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FORMATION OF SPICULES BY SCLEROCYTES FROM THE FRESHWATER SPONGE *EPHYDATIA MUELLERI* IN SHORT-TERM CULTURES IN VITRO

GEORG IMSIECKE, RENATE STEFFEN, MARCIO CUSTODIO, RADOVAN BOROJEVIC, AND WERNER E. G. MULLER'

Institut für Physiologische Chemic, Universität, Duesbergweg 6, *D-55099 Mainz, Germany (G. I., R. S.,* W. E G. *M.) and Departamento de Bioquimica, Instituto de Quimica, Universidade Federal do Rio de Janeiro, Caixa Postal* 68021, *21944* – 970 *Rio de Janeiro, Brazil (M. C., R. B.)*
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SUMMARY

Cells from the freshwater sponge *Ephydatia muelleri* were isolated by dissociating hatching gemmules. During the first 24 h the cells reaggregated, but the aggregates progressively disintegrated again to single cells, among which the spicule,– forming sclerocytes were recognized. Such cultures were used to study spicule (megascleres) formation in vitro. The isolate J sclerocytes formed the organic central axial filament onto which they deposited inorganic silicon. The size of the spicules (200 to 350 μm in length) as well as the rate of spicule formation (1 to 10 μm/h) under in vitro conditions were similar to the values measured in vivo. Immediately after completion of spicule formation, or even before, the sclerocyte could start formation of a new spicule; 5% of the cells were in the process of forming two spicules simultaneously. Cultivation of sclerocytes in the absence of silicon resulted in the formation of the axial filament only. We succeeded in maintaining the sclerocytes in a proliferating and spicule-forming state for up to 3 mo. These results demonstrate that the establishment of short-term cell cultures from *E. muelleri* is possible; however, future studies must be undertaken to identify the growth factors required for a permanent culture of sponge cells.

Key words: sponges; *Ephydatia muelleri;* spicules; sclerocytes; cell culture.

INTRODUCTION

The phylum Porifera (sponges) is considered to be the lowest multicellular Metazoa. Sponges comprise several thousand species, among which approximately 120 species live in freshwater (Penney and Racek, 1968). Marine forms have been known since the Proterozoic period, >1 billion yr ago (Orlov, 1971). The oldest fossil freshwater sponge, *Spongilla gutenbergiana,* was described from the Middle Eocene (Müller et al., 1982), whereas spicules from freshwater sponges were already known from the Cretaceous of Patagonia (Ott and Volkheirner, 1972).

The body of the sponges is permeated by water, entering through inhalant pores, flowing through the frequently very complex aquiferous system of channels and choanocyte chambers, and leaving through oscule(s). The sponge's internal environment is separated from the external water by exopinacocytes, and from the water flowing in the aquiferous system by endopinacocytes and choanocytes. Sponges are classified by the chemical nature of their skeleton. The largest group, Demospongiae, which are found in all aquatic environments, are characterized by siliceous (SiO,) spicules, supplemented with collagen and, often, spongin, which also belongs to the collagen family. The spicules have a one-(monactine) to four-(tetractine) rayed organization (Bergquist, 1978; Simpson, 1984).

Little is known about the course and the control of spicule development, in either marine and freshwater sponges, Besides electron microscopic studies, light microscopic investigations using sandwich cultures have been performed to study the spicule formation. Using the freshwater sponge E*phydatia fluviatilis*, Weissenfels and Landschoff (1977) and Weissenfels (1989) demonstrated that the formation of spicules starts in sclerocytes within a specific vesicle. After the production of an axial organic filament, silicon is deposited around it, and the whole process of forming a spicule (190 μm in length and 6 to 8 μm in diameter) is completed after 40 h, at 21° C.

Several aspects of spicule formation still remain unclear: a) It is not known if a single sclerocyte or several sclerocytes together with "helper cells" contribute to spicule formation (Simpson, 1984). b) The final fate of the sclerocyte after the formation of the spicule is unknown; Minchin (1909) assumed that sclerocytes degenerate after completion of the spicules. The alternative view would be moving away from the recently formed spicules, and initiating a new cycle of spicule formation. c) Moreover, since the contribution of Evans (1899), it remains unclear if sclerocytes represent a specific cell type, or may originate from more than one type of precursor cells. To approach an understanding of spicule formation under more defined conditions, primary cultures of cells from the freshwater sponge *F.. muelleri* have been established. We used the gemmules – asexual reproduction bodies – as starting material for the establishment of primary cultures of sclerocytes.

