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Macrophage involvement for successful degeneration of apoptotic organs in the colonial urochordate *Botryllus schlosseri*

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Summary

Apoptosis is an important tool for shaping developing organs and for maintaining cellular homeostasis. In the colonial urochordate *Botryllus schlosseri*, apoptosis is also the hallmark end point in blastogenesis, a cyclical and weekly developmental phenomenon. Then the entire old generation of zooids are eliminated (resorbed) by a process that lasts 24–36 h. Administration of the antioxidant butylated hydroxytoluene (BHT) resulted in resorption being arrested by 1–8 days on average. At high doses (2.5–15.0 mg BHT l^{-1}) resorption was completed only after removal of BHT. Colonies that were not removed in time, died. In treated colonies, although DNA fragmentation was high, tissues and organs that would normally have died, survived, and the general oxidative levels of lipids were reduced. Blood vessels were widened, containing aggregates of blood cells with a significantly increased proportion of empty macrophage-like cells without inclusion. In colonies rescued from BHT treatment, resorption of zooids started immediately and was completed within a few days. We propose three possible mechanisms as to how BHT may affect macrophage activity: (1) by interrupting signals that further promote apoptosis; (2) through the respiratory burst initiated following a phagocytic stimulus; and (3) by reducing lipid oxidation and changing cell surface markers of target cells. Our results point, for the first time, to the role of phagocytic cells in the coordination of death and clearance signals in blastogenesis.

Key words: apoptosis, phagocytosis, macrophage, BHT, antioxidant, tunicate, *B. schlosseri.*

Introduction

Apoptotic cell recognition, engulfment and cell corpse digestion are the final stages in the process of apoptosis. This selective elimination pathway of dying cells is carried out by a variety of phagocytic cells, including macrophages. Studies on *C. elegans* (Hoeppner et al., 2001; Reddien et al., 2001) and mammals (Diez-Roux et al., 1997; Boyle et al., 2001) revealed that in addition to their classic role of corpse elimination, macrophages may also have a role in the induction of apoptosis in normal cells *in vivo* or, by engulfing cells, they act to ensure that cells triggered to undergo apoptosis will die, rather than recover after exhibiting initial stages of death. This novel exciting role of macrophages as mediators of the apoptotic process has not been yet documented in whole body apoptotic events. The weekly, colony-wide apoptotic process, characteristic to the urochordate *Botryllus schlosseri*, offers a unique whole-body apoptotic model system to study this process.

Botryllus schlosseri is a colonial marine organism; each colony is composed of numerous genetically identical modules (zooids) and is derived from a single, sexually produced tadpole larva, which immediately settles upon release. The colony grows through a highly synchronized and cyclical developmental phenomenon called blastogenesis (Berrill, 1950). Blastogenesis is divided into four major stages, A–D (Mukai and Watanabe, 1976), and lasts about 1 week (at 18–20°C). It is a highly tuned cycle in which: (1) a new set of zooids is established through the development and maturation of 1–4 primary buds per zooid, and (2) the parent set of zooids deteriorates and is morphologically eliminated. During stages A–C, bud tissues are differentiated and internal organs are formed, while four of the zooids remain active. At stage D, lasting 24–36 h, all zooidal tissues in the colony die, mainly by an apoptotic process, and are phagocyted by specialized blood cells, the macrophages (Lauzon et al., 1992, 1993), which, at this stage, increase infrequency among circulating blood hemocytes (Ballarin et

al., 1998). The developing primary buds then, simultaneously, mature into the new generation of functional zooids, replacing the old generation of zooids. Zooid apoptosis proceeds in *B. schlosseri* colonies in a wave-like fashion, beginning at the anterior end of each zooid and gradually advancing towards the posterior end (Lauzon et al., 1992). This unique developmental phenomenon, in which every week all functional soma go through an apoptotic process, remains an empirical and theoretical challenge. To date, only a limited number of methodologies have been found to successfully alter the blastogenic rhythm, including changes of water temperature regimen (Boyd et al., 1986; Rinkevich et al., 1998), use of allogeneic fusions (Rinkevich and Weissman, 1987a), or zooid/bud removals (Sabbadin, 1956a,b; Lauzon et al., 2002) or by employing ionization radiation (Rinkevich and Weissman, 1990). All the above protocols, however, had not revealed much of the nature of this unique phenomenon.

Recently, we (Voskoboynik et al., 2002) showed that blastogenesis was arrested and colonies deteriorated to a morphologically chaotic state in clonal replicates of *B. schlosseri* that had been treated with high doses of the anti-oxidant butylated hydroxytoluene (BHT). Rescued colonies resorbed BHT-treated zooids, regenerated entirely new sets of zooids, and then revealed enhanced growth rates and also, in many cases, significant extension of post-treatment life expectancies. Here, we further analyze blastogenesis in *Botryllus schlosseri* colonies. The results reveal that macrophages, in addition to their role in corpse removal, play an important role as mediators in this whole body apoptotic event.

