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Progress towards cell cultures from a marine sponge that produces bioactive compounds

Micha Ilan,[1] Hava Contini,[1,2] Shmuel Carmeli,[3] and Baruch Rinkevich[2]

[1]Department of Zoology, Tel-Aviv University, Tel-Aviv 69978, Israel; [2]National Institute of Oceanography, P.O. Box 8030, Haifa 31080, Israel; and [3]School of Chemistry, Tel-Aviv University, Tel-Aviv 69978, Israel

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Abstract. The feasibility of initiating cell cultures from the tropical sponge, *Latrunculia magnifica*, was evaluated by testing sponge collection procedures, four different dissociation approaches, the procedures for contamination control, and the quality of two commercial media. This sponge is of pharmaceutical interest because of the production of very potent bioactive metabolites. We found that holding the sponge fragments at 0–4°C (up to four days) before tissue dissociation improved the quality of primary cultures. Using the mechanical dissociation of sponge fragments from which the cortex layer was removed was found to be superior to all other procedures tested. Diluted media significantly reduced bacterial contamination. Primary cultures were kept for more than six months. During this period all cultures were taken over by thraustochytrid cells, a group of eukaryotic heterotrophic protists, very common in the marine environment. Although we do not yet have established cell cultures from *L. magnifica*, the above results clearly indicate that this approach is feasible, after solving the difficulties that are common to all other marine invertebrate cell cultures.

One of the main reasons for investigating sponge cells (in addition to their use as model systems in cell biology) is that a broad spectrum of natural bioactive metabolites with high pharmacological potential have been extracted from marine species (Jacobs et al. 1985; Faulkner 1991). Sponges provide a rich source of novel bioactive compounds, more than any other invertebrate phylum (Ireland et al. 1988). An example of such a promising compound is latrunculin A, a 2-thiazolidinone macrolide (Kashman et al. 1980), isolated from the sponge *Latrunculia magnifica* Keller 1889.

L. magnifica is a bright-red branching species found in shallow-water coral reefs from the Gulf of Aden, Indian Ocean to Eilat, northern Red Sea (Ilan 1995). Its major bioactive compounds, latrunculins, were found to be potent inhibitors of microfilament-mediated processes, such as cell division and fertilization in a variety of animals, and highly cytotoxic agents with promising antitumor activities (Schatten et al. 1986; Spector et al. 1989; Longley et al. 1993). However, the supply of large enough quantities of latrunculins for research is hampered due to a combination of three major obstacles: (1) The sponge is relatively uncommon. It is often found in protected areas (mostly nature reserves), whereas the collection of sufficient amounts from other localities is not feasible (personal observations). (2) The yield of latrunculins is very low, up to 0.35% of sponge dry weight (Groweiss et al. 1983). (3) Total synthesis of latrunculins in the laboratory has been achieved with poor yields of only 1–2% (Smith et al. 1992; White and Kawasaki 1992).

One approach to improve the production of natural products directly from sponge tissues or from chemical synthesis is the establishment of sponge cell cultures (Pomponi and Willoughby 1994). However, continuous cell lines from marine sponges are not available, and the methodology for the establishment of such cell lines is in the preliminary stages. Recent studies (Pomponi and Willoughby 1994; Rinkevich et al. 1994) have revealed and discussed several major difficulties that hampered the development of tissue cultures from marine invertebrates, including contamination and lack of appropriate media and conditions. Studies from the beginning of this century (reviewed by Gomot 1971) revealed that the development of specific techniques for sponge cell culture is a long and tedious process, although their significance for commercial production of natural bioactive compounds is beyond dispute.

Here we present several fundamental steps for the initiation of cell cultures from the tropical sponge *L. magnifica* and discuss some of the difficulties involved.

Correspondence to: M. Ilan. Fax: 972 3 640 9403.

Materials and methods

Sponge collection

Samples from *L. magnifica* colonies were collected by SCUBA diving in the vicinity of the Interuniversity Institute, Marine Biological Laboratory, Eilat, Red Sea (5–14 m depth). Each sponge fragment (2–14 cm^3) was carefully detached from a healthy specimen by a surgical blade and transferred to a 50-ml plastic vial containing sterile seawater (SSW; 0.22 μm filtered) supplemented with 1% v/v gentamicin sulfate from a stock solution of 0.5 mg/ml. Fragments were shipped to the laboratory at Haifa within 24 h of collection. Special attention was given during collection and handling to avoid air exposure of sponge fragments.