MATERIALS AND METHODS

Animals

Gemmules of the freshwater sponge *E. muelleri* (Lieberkühn, 1855; Porifera: Demospongiae: Spongillidae) were collected in the river Wied

'To whom correspondence should be addressed

near Neustadt (Bonn region; Germany) in October 1992, and kept at 4° C in tap water until use. After a short treatment of gemmules with 5% NaOC1 and 70% ethanol =100 gemmules were incubated in plastic petri dishes (Greiner, Frickenhausen; Germany) containing 10 ml deionized and autoclaved water. Under these conditions, 90% of the gemmules started to hatch after 48 h, at 20° C.

For the histologic study of spicules, the freshwater sponge *S. lacustris* (Linnaeus, 1758; Porifera: Demospongiae: Spongillidae) was prepared as described by Imsiecke (1993).

Dissociation of cells from germinating gemmules and culture method, Approximately 100 gemmules were transferred to 20° C to initiate the hatching process. After 48 h they were disrupted by squeezing them through a 10-ml syringe. Immediately after the mechanical dissociation, the cells were suspended in the culture medium consisting of the sterile "M" medium (Rasmont, 1961), supplemented with 10 mM HEPES, pH 7.5, and 0.1% of BME-amino acids (nr. K 0263; from a 100 X stock solution; Biochrom, Berlin, Germany), BME-non-essential amino acids (nr. K 0293), and BME-vitamins (nr. K 0273), as well as 0.1% chicken embryo extract (nr. 061 – 05115C; GIBCO/BRL, Grand Island, NY). To avoid bacterial contamination antibiotics in a final concentration of 100 U/ml penicillin and 100 mg/ml streptomycin (Biochrom) were added to the medium. The cell suspension was transferred into six-well tissue culture plates (Nunclon, Roskilde, Denmark), and the cell density was adjusted to 2 X 10^4 cells/well. Cells were maintained in darkness at 20° C,

If not mentioned otherwise, the culture medium contained 15 mg/liter of Na-silicate; in one series of experiments silicon was omitted from the medium.

Determination of cell viability and mitotic index. For the determination of the mitotic index, the cells attached onto cover slips were fixed in 90% methanol/10% 0.5 *M* EGTA at – 20° C for 30 min. After washing with medium M, staining was performed for 30 min with the fluorescent dye Hoechst N 33258 (bisbenzimid) at a concentration of 1 µg/ml (Russell et al., 1975).

Microscopy. Light microscopic observations were performed with an Olympus IMT-2 inverted microscope equipped with phase contrast. Thin and semithin sections were produced as described (Simpson and Vaccaro, 1974). The silicon was removed from the thin sections applying a 2.5% aqueous solution of hydrofluoric acid (45 min; 2 h). Micrographs were taken with a Phillips KM 300 electron microscope. The confocal laser scanning microscopy was performed with a Zeiss LSM 10 as described (Bachmann et al., 1992). Lysosomes were stained with 0.0001% acridine orange as described (Harrison and Cowden, 1983).

RESULTS

Establishment of a short-term culture. Gemmules are the dormant stages of the spongillids in which they can resist low temperatures during winter season. This dormancy is interrupted after the rise of temperature in spring (approximately at > + 10° C). This interruption can be prevented under experimental conditions if the gemmules are kept at + 4° C. After transfer to 20° C, the gemmules of *E. muelleri* start to hatch after an incubation period of 48 h. During this phase, the high osmotic pressure within the gemmules is reduced, and their shells open at a preformed opening, the micropyle. At this stage, the gemmules still contain the dormant thesocytes, the physiologically active archeocytes, and the differentiated histoblasts. The archeocytes derive from thesocytes (cells containing two nuclei). The thesocytes are rich in vitelline platelets which are storage organelles that serve as energy source for the archeocytes. The archeocytes further differentiate into histoblasts, which contain very few or no vitelline platelets and only one nucleus (Zeuthen, 1939; Schmidt, 1970). They can subsequently differentiate to pinacocytes, which form the pinacoderm, an epithelial-like cell layer. This layer mediates the attachment of the sponges primordium to the substratum.

At this stage, the hatching gemmules were mechanically dissociated. The resulting single-cell suspension reaggregated during the following 24 h, forming aggregates of 20 to 4000 cells (Fig. 1 a). In addition to archeocytes, which contained a large nucleolus (Fig. 1 d), the 5-day-old aggregates in the cultures also contained sclerocytes, characterized by the presence of an axial filament and by their capacity to form spicules (Fig. 1 *e-g)* and pinacocytes (Fig. 1 b).

