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RESEARCH ARTICLE

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Cellular dynamics of in vitro allogeneic reactions of *Hymeniacidon heliophila* (Demospongiae: Halichondrida)

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Abstract Allograft reactions of the sponge *Hymeniacidon heliophila* Parker, 1910 have been studied and quantified at the cellular level in vitro. Cell-layer assays were used to record the timing of rejection between allogeneic individuals and to investigate changes in the cellular subpopulations of primmorphs belonging to different individuals and placed in direct contact. The initial contact on both cell layers and primmorphs was followed by partial fusion and ended by their isolation with formation of a defined barrier but without marked collagen deposition. Using cytospins, cellular subpopulations from primmorphs were morphologically distinguished based on overall shape and on nuclear and cytoplasmic characteristics. In the beginning of the cultures up to 17 cell types could be recognized. Within 5 days, following reaggregation and primmorph formation, populations of eight of these types suffered marked reduction, whereas nine other cell types were either maintained or reduced at a slower pace. When submitted to allogeneic contact, the cellular dynamics of the four remaining types were altered. Surprisingly, the percentage of archeocytes is reduced, probably depleted by differentiation into other, differentiated cell populations. Cellular responses to allogeneic contact were characterized mainly by a transient increase in globoferous cells, a larger participation of presumed collagen-secreting cells, and by a remarkable expansion of the collencyte population. The collencyte population remained altered after the isolation of the individuals by a collagen barrier, and this can represent a mechanism of short-term immune memory.

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Introduction

The ability to distinguish self and non-self components is one of the bases of multicellularity. In all animals, an immune response is the result of multiple processes through which the organism tries to exclude, by killing or isolation, the signal that started the recognition reaction. From simple self/non-self, or innate recognition at cellular level, these reactions have evolved to adaptive immune systems controlling, not only immune reactions, but also homeostasis. The multiplicity of these mechanisms can be large within the same phyla or even within one individual, as a consequence of the importance of the task to be accomplished. In higher animals such important functions are rarely accomplished by a single mechanism, and there are frequently additional alternatives (Mushegian and Medzithov 2001; Pasquieur 2001).

Sponges are the oldest animals with multicellular organization (Müller 1998), and several authors have dealt with sponge cell behavior in immune reactions. Using classical grafting techniques, numerous studies described the processes following either allo- or xenogeneic contact at a histological level (van de Vyver 1979; Jokiel et al. 1982; Buscema and van de Vyver 1984a, 1984b; Smith and Hildemann 1986a, 1986b; Amano 1990; Fernàndez-Busquets et al. 1998, 2002; Müller et al. 2002). These studies evidenced the capability of self/non-self recognition in sponges, not only among different species, but also between co-specific individuals. A few authors demonstrated the ecological importance of fusion of lar-

vae and juveniles forming chimaeric sponges, a phenomenon also mediated by self/non-self recognition (Ilan and Loya 1990; Maldonado 1998; Maldonado and Uriz 1999). In addition, other studies showed that sponges have the capacity to differentiate among distinct inert materials (Buscema and van de Vyver 1985; Gaino et al. 1993). Relative to cell–cell interactions, the basic selective aggregation seems to be mediated by a large proteoglycan complex containing several calcium-binding sites (Blumbach et al. 1998; Fernàndez-Busquets and Burger 1997; Jarchow et al. 2000). Besides playing a role in basic self/non-self recognition, it is probable that this element and its membrane receptors also act in the sorting and organization of structures, such as the choanocyte chambers (Burkart et al. 1979). In addition, several other molecular components of the cell adhesion system were identified through molecular and biochemical techniques, such as tenascin, integrin, galectin, receptor tyrosine kinase, immunoglobin-like proteins and laminin (Humbert-David and Garrone 1993; Brower et al. 1997; Müller 1997; Pancer et al. 1998; Müller et al. 1999a).

Some basic recognition events in these immune reactions are still not understood. The evaluation of rejection processes is often complex and subjective, mainly due to the characteristics of the sponge tissue, cellular plasticity, the presence of other components such as contaminant organisms, and the reorganizing skeleton and channel systems. There is little evidence of the precise cellular mechanisms triggered by the contact with allogeneic tissues. Currently, the most accepted model states that reactions are initially of simple macrophagic type. The contact triggers intense cell migration and ends either by the secretion of a collagen barrier or by cytotoxicity (van de Vyver and Barbieux 1983; Buscema and van de Vyver 1984a, 1984b, 1985; Smith and Hildemann 1986a, 1986b; Amano 1990; Müller et al. 1999a). However, it appears that the cell types and mechanisms involved in the basic immune response in Porifera are varied among different species and still have not been clearly determined (Smith and Hildemann 1986a; van de Vyver and Barbieux 1983; Yin and Humphreys 1996; Fernàndez-Busquets and Burger 1999; Fernàndez-Busquets et al. 2002).

In the present work we used in vitro cell-layer assays and three-dimensional cultures (primmorphs: Custódio et al. 1998) to investigate the cellular processes triggered by allogeneic contact in the sponge *Hymeniacidon heliophila* Parker, 1910 (Halichondrida: Halichondriidae) from southeastern Brazil. The cellular subpopulations involved were identified, and their variations during this process were quantified.

Materials and methods

Sponges

A total of 28 different individuals of the sponge *Hymeniacidon heliophila* were collected in the CEBIMar area, São Sebastião (São Paulo state, Brazil), within distances varying from 3 to 1500 m from each other. After collection, the samples were brought immediately to the laboratory and submitted to tissue dissociation. *H. heliophila* is an encrusting sponge growing in the intertidal zone to 14 m depth, sometimes covered with debris and leaving only the papillae exposed. A detailed description of this species can be found in Wiedenmayer (1977) and in situ photographs are available in Custódio et al. (2000).

