Abstract  Associations with pathogenic bacteria have recently been shown to initiate apoptotic programs in the cells of their animal hosts, where host cell death is hypothesized to be a response of the immune system, either initiated as a mechanism of host defense or bacterial offense. In this study, we present evidence that bacterial initiation of apoptosis is neither restricted to pathogenesis nor to the initiation of an immune response. In the cooperative association between the sepiolid squid Euprymna scolopes and the luminous bacterium Vibrio fischeri, the bacteria induce a dramatic morphogenesis of the host tissues during the first few days of interaction between these partners. The most striking change is the bacteria-triggered loss of an extensive superficial epithelium that potentiates the infection process. Our analyses of these tissues revealed that the bacteria induce apoptosis in the cells that comprise this epithelium within hours of the interaction with bacteria. Ultrastructural analysis revealed that after 24 h the integrity of the epithelium had been lost, i.e., the basement membrane had degenerated and the majority of the cells exhibited signs of apoptosis, most notably chromatin condensation. Analysis of these tissues with probes that reveal intracellular acidification showed that the cells first undergo an initial acidification beginning about 6–8 h after exposure to V. fischeri. As determined by end-labeling of DNA fragments, extensive endonuclease activity was detected at approximately 16–20 h post-infection. These data provide evidence that cooperative bacteria can participate in the remodeling of host tissues through the induction of host apoptotic programs.

Key words  Euprymna scolopes · Vibrio fischeri · Apoptosis · Symbiosis

Introduction  A central role for programmed cell death, or apoptosis, in developmental processes has been recognized for several years as a result of research on a number of vertebrate and invertebrate systems (White 1996). Most of these studies have focused on the cell-cell signals that trigger apoptosis, the biochemical pathways that characterize the progression through the process, or the genetic basis by which cells either affect or inhibit the program itself. The results of these analyses have indicated that there exist conserved mechanisms underlying cell death programs throughout the animal kingdom (Vaux et al. 1994).

Several inducers have been implicated in potentiating these programs, including intrinsic signals within the animal (Milligan and Schwartz 1997), abiotic environmental triggers (e.g., Ojeda et al. 1994), or biotic influences, thus far limited to microbial pathogens (Norimatsu et al. 1995; Guichon and Zychlinsky 1996; Garcia-del Portillo et al. 1997; Shen and Shenk 1997). The involvement of native, beneficial (i.e., nonpathogenic) bacteria in the development and homeostasis of the host tissues with which they interface, such as the intestinal epithelium and the immune system, as well as the role of symbiotic bacteria-induced apoptosis as a component of these processes, has not been extensively addressed. However, recent studies with germ-free mice have indicated that bacteria exert a profound effect on the biochemistry and microanatomy of these tissues during their maturation, and it is suspected that the native microbiota play a critical role in the induction of the extensive apoptotic programs that characterize these tissue types (Gordon et al. 1997).

Bacteria-induced cell death has been reported in the normal development of the tissues of another animal model, the Hawaiian sepiolid squid Euprymna scolopes (Montgomery and McFall-Ngai 1994). Adult members of this species house the luminous bacterium Vibrio fischeri extracellularly, deep within epithelial crypts of the symbiotic organ that are similar in microanatomical arrangement to that described for the more complex intes-
tinal consortial symbioses characteristic of most, if not all, animal species (McFall-Ngai and Montgomery 1990). Studies of the development of this light-emitting organ have shown that the adult morphology differs markedly from that of the juvenile, and that the bacteria are essential for the normal developmental process (McFall-Ngai and Ruby 1991; Montgomery and McFall-Ngai 1994). Specifically, during embryogenesis, the host develops an organ morphology that appears to potentiate the infection process (Fig. 1). On either side of the organ of a newly hatched animal (Fig. 1A) a superficial, ciliated, microvillous field of cells (Fig. 1B) serves to bring environmental *V. fischeri* into the vicinity of three pores. Each field is comprised of an anterior and a posterior epithelial ‘appendage’ extending from a basal pad of epithelial cells whose medial edge bears a ridge with elongated cilia. The bacteria enter the pores and travel down ducts to sac-like crypt spaces, the site of symbiotic colonization (Montgomery and McFall-Ngai 1993). Previous studies of the *E. scolopes-V. fischeri* system have shown that the bacteria induce a series of morphogenetic changes in the host tissues that mediate the transition from the initiation morphology of the juvenile light organ to the functional, maintenance morphology characteristic of the adult condition (Montgomery and McFall-Ngai 1994; Doino and McFall-Ngai 1995; Lamarcq and McFall-Ngai 1998).

