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In vitro delayed senescence of extirpated buds from zooids of the colonial tunicate *Botryllus schlosseri*

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Summary

In the colonial growth of botryllid ascidians, blastogenesis (bud formation) is a cyclical and synchronized developmental process characterized by a weekly rhythm of budding and apoptotic events. Very little is known about this cycle regulation and its control. In this study, the *in vitro* fate of developing buds and regressing zooids extirpated from *Botryllus schlosseri* colonies at different blastogenic stages were examined, revealing that stages 'B' to 'D' buds (but not stage 'A' buds) developed new structures under *in vitro* conditions. These were mainly spheres (up to 1 mm in diameter) and epithelial monolayers around the attached buds. We also found that: (1) when attached spheres and epithelial monolayers appeared, the life expectancy of an isolated bud *in vitro* reached 50–60 days, five times the life expectancy of intact, *in vivo* developing zooids; (2) the life expectancy of *in vitro* buds that remained unattached to the substrates was at least 150 days; (3) after attaching to the substrates, buds obeyed a newly imposed developmental clock dictating up to 35 survival days for spheres and up to 14 days for epithelial monolayers; (4) the prevailing mode of death *in vitro* was necrotic, in contrast to the apoptotic mode of zooidal deterioration at the takeover phase of blastogenesis; (5) under *in vitro* conditions, degenerating zooids surprisingly produced epithelial monolayers within 3 weeks of culturing. Monolayers survived for up to 10 additional days, extending the lifespan of the degenerating zooids from a few hours to up to 1 month. We conclude that under *in vitro* conditions, not only are the underlying colonial growth mechanisms replaced by different developmental pathways, but also the internal colonial-level clocks programming death, are replaced by a new biological mechanism with different timetables.

Key words: apoptosis, ascidian, *Botryllus schlosseri*, blastogenesis, budding, epithelial monolayer, life extension, senescence.

Introduction

Blastogenesis, which in some colonial tunicate species is a cyclical and synchronized organogenesis process, is an indispensable developmental operation in modular organisms. This phenomenon is particularly important in the life history of the botryllid ascidians (Family Styelidae), most thoroughly studied in the cosmopolitan species *Botryllus schlosseri* (Berrill, 1941a,b, 1951; Oka and Watanabe, 1957; Watkins, 1958; Milkman, 1967; Izzard, 1973; Sabbadin et al., 1975; Burighel and Schiavinato, 1984; Lauzon et al., 1992., 1993, 1996, 2002; Voskoboynik et al., 2002; Cima et al., 2003; and literature therein). Each botryllid colony develops from a sexually produced tadpole larva that settles and undergoes metamorphosis into a sessile body (an oozooid). The settled oozooid begins a lifelong synchronized series of budding (blastogenic cycles, approximately 1 week each, under a 20°C regimen), leading to several or many, even thousands, of asexually derived modules (zooids) per colony. All zooids and buds are interconnected by a ramifying network of blood vessels embedded together within the matrix of a common translucent tunic (Milkman, 1967). Each blastogenic cycle starts as a set of evaginations and invaginations of the zooid lateral walls, forming double-walled vesicles that develop as 1–4 buds per zooid. The perivisceral epithelium of the zooids, the covering epidermis and blood cells, all participate in this developmental process (Berrill, 1941a). Each blastogenic cycle culminates in a massive somatic death (takeover). During the takeover process, all matured zooids synchronously die, primarily through apoptosis. They are cleared by circulating professional phagocytes and are replaced by developed buds that mature into a new generation of functional zooids (Mukai, 1974; Burighel and Schiavinato, 1984; Lauzon et al., 1992, 1996, 2002; Cima et al., 2003).

The life history of a *Botryllus schlosseri* colony is characterized, therefore, by multiple, blastogenic cycles, succeeding one another periodically and each terminating in the takeover process. Blastogenesis is intrinsically regulated and probably controlled by blood-borne elements (Oka and Watanabe, 1957; Lauzon et al., 1992). Thus, Watkins (1958)

demonstrated that surgical removal of all buds from a colony did not affect the lifespan of its functional zooids. Conversely, extirpation of all adult zooids in a colony (Zaniolo et al., 1976), although resulting in a considerably belated development of many buds, did not affect the lifespan and survival of its buds. Vascularization is an imperative condition for this type of bud survival. When both functional zooids and buds are removed simultaneously, vascular budding of replacement zooids may occur (Milkman and Therrien, 1965; Sabbadin et al., 1975). Bud primordials then arise from the peripheral blood vessels (the ampullae). This capacity is characteristic of many other polystyelid ascidians (Rinkevich et al., 1995) and in some other colonial sytelids (Watanabe and Newberry, 1976).

Studies on blastogenesis have been performed at morphological levels (Berrill, 1941a,b, 1951; Oka and Watanabe, 1957; Watkins, 1958; Milkman, 1967; Izzard, 1973; Sabbadin et al., 1975; Zaniolo et al., 1976; Burighel and Schiavinato, 1984), cytological ones (Lauzon et al., 1992, 1993, 2002), and molecular–biochemical ones (Chang and Lauzon, 1995; Lauzon et al., 1996; Voskoboynik et al., 2002; Cima et al., 2003). These studies have revealed that blastogenic duration is temperature-dependent and is shortened at elevated temperatures (Milkman, 1967; Sabbadin, 1969; Rinkevich and Shapira, 1998; Rinkevich et al., 1998). Particularly severe conditions may involve regression of adult zooids, irrespective of their blastogeneic stage (Sabbadin, 1958; Rinkevich et al., 1996). When two ramets of the same genet or two allogeneically compatible genets at different blastogenic phases are fused together, this difference is invariably equalized (Watanabe, 1953; Rinkevich and Weissman, 1987). Ionization radiation (Rinkevich and Weissman, 1990) or acute administration of the anti-oxidant butylated hydroxytoluene (Voskoboynik et al., 2002) may arrest blastogenesis, inducing a morphologically chaotic state and deterioration that eventually lead to colony death. Nevertheless, despite these several lines of intriguing observations, very little is known about the mechanisms governing regulation of blastogenesis and its control.

