Visceral Regeneration in Holothurians

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ABSTRACT   Holothurians, or sea cucumbers, exhibit two processes that have intrigued biologists for decades: autotomy and regeneration. Autotomy includes the loss of body parts by evisceration or fission, and regeneration is the extraordinary process by which the lost organs are replaced. In this article, we review the literature on evisceration, transection, and visceral regeneration in holothurians and compare these processes in different orders and lower taxa. Focusing mainly on the digestive tube, we analyze regeneration from a cellular perspective, considering especially the origin, migration, and proliferation of the cellular components of the regenerated organ. The data highlight the most interesting aspects of holothurian regeneration and indicate those critical problems requiring new information and new approaches. *Microsc. Res. Tech. 55:438–451, 2001.*   © 2001 Wiley-Liss, Inc.

INTRODUCTION

The regeneration of lost body parts is well developed in most echinoderms, and some examples of this capacity are even well known among the general public. The regeneration that follows the autotomy or amputation of one or more arms of starfish (Asteroidea) and brittle stars (Ophiuroidea) is certainly notorious and this phenomenon has also been a frequent subject of experimental investigation (reviewed by Hyman, 1955; Swan, 1960; Emson and Wilkie, 1980; Thorndyke et al., 1999). Other examples of echinoderm regeneration—such as the replacement of crinoid (Crinoidea) arms and stalk and of sea urchin (Echinoidea) spines—are less well known and have been studied only occasionally by the scientific community (see Candia Carnevali et al., 1993, 1995a, 1997, 1998; Candia-Carnevali and Bonasoro, this issue; Ebert, 1967).

Although not so widely known as that of asteroids, the regenerative capacity of sea cucumbers (Holothuroidea)—known from ancient times—is extraordinarily well developed. For example, a disk of tissue from *Leptosynapta crassipatina* containing the calcareous ring, the nerve ring, and the mouth will regenerate the whole animal (Smith, 1971b). Equally remarkable, a holothurian that has undergone evisceration and has ejected most of its viscera, including the digestive tube, the hemal system, and the respiratory trees, will slowly regenerate all of these lost internal organs (reviewed by Hyman, 1955; Emson and Wilkie, 1980). The first organs to regenerate, in all species documented to date, are associated with the digestive tract. Thus, most of our knowledge about visceral regeneration in holothurians concerns this system and is therefore the focus of this review.

We begin this review with a consideration of the evisceration process in three orders of the class Holothuroidea and then examine the course of the regeneration that follows evisceration, as well as experimental transection and transverse fission. Because the digestive tube is central to the story, we set out the details of the normal histology of the system. Cellular aspects of regeneration are then considered in terms of the critical role of the mesentery, the source of the cells that form the regenerate, and the presence and number of cell divisions.

Electron microscopy has rarely been applied to the study of evisceration and regeneration in holothurians. Nevertheless, we focus here upon the studies that are available and that illustrate the cellular events associated with visceral ejection and the formation of new viscera. We hope that this review, by highlighting the formidable regenerative capacity of holothuroid species, will stimulate other scientists, particularly microscopists, to employ these animals in investigations of the mechanisms of evisceration and regeneration.

THE EVISCERATION PROCESS
Patterns of Evisceration

Members of the class Holothuroidea differ dramatically in the way they eviscerate, in the organs that are expelled, and in the order of the subsequent regeneration. Yet at the ordinal level, a general pattern emerges.

Dendrochirota. Species in this order (e.g., *Sclerodactyla briareusl,* previously known as *Thyone briareus)* eviscerate through the anterior end. The lantern retractors rupture near the body wall; the longitudinal body wall muscles contract; the introvert wall softens and ruptures; and through the opening are expelled the oral structures (the tentacles with their ampullae, the lantern and its retractor muscles, the nerve ring, and water vascular ring), followed by the attached stomach and intestine, hemal system, and part of the gonad (Scott, 1914; Tracey, 1972). The remnants—the body wall complete with its muscles and innervation, and the cloaca—survive and regenerate every lost part, including the oral structures.

Both *Sclerodactyla* (Kille, 1935, 1936) and *Thyonella gemmata* (Reinschmidt, 1969) have been transected in

the laboratory into anterior and posterior regions. In those experiments the posterior fractions regenerated the entire animal (except the gonad in *Sclerodactyla*), whereas anterior portions died. Fragments retaining the cloaca could regenerate an entire animal. Thus, the regenerative capacities in these two dendrochirotes appear to be associated with the posterior end, although *Thyonella* (in spite of long-term observation and testing) never shows self-induced evisceration. In a recent review, Dolmatov (1999) suggests that 1-year-old *Eupentacta fraudatrix* can regenerate both anterior and posterior halves after transection, whereas adults regenerate only the anterior portion. Thus, the regenerative potential of dendrochirotes might change during development and as a function of age.

Aspidochirota. In members of this order, evisceration typically begins with the rupture of the cloaca; but some species of *Holothuria*—e.g., *H. surinamensis* (Crozier, 1915) and *H. parvula* (Kille, 1937)—eviscerate through an opening torn in the body wall. Contractions of the body wall expel both the intestine and associated viscera, but not the organs of the oral complex (Hyman, 1955). The inventory of eviscerated organs varies with species: *Stichopus mollis* and *S. regalis* lose both respiratory trees with the digestive tube and hemal system. Parts of the gonadal tubules may also be eviscerated, but the extent of this loss depends on the reproductive stage (Bertolini, 1930; Dawbin, 1949). Species of *Holothuria* eviscerate the same organs but retain the right respiratory tree (Bertolini, 1932; Kille, 1935; Bai, 1971; García-Arrarás et al., 1998).

When aspidochirotes in the laboratory are subjected to even mild trauma, the body wall disintegrates; thus, transection experiments are usually impossible (Kille, 1942; Motokawa, 1988). Nevertheless, some species of aspidochirotes can be transected, and when these animals were cut into 2–3 portions the fragments regenerated well (see Dolmatov, 1999). Moreover, fission apparently occurs in nature in some aspidochirotes as a method of asexual reproduction (Kille, 1942; Emson and Wilkie, 1980; Emson and Mladenov, 1987). In these cases both the anterior and posterior ends can regenerate.

Apoda. Only one genus in this order, *Leptosynapta,* has been investigated, and evisceration has never been observed in these species. In laboratory transection experiments, anterior pieces that include the oral complex can survive and regenerate the rest of the animal, whereas posterior pieces do not survive (Clark, 1901; Smith, 1971a; Gibson and Burke, 1983). However, a few posterior halves survive long enough to suggest their potential to regenerate posterior parts (Smith, 1971b). Thus, the polarity of apodan regeneration seems to be directly opposite to that of the dendrochirotes.

Mechanisms and Functions

Evisceration in holothurians is a complex process. It involves 1) a rapid softening of the attachment ligaments of the viscera and of the tendons of selected muscles to the cloaca, the mesenteries, and the body wall; 2) an intense, localized softening of the body wall or the cloaca; and 3) a contraction of the muscles that easily stretches and ruptures the weakened structures, expelling the disconnected viscera.

Ultrastructure studies have been done by Byrne (2001) on the dendrochirotid *Eupentacta quinquesemita* during evisceration (Fig. 1). These studies provide a rare glimpse into the histological and cellular changes that occur in the structures that fail during autotomy: i.e., the introvert, the tendon that links the retractor muscle to the body wall muscle, and the intestine–cloacal junction. A common finding in the three sites studied was that the peritoneum dissociated from the autotomizing tissue, the structure of the collagen fibrils remained intact, and rather small changes were observed in the axons and their vesicles. In every structure, the loss of tensility and the dramatic change from a gel-like state to a fluid-like state was associated with a breakdown of the ground substance in the connective tissue.

The rapid loss of tensile strength is a manifestation of a unique echinoderm feature—a connective tissue that is variable in its mechanical properties (catch connective tissue, Motokawa, 1984; mutable connective tissue, Wilkie, 1979, 1984, 1996). The mutable properties of this connective tissue are regulated by the nervous system; thus, appropriate stimuli can cause softening or stiffening and induce complex behaviors like autotomy or evisceration. Impure "evisceration factors" have been reported in dendrochirotes (Smith and Greenberg, 1973, *Sclerodactyla*; Byrne, 1986, 2001, *Eupentacta quinquesemita*). More recently, three novel peptides—isolated and sequenced from the body wall of *Stichopus japonicus*—were found to affect the dermal stiffness of *Stichopus japonicus* and *Holothuria leucospilota* (Birenheide et al., 1998). Although these small molecules were interpreted as possible neuropeptides, acting in vivo as connective tissue modulators, neither their cellular source nor their specific functions are clear. Two proteins have also been partially purified from the dermis of *Cucumaria frondosa*, one stiffening, and the other plasticizing the tissue. Both of these macromolecular agents seem to be released from cellular components of the dermis, but probably not from neurons (Koob et al., 1999).

