tips of marginal ampullae of paired noncompatible encounters actively extended towards each other in a tip-tip orientation, without forming true tunic-matrix fusion. This continued in the production of PORs without penetration of ampullae into the tunic of the confrere genotype. In WH noncompatible pairs, however, the cortical layers of both tunics usually become enmeshed and are dissolved in limited areas near the ampullar tips. This results in reciprocal penetration of ampullae into the tunic of the facing colony. On the other hand, all other *B. schlosseri* allospecific phenomena, including the morphology of PORs, are similar to all 5 studied populations, except for the intensive aggregations of yellow blood cells in tips of interacting ampullae, which is also characteristic of Ja intrapopulational interactions [13].

Four interpopulation combinations were studied until now, including the WH vs Mon [3, 4] and Ja vs Mon, Is vs Ja, Is vs Mon interactions (this study). We [3, 4 and unpubl.] established more than 150 WH vs Mon CAAs, which resulted in zero fusions, similarly to the 48 Is vs Ja CAAs studied here. It is therefore very surprising that fusions were obtained in high proportions in Is vs Mon interactions (4.4%) and Ja vs Mon pairs (12.0%). In the 25 CAAs of Ja vs Mon, we used 25 Mon and only 11 Ja genotypes, which revealed 3 fusions (done with Ja colonies Nos. 5, 9, 11 [13]). The allelic pattern of the Fu/HC locus of these 3 fusible Ja genotypes [13] reveals that at least 2–3 alleles on the fusibility haplotype are common to both Ja and Mon populations. Therefore, it is not only that these three extremely separated populations (eastern Mediterranean vs eastern Pacific Ocean and eastern Pacific Ocean vs Japan Sea, respectively) belong to the same cosmopolitan species, namely *B. schlosseri* (as was concluded when comparing WH vs Mon populations [3]), but also these populations are found to possess the same histocompatibility alleles in each populational genetic pool (from a pool of a size of at least 100 allorecognition alleles [9, 19]). This is probably the reason for the high percentage of interpopulational fusions recorded here.

The results of the present study and of earlier studies on *B. schlosseri* interpopulation specific responses [3, 4] indicate that there are some differences in allogeneic responses (ampullae penetrations into confronting tunic matrices and the gathering of yellow pigment cells in tips of interacting ampullae). These responses appear to be

characteristic of distinct populations. Additional studies on other *B. schlosseri* populations may elucidate the repertoire of alloresponses characteristic of this cosmopolitan species.

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