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Responses of Host Hemocytes During the Initiation of the Squid-Vibrio Symbiosis

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Abstract. Within hours after colonization of the light organ of the squid *Euprymna scolopes* by its bacterial symbiont *Vibrio fischeri,* the symbiont triggers morphogenesis of the light organ. This process involves the induction of apoptosis in the cells of two superficial ciliated epithelial fields and the gradual regression of these surface structures over a 96-h period. In this study, microscopic examination of various squid tissues revealed that host hemocytes specifically migrate into the epithelial fields on the surface of the light organ, a process that begins before any other indication of symbiont-induced morphogenesis. Experimental manipulations of symbiont-signal delivery revealed that hemocyte infiltration alone is not sufficient to induce regression, and high numbers of hemocytes are not necessary for the induction of apoptosis or the initiation of regression. However, studies with mutant strains of *V. fischeri* that show a defect in the induction of hemocyte infiltration provided evidence that high numbers of hemocytes facilitate the regression of the epithelial fields. In addition, a change in hemocyte gene expression, as indicated by the up-regulation of the C8 subunit of the proteasome, correlates with the induction of light organ morphogenesis, suggesting that bacteria-induced molecular changes in the hemocytes are required for the participation of these host cells in the regression process.

Introduction

Hemocytes, the circulating blood cells of invertebrate hemolymph, are active participants in tissue remodeling. This process has been studied extensively in arthropods during larval metamorphosis and tissue morphogenesis, and in molluscs during wound repair. For example, arthropod hemocytes phagocytose apoptotic cells and tissue debris during metamorphosis, and they participate in both the formation and the destruction of the extracellular matrix during larval metamorphosis and adult tissue remodeling (Whitten, 1964; Scharrer, 1966; Crossley, 1968; Nardi and Miklasz, 1989; Uhrik *et al.,* 1989; Kurata *et al.,* 1992a, b; Rheuben, 1992; Govind and Pearce, 1994; Kiger *et al.,* 2001). Molluscan hemocytes phagocytose cells and tissue debris and produce new extracellular matrix to repair damaged tissues during wound repair (DesVoigne and Sparks, 1968; Sminia *et al.,* 1973; Cheng, 1981; Cowden and Curtis, 1981; Feral, 1988; Franchini and Ottaviani, 2000; Farr *et al.,* 2001).

Arthropod and molluscan hemocytes are also key mediators of innate immune responses (Ratcliffe *et al.,* 1985). During immune challenge, hemocytes from both phyla are known to phagocytose or encapsulate foreign invaders and produce antimicrobial peptides and reactive oxygen species to eliminate the threat (Cheng, 1981; Cowden and Curtis, 1981; Ford, 1992; Pipe, 1992; Cociancich *et al.,* 1994; Carton and Nappi, 1997; Nappi and Vass, 1998; Nappi *et al.,* 2000; Nappi and Ottaviani, 2000; Anderson, 2001; Bachere *et al.,* 2004). Further, as part of the immune response in molluscs, hemocyte-associated remodeling of pathogenic tissue has been reported (Mackin, 1951; Farley, 1968; Villalba *et al.,* 1997; Lee *et al.,* 2001).

Although little is known about cephalopod blood cells, current evidence suggests that there is only one type, the macrophage-like hemocyte (Cowden and Curtis, 1981; Nyholm and McFall-Ngai, 1998). Like the hemocytes of other molluscs, these cells participate in immune responses by phagocytosing or encapsulating bacteria and other foreign particles (Cowden and Curtis, 1981; Ford, 1992; Beuerlein and Schipp, 1998; Malham and Runham, 1998; Nyholm and

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Abbreviations: CAM, chloramphenicol; FSW, filter-sterilized seawater; PGN, peptidoglycan; WT, wild type.

McFall-Ngai, 1998). Cephalopod hemocytes also participate in tissue morphogenesis, repairing damaged tissue by forming cellular clots, or secreting collagen to block epithelial barrier disruptions (Jullien, 1928; Cowden and Curtis, 1981; Polglase *et al.,* 1983; Feral, 1988; Ford, 1992). Some evidence also suggests that they can induce tissue histolysis during inflammatory reactions (Jullien, 1927; Jacquemain *et al.,* 1947; Cowden and Curtis, 1981; Feral, 1988).

The association between *Euprymna scolopes,* the Hawaiian bobtail squid, and *Vibrio fischeri,* its bioluminescent, gram-negative bacterial symbiont, provides an opportunity to study the dual roles of cephalopod hemocytes as both immune sentinels and developmental mediators. In this symbiosis, the squid light organ houses a culture of *V. fischeri* within epithelium-lined crypts. The nocturnal host is thought to use the light produced by the symbiont to obscure its shadow cast by moon and starlight, thus helping it avoid predation (Jones and Nishiguchi, 2004). In return, the host provides the symbiont with nutrients to facilitate its proliferation within the crypt spaces (Graf and Ruby, 1998); however, measures must also be taken to prevent overpopulation. One such measure involves the expulsion of the crypt contents each dawn (Ruby and Asato, 1993; Boettcher *et al.,* 1996). This daily venting reduces the number of symbionts within the crypts by 90%–95%, leaving the remaining cells to repopulate the crypts over the following day. The host's hemocytes, which patrol the crypt lumen and phagocytose the bacterial contents, provide another possible mechanism of population control (Nyholm and McFall-Ngai, 1998).