Although the cellular mechanisms underlying evisceration are becoming clear, the significance of the phenomenon itself is still obscure. In their 1980 review, on page 239, Emson and Wilkie wrote: "The ease with which some holothurians eviscerate, the invariability of the points of rupture and breakage planes, and the reliability and rapidity with which the whole organ system is regenerated clearly demonstrate that evisceration is a highly developed behavioral response and not an incidental pathological symptom. Nevertheless, the cause and function of evisceration in nature remain unsolved problems."

Evidence in support of the notion that evisceration actually occurs in nature is patchy. On the one hand, the observations of Bertolini (1932) and Swan (1961) strongly suggest that *Stichopus regalis* and *Parastichopus californicus* eviscerate seasonally in Naples and Friday Harbor, respectively. On the other hand, evisceration in the field is uncommon in other species of Aspidochirota (Kille, 1936; Dawbin, 1949). If evisceration is a seasonal phenomenon in some aspidochirotes, the timing of the event could be due to seasonal
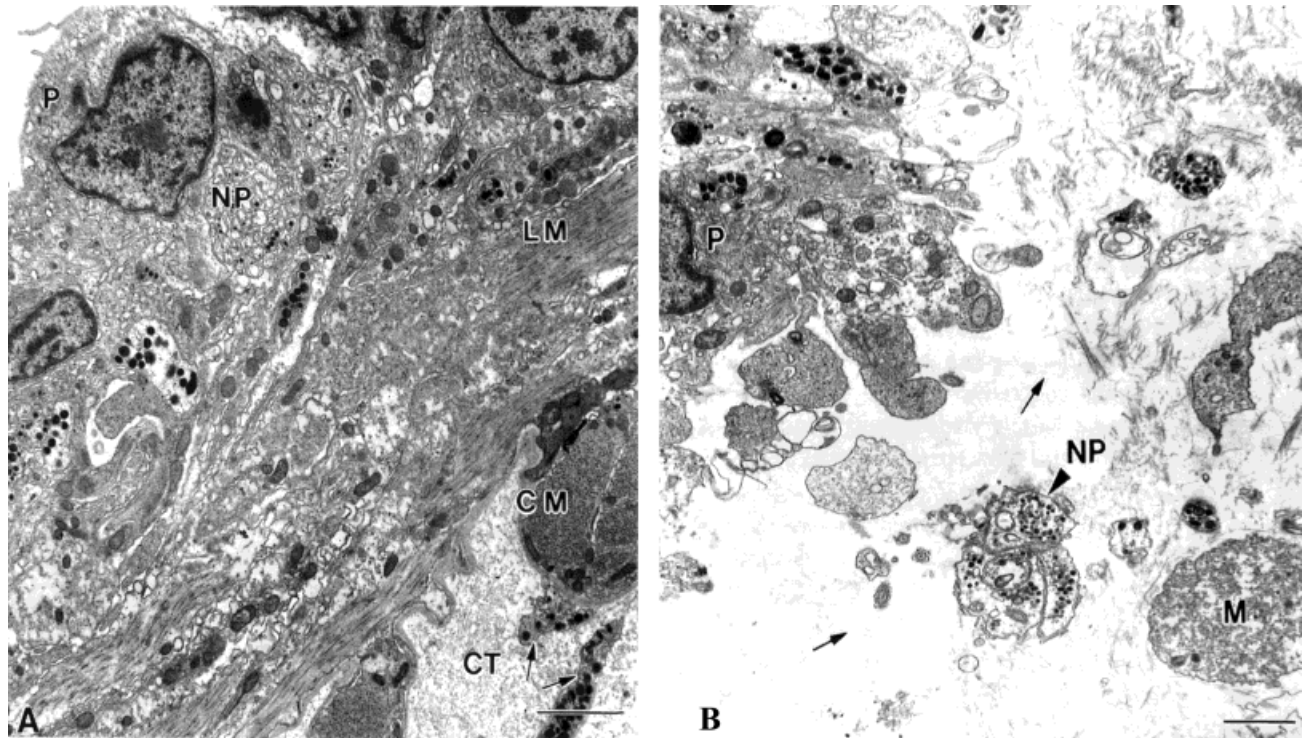
Fig. 1. Transmission electron micrographs of (**A**) uneviscerated and (**B**) eviscerating intestines of *Eupentacta quinquesemita*. The different tissue layers of the intestine: peritoneocytes (P), longitudinal muscle (LM), circular muscle (CM) and connective tissue (CT), as well as some nerve processes (NP), are observed in the noneviscerated intestine. During evisceration, these layers become highly disorganized, the peritoneum detaches from the remaining tissues, and coelomic fluid infiltrates the connective tissue (from Byrne 2001). Scale bars: **A**, 2 µm; **B**, 1 µm.

changes in the reproductive system or in the environment. And the environmental variables could be chemical, nutritional, symbiotic, or even pathogenic (see Pierce, 1999, for an illuminating example from another phylum). None of these possibilities has been investigated in Holothuroidea. The question of natural evisceration is further complicated by reports that some species (i.e., *Parastichopus californicus*) lose their viscera by atrophy, and not by seasonal evisceration, as previously believed (Fankboner and Cameron, 1985). In these cases, the viscera eventually regenerate by a process similar to that described for regeneration following evisceration.

Self-induced evisceration in holothuroids was assumed to be a natural response to noxious stimuli, such as high temperatures, low oxygen levels, and foul water (Scott, 1914; Kille, 1935; Dawbin, 1949; Swan, 1961). In fact, in laboratory experiments a variety of agents could induce evisceration when injected into the coelomic cavity, but survival was not often part of the outcome of these experiments (reviewed by Hyman, 1955). Many of these treatments were probably acting on the nervous system, possibly indirectly affecting the mechanisms controlling the mutable connective tissue. More recently, they have been supplanted by KCl injections, mild electrical stimulation, or pharmacological agents aimed more specifically at the nervous system or the connective tissue (Smith and Greenberg, 1973; Byrne, 1986, 2001).

Evisceration has also been put forward as a gambit, distracting a predator with a tasty morsel while the individual quietly regenerates; but simple softening or stiffening may be sufficient to ward off predation (Motokawa, 1988). *Thyonella gemmata*, for example, has never been induced to eviscerate, but has a high rate of regeneration in the field. In one month, nine of 75 animals obtained from a heavily trafficked collecting area had not eviscerated, but were clearly regenerating, "probably the victims of mutilation by predators or shovels" (Reinschmidt, 1969).

The parable of *Thyonella* suggests that our concern with evisceration may be misplaced. What *Thyonella* has in common with other holothuroids is not evisceration, but a high capacity for regeneration and a mutable connective tissue. Thus, we should be asking how this unusual connective tissue—and the collagen chemistry and biophysics underlying its mutability (Motokawa, 1984; Bailey, 1985, Trotter and Koob, 1995; Thurmond and Trotter, 1996; Trotter et al., 2000; Wilkie, this issue)—enhances the regenerative capacity of holothurians, and indeed of all echinoderms. In other words: what is the relationship between the regeneration potential of echinoderms and the mutable connective tissue?