Cells

Cell dissociation and primmorph formation followed protocols described elsewhere (Custódio et al. 1998; Müller et al. 1999b). Briefly, sponge tissues were cut in 2–3 cm^3 pieces, washed in seawater, and incubated with CMFSW + E (calcium and magnesium-free seawater with EDTA: 460 mM NaCl, 7 mM Na_2SO_4, 10 mM KCl, 10 mM HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid], 2.5 mM EDTA (ethylenediaminetetraacetic acid, pH 8.2), for 30 min in a low-speed rotary shaker. The supernatant was discarded, and the fragments resuspended in fresh CMFSW + E solution. After continuous shaking for 45 min the supernatant was collected and filtered through a 40 μm nylon mesh. The cells were then pelleted by centrifugation (250 *g*, 10 min) and washed once with CMFSW without EDTA. The final pellet was resuspended in sterile, filtered, natural seawater (0.22 μm) with antibiotics (kanamycin 100 mg l^{-1} and tylosin 8 mg l^{-1}; Calbiochem) and phenol red (16 mg l^{-1}; Sigma). This solution was used as media for culture maintenance in all experiments, and cell concentration was adjusted as necessary for the assays using a Neubauer chamber.

Histocompatibility reactions

Cell-layer assay

Culture dishes (60 mm) were prepared with their bottom gently scratched by a plastic pipette tip to enhance cell adhesion (Custódio et al. 1998; Müller et al. 1999b) and divided transversally by a polyester strip. The dishes were then filled with isopropyl alcohol as an additional decontamination measure and left in place for 30 min, after which the alcohol was drained and the plates allowed to dry in a sterile flow chamber. Although no chemical effect on the plastic surface was expected, this treatment enhances cell adhesion remarkably. Dissociated cell suspensions from two different individuals, adjusted to 5×10^8 cells ml^{-1}, were plated in each halve. The dishes were left in place for 6 h at 20°C to allow cell adhesion in the bottom, and then the polyester strip was removed to allow contact of the cell layers. A total of 4 isogeneic and 12 allogeneic pairs were prepared using this method and investigated for 14 days. From these, 3 isogeneic and 7 allogeneic pairs presented cell-layer contact. The media was changed daily, carefully to avoid layer damage and loss of cells.

Primmorph assay

Cell suspension was adjusted to 1×10^8 cells ml^{-1}, and 8 ml was added to 60 mm culture dishes. The medium was replaced daily, with the cell clumps being resuspended to avoid adhesion to the plate. After 2 days in culture, these cell aggregates reached the stage of primmorph and were used in histocompatibility tests. Two primmorphs of similar size (< 1 mm) and from different individuals were put in contact in 60 mm petri dishes and kept in place with the help of a small glass device (Fig. 1A). This apparatus was made of three 2×7 mm coverslip sections fixed with silicon rubber in an "H" shape. Pairs of primmorphs from the same individual were used as controls. As for the initial culture, 3 ml of the media was changed daily. Since the primmorphs are well-defined structures (cell masses surrounded by pinacoderm), there was no risk of cell loss due to the media changes. To obtain the cytospins, the primmorphs were dissociated with CMFSW + E, the cell concentration was adjusted