Hatchling squid (Fig. 1A) are nonsymbiotic, and cells of *V. fischeri* must be harvested from the environment (Nyholm and McFall-Ngai, 2000). The initiation of the symbiosis is facilitated by two fields of epithelia on the ventral surface of the light organ; each field is a ciliated epithelial monolayer that protrudes into two prominent appendages from a base containing three pores (Fig. 1B, C). Within 1–2 h of hatching, these epithelia are induced to shed mucus onto the organ's surface in response to fragments of pepti-

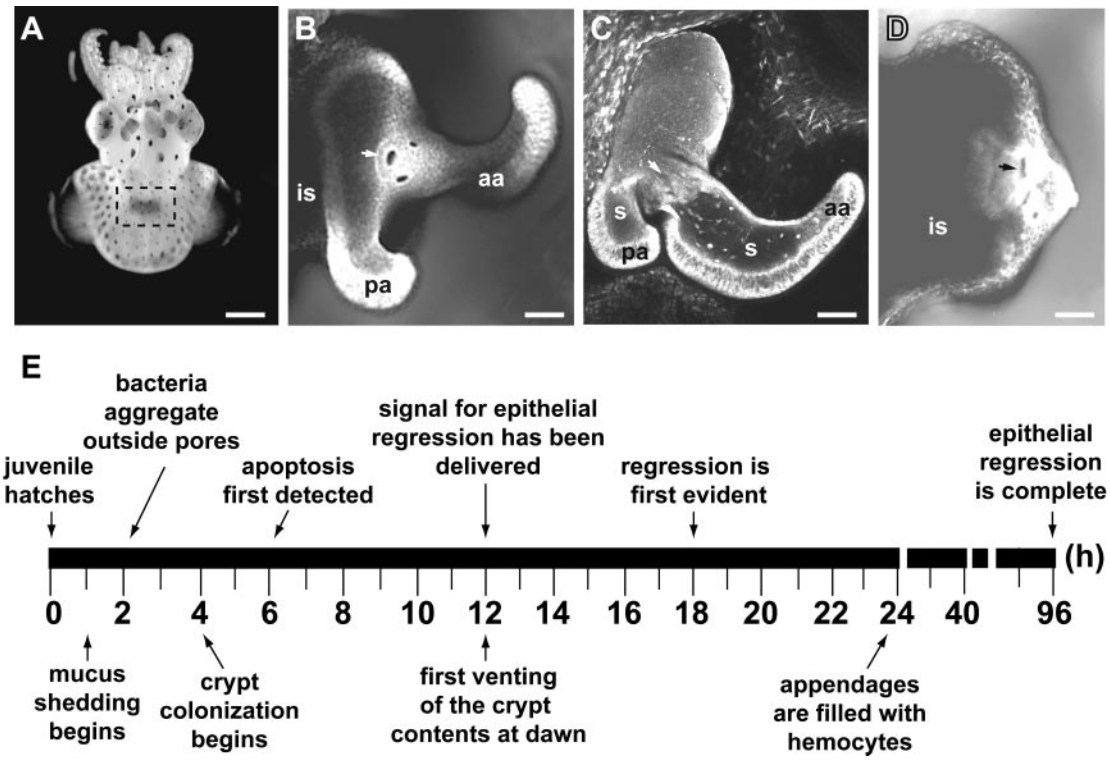

Figure 1. The light organ of the juvenile *Euprymna scolopes.* (A–C) Images were taken at 0 h on the timeline shown in (E), (A) A dorsal view of a newly hatched juvenile squid. The boxed region indicates the location of the light organ within the mantle cavity, which is associated with the ink sac (dark shadow). Scale bar, 500 μm. (B) The ventral surface of one half of a hatchling light organ, showing one epithelial field. Each field contains an anterior (aa) and a posterior (pa) appendage and a base with 3 pores (arrow indicates the medial pore). is, ink sac. Scale bar, 50 μm. (C) A section through one epithelial field reveals that each appendage is composed of a monolayer of epithelial cells overlying a central sinus (s) space. Scale bar, 50 μm. (D) The ventral surface of one half of a 96-h symbiotic light organ. The appendages have been lost during the process of epithelial regression. Scale bar, 50 μm. (E) Timeline illustrates the order of relevant events during symbiotic initiation.

doglycan (PGN), a common component of the bacterial envelope (see Fig. 1E for timeline of events) (Nyholm *et al.,* 2002). Bacterial cells present in the seawater enter the mantle cavity during ventilation, and some of these are captured in the mucus as they pass over the light organ in currents created by the ciliated surface. By 4 h, cells of *V. fischeri* that have aggregated in the mucus begin to migrate through the pores and down ciliated ducts to colonize the crypts deep within the light organ (Nyholm *et al.,* 2000).

After light organ colonization, fragments of PGN and another bacterial envelope component, lipopolysaccharide, act in synergy to induce the morphogenesis of the ciliated epithelial fields on the surface, a process that culminates in the complete regression of the epithelial appendages by 96 h (Fig. 1D) (Montgomery and McFall-Ngai, 1994; Koropatnick *et al.,* 2004). Symbiont-induced changes in the epithelial fields are evident shortly after crypt colonization, starting with the induction of widespread apoptosis by about 6 h after hatching (Foster and McFall-Ngai, 1998). However, regression is not induced until the squid has been incubated with the symbiont for longer than 8 h, and most light organs require a 12-h incubation for the induction of morphogenesis (Doino and McFall-Ngai, 1995). Regression itself is first evident at about 18 h, when cells of the epithelial fields begin to detach and slough into the mantle cavity (unpubl. data).

In addition to their interactions with the bacterial culture within the crypts, the squid's hemocytes are also present in high numbers in the morphogenic epithelial fields of 24-h symbiotic light organs, and this infiltration is induced by a derivative of PGN, the same factor required for the induction of epithelial regression and mucus shedding (Nyholm *et al.,* 2002; Koropatnick *et al.,* 2004). These cells are also the source of transcript expression for a key subunit of the proteasome that is up-regulated in 12-h symbiotic light organs (Kimbell *et al.,* 2006).