HISTOLOGICAL ORGANIZATION OF THE DIGESTIVE TUBE

Since this review focuses on some of the cellular and histological events that occur during regeneration of the viscera, the histology of the digestive tube, and particularly the organization of the intestinal tissues, must first be described. We focus on the digestive tract
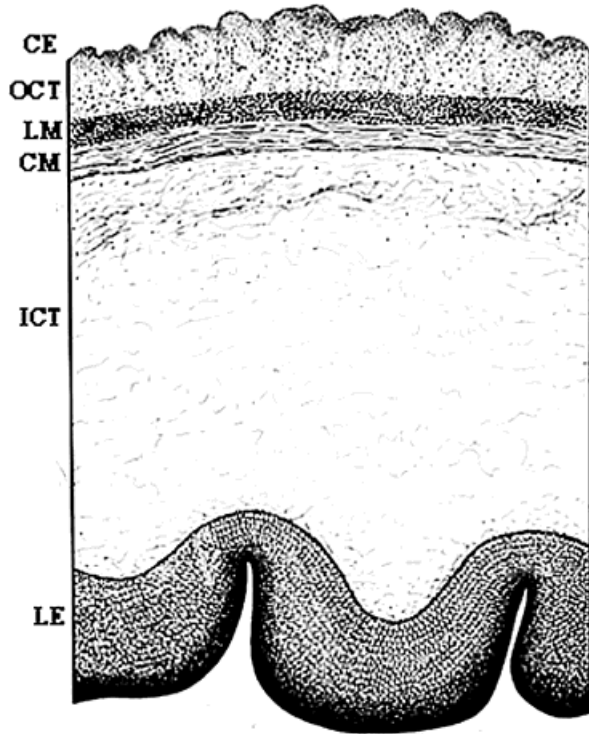
Fig. 2. Schematic diagram of the tissue layers of the holothurian digestive tract. The diagram corresponds to a cross-section of the large intestine and shows coelomic epithelium (CE), circular muscles (CM), inner connective tissue (ICT), luminal epithelium (LE), longitudinal muscles (LM), and outer connective tissue (OCT).

for two main reasons: first, the gut is the major visceral system within the holothurian body cavity, and, second, it is—in all species studied to date—the first organ system to regenerate after evisceration.

The digestive tract of holothurians is a continuous tube that runs posteriorly, from mouth to cloaca. It can be subdivided into seven specialized areas: pharynx, esophagus, stomach, small intestine, large intestine, and cloaca. Nevertheless, because the relative size and structure of each region in any species is determined by its specific feeding behavior and digestive physiology, there is significant species-specific variation. Indeed, some areas of the gut cannot be readily identified in some species; e.g., *Holothuria glaberrima* lacks a defined stomach (García-Arrarás et al., 1998).

The histology of this system has been described by several investigators in different holothurian species (Hyman, 1955; Fish, 1967; Farmanfarmaian, 1969; Nace, 1971; García-Arrarás et al., 1998). In general, the seven areas of the digestive tract are similar in their histology; a certain variability is limited to the thickness of the specific tissue layers, or to the extent of folding of the intestinal mucosa. The intestinal tube, in particular, consists of a coelomic epithelium, an outer connective tissue layer, a muscle layer, an inner connective tissue layer, and a digestive epithelium lining the lumen (Figs. 2, 3). Each tissue layer contains nervous elements, including, for example, neuronal cells within the coelomic epithelium, nerve fibers associated with the muscle cells, and neuroendocrine-like cells within the luminal epithelium (García-Arrarás et al., 2001).

In many ways, the histological organization shows morphofunctional analogies with that of vertebrates. The coelomic epithelium, together with the adjacent outer connective tissue, is comparable with vertebrate serosa. The muscle layer is disposed in two layers with distinct orientations, longitudinal and circular. The inner connective tissue layer, which contains only a few cells within its matrix, is analogous to vertebrate submucosa. Finally, the pseudostratified epithelium, which lines the intestinal lumen in holothurians, is similar to vertebrate mucosa.

Although the histology of the holothurian digestive tract is rather simple and unexceptional in its structure, the organization of the different layers, as presented above, is slightly controversial. Some investigators, in fact, consider the coelomic epithelium (also called the peritoneocyte layer), the outer connective tissue, and the related muscles as components of an independent lamina the "perivisceral mesothelium" (Smiley, 1994). According to some authors, the mesothelium can be regarded as a single layer, whereas others consider its three components as independent units. Apart from these nomenclatural problems, the mesothelium appears to be a key element in the regenerative properties of holothurians, and this will be discussed below.

The digestive tract lies within the coelom and is anchored to the body by a series of mesenteries. Although these structures are designated "dorsal," "ventral," and "lateral," they actually constitute a continuous mesenterial sheet that changes its areas of attachment to the body wall at different levels of the digestive tract. The mesenteries have a central role in the regeneration of new viscera and their histological organization clarifies that function. They are composed of a thin, central connective tissue layer sandwiched between two muscle layers which are, in turn, tightly covered by the coelomic epithelium (see García-Arrarás et al., 1998). Nervous elements, primarily neuronal fibers of the visceral plexus (see García-Arrarás et al., 2001), are also present in the mesenteries. Thus, except for the luminal epithelium, the mesenteries contain all the histological elements that constitute the digestive tract.

REGENERATION

Visceral regeneration in holothurians, long known as a curious phenomenon of natural history, has been well documented since the 19th century. But it was Fausta Bertolini, working in the 1930s at the Stazione Zoologica de Napoli, who explored the cellular events that enable regeneration of the digestive tract and first accurately analyzed the histological aspects of the regenerative process. She compared regeneration in different holothurian species, identified the tissues involved in the process, and described their activities. These pioneering histological studies were, for the past 70 years, the basis for subsequent investigations. Many of Bertolini's findings have been widely confirmed by other investigators, but others are still controversial.
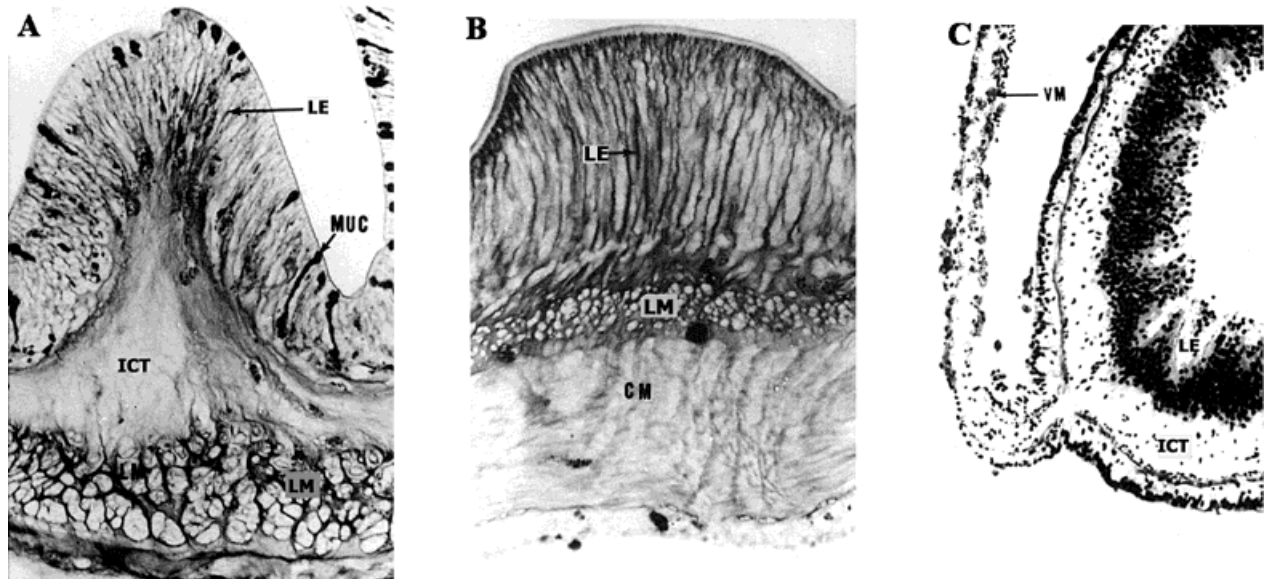
Fig. 3. Cross-sections of the digestive tube of *Thyonella gemmata* at three different levels: (**A**) esophagus, (**B**) stomach, and (**C**) large intestine (from Nace, 1971). Coelomic epithelium (CE), circular muscles (CM), inner connective tissue (ICT), luminal epithelium (LE), longitudinal muscles (LM), ventral mesentery (VM). **A**: ×230; **B,C**: ×120.