Materials and methods

Animals

Sexually mature colonies of *Botryllus schlosseri* Pallas were collected from Monterey Marina, CA, USA and Rovinjtown, Croatia. Hatched larvae were settled and maintained as described (Rinkevich and Weissman, 1987b; Rinkevich and Shapira, 1998). Colonies that reached a minimum size of six zooid systems were divided to three groups (BHT-treated, ethanol control and un-manipulated control ramets) and subjected to experimental procedures as described (Voskoboynik et al., 2002). The BHT concentrations used here were 0.03–15 mg l^{-1} seawater. Observations were performed daily under the compound stereomicroscope. Zooid, bud, blood vessel measurements and cell sizes were preformed by image analysis program: Imagepro plus (Media Cybernetics, Silverspring, MD, USA).

Histology

Specimens were fixed in Bouin's fixative (Gretchen, 1967) for 40–60 min at room temperature, dehydrated in a graded ethanol series (70–100%) and butanol, and embedded in Paraplast (Sigma, Israel). Cross sections (5 μm) were cut by hand-operated microtome (Leica, Nussloch, Germany) and were stained with Azan Heidenhains (Gretchen, 1967) for general morphology, or were used in the Klenow fragEL assay (see below). Outcomes were observed under an Olympus (Tokyo, Japan) BX50 microscope.

Klenow fragEL assay

Apoptotic nuclei were stained using a Klenow fragEL DNA fragmentation detection kit (QIA21 Calbiochem, Darmstadt, Germany), alkaline phosphatase conjugate (Zymed, San Francisco, CA, USA) and BCIP/NBT substrate kit (Zymed), according to the manufacturer's protocols. Endogenous alkaline phosphatase was inhibited by Levamisole solution (Zymed). Each slide contained six sections, three for DNA fragmentation detection and three for negative control. Negative controls were generated by substituting the Klenow in the reaction mixture with dH_2O.

HPLC thiobarbituric acid test

Fatty acid oxidation in *B. schlosseri* homogenates from different blastogenic stages, BHT-treated ramets, and their controls, were determined by using high performance liquid chromatography based on thiobarbituric acid tests (Chirico, 1994). The thiobarbituric acid (TBA) test measures malondialdehyde (MDA), which is formed in peroxidizing lipid systems (Chirico, 1994). *B. schlosseri* homogenates were prepared as described. Protein concentration was estimated using a Bio Rad Protein Assay Dye Reagent (Bio Rad, Hercules, CA, USA).

Results

Blastogenic changes under BHT treatment

Ten *Botryllus* genotypes were subcloned into 68 ramets that were divided into three experimental groups: non-manipulated controls (23 ramets), ethanol controls (15 ramets) and a BHT-treated group (30 ramets). Two weeks preceding the first BHT administration, all ramets, including controls, were carefully monitored daily for their blastogenic stage. This served as an additional control for the pretreatment blastogenic rhythm. BHT-treated ramets were exposed to daily administrations of BHT for 6–19 days, depending on the ramets' reaction to the treatment and the severity of the outcome.

Stage D zooid apoptotic resorption was significantly delayed at all tested BHT doses (0.03–15 mg l^{-1}). While treatment with alcohol did not affect the duration of this stage (regular colonies, 1.0±0.2 days, N=23 ramets; ethanol-treated colonies, 1.1±0.3 days, N=15; P>0.05), under BHT treatment, stage D was significantly prolonged to 2.7±0.7 days (P<0.05; N=9) at low BHT doses (0.03–1.2 mg l^{-1}), and up to 8.4±3.3 days at higher BHT doses (2.5–15.0 mg l^{-1}, N=21; one-way ANOVA, $P \ll 0.001$; Duncan's test, P<0.05). Exposing *B. schlosseri* ramets to low BHT doses (0.03–1.2 mg l^{-1}) resulted in the lengthening of stage D, although it did not affect the successful outcome of the resorption process. At higher BHT doses, however, morphological resorption of zooids was completed only after the colonies were removed from the BHT environment.

Morphological changes in colonies arrested in stage D

Exposure of the colonies to doses >2.5 mg BHT l^{-1} seawater dramatically arrested blastogenesis, followed by the development of a chaotic morphology (Fig. 1) and zooid deterioration that eventually led to their death. Arrested blastogenesis was further accomplished by an accumulation of pigment cells in the zooids, buds and blood vessels with peripheral ampullae shrinkage, sluggish blood flow rates throughout the colony, dispersion of zooids within the tunic matrix and abnormal development of primary and secondary buds. Zooids at arrested stage D remained inactive (closed siphons) but alive (beating hearts) within the tunic matrix. The primary buds continued their development and grew into the stage where they are supposed to replace the old generation of zooids (with opened siphons). Then, instead of creating new systems, they were dispersed in the tunic as abnormal functional zooids together with the old generation zooids (Fig. 1D,F). After several days, the old generation of zooids became very condensed and partly resorbed, while the newly formed functional zooids and their buds developed additional, abnormal morphologies such as round zooids instead of the regular pearl-like shapes. At the same time, the typical synchronization between successive generations in the colony was lost, and zooids and buds of different sizes and generations survived together within the tunic. The tunic became softer and opaque (Fig. 1F). Ethanol control colonies developed none of these morphological changes, with the exception of light pigmentation of zooids and ampullae and an opaque tunic in a few ramets.

