Zoomorphology (2002) 121:203–211
DOI 10.1007/s00435-002-0057-9

ORIGINAL ARTICLE

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In vivo study of microsclere formation in sponges of the genus *Mycale* (Demospongiae, Poecilosclerida)

Received: 14 August 2001 / Accepted: 16 January 2002 / Published online: 24 May 2002

Abstract The process of microsclere secretion was examined in vivo through glass coverslip implants in three species of the genus *Mycale* from São Sebastião channel, southeastern Brazil: *Mycale* (*Aegogropila*) *angulosa*, *Mycale* (*Arenochalina*) *laxissima*, and *Mycale* (*Carmia*) *microsigmatosa*. All three species adhered well to coverslips and developed normally through at least 2 weeks. Similar experiments with different species (*Cinachyrella alloclada*, *Amphimedon viridis*, *Haliclona melana*, and *Aplysina caissara*) were also successful with one exception (the cartilaginous *Chondrilla nucula*), indicating that the method can be applied to most demosponges. Microsclerocyte size varied according to the type of microsclere secreted, but all were elongated to fusiform and had small, anucleolated nuclei. Spicules were transported by microsclerocytes alone, without any other cell type ("helper cells") involved. Secretion of a microsclere was performed by a single sclerocyte. Although some axial filaments were found free in the mesohyl, all microsclere secretion in these animals was fully intracellular. Normal axial filaments were observed in most types of microscleres of the *Mycale* species (sigmas, toxas, and microxeas). Timed observations of sclerocytes suggest that immature spicules with the aspect of short straight rods with thick ends might be the precursors of the anisochelae. Observed differences in the size versus number of toxa secreted may indicate either the presence of two distinct subpopulations of toxa-producing microsclerocytes or that the initial number of axial filaments at the beginning of silica deposition may determine the final size of the spicules. Although other microscleres such as sigmas and chelae are secreted in a one cell–one spicule basis, several toxas and microxeas can be secreted simultaneously in a single cell.

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Introduction

Many species of Demospongiae (Porifera) have skeletons composed of small siliceous spicules, which are organized in various arrangements. Based on their relative size and inferred structural importance, spicules are divided in two types, megascleres and microscleres. Both types show a wide variety of shapes and sizes, and are a major source of taxonomic characters. In recent times there is a growing interest in the mechanisms of spicule formation. The biopolymerization of silicates at ambient temperatures and pressures and at near-neutral pH, in contrast to those harsh conditions required in geological or human-made processes, has attracted considerable attention due to its possible industrial applications (Shimizu et al. 1998; Cha et al. 1999; Morse 1999).

The mechanisms of spicule formation in demosponges are not yet fully understood. Megasclere formation in Demospongiae has received considerable attention and several works dealt with aspects of their production and composition (see, for example, Weissenfels and Landshoff 1977; Simpson 1969, 1978; Simpson et al. 1979; Hartman 1981; Holvoet and Van de Vyver 1986; Imsiecke et al. 1995; Uriz et al. 2000). On the other hand, only a few studies provided data on microsclere formation (Simpson 1968, 1978, 1984; Rützler and Macintyre 1978; Wilkinson and Garrone 1980; Garrone et al. 1981; Poirrier et al. 1987). In both cases, basic questions such as the transport of silica, helper cell participation, or whether spicule secretion is intra- or extracellular are still debated.

Despite recent advances in the development of techniques to allow observations in intact sponges (Wyeth et al. 1996) or at the ultrastructural level (Uriz et al. 2000) there are, nevertheless, only a few systems available for the study of cellular processes in these animals. For in

Table 1 Descriptive characteristics of the *Mycale* species used in this work

Species Vouchers	*Mycale angulosa* MNRJ 1324	*Mycale laxissima* UFRJPOR 3636	*Mycale microsigmatosa* MNRJ 1308
Habit	Thick incrustations to ramose specimens up to 50 cm in height	Massive to tubular, with one to several tubes	Incrusting
Ectosomal architecture	Reticulate	Unspecialized. Projecting brushes of megascleres of terminating choanosomal ascending tracts may be seen	Unspecialized. Projecting brushes of megascleres of terminating choanosomal ascending tracts may be seen
Choanosomal architecture	Plumoreticulate	Stout quadrangular reticulation with abundant spongin	Plumose
Spicules[a] (data assembled from several specimens from the study area and vicinities)	Subtylostyles 276–341/4.5–6.9/6.5–10.2 Anisochelae I 46.9–54.7 Anisochelae II 17.4–22.4 Isochelae 10.1–13.9 Sigmas I 72.8–82.5 Sigmas II 13.4–21.6 Sigmas II 20.0–33.6 Toxas I 54.7–78.7 (94.3[b]) Toxas II 25.0–43.8 (77.0[b]) Microxeas 24.8–37.6	Subtylostyles 227–286/2.8–10.2 Anisochelae 17.6–28.6 Sigmas 67.2–98.0	Subtylostyles 185–246/2.1–4.2 Anisochelae 14.0–21.0 Sigmas 23.0–34.0

[a] Spicule type followed by the smaller and larger length in microns (subtylostyles includes the diameter)
[b] Data extracted from live preparations made from other specimens

vivo observations, both larval cultivation (Maldonado et al. 1999) and the classic method of gemmule development in sandwich cultures (Weissenfels 1990, 1992) are applicable to only a few species and/or are season dependent. In explant techniques sections of sponges are tied to slides where they can attach and develop, allowing the outgrowth region to be observed (Simpson 1978). Theoretically, this technique can be used in most or all species of sponges, but in practice it requires slides being fixed in suitable locations with sponge fragments being tied up, attaching, and developing.