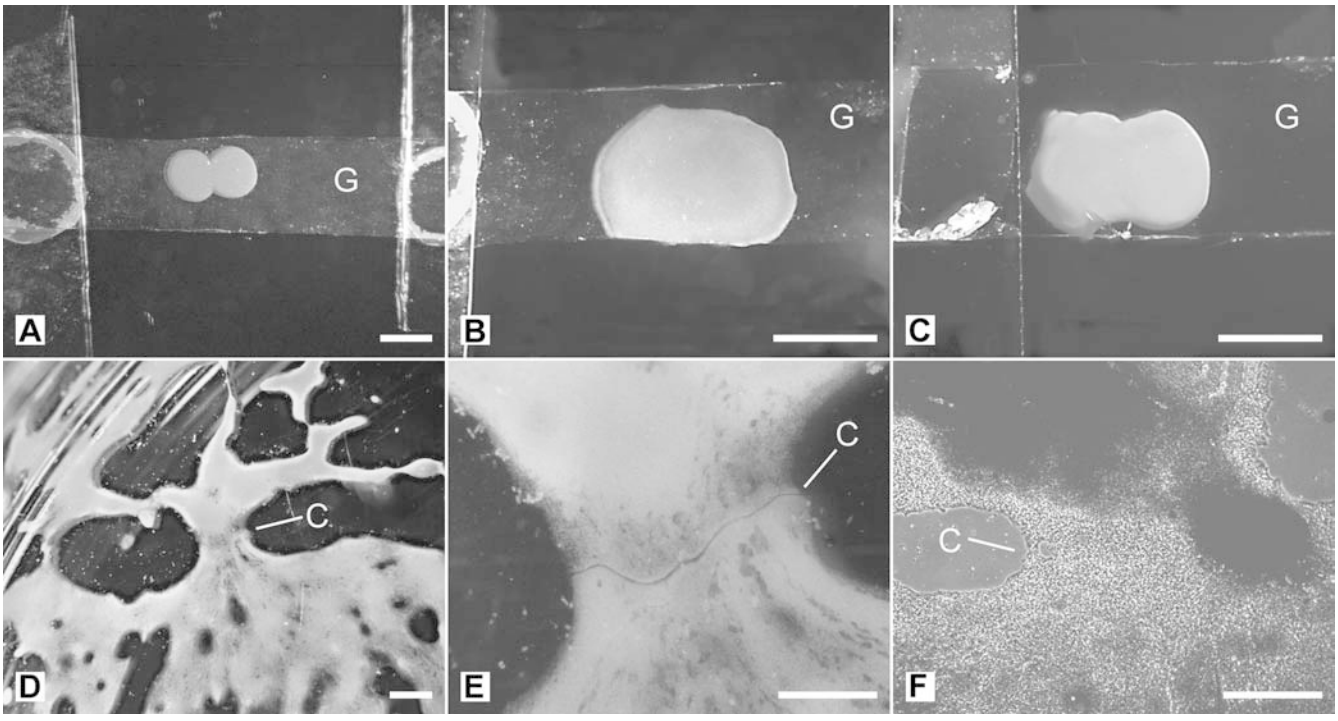

Fig. 1A–F *Hymeniacidon heliophila*. **A** View of the beginning of the allogeneic reaction in the primmorph assay. The coverslip section is placed over the primmorphs to keep them in contact. **B** Isogeneic pair after 5 days of contact. Both individuals fused completely. **C** Allogeneic pair after 7 days of contact. The individuals are attached, but the contact zone between the original primmorphs is clearly discernible. **D** Cell-layer assay after the contact of allogeneic layers (5 days in culture). **E** Dark-field detail of the contact zone depicted in panel D, showing the separation between the individuals. **F** Contact area in isogeneic pairs after 5 days in culture. The cell layers are completely fused (*G* glass; *C* contact zone). *Scale bars*: 1 mm (A–D: stereomicroscope; E, F: inverted microscope)

to 5×10^5 cells ml^{-1}, and slides were made using a cytocentrifuge (100 µl, 5 min, 80 *g*; citospin 248; FANEM). The slides were fixed with formaldehyde sublimate (1 h) and stained with Mallory's trichrom (Behmer et al. 1976). Ziehl's fucsin and PAS reaction were also used to investigate some particular cellular characteristics. Cell-type determination and counting were carried out using a Leitz Labophot microscope. The subpopulations were determined by the morphology presented in the cytospins, based on analysis of: (1) overall shape (irregular/regular, presence of filopodia or special characteristics such as flagellum or filament); (2) nuclear characteristics (size, shape, presence/absence of nucleolus); and (3) cytoplasmic characteristics (presence/absence, size, position and contents of inclusions). For the analysis performed in this work, the cells were considered morphotypes and numbered from I to XVII according to their behavior in the histocompatibility reaction. There was no intention to propose new cell-type designations. The actual functional role played by all cell types in a normal physiological state was not investigated, and presumed designations from the literature were given only to those types with remarkable morphological characteristics. For determination of the cellular dynamics, four different individuals were used in two separate experiments consisting of series of allogeneic and isogeneic pairs. During 11 days in each experiment, two pairs from each series were collected, polled and dissociated. Cell density was adjusted, and cytospins were prepared as described above. Counting was made with a reticulate ocular in 20 random fields in each cytospin, and the average of four counts (two from each experiment) was used. Abundance of each cell type was plotted as a percentage of the total, and the statistical significance of the subpopulation variations was verified using Mann–Whitney tests ($P < 0.05$).

Histology

Eight allogeneic and four isogeneic pairs of reacting primmorphs were fixed using MFAA (methanol 85%, formalin 10%, acetic acid 5%; Reite 1997) and embedded in paraffin following the usual methods (Behmer et al. 1976). Sections (8 µm thick) were cut with a microtome and stained with PAS and Picro-Sirius, using hematoxylin/eosin as a counterstain to verify the cellular arrangement, glycogen content and collagen deposition in the contact zone.

Results

Cell types

After tissue dissociation, 17 cell subpopulations could be morphologically distinguished (Fig. 2; Table 1). Although no further analyses were made to verify the material left in the sponge matrix after dissociation, these cell types are presumed to be present, in similar concentrations, in the living sponge. Type I cells can be identified as choanocytes by the presence of flagella and collar remains shortly after the dissociation and, later, by size, the eccentric nucleus and the irregularly vacuolized cytoplasm. Cell types II, III and V have no remarkable morphological features associated to possible functional roles or previous descriptions in literature and therefore remained unidentified. Type II shows well-defined vacuoles with clear content in cytospins and a distinct edge free of inclusions. Type III shows an unusual elongated shape and apical nuclei that resemble

characteristics described for larval secretory cells (''cellules en urne'': Lévi and Porte 1962; Boury-Esnault 1976a), but those have not been found in adults so far. Alternatively, this morphotype and type V might represent cells under apoptotic/necrotic processes due to apparent membrane blebbing or disruption. Such events could be responsible for the peaks observed in the participation of these cell types within a few days after dissociation and for their decline after primmorph organization. Type IV is distinguished from type I (choanocytes) by the larger size of the nucleus and the presence of small vacuoles around it. A large nucleus in relation to cell volume is characteristic of polyblasts, suggested to be either an intermediate stage between choanocytes and archeocytes or derived from other cell types following tissue alterations (Connes et al. 1972, 1974; Diaz 1977; see Simpson 1984). Type VI shows similar morphology to type XVI (archeocytes), with its cytoplasm loaded with several varied inclusions, but with a homogenous nucleus without nucleolus. In addition, the inclusions permeate the cytoplasm, without the smooth edge found in type XVI.