All the above studies were performed *in situ* or *in vivo*. In this study, we examined the *in vitro* fate of developing buds and regressing zooids isolated from *B. schlosseri* colonies at different blastogenic stages. Lifespan, apoptotic events, and cytological and morphological characteristics were compared with non-manipulated buds developing *in vivo*.

Materials and methods

Isolation of buds

We used large, laboratory-bred colonies of *Botryllus schlosseri* Pallas (each containing several scores of zooids) growing on glass slides (Rinkevich and Shapira, 1998). Before bud excision, the colonies were cleaned by soft, small brushes under a binocular stereomicroscope (Olympus Optical Co., Germany), briefly immersed in 70% ethanol and then incubated (2 h) in 0.2 μm-filtered seawater (FSW) supplemented with 50 μg ml^{-1} of Gentamycin (FSW-G, Biological Industries, Kibbutz Beit-HaEmek, Israel). Glass slides carrying *Botryllus* colonies were immersed in the FSW-G solution in glass dishes (15 cm diameter). During bud excision, slides were fitted into grooved Perspex platforms that were designed to immobilize them.

Primary buds at blastogenic stages 'A' to 'D' (*sensu* Mukai and Watanabe, 1976) were excised under the dissecting microscope by a sterile, pyrogen-free syringe, equipped with a 28-G needle. Using the needle tip, we carefully peeled off the uppermost layer of the tunic coating from around the buds without injuring the buds' epidermis. During this manipulation, most secondary buds were accidentally detached from the isolated primary buds. Buds from the same colony were collected by sterile Pasteur pipettes and incubated for 30 min in a 10 cm tissue culture Petri dish containing FSW-G at room temperature. The buds were then transferred to a sterile nylon cell strainer (100 μm pore size, Falcon, Becton Dickinson Labware, NJ, USA), immersed in FSW-G, extensively rinsed with 200 ml FSW through the nylon filter and transferred into a single 6-well culture dish (TPP, Switzerland) containing FSW supplemented with 10% tunicate culture medium (TCM) for an overnight incubation (20°C, 5% CO_2 humidified incubator).

TCM was prepared as follows: 25 ml RPMI, HAM F12 or Fischer synthetic liquid medium (single strength) was mixed with 19 ml double-strength artificial seawater (ASW; stock solution 2 in Rinkevich and Rabinowitz, 1993) and was supplemented with 4 mmol l^{-1} L-glutamine, 20 mmol l^{-1} Hepes, 3% heat inactivated foetal calf serum (HI-FCS) and 1% mixed antibiotic solution containing penicillin (10 000 U ml^{-1}), streptomycin (10 mg ml^{-1}) and amphotericin B (25 μg ml^{-1}) (PSA). A final volume of 50 ml was achieved by the addition of tissue culture grade water. All media and medium supplements were purchased from Biological Industries (Kibbutz Beit-HaEmek, Israel). All other chemicals were purchased from Sigma (St Louis, MO, USA).

Buds, 2–6 per well, were then transferred into collagen/fibronectin pre-coated TPP 24-well plates or activated ProNectin 24-well dishes (Polymer Technologies Inc., San Diego, CA, USA), and incubated in a humidified incubator. An equal volume of 200 μl/well ASW supplemented with 3% HI-FCS, 2 mmol l^{-1} glutamine and 1% PSA solution combined with 200 μl/well TCM, served as the initial growth medium. In the first 2 weeks, 50 μl of TCM were added to each well once every week, increasing the total volume to 500 μl/well. Thereafter, 50 μl of TCM/well were replaced once every 2 weeks, increasing nutritional concentration gradually.

Preparation of collagen and fibronectin-coated TPP-culture dishes

Type VII collagen from rat-tail (Sigma) was dissolved at a concentration of 1 mg ml^{-1} in 0.1% acetic acid diluted with tissue culture grade water. Collagen was allowed to dissolve overnight at 4°C. The collagen solution was adjusted to a final concentration of 10 μg ml^{-1} by the addition of sterile tissue culture grade water. A volume of 0.5 ml was pipetted to each

of the 24-well culture plates, vacuum-aspirated, air-dried and then polymerized in a laminar flow-hood under UV light for 60 min. The plates were washed twice with sterile PBS and stored at 4°C until use (up to 6 months). Prior to use, the collagen pre-coated plates were incubated with medium containing fibronectin (5 μg ml^{-1}) at 37°C for 30–60 min. There was no further addition of fibronectin whenever the medium was replaced.

MTT-colorimetric cell proliferation assay

Dissociated buds were incubated in 96-well plates with MTT-tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma), at a final concentration of 1 mg ml^{-1} (4 h, 20°C, 175 μl/well). MTT salt was dissolved in TCM and then sterilized by filtration (0.2 μm). The tetrazolium ring of MTT was reduced by active reductase system (active only in viable cells) to water-insoluble formazan, blue in color, that was subsequently solubilized by adding 100 μl/well extraction buffer (total volume of 275 μl/well). Extraction buffer was prepared as follows: 20% SDS was dissolved at 37°C in a solution of 50% dimethyl formamide in DDW. The pH was adjusted to 4.7 by adding 2.5%, from 80% acetic acid solution, and 2.5%, from 1 mol l^{-1} HCl. Extraction buffer releases the cell-bound dye when incubated overnight at 37°C. Absorbance was read at 570 nm using an ELISA plate reader (TECAN, Spectra Image, Austria). Background absorbance was established with wells containing either extraction solvent and MTT without cells or extraction buffer with cells only, omitting MTT. Five sets of experiments were performed and 200 buds per experiment (at blastogenic stages 'B' or 'C') were isolated from subclones of the same genotypes. Buds were enzymatically and mechanically dissociated, generating approximately 10^4 cells per bud. Cells at a concentration of 4×10^4 per well were cultured in 96-well plates using two medium types: HAM F12 and Fischer.

