Original Article

The fate of larval flagellated cells during metamorphosis of the sponge *Halisarca dujardini*

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ABSTRACT Sponge larval flagellated cells have been known to form the external layer of larva, but their subsequent fate and morphogenetic role are still unclear. It is actually impossible to follow flagellated cell developmental fate unless a specific marker is found. We used percoll density gradient fractionation to separate different larval cell types of *Halisarca dujardini* (Demospongiae, Halisarcida). A total of 5 fractions were obtained which together contained all cell types. Fraction 1 contained about 100% FC and its polypeptide composition was very different to that of the other fractions. Of all larval cell types, flagellated cells displayed the lowest *in vitro* aggregation capacity. We raised a polyclonal antibody against a 68 kDa protein expressed by larval flagellated cells. Its specificity was tested on total protein extract from adult sponges by Western blotting and proved to be suitable for immunofluorescence. By means of double immunofluorescence using both this polyclonal antibody and commercial anti-tubulin antibodies, we studied the distribution of the 68 kDa protein in larval flagellated cells and its fate at successive stages of metamorphosis. In juvenile sponges just after metamorphosis the choanocytes and the upper pinacoderm were labelled with both antibodies. In larval flagellated cells, the 68 kDa protein was found all over the cytoplasm appearing as granules, while in adult sponges, it was present in the apical part of choanocytes in the vicinity of collars. Direct participation of the larval flagellated cells in the development of definitive structures was demonstrated.

KEY WORDS: *porifera, larva, metamorphosis, protein marker*

Introduction

Adult sponges are sessile animals, so their free-swimming larval period is a critical one for habitat selection. After the free-swimming period, larvae settle on the substrate and initiate metamorphosis. The layer of the flagellated cells (FC) is a larval swimming organ, therefore those cells disappear soon after settlement. The fate of flagellated cells has long been discussed for their role in forming germ layer derivatives (for review, see Efremova, 1997). In 1892, Y. Delage proposed the theory of "inversion of the germ layers" in sponges. In some freshwater and marine Demospongiae he described the migration of the external larval flagellated cells (ectoderm) inward during metamorphosis to form the choanoderm (entodermal layer) of the adult sponge. Such cell behaviour gave an occasion to oppose sponges to other Metazoa as to be called Enantiozoa that signified "turned inside out". The origin of choanocytes from the larval flagellated cells did not raised doubts in calcareous sponges (Tuzet, 1973) and recently was confirmed both for the Calcaronea and the Calcinea

(Amano and Hori, 1993, 2001). As regards the demosponges, the developmental fate of larval flagellated cells in parenchymellae has long been debated. One view holds that the FC are transformed into the choanocytes in juvenile sponges *Mycale contarenii* (Borojevic and Lévi, 1965), *Hamigera hamigera* (Boury-Esnault, 1976), *Haliclona permollis* (Amano and Hori, 1996), *Reniera sp.* (Leys and Degnan, 2003) but the opposing view contends that they are lost by exfoliation or phagocytosis during metamorphosis as it was shown in *Spongilla lacustris* (Brien and Meewis, 1938), *Ulosa sp.*, *Halichondria moorei* and *Microciona rubens* (Bergquist and Green, 1977; Bergquist and Glasgow, 1986), *Microciona prolifera* (Misevic *et al.*, 1990) and commercial sponges *Hippospongia* and *Spongia* (Kaye and Reswig, 1991). Large phagocytes, presumably, archaeocytes engulf them near the time of settlement (Bergquist and Glasgow. 1986; Misevic *et al.*, 1990).

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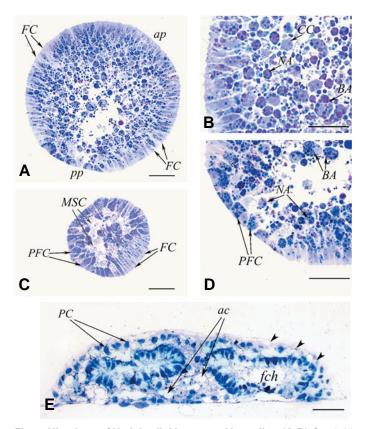
Fig. 1. Histology of H. dujardini larvae and juveniles. (A-D) *Semi-thin sections of the free-swimming larva;* **(A)** *longitudinal section,* **(B)** *longitudinal section of lateral area,* **(C)** *tangential section of posterior pole,* **(D)** *longitudinal section of posterior pole. Abbreviations: ap, anterior pole; pp, posterior pole; BA, blastomere-like nucleolated amoebocytes; CC, collencyte-like cells; FC, flagellated cells; MSC, maternal spherulous cells; NA, nucleolated amoebocytes; PFC, posterior flagellated cells. Scale bar: 20 μm.* **(E)** *Semi-thin cross-section of post-settlement juvenile sponge 3 days after metamorphosis. Abbreviations: ac, aquiferous canals; fch, flagellated chambers; PC, T-shaped pinacocytes. Scale bar: 20 μm.*

H³-thymidin autoradiography demonstrates that the larval FC of the Baikal sponge *Baikalospongia bacillifera* do not synthesise DNA and are possibly at the terminal state of differenciation, which is an evidence for their temporary ("provisional") nature (Efremova and Efremov, 1979). It has been shown by concanavalin A staining that in *Microciona* larvae, mannose is the only terminal, lectin binding sugar on the FC surface that enhances apoptosis and engulfment. The former was confirmed by dark staining of fragmented DNA (TUNEL assay) (Kaltenbach *et al.,* 1999).