Cell type VII was designated cystencyte A, due to the presence of a single vacuole occupying most of its cytoplasm (sensu Boury-Esnault and Rützler 1997). The vacuole content is homogenous and in some cases appears condensed; forming an ovoid structure slightly separated from the enclosing cell. Type VIII cells could be identified as sclerocytes by the presence of a long axial filament. Type IX cells were identified as pinacocytes by their relatively small size, central nucleus and the tendency to be found in groups in incomplete dissociation. Type X is a structurally similar but larger counterpart of type VII (cystencyte A), described above, and was named cystencyte B. The following three types comprise all of the cells in the cell population with small homogeneous inclusions present. Types XI and XIII contain cytoplasm with several PAS-negative, elongated inclusions. In type XI, they are scattered and thinner, with pointed ends in a way which resembles rhabdiferous cells (Simpson 1968, 1984). In type XIII, they are bacterium-like, cylindrical and with rounded ends. These densely packed inclusions fully occupy the cytoplasm, sometimes obscuring the nucleus. Type XII cells show well-defined, round inclusions, and were identified as glycocytes by the positive PAS reaction of these granules. The number of inclusions is highly variable, but the cytoplasm is never as loaded as type XIII.

Type XIV fits the description of the morphology of globoferous cells, presenting a variety of small inclusions and a single, large vacuole, which was stained with methyl blue. Cell subpopulation type XV shows a stellate to fusiform shape and vacuolated cytoplasm, which are general characteristics of collencytes. Type XVI cells are easily identified as archeocytes by the characteristic nucleolus and vacuolated cytoplasm. Typically, the cytospin image shows cytoplasm with edges without inclusions. Type XVII includes small cells with a collagen-secretion function, as expressed by their strong methyl-blue staining. The images from cytospins show no direct morphological correspondence to the classical transmission electron microscopy figures of lophocytes (polarized cells with fibrilar collagen tail: see Simpson 1984). Dissociated cells, such as those used in cytospins, no longer have the attachment to physical structures (extracellular matrix) that is probably required to assume such an arrangement. Nevertheless, their functional role as collagen-producing cells supports their tentative identification as lophocytes.

Cell dynamics

Cell-layer assay

Once in the culture dishes, cell suspensions attached to the bottom within 6 h. After the initial adhesion, the cells assume a monolayer-like distribution that later retracts in a patchy organization (Fig. 1D), with dense cell streams and monolayer areas mixed with empty spaces. This organization is dynamic, and the cell layers from the different individuals connect soon after barrier removal. The initial contact zone of both isogeneic and allogeneic cell layers is characterized by adhesion and accumulation of a cell mass. Although no cells were actually observed crossing the tissues in allogeneic reactions, it is probable that a mixture of cell populations occurs to some extent, since there is no detectable special organization or boundary between the individuals. During the following days, this initial cell accumulation in both isogeneic and allogeneic pairs dissolved. A well-defined barrier between the individuals of allogeneic pairs was completely formed 4 days after initial contact (Fig. 1E), whereas the contact zone of isogeneic pairs became part of the layer (Fig. 1F). Observations during this process using an inverted microscope did not detect cytotoxic or necrotic reactions in the contact zone of allogeneic pairs.

Primmorph assay

All allogeneic pairs showed rejection, and no relevant differences in cell populations were observed between those attached to the culture plates or glass devices and non-adherent primmorphs. Similar to results of the cell-layer assay, initial contact of both isogeneic and allogeneic primmorphs was followed by adhesion of the structures. The difference is that isogeneic primmorphs fuse completely after 2 days and become a single, round structure (Fig. 1B). In contrast, the boundaries of allogeneic primmorphs are always discernible, in spite of remaining strongly attached (Fig. 1C).

The primmorphs of both the isogeneic and allogeneic pairs showed the usual cell mass surrounded by a single layer of pinacocytes. In isogeneic reactions the prim-

►

Fig. 2 *Hymeniacidon heliophila*. Cell types I–XVII. See Table 1 for descriptions (*F* flagellum; *N* nucleus; *V* vacuole; *A* axial filament; *I* inclusion). *Scale bars*: 5 μm

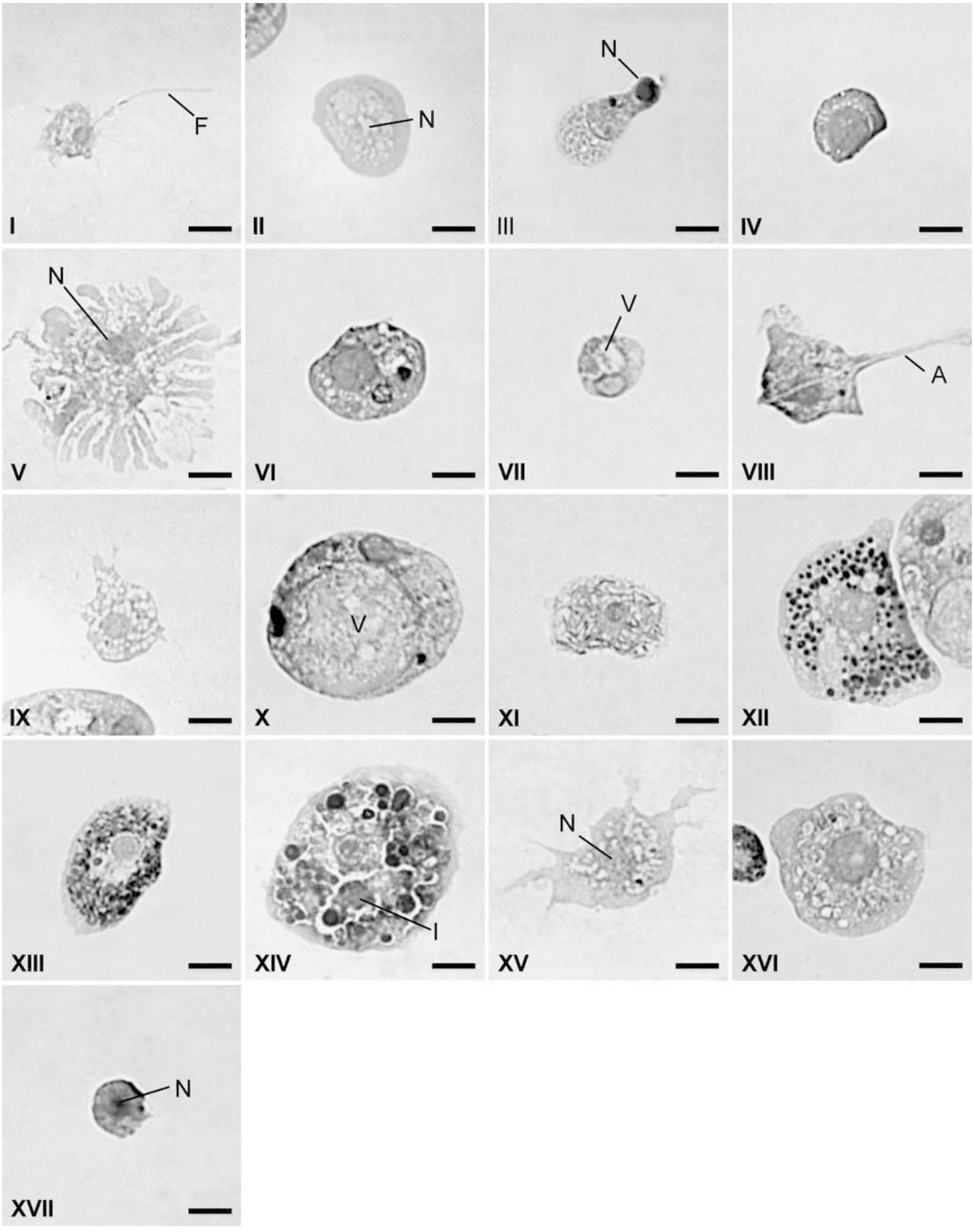
F
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Table 1 *Hymeniacidon heliophila*. Description and presumed identification of cell types from reactive primmorphs