The hemocyte population within the morphogenic epithelial fields of the juvenile light organ may serve an immunological function, guarding against breaches in the epithelial monolayer that may occur during appendage regression. Alternatively, these cells may be active participants in the developmental remodeling of the light organ's surface. As a first step in understanding their purpose in the morphogenic epithelial fields, we described the temporal and spatial pattern of hemocyte infiltration into light organs of juvenile *E. scolopes,* and experimentally manipulated the delivery of the morphogenic signal to determine whether the hemocytes are involved in the induction of other hallmarks of light organ morphogenesis.

Materials and Methods

General procedures

Adult squid were collected from shallow sand flats of Oahu, Hawaii, and breeding colonies were maintained as previously described (Doino and McFall-Ngai, 1995; Foster *et al.,* 2000). Newly hatched juvenile squid were transferred through three rinses of filter-sterilized seawater (FSW) to prevent premature inoculation with the symbiont. Animals were maintained as nonsymbiotic in FSW or made symbiotic by placing them in FSW containing 5000 cells of *Vibrio fischeri* per milliliter. Light organ colonization was monitored by measuring squid luminescence with a photometer (Turner Designs, TD-20/20). To cure light organs of *V. fischeri,* animals were transferred to fresh FSW containing the bacteriostatic antibiotic chloramphenicol (CAM) at 30 μg/ml, as previously described (Doino and McFall-Ngai, 1995). Water was changed daily in experiments with incubations longer than 24 h. For all assays involving dissections or whole-animal fixations, squid were first anesthetized using 2% ethanol in FSW. All data presented in this study are representative experiments selected from 2–4 replicates ($n = 6–10$ animals per treatment for each replicate experiment).

Bacterial strains

V. fischeri strain ES114 (Boettcher and Ruby, 1990) was used for all wild-type (WT) symbiotic bacterial inoculations. Several other bacterial strains were used at concentrations of 5000 cells/ml in FSW for certain experiments. Specific assays for hemocyte infiltration also used either the *V. parahaemolyticus* strain KNH1 (Nyholm *et al.,* 2000) or the nonluminous *V. fischeri* strain LKS4, a site-directed (H45A) point mutant in the *luxA* gene of *V. fischeri* ES114, which results in an enzymatically inactive LuxA luciferase subunit (L. Sycuro and E. Ruby, University of Wisconsin, pers. comm.). The ES114-derived CAM-resistant mutant JRM200 (McCann *et al.,* 2003) was used to inoculate animals as a symbiotic control in the antibiotic-curing experiments.

Reagents

Staphylococcus aureus PGN (Fluka, Milwaukee, WI) was prepared as previously described (Nyholm *et al.,* 2002). Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Fluorochromes were purchased from the Molecular Probes branch of Invitrogen (Carlsbad, CA).

Live tissue studies

To identify and count hemocytes in the central sinuses of the light organ appendages and the branchial vessels of the gills, and to examine epithelial fields for evidence of epithelial morphogenesis, live animals were co-stained with 0.001% acridine orange, a fluorochrome that binds nucleic acids, and 0.008% LysoTracker Red, a fluorochrome that labels acidic vacuoles, in FSW for 30 min at room temper-

ature. Ventral dissections were then performed, and animals were mounted on glass slides in 2% ethanol in FSW and analyzed using an LSM 510 confocal microscope (Zeiss, New York, NY). Apoptotic cells were identified by their pycnotic nuclei, which label strongly with acridine orange, and counted in the epithelial fields by fluorescence microscopy as previously described (Foster and McFall-Ngai, 1998).

Regression analysis

Light organs were processed for epithelial regression analysis as previously described (Doino and McFall-Ngai, 1995; Koropatnick *et al.,* 2004). The stage of regression was evaluated by light microscopy, using a scoring system based on the extent of regression induced by the intact symbiont from 0 d (hatchling) to 4 d (0 = no regression, 1 = ciliated ridge absent, 2 = appendages shortened, 3 = posterior appendage absent, 4 = complete regression).

Immunocytochemistry

Acridine orange and LysoTracker Red do not penetrate tissues sufficiently to label the crypt region of the light organ, which is 100–200 μm below the surface. Therefore, we used immunocytochemistry to determine the distribution of hemocytes in these deeper regions by labeling the tissue with a polyclonal antibody to β-actin. This antibody displays a distinctive cross reactivity in hemocytes, and coupled with the extensive permeabolization of the tissue required for whole-mount immunocytochemistry, this technique allows for easy identification of hemocytes deep within the light-organ body. Nonsymbiotic and symbiotic light organs were processed as previously described (Kimbell and McFall-Ngai, 2004).

In situ *hybridization*

Full-length sense and antisense riboprobes for the C8 subunit of the 28S proteasome were constructed, and light organs were processed for *in situ* hybridization as previously described (Kimbell *et al.,* 2006).

Statistics

Experiments were analyzed either by one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons (95% confidence interval), or by two-sample Student's *t* test using Minitab version 13 (Minitab Inc., State College, PA).

Results

Quantification of hemocytes in specific squid tissues

Hemocytes were identified by their characteristic size (10 μm in diameter), their distinctive U-shaped nucleus, and the presence of large, acidic compartments (Nyholm and McFall-Ngai, 1998). These cells infiltrated the appendage sinuses of symbiotic light organs in large numbers and were thus largely responsible for the filling of the sinus spaces of morphogenic appendages (Fig. 2A), a phenomenon previously attributed to the collapse of the basement membrane during appendage regression (Foster and McFall-Ngai, 1998).

To investigate the infiltration of hemocytes into juvenile light organs, hemocytes were counted in the appendage sinuses during the early hours of the symbiosis (Fig. 2B). As early as 2 h after the inoculation of the FSW with cells of *V. fischeri,* a statistically significant ($P < 0.05$) increase in hemocyte numbers could already be detected in symbiont-exposed light organs in 2 of 4 replicate experiments. As the light organ is not yet colonized by 2-h post-inoculation (Fig. 1E), the induction of hemocyte infiltration at such an early time point suggests that the initial signal for infiltration was received from outside the crypt spaces. In support of this theory, the nonsymbiotic *V. parahaemolyticus,* a bacterium that can aggregate in the mucus outside the pores but cannot colonize the light organ (Nyholm *et al.,* 2000), was able to induce significant hemocyte infiltration (Fig. 2C). Further, PGN fragments derived from the gram-positive bacterium *S. aureus* also induced significant infiltration after the 2-h incubation.