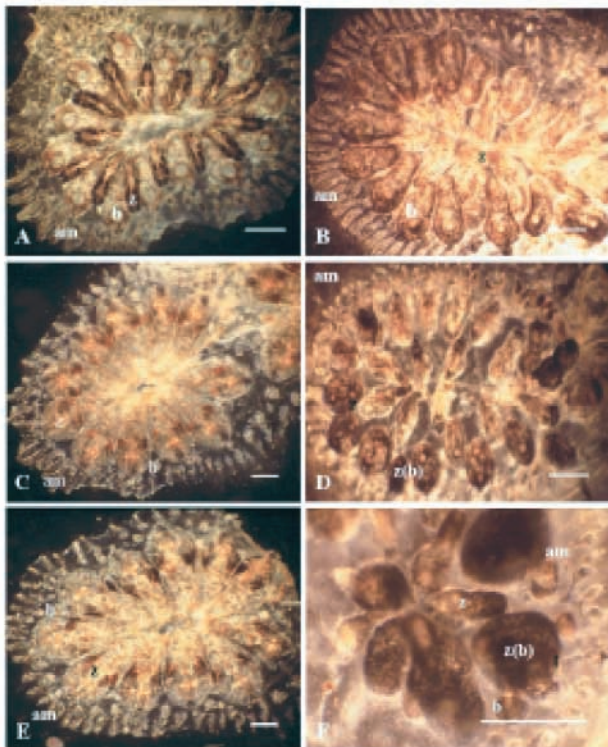

Fig. 1. Control *B. schlosseri* ramet (A,C,E) and a 6 mg BHT l^{-1}-treated ramet (B,D,F). (A) Control ramet in stage D. The zooid resorption has already started and fully developed buds ready for the takeover stage are visible; (B) BHT-treated ramet after 5 days under BHT administration, reaching the first day of blastogenic stage D; (C) control ramet 1 day later, already at blastogenic stage A. The old generation of zooids was completely resorbed. (D) The corresponding BHT-treated ramet, 1 day later (6th day of BHT exposure). The old gene ration of zooids in the center still exists. The new generation of zooids dispersed in the tunic matrix without the typical patterning of systems. (E) The control ramet 1 day later, at normal blastogenic development, already at stage B; (F) the BHT-treated ramet after 7 days of BHT exposure. The old generation of zooids was not resorbed, while the new generation of zooids developed abnormal round shapes of zooids with open, inhalant siphons and accumulated deep pigmentation. The tunic looks opaque. z, zooid; z (b), new generation zooid; b, bud; am, ampulla; t, tunic. Scale bars, 1 mm.

Histological characteristics of colonies arrested at stage D

The morphological changes in ramets arrested in stage D were studied at various stages following the onset of treatment. A histological comparison of a stage D control colony *vs.* a 3-day arrested stage D (6 mg BHT l^{-1}, Day 10 exposure) is

