RESEARCH ARTICLE

Spherulocytes in the Echinoderm *Holothuria glaberrima* and Their Involvement in Intestinal Regeneration

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The holothuroid echinoderm *Holothuria glaberrima* can regenerate its intestine after a process of evisceration. Spherule-containing cells, the spherulocytes, appear to be associated with intestinal regeneration. We have used histochemistry and immunocytochemistry to characterize these cells and their role in the regeneration process. Spherulocytes are 10–20 μm in diameter with an acrocentric nucleus and spherule-like structures within their cytoplasm. They are found in the connective tissue of the intestine and mesentery of noneviscerated and regenerating animals. During the second week of regeneration, the number of spherulocytes in the regenerating intestine increases and a dramatic change in their morphology occurs. Together with the morphological change, the immunohistochemical labeling of the cells also changes; the antibodies not only recognize the spherule structures but also label the cellular cytoplasm in a more homogeneous pattern. Moreover, immunohistochemical labeling also appears to be dispersed within the extracellular matrix, suggesting that the cells are liberating their vesicular contents. Spherulocytes are found in other tissues of *H. glaberrima*, always associated with the connective tissue component. Our data strongly suggest that spherulocytes are involved in intestinal regeneration but their specific role remains undetermined. In summary, our data expand our knowledge of the cellular events associated with regeneration processes in echinoderms and provide for comparisons with similar processes in vertebrates. *Developmental Dynamics 235:3259–3267, 2006.* © 2006 Wiley-Liss, Inc.

Key words: wound healing; regeneration; echinoderm; digestive tract; organogenesis

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INTRODUCTION

All metazoans show a capacity to regenerate cells and tissues after wounding or physical trauma. This regenerative ability, however, varies greatly among animals. Some well-studied organisms such as the fruit fly *Drosophila* or the nematode *Caenorhabditis elegans* have very limited regeneration capacity, whereas others, such as the coelenterate *Hydra*, the flatworm *Planaria*, and the echinoderms show superb regeneration feats (Sanchez Alvarado, 2000, 2004; Tsonis, 2000; Brockes et al., 2001; Candia Carnevali and Bonasoro, 2001; García-Arrarás and Greenberg, 2001).

Our laboratory has been studying the regeneration phenomenon by focusing on a novel model system that shows striking regeneration capacities, the sea cucumber *Holothuria glaberrima*. Holothurians can undergo a process of evisceration, wherein most of the viscera are expelled (Hyman, 1955; García-Arrarás and Greenberg, 2001; Byrne, 2001). Evisceration is followed by a process of regeneration whose outcome is the formation of new organs. We have focused our studies on the regeneration of the intestine to understand the cellular and genetic mechanisms that

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are key to the regeneration process (García-Arrarás et al., 1998, 1999).

The process of intestinal regeneration in *H. glaberrima* has been well documented elsewhere (García-Arrarás et al., 1998; Quiñones et al., 2002). In brief, the animals regenerate their digestive tract following its loss by chemically induced evisceration. The first organ to regenerate is the intestine, which originates from a thickening of the mesenterial edge. A blastema-like structure forms at the tip of the mesentery during the first week of regeneration. This intestinal primordium continues its growth during the second week, and proliferation and migration of luminal epithelial cells from the cloaca and from the esophagus form the new lumen within the primordium. Cellular differentiation and reorganization occurs during this process, and by the fourth week of regeneration a smaller, but apparently functional, replica of the normal intestine has formed.

During our experimental work, we have observed the presence of spherule-containing cells within the regenerating intestine. These cells, from here on named spherulocytes, have been described previously in the coelomic fluid and connective tissues of several holothurian species with multiple names, including colorless morula, migratory plasma cell, cellules muriformes, colorless amoebocyte with spherules (see Endean, 1966), large spherule amoebocytes (Cowden, 1968), spherule cells (D'Ancona and Canicatti, 1990), or spherulocytes (Jans et al., 1996). Spherulocytes have been proposed to play roles associated with defense mechanisms such as production of chemotactic or antibacterial agents, cell clumping, and encapsulation (Dybas and Fankboner, 1986; Jans et al., 1996; Pagliara et al., 2003) and roles associated with the formation and maintenance of extracellular matrix (ECM; Endean, 1966; Menton and Eisen, 1973; Fontaine and Lambert, 1977; Byrne, 1986).