DNA synthesis assay

Blastogenic stage 'C' buds, isolated from the same colony, were grown in a 24-well collagen and fibronectin-coated dishes, three per well. To analyze DNA synthesis, we exposed cultured whole buds to 5 μCi ml^{-1} methyl-^{3}H-thymidine (83 Ci mmol l^{-1}, 1 mCi ml^{-1}; Nahal Sorek, Israel) (1 mCi=37 MBq) for 24 h at various time points following bud extirpation. Buds were then repeatedly (×3) rinsed with a total of 50 ml ASW/sample followed by a rinse of 20 ml ice-cold 10% trichloroacetic acid through a glassfibre profilter (25 mm, Sartorious, AG W-3400, Goettingen, Germany). Each filter was then rinsed twice with 10 ml anhydrous ethanol (–20°C), air dried, placed in a separate vial and 3 ml per vial of liquid scintillation cocktail (Safe Fluor S Lumac/3M, Netherlands) added. Tritiated thymidine incorporated into newly synthesized DNA was measured by liquid scintillation counting.

PKH-26 assay for cell proliferation

Whole isolated buds were exposed to red fluorescent Zyn-linker PKH-26 (prepared for Sigma by Zynaxis, Inc.) as follows. Buds were rinsed twice with 1 ml of calcium and magnesium-free ASW in 15 ml polypropylene centrifuge tubes (Greiner, Austria). PKH-26 solution was prepared at a concentration of 4×10^{-6} mol l^{-1} diluted with Diluent-C, which was provided with the kit. PKH-26-Diluent-C solution was added to the buds, which were incubated for 10 min with gentle swirling to ensure a homogeneous staining throughout bud tissues. Labeling was stopped by adding an equal volume of 100% serum (foetal bovine serum, previously adjusted to sea-water osmolarity with NaCl), diluting the stain by half, and by adding an equal volume of TCM. Finally buds were rinsed with TCM (×3, 10 min each). All these procedures were carried out at room temperature. Thereafter, the buds were cultured and observed under an inverted Olympus fluorescence microscope equipped with an excitation filter of 550 nm and a barrier filter of 590 nm. Zyn-Linker incorporated itself into cell membranes of the buds, and remained stably incorporated for up to 2 months (C.R. and B.R., personal observation). Proliferation is detected by the dilution of Zyn-Linker fluorescence in daughter cells.

DNA fragmentation assay – TUNEL, on attached monolayers

Isolated blastogenic stage 'C' buds were cultured as described. The medium from wells with developed epithelial monolayers was carefully aspirated under a stereomicroscope and tissues were fixed in the well by adding 4% paraformaldehyde solution (room temperature, 30 min). Paraformaldehyde was first prepared at a concentration of 8% in DDW and then mixed with an equal volume of 2× ASW. The fixative was replaced with 80% ethanol and the plates were stored at 4°C until used. Staining was performed using the Klenow-FragEL DNA Fragmentation Detection Kit (Oncogene Research Products, MA, USA) basically following the manufacturer's procedures, with adjustments to 24-well plastic culture plates. Most solutions were added at a volume of 150 μl (the supplier recommends 100 μl). A minimum of 100 μl of the Klenow Labeling Reaction Mixture was added (supplier recommends 60 μl) with the aid of a coverslip. The coverslip was made of a round piece of parafilm cut just slightly smaller in diameter than the well diameter. One edge of the parafilm was folded up and was carefully applied on to the fixed tissue, permitting the small volume of liquid in the well, to spread evenly. Biotinylated nucleotides were detected using a streptavidin-horse radish peroxidase conjugate. Dehydration was performed with absolute ethanol, eliminating the step of xylene washes. A mounting medium was prepared by the addition of nine volumes of glycerol to one volume of PBS; 200 μl of mounting medium was added per well.

Histology of attached bud-bearing spheres

For histological observations and TUNEL staining, 1-month old *in vitro* cultured buds (isolated at stage 'C') with developed spheres were fixed in tissue culture wells by 4% paraformaldehyde/artificial seawater for 30 min at room temperature. The paraformaldehyde was then replaced by 80%

Table 1. *Fate of* in vitro *cultured* B. schlosseri *buds isolated at different blastogenic stages: spheres and attached epithelial monolayers*

Blastogenic cycle	Bud size (μm)	Number of experiments	Number of isolated buds	Developmental stage				Ratio of monolayers/spheres
				Sphere		Monolayer		
				N	%	*N*	%	
A	<300	5	164	0	0	0	0	0
B	300–600	19	430	139	32.3	37	8.6	0.27
C	600–1000	11	429	116	27.0	28	6.5	0.24
D	1000–1300	17	201	8	4.0	41	20.4	5.12

ethanol. Specimens were subsequently dehydrated in a graded ethanol series and embedded in paraplast. Sections 4–5 μm thick were cut with a rotary microtome and stained either with Azan Heidenhain and Alum Hematoxylene and Eosin or processed for TUNEL as described above.