Tissue dissociation protocols

We used four different dissociation protocols: mechanical, chemical, enzymatic, and spontaneous dissociations, as described for marine invertebrates (Frank et al. 1994; Rinkevich and Rabinowitz 1994). Published protocols were modified according to the nature of the sponge material. Before dissociation, sponge tissue was cut into small sections with a surgical blade and incubated in fresh sterile seawater (SSW) with 1% v/v gentamicin for 1 h. For mechanical dissociation, each individual small sponge fragment was put into a 1.5-ml Eppendorf vial in SSW with gentamicin and carefully minced by a homogenizer rod against the vial walls. Cell suspensions were collected using Pasteur pipets and filtered (30 μm) into 15-ml tubes. For chemical dissociation, sponge fragments were soaked in 5 ml calcium- and magnesium-free seawater (CMF-SW) (Müller et al. 1978) for 1 h. Fragments were then gently filtered through a fine mesh (30 μm). Enzymatic dissociation was achieved with 0.05% collagenase in SSW for 3 h, in 6-cm Petri dishes with magnetic stirring (60 rpm). In the above three protocols, cell suspensions were rinsed three to six times and resuspended in 1–2 ml SSW with gentamicin (300–500 g, 10 min, 4°C). Centrifugation was completed when most cell debris was removed. Cells were then diluted to 10[illegible]–10[illegible] cells/ml, and incubated in 24-well culture plates (0.5 ml medium). Spontaneous dissociation (sensu Frank et al. 1994) was performed on small fragments that were transferred to flat-bottom tissue culture plates (0.5 ml medium or SSW, 24-well plates). Sponge fragments were left for three days, during which many cells migrated from the sponge tissue into the medium. Thereafter, the fragments were discarded and the medium was replaced. Cell viability was evaluated by the trypan blue protocol, done on samples from each dissociation (0.1% in SSW, 1 min).

Culture conditions and media

Experiments were performed under aseptic conditions, carried out in a sterile laminar-flow hood. All liquids were sterilized by filtration (0.2 μm), glassware was autoclaved, and only sterilized plasticware was used. Two basic commercial media were used as nutrient sources: (1) Leibowitz L15 (Biological Industries, Kibbutz Bet HaEmek, Israel) supplemented, as described by Frank et al. (1994), and (2) Landureau (Landureau and Grellet 1972). We used two concentrations of each medium. A final "concentrated" medium consisted of 90% of either basic medium and 10% of heat-inactivated fetal calf serum (HIFCS). A final "diluted" medium consisted of 5–10% of either basic medium, 5–10% HIFCS, and adjusted to 100% with SSW. Antibiotic stock solutions (0.5 mg/ml gentamicin sulfate, 10[illegible] IU sodium penicillin, 1 μg/ml streptomycin, 2.5 μg/ml amphotericin, 5 μg/ml nystatin; final concentration in the wells 1% v/v except for gentamicin, 0.1%) were added routinely as a mixture or individually, depending on the level of contaminations. Chemicals were purchased from Sigma (St. Louis, MO, USA), and plasticware from Nunc and Corning. Cells were incubated in a partly humidified incubator (Leek, UK; 23°C) under normal atmosphere and observed through Nikon X, an inverted phase-contrast microscope.

Results and discussion

Shipment of sponge fragments

Improved conditions for the shipment of living material is one of the requirements for the successful establishment of cell lines from marine invertebrates (Rinkevich et al. 1994). In the case of sponges, where all internal and external surface areas of the animals may be colonized by a variety of micro- and macroorganisms, this point should be taken into further consideration. Proliferation of these symbionts/epibionts may develop stress conditions in cell cultures. *L. magnifica* is a tropical sponge species, typically inhabiting warm water, more than 20°C. Shipment from Eilat to Haifa (about 400 km distance) was done in two ways: (1) at ambient temperature (20°C), and (2) at 4°C. All sponge fragments shipped at 20°C were characterized by low cell viability and high rates of contamination (Contini 1995). In contrast, shipping at 4°C (24 h) and holding the sponge fragments in the refrigerator until use (up to an additional three days) did not affect cell viability and, with proper treatment, resulted in a low rate of contamination.

