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Letter to the Editor

INITIATION OF EPITHELIAL CELL CULTURES FROM PALLEAL BUDS OF *BOTRYLLUS SCHLOSSERI*, A COLONIAL TUNICATE

Dear Editor:

While expertise in invertebrate tissue culture is still limited largely to terrestrial arthropods, the recent extensive studies on marine invertebrate immunology and developmental and cellular biology have initiated parallel efforts to develop novel tissue culture techniques in a variety of marine taxa. This is also true for tunicates (Urochordata), one of the most highly evolved invertebrate taxa, in which recent studies have shown that different types of cells are amenable to culture (5–7). As such, proliferative cultures were initiated from pharyngeal explants (5), blood cells (6), and blastomers (7). Here, we present the initiation of epithelial cell cultures from isolated palleal buds of the colonial tunicate, *Botryllus schlosseri*.

B. schlosseri is a cosmopolitan shallow water styelid ascidian (subfamily Botryllinae), a common inhabitant of hard-bottom communities. A colony, composed of up to thousands of modular units called zooids, develops from the metamorphosed larva. The zooids are embedded within a common organic matrix (the tunic) and ordered in star-shaped structures (the systems). The tunic is traversed by a ramified vascular network connecting the zooids to each other, and to a marginal vessel from which a crown of peripheral blind projections (ampullae) are expanded. This species is characterized by a complex synchronized developmental cycle (called blastogenesis), in which at 18–20° C all functional zooids degenerate once a week and a new generation of developing buds "take over" the colony (3). This blastogenic cycle has been arbitrarily divided into four (termed as A–D) developmental stages (4), where stage D is represented by the degeneration phase of the old generation of zooids, and the maturation of the developing buds into the new generation of functional zooids. As a matter of fact, there are three sequential generations of zooids that are simultaneously presented: functional adult zooids and two generations (primary and secondary) bilateral buds, which are attached to the parental zooids by vascularized epidermal stalks. Zooids of the same generation are of the same developmental stage. Budding is palleal. A secondary bud is developed from the atrial epithelium of the primary bud and is overlaid by the epidermis (1).

The present communication describes short-term proliferating epithelial cell cultures initiated from isolated primary palleal buds of 6-wk-old *B. schlosseri* colonies. We used laboratory bred colonies, attached to 5 × 7.5 cm glass slides at the metamorphosing larval stage. Colonies were reared in a culture room (20° C, 14/10 light/dark regimen) within 17-l glass tanks supplied with standing sea water, preconditioned in 300 l plastic holding tanks. Detailed mariculture procedures and preparations of the animals for collection of tissues are described elsewhere (6,7).

Developing buds at blastogenic cycles A to C (sensu ref. 4) were dissected from well-developed *Botryllus* colonies. Sampling of buds from the selected zooids was made under the dissecting microscope using a sterile syringe equipped with a 28 G size needle. Using the needle tip, primary buds with attached secondary buds were carefully isolated from the adult zooids. They were collected by sterile Pasteur pipettes and transferred to 15 ml plastic tubes containing sterile seawater (SSW). The isolated buds were rinsed up to 10 times with SSW and transferred to 24-well plates (Nunc, Denmark), several buds from the same colony/well. For the first 48 h, the buds were incubated in incubation medium at pH 7.5, containing per 100 ml: 45.5 mg RPMI-1640, 5% dialyzed fetal calf serum (FCS), 2 ml HEPES in artificial sea water (made of two stock solutions: A = 13.67 g NaCl, 0.412 g KCl, 0.721 g $CaCl_2 \cdot 2H_2O$ in 125 ml double distilled water (DDW); B = 5.57 g $MgSO_4 \cdot 7H_2O$, 3.05 $MgCl_2 \cdot 6H_2O$ in 125 ml DDW. The stock solutions were diluted 1:2 in DDW before use and supplemented with a "cocktail" of antibodies (50 mg/ml streptomycin sulphate, 5.10^4 IU/ml sodium penicillin salt, and 6250 IU/ml nystatin; 1 ml of antibiotic cocktail per 100 ml incubation medium). Collagenase (final concentration 0.01%) was then added for partial chemical dissociation of the buds. Following the first 48 h, the buds and medium were transferred to 2 ml sterile Eppendorf vials and centrifuged (1700 RPM, 5 min) in a bench top centrifuge. The buds were then further dissociated mechanically by hand, using a fitted homogenizer. The resultant cultures contained single cells, cell clumps, and partly dissociated bud fragments, 50–300 μm in size.

Samples of dissociated tissues were transferred to 24-well plates within 0.5 ml incubation medium per well, three to four buds/well; 0.2 ml of the medium was replaced with fresh medium every other day. We analyzed six different treatments (Table 1) in which the incubation medium was supplemented by: 1. Fibroblast Growth Factor (FGF) (1 ng/ml); 2. heat inactivated (HI) crab (*Portunus pelagicus*) hemolymph (5%); 3. additional 5% (total 10%) HI dialyzed FCS; 4. fibronectin from bovine plasma (60 μg/ml); 5. Lipopolysaccharides (LPS) (10 mg/ml); 6. HI sea cucumber (*Holothuria forskali*) hemolymph (5%). Two experiments were run. In each, four wells/treatment were established; two wells with buds at stage B and two wells with buds at stage C. In preliminary experiments, we found that stage A buds did not develop epithelial cell cultures (data not shown).