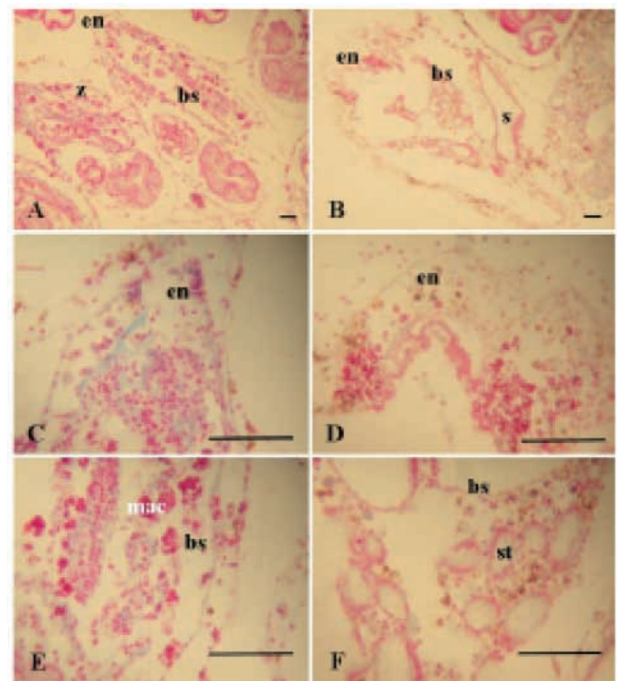

Fig. 2. Azan Heidenhains stain of sections from stage D control zooids (A,C,E), compared to 6 mg BHT l^{-1}-treated zooids (B,D,F) after 3 days arrested at stage D. (A) A control ramet with two resorbed zooids at stage D. (B) BHT-treated zooid after 3 days arrested at stage D. Internal organs, such as endostyle, branchial sac and siphon, are intact. (C) Enlargement of the deteriorating endostyle area of a control zooid. (D) Enlargement of the BHT-treated zooid endostyle area with unspoiled structures including morphologically normal epithelial cells. (E) Stage D 22 control, the deteriorated zooid branchial sac stigmata area. Leftover cells and macrophages carrying debris are seen. (F) Third day arrested stage D zooid, branchial sac stigmata area. The organ is intact. z, zooid; bs, branchial sac; en, endostyle; s, siphon; st, stigmata; mac, macrophage. Scale bars, 5 µm.

shown in Fig. 2. In normal blastogenic stage D zooids (mid takeover stage; Fig. 2A,C,E) the internal organs are already at various degrees of deterioration (Fig. 2A). However, the tissues of BHT arrested stage D ramets seemed to be functional, even after 3 days (Fig. 2B). That was further confirmed by examining, at higher magnification, organs such as the branchial sac stigmata, endostyle and siphons, which still existed after 3 days arrested at stage D (Fig. 2D,F). In the control (Fig. 2C,E), a few hours after the onset of stage D, the general structure of epithelia was in advanced deterioration phase and macrophages carrying debris were seen all over. It is evident that the typical apoptotic wave of stage D colonies (Lauzon et al., 1992) was either suspended or failed to start under the impact of BHT treatment.

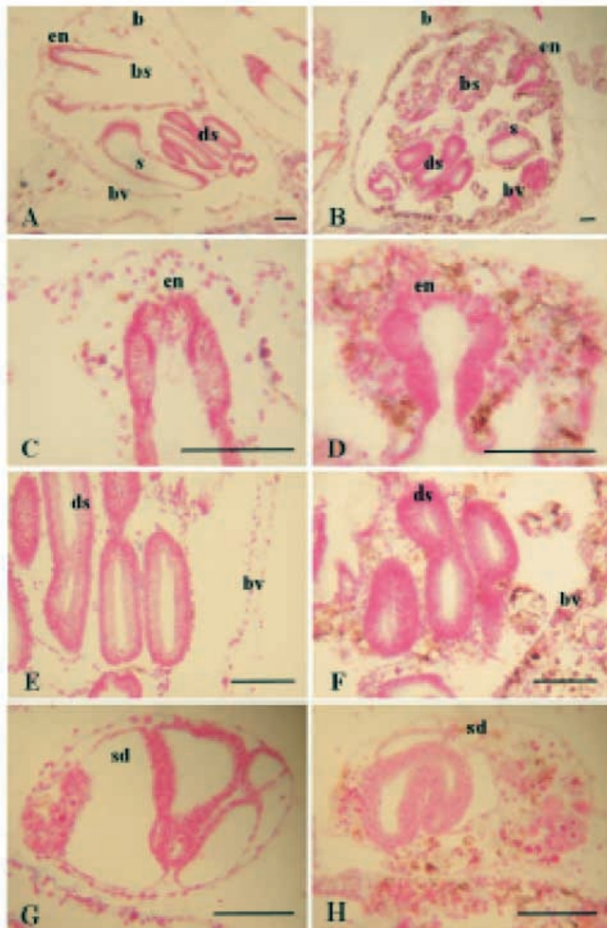

Fig. 3. Histological sections of control blastogenic stage D primary and secondary buds (A,C,E,G) as compared to 6 mg BHT l^{-1}-treated primary and secondary buds after 10 days exposure, on third day of arrested stage D (B,D,F,H). Azan Heidenhains stain. (A) Control ramet, primary bud at blastogenic stage D. Only a few cells can be seen in the bud blood vessels. (B) BHT-treated primary bud after 3 days arrested at blastogenic stage D. The bud periphery blood vessels are thick and packed with enormous numbers of cells. (C) Enlargement of the endostyle area in the primary control bud; only few blood cells can be seen near the endostyle epithelium. (D) The endostyle of the treated primary bud, surrounded by an unusual mass of cells. (E) A few cells circulating in the peripheral blood vessel of the primary control bud and the lacunas around its digestive system. (F) Large numbers of blood cells circulating in the treated primary bud peripheral blood vessel and its digestive system. (G) Secondary bud control ramet has large empty spaces (subdivisions of the future organs) lined by epithelium of thin blood vessel. (H) BHT-treated secondary bud has dilated blood lacuna filled with m any cells. b, bud; bs, branchial sac; bv, blood vessel; ds, digestive system; en, endostyle; s siphon; sb, secondary bud.