We have now obtained several monoclonal antibodies that label the spherulocyte population. With these antibodies on hand, we have aimed to determine whether these cells might be playing a role in the intestinal regeneration process. Our findings are consistent with such a role. We have found that spherulocytes increase in numbers during the regeneration process and undergo morphological changes that are associated with the secretion of their granule contents. The discovery of a cell population associated with the regeneration of the intestine may provide an important insight on the cellular and molecular events associated with the regenerative capacity of echinoderms.

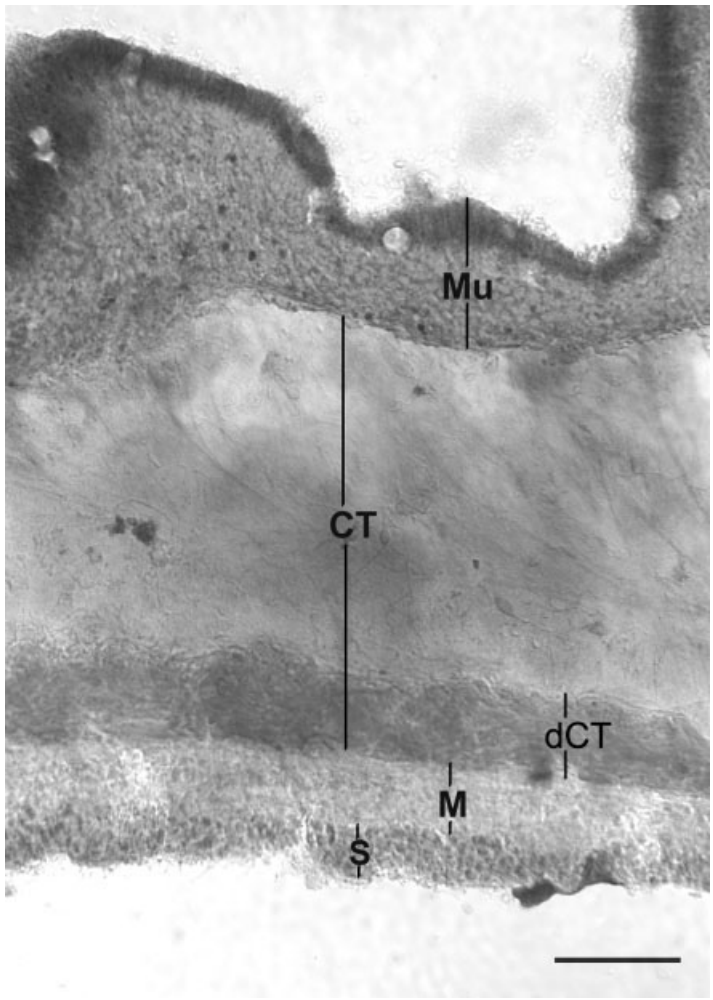


Fig. 1. Transverse section of a noneviscerated (normal) large intestine using toluidene blue dye. The four main layers of the intestine are shown: serosa (S), muscle (M), connective tissue or submucosa (CT), and mucosa (Mu). Staining is stronger in the dense connective tissue (dCT) area within the submucosa that can be observed adjacent to the muscle layer. Increase staining is also observed in the apical part of the luminal epithelia. Scale bar = 50 μm.

RESULTS

To understand the results of this work, it is important to have a clear understanding of the intestine's overall structure. This structure is presented in Figure 1, showing that the holothurian intestine is composed of four main layers: a serosa or coelomic epithelial layer, a muscle layer, a connective tissue layer or submucosa, and a luminal epithelial layer or mucosa. The muscle layer itself is made up of two individual layers: a longitudinal muscle layer and a circular muscle layer. In the posterior or large intestine, the submucosa can also be divided into two layers or areas, a dense connective tissue area close to the muscle layer and a wider area of connective tissue that appears to contain fewer cells and ECM fibers.

Characterization of Cell Types

Immunocytochemistry.