Mesentery as the "Center" of Visceral Regeneration

When the evisceration process is activated, the digestive tract and the mesenteries separate from each other along the line of their attachment. Once the process is completed, most of the mesenteries remain intact, with their proximal edge still attached to the body wall, and their distal edge—now free of the gut—hanging loose in the coelomic cavity. In all species of holothurians studied to date, the free edge of the mesentery plays a key role in the regeneration of the digestive tract (Bertolini, 1930, 1932; Kille, 1935; Dawbin, 1949; Mosher, 1956; Bai, 1971; Smith, 1971; Tracey, 1972; Byrne, 1983; Leibson, 1992; García-Arrarás et al., 1998). Indeed, the primordium of the regenerating digestive tract develops from a thickening of the free edge (Fig. 4). Initially, this thickening appears as a series of localized swellings that show considerable variation in width along the mesentery. Moreover, the thickenings develop only in certain areas of specific mesenteries. In *Holothuria glaberrima*, for example, they are prominent only in the lateral and ventral mesenteries, the dorsal tissue showing little or no change (García-Arrarás et al., 1998). As regeneration advances, the localized thickenings lengthen, coalesce, and finally form a continuous, linear, solid cord, homogeneous in width, extending from the buccal or esophageal area to the cloaca.

In histological cross-sections the thickening resembles a blastema. It consists of a large number of cells within a loose connective tissue matrix (Fig. 5), and the entire structure is invested by a coelomic epithelial layer that first develops during the wound-healing stage to cover the site of gut ablation. The phenotype of these cells is difficult to ascertain because, in contrast to studies of other echinoderms—particularly in the

regenerating arms of crinoids (see Candia-Carnevali et al., 1993, 1995a, 1997; Candia-Carnevali and Bonasoro, pages 403–426, in this issue)—ultrastructural studies of holothurian visceral regeneration are lacking. Nevertheless, light microscopy and, in particular, immunohistochemical studies have clearly shown different types of cells within the mesenterial thickenings, including peritoneocytes, myocytes, neurons within the mesothelium, as well as migrating cells that are scattered in the connective tissues and have been described as amebocytes, hemocytes, lymphocytes, and morula cells (García-Arrarás et al., 1998, 1999). Moreover, some of these cell populations do undergo changes during regeneration. Among these, a surge in the number of hemocytes and amebocytes has been documented within the blastema-like structure (García-Arrarás et al., 1998).

Origin of Cells Within the Mesenterial Thickenings

Although there is agreement that the mesenterial thickenings represent the primordia for the regenerating intestinal tube, the origin of the cells that form the thickenings in the mesenterial edges is still obscure. Most investigators have regarded the blastemal thickening as the result of cell migration from within the mesentery (Tracey, 1972; Dawbin, 1949), but the specific migrating cells that might give rise to the new tissues are not known. There are two obvious hypotheses: 1) independent cell lineages might originate from differentiated cells by dedifferentiation, or 2) undifferentiated stem cells might be involved as precursors of all differentiated cell types.

In contrast to these views, other investigators have proposed that the primordia are formed by dedifferentiated cells that originate in, and migrate from, tissues
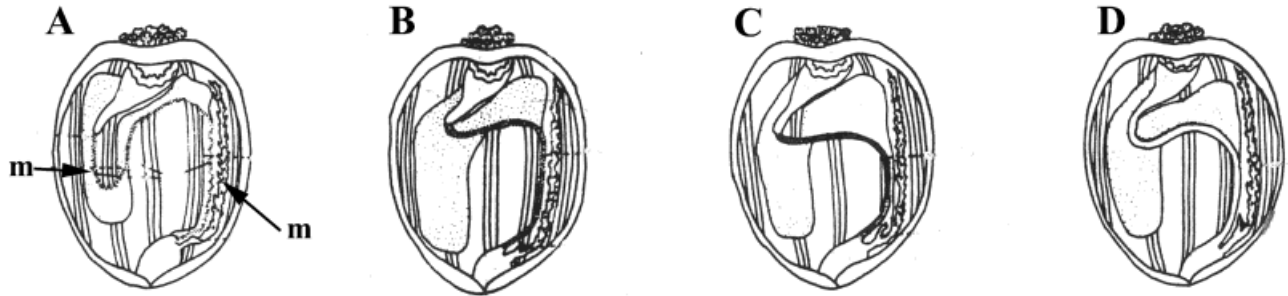
Fig. 4. Schematic diagram depicting the sequence of events during visceral regeneration of *Holothuria scabra*. **A**: After evisceration, the free mesenterial edges (arrows) remain in the coelomic cavity. The mesenteries are attached to the body wall and to the remaining digestive structures; the stomach (s) and the cloaca (c). **B**: A thickening of the mesenteries (arrow) appears in the ventral and lateral mesenteries. **C**: Lumen formation starts from the stomach and cloacal ends (arrows) of the tube. **D**: The lumen is completely formed in 7–9 days (modified from Bai, 1971).

or organs peripheral to the mesenteries: e.g., from the body wall, the coelomic cavities (somatocoelic or hydrocoelic), and the radial nerve cord (Dolmatov, 1992; García-Arrarás et al., 1998). Excellent descriptions were provided by Nace (1971) of the dedifferentiation of cells and their migration to the regenerating lantern complex and digestive tract of *Thyonella gemmata*, and by Dolmatov (1992), of similar events during the regeneration of the aquapharyngeal complex in *Eupentacta fraudatrix* (Fig. 6). In these species, the cells involved in the regeneration process appear to migrate to the wound area along the free mesenterial edge. Some of these cells may be derived from dedifferentiated myocytes that have lost their contractile apparatus before starting to migrate (Dolmatov, 1992). This author also proposed that cells migrating from both the water coelomic canal and the nerve cord could contribute to the formation of the blastemal thickenings along the mesenterial edge. The low level of cell division observed within the regenerating tissues in several species (see below) strongly supports the view that the origin of the blastema-like structure—not only in *E. fraudatrix*, but also in all holothurians—is via cell migration. Nevertheless, the crucial problem—whether the migrating cells originate from dedifferentiated cells, or from undifferentiated stem cells located within the mesentery—is still unresolved.

Recent experiments from our laboratory have revealed that, during the early stages of regeneration, dramatic changes occur in the components of the mesentery (Quinones-Rivera et al., submitted). These changes include the disappearance of extracellular matrix molecules, such as collagen and laminin and of the muscle cells close to the mesenterial thickening. The changes in the mesentery can be visualized with molecular labels, such as muscle-specific or matrix-specific antibodies (Fig. 7). As was shown by Dolmatov (1992) in the regenerating aquapharyngeal complex of *Eupentacta fraudatrix*, muscle cells and peritoneocytes are probably dedifferentiating. We expect that these cells eventually migrate into the blastema-like structure, producing the surge in amebocytes observed in animals that had been regenerating for 1 week (García-Arrarás et al., 1998).

The experimental evidence from other echinoderm species, together with the recent data from our laboratory, could well explain the cellular events that take place during the initial stages of intestinal regeneration in *Holothuria glaberrima*. Our working hypothesis is as follows.

The cells of the coelomic epithelia are the most likely candidates to provide the precursors for the intestinal primordium. The coelomic epithelial cells (or peritoneocytes) have been described in various echinoderm species (Fig. 8), as forming a continuous epithelial layer that covers the coelomic cavity and the visceral organs. These cells have also been proposed to give rise to the other cell types within the mesothelium, including muscle and neurons. After evisceration, the coelomic epithelial and muscle cells adjacent to the free edge of the mesentery dedifferentiate; they lose structures such as the cilium and intracellular filaments, assume a cuboidal morphology, and migrate toward the mesenterial edge, where they form the intestinal primordium. These cells are now identified as amebocytes; they probably assume new roles that are necessary for the organogenesis of the new intestine, especially phagocytosis of cellular debris left over from the evisceration and from the remodeling processes and deposition of extracellular matrix proteins. Once the early regenerative events end, these cells redifferentiate into other cellular phenotypes. Whether the original muscle cells differentiate into other cellular types or redifferentiate into muscle cells is unknown. Candia Carnevali et al. (1998) suggest that two lines of stem cells are involved in crinoid arm regeneration: one originates from the brachial nerve and the other from the coelomic epithelium. The latter stem cells give rise to differentiated cells of the mesothelium and other coelomic derivatives within the regenerating structure. In our holthuroid visceral system, it is this coelomic stem cell line that would likely be involved in the formation of the mesenterial thickenings, and thus of the intestinal primordium.