In the present work we have examined in vivo the process of microsclere secretion in three species of the genus *Mycale* Gray, 1867 from São Sebastião channel, southeastern Brazil. All species are common in tropical Western Atlantic shallow waters, and produce a range of microscleres including anisochelae, isochelae, sigmas, toxas, and microxeas. We have performed our observations through glass implants, used here for the first time in cell biology studies of invertebrates. This method allows unrestrained observation of the living tissues of functional sponges in vitro, without the dependence on gemmule formation, larval release, or explant fixation. We aimed at answering the following questions:

1. Is the glass implant method suitable for studies of cell behavior in sponges?
2. Are there differences in the formation of microscleres of different types or in different, although closely related, species?
3. How many cells are involved in the formation and transport of a spicule or a group of spicules?
4. Is the formation of microscleres in *Mycale* species intra- or extracellular?

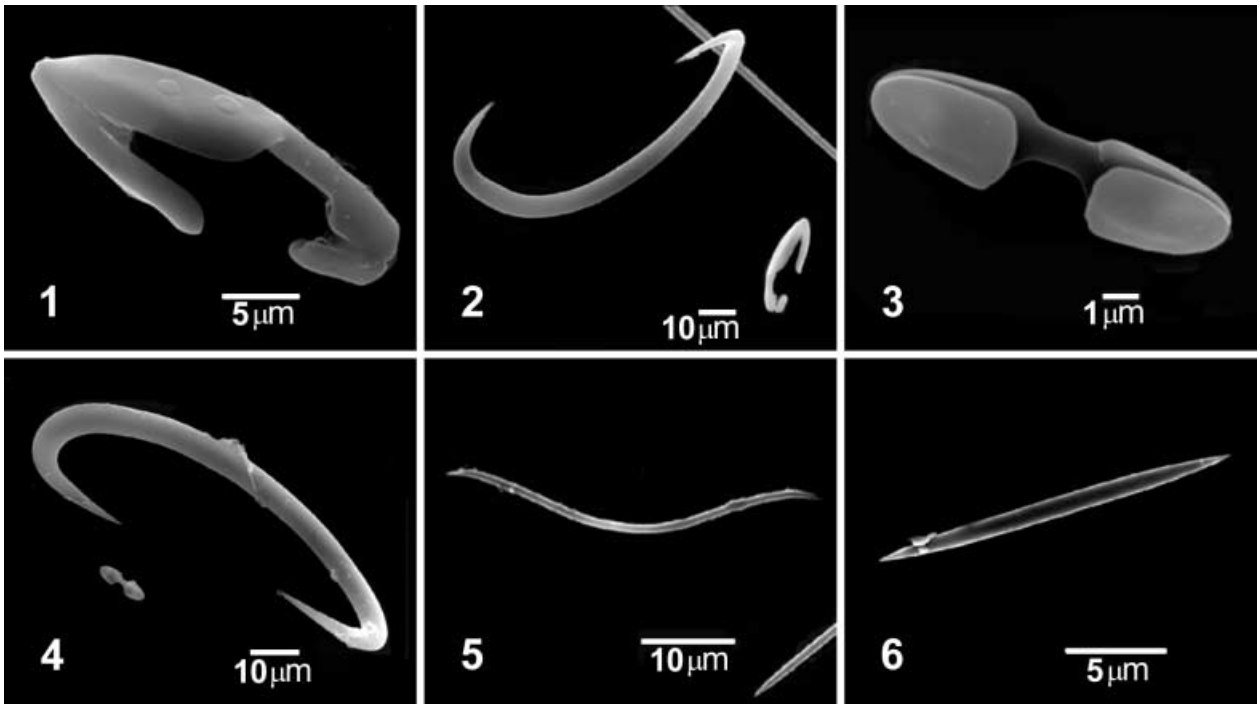
Materials and methods

Species

Four to six individuals of each of three species of *Mycale* found in São Sebastião (São Paulo State, Brazil) were used in the experiments: *Mycale* (*Aegogropila*) *angulosa* (Duchassaing and Michelotti, 1864), *Mycale* (*Arenochalina*) *laxissima* (Duchassaing and Michelotti, 1864), and *Mycale* (*Carmia*) *microsigmatosa* Arndt, 1927 (Table 1; Figs. 1, 2, 3, 4, 5, 6). *Mycale angulosa* varies from thick incrustations to ramose specimens up to 50 cm in height. The skeleton is plumoreticulate, with ascending spicule bundles in the choanosome and a dense triangular reticulation in the ectosome. Anisochelae in rosettes and sigmas are abundant, together with isochelae, toxas (frequently organized in toxodragmas), and microxeas (frequently organized in bundles). *Mycale laxissima* varies from massive to tubular, with one to several tubes. Consistency varies from soft to firm, and the sponge produces abundant mucus. The ectosomal skeleton is unspecialized, and the choanosomal skeleton is formed by a quadrangular reticulation of thick spongin fibers cored with megascleres. Spicules are subtylostyles, anisochelae, and sigmas. *Mycale microsigmatosa* is usually found incrusting large areas. The consistency is soft and the skeleton is plumoreticulate with bundles of subtylostyles crossing the choanosome, with rare anisochelae and randomly distributed sigmas. Supplementary descriptions of these species can be found elsewhere (see, for example, Van Soest 1984; Hajdu and Boury-Esnault 1991; Hajdu and Rützler 1998). In situ photographs are available in Custódio et al. (2000).