After 30 days the cell aggregates formed in early stages of cultures disintegrated. At Day 30, many single cells were present in the culture (Fig. 2 *a, b),* among them the tetranucleated archeocytes which attached to the bottom of the culture dish (Fig. 2 a, c); the round cells have varying diameters ranging from 15 to 25 µm. In addition, about 1% sclerocytes characterized by their spindle-like shape and by their capacity to form siliceous spicules were present in the culture (Fig. 2 b). These cultures could be kept for up to 3 mo., but after this period the number of living cells decreased gradually.

The mitotic index was determined in the aggregates at Day 21. It was found to be between 0.5 and 1%. It is interesting to note that even after a cultivation period of 1 mo. a tetranucleated cell was observed (Fig. 2 c). Very likely this cell originated from a binucleated cell by karyokinesis. Trinucleated cells have not been observed.

Formation of spicules in sclerocytes. After 5 days in culture the first spicules, megascleres, appeared in the aggregates. Spiculogenesis continued (Fig. 1 *b* – at Day 13) until the aggregates began to migrate. The completed spicules were released at Day 22 (Fig. 1 c). They were only attached to the substratum but not to each other like a skeleton in a functional sponge (Fig. 1 c). In contrast the first sclerocytes present in the isolated state started to form spicules after 30 days in culture.

As in functional sponges, spicule formation started with the appearance of an organic axial filament in the sclerocytes. This axial filament is clearly visible by phase contrast microscopy in a histologic preparation of a developing sclerocyte (Fig. 3 b). The silicon was deposited around the axial filament, resulting in the full spicule formation (Fig. 1 *e, f*). During the process of spicule formation, the sclerocytes were fiat with a large contact area to the substratum or they were spindle shaped with only short filopodia (Fig. 1 *f*). Growing and fully developed spicules in sclerocytes are shown in the Fig. 1 *f* and *g*, respectively (s_2 in Fig. 1 g). The rate of increase in spicule length varied between 1 and 10 µm/h, a value that corresponds to that found in vivo (Weissenfels and Landschoff, 1977). The three-edged axial filament (diameter of about 300 to 500 nm) remained present and could be visualized electron microscopically after dissolving the spicule by treatment with hydrofluoric acid (Fig. 3 a). The fully formed spicules were embedded in a matrix, corresponding to the "spongin," containing typical collagen-fiber bundles. The spongin was always surrounded by pinacocytes (Fig. 3 a; P), which probably function as spongioblasts.

The sclerocytes were characterized by a well-developed rough endoplasmic reticulum, abundant mitochondria, and many small refracting inclusions (1 to 2 µm) (Simpson and Vaccaro, 1984); the latter structures could be readily seen by light microscopy (Fig. 1 g). Confocal laser scanning microscopy of a living sclerocyte revealed the spatial distribution of many small (1 to 2 µm) inclusions, probably lysosomes, which were stained by the fluorescent die acridine orange (Fig. 3 c – e). The enlargement (Fig. 3 c) demonstrates clearly that these cell organelles do not reach the tip of the intracellular spicule. At this late stage of spiculogenesis archeocytes (Fig. 3 c) were in close contact with the sclerocytes.

With the increase in size, the spicules protruded the body of sclerocytes (Fig. 1 *e–g).* However, they were still covered by the cell membrane until reaching the full spicule size. In 5% of the sclerocytes, the formation of a second spicule (s^2) started even at a stage during which the first spicule (s^2) was still associated with them (Fig. 3c).