In the overall array of bacteria-induced developmental alterations in the host tissue, the most dramatic of the early morphogenetic events is the loss of the extensive superficial field of cells over the first 4 days of the association with the symbionts (Fig. 1C; Montgomery and McFall-Ngai 1994; Doino and McFall-Ngai 1995). Acridine orange staining of the whole organ revealed areas of condensed chromatin in the cells of this remote superficial field in symbiotic animals only (Fig. 1D,E), which suggested that this tissue is lost as the result of an extensive cell death program (Montgomery and McFall-Ngai 1994). One important feature of this process is that the cells of this superficial field are not in direct contact with the growing culture of bacteria located in the crypts; the crypt epithelium with which the bacteria interface does not undergo cell death and is present throughout the symbiosis.

In the present study we have analyzed the symbiotic organ at the morphological, molecular, and biochemical levels to determine the precise nature of this superficial field of cells and to determine whether the bacterial symbionts trigger true apoptosis rather than necrosis in the cells of this tissue. Our results indicate that *V. fischeri*, a benign, cooperative symbiont, induces an apoptotic cell death program in this remote field of cells that results in the dramatic morphogenesis of the host’s superficial epithelium. Our data thus indicate that microorganisms are capable of inducing host cell apoptosis not only during the progression of a pathogenic infection, but also during the development of a cooperative association in a specific symbiosis.

### Materials and methods

#### General procedures

Adult *Euprymna scolopes* were collected and maintained under laboratory conditions as previously described (Montgomery and McFall-Ngai 1993; Lamarcq and McFall-Ngai 1998). The infection and successful colonization of host light organs with symbionts was performed using standard protocols (as reviewed by Ruben 1996). Briefly, immediately upon hatching, juveniles were placed individually in scintillation vials in offshore seawater, which does not contain infective strains of *V. fischeri* but does contain, on average, 10⁶ cells ml⁻¹ of a variety of bacterial species. To render juveniles symbiotic, they were exposed to offshore seawater to which 10⁴ cells ml⁻¹ of *V. fischeri* ES114 (a light organ isolate) had been added. Successful colonization with *V. fischeri* was determined by monitoring the onset of luminescence with a photometer (Model 3600, Biospherical Instruments, San Diego, California). [In the absence of *V. fischeri*, the light organ is not colonized by other bacterial species (McFall-Ngai and Ruby 1991), i.e., animals maintained in offshore seawater remained aposymbiotic.] All chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise noted.

#### Electron microscopy

To characterize the ultrastructural morphology of the cells comprising the ciliated microvillous field, tissues were analyzed by transmission electron microscopy (TEM). Live juvenile squid were fixed for TEM by placing them directly in 2.5% glutaraldehyde/2.5% paraformaldehyde in a buffer of 0.1 M sodium cacodylate with 0.45 M NaCl, pH 7.4, at 23°C for 1 h. The specimens were then rinsed three times for 15 min in the same cacodylate/NaCl buffer. After rinsing, the animals were post-fixed for 45 min with 1% osmium tetroxide in the cacodylate/NaCl buffer. The animals were then rinsed in buffer as before, and dehydrated with a graded ethanol (EtOH) series. Five percent uranyl acetate was added to the 30% EtOH dehydration step to increase the contrast of the tissue sections. Specimens were infiltrated with 100% propylene oxide for 15 min, followed by an overnight infiltration in a 1:1 ratio of propylene oxide and accelerated Spurr (Spurr 1969). The animals were then infiltrated with 100% accelerated Spurr for 3 days at room temperature, and embedded at 60°C for 24 h with freshly prepared accelerated Spurr. Thin sections were stained with a saturated solution of uranyl acetate and Reynolds lead citrate, then examined with a JEOL CX-100 transmission electron microscope.