Our hypothesis about the role of the coelomic epithelium in initial regenerative events receives further support from studies in other echinoderms. These echinoderm models include the regeneration of crinoid arms, where the regenerating cells originate from the brachial nerve and the coelomic epithelium (Candia Carnevali, 1993, 1995a), the regeneration of asteroid arms, where cell proliferation is mainly limited to the

Fig. 5.   Initial stages of regeneration in *Leptosynapta crassipatina*. **A**: Formation of the mesenterial thickening that resembles a blastemal structure (arrow). **B**: Regenerating gut in a more anterior segment, showing the developing lumen (from Smith, 1971b). **A**: ×70; **B**: ×200.

epithelial structures (Mladenov et al., 1989; Moss et al., 1998), and regeneration of longitudinal muscle (Dolmatov et al., 1996) and Cuvierian tubules (Vanden-Spiegel et al., 2000), both in holothurians.

Origin of the Luminal Epithelial Cells

After a solid cord has developed and has grown along the mesenterial edge from esophagus to cloaca, the intestinal lumen forms. As the lumen opens, epithelial cells are recognizable and begin to form a complete lining. The origin of these luminal epithelial cells is a subject of controversy, as might be expected. On the one hand, the mesentery consists of coelomic epithelia, muscles, and connective tissue—all mesodermally derived tissues; thus, we might readily assume that the new cells and tissues originate from precursors derived from the mesentery. During embryogenesis, on the other hand, the luminal epithelium is clearly derived from endoderm. So to propose that the luminal epithelial cells in the regenerated organ may be derived from mesodermal precursors is an apparent paradox. Two distinct mechanisms have been suggested for the regeneration of the luminal epithelium, and both have at least some experimental support. According to the first hypothesis, the new luminal cells could originate from endodermally derived cells of the luminal epithelia of the cloaca, esophagus, or water vascular system. According to the alternative explanation, the luminal cells could originate directly from the coelomic epithelium, a tissue layer that shows high histogenetic plasticity (Tracey, 1972; Dawbin, 1949; Mosher, 1956; Leibson, 1992; Candia Carnevali et al., 1995b).

In agreement with the first hypothesis, the luminal cells of Aspidochirota and Dendrochirota species have been shown to originate primarily from the remnants of the digestive tract, namely, the cloaca and the esophagus (Bertolini, 1932; Leibson, 1992; Kille, 1935; Smith, 1971; Nace, 1971; García-Arrarás et al., 1998). Cells from all of these remnant tissues appear to migrate and invade the mesenterial thickenings, thereby giving rise to the digestive tract, including its luminal epithelium (Fig. 9A). Kille (1935) also proposed that, in *Sclerodactyla briareus*, migrating cells from the water vascular system (enterocoelic in origin) might contribute to the regenerating luminal epithelium. In this scenario, therefore, the luminal epithelium is derived from cells with a presumptive endodermal origin.

In contrast, the intestinal lumen in several species develops either as a continuous structure with no evidence of cell invasion from the remnant digestive structures, or from scattered cell clusters that form discontinuous microcavities, which eventually fuse into a continuous canal (Tracey, 1972; Dawbin, 1949; Mosher, 1956; Leibson, 1992) (Fig. 9B). In fact, in *Actinopyga agassizi*, the lumen forms at first in the center of the solid primordium—apparently originating from infolds of the coelomic epithelial thickening—before finally appearing at the ends (Mosher, 1956). In these cases, the luminal epithelium appears to derive from the coelomic epithelium or mesothelium without any participation of the proper endodermal cells and the new luminal epithelium would therefore have a mesodermal origin. The echinoderm mesothelium is a complex tissue that has been proposed to contain precursors, not only for epithelial cells, but also for myocytes and
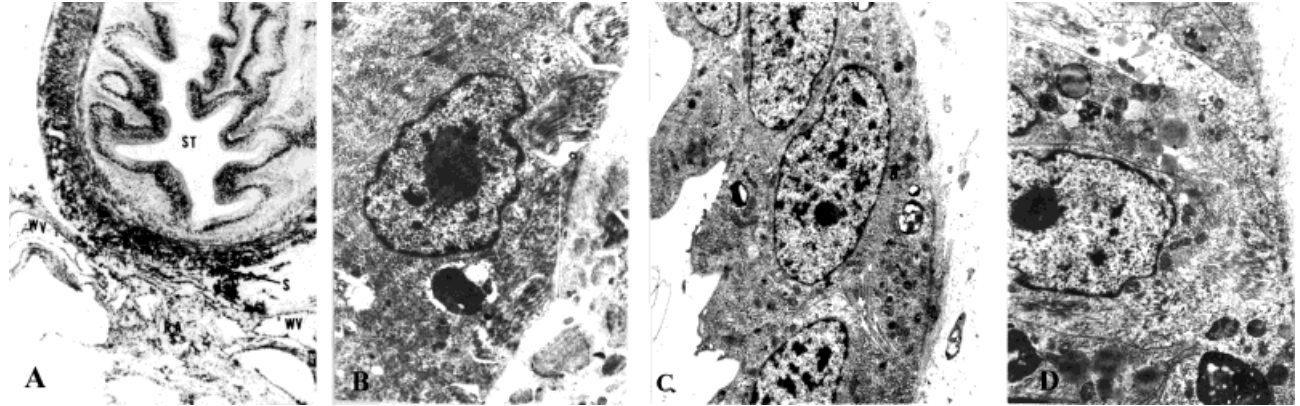
Fig. 6. **A**: Longitudinal section through the regenerating digestive tube of *Thyonella gemmata* showing changes in the muscular layer adjacent to the regeneration area. Muscle cells in this area have become largely disorganized, losing their layered structure. The cells have partially dedifferentiated, their contractile apparatus reduced to spindles that will eventually be eliminated (from Nace, 1971). Regenerating area (RA), spindles (S), stomach (ST), water vascular canals (WV). **B–D**: Transmission electron micrographs showing epithelial cells from the ambulacral canal of *Eupentacta fraudatrix* at different stages of regeneration. The photos show the proposed sequence of events in the dedifferentiation of the coelomic epithelial cells. **B**: Spindle formation early in regeneration. **C**: Cells without contractile apparatus during the middle stages of regeneration. **D**: Formation of new fibrils in redifferentiating cells (from Dolmatov, 1992). **A**: ×40; **B**: ×5,750; **C,D**: ×4,600.

neurons (Smiley and Cloney, 1985; Rieger and Lombardi, 1987; Candia Carnevali et al., 1995b; Dolmatov et al., 1996). Whether the precursor cells in this tissue layer may also give rise to the luminal epithelium is a tantalizing, but still unanswered question.

Cell Divisions

We generally assume that biological processes involving regeneration and growth should be accompanied by a relevant increase in cell division. On the other hand, cell division is not absolutely required, and regeneration might depend primarily upon other types of mechanisms, such as cell migration. Several investigators studying visceral regeneration in echinoderms referred to 'proliferation' without performing experiments to monitor the levels of cell division (Tracey, 1972; Dolmatov, 1992). As a result, our ideas about the involvement of cell division in echinoderm regenerative processes—particularly in visceral regeneration—remain unclear.

In transection experiments, little ^{3}H-thymidine incorporation was observed (Gibson and Burke, 1983), suggesting that cell division is not an important aspect in this type of regeneration. In contrast, several studies involving various techniques showed that cell division might play a significant role in regeneration following evisceration. Cell division was quantified 1) in *Stichopus mollis* by measuring mitotic figures (Dawbin, 1949); 2) in *Eupenctata fraudatrix* by measuring radioactive thymidine incorporation (Leibson, 1992; Dolmatov, 1993); and 3) in *Holothuria glaberrima* by incorporation of the nonradioactive thymidine analog bromodeoxyuridine (BrdU) (García-Arrarás et al., 1998) (Fig. 10). A common pattern of cell proliferation is reported in these three species. First, in the early stages of regeneration few divisions occur in the cells that populate the internal connective tissue of the mesenterial thickening. Second, most of the cellular divisions occur in the coelomic epithelia and in the mesentery adjacent to the thickening. Thus, the mesenterial thickening does not seem to correspond to a "true blastema," usually defined as a mass of proliferating undifferentiated cells. In contrast, the highest rates of cell division were found at the tip of the invading luminal epithelium (García-Arrarás et al., 1998), suggesting that the invading cells constantly divide and thus provide a complete lining of the luminal cavity, even as this structure is developing. Third, once the tissue layers of the regenerating intestine are formed, cell divisions continue and increase in number as the organ grows in size. These later divisions continue to occur mainly in the luminal and coelomic epithelia (García-Arrarás et al., 1998).