Glass implants

Living tissues from each species were obtained using glass implants. Coverslips (Corning) were cut in 5×20 mm sections, cleaned with isopropanol, and rinsed with sea water before use. A thin steel blade (5×30×0.3 mm) was dug into the sponge tissue and two coverslips aligned by its larger side were inserted up to 15 mm through the cut. The blade was then removed and the



Figs. 1–6 Representative microscleres of *Mycale* species

Fig. 1 *M. laxissima*: anisochelae

Fig. 2 *M. laxissima*. Sigma and anisochelae

Fig. 3 *M. angulosa*. Isochelae

Fig. 4 *M. angulosa*. Sigma and isochelae

Fig. 5 *M. angulosa*. Toxa

Fig. 6 *M. angulosa*. Microxea

coverslips were left in place for 24–72 h. This arrangement in pairs hinders the growth of tissue in one side of each coverslip and avoids the interference during the posterior observations at the microscope. After removal, the coverslips were placed with the clean side down in 60-mm cell culture dishes (Corning). The dishes were then taken to the laboratory and kept in running seawater for up to 2 weeks. The observations were made in a Nikon Diaphot inverted microscope using phase contrast.

To perform cytological and histological observations the coverslips were fixed with a mixture of methanol, formalin (37–40% formaldehyde), and acetic acid in the volume ratio of 85:10:5 for 2 h at 4°C (MFAA; Reite 1997). The fixed tissues were then stained either with Ziehl's fucsin (Martoja and Martoja 1967) or Mallory's trichrome (Behmer et al. 1976). After staining, coverslips were dehydrated, inverted, and mounted directly on microscopic slides with Entellan (Merck). Observations were made with a Zeiss Labophot microscope.

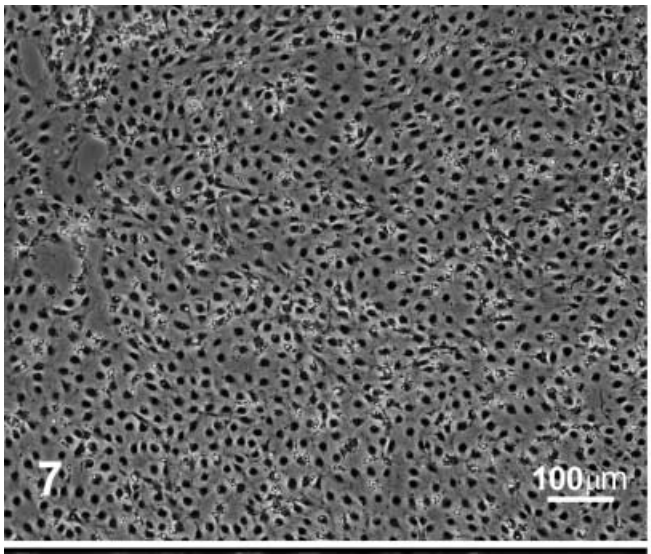
Results

Glass implants

The three species studied showed some variation of responses to coverslip implants. The initial cell adhesion is quick. After 2 h large groups of pinacocytes can already be seen attached to coverslips. After 24 h the coverslips showed large areas covered with pinacocytes, with only a few other cells (Fig. 7). The time necessary for enough tissue formation on the glass to remove some choanosome with the coverslip varied from 48 h in *M. microsigmatosa* to 72 h in *M. angulosa* and *M. laxissima* (Fig. 8). Given longer times the sponges grow tightly around the glass, and either the intact coverslip recovery was impracticable or the amount of tissue recovered with it was too thick to allow any observation.

Soon after removal the tissue in the coverslips was partially disorganized and active cell migration could be seen. After 12 h reorganization was evident, with a concentration of cells in the central area surrounded by a fringe of pinacoderm. At this stage there was already a distinction between baso- and exopinacocytes and some newly formed, isolated choanocyte chambers were present. Cell streams were evident, moving actively (approximately 11–12 μm/min). After 36 h, organized groups of choanocyte chambers lined an incipient canal system. Cell streams and groups of 15–20 choanocytes were already arranged in the shape of small chambers at the periphery of the tiny reorganizing sponge, but still without the beating flagella. The growing stages of the chambers can be seen along the canal toward the center of the sponge. After the canal system was completely formed (Fig. 9), this stage remained in running seawater for at least 5 weeks. During this time the whole sponge moved on the coverslip, leaving behind a trail of basal spongin and spicules. Contamination by other organisms (mainly diatoms and protozoans) was controlled by washing the coverslip sections with filtered seawater and changing the culture dishes.