Significant morphological changes were observed in the primary and secondary buds of the BHT-treated colonies (Fig. 3A–H). Whereas only a few cells were found circulating in the blood vessels and lacunas around organs of stage D control buds (Fig. 3A), large numbers of blood cells appeared in those blood vessels during BHT treatment (3 days arrested at stage D colonies; Fig. 3B). Three examples are depicted here: (1) the bud endostyle of the treated colonies surrounded by a mass of blood cells, as compared to the relatively empty space around the control bud endostyle (Fig. 3C,D); (2) the digestive system area of the treated buds packed with blood cells, as compared to the control section (Fig. 3E,F; a high number of blood cells found in the peripheral-treated bud blood vessels, as compared to the control (bv in Fig. 3E,F). (3) The internal morphology of the secondary bud in the BHT-treated colony (Fig. 3H) was adversely affected with dilated blood lacunas filled with many cells. In the control, the secondary bud had large empty spheres lined by the epithelium of thin blood vessels (Fig. 3G).

Changes in the vascular system in colonies arrested at stage D

Various modifications resulting from BHT treatment were observed within the vascular system. The thickness of the peripheral blood vessels epithelium at stage D, which in control zooids averaged 0.8±0.5 μm (N=25), increased to 5.6±3.6 μm (N=23) in zooids on the third day of arrested stage D ($P\ll 0.001$; t test). The thickness of the bud's peripheral blood vessel epithelium increased from 0.9±0.6 μm (N=28) in stage D control colonies, to 4.9±2.8 μm (N=30) in third-day arrested buds ($P\ll 0.001$; t test). Dilated peripheral blood vessels in the 3-day stage D arrested zooids and buds were 9.5- to 10-fold wider than in the controls. The number of blood cells within the lumen of peripheral blood vessels also increased from an average of 11±6 cells (N=6) per 100 μm^2 section area of control stage D peripheral blood vessels lumen to 192±125 in stage D arrested ramets (N=12; P=0.002, t test).

We further examined the appearance and number of macrophages in histological sections. The following numbers reflect counts per 3500 μm^2 section area. Empty macrophage-like cells (cells without phagocyte inclusions) were observed only in stage D arrested ramets [11.7±5.8 (N=5) and 8.2±5.3 (N=7) zooid and bud sections, respectively; Fig. 4] and could not be recorded in either the zooid or bud section of stage D control ramets. Conversely, the number of macrophages with phagocytic inclusions reached 6.6±1.5 (N=4) and 4.2±2.8 (N=4) per section area, in stage D zooid and bud control ramets, respectively, as compared to 1.3±1.9 (N=5; P=0.002,

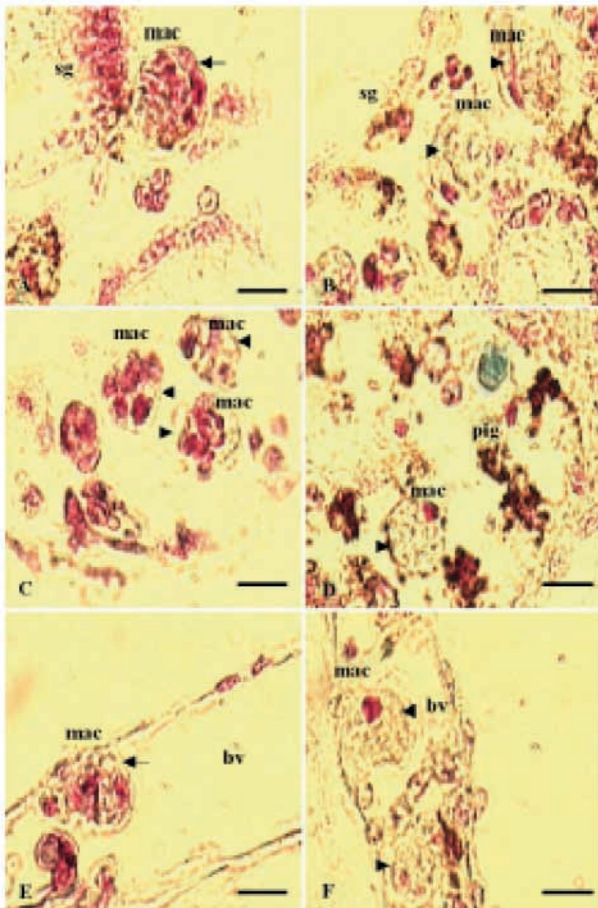

Fig. 4. Histological section of a control ramet at blastogenic stage D and its corresponding BHT-treated ramet, 3 days arrested at stage D. (A) A 'packed' macrophage (arrow) is seen with ingested inclusions between the control zooid 23 stigmata; (B) BHT-treated ramet, empty macrophages (arrowheads) are located between zooid stigmata; (C) packed macrophages (arrowheads) in the control ramet bud; (D) BHT-treated bud with an empty macrophage (arrowhead); (E) a packed macrophage (arrow) in control zooid blood vessel; (F) BHT-treated zooid blood vessel with empty macrophages (arrowheads). Azan Heidenhains stain. bv, blood vessel; mac, macrophage; pig, pigment cell; sg, stigmata. Scale bars, 1 μm.