Conditions for cell maintenance

The control of microbial contamination is one of the major problems in establishing cell lines from marine invertebrates. Contamination results either from an imperfect disinfection of the animal surface area or from the appearance of intracellular and intercellular microorganisms and cryptoorganisms (Le Douarin 1971; Vaughn 1971; Rinkevich et al. 1994; and literature therein).

The two media tested in the present study (Leibowitz L15 and Landureau) revealed similar results for the maintenance of sponge cells, and neither was found to be superior (Contini 1995). A study was performed to evaluate the effects of concentrated versus diluted media (Table 1). For that purpose we used Leibowitz L15 with sponge cells dissociated mechanically (five experiments). The results clearly indicate that enriching the environment is not favorable for the sponge cells in vitro. While about 1/3 of the wells were free of contaminants after one week in the diluted medium, all the wells were contaminated within four days in the concentrated medium ($P < 0.01$, testing of quality of two percentages, Table 1). Even when using high antibiotic concentrations and multiple rinsing of sponge fragments, enriched medium should not be employed for *L. magnifica* primary cultures, before establishing axenic conditions. This conclusion is in accordance with past results on initiating cell lines from marine cnidarians (Frank et al. 1994).

Sponges are probably an extreme example of "infested" organisms because, unlike most other invertebrates, there are no sterile areas in a sponge (Pomponi and Willoughby 1994). The upper surface area of the sponge (the cortex) is particularly exposed to contamination. In *L. magnifica*, this area is about 1 mm thick and is covered externally by exopinacocytes, which have the appearance of a squamous epithelium. Only few cells are found within the matrix of the cortex. This layer partly isolates the sponge interior area (the mesohyl) from the environment (Bergquist 1978) and may be easily peeled off by the use of thin forceps (Contini 1995).

Four different dissociation protocols were employed on sponge samples with and without the cortex layer in diluted media (Fig. 1a,b). Primary cultures from sponge samples with the cortex (12, 6, 6, and 6 wells of mechanical, chemical, enzymatic, and spontaneous dissociation, respectively) were maintained from one day (chemical and spontaneous dissociation) to five days (mechanical dissociation; Fig. 1a.) Afterwards, cultures were stopped by the development of micro-

Table 1. Rate of bacterial contamination in short-term primary sponge cultures grown in concentrated vs. diluted Leibowitz L15 supplemented medium.

Type of medium	No. of wells	Percentages of uncontaminated wells after (days) 1	2	3	4	5	6	7
Concentrated	60	83.3	33.3	16.7	0			
Diluted	126	95.2	87.3	79.4	55.6	47.6	31.7	29.4

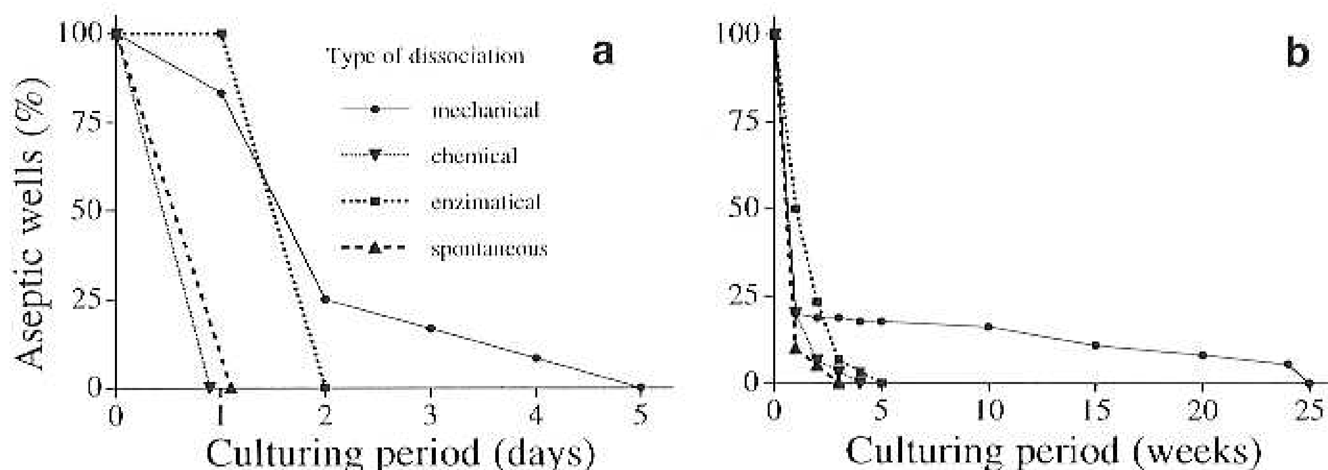

Fig. 1. Rates of contamination in *L. magnifica* primary cultures after different dissociation protocols of sponge fragments: (a) containing their cortex layer, (b) without the cortex layer.