The main results are summarized in Table 1. Out of the six different treatments, only cultures supplemented with FGF and with crab hemolymph resulted in cellular proliferations, characterized by the formation of flattened epithelial cell monolayers, which lasted up to 1 mo. after bud samplings (Fig. 1 *a–c*). No differences were observed between buds of blastogenic stage B as compared to stage C. Monolayers of epithelial cells were initiated from partly dissociated buds, not from single cells or cell clumps, and only from fragments that attached to the substrate (Fig. 1 *a* and *b*). Attachment of tissue fragments to the plastic substrate (in FGF treatment) or to the overlayed organic substrate (in crab hemolymph treatment) was recorded within a few days. Within 1 wk after attachment, some of the attached fragments developed large (up to 0.4 mm) hollowed spheres. Only

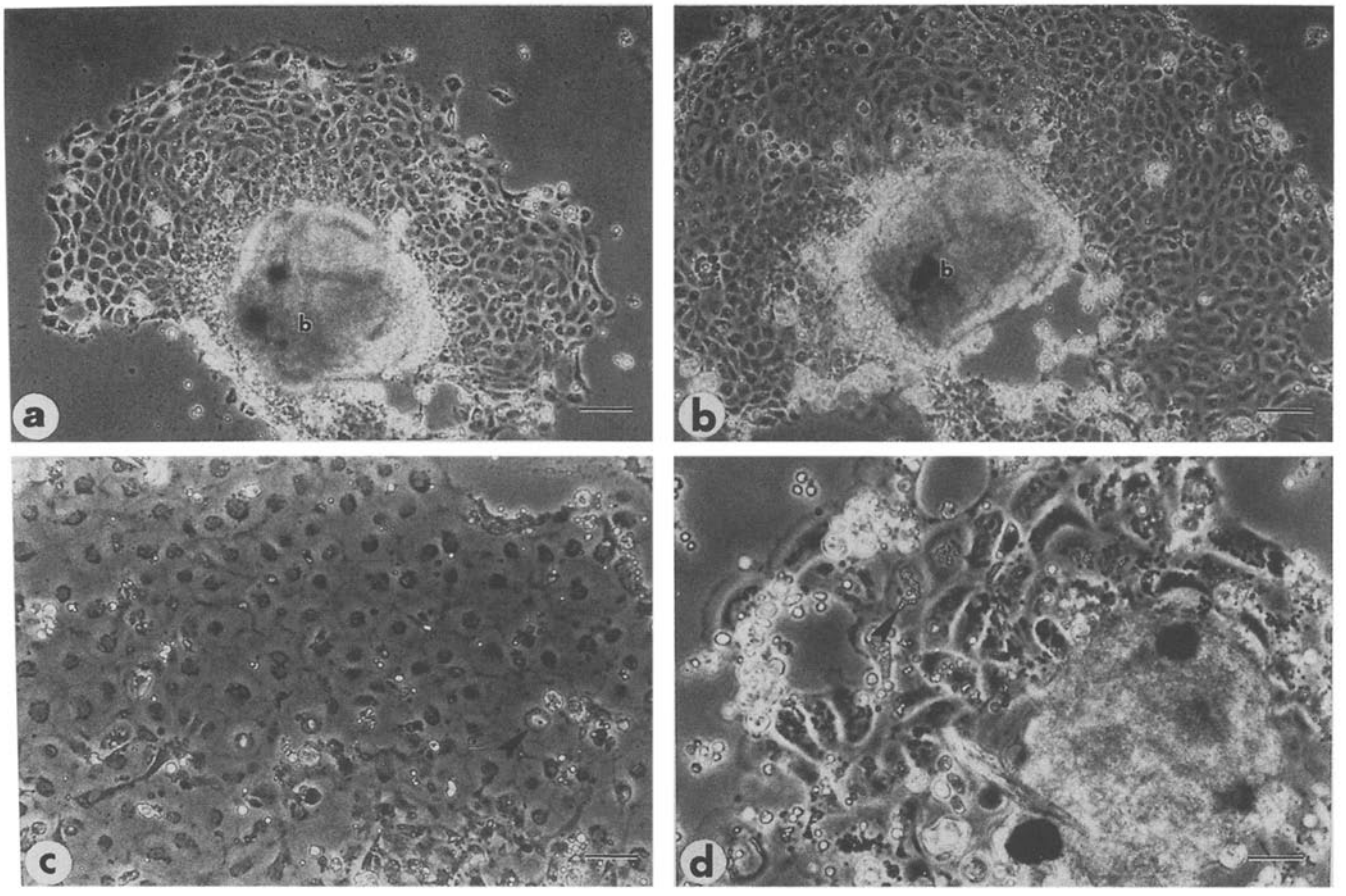

FIG. 1. The initiation of epithelial cell cultures from *Botryllus schlosseri* palleal buds. (*a*) A 2-wk-old epithelial cell culture around an unattached, partly dissociated bud (*b*) at blastogenic stage B. A day before, only 110 cells were counted in the adhered monolayer of epithelial cells. (*b*) One day thereafter, same magnification as in Fig. 1 *a*. (*c*) A 3 wk well-developed monolayer of epithelial cells. First sign of degeneration is characterized by the appearance of small vacuoles around the cell nucleus (*arrowhead*). (*d*) Epithelial monolayer in advanced degenerative stage. Accumulation of small vacuoles in all parts of the cell (*arrowhead*). Degenerated cells rapidly detach from substrate. *Scale bars* = 50 μm.

TABLE 1

INITIATION OF EPITHELIAL CELL CULTURES FROM *BOTRYLLUS SCHLOSSERI* PALLEAL BUDS (SUMMARY OF RESULTS AFTER 3 WK)

Medium Supplemented by[a]	Summary of Main Results	Epithelial Monolayer Formation[b]
FGF	Within 1 wk cells and tissue fragments were attached to the substrate. After 2 wk, part of the attached fragments developed large spheres (50% of the wells) followed by the formation of a fast-growing monolayer. After 3 wk, monolayers appeared in all wells. Earlier-formed monolayers started to degenerate.	+
Crab hemolymph	The crab hemolymph produced an overlayed organic matter on the plastic substrate, with enhanced cells and tissue attachments. Main results as in the FGF treatment.	+
FCS	No cell or tissue fragments attachment to the substrate. No initiation of any cell proliferation. Cells in bad condition after 3 wk.	−
Fibronectin	Cells and tissue fragments attached to the substrates within the first week. Cultures started to degenerate. No cellular proliferation. Cultures started to degenerate after 2 wk.	−
LPS	After 10 d, part of the tissue fragments were attached to the substrate. After 2 wk, one case of large sphere development was followed by initiation of an epithelial cell monolayer. After 3 wk most of the cells degenerated. The single sphere detached from the substrate.	+ −