The morphology of larval FC changes radically in early metamorphosis. For this reason, it is almost impossible to follow their developmental fate without use of an identification marker. To date, cytological markers have allowed reliable description of differentiation pathways of sponge cells (Boury-Esnault and Rützler, 1997; Amano and Hori, 1996). Molecular markers are only available for sclerocytes (skeletal cells) (Müller *et al.,* 2003) and germ cells (Perovic-Ottstadt *et al.,* 2004). The vital fluorescent non-specific tracer CMFDA was applied for labelling larval

peripheral cells (Leys and Degnan, 2002). Annexin was shown to be a specific choanocyte marker and archaeocytes were found to be the precursors of choanocytes in the course of asexual reproduction of the fresh water sponge *Ephydatia fluviatilis* (Funayama *et al.,* 2005). However, the range of available markers is very limited. Thus, sponge cell lineages, especially those involved in development and reproduction, are only poorly understood.

The situation is complicated by the rationale underlying some experimental approaches: for sponge cells to retain developmental and differentiational potential, they must have capacity to aggregate. For example, to form a choanocyte chamber, individual cells must first aggregate (Borojevic and Lévi 1965; Bergquist and Green 1977). Berquist and Glasgow (1986) postulate that direct observation of phagocytosis or shedding of larval FC together with inability of isolated FC to aggregate, provides strong evidence that FC do not migrate inward at metamorphosis and that they are larval, yet terminally differentiated structures (Bergquist and Glasgow, 1986).

We separated different larval cell types of *Halisarca dujardini* using density gradient fractionation, isolated a specific 68-kDa protein, expressed in larval flagellated cells and raised polyclonal antibodies against it (AB S). The specific localization of the protein in cytoplasm of FC was confirmed by double staining using AB S and anti-tubulin antibodies (AB T). AB S were used as a cell-type specific marker and anti-tubulin antibody (AB T), for revealing cell structure peculiarities. Double staining using both antibodies showed that FC are the only cell type containing 68-kDa protein and revealed the distribution of the protein in FC cytoplasm. This allowed us to demonstrate the participation of larval FC in the formation of choanocyte chambers and of a dermal layer in juvenile sponge during metamorphosis. We studied also the aggregation properties of different larval isolated cells.

Results

Cytological analysis of swimming larvae

The cell types recognizable in semi-thin sections of swimming larva are shown (Fig. 1 A-D). Flagellated cells were concentrated on the surface of the larvae (Fig. 1A). The cells of the anterior lateral areas were elongated and contained apical drop-like nuclei with small nucleoli. Few yolk granules were seen in the basal part of the cell, while the apical cytoplasm was free of the yolk. Posterior flagellated cells were more voluminous with rounded nuclei and prominent nucleoli. Their yolk granules were distributed evenly in cytoplasm (Fig. 1 C,D). During larval free-swimming period, these wedge-like cells flattened and lost the flagella. Nucleolated amoebocytes with several yolk granules in the cytoplasm and prominent nuclei and nucleoli occurred immediately below the FC layer (Fig. 1 B,D). Blastomere-like amoebocytes were seen in the central part of the larva and near the posterior pole appearing as large blastomere-like nucleolated cells with numerous yolk granules and large nuclei and nucleoli (Fig. 1, B,D). Collencyte-like cells of polygonal shape were yolk-free, but their clear light-coloured cytoplasm contained small inclusions. They are found in the internal cell mass of the larvae (Fig. 1B). Maternal spherulous cells were mainly concentrated at the posterior pole, especially at the end of free-swimming period. Their nuclei and cytoplasm were light-coloured. After three-colour stain-
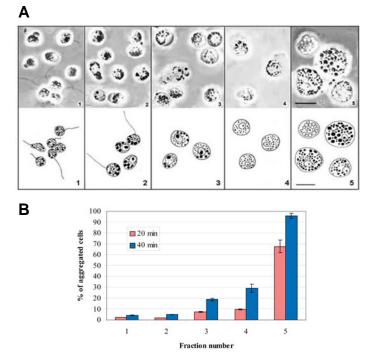
A

B

Fig. 2. Phase contrast microscopy of fractions of fixed H. dujardini cells *obtained by Percoll density gradient centrifugation (A) and its schematic representation immediately below. Illustrations 1 to 5 correspond to fraction numbers 1 to 5. The cell composition of each fraction is described in the text. Bar 5 mm.* **(B)** *Aggregation capacity of different fractions. Abscissa, proportion of aggregated cells to total cell number (%); ordinate, fraction numbers. Counting was performed 20 min and 40 min after loading in filtered sea water. Bars designate standard errors of mean.*

ing, large light-rose granular inclusions were observed in the cytoplasm (Fig. 1C).