Alternatively, the plates were kept in the incubator without running seawater. The process of tissue reorganization in these plates was similar to those in running sea-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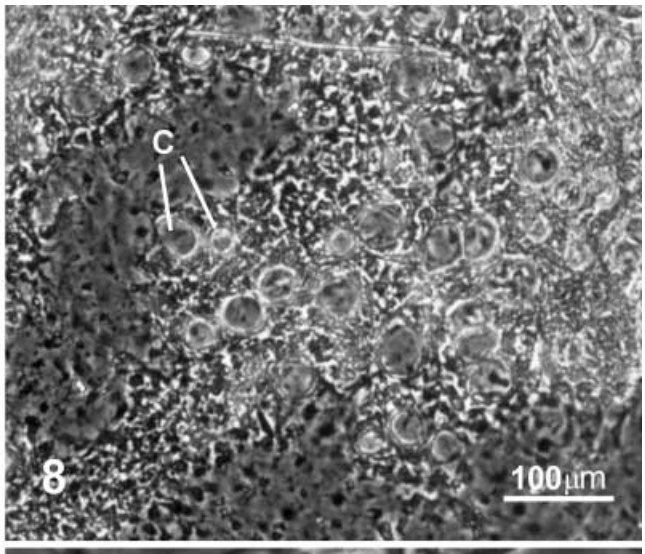
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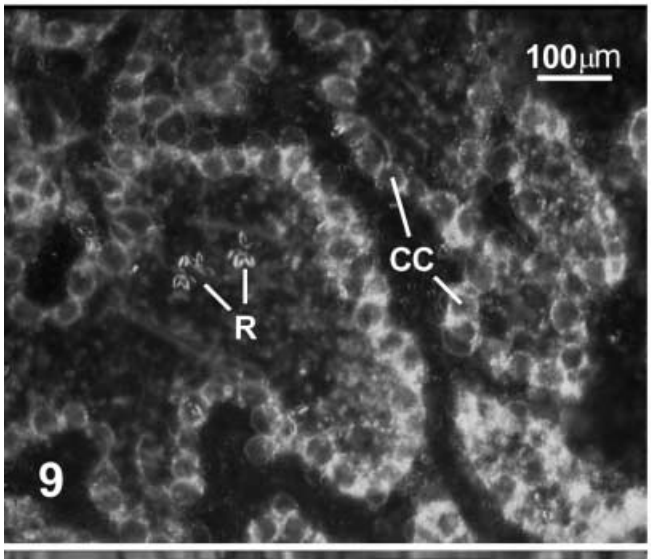
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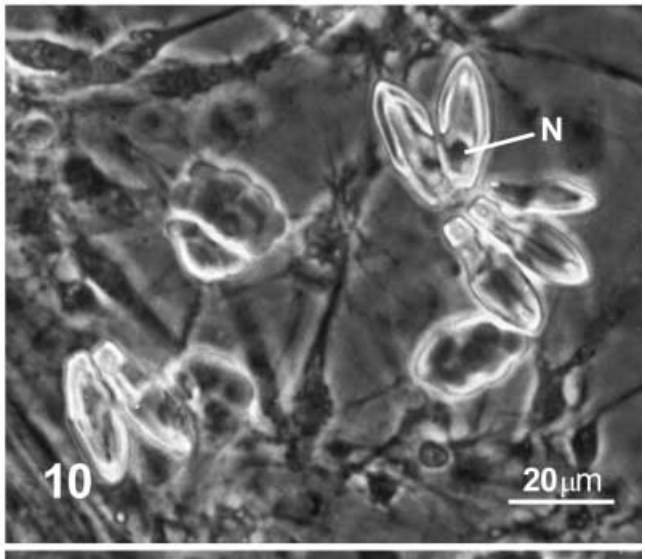
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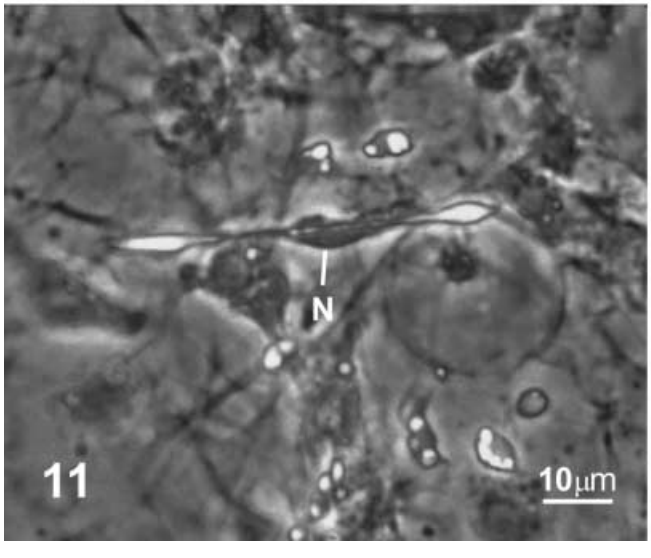
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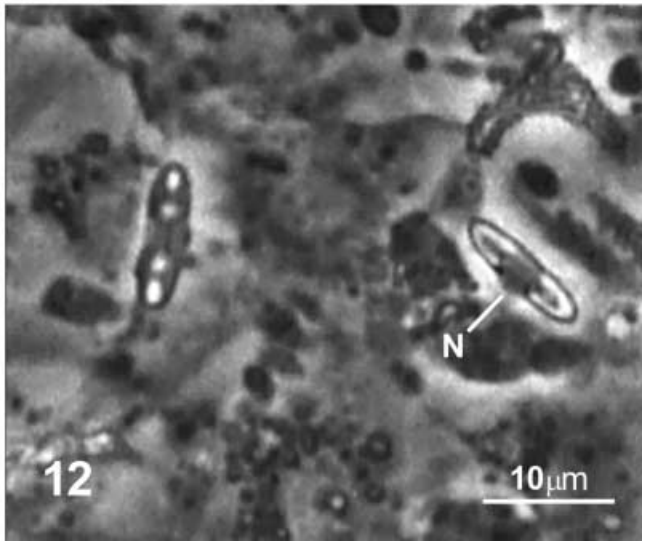
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water, and small sponges were also formed. The sponges kept in these plates stood healthy, as recognized by the functional aquiferous system, for approximately 3 weeks without supplementary food. At the end of this period the tissue started to retract and eventually reached a stage of diamorph (Borojevic et al. 1968), loosing the attachment to the glass.