bial contamination. Contamination was lower in samples taken from sponges without the cortex (186, 30, 30, and 20 wells of mechanical, chemical, enzymatic, and spontaneous dissociation, respectively; Fig. 1b). Mechanical dissociation proved to be more appropriate than other dissociation methods in terms of contamination control, as more than 16% of the wells remained aseptic 10 weeks after the initiation of the culture. In this set of experiments, sponge cells survived in 5.4% of the wells up to 24 weeks after dissociation.

Development of primary cultures

High plasticity of shapes and structures of invertebrate cells resulted in difficulties of cell classification, especially in in vitro conditions (Gomot 1971; Rinkevich et al. 1994). The morphology of cells in culture conditions may also differ from those in vivo or as observed in histological section (Rinkevich et al. 1994). Concerning sponge cells, there is also much confusion for in vitro identification of cells. This could only be cleared up through ultrastructural and histochemical studies (Bergquist 1978). In the Demospongiae, more than 20 basic cell types were characterized in vivo (Bergquist 1978; Simpson 1984). It was difficult to identify these basic cell types in our primary cultures, since no histochemical and ultrastructural studies were performed. However, even simple observation of cells under the microscope revealed that the use of four different dissociation protocols resulted in differences in the number of sponge cell types within primary cultures (Table 2). Cells obtained by the different dissociation protocols were up to 12 μm in size. Cells differed in size and internal inclusions. Qualitatively, we can summarize these observations as follows: number of cell types that appear in spontaneous dissociation > mechanical dissociation > chemical dissociation > enzymatic dissociation (Table 2).

We followed >40 wells for more than six months after dissociation. Most of the cell types observed in the wells were in a resting stage, since cell divisions were observed only occasionally. From two weeks after dissociation, we observed the appearance of several types of cells (between 3 and 30 μm) which slightly dominated the cultures. Histochemical and electron microscope studies (Contini 1995) (Figs. 2 and 3) indicated that these cells belong to the Thraustochytriidae, a group of marine eukaryotic microorganisms of the phylum Labirinthulomycota (Porter 1990). These common heterotrophic protists (Raghukumar and Schauman 1993) have multilamellate (Chamberlain 1980) or thin cell walls (Fig. 2a,b) from which ectoplasmic nets (Fig. 3a,b) or organic matrices (Fig. 2b) are extended or secreted. These organisms were found to develop in most of the marine invertebrate cell cultures such as bivalves (Ellis and Bishop 1989), corals (Frank et al. 1994) and tunicates (personal observation), in addition to their appearance in living sponges (Höhnk and Ulken 1979). All cultures containing thraustochytrid cells that proliferated were discarded within weeks, since sponge cells were significantly reduced in number. The attendant difficulty in obtaining sponge cells free

Table 2. Qualitative summary for relative applicability of each of four different dissociation protocols in *L. magnifica* cell cultures.

Dissociation approach	Relative applicability for[a]			Remarks
	No. of cell types	Aseptic conditions	Cell survivorship	
Mechanical	−++	++	−+++	The best protocol for sponge cell culture (this study)
Chemical	+−	++	+	
Enzymatic	+	+−++	+	Commonly used for many invertebrate cell cultures [refs. in Frank et al. (1994) and Rinkevich et al. (1994)]
Spontaneous	++−+	+	+	Highly recommended for cnidarian (Frank et al. 1994) and tunicates (Raftos et al. 1990) cell cultures.

[a] + to ++++ is from the less successful result to the best outcome.