Cells were mainly found within the connective tissue of the intestine and of the mesentery (Fig. 2). Although

they were distributed throughout the connective tissue, they were most frequently found in the dense connective tissue layer adjacent to the muscle layer or bordering the basement lamina of the luminal epithelium. When the number of immunoreactive cells was counted in different areas of the connective tissue, between three and five spherule cells were labeled per microscope field of view (3 ± 1.4; mean ± SE for Sph1; 4 ± 1.3 for Sph2; 4.6 ± 1.3 for Sph3). This finding accounted for approximately 8 to 10% of the cells found within the same area (as measured by double labeling where all cell nuclei in the section are labeled with Hoescht dye).

Four monoclonal antibodies, obtained from four different fusions, labeled the spherule-containing cells in the intestine of noneviscerated *Holothuria glaberrima*. The four antibodies appeared to label the same cellular phenotype: cells of a round or elliptical morphology that varied in size between 10 and 20 μm (Fig. 3). The antibodies clearly defined multiple vesicular structures or spherules within the cell cytoplasm. When these cells were observed in phase contrast, the cells showed a small but distinct acrocentric nucleus and round or elliptical morphology, however, the spherules were not clearly evident. Small differences in labeling between antibodies can also be appreciated. For example, Sph2 appeared to label the vesicles with a punctuated pattern, while the labeling by the other antibodies appeared to be more homogeneous.

To determine whether each antibody was truly labeling the same cells, combinations of two antibodies were used in several tissue sections of normal and regenerating animals and compared with sections where only single antibodies were used. No significant differences were observed when double labeled combinations were compared with single labeling (results not shown), suggesting that all four antibodies labeled the same cell population.

Histological dyes.

During the immunohistochemistry experiments, we consistently observed that the spherules present within the immunoreactive cells, although clearly visible with immunoreactivity, were not necessarily observed by phase contrast. At the same time, vesicle-loaded cells that were evident using phase microscopy were not necessarily labeled by our antibodies (Fig. 4A,B). In our previous experiments using Milligan Trichrome stain (García-Arrarás et al., 1998), we had defined a vesicle-loaded cell phenotype as morula cells. To determine the relationship between this cell type and the immunoreactive cells, we stained sections with toluidene blue or with thionine. This revealed two vesicle or spherule-containing cell populations: one that was intensively labeled by both toluidene blue and thionine and another that was either unlabeled or only lightly stained (Fig. 4C). In fact, the population of cells that was stained with the classic dyes accounted for only 3.7 ± 0.6% (mean ± SE) of cells within the tissue. Moreover, staining sections with both immunocytochemistry and the histological dyes showed that the morula cell, whose vesicles were clearly observed in phase contrast microscopy stained deep purple but was not usually recognized by the Sph antibodies (Fig. 4D,E). In contrast, the cells labeled with the Sph antibodies were either lightly stained with toluidene blue and thionine or not stained at all. Cell counts in sections from the large intestine showed that less than 10% of the toluidene blue-labeled cells were also labeled by the Sph3 antibody, and that less than 5% of the Sph3 immunoreactive cells could be recognized using toluidene blue labeling.

Temporal Changes During Intestinal Regeneration

We studied the spherule cell population labeled by our antibodies during the process of intestinal regeneration. Because similar results were obtained with the three antibodies that were tested (Sph1, Sph2, and Sph3), the results are presented together. Thus these results highlight the changes in cell numbers and cell morphology and not differences among the antibodies themselves.

In the 7-day regenerating structure, we observed a slight increase in the number of immunoreactive cells within the connective tissue when compared with the number present within the connective tissue of normal, noneviscerated intestine (Fig. 5A). The number of cells continued to rise, peaking at 14 days of regeneration and then decreased during the following 2 weeks to levels similar to those during early regeneration stages.

We also measured the number of spherule cells found within the mesentery, next to the regenerating intestine, at various stages of regeneration. Similar to what was found in the regenerating intestine, the number of spherule cells within the mesenterial connective tissue also increased during the second week of regeneration, with the largest increase observed at day 14 of regeneration when the number of cells more than doubles (Fig. 5B). This peak was followed by a decrease in cell number to levels similar to those of the first week.