Three days after larva settlement and metamorphosis, the juvenile sponge displayed a covering formed of T-shaped pinacocytes (Fig. 1E). Star-shaped bodies of these cells did not form a close monolayer; however, their histological and TEM investigation revealed thin cytoplasmic processes, covering the animal surface (Lévi, 1956; Boury-Esnault, 1973). Choanocyte flagellated chambers (Fig. 1E) and the anlages of aquiferous canal system (Fig. 1E) could also be observed at this stage. The inner cells of the swimming larva gave rise to dispersed cellular elements of the mesohyl, an internal sponge compartment, bounded by the pinacoderm and choanoderm (Borojevic *et al.,* 1967).

Analysis of the density gradient, cell distribution and aggregation capacity

The internal cell mass together with the posterior FC detached 3–5 min after larvae placement in Ca++, Mg++ -free artificial sea water (ASW), with the remaining layer of the anterior lateral FC forming unclosed translucent bubbles. Soon after the detachment the larvae dissociated into single cells.

Percoll density gradient centrifugation separated the cell mixture into the upper (10%) zone, which contained debris, bacteria etc., but lacked cells and 5 cell fractions. The first layer was kept in the gradient for security reasons, in order to prevent the

contamination of the subsequent fractions.

Fraction 1 (20% Percoll) contained anterior and lateral FC, which became round-shaped after centrifugation (Fig. 2A.1). Their diameter ranged between 2.4–3.0 μm. They had 0.8-1 μm nuclei with small nucleoli. Their cytoplasm contained numerous small granules. When transferred to filtered SW, they recovered the ability to flagellar movement. A wave of hyaline cytoplasm ran around the cell as a result of lamellipodium formation; however, no cell movement such as aggregation or spreading was observed on the substrate.

Fraction 2 (25% Percoll) comprised posterior FC (~60 %) and nucleolated amoebocytes (~40%) (Fig. 2A.2). These flagellated cells were larger (3–4 μm in diameter). Some of them lacked flagella, which is normally typical of posterior pole FC (Fig. 5H). Immotile rounded cells were observed attached to the substrate separately or in groups of 2 to 3. Nucleolated amoebocytes were 4.5–4.8 μm in size. Their cytoplasm contained yolk granules and their nuclei were 2 μm in diameter with large excentric nucleoli. Ten minutes after placement in filtered SW, they protruded long pseudopodia and began to move along the substrate. In 25–30 min, they formed aggregates of 3–7 cells.

Fraction 3 (30% Percoll) contained only nucleolated amoebocytes (Fig. 2A.3). They differed from the amoebocytes of the previous fraction in a lower number of yolk granules. However, they behaved much alike those of fraction 2.

Fraction 4 (35% Percoll) consisted of collencyte-like cells. These cells were 4.4–4.5 μm in diameter. They had a centrally
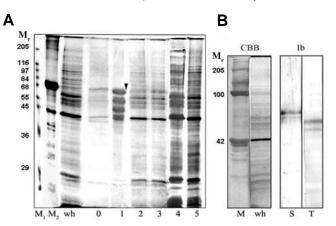
A   **B**

Fig. 3. Polypeptide pattern of cell fractions and AB testing. (A) *Polypeptide patterns of H. dujardini cell fractions obtained by Percoll density gradient centrifugation. Protein samples were applied to the 12, 5% SDS-PAGE and the gel was CBB stained. The probes loaded: M₁, commercial marker; M₂, second marker (rabbit myofibrils with BSA (68 kDa)); wh, joint sample of larval cells; Lane 0, top empty fraction obtained after Percoll gradient fractionation. Lanes 1-5 correspond to fractions 1 to 5. Numbers on the left designate Mr of the marker proteins in kDa. The mark in Fraction I corresponds to the 68 kDa zone, which was used for the production of antibodies against the S antigen. Note the prominent difference between fraction I and the remaining fractions in polypeptide composition.* **(B)** *Antibody labelling of adult sponge cells. Adult sponge samples were subjected to 10% SDS-PAGE (wh); the gel was CBB stained (CBB) or processed for immunoblot with the corresponding AB (Ib). Numbers on the left designate Mr of the marker proteins in kDa; M, marker, rabbit myofibrils without BSA. Lane wh, adult sponge cells in CBB probed with antibodies against tubulin (AB T) and against S antigen (AB S) (A, arrowed zone in column 1).*

positioned nucleus 1.5 µm in diameter with a small nucleolus (Fig. 2A.4). Their distinctive feature was the presence of numerous tiny (0.1–0.2 µm) granules in the cytoplasm. The cells formed short pseudopodia and were capable of rapidly attaching to and spreading on the substrate. However, they were less motile than nucleolated amoebocytes.