From 4 h to 40 h, all replicates consistently showed significantly more hemocytes in the appendages of symbiotic organs than in those of nonsymbiotic organs of the same age (Fig. 2B). Between 2 and 10 h, there were about 2-fold higher levels of hemocytes in symbiont-exposed organs, and the difference increased to between 3- and 4-fold between 12 h and 40 h of symbiont inoculation. Infiltration slowed after the second surge of hemocytes entered the epithelial fields at around 12 h, and the maximum number of hemocytes within the appendage sinuses was reached between 18 and 24 h. By 40 h post-inoculation, the number of hemocytes remained high, even though the process of regression had begun to reduce the volume of the appendage sinuses.

To determine whether the symbiont-induced increase in hemocyte population was specific to the light organ, hemocytes were counted in the central blood vessels of the gills (*i.e.,* branchial vessel) of symbiotic and nonsymbiotic animals (Fig. 2D). Levels of hemocytes within the branchial vessels of 24-h nonsymbiotic and symbiotic animals were indistinguishable. These data suggest that the induction of symbiosis does not stimulate a general increase in circulating hemocytes, a response known to occur during some pathological infections in molluscs (Feng *et al.,* 1971; Jeong *et al.,* 1980; Amen *et al.,* 1991; Oubella *et al.,* 1994; Malham *et al.,* 1998).

Although the sinus spaces within the appendages filled with hemocytes in response to symbiotic initiation, it was

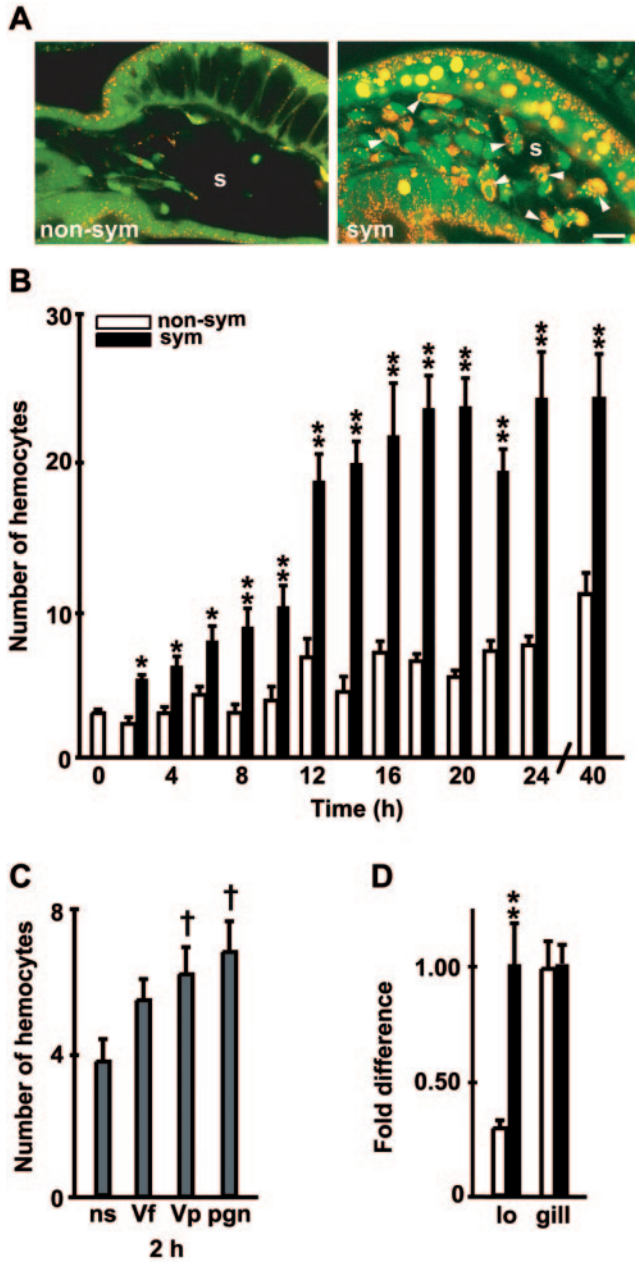

Figure 2. Hemocytes infiltrate the epithelial fields of symbiotic light organs. (A) Confocal micrographs of anterior appendages stained with acridine orange (green), which labels nuclei, and LysoTracker (red), which labels the acidic vacuoles of the hemocytes. Although the sinus spaces (s) within nonsymbiotic (non-sym) organ appendages contain few cells, hemocytes (arrowheads) are present in high numbers within the sinuses of symbiotic (sym) organ appendages by 24 h post-inoculation. Yellow foci, apoptotic cells. Scale bar, 15 μm. (B) Hemocytes were counted in the sinuses of anterior and posterior appendages of one epithelial field per light organ for each time point. (C) Hemocytes were counted in one epithelial field per light organ after a 2-h incubation with the symbiont *Vibrio fischeri* (Vf), the noninfective bacterium *V. parahaemolyticus* (Vp), or peptidoglycan (pgn). ns, nonsymbiotic control. (D) Hemocytes were counted within the central branchial vessel of one gill for each nonsymbiotic and symbiotic animal at 24 h post-inoculation. Hemocytes were also counted in the appendage sinuses of one light organ epithelial field (lo) in the same animal for comparison. The fold difference of hemocyte levels in gills and light organs was calculated by normalizing the symbiotic level to 1. Data are means ± s.e.m. (*) indicates significant ($P < 0.05$) difference compared to the non-sym treatment at the same time point (two-sample *t* test); (**) indicates significant ($P < 0.001$) difference compared to non-sym (two-sample *t* test); (†) indicates significant ($P < 0.05$) difference compared to the non-sym treatment (one-way ANOVA).