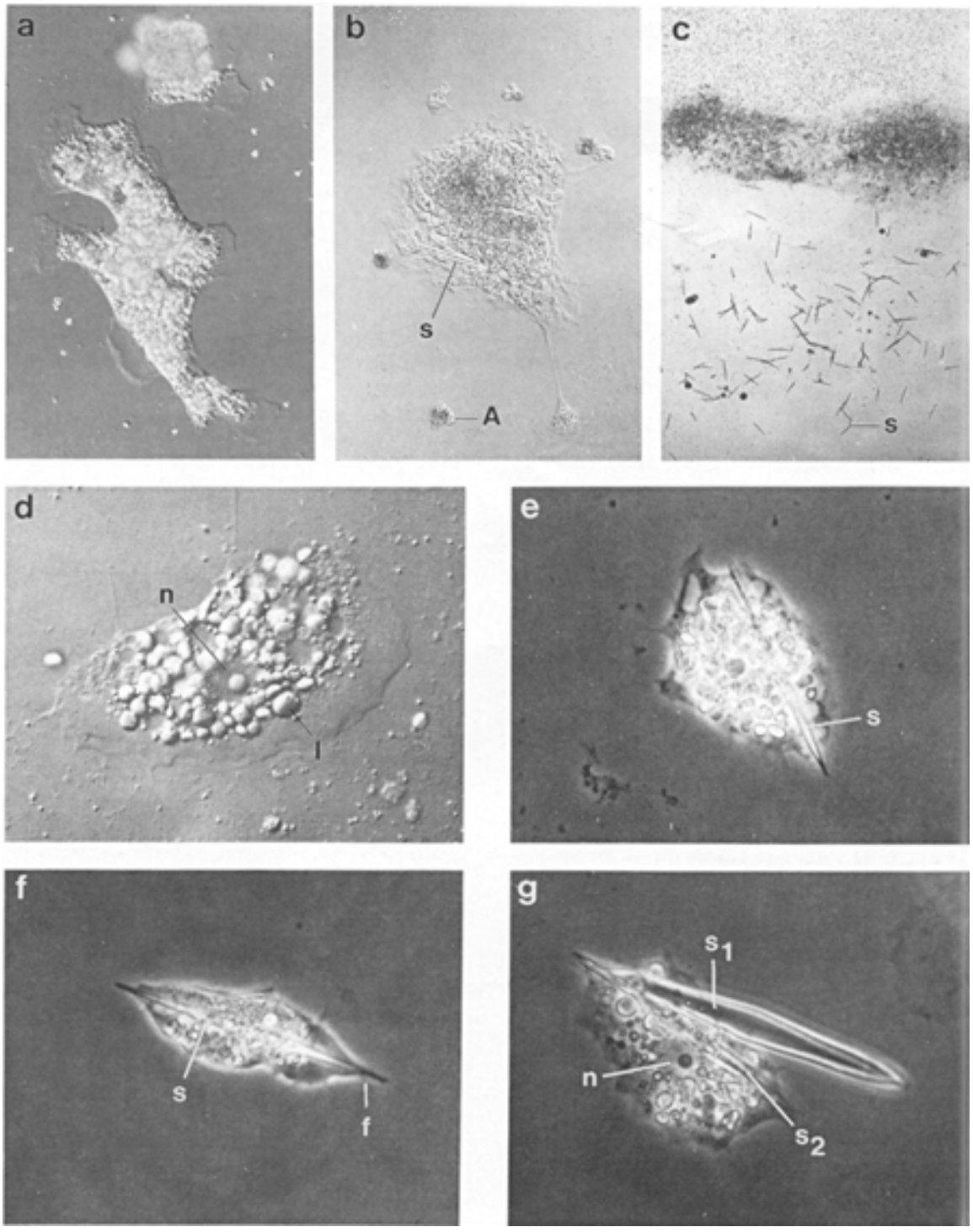

Fig 1. Cells obtained from hatched gemmules of the freshwater sponge *E. muelleri.* After mechanical dissociation, aggregates are formed from singe cells 24 h later (a). After 13 days in culture, spicules are formed in the aggregates (b). Aggregates migrate and leave behind the spicules formed by them (22 days) (e). Archeocyte containing lysosomes and nucleolated nucleus (d); sclerocyte with a single (e and *f*) or with a fully developed spicule just released (s,) together with a second, newly formed spicule (s.,) (g). *a, b,* and *d:* differential interference contrast imaging; c: bright field analysis; *e, f,* and *g:* phase contrast microscopy. *s:* spicule; *n:* nucleus; *l:* lysosome; *f:* filipodium; *A:* archeocyte. *a:* X 215; *b:* X 75; c: X *25; d – g:* X 650.

Types of spicules formed. In *E. muelleri* the developed sponge contains only megascleres which are straight-to-slightly curved and stout amphioxea; their length reaches 350 μm with a width of up to 20 μm (Penney and Racek, 1968). These values are higher than those found in cultured single cells and aggregates: up to 150 μm.

In culture, normal amphioxea were formed which have about the same shape but a smaller size as those found in vivo. However, very regularly abnormal spicules were formed as have also been isolated and described from whole sponges (Schulze, 1923); their shape varies depending on the type of the style (Fig. 4 b), from the centrostyl (Fig. 4 c, g) to the triactine (Fig. 4 d) and even to an almost ball-like type (Fig. 4 e). The spicules were often encountered in groups of 8 to 10 then having an almost uniform (abnormal) shape (Fig. 4 f, g). We observed also spicules in which two axes gave a shape similar to irregular triactines (Fig. 4 d). We interpret these as examples of an imperfect synchronization between the organic axis formation and the completion of the spicule formation, in which the formation of a new spicule began while the first was still being silicified. At this stage of culture, the size of newly formed spicules decreased only slightly to 130 μm (Fig. 4 *f*), and the spicules often became more compact, indicating that the elongation of the spicule along the central filament was decreased, while the deposition of silicon was still fully active.