Results

Isolated buds

A total of 1224 primary buds were extirpated at blastogenic stages ‘A’ to ‘D’ (Table 1). Isolated stage ‘A’ primary buds (*N*=164; Table 1) consisted of double-layered vesicles of ectodermal origin (Berrill, 1941a). None grew to more than 300 μm in diameter. A total of 430 stage ‘B’ primary buds, ranging 300–600 μm in diameter, were cultured (Fig. 1A, Table 1). At this stage, rudiments of visceral organs of the eventual adult zooid are already present, consisting of various differentiated cell types. ‘B’ buds are already carrying secondary bud discs, double-layered vesicles that expand from the atrial epithelium. Almost 430 early and late stage ‘C’ primary buds (600–1000 μm) were grouped together. In blastogenic stage ‘D’ buds (*N*=201; 1000–1300 μm in diameter) it is imperative to isolate the buds within a time frame of 24 h before the opening of their siphons. In addition, 86 stage ‘D’ regressing zooids (Fig. 1B) were isolated and cultured. Free-floating hemocytes were found in all wells of cultured extirpated buds immediately following bud isolation. They remained in the cultures without proliferating, as described (Rinkevich and Rabinowitz, 1993).

In our *in vitro* protocols, buds did not attach to the substrates within the first 48 h after extirpation. But on day 3, approximately 50% of the buds isolated at stages ‘B’ to ‘D’ were adhered to the substrate. All buds isolated at stage ‘A’ and some of the buds isolated at stages ‘B’ to ‘D’ remained unattached on the substrate. Both attached and unattached buds of stages ‘B’ to ‘D’ maintained their own heartbeat activity, which gradually ceased during the first week of explant cultures. With time, additional hemocytes migrated from lumens within the buds into the medium, appearing as aggregates or as single-cell suspensions (Fig. 2A). Elongated test cells with long pseudopodial extensions also migrated from explants (Fig. 2B), most abundantly from stage ‘D’ buds, adhering tightly to the culture vessels. A few of them developed a dendritic morphology with several filamentous extensions. With time, small tissue fragments spontaneously dissociated from some explants. However, most buds maintained their external spherical integrity for the entire culture duration (up to 5 months).

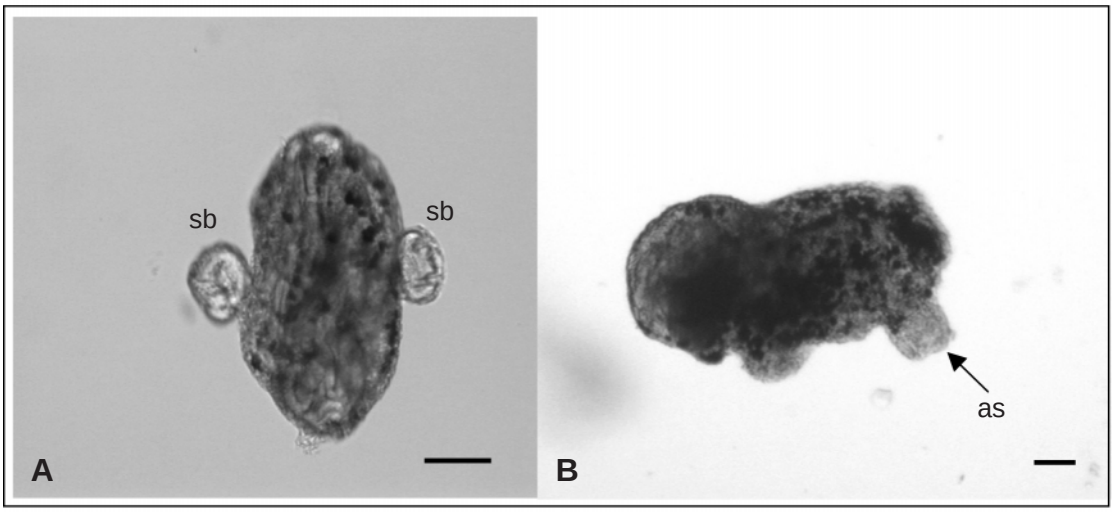


Fig. 1. General morphology of (A) an extirpated *Botryllus* bud *in vitro* at blastogenic stage ‘B’ (×100) and (B) extirpated *Botryllus* zooid at blastogenic stage ‘D’ (×40). as, atrial siphon; sb, secondary bud. Bars, 100 μm.

Epithelium growth

Within 3–12 days of explant culture, we recorded growth of hollow spheres (1–3 per bud; Fig. 2C) of a single epithelial layer in some isolated buds. Each sphere was first noticed as a small vesicle that started to expand at an average rate of 60 μm (diameter) per day, reaching up to 1000 μm (2 weeks). The spheres that remained unattached to the substrates deteriorated, a process that developed within 50 days after they attained their full size. Other spheres attached to substrates within 1–17 days of initial appearance (attachment was also observed once in a 5 month-old culture) and usually developed discoid monolayer of epithelial cells (Fig. 2D). Monolayers grew rapidly, primarily through cycles of cell division (PKH-26 stain, MTT assay and thymidine incorporation, see below). The fast augmentation in cell numbers initially formed confluent monolayer sheets (15–20 μm cell size) that expanded, especially at the periphery, into a monolayer of dilated (100 μm) cells. Cells in the monolayers exhibited typical epithelial morphology, with flattened polygonal lattice-like structures (Fig. 2E). Peripheral cells developed irregular structures, with long, thin cytoplasmic projections. Epithelial sheets grew at an average rate of 230 μm diameter/day for up to 2 weeks and then ceased, starting a phase of cell deterioration. Cellular deterioration was initially noticed by the appearance of a small number of vacuoles around the nuclear membrane that expanded into the entire cytoplasm. The attached intact monolayers began to dissociate within the next