possible that these spaces served as an "overflow" site, and the target of hemocyte migration was the bacteria-containing crypt spaces. To identify the target of hemocyte migration within the light organ, hemocytes were counted within the ventral surface and crypt regions of the body of the light organ at 12-h increments over the first 3 days of the symbiosis (Fig. 3). In all light organs examined, significantly ($P < 0.001$) higher numbers of hemocytes were found at the ventral surface than in the crypt regions at all time points. Comparisons of nonsymbiotic and symbiotic light organ revealed no detectable differences in hemocyte numbers in either the surface or crypt regions at 12 h post-inoculation (Fig. 3C). However, by 24 h, an increased number of hemocytes were detected in the surface region of symbiotic organs in comparison to nonsymbiotic organs, and this trend continued until 72 h, when the morphogenesis was nearly complete. Although there was a trend showing the numbers of hemocytes in the crypt regions of symbiotic light organs to be higher than in nonsymbiotic organs between 36 and 60 h, the difference was not statistically significant ($P > 0.05$). Taken together, these data suggest that hemocyte infiltration was specific to the epithelial fields on the ventral surface of symbiotic light organs.

Hemocyte infiltration in relation to other aspects of morphogenesis

Although the principal target of hemocyte migration was determined to be the epithelial fields of symbiotic organs, the purpose of the hemocytes within these fields remained unclear. To investigate whether hemocyte infiltration was necessary and sufficient for any or all aspects of light organ morphogenesis, we next screened several mutant strains of *V. fischeri* to find a strain that was defective in the induction of infiltration. The *luxA* mutant is unable to produce light, as it is defective in a gene encoding one of the subunits of luciferase, the enzyme responsible for luminescence in *V. fischeri* (Visick *et al.,* 2000). This strain initially colonized the light organ at wild-type (WT) levels, and organs infected with the *luxA* mutant showed most of the characteristics of early-stage epithelial morphogenesis by 22 h post-inoculation. Specifically, the *luxA* mutants induced a considerable amount of apoptosis in the epithelial fields (Fig. 4A), as well as epithelial cell detachment from the appendages (data not shown). Although *luxA* mutant-infected light organs did not contain a significant number of hemocytes in the epithelial

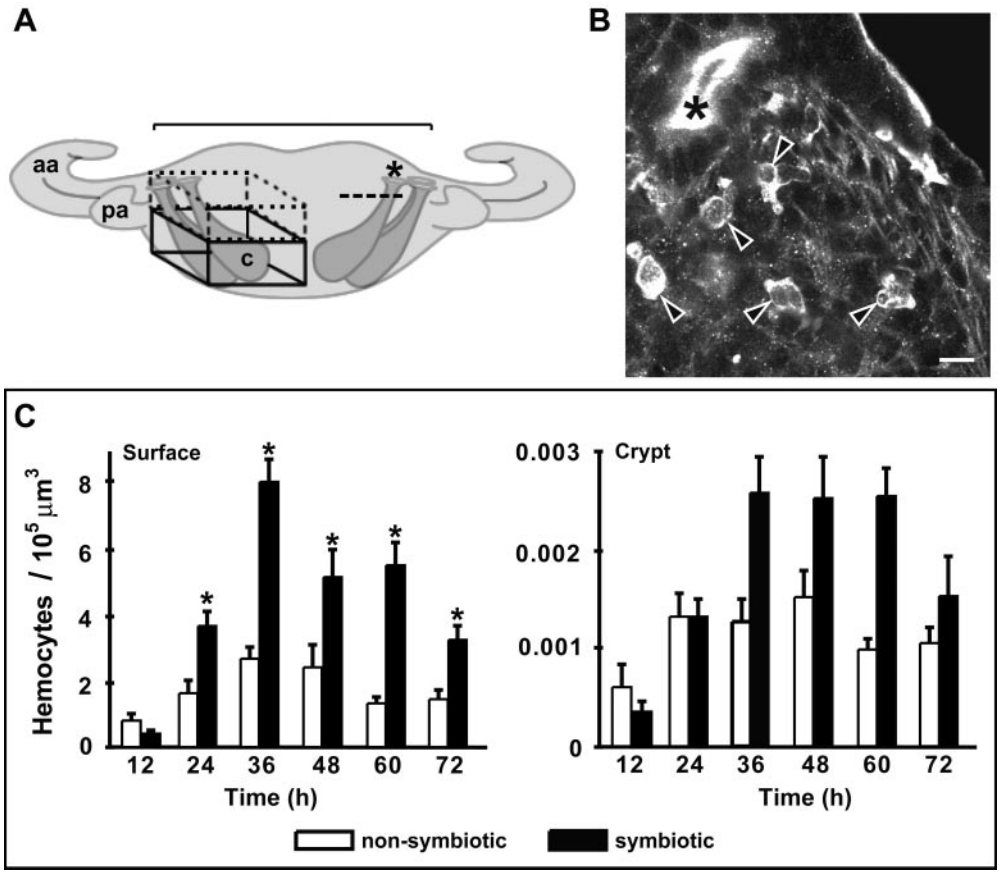

Figure 3. Quantification of hemocytes in the body of the light organ during the initiation of symbiosis. (A) Diagram shows a transverse view of a juvenile squid light organ. The surface of the organ is covered with 2 fields of epithelia that protrude laterally into anterior (aa) and posterior (pa) appendages. These fields also contain the pores (asterisk indicates one medial pore) that lead down ducts into bacteria-containing crypt (c) spaces. The main body (bracket) of the organ can be divided into ventral surface (upper hatched cube; average volume = 3.5×10^5 μm^3) and crypt regions (lower solid cube; average volume = 4.8×10^5 μm^3). Dashed line indicates the location of the confocal micrograph shown in (B). This diagram was contributed by M. McMahon, used with permission. (B) Hemocytes (arrowheads) in the body of the light organ were identified by their characteristic size, shape, and U-shaped nucleus when the actin cytoskeletons were labeled with a polyclonal antibody to β-actin. Asterisk indicates a section through a duct. Scale bar, 10 μm. (C) Hemocytes were quantified in the surface and crypt regions on one side of each nonsymbiotic and symbiotic light organ at 12-h intervals. Data are means ± s.e.m. (*) indicates significant ($P < 0.001$) difference compared to the nonsymbiotic control at the same time point (one-way ANOVA).