Fraction 5 (40% Percoll) comprised maternal spherulous cells (MSC) and large blastomere – like nucleolated amoebocytes (Fig. 2A.5). The latter were 6–10 µm in diameter. Their cytoplasm was filled with prominent yolk granules. Blastomere-like amoebocytes moved actively along the substrate using a short lobopodia. They formed aggregates of up to 15–20 cells, to which MSC could be involved. MSC were 4.5–5 µm in diameter. Their cytoplasm contained refractive granules and an excentric 2- µm nucleus with small nucleolus.

The aggregation capacity of different fractions was estimated as a ratio of the number of aggregated cells to the whole number of cells in 20 and 40 min after placement to cell culture dish (Fig. 2B). The aggregation capacity of cells decreased considerably from fraction 5 to fraction 1. Of all cell types examined, FC displayed the lowest ability to *in vitro* aggregation. Blastomere-like nucleolated amoebocytes exhibited the highest aggregation capacity accounting for rapid aggregation of fraction 5 cells (Fig. 2B).

Polypeptide fraction composition

Polypeptide composition of the fractions was determined with SDS-PAGE (Fig. 3A). In all cell-containing fractions, 3 zones of 16–19 kDa could be distinguished, which probably corresponded to the histones based on their apparent mobility in SDS-PAGE (Podgornaya and Shaposhnikova, 1998). Two bottom fractions contained a large amount of 42 kDa actin. Cells of the same fraction displayed the highest rate of *in vitro* motility. Fractions 2 (nucleolated amoebocytes and posterior FC) and 3 (only nucleolated cells) were very similar in polypeptide composition, as were fractions 4 (collencyte-like cells) and 5 (blastomere-like amoebocytes and maternal spherulouse cells). Fractions 2-5 display patterns typical of Metazoa, with the polypeptide composition covering the whole range of molecular masses (M_r), while the protein composition of fraction 1 (FC) differed drastically from that of other fractions. There are 4 prominent major zones in the range of 42-68 kDa (Fig. 3A, lane 1). One band of the double zone close to 42 kDa corresponds to actin. The double zone of about 55 kDa represents α and β tubulin isoforms, originating, obviously, from FC flagella. The cell-type specific zone of unknown nature with apparent M_r of 68 kDa (protein S) was chosen for AB production due to the highest M_r of 4 majors of FC (Fig. 3A, lane 1, arrow).

The breeding season of the sponge H. dujardini is rather short lasting for about 2 weeks, but AB production takes at least 2 months, so we were forced to use adult sponge for AB testing (Fig. 3B). The AB obtained against p68 kDa protein (AB S) and commercial AB against tubulin (AB T) were tested. Western blot after SDS-PAGE shows that both ABs recognize proteins of the corresponding M_r among adult sponge cell proteins. AB S works in the serum dilution of 1:5000 (Fig. 3B, lane S). So both ABs are specific enough and suitable for immunofluorescence.

Immunofluorescence

Both ABs were tested on whole mounted swimming larvae. Using phase contrast combined with fluorescence optics, we found that the S protein was concentrated in the apical part of the FC cytoplasm and was absent in the flagellum (Fig. 4A). In paraffin sections, AB S stained apical, central and slightly basal parts of anterior and lateral FC and, less intensively, the posterior FC (Fig. 4B, pp). The inner cells of the swimming larva were not stained by AB S (Fig. 4B) suggesting that it is sufficiently specific for FC.
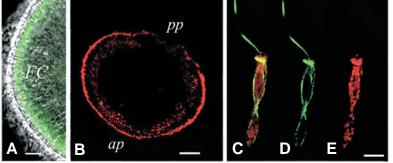
Mechanically isolated flagellated cells (see "Material and Methods", part "Immunofluorescence") retained elongated shape (Fig. 4C) unlike those separated in Percoll gradient (Fig. 2A.1). Double-staining of isolated FC with both ABs confirms the above proposal that protein S appearing in granules in the cell body is absent in the flagellum (Fig. 4E). On the contrary, AB T stained brightly the flagellum and basal body and only weakly the plasma membrane visualizing submembrane cytoskeleton structures; no staining of cytoplasm or AB S-labelled structures was observed (Fig. 4 D,E). Thus, AB T and AB S stain different compartments in the flagellated cells and, when used together, are suitable to trace flagellated cells fate during metamorphosis.

All external cells bore flagella immediately after larva release (Fig. 5 A-F). Before settlement, posterior flagellated cells lost flagella, anterior – lateral FC retained them but these flagella adhered to the larval surface just after attachment to the substrate (Fig. 5, G,H). The area of AB S staining of flagellated cells bodies appeared significantly smaller than that of AB T staining (Fig. 5I).

Flagellated cells were directly involved into metamorphosis. On the middle sections of metamorphosing larvae (Fig. 5, J-L), both AB S and AB T staining were confined to internal cell mass. This implies that former external cells (Fig. 5, A-C and D-F) rearrange to form flagellated chambers or line aquiferous canal anlages. In 12 hours post-settlement, the canals could be distinguished from flagellated chambers by the lack of AB T staining (Fig. 5J).