Spiculogenesis

In reorganizing sponges the process of microsclere secretion can be accompanied in vivo since the early formation of the axial filament. Microsclerocytes have a small anucleolated nucleus and a cytoplasm with relatively few vacuoles. Cell dimensions varied both according to the type of spicule secreted as well as dynamically, due to transient cell projections several microns long. The largest cells were those producing sigmas (15–110 µm long), followed by toxa-secreting cells (25–90 µm long); the smallest were the isochelae- (8–18 µm long) and the anisochelae-producing microsclerocytes (12–58 µm long; Table 1).

In anisochelae-producing microsclerocytes, the small and anucleolated nucleus is always positioned inside the chelae. These cells can be frequently found associated in pairs and moving through the mesohyl, either arranged side by side or at different angles (Fig. 10), but always with the feet of the chelae (*sensu* Hajdu and Desqueyroux-Faúndez 1994) positioned together. The formation of rosettes of anisochelae begins with a pair of spicules, which marks the focal point. Additional anisochelae are transported singly or in pairs to this area, and are positioned with the feet toward the center of the rosette only after they reach the structure. Although the individual spicules forming the rosette can be seen moving sideways, the entire structure maintains a stable position in the tissue. Staining of fixed tissues by Mallory's trichrome showed that the anisochelae feet are held together by a dense collagen deposition, and the nuclei of microsclerocytes can be detected even after the rosette formation is completed. No other cells were observed participating in this process.

Young stages of anisochelae, identified by an axial filament without visible silica deposition, as observed in other microscleres, were never detected. However, in *M. laxissima* we observed several sclerocytes containing immature spicules with the aspect of short straight rods with thick ends (Fig. 11). Such microsclerocytes clearly differed from subtylostyle-producing megasclerocytes, which showed long and thin axial filaments with homogeneous width.

The smaller isochelae-secreting microsclerocytes in *M. angulosa* show the same structural characteristics of the cells producing larger anisochelae, with small anucleolated nuclei, which keep a stable position inside the chelae (Fig. 12).

Sigma-producing microsclerocytes also have small anucleolated nuclei, which were always positioned inside the arch of the spicule. In all species the axial filament, although flexible, was already curved in the final shape in the early stages of spicule formation (Fig. 13), but flat in the same plane. Later, as the silica deposition becomes visible, the final twist in the pointed ends is acquired. Remarkably, in this stage the cell membrane seems to be tensioned by the spicule, and projections outside the spicule's curvature are rarely observed (Fig. 14).

In toxa-producing microsclerocytes the early stages of the axial filaments, although already tending to the final shape, are flexible and can be considerably curved as the cell moves through the mesohyl (Fig. 15). At this stage the nucleus is usually placed in the center of the cell, surrounded by young spicules positioned with the curved sides in opposition. Later, as the spicules become bigger and the cell more loaded, the nucleus acquires a peripheral position and the spicules have a tendency to assume a distinct arrangement (toxodragma), all with the curves to the same side (Fig. 16). Several groups of toxa-secreting microsclerocytes in diverse stages of spicule formation were observed in the periphery of the sponge. The larger toxas of *M. angulosa* are usually found alone or in pairs, whereas the smaller ones are secreted in groups of up to 11 spicules, all inside a single cell.