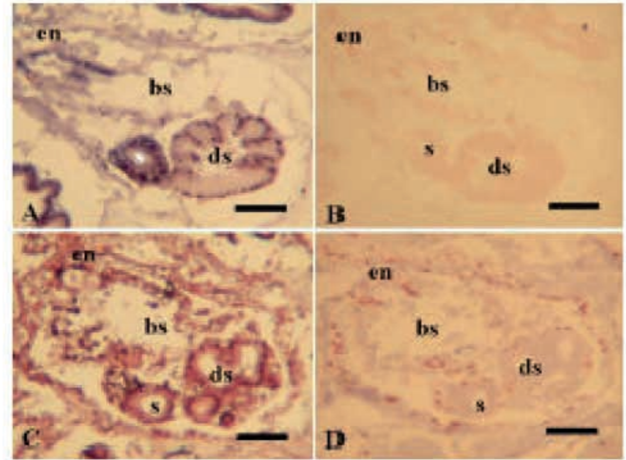

Fig. 5. DNA fragmentation detection staining (fragEL) of control ramet (A,B) and BHT-treated ramet, 3-day blastogenic stage D arrested (C,D). A and C reveal DNA fragmentation staining. B and D are, respectively, their negative control for background staining. bs, branchial sac; ds, digestive system; en, endostyle; s, siphon. Scale bars, 10 μm.

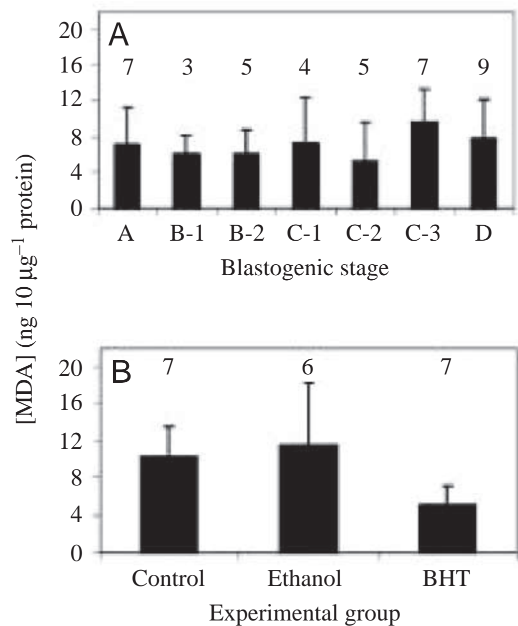

Fig. 6. Effect of BHT on lipid oxidation levels, (A) at different stages of the blastogenic cycle of control *B. schlosseri* colonies; (B) in BHT (6 mg l^{-1})-treated, ethanol-treated and control ramets at blastogenic stage D. Lipid oxidation was determined as MDA using the HPLC based Thiobarbituric acid test (see Materials and methods for details). The MDA levels were standardized to 10 μg protein. n, ramets number. Data are mean ± S.D. *N* values are given above each bar.

t test) and 0.35±0.6 (N=7; P=0.005, *t* test) at arrested stage D colonies, zooid and bud, respectively.

Effect of BHT on apoptotic processes

We followed the apoptotic status of five different colonies arrested at stage D (6 mg BHT l^{-1}), and their corresponding stage D control ramets using Klenow FragEL, a DNA fragmentation detection staining kit. Typical sections representing a control stage D zooid and a 3-day arrested stage D zooid are shown in Fig. 5. Surprisingly, a similarly intensive staining was documented in both types of treatments (Fig. 5A,C).

Effect of BHT on lipid oxidation

Only slight and insignificant (one way ANOVA, P>0.05) changes in the lipids oxidative MDA levels were recorded in 40 tissue samples taken from regular colonies at different blastogenic stages (Fig. 6A). The minimal level was recorded on the second day of blastogenic stage C (5.2±4.4 ng 10 μg^{-1} protein), whereas the maximum level was found on the third day of blastogenic stage C

(9.5±3.8 ng 10 μg^{-1} protein). After BHT treatment (6 mg l^{-1}, 1–3 days at arrested stage D) MDA levels decreased significantly to a level of 4.8±2.2 ng MDA 10 μg^{-1} protein (7 ramets; one-way ANOVA, $P<0.05$), as compared to the average MDA level of additional seven control ramets at blastogenic stage C, day 3, (10.1±3.4 ng MDA 10 μg^{-1} protein) and 11.4±6.7 ng MDA 10 μg^{-1} protein in six ethanol controls (Fig. 6B).