Sea cucumber hemolymph	Only a single case of tissue fragment that attached to the substrates. More attached in the following 3 d. After 2 wk, only one tissue fragment had been attached, without sphere formation. Cultures degenerated after 3 wk.	−

[a] FGF = Fibroblast Growth Factor; FCS = fetal calf serum; LPS = Lipopolysaccharides.

[b] + = Formation of epithelial monolayers; ± = development of a large sphere tissue, without the formation of proliferating epithelial monolayers; − = No cellular proliferation.

spheres adhered to the substrate have initiated monolayers of epithelial cells. These monolayers grew rapidly (Fig. 1 *a* and *b*), probably by cellular divisions and cell movement from the tissue fragments. Cells in the flattened epithelial monolayers were polygonial, and reached up to 50 μm in diameter, with 5 μm nuclei (Fig. 1 *c*). The fast growth rate ceased within the following week where cultures gradually degenerated. The first sign of degeneration was evident by the appearance of small endoplasmatic vacuoles, first concentrated around the cell nucleus (Fig. 1 *c*) and thereafter all along the cell cytoplasm (Fig. 1 *d*).

Degenerated cells were detached from the substrates. Work with some wells was discontinued due to the appearance of thraustochytrids, a group of eukaryotic achlorophyllus microorganisms, as was recorded in other invertebrate cell cultures (2). Colonies of thraustochytrids appeared in these cultures, also at the periphery of the epithelial monolayers, and were characterized by dense masses that produced ectoplasmic nets of filopodia around them. These microorganisms continued to grow causing epithelial cell detachment. Detached cells rapidly degenerated.

Out of the other four treatments, in two (the FCS- and the fibronectin-supplemented media) no cell proliferation or tissue fragment attachment to the substrate was recorded (Table 1). In the sea cucumber hemolymph treatment, only a single case of unattached sphere formation was recorded, a much poorer result as compared to the use of this component in culturing of blood cells from *Botryllus* (6). With LPS-supplemented medium, only one case of limited epithelial cell proliferation was recorded (Table 1).

For the first time, epithelial cell primary cultures derived from the palleal buds of the compound tunicate *Botryllus schlosseri* have been successfully established, and kept viable for up to 1 mo. after sampling. We demonstrated that under specific conditions epithelial cells not only were maintained viable *in vitro*, but also that fast-growing monolayers of these cells could be repeatedly obtained. We have not yet been successful in subculturing the epithelial cell monolayers during the phase of rapid growth (data not shown). However, improving the technology involved in the isolation of buds from zooid tissue, the partial dissociation of buds and especially the improvement of the medium may result in the establishment of long-term epithelial cell cultures available and suitable for different purposes, including the transfection of these cells with appropriate plasmid vector-carrying immortalizing genes.

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