Regulation of spicule formation by sodium silicate. Reducing the silicon concentration in the culture medium from 15 mg/liter to 0 mg/ml prevented the formation of spicules. When maintained under normal culture conditions, but without silicon, the cells produced only the axial filament (Fig. 5 a, b). The length of the released filaments is only about 75 μm and does not reach the length of the spicules produced in the presence of silicon.

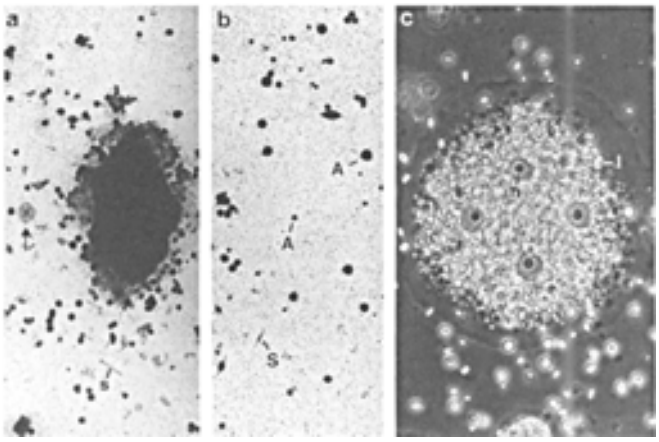

FIG. 2. Increase of the portion of single cells on expense of aggregates. Culture after 30 days. Aggregate is disintegrated into single cells; among them is an attached tetranucleated archeocyte (arrow) (a); the latter cell is shown at higher magnification in (c). (b), Round-shaped archeocytes of different sizes and single spindle-shaped sclerocytes. *a* and *b:* bright field; c: phase contrast microscopy. *a* and *b:* X 50; c: X 490. *A:* archeocyte; *S:* sclerocyte; *n:* nucleus; *s* spicule; *l:* lysosome.

DISCUSSION

Culture conditions for long-term growth of isolated sponge cells in vitro are not yet defined. As a first step, we maintained dissociated cells from the freshwater sponge *E. muelleri* in a proliferating and functionally active state for at least 3 mo. The culture medium contained, in addition to amino acids and vitamins, low concentrations of chicken embryo extract. The growth rate of the cells in vitro was low, and a mitotic index of only 0.5 to 1% was measured. This value is much lower than that observed in vivo, which was found to be 12% in gemmules, on the first day after hatching (to be published). Previously it was reported that ~2.5% of the archeocytes are in the proliferating state during germination of *Ephydatia fluviatilis* (Berthold, 1969) or in 14-day-old *S. lacustris* (Imsiecke et al., 1994). This fact is taken as an indication that the cells require either metabolites supplied by other cell types, or growth hormones which have not yet been identified. Both would indicate that the sponge internal environment is strictly controlled, despite the lack of specific cell structures at the interface between the internal and external environments. Recent studies on marine sponge cells have detected the molecular pathways of signal transduction as those used for intercellular signaling in higher multicellular organisms, including phosphatidyl-inositide cascade, tyrosine kinases, etc. (Müller et al., 1987; Schäcke et al., 1994). This suggests the existence of intercellular messengers and their corresponding functional receptors, which may be required for optimal growth stimulation of sponge cells.

The cultures were suitable for the first time, to follow in vitro, the phases of spicule formation in sclerocytes. The results revealed that sclerocytes synthesized under in vitro conditions spicules at a rate of 1 to 10 μm/h; the same value was found in vivo (Weissenfels and Landschoff, 1977). In early cultures, the sclerocytes followed completely the spicule-formation program, and the spicules formed in vitro were comparable, both in form and in size, to those produced in vivo. From the observation that the spicule formation occurs in isolated sclerocytes, it can be deduced that "helper cells" are not required for the individual spicule formation, as was proposed previously (Weissenfels and Landschoff, 1977). Taken together, our observations indicate that the information for spicule formation is inherent to a single cell, which can fully execute the program of organic axis formation and the subsequent silicon deposition. This is in agreement with earlier observations indicating that the differentiation of sclerocytes is the "default" option of cell differentiation in morphogenesis of Spongillidae from the hatching gemmules. Rozenfeld (1980) has shown that archeocytes from hatching gemmules initiate spiculogenesis in the presence of puromycine, at concentrations that inhibit or severely decrease protein synthesis, resulting in an increase of the spicule production as compared to controls. A similar increase of spiculogenesis under conditions that inhibit the differentiation of other cell types was also described in other experimental models (Holvoet and van de Vyver, 1985a). As suggested by Pé (1973), this may indicate that a production of a proteid factor(s) by other differentiated cells mediates the interruption of the spiculogenesis program and induction of an alternative program of cell differentiation. We have recently shown that the formation and maintenance of a functional aquiferous system depends on the presence of a classical soluble morphogen, the retinoic acid (Imsiecke et al., 1994), and the interplay between positive and negative controls of the differentiation can possibly account for a sequential differentiation of cells in gemmule hatching.