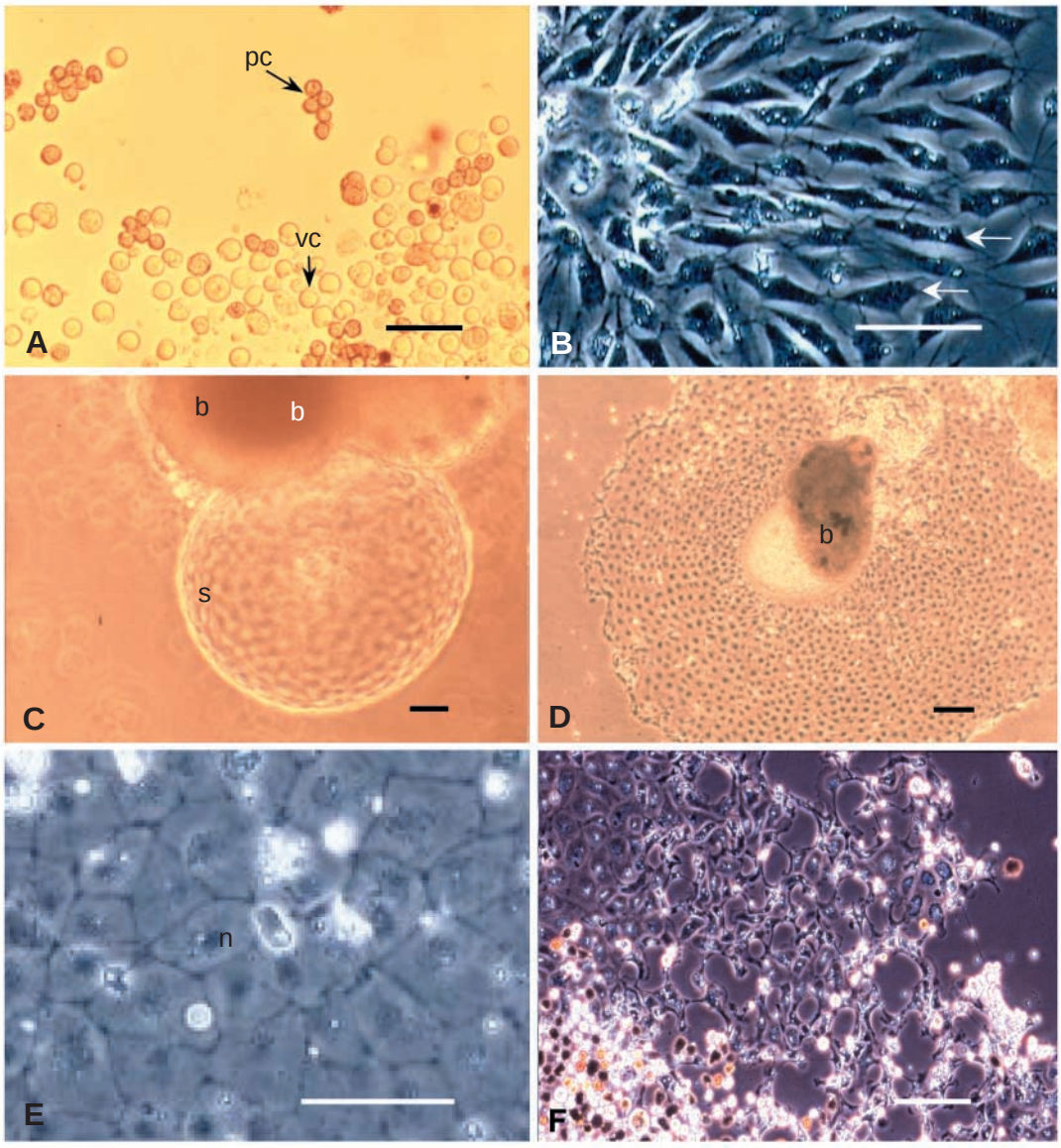
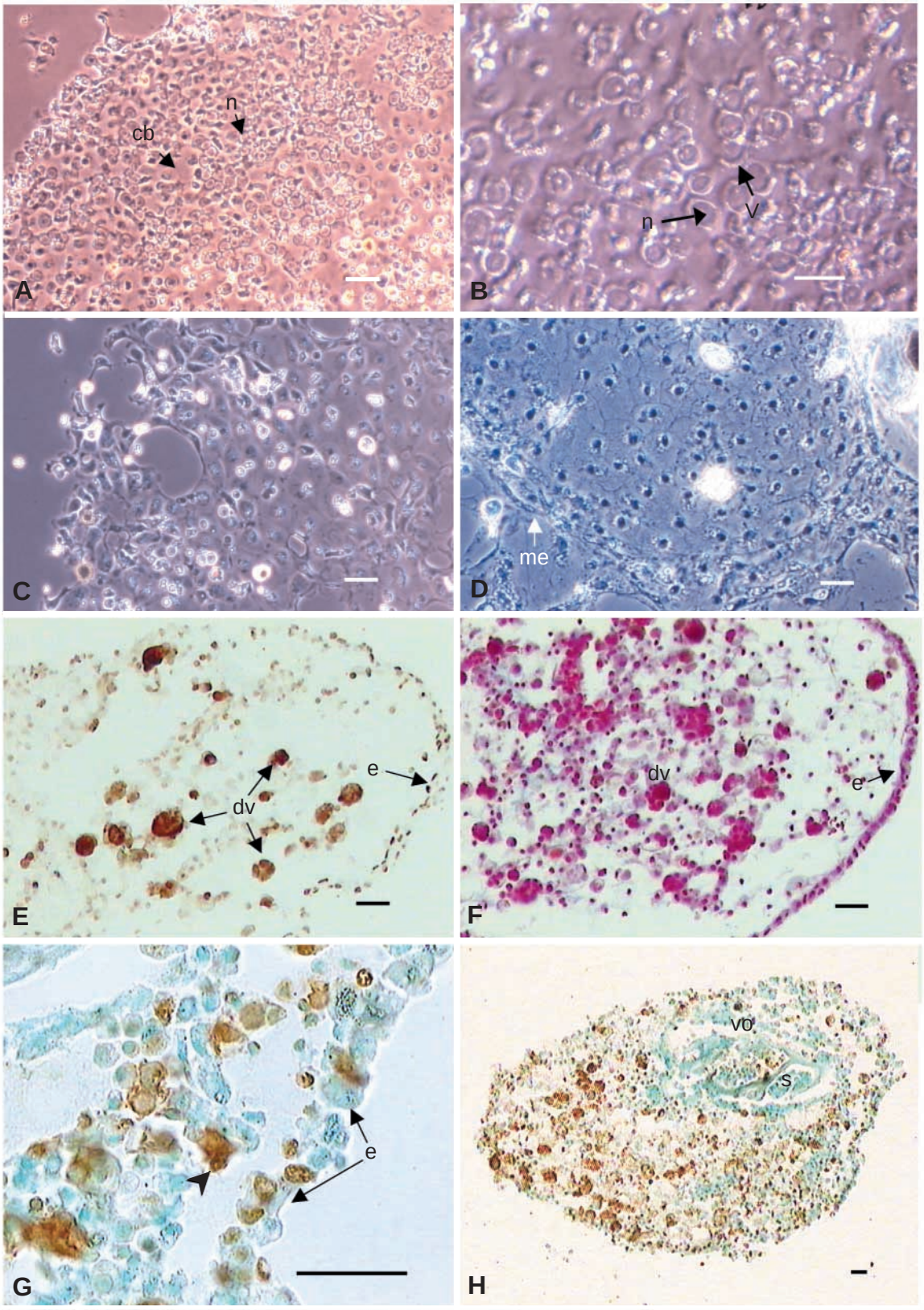