Similar to the toxas, the microxeas in *M. angulosa* were secreted in tight bundles of 5–10 spicules and showed a uniform length. In the earliest stage of its formation, thin filaments were arranged in parallel and regularly spaced inside a single cell (Fig. 17). Microxea-producing microsclerocytes also had small anucleolated nuclei, but their cytoplasm was more vacuolated than in

◀ **Fig. 7** *M. angulosa*. Glass implant removed after 24 h inside the sponge tissue. Large areas are covered by a pinacoderm, similar to a monolayer in confluent cell cultures in higher invertebrates. Few other cell types are found in this stage

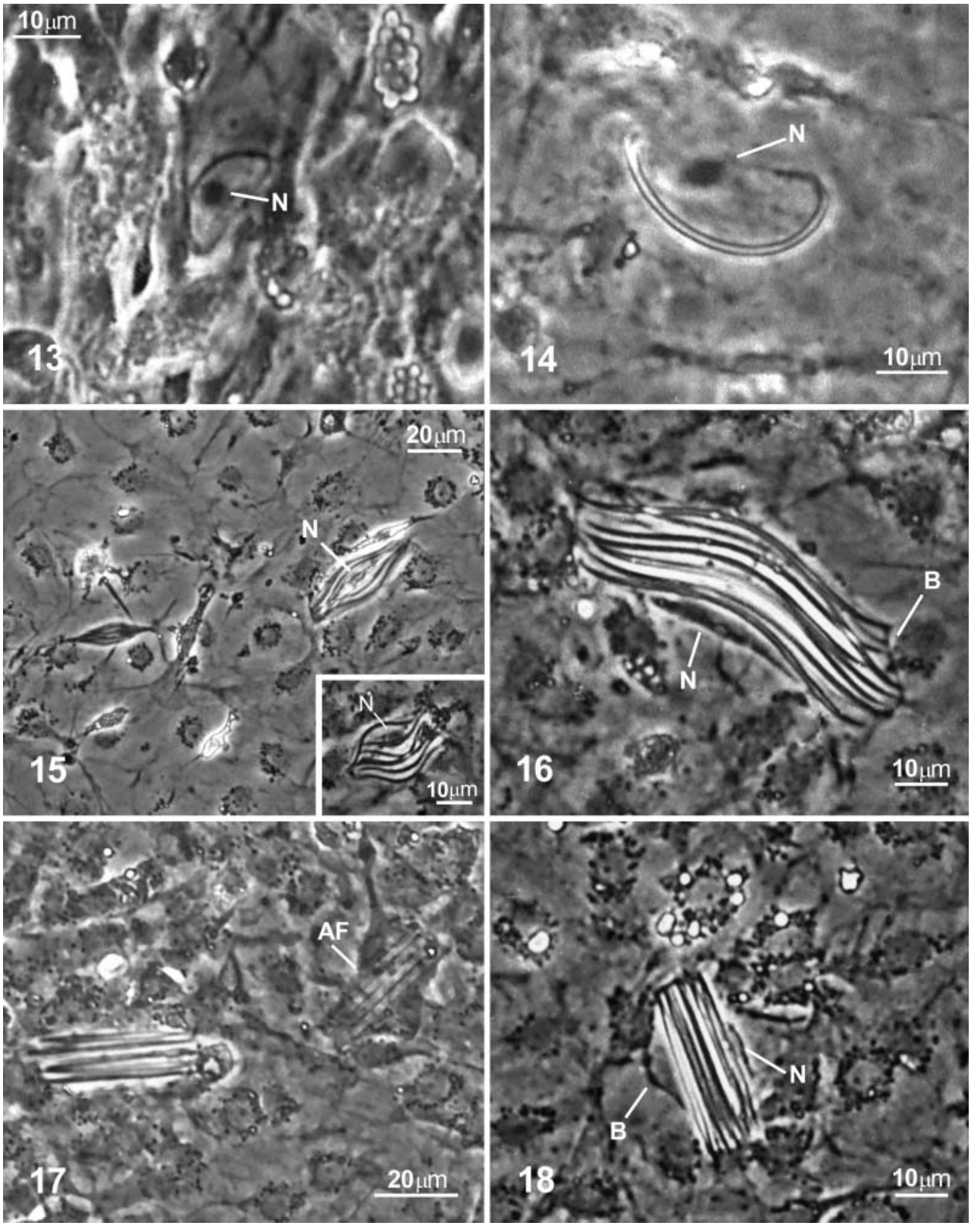
Fig. 8 *M. angulosa*. Coverslip removed from sponge tissue after 48 h. At this stage part of the sponge choanosome is well connected to the pinacoderm beyond and is pulled out with the coverslip. We can observe several cystencytes (*C*), large cells with a single vacuole. This timing is approximately the same for *M. laxissima*, but *M. microsigmatosa* takes only 24 h to reach this stage

Fig. 9 *M. laxissima*. Coverslip removed from the sponge after 48 h and kept in running seawater for an additional 72 h. The sponge tissue is totally regenerated and the aquiferous system, complete with channels lined with choanocyte chambers (*CC*), is fully functional. Some rosettes of anisochelae are being formed (*R*)

Fig. 10 *M. laxissima*. A pair of anisochelae moving sideways in the mesohyl toward a rosette. This arrangement is frequently found. While the larger extremity of the spicules is free to move, their feet seem already aggregated. The position of the nucleus inside the chelae is constant (*N*) as seen in the anisochelae forming the rosette

Fig. 11 *M. laxissima*. View of the cell with the rod, which can represent the early stages of anisochelae secretion. The nucleus is already positioned in the middle (*N*) and the structure keeps its shape even though the cells move actively in the mesohyl

Fig. 12 *M. angulosa*. Two tiny isochelae-producing cells. These cells are always found isolated and move actively in the mesohyl while the nucleus (*N*) keeps a stable position inside the chelae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toxa-producing sclerocytes (Fig. 18). The nucleus was flattened and located in the periphery of the cell, even after the spicules reached full development. In spite of appearing tightly held together after fixation and acid oxidation when prepared for dissociated spicule mounts, in vivo observations indicated that each spicule within these bundles is capable of independent movement during the cell activity.