Specimens prepared for scanning electron microscopy (SEM) underwent a similar fixation procedure as those prepared for TEM. However, after the ethanol dehydration series, specimens were critical-point dried, mounted on an SEM stub, and the mantle was dissected away exposing the light organ. The sample was then sputter coated with gold and examined with a Cambridge 350 scanning electron microscope.

#### Visualization of condensed chromatin

To examine the pattern of cell death in symbiotic light organs, juveniles were anesthetized in a 1:1 solution of 0.37 M MgCl₂/sea-water, then incubated for 1 min in a 5-ng/ml solution of acridine orange, a fluorescent probe that specifically highlights areas of condensed chromatin (Delic et al. 1991). After staining, the mantles and funnels of the squid were removed, exposing the light organ, and the animals were examined either with an Olympus B-MAX50 confocal microscope or a Nikon HFX-11 epifluorescence microscope.

#### Probing for DNA fragmentation

To determine whether cell death involved the type of DNA fragmentation characteristic of apoptosis [i.e., cleavage of the linker
**Fig. 1A–E** The juvenile light organ of the host *Euprymna scolopes*.  

**A** Ventral dissection of a newly hatched squid showing the organ’s location (arrow) in the center of the mantle cavity where it is invested in the black ink sac (*bar* 500 µm). In this dissection, the transparent ciliated field cannot be seen against the background of other tissues.  

**B** Scanning electron micrograph (SEM) revealing the conspicuous ciliated, microvillous field on each lateral face of a hatchling light organ. The white arrow corresponds to location of the black arrow in Fig. 1A [aa anterior appendage, pa posterior appendage, cr ciliated ridge (arrowhead), bar 100 µm].  

**C** SEM of a 4-day symbiotic animal demonstrating the bacteria-induced loss of the lateral fields. (*bar* 100 µm).  

**D** Acridine orange (AO)-stained, 14-h aposymbiotic juvenile (i.e., not exposed to symbiosis-competent *Vibrio fischeri*) showing no signs of cell death (*bar* 100 µm).  

**E** AO-stained, 14-h symbiotic juvenile revealing a predictable pattern of cell death. Cells with condensed chromatin appeared along the ciliated ridge (arrow) and throughout the associated appendages (*bar* 100 µm).

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**Fig. 2A, B** Structure of the ciliated, microvillous field upon hatching.  

**A** A confocal micrograph of one half of the light organ revealing that each appendage is comprised of cells overlying a basement membrane-lined sinus. The sinus extends to the tips of both appendages. Animal cells (arrows) free in the sinus accumulate toward its base. Also visible in this micrograph are three pores (1, 2, 3) through which the bacterial symbionts enter the light organ and travel to crypts deep in the light organ’s interior (not shown; aa anterior appendage, pa posterior appendage, bar 50 µm).  