Fig. 2. Morphological consequences for *in vitro* maintenance of extirpated buds and migrating cells. (A) Aggregations of hemocytes released from an isolated buds, day 7 of culture (×200). (B) Test cells attached to the plate (arrows) after migrating from an isolated bud, day 3 of culture (×200). (C) A hollow sphere development from an extirpated stage ‘B’ bud (×200). (D) Epithelial monolayer originating from isolated and cultured buds, in 14 day-old culture (×100). (E) Phase contrast photomicrographs of 1-month old culture, with confluent epithelial monolayer grown from a bud isolated at stage ‘D’, showing cuboidal cell morphology with a polygonal shape (×400). (F) A deteriorating monolayer, day 21 (×200). b, bud body; n, nucleus; pc, pigment cell; s, sphere; vc, vacuolated cell. Bars, 50 μm.



3 days leading to fragmentation of the sheet into small patches of cells (Fig. 2F). Cells finally rounded up and detached from the substrate.

Over a 2-month culture period, neither spheres nor attached epithelial monolayers developed from buds isolated at blastogenic stage ‘A’ (5 experiments; 164 buds; Table 1). In

Fig. 3. Histology and cytochemistry of extirpated buds and degenerating zooids isolated from *Botryllus schlosseri* colonies. (A–C) An epithelial monolayer that developed from an isolated zooid in mid-takeover, 14-day culture period (A, ×200; B, ×400). At 3 weeks incubation (C, ×200), the monolayer has started to deteriorate. TUNEL assay did not reveal positive nuclei. (D) An epithelial monolayer originated from stage 'C' bud (×200) at the deteriorating stage, 4 weeks in culture. TUNEL assay performed on this monolayer did not reveal positive nuclei. (E,F) Apoptotic cells in histological section carried out on the extirpated stage 'D' and TUNEL-stained zooid (E, ×200); also stained for general morphology by Azan (F, ×200). (G,H) TUNEL-positive cells (arrowhead) in histological sections of 5-week old cultured buds, counterstained by Methyl Green (G, ×1000; H, whole bud, ×200). cb, cell boundary; dv, disintegrated visceral organs; e, epithelium; me, monolayer edge; s, stomach; v, vacuole; vo, visceral organ; n, nucleus. Scale bars, 50 μm.

isolated stage 'D' buds (17 experiments; 201 buds), 36 of the 41 attached epithelial monolayers developed directly without going through a sphere stage. Approximately 27–33% of buds isolated at stages 'B' and 'C' (*N*=430 and 429, respectively) developed spheres, of which only 8.6% and 6.5%, respectively, further developed attached epithelial monolayers (Table 1). These outcomes are also reflected by the ratios of monolayers to sphere, which are in an order of magnitude higher at stage 'D' (5.12) than the 0.24 and 0.27 values at stages 'C' and 'B', respectively. In total, isolated stage 'D' buds developed almost 3 times more epithelial monolayers than stages 'B' and 'C' buds. Surprisingly, some of the zooids isolated at mid takeover stage, just prior to parental heartbeat cessation, also developed epithelial monolayers within 8–20 days in culture (Fig. 3A,B; Table 2). These monolayers developed directly, as in most stage 'D' isolated buds, without going through sphere development, and they were characterized by cells with larger nucleus-to-cytoplasm ratios (Fig. 3B) than in cells spreading from isolated buds. The deterioration of attached monolayers that developed from resorbing zooids (up to 10 days after development), and from isolated buds, is due to a non-apoptotic process, as revealed by the TUNEL assay (Fig. 3C). On the other hand, cells from other parts of cultured stage 'D' zooids stained positive for apoptosis (Fig. 3E,F), including cells from the visceral tissues (pharynx, esophagus, stomach, intestine, endostyle), which had already been in an apoptotic state when isolated from the mother colonies.