Discussion

Glass implants

The technique of glass implants used in this work is suitable for studies of cell biology of sponges, since it can be applied to any specimen thick enough to hold a coverslip section, either in the field or in aquaria. Similar implant techniques have been used in cell biology studies of vertebrates. Introduction of glass coverslips into the peritoneal cavity of mice was used to induce granuloma formation and allow the recovery of distinct cell populations to be cultivated in vitro (Mariano and Spector 1979; Borojevic et al. 1984).

The behavior of dissociated sponge cells in contact with foreign materials has been well studied. The initial adhesion seems to be weak, even when the substrate is coated with adhesion molecules such as laminins, collagens, or fibronectin (Gaino et al. 1993; Gaino and Magnino 1994). However, as soon as the basal spongin lamina is secreted, the sponges follow their normal development.

◀ **Fig. 13** *M. microsigmatosa*. Cell carrying a sigma in the early stages of formation. Although the axial filament still show some flexibility, the nucleus (*N*) keeps a stable position inside the arch as the cell moves through the sponge tissue

Fig. 14 *M. laxissima*. Cell carrying a sigma with visible silica deposition around the axial filament. At this stage the overall shape of the axial filament is stable, although the spicule still grows. The cell boundary can be observed between the nucleus (*N*) and the spicule end to the right

Fig. 15 *M. angulosa*. Two cells showing different stages of toxa secretion. The axial filaments in the smaller cell on the left are still flexible and bow considerably as the cell moves (*inset*). Both cells where moving fast to the *upper right* in this photograph, and nucleus (*N*) can be observed in the center of the cell on the right, with the toxas disposed around it

Fig. 16 *M. angulosa*. Cell showing toxa secretion. Each spicule in the bundle moves independently, sometimes forcing the cell border (*B*) as in this picture

Fig. 17 *M. angulosa.* Microxeas being secreted in bundles. The axial filaments (*AF*) are visible on the left, already organized in parallel in the early stages of spicule formation. As to the toxa-secreting microsclerocytes, these cells move actively in the sponge mesohyl

Fig. 18 *M. angulosa*. Group of microxeas being secreted in a single cell, with a projecting border (*B*) while moving over the pinacoderm. The nucleus, positioned to the right (*N*), always keeps position in the cell periphery

Apart from sponges belonging to *Mycale*, pilot experiments were conducted with five other different species: *Chondrilla* aff. *nucula* Schmidt, 1862 (Hadromerida, Chondrillidae), *Cinachyrella alloclada* Uliczka, 1929 (Spirophorida, Tetillidae), *Amphimedon viridis* Duchassaing and Michelotti, 1864 (Haplosclerida, Niphatidae), *Haliclona melana* Muricy and Ribeiro, 1999 (Haplosclerida, Chalinidae), and *Aplysina caissara* Pinheiro and Hajdu, 2000 (Verongida, Aplysinidae). These species showed good cell adhesion and formation of small functional sponges, although with some differences in timing. The only exception was *C. nucula*, in which the coverslip sections showed no cell adhesion, even after 8 days of insertion. Instead, a marked tissue necrosis and protozoan infection were observed in the implant area. The results of these experiments made with distantly related species indicate that the method can be applied to most demosponges, and possibly also to the Calcarea.

Therefore, the technique of coverslip implants is a simple way to obtain slides with adhered sponge cells, in which the tissues and cellular processes can be easily observed. Furthermore, the possibility of obtaining a number of replicas from a single animal adds the further advantage of clonal identity, avoiding genotypic differences among the samples.

Spiculogenesis

In our study, both the structure and the behavior of microsclerocytes in *Mycale* species was similar for all microscleres. Although their size varied according to the type of microsclere secreted, all microsclerocytes were elongated to fusiform, and all had small, anucleolated nuclei. The presence of anucleolated nuclei seems to be the rule in the microsclerocytes of demosponges (Connes 1968; Simpson 1968, 1984; Wilkinson and Garrone 1980). The only exceptions documented so far are the gemmosclere-producing sclerocytes of *Ephydatia fluviatilis* (Linnaeus, 1758) and *Spongilla lacustris* (Linnaeus, 1758) (Haplosclerida, Spongillidae) (see Leveaux 1939), and the spheraster-producing cells of *Tethya lyncurium* Linneus, 1767 (Spirophorida, Tetillidae) (see Connes 1968). This contrasts with the nucleolated megasclerocytes of both Calcarea and Demospongiae, supporting the hypothesis that microsclerocytes and megasclerocytes have different cellular origins (Simpson 1984).

Microsclerocytes of *Mycale* species, either with complete or immature spicules, move actively over the pinacoderm and inside the mesohyl, without any other cell type (“helper cells”) involved in the transport of spicules. Similar conclusions were reached for *Ephydatia muelleri* (Lieberkühn, 1855) (see Bond 1992) and *Crambe crambe* (Schmidt, 1862) (Poecilosclerida, Crambiidae) (see Uriz et al. 2000), using different methods. The secretion of a microsclere is performed by a single sclerocyte, which can also be the only one re-

sponsible for its arrangement in multispicular structures, as in the formation of rosettes of anisochelae. All microsclere secretion in these sponges is intracellular and no extracellular process was observed. This contrasts to production of toxa in *Microciona prolifera* (Ellis and Solander, 1786) (Poecilosclerida, Microcionidae), in which the process seems to be performed in both ways (Simpson 1968, 1978).