Sponge spicules can have a rather simple form, as those observed in Spongillidae, or a very elaborate one with a complex symmetry, shape, and superficial ornamentation, as observed, for example, in Poecilosclerida and Tetractinomorpha. The details of the spicule

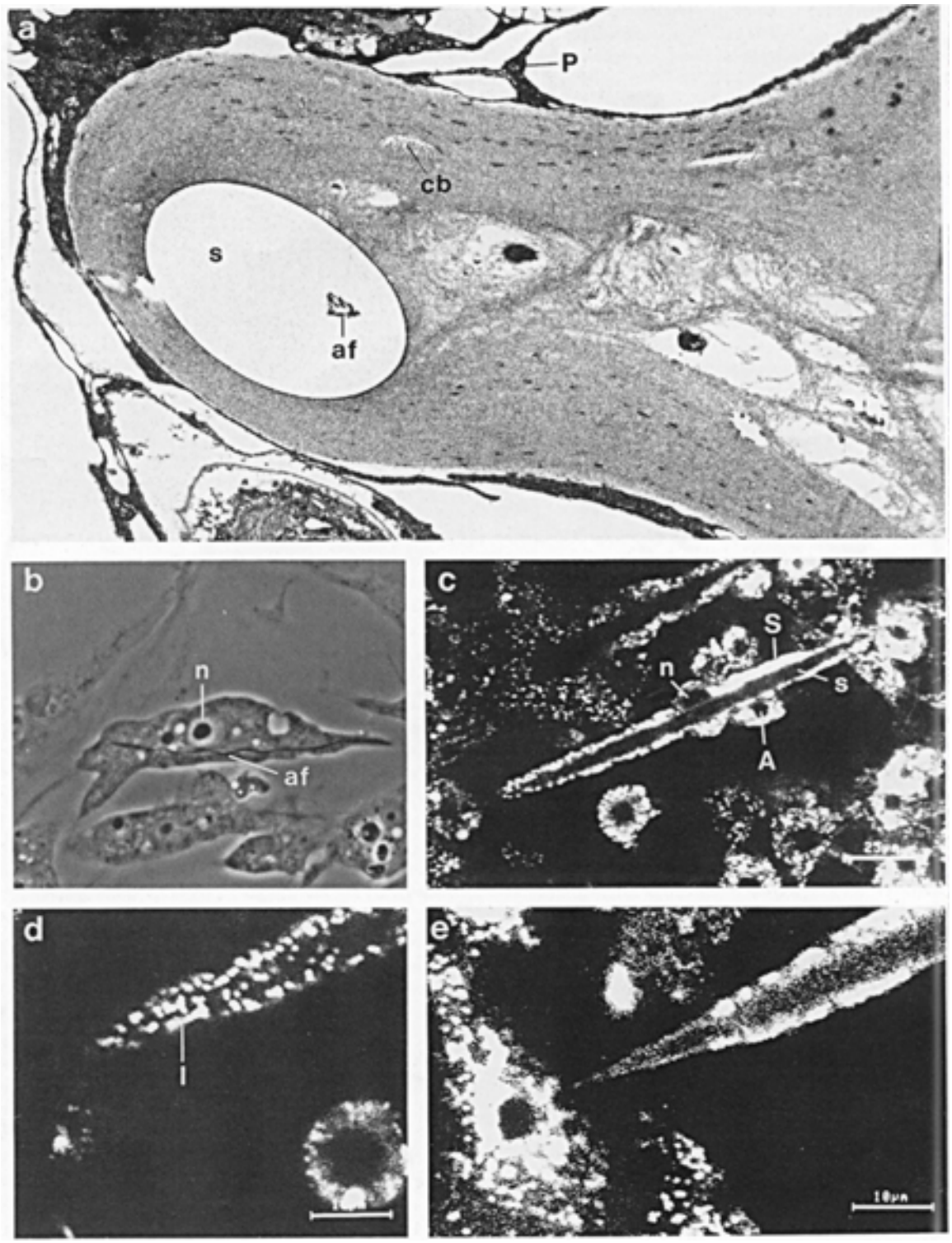

FIG. 3. Spicules and sclerocytes in vivo. a, Ultrathin cross section through a megasclere of *S. lacustris,* treated with aqueous hydrofluoric acid (transmission electron micrograph); *b,* semithin section through a young sclerocyte; c – *e,* sclerocyte of *E. muelleri* stained with acridine orange and analyzed by confocal laser scanning microscopy. Same aspect was cut into horizontal sections. sagittal through the middle of the spicule at lower (c) and higher magnification (e) and cutting the surface of the spicule, showing the discretely arranged lysosomes (d). *A:* archeocyte; *S:* sclerocyte; *P:* pinacocyte; *S:* spicule; *af:* axial filament; *cb:* collagen bundle; *n:* nucleus; *a:* X 11 660; *b:* X 1200; c: X 500; *d* and *e:* X 1200.