fields at 22 h, by 40 h they were similar in numbers to WT-infected organs (Fig. 4B). These data suggest that hemocyte infiltration was simply delayed in organs colonized with the *luxA* mutant. By 48 h, light organs colonized with either WT or the *luxA* mutant showed similar levels of epithelial regression; however, by 96 h WT-induced regression was significantly more extensive than the level induced by the *luxA* mutant (Fig. 4C). Taken together, these data suggest that the WT-induced level of hemocyte infiltration is not necessary for the induction of apoptosis or the early stages of epithelial morphogenesis, but high numbers of hemocytes may be required for, or may facilitate, the process of regression.

Because WT cells of *V. fischeri* induced hemocyte infiltration at a faster rate and also induced a significantly more advanced stage of regression in comparison to the *luxA* mutant, we next investigated whether the delivery of the morphogenic signal for regression was required for the infiltration of peak numbers of hemocytes into the appendage sinuses. Animals were treated with the antibiotic chloramphenicol (CAM) at 8 h post-inoculation to cure the light organ of symbionts. By 8 h, the crypts were colonized (Fig. 1E) and hemocytes were infiltrating the epithelial fields (Fig. 2B); however, the signal for epithelial regression is not delivered until about 12 h post-inoculation (Doino and McFall-Ngai, 1995). When symbiotic light organs were cured at 8 h, there was little or no evidence of epithelial regression by 4 days (data not shown); however, by 40 h

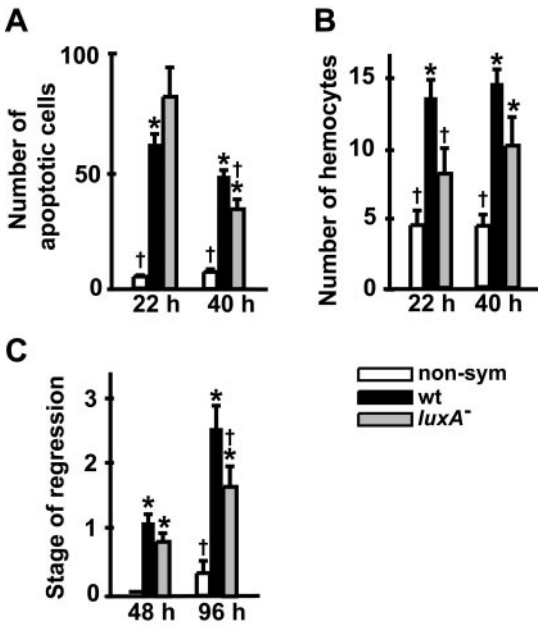

Figure 4. Effects of the *luxA* mutant on light organ morphogenesis. (A) The condensed nuclei of apoptotic cells were counted in one epithelial field per light organ at 22 and 40 h post-inoculation. (B) Hemocytes were counted in the sinuses of anterior and posterior appendages of one epithelial field per light organ at 22 and 40 h post-inoculation. (C) Light organs were scored for epithelial regression at 48 and 96 h post-inoculation. Data are means ± s.e.m. (*) indicates significant ($P < 0.001$) difference compared to non-sym at the same timepoint; (†) indicates significant ($P < 0.001$) difference compared to wild type (wt) at the same timepoint (one-way ANOVA).

post-inoculation hemocytes had infiltrated the appendage sinuses at levels indistinguishable from those reached in organs infected with CAM-resistant cells of *V. fischeri* (Fig. 5). A slight reduction in hemocyte infiltration in all symbiotic organs treated with CAM may indicate subtle effects of the antibiotic on the host; however, the level of infiltration achieved was not out of the range of levels naturally induced by WT cells of *V. fischeri.* Taken together, these data demonstrate that the delivery of the morphogenic signal was not required to induce peak numbers of hemocytes in the epithelial fields, and the presence of high numbers of hemocytes within the appendage sinuses was not sufficient to trigger epithelial regression.

A change in gene expression in the hemocytes of morphogenic light organs

Because high numbers of hemocytes could infiltrate the appendage sinuses without triggering morphogenesis in CAM-cured symbiotic light organs, we next investigated whether differences exist between the hemocyte population of morphogenic appendages and the hemocytes of the non-morphogenic appendages of CAM-cured organs. Previously we reported that a key subunit of the proteasome is transcribed in abundance in hemocytes within the morphogenic epithelial fields of 12-h-symbiotic light organs (Kimbell *et al.,* 2006). Here, symbiotic animals were CAM-cured at 8 h, and *in situ* hybridization was performed to look for this transcript in hemocytes within the epithelial fields at 40 h post-inoculation (Fig. 6). Although nearly all the hemocytes in the sinuses of morphogenic light organs infected with CAM-resistant *V. fischeri* expressed the proteasome transcript in abundance (Fig. 6A), most of the hemocytes filling

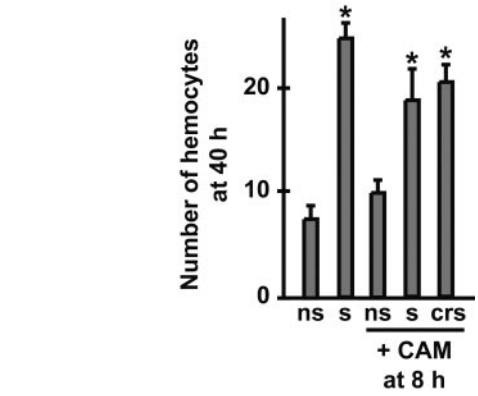

Figure 5. Effects of antibiotic curing on hemocyte infiltration. A cohort of animals was treated with the antibiotic chloramphenicol (CAM) at 8 h to cure the light organ of the symbiont before the delivery of the signal for epithelial regression. Hemocytes were counted in the sinuses of anterior and posterior appendages of one epithelial field per light organ at 40 h post-inoculation. ns, nonsymbiotic; s, symbiotic; crs, light organs colonized with CAM-resistant cells of *Vibrio fischeri.* Data are means ± s.e.m. (*) indicates significant ($P < 0.001$) difference compared to both non-sym controls (one-way ANOVA).