Thick paraffin sections (12 µm) of the sponge 3 days post-settlement were double stained and optically sliced at a 0.75 µm increment using confocal microscopy. Two optical sections ob-

Fig. 4. Immunoflurescence of AB S on whole-mount (A), paraffin section (B) and separate flagellated cell (C) of *H. dujardini* larvae just released from the sponge. (A) *Epifluorescent microscopy combined with phase contrast. Green fluorescence is produced by FITC-conjugated secondary antibodies. Scale bar: 5 µm.* **(B)** *Confocal microscopy of thick paraffin section. Texas red-conjugated (red) secondary antiserum was used here and in all the subsequent images for AB S. ap, anterior pole; pp, posterior pole. Scale bar: 20 µm.* **(C-E)** *Single flagellated cell (squash preparation) double stained with tubulin AB (T) and AB S.* **(C)** *The merged image;* **(D)** *AB T only;* **(E)** *AB S only. Scale bar: 2 µm.*
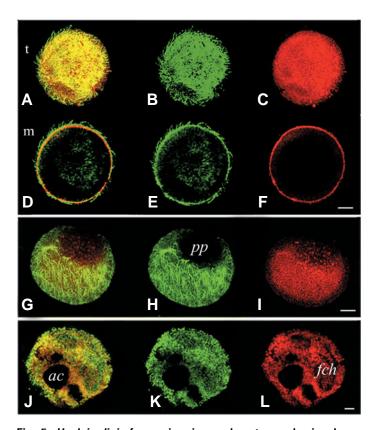
Fig. 5. *H. dujardini* **free-swimming and metamorphosing larva double stained with AB S and T.** *Laser confocal microscopy of the whole mount larva. (A,D,G,J) The merged image; (B,E,H,K) AB T (green) only and (C,F,I,L) AB S (red) only.* **(A-F)** *Free-swimming larva; t, top optical section; m, midsection.* **(G-I)** *Larva just after attachement to the substrate.* **(J-L)** *12 hours post-settlement. Note newly-formed choanocytes chambers (fch) and aquiferous canal (ac). Scale bars: 20 μm.*
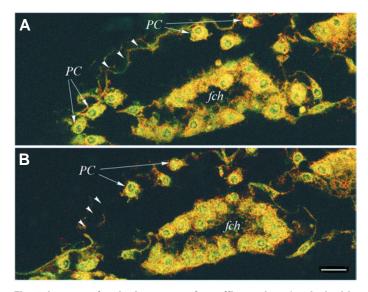

Fig. 6. Laser confocal microscopy of paraffin sections (optical midsections) of *H. dujardini* **juvenile** *3 days post-settlement double-stained with anti-tubulin and anti-p68 antibodies.* **(A,B)** *Sequential optical sections at a distance of 3 μm; abbreviations: fch, flagellated chambers; PC, pinacocytes. Scale bar: 5 μm.*

tained at a distance of 3 μm between them are shown in Fig. 6. As it is seen, most of former flagellated cells composed the newly formed choanocyte chambers. AB S labels granules in the cell body, as it did in premetamorphic flagellated cells. AB S and AB T staining is visible also in T-shaped dermal pinacocytes on sponge surface. Still, these cells do contain both antigen characteristics of flagellated cells.

A newly-formed adult sponge develops the definitive choanocyte chambers within one month after the settlement (Fig. 7A). At that time AB S label is seen in the choanocytes, but it disappears in the pinacoderm cells (the T-shaped pinacocytes) (Fig. 7B). Choanocyte protein S is concentrated at cells' apical part as circles, curves or polygonal structures (Fig. 7 C,D). A peculiar AB S staining pattern is caused by cutting apical cell parts in the vicinity of collars at different angles. Choanocyte flagella appear as an AB T-stained bright ribbon in chamber cavity. Amoebocytes - the other cell type visualized in the sections – display no AB S staining (Fig. 7, AmC)

Discussion

Protein

There are many examples of choanocyte and pinacocyte derivation from inner cells of larva, mostly from multivalent archaeocytes (Meewis, 1939; Brien and Meewis, 1938; Boury-Esnault, 1976; Bergquist and Green, 1977; Bergquist and Glasgo, 1986; Weilsputz and Saller, 1990). The diverse fate of larval flagellated cells may depend on their state of differentiation before metamorphosis. The terminal character of the differentiation of larval external cells was shown for some fresh-water (Efremova and Efremov, 1979) and marine Demospongiae (Bergquist and Glasgow, 1986; Kaltenbach and al., 1999).