We next measured the percentage of cells that were labeled with our antibodies to determine whether the number of spherule-containing cells really increased during the regeneration period and is not merely a reflection of a general increase in cell number. The number of Sph3 antibody labeled cells was compared with the number of Hoescht-labeled nuclei within the field of view of the microscope to determine the percentage of spherule-containing cells. In the connective tissue of the regenerating intestine, the number increased from 5.5 ±1.7% (mean ± SE) at day 7 to 12.5 ± 2.6% day 14. In the mesentery, the corresponding percentages were 4.9 ± 0.2% at day 7 and 7.9 ± 1.6% at day 14. It is interesting that, during this same period of time (as has been reported by our group, García-Arrarás et al., 1998), the number of cells in the regenerating structure that is stained with the classic dyes decreases to 2 ± 0.2% at 7 days post evisceration (dpe) and 2.2 ± 0.2% at 14 dpe, increasing at 28 dpe (3.0 ± 0.5%) toward the values found in noneviscerated intestines (3.7 ± 0.6%).

Although a significant increase in the number of immunoreactive cells was observed during the second week of regeneration, the most striking changes were observed when one focused on the morphology of the spherule-containing cells. The morphological transformations paralleled the changes observed in cell number. Dur-

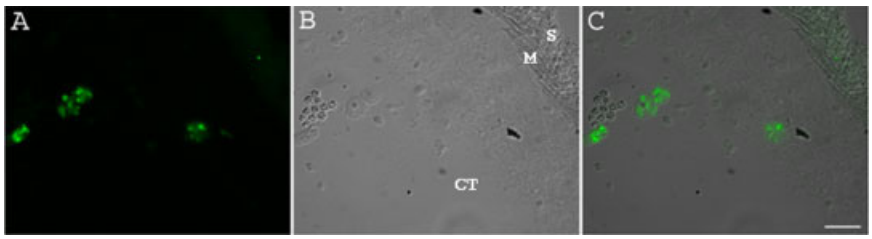


Fig. 2. Tissue localization of spherulocytes. **A–C:** A lower magnification of a transverse section of *Holothuria glaberrima* large intestine shows the immunoreactivity to Sph4 (A), the same section in phase contrast (B), and an overlay of both (C). Immunoreactive spherulocytes can be seen within the intestinal connective tissue (CT) or submucosa in the area close to the muscle layer (M) and serosa (S). Note that the immunoreactive spherules are difficult to define by phase contrast. Scale bar = 25 μm.

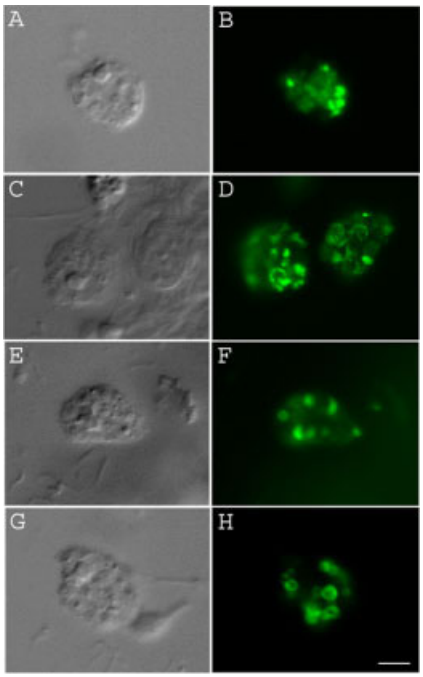


Fig. 3. Spherulocytes in *Holothuria glaberrima* labeled with monoclonal antibodies. **A–H:** Phase contrast (A,C,E,G) and immunocytochemistry with four monoclonal antibodies, Sph1 (B), Sph2 (D), Sph3 (F), Sph4 (H). The labeled cells are round or oval and contain numerous vesicular or spherule-like structures in their interior that are mainly seen by the immune reaction. Scale bar = 10 μm.