Recovery from the BHT treatment

In ramets rescued from BHT treatment, zooid resorption was immediately initiated and completed within 1–7 days through a massive phagocytosis process. In most cases, old generations of zooids and buds were cleared at once, even before the first new takeover stage. Blood vessels were cleared of their unusually deep pigmentation after 4–8 days; the excess zooidal pigmentation cleared within 1–3 weeks and regular sausage-like ampullae developed after 3–7 days. Few remaining active zooids created the new colonies. Those post-BHT-treated ramets revealed higher growth rates, fast blood flow, pale pigmentation and were characterized by active and long peripheral ampullae, all resembling young colonies (Voskoboynik et al., 2002).

Discussion

The blastogenic cycle in botryllid ascidians, where, once a week, the old soma is eliminated by apoptosis (‘organopoptosis’ *sensu* Skulachev, 2001) is probably one of the most prominent instance in which apoptosis is not only used as an important key factor for normal cellular homeostasis, or as a crucial element in shaping developing organs, but is also a key apparatus for establishing normal growth patterns in mature organisms. The nature of this phenomenon is not yet fully understood.

By exposing *B. schlosseri* colonies to high doses of the antioxidant BHT, we significantly affected this organopoptosis phase. This stage was arrested while the supposedly resorbed zooids remained alive in the tunic matrix without completing their programmed death. The apoptotic process was completed only when the colonies were taken out of the BHT treatment. Prolonged periods of exposure to BHT have always caused the death of treated ramets. BHT was not the only antioxidant agent that had an impact on the apoptotic stage. Administration of the antioxidant Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, gave similar results (Voskoboynik, 2001).

The analysis of arrested blastogenesis has revealed the possible important role of the blood-borne phagocytic activity in blastogenesis. As in our experiments, BHT treatment in mice also increased the number of phagocytes. Following four weekly high doses of BHT injections to BALB mice, alveolar macrophage numbers increased fivefold over basal levels (Bauer et al., 2001). This study did not examine the phagocyte state (empty or full). In our study, however, the activity of phagocytosis was clearly reduced by BHT treatment, as large numbers of ‘empty’ macrophage-like cells were observed in blastogenesis stage D arrested zooids. In these colonies, significantly, higher numbers of empty phagocytic cells were found to circulate in wider blood vessels and lacunae and zooid organs, such as the stomach, intestine and the branchial sac, remained intact days after the expected date for their regression and resorption. Phagocytic activity, however, was regained immediately following BHT elimination and zooid resorption was completed within a few days.

In botryllid ascidians, two hemocyte types, the uni-or multi-vacuolated macrophage-like cells and hyaline amoebocytes are involved in phagocytosis (Ballarin et al., 1994; Cima et al., 1996, 2001). Studies suggested that both cell types represent two functional stages of a single cell type (Ballarin et al., 1994; Cima et al., 1996) and both share common cytochemical properties, a common content of hydrolytic enzymes, and are positive for specific lectins such as WGA and ConA (Cima et al., 2001). The macrophage-like cells are usually large (10–15 μm in diameter) round cells containing one to a few vacuoles, ingested material of heterogeneous appearance and, in some cases, such as during the takeover process of blastogenesis, these phagosomes occupy most of the cell volume (Lauzon et al., 1993). The macrophage-like cells in *Botryllus* are also classified by several authors as macrophages (literature cited in Cima et al., 2001).

We propose four possible mechanisms as to how BHT may affect *Botryllus* macrophage activity. The first possibility is that these cells, in addition to their classical role in cellular elimination, have a role in providing signals that promote apoptosis. Mutations in *C. elegans* engulfment genes allow the survival and differentiation of cells that are programmed to die (Hoeppner et al., 2001; Reddien et al., 2001). Earlier, Diez-Roux and Lang (1997) had also shown that elimination of macrophages from the anterior chamber of the rat eye resulted in the survival of endothelial cells that were normally programmed to die. Boyle et al. (2001) also found that human macrophages induced apoptosis in plaque derived vascular smooth muscle cells. Similarly, we propose that in the *Botryllus* system, macrophages are needed *in vivo* for the activation and the completion of apoptosis. This can be developed through reactive oxygen species. It is well known that macrophages produce high levels of the reactive oxygen species NO·, which can initiate apoptosis (Chandra et al., 2000; Mates and Sanchez-Jimenez, 2000). Activated macrophages were also found to direct apoptosis of mesangial cell population *in vitro via* the release of NO· (Duffield et al., 2000). The antioxidant BHT could interfere with this process in the *Botryllus* system. It is also documented that various cellular antioxidants, such as catalase and *N*-acetylcysteine, can block apoptosis induced by diverse agents other than oxidants (Mates and Sanchez-Jimenez, 2000). The precise mechanism of action of ROS in the cascade leading to cell death is largely unknown (Feinendegen, 2002; Kagan et al., 2002; Martin et al., 2002). They represent attractive candidates for final common mediators of apoptosis, yet a specific role for ROS in the execution or resolution of the apoptotic program

has not been established (Ragan et al., 2002). However, it is interesting to note that high doses of BHT in plants (wheat seedlings) also blocked the apoptotic process (Bakeeva et al., 2001).