We have demonstrated that the major protein (antigen S) from the anterior-lateral FC of the larvae is cell-type specific and that an antibody against it could be used for tracing FC fate in metamorphosis. In swimming larvae of H. dujardini all external cells have flagella (Lévi, 1956; Korotkova and Ermolina, 1982; Ereskovsky and Gonobobleva, 2000). We have presented the evidence of their disappearance in the posterior cells before metamorphosis (Fig. 5 G-I). This also appears to be the case in newly metamorphosed larvae of the Calcinea (Amano and Hori, 2001). In juvenile Halisarca, which develops within several days after metamorphosis, both flagellated cell markers retain in choanocytes of flagellated chambers. This agrees with the ultrastructural data obtained upon the larvae metamorphosis in H. dujardini by Gonobobleva and Ereskovsky (2004). They testify to the migration inward and transdifferentiation of the anterior cells into choanocytes. The authors report also the origin of the upper pinacoderm from the posterior flagellated cells. In our experiments major protein S is found in the external T-shaped pinacocytes in juvenile sponge but it disappears in the pinacocytes of the adult sponges (Fig 6, A,B; Fig. 7 A,B).

The problem of stability of the antigen through metamorphosis is a common one for such an approach to cell fate investigation. Nevertheless, our successive observations of cultured metamorphosing larvae provided evidences against destruction and *de novo* production of the antigen in different cell types during the early steps of the metamorphosis. The first evidence deals with the observation of consecutive centripetal dislocation of the
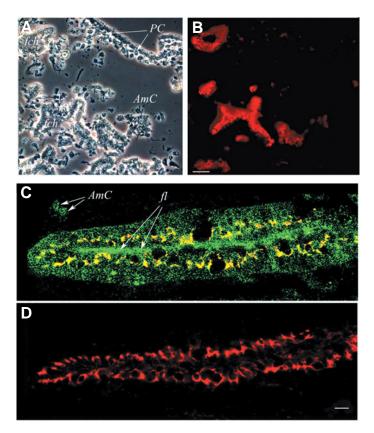
Fig. 7. Adult sponge stained with AB S and AB T. (A) *Cross-section of a body fragment; (a) phase contrast; (b) anti-p68 staining (red). Abbreviations: fch, flagellated chambers; PC, pinacocytes. Scale bar: 20 mm.* **(B)** *Section of choanocyte chamber. (a) double staining with AB T and AB S; (b) only AB S staining. AmC, amoebocytes; fl, flagella. Scale bar: 5 μm.*

marked area at the beginning of metamorphosis, characterized by peripheral localization of S protein in free-swimming larvae and label positioning in the internal structures of metamorphosing larvae. It appears as centripetal minimization of a circular fluorescent AB S area in larvae just after their attachment to the substrate. Fig. 5 G-I illustrates the first step of the internalization of both AB S and AB T labels; AB S label is localized closer to the center and away from the surface boundary, adhered by the flagella. The second evidence is a correlation between partial disappearing of cells labeled by both antibodies in larva periphery with appearing of marked cells in internal structures and finally, in choanocyte chambers. All of peripheral cells of juvenile sponge have large amount of both AB S and AB T antigens. We found no bleaching of fluorescent labels in those cells, but observed a reduction in their number during the process. What is the aim of this reduction? In the absence of cell death, which is not demonstrated for the metamorphosis of H. dujardini, only cell rearrangement, i.e. inward migration, could explain this process.

So the larval flagellated cells are the source for internal and surface cells in juvenile H. dujardini. This conclusion is not in full agreement with Y. Delage's classical description of "inversion of germ layers" (Delage 1892). The external cells of the larva transdifferentiate into the collar cells which are the inherent part of the internal environment, but inner cells of the larva do not organize the upper pinacoderme. According to Ivanova (2002),

even in fresh-water sponges of the family Spongillidae, whose larvae have prematurely developed choanocyte chambers, larval external cells may transform into choanocytes and pinacocytes of a juvenile animal. A treatment of the haplosclerid sponge *Reniera sp.* larvae with fluorescein-containing marker CMFDA, labelling only surface FC, revealed a similar FC behaviour (Leys and Degnan, 2002). In a juvenile sponge at the stage 3 days after metamorphosis, the marker was localized mainly in choanocytes but some elongated cells on the sponge periphery were also labelled. One can not exclude that these cells belong to pinacocytes category. Transdifferentiation of larval flagellated cells into choanocytes was shown with TEM in the demosponge *Haliclona permolis* (Amano and Hori, 1996). In calcareous sponges the cytological transformations of the larval flagellated cells into the choanocytes occur during metamorphosis of the amphiblastula and coeloblastula larvae as well (Amano and Hori, 1993, 2001) in spite of the paraphyly of the sponges demonstrated recently by Borchiellini *et al.* (2001).

Larval flagellated cells and the collar cells, choanocytes of the adult sponge, differ significantly by their morphology and the flagellum basal apparatus organization (see Simpson, 1984; Woollacott, 1993). The distribution of protein S in larval cells differs also from that in choanocytes. In FC, it is spread all over the cytoplasm as granules, while in definitive choanocytes it is confined to the apical part of the cell, namely, to its collar which composed of a row of microvilli. The very unusual polypeptide composition of larval external cells with few prominent major proteins (Fig. 3A) might suggest their terminal differentiation. However, we found no evidence for phagocytosis of FC by other cells or their shedding within short period of metamorphosis. We therefore believe that the unique protein composition of FC rather suggest their preparation for rapid and substantial changes evoked by transdifferentiation. If it is the case, the presence of protein S in flagellated cells is accounted for its anticipatory expression and storage for subsequent use in apical cytoplasm structure formation. The attempt to identify protein S by MALDI procedure has been done. The control sample of the 42 kDa zone was identified as β-actin with highest scores of similarity of invertebrate actin. At present the protein S spectrum obtained does not allow its unambiguous identification. Future investigation using TEM and immunoprecipitation are needed to confirm protein S distribution and identity.