Duration of Regeneration

The time required for visceral organs to regenerate also varies from species to species. Typically, experimental species regenerate the digestive tube in about 1 month, but some need less time, and some much more. For example, *Holothuria scabra* requires only 7 days to complete most of the regeneration process (Bai, 1971); but *Sclerodactyla briareus* (Kille, 1935) and *Holothuria glaberrima* (García-Arrarás et al., 1998) regenerate their lost body parts within 20–30 days; and *Stichopus mollis* requires 145 days (Dawbin, 1949). The time to regeneration of transverse sections of *Thyonella gemmata* varies markedly with the relative length of the segment and with its position along the anterior–posterior axis of the animal (Reinschmidt, 1969). Most of these findings, however, were obtained with animals kept in the laboratory—and not necessarily under conditions that mimicked the natural habitat. Our own experiences and those of others (Kille, 1935; Dawbin, 1949; Reinschmidt, 1969) indicate clearly that regeneration is influenced by aquarium conditions, as well as by experimental variables.
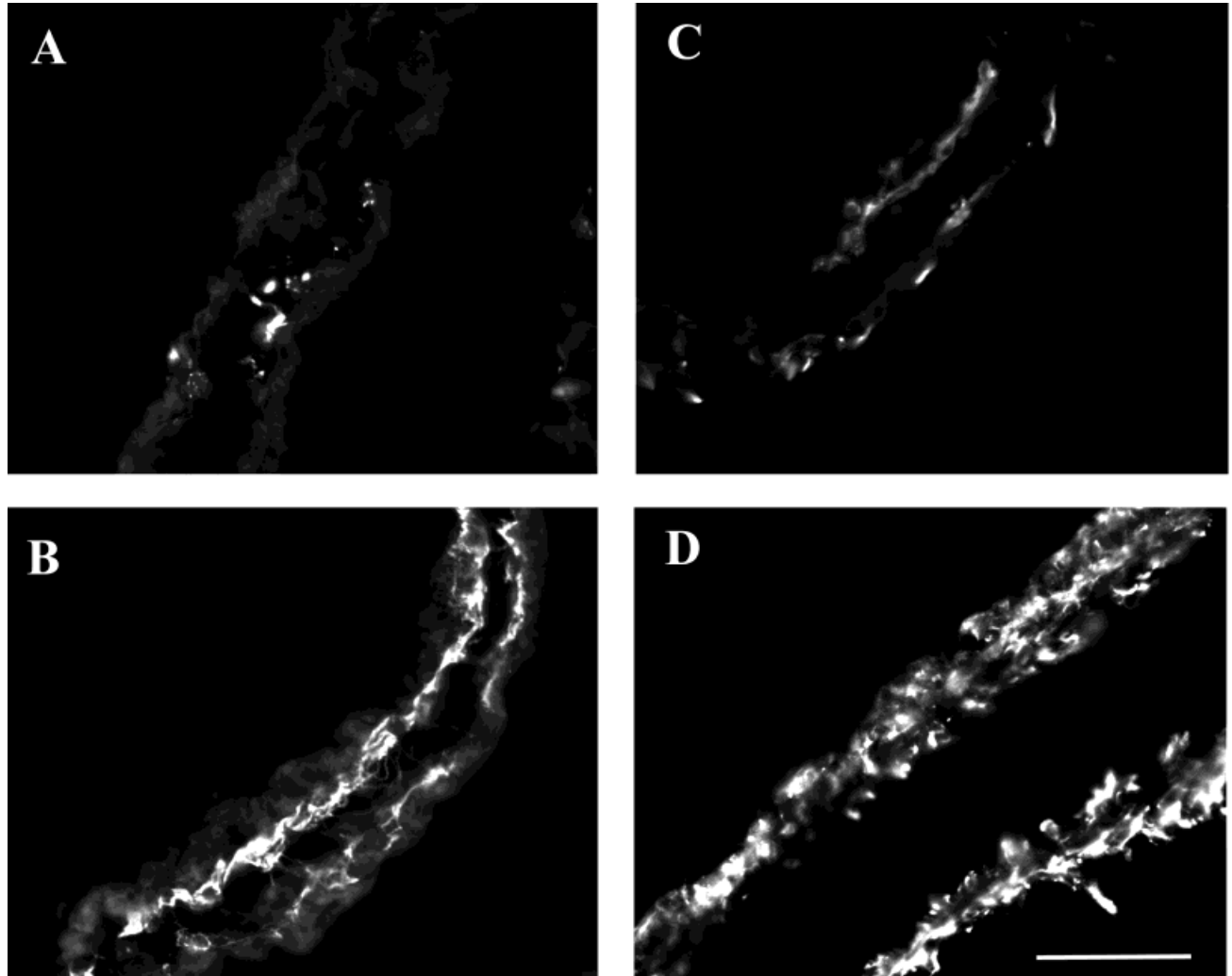
Fig. 7. Changes in the components of the mesentery occur during visceral regeneration. These changes can be observed at the level of extracellular matrix molecules, such as collagen (**A,B**), or at the level of the cells, such as muscle cells (**C,D**). In 7-day regenerating animals, the mesentery adjacent to the intestinal primordial loses most of its collagen (**A**) and muscle cells (**C**). In contrast, the same mesentery at the extreme adjacent to the body wall maintains the collagen (**B**) and muscle layer (**D**) pattern observed in noneviscerated animals. These images were obtained using an anticollagen (**A,B**) and a muscle-specific antibody (**C,D**). The labeling of muscle cells or the organized actin in the contractile apparatus is greatly decreased or completely absent after 7 days of regeneration. Bar = 50μm

Regeneration of Other Viscera

As mentioned above, the digestive tract is the first visceral organ to regenerate after evisceration and the regeneration of other visceral structures follows. The regeneration of the digestive tract-associated viscera, such as the hemal system and respiratory trees, has been studied mainly in aspidochirotes. These organs usually start to regenerate only after a primordium of the digestive tube has formed, or when gut regeneration is highly advanced.

Hemal System. In *Stichopus mollis*, the ventral vessel of the hemal system regenerates from the ventral side of the regenerating digestive tube (Dawbin, 1949). Several weeks after the ventral vessel appears, the dorsal vessel begins to form near the junction between the mesentery and the intestine. In contrast, the dorsal vessel develops first in *Holothuria scabra* (Bai, 1971).

In both species, however, the hemal vessels form within the regenerating gut and then extend anteriorly to connect with the original remnants of the esophagus. The cells of the regenerating hemal system may presumably be derived from both the coelomic epithelium and the blastemal-like cells within the mesenchyme of the intestinal primordium (Dawbin, 1949).

Respiratory Trees. The respiratory trees form as solid projections from the anterior part of the cloaca and eventually acquire an internal lumen (Dawbin, 1949; Bai, 1971). These tubules subsequently branch into smaller ampullae. The origin of the cells that form the respiratory trees, particularly the luminal epithelia, is unknown. However, Dawbin (1949) describes regeneration of the respiratory trees in *Stichopus mollis* as proceeding similar to regeneration of the digestive tract in this species. In that case, regenerated
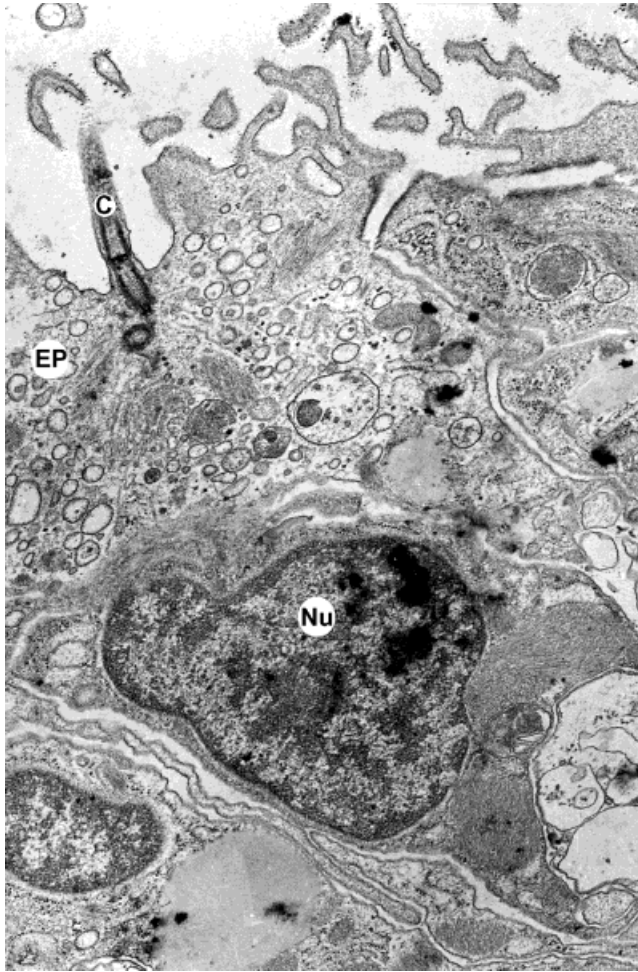
Fig. 8. Transmission electron micrograph of a coelomic epithelial cell or peritoneocyte of *Holothuria glaberrima*. These cells are characterized by their apical cilia, large nuclei and fibrilar structures that extend from the area of the nuclei. Dedifferentiated peritoneocytes are thought to constitute the precursor cells responsible for many of the echinoderm regenerative properties. ×17,300.