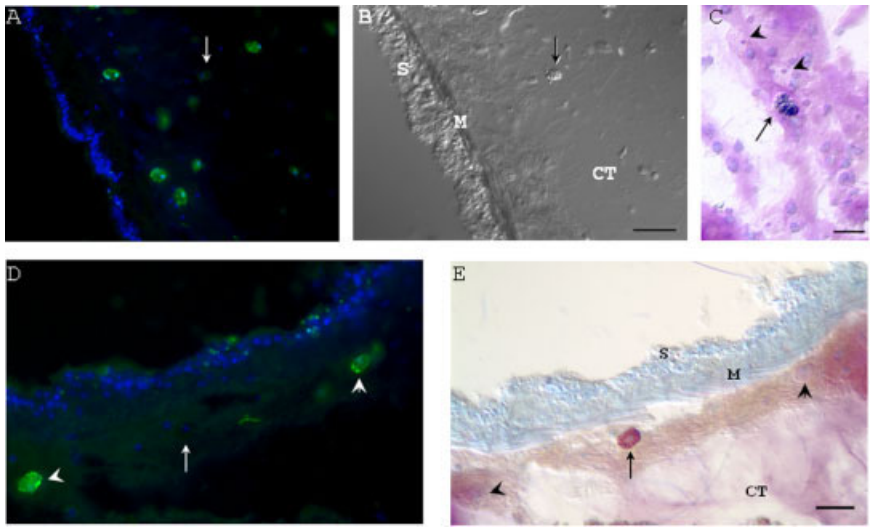


Fig. 4. Immunohistochemistry and histochemical characterization of spherulocytes. **A,B:** Immunoreactivity to Sph2 (A) and phase contrast of the same intestinal tissue section of *Holothuria glaberrima* (B) demonstrate that some spherule-containing cells (arrows) that are clearly seen by phase contrast are not labeled by the antibody. In contrast, immunoreactive cells are not easily identified in phase contrast as spherule-containing cells. **C:** Thionine dyes serve to identify two spherule containing populations, a strongly labeled cell population that stains from magenta to deep purple (arrow) and a lightly stained population (arrowheads). **D,E**: Staining with both Sph2 immunohistochemistry (D) and thionine (E) shows that the immune-labeled cells (arrowheads) are either lightly or not stained with the histological dyes, whereas the cell population that stains heavily with the classic dyes (arrow) are not labeled by the antibodies. S, serosa; M, muscle; CT, connective tissue. Scale bar = 50 μm in A,B, 25 μm in C–E.

ing the early regeneration stages (first week), most of the cells showed the spherical or elliptical morphology and the labeled vesicles described earlier (Fig. 6A–F). Nonetheless, during the second week of regeneration, the cells increased in size, sometimes to 30–40 μm (Fig. 7A–I) and lost their spherical or oval morphology, acquiring an irregular morphology where the cells appeared to spread out. In some cases, the spherules were no longer evident and the immunoreactivity seemed to have spread into the ECM. Many cells could be found in the connective tissue under the basal lamina of the luminal epithelium, where they appeared to release their vesicular contents. In fact, in some cases the boundary between the cells and the ECM appeared to be poorly defined. During the next 2 weeks of regeneration, cells in the connective tissue of the regenerating intestine once again returned to their normal morphology and spherules could be again observed within their interior (Fig. 8A–F).

In an effort to determine the vesicular content of the spherulocytes, histological stains known to label some ECM components (particularly glucosaminoglycans) were used, including toluidene blue, thionine, Alcian blue pH 2 and pH 3.4, Alcian green pH 4, Alcian yellow pH 5, periodic acid–Schiff. In addition, detection of hyaluronic acid was done using a hyaluronic acid binding protein. However, although some spherule-containing cells were labeled by some of these markers, none of them consistently labeled the large number of cells that is recognized by our monoclonal antibodies. In fact, double labeling of sections with both immunoreactivity and classic dyes again demonstrated that the immunoreactive cells in the regenerating intestine were not the same cell type as those labeled with either thionine or toluidene blue (results not shown). This finding was clearly evi-

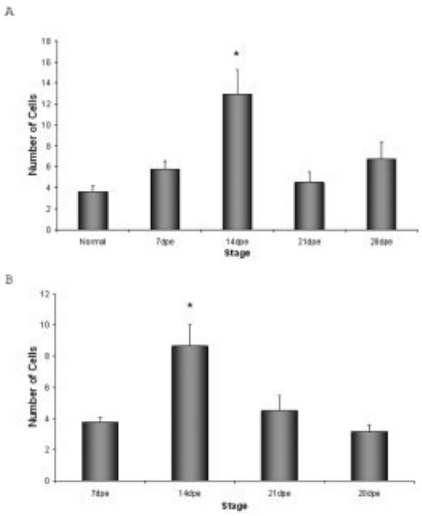


Fig. 5. Changes in the number of immunoreactive spherulocytes in the intestine of *Holothuria glaberrima* during the regenerative process. **A,B:** The number of cells in the connective tissue of the intestine (A) and mesentery (B) increases at day 14 postevisceration when compared with other stages. At least three fields of view per animal were analyzed. Values are expressed as the mean ± SE of at least three different animals per stage. Measurements made with each individual antibody were similar among themselves for each stage; therefore, they were pooled together. $^*P < 0.05$.