Aggregation

Cells locomotion and interaction are necessary components of morphogenesis in sponges both in normal and experimentally provoked development (for review, see Gaino and Burlando, 1990).

The tracing of flagellated cells with cell-type specific AB S and AB T, forces us to conclude that these cells migrate inward. On the other hand, when separated in Percoll gradient and placed on glass surface, they show the lowest ability to active movement and aggregation among all cell types. Apparently the extracellular matrix (ECM) produced by other cell types is necessary for FC directed movement as it is a case in higher Metazoa (Behrendtsen *et al.,* 1995; Solnica-Krezel and Eaton, 2003)

Collencyte-like cells spread and flatten on the substrate, move or aggregate to lesser extend than nucleolated amoebocytes (Fig. 2B). At the same time, *in situ* the collencytes move actively

in the mesohyl, which was demonstrated by time-lapse filming in a fresh-water sponge (Kilian and Wintermann-Kilan, 1979). Using time-lapse kinetic analysis, diverse locomotory patterns were shown in different cell types of *Clathrina clathrus* (Burlando *et al.,* 1979). In the present study, nucleolated amoebocytes and blastomere-like cells (differing from nucleolated amoebocytes in the presence of numerous yolk granules) displayed high aggregation capacity (Fig. 2). They moved actively using short lobopodia and formed conglomerates together with maternal spherulous cells. Possibly, the presence of spherulous cells facilitates blastomere-like cells aggregation *in vitro*. In *Geodia cydonium,* granules of spherulous cells were shown to contain galectin, which is an important component of the system of adhesion molecules (Wagner-Hülsmann *et al.,* 1996; Müller, 1997). On the other hand, *Ircinia muscarum* spherulous cells isolated using Ficoll gradient fractionation, effectively inhibited cell aggregation in *G. cydonium* (Müller *et al.,* 1976). Thus, in addition to chemical defence, (Krylova *et al.,* 2003) these cells may play some role in *H. dujardini* morphogenesis.

The next challenge to address will be to determine whether nucleolated amoebocytes and spherulous cells are involved and whether they produce ECM proteins which could direct FC movement.

Materials and Methods

Collection of sponges and larvae cultivation

The sponges *Halisarca dujardini* were collected from the alga *Fucus vesiculosus* in the bays of Island Sredniy, Chupa Inlet, Kandalaksha Bay, White Sea, at a depth of 0.5 – 5 m in July 2001- 2004. The specimens were transported to the laboratory of White Sea Biological Station of Zoological Institute RAS and maintained in the aquarium with aerated seawater at 14⁰C. The released larvae were placed in Petri dishes, coated with 2% bactoagar (DiFCo Laboratories, USA) and containing sterile-filtered seawater (FSW) with 0.003% sodium cefazoline added. Three to six days after that, free-swimming larvae attached to the substrate and flattened.

Light microscopy

For whole mount preparations, free-swimming and metamorphosing larvae were fixed in 4% paraformaldehyde in 0.02 M phosphate buffer (pH 7.4). Sucrose was added to the fixative mixtures to reach isoosmotic condition of 625 mOsM.

For semi-thin sections, larvae were prefixed in 1% OsO_4 in 0.1M phosphate buffer (pH 7.4) for 10 min., washed 3 times in the same buffer, fixed in 2.5% glutaraldehyde and postfixed in 1% OsO_4 in the same buffer. The normal osmolality of the solutions was maintained as stated above. Samples were dehydrated through an ethanol series and embedded in Epon.

For paraffin sections, juvenile sponges and the fragments of adult sponges were fixed in Bouin's solution for 4 hours, dehydrated through an ethanol series, transferred into xylene and xylene-paraffin mixture and embedded in paraffin.

One micrometer thick semi-thin sections were stained with methylene blue – azure II – basic fuchsin according to Humphrey and Pittman (1974).

Cell separation

Discontinuous density gradients were manually preformed using Percoll media (Parmacia, Sweden) in the tube from bottom to the top (Bergquist and Glasgow, 1986) Larvae were treated with artificial seawater (ASW), containing 0.3 M NaCl, 20 mM KCl, 45 mM EDTA, 10 mM Tris-HCl at pH 7.7, appropriate for cell dissociation with high viability. In 3 – 5 min, they were shaken and pipetted to ensure complete cell dissociation.