Fig. 9. **A**: Proliferating cells from the stomach remnant growing into the mesenterial thickening of *Thyonella gemmata* (from Nace, 1971). Mesentery (MES), proliferating tube (PT), spindles (S), stomach (ST), water vascular canals (WV). **B**: Microcavities formed within the mesenterial thickening in *Eupentacta fraudatrix*. These cavities are lined by cells apparently originating from the coelomic epithelia. The cavities eventually fuse to form the intestinal lumen (from Leibson, 1992). Lumen (Lu), mitosis (M), stroma of mesenterial edge (Mstr), splits or microcavities (Spl). **A**: ×68; **B**: ×550.

respiratory trees would be purely mesodermal in origin.

Gonads. The loss of the gonads during evisceration and the course of their subsequent regeneration are influenced, at least in part, by the seasonal reproductive condition of the organism. In holothurian species that eviscerate through the cloaca (Aspidochirota), only the gonadal tubules are ejected; the gonadal base, which contains the germinal cells, is retained (Dawbin, 1949; Mosher, 1956; Leibson, 1992; García-Arrarás et al., 1998). Thus, the new gonads are produced by the remaining tissues and this process is similar to what occurs seasonally under natural breeding conditions. In dendrochirotes, which eviscerate anteriorly (e.g., *Sclerodactyla briareus)*, the gonadal basis and even the tubules remain attached to the dorsal mesentery, thus serving as primordia for the formation of the new gonad. But if the gonad base containing the germinal cells is surgically extirpated from eviscerated animals, then all the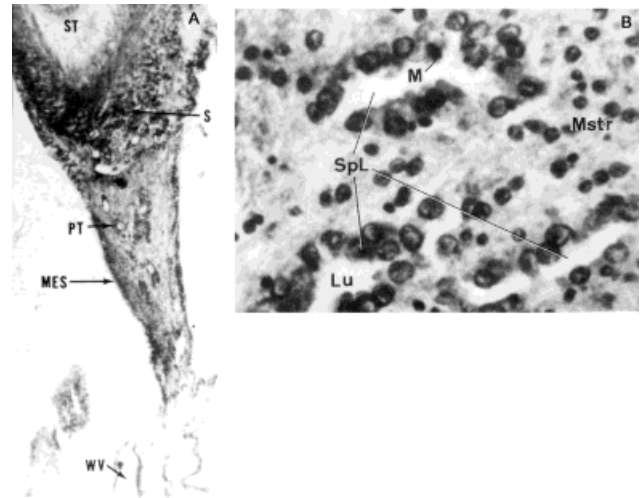 viscera can regenerate after 120 days, except for the gonads themselves (Kille, 1939). This finding might suggest that germinal cells are essential for gonad regeneration; but the alternative explanation, that insufficient time was allowed for regeneration to occur, has yet to be tested. If another dendrochirote species (*Thyonella gemmata*) is transected serially, pieces located well posterior to the gonad and measuring about one-eighth the length of the animal, can still regenerate a new gonadal basis and tubules—but only after 243 days (Reinschmidt, 1969). Thus, the new gonad in these animals must have arisen from newly committed cells.

Animals that lose their gonadal base during evisceration or transection and are able to regenerate the gonad have significant experimental potential. In particular, they can be used to determine the origin of the stem cells that are responsible for germinal cell production in the regenerated gonad. Kille (1942), studying asexual reproduction by fission in *Holothuria parvula*, proposed that the germ cells involved in the gonadal regeneration originate in the coelomic epithelium. If this idea is correct, the cells of the coelomic epithelium of holothurians (and probably of echinoderms in general) would emerge as one of the most pluripotent cell types in nature, possibly providing precursors not only for specialized somatic tissues such as muscles and nerves, but also for germ cells.

Two additional studies might help to elucidate visceral regeneration: i.e., regeneration of Cuvierian tubules (VandenSpiegel et al., 2000), and that of digestive tract organs in starfish (Anderson, 1962, 1965a,b).

Cuvierian Tubules. These holothurian defensive structures are released from their attachment site at the base of the respiratory trees, expelled from the cloaca, and then replaced. This phenomenon can thus
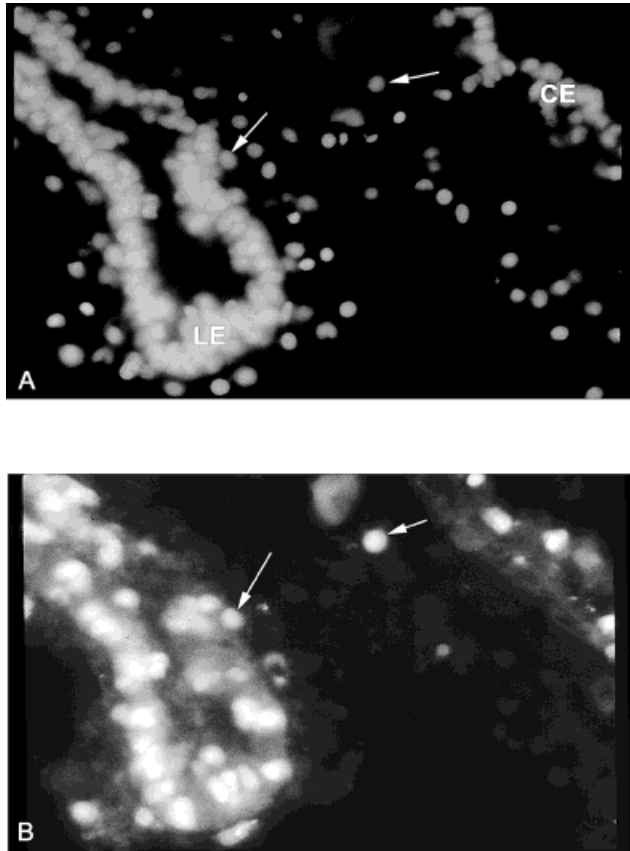
Fig. 10. Double-labeling of regenerating *Holothuria glaberrima* intestine for (**A**) cell nuclei (Hoechst labeling) and (**B**) dividing cells (BrdU labeling). **A**: The total number of cells present in the section can be observed with the Hoechst nuclear labeling. **B**: The BrdU labeling shows the dividing cells that have incorporated the thymidine analogue in a 2-week-old regenerating intestine. Arrows indicate some examples of double-labeled nuclei. Observe that most of the dividing cells are localized to the coelomic (CE) or luminal (LE) epithelia, with few dividing cells found in the inner connective tissue (ICT). **A,B**: ×1,000.

be seen as a model of partial evisceration, since it follows a sequence of events similar to that of the evisceration process described above. In an elegant ultrastructure study, VandenSpiegel et al. (2000) showed that the mesothelium plays a key role in the regeneration of the Cuvierian tubules of *Holothuria forskali*. As in regeneration of the digestive tract, cells from the mesothelium undergo proliferation, migration, and differentiation (Fig. 11). The formation of a pseudostratified mesothelium containing peritoneocytes and undifferentiated cells is the first step in the regeneration of the Cuvierian tubule. The undifferentiated cells within this mesothelium probably arise from peritoneocytes and will give rise to granular cells of the connective tissue, as well as to myocytes. Although the initial step occurs mainly by cell migration and cell division in the mesothelial layer, later events include extensive proliferation by the cells of the inner (luminal) epithelium. In contrast to the regeneration of the digestive tube, however, many dividing cells were found within the connective tissue, particularly in the later stages of Cuvierian tubule regeneration.