Type	Overall shape	Cytoplasm	Nucleus	Remarks (presumed identification)
I	Small cell (5.2 μm), sometimes presenting thin and prolonged scattered projections	Reduced and vacuolated, with homogeneous border	Peripheric (2.1 μm), always placed on flagellum base	Presenting collar and flagellum after dissociation (choanocyte)
II	Irregular cell (10.1 μm)	Marked by irregular vacuoles (or a single vacuole), distinct from the cytoplasm and with marked borders. Vacuole contents are clear and homogeneous, giving them an empty appearance	Small (2.1 μm), also defined by a distinct border and with marked nucleolus	(unknown)
III	Elongate cell (13.6 μm)	Vacuolized cytoplasm, always with a palmate shape	Small, well-defined, homogeneous (1.9 μm), typically located in the small end; stains with orange G	(“cellules en urne”?)
IV	Remarkably homogeneous small cell (7.4 μm), with smooth borders	Reduced. Sometimes uniformly stained with orange G and with infrequent perinuclear vacuoles with clear content	Regular, inconspicuous, and sometimes very large in relation to the cellular volume (4.2 μm)	(polyblasts?)
V	Large cell (17.8 μm), with extremely irregular borders with several thin projections	With small, irregular vacuoles with clear content. The projections are usually free of inclusions	Large and irregular (4.7 μm), vacuolized and inconspicuous	Remarkably irregular appearance. Possible membrane blebbing (unknown)
VI	Ovoid (13.9 μm), with regular border	Loaded with inclusions, varied in size and content	Well defined (2.3 μm)	Similar to type XVI, but without the vesicular nucleus and the typical nucleolus (unknown)
VII	Small ovoid cell (7.5 μm)	Mostly occupied by a single vacuole (3.6 μm) of homogeneous content; stains with orange G	Small, peripheric (1.9 μm)	Similar and perhaps different stage of type XIV (cystencyte A)
VIII	Irregular cell (10.6 μm), cell border displays several filopodia of varied size	With several inclusions of mixed content and size. Typically with a single long and thin structure (20.2 μm)	Regular, well marked (2.9 μm); stains with orange G	The long thin structure is probably an axial filament (sclerocyte)
IX	Small and irregular, with short pseudopodia (6.3 μm)	Irregular with several vacuoles with varied dimensions and formats but always with homogeneous content without special coloration	Small and well marked (1.9 μm); stains with orange G	Frequently found in groups up to 6 cells (pinacocytes)
X	Large, regular, oval cell (17.6 μm)	Small peripheric inclusions varied in shape and content and always occupied by a large, single vacuole (9.0 μm), the content of which is a heterogeneous round structure; stains with methyl blue and fucsin	Peripheric irregular (3.2 μm); stains with orange G	Perhaps different stage of type VII (cystencyte B)
XI	Ovoid irregular (15.1 μm)	Loaded almost exclusively with elongated inclusions (1.0 μm length); stains well with fucsin	Round (2.5 μm), with small nucleolus	Number of inclusions is highly variable: usually high at the beginning of culture, becomes scattered after 1 week (rhabdiferous cells?)

Table 1 (Contd.)