**B** Cross section through an anterior appendage of a light organ showing that it is comprised of a single layer of epithelial cells. Numerous vacuoles (v) could be seen in this epithelial tissue, usually at the base of these cells. This section also captured a free-floating cell (arrow) in the sinus (s, *bar* 25 µm).
DNA between nucleosomes producing fragments of multiples of approximately 300 base pairs (bp), we employed the terminal deoxytransferase-mediated dUTP-biotin nick end labeling (TUNEL) method for tagging the fragmented ends of the DNA. To prepare the sample for the TUNEL reaction, tissue was fixed and embedded in paraffin. Briefly, whole squid were fixed in 2.5% glutaraldehyde/2.5% paraformaldehyde in an 0.1 M sodium cacodylate buffer with 0.45 M NaCl, pH 7.4, for 1 h. The samples were rinsed three times for 15 min each in the cacodylate/NaCl buffer and dehydrated with a graded ethanol series, then transferred to toluene for 30 min. The tissue was then infiltrated overnight at 56°C with a 1:1 ratio of toluene and paraffin. The animals were subsequently moved to 100% paraffin for 24 h at 56°C, then embedded in paraffin molds at 23°C. Sections of 4-µm thickness were cut on a Carl Zeiss HM 340 E microtome and mounted on TESPA (3-aminopropyl triethoxysilane)-coated slides.

Sections were dewaxed by heating the slides for 30 min at 60°C and then immersing the slides in 100% xylene for 10 min. Once dewaxed, the sections were rehydrated by placing the slides for 3 min intervals in 95 (× 2), 90, 80% EtOH, and then finally in deionized water. To protect the DNA from DNAses, sections were incubated in a solution containing 20 µg of proteinase K per ml of 10 mM TRIS-HCl, pH 7.2, for 30 min at 37°C, then rinsed in a 0.1 M sodium phosphate buffer containing 0.45 M sodium chloride, pH 7.2 [marine phosphate buffered saline (PBS)]. To inactivate the endogenous peroxidases, the sections were treated with 0.3% H2O2 in methanol for 30 min at 23°C, then rinsed in marine PBS. The DNA fragments were visualized using a commercially available kit ("In Situ Cell Death Detection Kit, POD", Boehringer Mannheim, Indianapolis, Ind.) according to manufacturer's instructions.

Evidence of endonuclease activity

Another means of determining whether the cells are undergoing apoptotic or necrotic cell death is to assay for characteristics of these types of cell death using molecular markers. One well defined characteristic of apoptosis is the breakdown of nuclear DNA by endonucleases into DNA fragments in multiples of 300 bp (Gavrieli et al. 1992). Typically this phenomenon is observed as a ladder pattern of the DNA when it is run on an agarose gel. In preliminary experiments with cells of whole juvenile light organs from symbiotic animals, a DNA ladder was not observed at any time during the first 3 days of colonization by V. fischeri (data not shown). These data suggest that either the DNA does not fragment or that the ratio of healthy to dying cells in the light organ precludes the detection of a visible ladder on agarose gels. This type of complication has been noted in other systems (Oberhammer et al. 1993). As an alternative approach, we employed another commonly used technique, the TUNEL method, which specifically labels the fragmented DNA in the apoptotic cells of tissue sections (Gavrieli et al. 1992; Sgonc et al. 1994). This technique allowed us to identify both the extent and specific location of endonuclease activity at any given time point during our experiments. Using this method, no evidence for endonuclease activity was detected in the cells of the superficial epithelium in aposymbiotic animals (Fig. 5A). However, beginning at 14–18 h after inoculation with symbiosis-competent bacteria, nuclei exhibiting endonuclease activity began to appear in the anterior and poste-
Fig. 3A–C  Ultrastructure comparisons of aposymbiotic and symbiotic light organ appendages in cross section and by scanning electron microscopy (SEM; only left half shown). A A 24-h aposymbiotic juvenile revealing that the overall ultrastructure of the appendage remained unaltered (bar 20 µm). Inset: An SEM showing that the superficial field, including the ridge of elongated cilia (arrow), also remained unchanged (bar 100 µm). B A 24-h symbiotic juvenile exhibiting a loss of polarized organization of the epithelium (bar 20 µm). Inset: An SEM showing a similar superficial field to that of the aposymbiotic juvenile except for the loss of the prominent ciliated ridge (arrow, bar 100 µm). C A 72-h aposymbiotic juvenile revealing that the ultrastructure of the appendage is unchanged from that of a newly hatched animal (bar 20 µm). Inset: An SEM showing the fully intact superficial field (bar 100 µm).