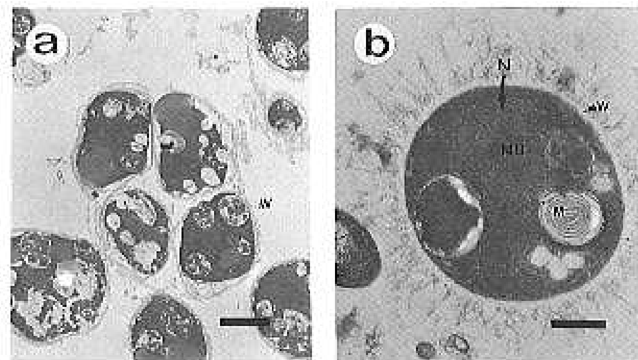

Fig. 2. EM sections of thraustochytrids from *L. magnifica* primary cultures. (**a**) Several protist cells, one sporangium with four daughter cells covered by common multilamellate cell wall (w). Scale bar = 1 μm. (**b**) A protist cell secreting organic matrix through a fibrillar, nonlamellate type of cell wall, characterized by poor staining by osmium tetroxide and lead citrate (see also Chamberlain 1980). M = possibly reserve material. N = cell nucleus. NU = nucleolus. W = cell wall. Scale bar = 0.5 μm.

of contaminants may be further demonstrated in the attempt to establish long-term cell cultures from several sponge species (Klautau et al. 1993, 1994). Cells that were isolated and maintained in culture for long periods of time were identified as the protozoan *Neoparamoeba aestuarina* (Custodio et al. 1996, and unpublished data).

Several important points for the initiation of cell cultures from *L. magnifica* were elucidated here, further emphasizing the major obstacles for developing cell cultures from sponges (see also Pomponi and Willoughby 1994). The mechanical dissociation using the methodology presented in this communication yielded the best results (Table 2). Alternatively, because it provides the highest diversity of cell types, spontaneous dissociation should also be used as a promising protocol for cell culture, as microbial contamination will be controlled (Table 2). The above results suggest that sponge cell culture is a feasible approach for future mass generation of cells, which may produce large quantities of bioactive metabolites and may become an alternative source for tissue extraction of these pharmaceutically interesting compounds. The results of the present study provide five main recommendations for the initiation of cell cultures from marine sponges: (1) to hold the sponge fragments at 0–4°C until dissociation (up to four days after collection), (2) to dissociate fragments from which the cortex has been removed, (3) to employ the mechanical dissociation protocol, (4) to use diluted media only, and (5) to pay special attention to the possible appearance of thraustochytrid and other symbiont cells in cultures.

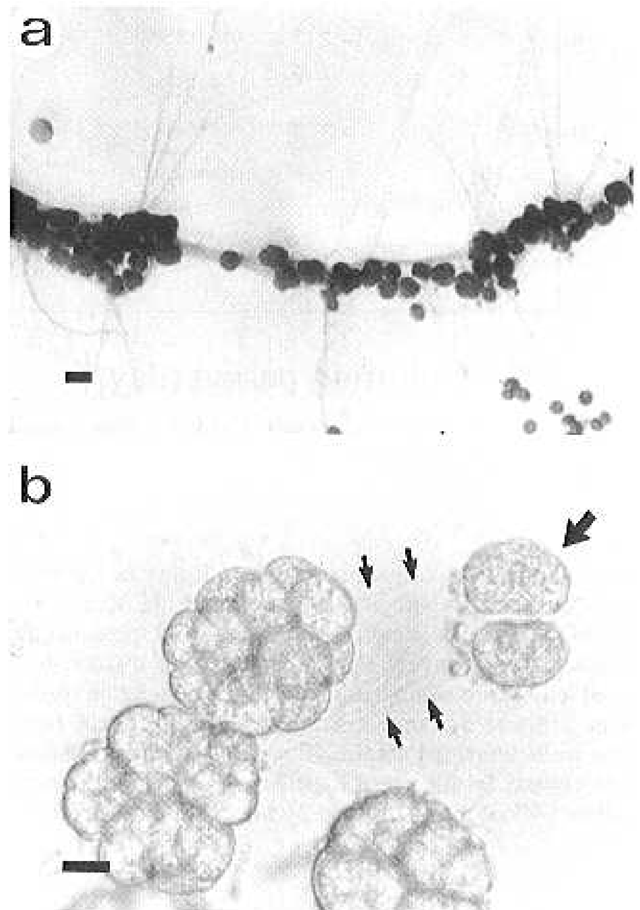

Fig. 3. Histochemical evaluation of thraustochytrids from the sponge primary cultures. (**a**) Ectoplasmic networks connecting between protist cells in an early stage are also extended outside on the substrate (a toluidine blue 0.01% staining). Scale bar = 10 μm. (**b**) Three developed sporangia and one still in the primary state of binary fission (thick arrow). Note the ectoplasmic matrix extended from the developed sporangium (small arrows). Scale bar = 10 μm.

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