Percoll media was mixed with the appropriate volume of ASW to make solutions of decreasing densities (45, 40, 35, 25, 20 and 10%) of initial concentration. Aliquots of 2 ml of each solution were overlaid sequentially into 15 ml polypropylene centrifuge tubes (Sarstedt, Germany). Each pair of tubes was centrifuged in a swing rotor at 800 g for 10 min. Cell layers were collected by gentle aspiration and washed twice in ASW. Then cell viability and the quantitative and qualitative composition of cell fractions were estimated under a LUMAM I-2 microscope (LOMO, Russia) using phase-contrast optics. Five hundred cells per sample of each fraction were examined. All manipulations were carried out at 14˚ C.

Estimation of cell aggregation potency

Cells fractions were analysed for aggregation potential. Samples of each cell fraction were transferred to FSW. Cell concentrations were equilibrated in the range of 2500±35 cells per ml. Cells were placed in cell culture Petri dishes (Nunc, USA) and their aggregation potency was estimated in 20 and 40 min under the same microscope using phase contrast optics. The number of cells in aggregates of 3-25 cells in 15 microscope field was counted and its proportion to the whole number of cells was estimated (Fig. 2B).

Electrophoresis and Western-blotting

The protein composition of cells in fractions was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Gels were Coomassi Brilliant Blue (CBB) stained. High molecular weight mixture of proteins (HMW, Sigma) and rabbit myofibrils preparation were used as markers. Western-blotting was done according to Towbin *et al.* (1979). Methanol activated polyvinylidene difluoride membranes (pore size - 0.45 μm, Millipore Corporation, USA) and modified Laemmli's running buffer (25 mM Tris, 192 mM glycine, pH 8.34, 0.001% SDS, 10% methanol) were applied for protein semidry transfer. Membranes with immobilized proteins were inactivated with a solution of 1% sodium caseinate, 3% bovine serum albumin, 150 mM NaCl, 0.02% Tween-20 (TBS-Tw) in 20 mM Tris-HCl at pH 7.5. Antibody diluting and membrane washing procedures were carried out in TBS-Tw. Bands were visualized by 3,3'-diaminobenzidine tetrahydrochloride staining of horse radish peroxidase conjugated with secondary goat anti-guinea pig and goat anti-mouse antibodies (MP Biomedicals, USA). Adult sponges were homogenized and loaded to SDS-PAGE as well.

Antibody production

Polyclonal antibodies were raised against the major 68-kDa protein S from larval flagellated cells. The protein was cut out from the slab gel, destained, homogenized with 20 mM PBS at pH 7.5 and mixed with complete Freund's adjuvant (1:1). The mixture was injected hypodermically into a male guinea pig. The boost was done in a week by an intramuscular injection with incomplete Freund's adjuvant. All the following immune response stimulations were developed by multipoint intracutaneous injections without adjuvant. Four weeks after the initial immunization, about 5 ml of blood from the animal's heart ventricle was taken. The antiserum was aliquoted and stored at -20⁰C. Working antibody dilutions for immunoblotting were estimated as 1:5000 for specific antibodies that marked sponge flagellated cells (AB S) and 1:20000 for commercially purchased anti-α, β-tubulin (AB T) (raised in mouse, Sigma, USA).

Immunofluorescence

Twelve micrometer thick paraffin sections were placed on poly-L-lysine coated slides, paraffin was removed with o-xylene treatment and after stepwise hydration in ethanol series (from 96% to 30%) the slides were put in TBS-Tw. In order to block non-specific reactions the slides were pretreated with 5% bovine serum albumin solubilized in TBS. AB S was used in 1:350 dilution. AB T was diluted with TBS in 1:2000 ratio. Fluorescein isothiocyanate (FITC) – or Texas red-conjugated goat anti-guinea pig or goat anti-mouse antibodies were used as secondary

antibodies (Sigma, USA). The final dilutions of secondary antibodies corresponded to recommendations provided. All the procedures were done at room temperature. Sections were mounted in Moviol 4.88 media and analysed under a LEICA fluorescent microscope and LSM 5 PASCAL confocal laser scanning microscope (Carl Zeiss, Germany), equipped with Ar (488 nm) and He-Ne (543 nm) laser sets. The images were processed by the LEICA Q-FISH or the LSM 5 Image Browser. For control, some sections were incubated in non-immune serum and processed otherwise as above. They were free of label.

Fixed preparation of swimming and metamorphosing larvae were washed three times in TBS (30 min each time), permeabilized with 0.2% Triton X-100 in TBS for 5 min at room temperature to prepare the whole mount. Non-specific binding sites were blocked with 5 % bovine serum albumin in TBS for 1 hour. Primary antibodies against major protein of flagellated cells (AB S, final dilution 1:500) and anti-α, β-tubulin (AB T) were incubated with larvae on poly-L-lysine coated multiwell slides at 4° C during 12 hours. The samples were washed three times in TBS (30 min each time) and stained with secondary antibodies as above, the whole procedure taking 2 hours. Individual flagellated cells were prepared by squeezing of paraformaldehyde-fixed larvae. Adobe Photoshop 6.0 software was used for image processing for publication.

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