The second way with which BHT may interrupt macrophage activity is through the respiratory burst initiated at the presence of a phagocytic stimulus. In this process, NADPH oxidase, a membrane-associated enzyme that is dormant in resting phagocytes, is activated and then catalyzes a reaction that produces superoxide anion (O_2^-; Halliwell and Gutteridge, 1999; Forman and Torres, 2001). Phagocytosis in *B. schlosseri* is also associated with a respiratory burst, as evidenced by superoxide anion production (Ballarin et al., 1994). During blastogenic stage D, phagocytic cell frequencies increased among circulating *B. schlosseri* hemocytes (Ballarin et al., 1998). Simultaneously, a significant increase in the levels of hydrogen peroxide, acid phosphatase, reactive oxygen metabolite production, and nitrite ion release were recorded (Cima et al., 1996). *In vitro* studies further revealed that phagocytosis by *B. schlosseri* blood cells was significantly reduced in the presence of superoxide dismutase, which decreased superoxide levels (Ballarin et al., 1994). It is therefore possible that the antioxidant capacity of BHT interferes with the generation of superoxide anion, or NO·, thus inhibiting the phagocytic activity.

The third way in which BHT can affect phagocyte activity is by inhibiting lipid oxidation. Phagocytic cells such as macrophages recognize apoptotic cells by specific changes in their cell surface markers through receptors like the scavenger receptors (SRs). SRs are lipoprotein receptors, which have high binding affinity for chemically modified, acetylated or oxidized lipoproteins, and can recognize oxidized low-density lipoproteins (Sambrano et al., 1994). Their wide ligand binding activity makes SRs attractive candidate receptors for the recognition and phagocytosis of apoptotic cells (Platt et al., 1998). Moreover, recent studies (Chang et al., 1999; Tyurina et al., 2002) have demonstrated that apoptotic cells express oxidation-specific epitopes, including oxidized phospholipids, on their cell surfaces. These epitopes serve as ligands for recognition and phagocytosis by elicited macrophages. We found here that lipid oxidation was significantly decreased in BHT-treated *B. schlosseri* ramets. It is likely, therefore, that BHT, a good antioxidant of the lipid phase, also blocks the scavenger receptor. A decrease in phagocytosis by macrophages was seen in guinea pigs after feeding them with high doses of vitamin E, which, like BHT, is an efficient antioxidant of lipid peroxidation (De la Fuente et al., 2000). Then again, *in vitro* treatment with antioxidants such as vitamin E, ascorbic acid, glutathione, *N*-acetylcysteine and thioproline causes an increase in various macrophage functions, such as ingestion and superoxide anion production (Del Rio et al., 1998). This may be the result of reduced levels of antioxidant doses used and the *in vitro* system, as opposed to the *in vivo* system of *Botryllus.*

The observation of high levels of DNA fragmentation in stage D arrested zooids as well as reduced Bcl-2 levels (anti human monoclonal; details in Voskoboynik, 2001) suggests tissue specific apoptotic signals. However, in the presence of BHT, when the first sets of dying cells are not engulfed and removed by the phagocytes, the apoptotic process is arrested. We therefore suggest, that the apoptotic process (the takeover phase) can proceed only in the presence of active phagocytes. These results indicate that proper functioning of blastogenesis requires continuous removal of dying cells and tight coordination between death signals and clearance signals. Recently, we have demonstrated a rapid growth and significant extension of life expectancies in colonies rescued from BHT (Voskoboynik et al., 2002).

Additionally, one need not necessarily invoke defects in macrophage recognition and/or engulfment of apoptotic cells. A fourth possibility is that BHT could be affecting the ability of cells from adult tissues and organs to commit suicide. It is well documented that apoptosis requires energy input from the cell. While the observation of TUNEL-positive cells implies some attempts at initiating the death program, BHT could be affecting other intrinsic aspects of the dying cell, independent of the phagocytic process. This could explain why organs appear morphologically intact in day 3-arrested BHT animals. At this stage, in the absence of other markers of apoptosis (such as annexin staining), it is rather difficult to explore the idea. It is also possible that BHT treatment has additional impacts on either stem cells or on the programmed life span characteristic to colonies of this species (Rinkevich et al., 1992). These aspects need to be further elucidated.

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