In cultures of stages 'B' and 'C' buds, spheres appeared at 3–12 days after isolation and survived for up to an additional 35 days. Attached epithelial monolayers appeared 3–25 days after bud isolation and survived for an additional 4–14 days (Table 2). Therefore, epithelial monolayers were present in cultures for up to 40 days after bud isolation, approximately a 1-month extension of their lifespan, compared to that of intact buds. This is equal to the duration of 4–5 blastogenic cycles. The attached bud's epithelium did not go through any massive apoptotic event (TUNEL; Fig. 3D). In some histological preparations of *in vitro* cultured buds with spheres, the majority of cells exhibited apoptotic nuclei; in other cases, they did not (Fig. 3G–H). Internal morphology of these buds, both those with apoptotic and those with non-apoptotic cells, was distorted, exhibiting the loss of most visceral organ organization.

Cell proliferation

Exposing buds to PKH-26 resulted in a universal and uniform distribution of dye binding to cell membranes. During cell divisions, the bound dye was partitioned evenly between daughter cells, and so it gradually faded with repeated divisions. In sphere tissues, cells lost fluorescent markers (Fig. 4A,B), indicating cell divisions there. Faint fluorescence was also observed at the base of each sphere, at its point of contact with the buds (Fig. 4B).

Cell proliferation was also documented by the MTT-assay in one of the five experiments performed. In this experiment (Table 3), epithelial growth was observed in a single well at day 9 of culture and was characterized by a significant increase (by a factor of 1.63) in enzymatic activity ($P<0.05$; Duncan multiple range test), as compared to the other wells at 1, 6 and 9 days. In the other four experiments, cells in cultures exhibited low metabolic activities with average absorbance levels of 36. A 24 h exposure of buds to ^{3}H-thymidine for 24 h at different time-frames *in vitro* revealed low rates of incorporated thymidine in cultures that did not develop spheres or attached epithelial monolayers, indicating a low rate of cell division. After the first day in culture, the isolated explant exhibited some proliferative activity (Fig. 5); this was reduced during prolonged *in vitro* conditions, as recorded previously (Rinkevich and Rabinowitz 1993, 1997). However, when sphere development was observed, the cultures revealed a significant thymidine incorporation (Fig. 5).

Table 2. *Culture properties of sphere and attached epithelial monolayer developments from* B. schlosseri *explants isolated at different blastogenic stages*

		Sphere		Epithelial monolayer	
Explant	Blastogenic stage	Onset of appearance (days)	Culture duration (days)	Onset of appearance (days)	Culture duration (days)
Primary buds	A	–	–	–	–
Primary buds	B	3–11	Up to 35	5–12	Up to 7
Primary buds	C	3–11	Up to 35	3–20	4–14
Primary buds	D	9–12	Up to 15	6–25	3–14
Resorbing zooids	D	–	–	8–20	Up to 10

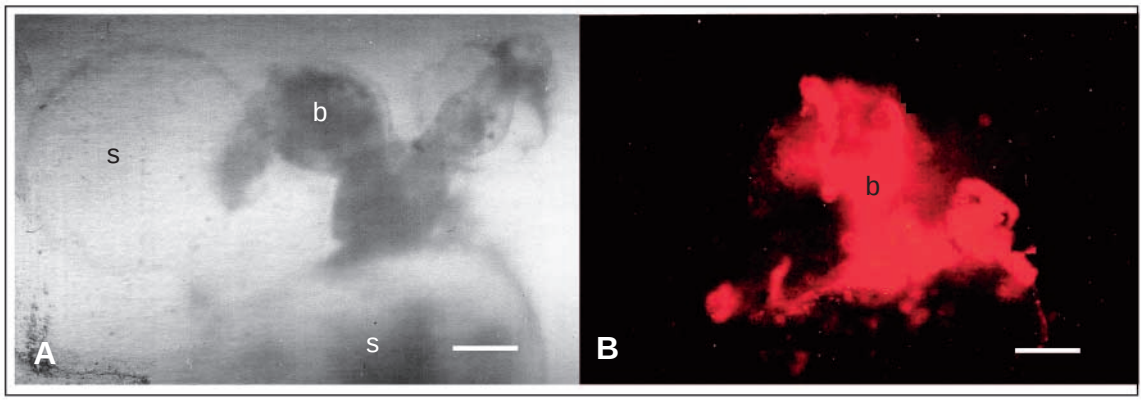


Fig. 4. Distribution of PKH-26 fluorescent dye in bud tissues. 2-week-old cultured buds developed two spheres clearly observed under light microscopy (A) but not under fluorescence microscopy (B). Bars, 100 μm (×100). b, bud; s, sphere.

Discussion

Growth of a *Botryllus* colony is executed by serial cycles of highly synchronized phases of development and death where three asexually derived successive generations, adult zooids, primary and secondary buds, coexist (Berrill, 1941a,b, 1951; Sabbadin, 1958, 1969; Izzard, 1973; Sabbadin et al., 1975; Mukai, 1974; Lauzon et al., 1992, 1993, 2002; Cima et al., 2003). Throughout their development buds remain intimately connected to their parent zooids by blood vessels that traverse the tunic (Burighel and Brunetti, 1971; Zaniolo et al., 1976; Mukai et al., 1978); buds exhibit developmental synchrony and their development and zooidal regression appear to be fine-tuned through these vascular connections (Watanabe, 1953; Rinkevich and Weissman, 1987). However, neither the specific mechanisms that regulate blastogenesis nor the nature of the 'death signal' that overwhelms parental zooids once a week (18–20°C; Rinkevich and Shapira, 1998) are well understood.