XII	Large, with varied shape, can be round to elongated (24.3 μm)	Irregular with spherical inclusions of varied size (1.0 μm)	Irregular (5.4 μm), slight differentiated and sometimes with nucleolus	Inclusions PAS positive (glycocytes)
XIII	Ovoid regular, with varied size (15.7 μm)	Completely loaded with round particles, similar in shape and size to bacteria, very abundant, sometimes masking the nucleus	Round, anucleolated (4.4 μm)	Inclusions differ from those in type XI as they are more dense and cylindrical (bacteriocytes?)
XIV	Large, ovoid, regular cells (20.2 μm)	Heavily loaded with inclusions varied in size and content	Central (4.2 μm), sometimes lost among the inclusions, with small nucleolus	Frequently with a larger inclusion; stains with methyl blue (globoferous cells)
XV	Irregular to fusiform and extremely spread in the cytospins (15.8 μm), giving the impression of little thickness	Irregular, with several vacuoles of varied dimensions and formats, but always with homogeneous content, without special coloration	Irregular (4.2 μm); stains with orange G	Frequently found in groups of 3–4 cells (collencytes)
XVI	Large and regular cells(18.2 μm)	Inclusions varied in form and content, generally showing a marked homogeneous border	Vesicular (4.5 μm) with marked nucleolus; stains well with orange G	Filled with vacuoles (archeocytes)
XVII	Small, regular, spherical cells (5.8 μm)	Dense cytoplasm stained with methyl blue with an irregular, perinuclear area, with tiny homogeneous vacuoles	Small and homogeneous (1.3 μm); strongly stains with fucsin	Strong methyl blue staining indicates high collagen content (lophocytes?)

morphs fused and became a single, rounded structure within 24–48 h after contact. In allogeneic pairs limited adhesion also occurs, but both original primmorphs remain distinguishable. Although the pair cannot be physically separated, the Picro-Sirius staining revealed a slight collagen deposition between the individuals. Beneath this contact zone the cells assumed a parallel arrangement, but no accumulation of specific cell types was observed.

Variations in cell abundance

The initial percentage of each cell type after dissociation varied from 26.4% (type IX) to as low as 0.6% (types V, VII, VIII and XII) of total cell counts (Fig. 3; Table 2). Within 6 days in culture, 8 of the 17 initial cell types (types I–VIII) suffered a marked reduction, for both isogeneic and allogeneic pairs. Cell types related to functional roles present in normal sponge structural tissues but absent in primmorphs, such as types I (choanocytes) and VIII (sclerocytes), were not detectable within 48–72 h after dissociation. Type VII (cystencyte A) could be a different stage of type X (cystencyte B); its decrease may have been caused by interruption of the maturation process and degeneration, since it was not accompanied by an equivalent enhancement in the participation of type X (cystencyte B).

Five cell types (IX–XIII) showed a slower reduction pace, were stable, or increased, following the same patterns for both isogeneic and allogeneic pairs. Within this group, the proportions of cell types with clear structural roles in both normal tissues and primmorphs, such as type IX (pinacocytes), remained higher. Most of the cells with putative storage functions, i.e. containing cytoplasm loaded with special inclusions, represented the other types found in this group.

During allogeneic reactions four cell types (XIV–XVII) showed significant modifications in their dynamics relative to isogeneic contacts (Mann–Whitney $P > 0.05$). The only cell subpopulation with a presumed storage role in this group, type XIV (globoferous cells), showed a marked enhancement after allogeneic contact, in contrast to its decline in isogeneic pairs. Type XV cells (collencytes) showed an increase after allogeneic contact, and proportions also remained higher (18.3%) up to the end of the observation period (11 days). The abundance of cell type XVI (archeocytes) was reduced following allogeneic contact when compared to isogeneic contact. Similarly, the percentage of type XVII (lophocytes?) also increased in allogeneic reactions, although only little collagen deposition was observed (see "Discussion").

Discussion

Cells kept under controlled conditions frequently lose some of their normal characteristics. Morphological changes and alterations in proliferative or secretory