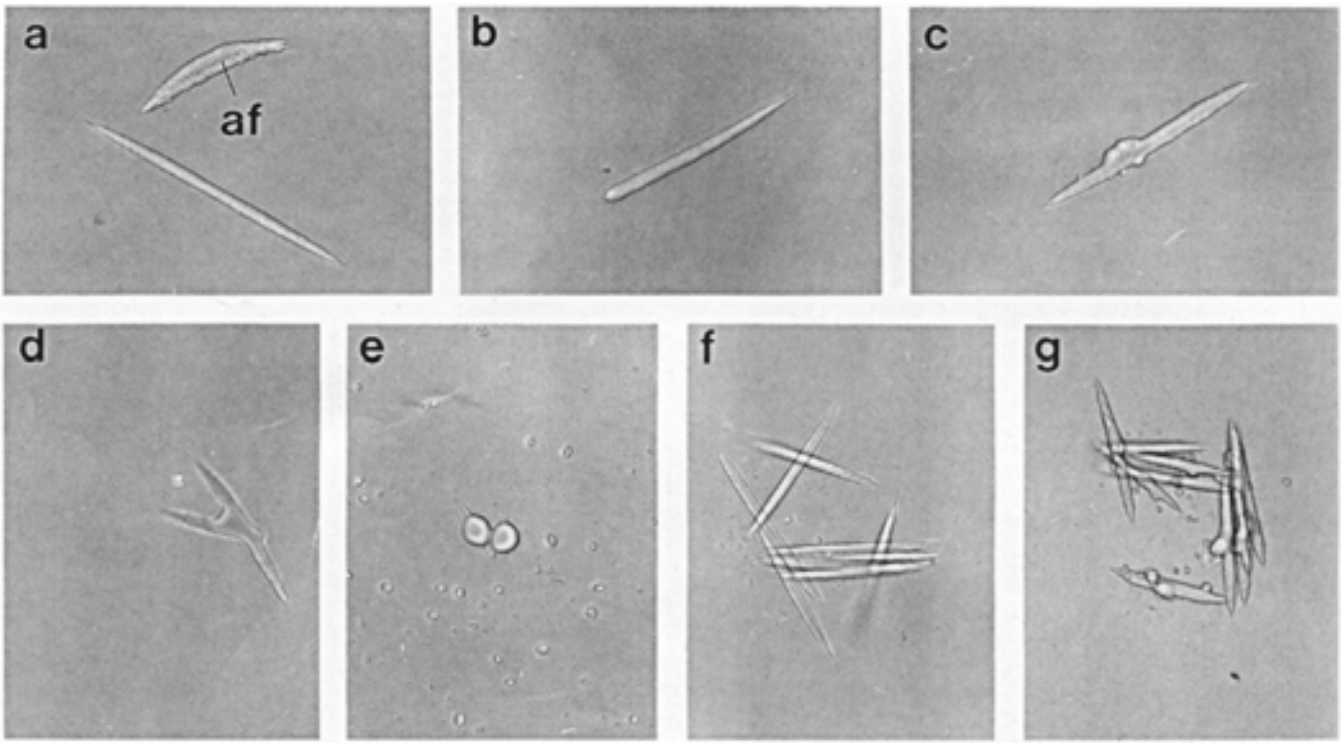

FIG. 4. Shape of the spicules (megascleres) formed by sclerocytes in vitro. a, Regular amphioxea together with a shorter and compact one. Abnormal spicules are shown: *b*, style-type; c and *g*, centrostyl-type; *d*, triactine-type; *e*, ball-like; *f*, and g, groups of 8 to 10 uniform spicules, probably produced by one sclerocyte each during the 2-mo. culture. *a – e:* X 245; *f:* X 180; g: X 147.

shape and size are genetically determined, and the spicule form is in general used to distinguish sponge species and to establish their classification both at the specific and supraspecific level. If the information concerning the spicule shape and size is inherent to a single cell, it should be controlled by an internal program executed by the cell during the formation of the organic axis and subsequent deposition of silicon. In higher animals, morphogenetic programs are frequently controlled by homeotic genes. Similar genes have recently been described both in freshwater and marine sponges (Coutinho et al., 1994; Müller et al., 1994). Similar to many Demospongiae, the adult Spongillidae have no defined antero-posterior nor dorso-ventral axis, neither a defined symmetry nor metamerization, and it is tempting to hypothesize that the homeotic genes in sponges may be involved in controls of their spicules shape. The in vitro culture of sclerocytes could make the spiculogenesis amenable to an experimental approach.