All previous studies (Oka and Watanabe, 1957; Watkins, 1958; Milkman and Therrien, 1965; Milkman, 1967; Sabbadin et al., 1975; Zaniolo et al., 1976; Rinkevich et al., 1995; Lauzon et al., 2002) have involved budectomized and/or zooidectomized assays, in which the developmental responses of the regenerating colonies were observed and documented. The present study reports the first experimental manipulations where buds and regressing zooids have been surgically removed and followed under *in vitro* conditions, away from any discrete colonial regulatory cue.

Table 3. *MTT enzymatic activity levels in cultured zooids*

Days in culture	Epithelium	Absorbance at 570 nm
1	–	41.6±7.8 (16)
6	–	26.9±6.3 (16)
9	–	74.0±5.9 (15)
9	+	127 (1)

Values are means ± S.D. (*N*).

The application of this *in vitro* approach reveals several unexpected and intriguing results. (1) When spheres and epithelial monolayers appeared, the life expectancy of an isolated bud reached *in vitro* periods of 50–60 days, exceeding by 5 times the life expectancy of intact, *in vivo* developing zooids. (2) The life expectancy of *in vitro* maintained buds that remained unattached to the substrates prolonged the above periods for up to 5 months. (3) After attaching to substrates, buds obeyed a newly developed 'death clock', permitting up to 35 survival days for developing spheres and up to 14 days of life for epithelial monolayers. (4) The prevailing mode of death *in vitro* was necrotic, as opposed to the apoptotic mode of blastogenic takeover. (5) Isolated degenerating zooids under *in vitro* conditions produce epithelial monolayers within 3 weeks of culturing. Monolayers survived for up to 10 additional days, extending the lifespan of degenerating zooids from a few hours to 1 month.

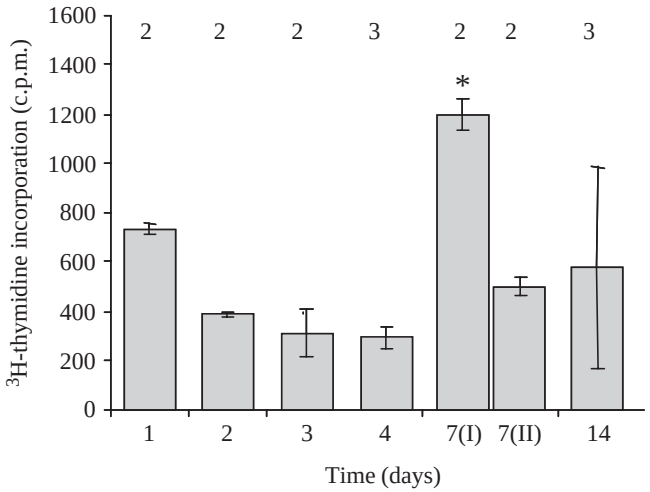


Fig. 5. ^{3}H-thymidine incorporation in cultures of stage 'C' extirpated buds assayed 1–14 days after isolation. Values are means ± S.D. The numbers of wells are indicated. On day 7, sphere growth was observed in 2 of 4 wells [7(I)]. Asterisk indicates significant difference (P<0.05, t test).

In addition to the colony-wide weekly apoptotic cycles, *B. schlosseri* colonies may also exhibit another type of colony-wide programmed mortality, a non-random senescence (Rinkevich et al., 1992; Lauzon et al., 2000). This type of death is expressed cytologically as a series of necrotic events that develop roughly simultaneously in multizooid clonal replicates experimentally separated from colonies months earlier. We recently showed (Voskoboynik et al., 2002) that the lifespan of the old, long-living phenotypes may further be extended significantly by acute administration of the anti-oxidant butylated hydroxytoluene.

The common vascular system (Burighel and Brunetti, 1971; Zaniolo et al., 1976; Mukai et al., 1978) permits synchronization of colony-wide developmental phenomena such as blastogenesis and non-random senescence (Mukai, 1974; Burighel and Schiavinato, 1984; Lauzon et al., 1992, 1996; Rinkevich et al., 1992; Voskoboynik et al., 2002). The determination of life or death is probably conveyed by blood-borne elements or cues (Oka and Watanabe, 1957; Lauzon et al., 1992; Cima et al., 2003) rather than by the recycling macromolecular components from old to newly developed soma (Lauzon et al., 2002). In experimentally manipulated colonies where different parts of the colony are surgically removed, the profiles of colony-wide developmental responses indicate a dynamic and synchronized central control for systemic changes within the colonial boundaries. On the other hand, isolated buds or isolated zooids at mid-apoptotic cycle are not liable to die as they are when integrated into the colony. Under *in vitro* conditions, not only are the underlying colonial mechanisms replaced by different developmental entities (spheres, epithelial monolayers) and pathways, but also the colonial level regulative longevity clocks are replaced by new biological clocks that feature different developmental timetables (such as the 14-day growth-to-senescence period for epithelial monolayers).

Studies that reveal alternative, non-apoptotic forms of programmed cell deaths (e.g. Sperandio et al., 2000; Leist and Jäätellä, 2001) or an array of genes that significantly affect the lifespans of multicellular organisms (Gems, 1999; Johnson et al., 1999) may lead to new insights into life-and-death programs and their roles in developmental pathways. The *in vitro* investigation of *Botryllus* developmental processes may provide an additional tool to understand clocks that act on the whole-colony level as compared to life-and-death processes expressed on the zooid level. These are two different tiers of developmental signals that shape organ development differently in modular organisms.

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