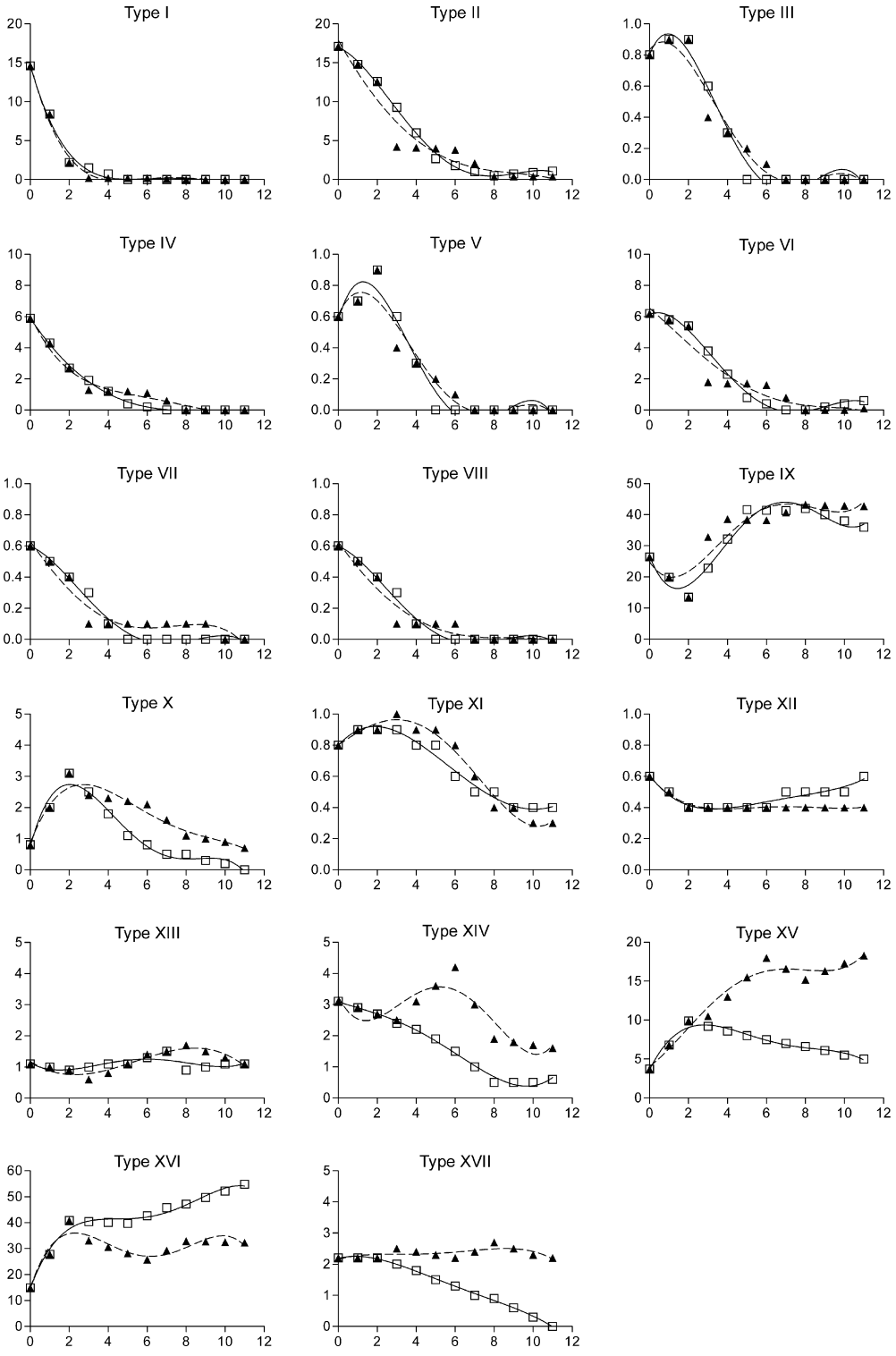

Fig. 3 *Hymeniacidon heliophila*. Cellular dynamics of allogeneic (*filled triangles*) and isogeneic pairs (*open squares*). The numbers are relative to the cell types in Tables 1 and 2. The percentage of each cell population is plotted against the days since dissociation (day 0) to the end of experiments (day 11). The allogeneic contact begins on day 2

capacity and even in DNA content are common problems in cultured cell lines (Harrison and Rae 1999; Freshney 2000). However, although they do not reproduce all of the conditions found in normal tissues, simplified in vitro systems allow a more direct comparison of cause and effect. In three-dimensional sponge cell cultures, the absence of standard physiological conditions leads to a lack of organized tissues and skeletal structures, such as the channel system, fibers and organized spicules. It also allows the elimination of bacteria, fungi and protozoans, which frequently overgrow sponge cells in open systems. Thus, an in vitro approach facilitates isolation of the events related exclusively to the contact with non-self tissues and allows more precise quantification of the changes and identification of the cell types involved. In allogeneic contacts, the

Table 2 *Hymeniacidon heliophila*. Initial and final percentage of each cell type in isogeneic and allogeneic pairs of primmorphs

Cell type	Initial (%)	Final (%)	
		Isogeneic	Allogeneic
I	14.6	0.0	0.0
II	17.1	1.1	0.4
III	0.8	0.0	0.0
IV	5.9	0.0	0.0
V	0.6	0.0	0.0
VI	6.2	0.6	0.1
VII	0.6	0.0	0.0
VIII	0.6	0.0	0.0
IX	26.4	36.0	42.7
X	0.8	0.0	0.7
XI	0.8	0.4	0.3
XII	0.6	0.6	0.4
XIII	1.1	1.1	1.1
XIV	3.1	0.6	1.6
XV	3.7	5.0	18.3
XVI	14.9	54.8	32.3
XVII	2.2	0.0	2.2

primmorphs of *Hymeniacidon heliophila*, despite the lack of organized multicellular structures, seem to be able to control the balance of their cell subpopulations and are capable of dynamic responses.

In sponges, cell-type identification is still a complex task, and no definitive terminology exists. There are some cell types with characteristic morphologies, such as archeocytes and choanocytes, and others that are identified by special inclusions or structures, such as glycocytes and sclerocytes (see Simpson 1984; Boury-Esnault and Rützler 1997). However, the significance of many cells and of their special cytoplasmic inclusions is still obscure, and the use of broad identification terms such as "cell with inclusions" or "amebocytes" is common. The determination of functional roles or ultrastructural characteristics is fundamental to correct cell-type identification (Simpson 1984). In the present work we intended to compare different responses (allogeneic vs. isogeneic) within a species, and therefore morphologically similar cell types were treated for the most part as numbered items, instead of according to designations which would indicate a certain function. Such precise cell identification would require transmission electron microscopy or cytochemistry techniques, which were not within the scope of the present study.

Using cytospins we were able to identify 17 morphologically distinct cell types, which is greater than the number usually cited for single species (e.g. Diaz 1977; Boury-Esnault et al. 1994; Muricy et al. 1996). As described previously for similar regenerative processes (Efremova 1970; Harrison et al. 1975; Harrison and Davis 1982), several cellular subpopulations of the sponge are quickly reduced after disorganization. These cells, represented here by types I–VIII, probably play specific roles in a normal sponge that are not strictly necessary in reorganizing tissues. Although their final fate (elimination or differentiation) is uncertain, the disappearance of types I (choanocytes) and VIII (sclerocytes) appears to illustrate this eventuality. In contrast, participation of totipotent cells (type XVI: archeocytes) and others with structural (type IX: pinacocytes) or potentially reserve/defense roles (type XII: glycocytes; type XIV: globoferous cells) are maintained or enhanced. These cells are required to ensure isolation and provide energy for primmorph maintenance and, thus, to allow the possibility of future regeneration of the organism.