On the other hand, the observation that the spiculogenesis is modified during a prolonged culture, without a decrease in the deposition of silicon, points toward complementary requirements for the full process of the skeleton formation. The culture conditions may be suboptimal, with a progressive depletion of the factors required for complete organic axis formation of the newly formed spicules. The fact that a normal spiculogenesis was previously observed in the presence of a protein synthesis inhibitor (Rozenfeld, 1980) does not support this hypothesis. Alternatively, other cells, the quantity of which decreases along the culture, may be required for promotion of cell-to-cell or cell-to-substrate adhesion, which is potentially necessary for extension of the central axis of the spicule. In anchorage-dependent cells, as is the case of sclerocytes, the adhesion to the substrate may be necessary for a proper organization of polymerizing molecules, along the tension lines or along the microtubule-centered axis. These questions are now open for experimental analysis. It would be interesting to see the influence of increased adhesion-modulating substances, e.g., retinoic acid or fibronectin-fragments, on the spicule form and size, as well as of the agents that modulate the microtubule polymerization, e.g., vinca alkaloids or colchicine.

From the reported findings we suppose that the formation of spicules is independent of the presence of a) the three-dimensional extracellular matrix to which the sclerocytes must bind and h) soluble morphogens. Recently we provided first evidence that soluble morphogens, known from studies with higher invertebrates and vertebrates, also regulate morphogenesis in freshwater sponges (Imsiecke et al., 1994). However, the effect of such morphogens on spiculogenesis and on the integration of spicules into a complete sponge skeleton remains to be established.

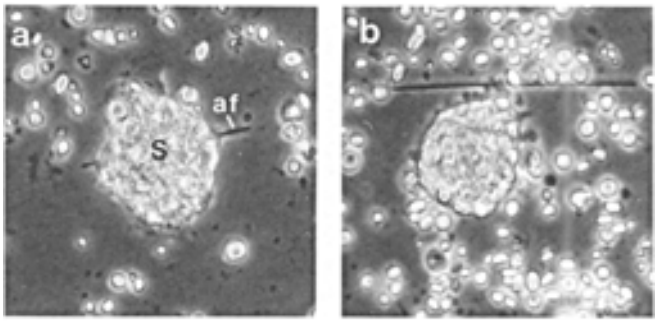

FIG. 5. Sclerocytes grown in medium lacking silicon. Axial filament (af) during (a) and after (b) release from the sclerocyte (S). Phase contrast microscopy. X 450.

Previous studies suggested that sclerocytes undergo death after spicule formation (Minchin, 1909; Yourassowsky and Rasmont, 1983). The in vitro studies reported here clearly show that these cells have the potency to produce more than one spicule.

Cultivation studies with low silicon concentrations in the medium showed that the axial filament, which is of organic, noncollagenous nature (Shore, 1972), is formed first and that the inorganic silicon is subsequently deposited around it. This result supports earlier findings (Weissenfels and Landschoff, 1977). The observation that in the absence of silicon in the medium formation of axial filaments is not present but the apposition of silicon is abolished suggests that the processes of formation of axial filaments and silicon deposition are under independent controls as suggested earlier (Yourassowsky and Rasmont, 1983). The malformations of spicules can be related to interference of the spiculogenesis at both levels and can occur also in vivo in freshwater sponges (Simon, 1953; Poirrier et al., 1989). Experiments showed that the formation of irregular spicules can be due to nonphysiologic silicon concentrations or competing ions or both, e.g., germanium ions (Simpson, 1981) as well as the metal chelator 2,2'-bipyridine (Holvoet and van de Vyver, 1985b). Alternatively, as indicated in earlier reports (Garrone, 1969) and in our short-term cultures, the production of the malformed spicules can be attributed to an irregular synthesis of the axial filaments.

In conclusion, the present study shows that short-term cultures obtained from gemmules of the freshwater sponge *E. muelleri* can be established and used for studies to answer physiologic questions, e.g., spicule formation in sclerocytes. Here we show that sclerocytes produce in vitro spicules in the same sequential manner as described for the in vivo situation. In future studies efforts must be undertaken to identify those growth factors required for a permanent culture of sponge cells in a similar manner as performed for intact organisms (Rasmont, 1961; Belas et al., 1992).

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