Digestive Tract Organs in Starfish. Regeneration of the pyloric caeca has been studied in several starfish species (*Henricia leviuscula, Asterias forbesi, Pisaster ochraceus,* and *Leptasterias pusilla*), whereas regeneration of the cardiac stomach has been studied in *Asterias forbesi* (Anderson, 1962, 1965a,b). In all cases, the mesentery appears to play a crucial role, providing guidance and support to the migrating cells that will constitute the regenerating caeca and also providing cellular components directly to the regenerating structures. A mechanism common in all of the species examined is that cells showing high mitotic activity accumulate in the growing tip and form the cecal lumen. Thus, mitotic activity occurs preferentially in the epithelial layers, both coelomic and luminal, as it does in the regenerating gut of holothurians.

MORPHALLACTIC VS. EPIMORPHIC REGENERATION

The various developmental processes that enable regeneration in echinoderms often show species-specific differences and these differences were, in the past, subjects of some controversy (Candia Carnevali and Bonasoro, pages 403–426, in this issue). On the one hand, a morphallactic mechanism—where lost parts arise from the redifferentiation and remodeling of existing tissues—has been often proposed for some species (Morgan, 1901; Wallace, 1981; Mladenov et al., 1989). And on the other hand, students of regenerating crinoids refer to the formation of a blastema—a mass of undifferentiated, proliferating cells that forms at the wound site and eventually gives rise to the regenerated structure (Candia Carnevali et al., 1993, 1995a, 1997). The formation of a blastema implies an epimorphic process. At present, the prevailing view is that both morphallactic and epimorphic mechanisms are involved in holothurian visceral regeneration (García-Arrarás et al., 1998, 1999).

However, the differences between morphallaxis and epimorphosis have become relatively unimportant to most students of the area and discussions about the distinction between these mechanisms seem rather sterile. The work presented here clearly shows that regeneration in any holothurian involves cellular differentiation, migration, and proliferation. Whether cell proliferation occurs locally, at the wound site, with subsequent dedifferentiation and later differentiation, or by dedifferentiation followed by migration and proliferation, is not critical to our general understanding of regeneration. Rather, the central phenomenon to be explained in this field is the rapid and radical, yet orderly, change in the genetic program of an adult tissue that switches the physiological state of the tissue from maintenance to renewed development. Thus, the questions that must be addressed, if holothurian regeneration is to be brought into the mainstream of 21st century biology, are those that will elucidate this cellular reprogramming. Because of their high regenerative capacity and their key position in metazoan evolution, holothurians and other echinoderms could surely provide answers to some of the essential questions regarding regeneration in both vertebrates and invertebrates (see Sanchez-Alvarado, 2000; Tsonis, 2000).
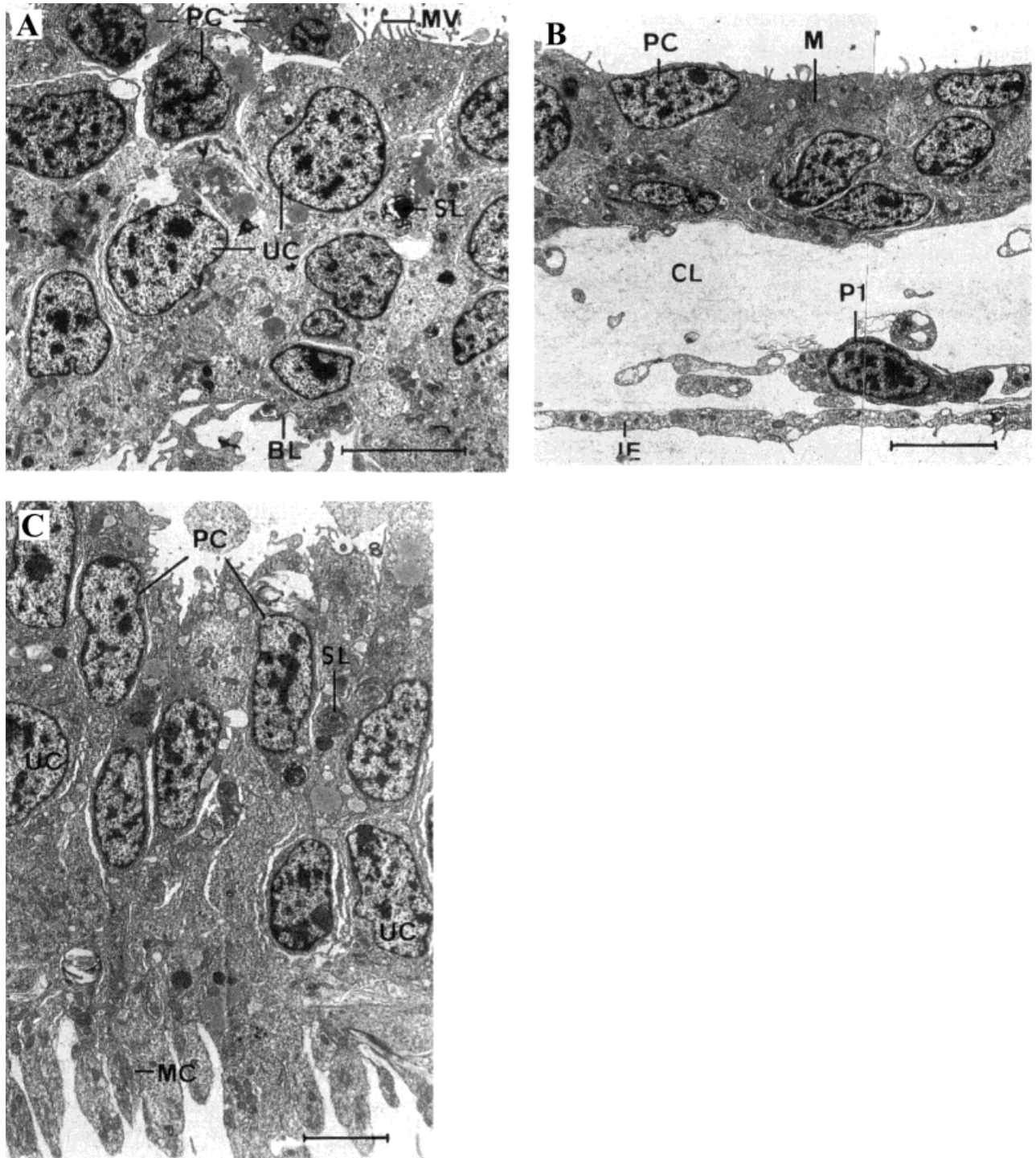
Fig. 11. Transmission electron micrographs of Cuvierian tubule regeneration in *Holothuria forskali*. **A**: Initial regenerative stage showing peritoneocytes (PC) with microvilli (MV), and undifferentiated cells (UC) with secondary lysosomes (SL), within a mesothelium defined by the basal lamina (BL). **B**: The three layers of the tissue are already defined in the following regeneration stage. The mesothelium (M) is adjacent to the connective tissue layer (CL) containing pseudopodial cells (P1), and a thin inner epithelium layer (IE) is observed. **C**: The next regenerative stage is characterized by changes in the mesothelium layer, particularly morphological changes of the peritoneocytes (PC) and the appearance of myocytes (MC) (from Vanden-Spiegel et al., 2000). Scale bars: **A,B**: 5 μm; **C**: 3 μm.

FUTURE PROSPECTS

We are interested in establishing the aspidochirote *Holothuria glaberrima* as a model system with which to study processes related to regeneration and organogenesis. Future steps will be to characterize with certainty the cellular phenotypes and the cellular and molecular mechanisms involved in regeneration; some preliminary studies have been made in this direction (García-Arrarás et al., 1998, 1999). Additional efforts have been also directed toward identifying the genes and gene products important for regeneration and some encouraging results have been obtained (Méndez et al., 2000; Santiago et al., 2000; Roig-López et al., 2001). Thus, we expect that other investigators will take advantage of the amazing regenerative properties of holothurians to expand our knowledge about the healing of wounds and the formation of organs in adult organisms.

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