Biochemical evidence of apoptosis

Apoptosis in many systems is associated with acidification of the cellular compartments (Barry and Eastman 1992; Barry et al. 1993). Using LysoTracker, which labels acidic compartments within cells, no significant fluorescent signal above background autofluorescence was observed in the ciliated, microvillous fields of aposymbiotic animals (Fig. 6A). However, beginning at 10 h post-inoculation with symbiosis-competent bacteria, a distinctive pattern of staining appeared along the superficial ciliated ridge in symbiotic animals. By 12 h the staining pattern extended throughout the anterior and posterior appendages, and this pattern strongly resembled the acridine orange staining of the organ at that time point (Fig. 6B). Double-labeling experiments using acridine...
orange and LysoTracker Red indicated that the staining of these two different fluorochromes was not coincident within these cells. These data suggest that cell death in the juvenile light organ is accompanied by acidification of cellular compartments. Although lysosomal rupture has been associated with the final stages of necrosis (Mundle and Raza 1995), there has been no documentation of sustained acidification in the intracellular environment of necrotic cells.

Discussion

The results of these studies indicate that the transient tissue comprising the surface of the newly hatched squid’s light organ is a simple epithelium that regresses as a result of apoptosis, and not necrosis, induced by interaction with the symbiont Vibrio fischeri. The progression of death events (Fig. 7) occurring in the symbiotic organ parallel those that are generally accepted as the characteristic series of apoptotic changes in other systems. First, there is an initial acidification of the cell, followed by extensive nuclear condensation, phagocytosis of fragmenting bodies, and finally activation of endonucleases. Regardless of the cell type or animal species, the order in which these events appear remains invariant (Arends et al. 1990; Kroemer et al. 1995). In addition, the timing of the apoptotic process correlates with that described in other systems, i.e., apoptosis does not begin for several hours (6 to 30 h) following an initial trigger in a number of other, well described programs, such as the neuronal death in Drosophila melanogaster and Manduca sexta (Schwartz 1992) and disease-related cell death in plants (Levine et al. 1994; Mittler and Lam 1997). Thus, although the full regression of this epithelial field takes place over a period of 4 days, most of the associated apoptotic program occurs within the first few hours of animal-bacterial interaction. The remaining regression appears to involve the gradual removal of cell debris over
Fig. 6A, B Fluorescence micrographs of 14-h aposymbiotic and symbiotic light organs stained for the presence of acidic organelles using the fluorescent probe LysoTracker Green (Molecular Probes, Eugene, Ore.; only one half of light organ shown). A Aposymbiotic light organs showed little evidence of acidification in the cells of the ciliated microvillous field; however, some of the free cells in the sinus appeared to contain acidified regions (aa anterior appendage, pa posterior appendage). B Symbiotic light organs had extensive labeling providing evidence for widespread acidification in the cells along the ciliated ridge and appendages (cr ciliated ridge, bar 50 µm).

Fig. 7 The time frame over which the bacteria-induced apoptotic events occurred in the superficial epithelial field. The bacteria, entrained by the beating of the ciliated, microvillous field, enter the light organ through superficial pores (open circles) within the first few hours after hatching. These bacteria then colonize the epithelial-lined crypts in the light organ interior (not shown) and initiate regression of the superficial field. At approximately 10 h post inoculation a sharp increase of cellular acidification (green areas) in the ciliated microvillous field could be observed. At 14 h the condensed chromatin (orange areas) reached its peak, occurring in a predictable pattern along the ciliated ridge and appendages. After 24 h of exposure to symbiosis-competent bacteria the majority of the cells of the ciliated microvillous field exhibited endonuclease activity (brown shading). All these described apoptotic events were initiated early on in the symbiosis, although the complete regression of the ciliated microvillous fields required an additional 72 h.
the following days, presumably by the ever-dwindling number of living cells in the field.