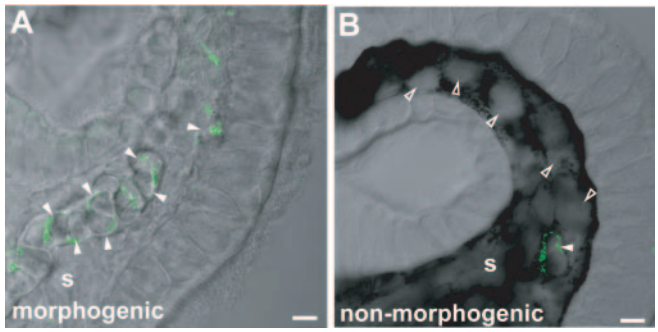

Figure 6. Expression of the C8 subunit of the proteasome in morphogenic and non-morphogenic light organs. Confocal micrographs superimposed over DIC images of anterior appendages show the localization of C8 subunit mRNA by *in situ* hybridization. Animals were treated with the antibiotic CAM at 8 h post-inoculation, prior to the delivery of the signal for epithelial regression, and *in situ* hybridization was performed at 40 h. (A) An appendage of a morphogenic organ that was infected with a CAM-resistant strain of *Vibrio fischeri.* (B) An appendage of a non-morphogenic organ that was cured of its symbionts prior to the 12-h morphogenic signal. Solid arrowheads, hemocytes that express detectable levels of C8 mRNA; open arrowheads, hemocytes that do not express detectable levels of C8 mRNA; s, sinus. Scale bar, 10 μm.

the appendage sinuses of non-morphogenic, CAM-cured organs did not express detectable levels of this transcript (Fig. 6B). These results provide the first evidence that the hemocytes respond specifically to the signal for epithelial regression.

Discussion

The results of this study show that the squid's macrophage-like hemocytes migrate specifically into the epithelial fields of symbiotic light organs. Further, although this hemocyte infiltration is not sufficient to induce the regression of the epithelial fields and not necessary for the induction of either apoptosis or epithelial cell detachment from the epithelial fields, high numbers of hemocytes may facilitate appendage regression. Finally, transcription of a key subunit of the proteasome is increased in hemocytes within light organs that have been induced to undergo epithelial regression, suggesting that the hemocytes respond to the morphogenic signal with a change in gene expression that may be required for the process.

Hemocytes begin to migrate into the sinuses of the light organ appendages as early as 2 h after the seawater is inoculated with symbiotic *Vibrio fischeri,* nonsymbiotic *V. parahaemolyticus,* or peptidoglycan (PGN) isolated from *Staphylococcus aureus* (Fig. 2B, C). This phenomenon is temporally correlated with another hallmark of symbiotic initiation, the induction of mucus shedding from the ciliated epithelial fields 1–2 h after the squid hatch, which is also induced by nonspecific bacterial PGN (Fig. 1E) (Nyholm *et al.,* 2002). Bacteria aggregate in this mucus during the harvesting process, resulting in the colonization of the crypts by symbiotic cells of *V. fischeri* several hours later (Nyholm *et al.,* 2000). Because both hemocyte infiltration and mucus shedding are induced at about the same time by the same bacterial factor, it is possible that these two host responses share the same host receptors. Receptors for PGN-induced mucus shedding were demonstrated to be localized outside the crypts (Nyholm *et al.,* 2002) and are presumably situated on the apical surface of the cells of the epithelial fields. These epithelial cells may be induced to shed mucus from their apical surfaces through direct interactions with environmental PGN, while at the same time they may also release an unknown chemotactic factor into the appendage sinuses to signal the infiltration of hemocytes.

Although hemocyte infiltration can be detected in light organ appendages as early as 2 h after the seawater has been inoculated with cells of *V. fischeri,* hemocyte numbers increase markedly at about 12 h post-inoculation (Fig. 2B), suggesting that a second, reinforcing signal for hemocyte infiltration is delivered. The timing of this second signal correlates with the delivery of the signal for epithelial regression, which takes place within the ducts or the crypts of the fully colonized light organ by 12 h (Doino and McFall-Ngai, 1995). Like this signal for morphogenesis, the reinforcing signal for hemocyte infiltration may be delivered within the ducts or the crypts, a process that would require some system of signal transduction to the epithelial fields on the surface. However, the surge in hemocyte infiltration at 12 h is also roughly coincident with the time of the first venting of 95% of the crypt contents at dawn (Lee and Ruby, 1994; Boettcher *et al.,* 1996). Thus, it is also possible that this second signal for infiltration may be triggered by the ventate as it is expelled through the ducts and onto the surface, where PGN fragments can interact directly with receptors on the epithelium and stimulate these cells to release more chemotactic factor into the appendage sinuses.