Our results showed that types XIV (globoferous cells), XV (collencytes), XVI (archeocytes) and XVII (lophocytes?) were the only cell types affected by allogeneic contact. Previous work with allografts described intense migration and accumulation of glycocytes, archeocytes and collencytes in the contact zone (van de Vyver and Barbieux 1983; Buscema and van de Vyver 1984a, 1984b, 1985; Yin and Humphreys 1996; Fernàndez-Busquets et al. 2002). We found no accumulation of special cells, nor enhancement in type XII (glycocytes) numbers. Previous research was done mainly using, relatively, larger sponge fragments cultivated in open systems. The in vitro system used in this work is more restrictive, since no nutrients are supplied with the culture media (sterilized seawater) and, thus, cells must depend on those nutrients carried by the cells before dissociation or obtained by phagocytosis during the reorganization process. Therefore, cell proliferation is possibly limited to those cell types strictly necessary. It is possible that the observed increase in the glycogen-accumulating glycocytes in reactive zones could be due to a secondary function; similar accumulation of glycocytes has been observed during the morphogenetic process (Boury-Esnault 1976b), and such events as those triggered by allogeneic contact presumably either require energy or produce by-products that need to be metabolized (excreted or accumulated).

During allogeneic recognition, cell type XIV (globoferous cells) showed a pattern distinct from all other cells, with its transitory increase and later diminution. No clear function has been identified for this cell type, and it is generally included within the "cells with inclusions" group, together with many other types, such as spherulous cells, sacculiferous cells and cystencytes. Some cells with similar characteristics are known to possess lectin in their vacuolar contents (Bretting and Königsmann 1979; Simpson 1984). Details on the role of lectins in sponges are still scarce, but speculation exists that they are involved in the elimination of non-self material and in defense, by accumulating bioactive substances (Bretting and Königsmann 1979; Arason 1996). In addition, they could be part of immune mechanisms, in analogy to their role in other modern animals (Smith et al. 1999). The beginning and end points of the peak shown for cell type XIV (Fig. 3, days 4–8) are roughly associated with initial tissue reorganization and final segregation of the individuals observed in the cell-layer assays. During this process, an increase in the direct contact with allogeneic material

could be expected, and the proliferation of this cell type could be triggered by this event. After separation, the signal initiated by the contact would cease and the cell dynamics would assume a similar pattern to that observed for the isogeneic pair.

Since Borojevic and Lévi (1965), several studies have depicted the fundamental role of collencytes in morphogenesis. The term collencyte has been used in the literature to refer to several fusiform or stellate cells found in the mesohyl, and it very likely represents a heterogeneous group (Simpson 1984). Cell type XV fits the definition of collencytes proposed by Borojevic (1966): stellate or fusiform cells with an ovoid nucleus without nucleolus and with cytoplasm containing few inclusions. Graft experiments have suggested the involvement of these cells in immune reactions (van de Vyver and Barbieux 1983; Buscema and van de Vyver 1984a, 1984b). It is also interesting to note that collencyte populations are maintained at higher levels in allogeneic pairs in comparison to their decline in controls. Some authors have suggested the existence of immune memory in sponges (Hildemann et al. 1979; Bigger et al. 1982, 1983). They have observed that a second allograft implant triggers a faster immune reaction, even though this does not appear to be a widespread feature within the phylum (van de Vyver 1980; Smith and Hildemann 1984). The maintenance of collencytes in greater numbers for some time after the initial contact could explain the presence of an immune memory, at least for some sponge species. This idea was expressed earlier by Curtis (1979), who stated that the amplification of specific cells and their persistence could be responsible for a faster second-set allograft rejection. Although possibly not antigen-specific, this mechanism of short-term memory may provide an advantage when dealing with the repeated contacts between individuals expected in the competition for substrate (Curtis 1979; Hildemann et al. 1979; van de Vyver 1980; Smith and Hildemann 1986b).

In contrast to other cell types affected by the allogeneic reaction, the number of type XVI cells (archeocytes) was reduced. Some functions of archeocytes in sponges are well established. They act as stem cells, are involved in nutrient transport, and are also phagocytes, and this ability lies on the basis of innate immune response. In both invertebrates and vertebrates, phagocytes play a similar role in defense mechanisms and in antigen-specific immune responses to foreign proteins and alloantigens (Leippe 1999; Diex-Roux 1997). Accumulation of this cell type in the contact zone of allogeneic tissues within 1–3 days has been reported previously using graft techniques (van de Vyver and Barbieux 1983; Fernàndez-Busquets et al. 2002). Such an initial increase was not observed in *H. heliophila* allogeneic contact. Moreover, the archeocyte participation in allogeneic pairs is maintained, but not enhanced as observed in isogeneic pairs. As for the glycocytes (type XII), the methodological approach may have been responsible for the pattern found; limitation of nutrient supply in the in vitro culture employed here possibly restrained growth, and the finite cell population may have been depleted by differentiation to other types. This interpretation is also supported by the corresponding fluctuation in type XIV (globoferous cells) and the increase in type XV (collencytes) participation.

The stability of a population of collagen-containing cells (type XVII: lophocytes?) in allogeneic pairs contrasts to its decrease in controls. Clearly the presumptive role of such cells is to secrete a collagen barrier between the two individuals, as observed previously in several other sponge species (van de Vyver 1979; Simpson 1984; Fernàndez-Busquets and Burger 1999). However, only slight collagen deposition was observed in the reacting primmorphs. In spite of an evident proliferative (or maintenance) signal activated by the contact, other systemic constraints could limit subsequent collagen secretion by this cell type.

In conclusion, the in vitro response to allogeneic contact in *H. heliophila* is characterized by a transient increase in the spherulous cells, by a larger participation of lophocytes, and by a remarkable expansion and later maintenance in the collencyte population, which could allow faster second-set reactions. In contrast to expectations, the archeocyte population is not expanded, possibly being committed to differentiation into other cell types. The accumulation of these cells at the allograft sites as observed in earlier studies, might be due to cell migration from other regions and not to local proliferation.

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