The initiation of a classic apoptotic program in response to interactions with a cooperative bacterium suggests the possibility that beneficial symbionts may participate in tissue remodeling during the normal development of tissues that typically interact with bacteria, such as the intestinal epithelium (Gordon et al. 1997). While, to our knowledge, this is the first report of cell death triggered by a cooperative symbiont, numerous studies have shown that certain microbial pathogens can initiate either apoptosis or necrosis in host animal and plant tissues (Levine et al. 1994; Stevens and Czuprynski 1996; Berninghausen and Leippe 1997; Mittler and Lam 1997; Garcia-del Portillo et al. 1997; Kurito-Ochiai et al. 1997; Thirumalai et al. 1997). For example, Bordetella pertussis, the causative agent of whooping cough, and Shigella flexneri, responsible for bacillary dysentery, both cause cell death of host macrophages (Khlef and Guiso 1995; Zychlinsky et al. 1996). In such cases, the data suggest that the pathogens induce apoptosis to avoid host defense responses. In other instances, it appears that apoptosis is a host defense mechanism to rid itself of the threatening pathogens (Zychlinsky et al. 1996). In such cases, the data suggest the possibility that beneficial symbionts may initiate apoptosis or necrosis in host animal and plant tissues (Levine et al. 1994; Stevens and Czuprynski 1996; Berninghausen and Leippe 1997; Mittler and Lam 1997; Garcia-del Portillo et al. 1997; Kurito-Ochiai et al. 1997; Thirumalai et al. 1997). For example, Bordetella pertussis, the causative agent of whooping cough, and Shigella flexneri, responsible for bacillary dysentery, both cause cell death of host macrophages (Khlef and Guiso 1995; Zychlinsky et al. 1996). In such cases, the data suggest that the pathogens induce apoptosis to avoid host defense responses. In other instances, it appears that apoptosis is a host defense mechanism to rid itself of the threatening microbial population; for example, dozens of genes have been implicated in the development of the symbiosis between nitrogen-fixing bacteria (rhizobia) and leguminous plants, in which the expression of these genes results from a reciprocal dialogue between partners of the association (for review see van Rhijn and Vanderleyden 1995).

Another interesting aspect of the triggering of apoptosis in this system is the spatial relationship between the bacterial symbionts and the cells destined to undergo apoptosis. Previous studies showed that, to signal the cell death process, the bacteria must enter and colonize the crypt spaces (Doiino and McFall-Ngai 1995), which are not in direct contact with the superficial field that undergoes apoptosis. When this epithelium is exposed to high concentrations (10^6 cells/ml) of mutant strains of V. fischeri that cannot enter the crypts (e.g., flagellar and motility mutants), no cell death is observed despite the opportunity of these bacterial cells to interact directly with the host’s superficial epithelial surfaces. These studies suggest that the interaction of V. fischeri cells, either with the host epithelial cells that line the crypts or with a population of unattached free animal cells in the crypt spaces, must remotely trigger the cell death of the superficial epithelium. Whether the bacteria themselves release a diffusible signal that interacts with superficial cells or they induce a host cell release of an inducer molecule, such as nitric oxide or tumor necrosis factor α, remains to be determined (Wang et al. 1994; Li et al. 1995). These aspects can be studied using a variety of methods, one of which is the production of bacterial mutants deficient in the induction of host development. While these mutant types are not yet available, efforts are underway to identify them from a pool of mutant strains (Visick and Ruby 1998).

The ultimate goal of this research is to determine the precise molecular language between the host and symbiont that mediates these morphogenetic changes. An essential first step toward this goal is to define the basic constituents of the tissues undergoing morphogenesis and to characterize the modifications in those host tissues that result from the interaction with the symbionts. The results presented here suggest that a cooperative bacterium triggers classic apoptosis in host epithelial cells from a distance. Future studies will be focused upon the nature of the bacterial trigger and how the activity of that trigger is translated into a remotely acting apoptosis program.

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