With the exception of anisochelae, whose immature stages were never positively observed, axial filaments were present in most types of microscleres of *Mycale* species (sigmas, toxas, and microxeas). Axial filaments have been already reported in microscleres of *Heteromeyenia* sp. (Haplosclerida, Spongillidae), *Geodia neptuni* (Sollas, 1886) (Spirophorida, Geodiidae), *Neofibularia irata* Wilkinson, 1978 (Poecilosclerida, Desmacellidae), and *E. muelleri* (respectively by Drum 1968; Rützler and Macintyre 1978; Wilkinson and Garrone 1980; Garrone et al. 1981). They are probably present in all demosponge microscleres, as they are in all megascleres (Reiswig 1971).

Some axial filaments were found free in the mesohyl, as observed in toxas of *M. prolifera* (see Simpson 1968) and styles of *C. crambe* (see Uriz et al. 2000), even days after the sponge tissue was completely regenerated. They probably correspond to spontaneously released filaments, and are not due to tissue damage. Although the presence of axial filaments free in the mesohyl has been related to the extracellular secretion of spicules in both *M. prolifera* and *C. crambe*, all spicules observed in *Mycale* species were secreted intracellularly.

In *Mycale* species, axial filaments with anisochelae shape were never observed. The youngest stage detected were spicules already with anisochelae appearance but lacking the lateral enlargement. However, in *M. laxissima* there were rod-carrying cells, which have no correspondence to other spicule types. The relative abundance of these structures, similar in numbers to the anisochelae, in contrast to other spicule types suggest that they can represent the early stages of anisochelae secretion. This is also supported by timed observations (15-min intervals) of distinct rod-carrying cells, followed in the moving tissues for over 5 h under the microscope in continuous flow culture chambers. On some occasions they were replaced in the area by immature anisochelae. Since the work of Minchin (1910) the anisochelae are usually considered to be derived from the simple sigma. In *Mycale* species the young sigmas already had the final sigmoid shape since the stage where no silica deposition was visible, in opposition to the straight rods. However, we failed to detect intermediate stages or observe the exact moment of transformation from the straight rods to the curved anisochelae, which could imply a fast transition between them.

Although *M. angulosa* is described as possessing only one class of toxa (Van Soest 1984), apparently two different kinds of arrangement were found in this species. Thus, smaller toxas (up to 77.0 µm long; up to 43.8 µm in MNRJ1326, cf. Table 1) can be found in large clusters of up to 11 spicules per cell whereas larger toxas (up to 94.3 µm long; up to 78.7 µm in MNRJ 1326, cf. Table 1) are found in smaller groups of up to 3 spicules per cell. As even the larger toxas were always observed inside microsclerocytes and never free in the mesohyl it seems unlikely that spicules can be completed and released independently from the bundle. It is not clear whether the observed differences in the size versus number represent two distinct subpopulations of toxa-producing microsclerocytes or the initial number of axial filaments on each cell at the beginning of the silica deposition determines the final size of the spicules. In *M. angulosa* the cells also lack the coiled material of unknown nature associated with toxa-producing microsclerocytes observed by Simpson (1968) in several other Poecilosclerid species.

The secretion of microxeas also starts with the organization of a bundle of axial filaments in a single cell. Microscleres are usually considered to be secreted one per cell. Until now the only known exceptions were microxeas in *N. irata* (see Wilkinson and Garrone 1980) and occasionally onychaetes in *Tedania ignis* (Duchassaing and Michelotti, 1864) (Poecilosclerida, Tedaniidae) (see Minchin 1910; Simpson 1968). In *Mycale* species it is clear that several axial filaments of toxas and microxeas are secreted inside a single cell, and not enclosed collectively in a cell after secretion in separate cells as suggested as a possibility for raphides in *N. irata* (see Wilkinson and Garrone 1980). Thus, although other microscleres such as sigmas and chelae are secreted in a one cell–one spicule basis, several toxas and microxeas can be secreted simultaneously in a single cell.

Our observations confirm most of the early findings by several authors in other species, the majority based on electron microscopy pictures. Nevertheless, there are some differences in those findings regarding the intra- and/or extracellular microsclere formation and the presence of extracellular material during its secretion. As it is true for many other physiological processes in Porifera, it seems that there is some degree of heterogeneity, and the available data on these mechanisms among the different groups still needs to be expanded.

Acknowledgements This work was supported by CAPES, CNPq, FAPERJ, and FAPESP. The authors thank Dr. Marcelo Alex de Carvalho (IBCCF – UFRJ) for the fundamental suggestions on the methodology, Dr. Márcia Attias and Noemia Rodrigues (IBCCF – UFRJ) for the provision of SEM facilities and help on SEM operation, and the staff of CEBIMar – USP for the provision of laboratory facilities and technical support during field trips.

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