The onset of hemocyte infiltration into the light organ precedes all signs of symbiont-induced morphogenesis, including apoptosis, and the cell sloughing that represents the onset of epithelial regression. Similarly, studies of microbial and parasitic infections in molluscs often report hemocyte infiltration prior to other signs of tissue remodeling (Mackin, 1951; Farley, 1968; Lauckner, 1983; Villalba *et al.,* 1997; Lee *et al.,* 2001). In these studies, the hemocytes appear to take an active role in the destruction of the basement membrane, cell sloughing, histolysis, or atrophy associated with pathogenic tissue remodeling. Likewise, several studies have documented the infiltration of hemocytes prior to, or concurrent with, signs of developmental morphogenesis in insects (Nardi and Miklasz, 1989; Rheuben, 1992; Kiger *et al.,* 2001; Nardi *et al.,* 2001). In these cases, the hemocytes participate in the destruction of the extracellular matrix in target tissues during metamorphosis-associated remodeling.

Blood cell infiltration can also be a late-stage event during both vertebrate and invertebrate larval metamorphosis (Weber, 1964; Whitten, 1964; Scharrer, 1966; Crossley, 1968; Kinoshita *et al.,* 1985). In these instances the blood cells participate as phagocytes that take up and dispose of the apoptotic cells and debris that are the remnants of previously degraded larval tissues. In the morphogenic squid light organ, previous work has described the symbiont-induced apoptosis of cells of the epithelial fields; however, we have found no evidence that these cells are removed by phagocytosis (Foster and McFall-Ngai, 1998).

Although hemocyte infiltration is not necessary for the induction of the early signs of light organ morphogenesis, it is possible that the basal population of hemocytes in the nascent epithelial fields can participate in these early morphogenic events. Additionally, a timely infiltration of hemocytes correlates with a more advanced stage of regression in wild-type *V. fischeri*-colonized organs (Fig. 4); thus hemocytes apparently facilitate the regression process. To determine whether these phagocytic cells are indeed required for organ morphogenesis, they might be eliminated using phagocyte-specific toxins such as phenylalanine

methyl ester (Little and Flores, 1993). Although preliminary experiments indicate that phenylalanine methyl ester (1 mmol l^{-1}) is an effective hemocyte toxin *in vitro,* this compound is highly toxic to juvenile *E. scolopes* (data not shown). Further experimentation using this and other cytotoxins awaits the development of techniques for culturing the light organ.

A transient, 8-h exposure to the symbiont is sufficient to induce the infiltration of peak numbers of hemocytes in appendage sinuses (Fig. 5). However, the appendages do not regress without the delivery of the morphogenic signal by about 12 h (Doino and McFall-Ngai, 1995). These data are in contrast to those reported in previous studies of bivalve molluscs, in which it is speculated that hemocyte aggregates block the circulation of oxygen and nutrients to tissues and thus induce the atrophy associated with pathogenic tissue infections (Villalba *et al.,* 1997; Lee *et al.,* 2001).

Most hemocytes that aggregate within the epithelial fields of non-morphogenic, 8-h-cured light organs fail to exhibit the characteristic increase in a key subunit of the proteasome that is abundantly expressed in the hemocytes of appendages undergoing morphogenesis (Fig. 6). These data provide evidence that although hemocyte infiltration is not sufficient for epithelial regression, hemocytes that have infiltrated the epithelial fields are responsive to a subsequent signal for regression, and a change in proteasome gene expression may be critical for the process. The proteasome, a multi-component enzyme complex, is an important regulator of many cellular processes (Coux *et al.,* 1996), and various subunits of the proteasome are known to be differentially regulated during both immunological challenge (Maksymowych *et al.,* 1998; Ichikawa *et al.,* 2000; Rosenberger *et al.,* 2000), and tissue remodeling (Temparis *et al.,* 1994; Samuels *et al.,* 1996; Llovera *et al.,* 1998; Price, 2003). Thus, the increased proteasome transcription observed in hemocytes within morphogenic light organs may indicate that these cells are participating in developmental or immune-related processes during the symbiont-induced morphogenesis.

The developmental morphogenesis of the squid light organ is intimately associated with the innate immune system. Hemocyte infiltration, apoptosis, and epithelial regression are all triggered by derivatives of the innate immune signal factors LPS and PGN (Foster *et al.,* 2000; Koropatnick *et al.,* 2004). Immunological responses to LPS and PGN are signaled through certain evolutionarily conserved pathways such as Toll/NF-κB (Hoffmann *et al.,* 1999). Transcripts coding for members of this pathway have been identified in juvenile light organs (Goodson *et al.,* 2005), and their involvement in the signaling of light organ morphogenesis is being investigated.

Links between development and immunity have also been established in other invertebrates. For example, the Toll-dorsal signal transduction pathway in *Drosophila melanogaster* directs dorsal-ventral patterning during embryogenesis, and it also mediates the production of antimicrobial peptides and hemocyte activation during immune challenge (Belvin and Anderson, 1996). Some evidence also suggests that certain defense-related proteins play a role in tissue histolysis during insect metamorphosis (Tryselius *et al.,* 1992; Natori *et al.,* 1999), and the onset of larval metamorphosis in an ascidian has been correlated with the differential expression of immune-related genes and the transepidermal migration of hemocytes into the larval tunic (Davidson and Swalla, 2002).

Here, we have demonstrated that hemocytes specifically infiltrate the epithelial fields of symbiont-exposed squid light organs and undergo a change in transcriptional activity that may be important for their ability to facilitate light organ morphogenesis. Although the purpose of the hemocytes in the morphogenic epithelial fields remains unclear, we know that the bacterial signal for hemocyte infiltration is a derivative of the potent immune signal factor PGN (Koropatnick *et al.,* 2004). Thus, one can speculate that the hemocytes may serve a defensive function, and their contribution to epithelial regression occurs as a consequence of inflammation- associated remodeling. As an alternative, we propose that the hemocytes infiltrate the epithelial fields specifically to participate in tissue remodeling, and the triggering of this developmental phenomenon by immune-related signal factors provides further evidence for the evolutionary link between the immune system and the regulation of developmental morphogenesis.

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