dent in the 14 day regenerate, where the immunoreactive spherule-containing cells increased in number and changed their morphology, whereas the cells stained with the toluidene blue and thionine remained oval and seemed to decrease in number. Additional efforts to characterize the spherulocytes and their vesicular contents were made using Western blots to characterize the antigens recognized by our antibodies. Although positive controls using anti-collagen antibodies showed that the technique worked (see Quiñones et al., 2002), we could not obtain definite labeling using the Sph antibodies even when different extraction procedures were used (results not shown). This result could be due to various reasons, including poor solubility of the antigens, changes in their tridimensional structure, or small size among others.

Tissue Distribution of Cell Types

The spatial distribution of the spherule cells within the organism could give us some clue as to the possible function of this cellular phenotype. Therefore, we next determined the tissues or organs of *H. glaberrima* where the spherule-containing cells were present. Immunoreactive spherule-containing cells were not restricted to the digestive tract, but on the contrary were found throughout the organism in a large number of tissues (results not shown). These tissues included the body wall, tentacles, respiratory tree, hemal system, and gonads. Nonetheless, one consistent finding was that spherule cells were mainly associated with the connective tissues layers. Immunoreactive cells were also found in the coelomocyte population in the coelomic fluid.

DISCUSSION

Which Cell Type Is Being Recognized?

It is obvious from our results that our antibodies are recognizing a population of spherule-containing cells. The general description of this cell type, usually named morula cell, is that of a spherical cell with a diameter of 8–20 μm and containing a large number of colorless refractile spherules or granules that are 0.5–6 μm (Hetzel, 1963). Many investigators have described these cells in various holothurian species as being intensely basophilic and have shown that they are stained with several dyes, including methylene blue and toluidene blue (Hetzel, 1963; Endean, 1966; Cowden, 1968). Other investigators have shown that the spherules display a metachromatic response staining an inner core pale blue and outer shell pink or violet (Hetzel, 1963; Menton and Eisen, 1970), whereas others describe the cells from ultrastructural observations using electron microscopy (Jans et al., 1996; Pagliara et al., 2003). However, a uniform description of the cell type is hampered by the use of different staining techniques that appear to label different subpopulations of spherule-containing cells or that might be specific for staining at different stages of their development. In fact, Byrne (1986) has shown that the spherule-containing cells within the connective tissues of *Eupenctata quinquesemita* vary in their response to dyes, with the staining intensity of the spherules decreasing as they mature.

We do know that our antibodies label spherule cells in other sea cucumber species, including *Holothuria mexicana* and *Stichopus badionotus* (unpublished observations). However, we have previously shown (and again repeated here using toluidene blue and thionine) that the number of morula cells (defined as spherule-containing cells stained with classic histological dyes) decreases within the regenerating intestine during the first 2 weeks after evisceration. This finding contrasts with the results reported here for immunoreactive spherule-containing cells that show an increase in their numbers and percentages. There are two possible explanations for our results. The first is that, similar to what has been described for other spherule-containing cells in other holothurians (Byrne, 1986), the different staining is stage-specific and that the antibodies are labeling epitopes mainly present in mature morulas, while the histological stains are principally labeling cells in early stages of development. Thus as the process of intestinal regeneration advances, the population of mature morula cells (identified by our antibodies) increases and few immature cells (stained with histological dyes) are present. However, the small percentage of cells labeled with both antibodies and histological stains in normal uneviscerated intestine and at different stages of regeneration suggest that this might not be the case. An alternate explanation is that the cells recognized by our antibodies correspond to a different cell type from those viewed using histological stains. This possibility is supported by the identification of three different spherule cell types in holothurians (D'Ancona and Canicatti, 1990; Jans et al., 1996; Pagliara et al., 2003). It is in view of this last possibility that we have favored the use of the name *spherulocytes* to identify the cells recognized by our antibodies, leaving the term *morula cell* to those recognized by histological stains.

Vesicular Content

What is the content of the spherules? The general view from various researchers is that the spherules con-

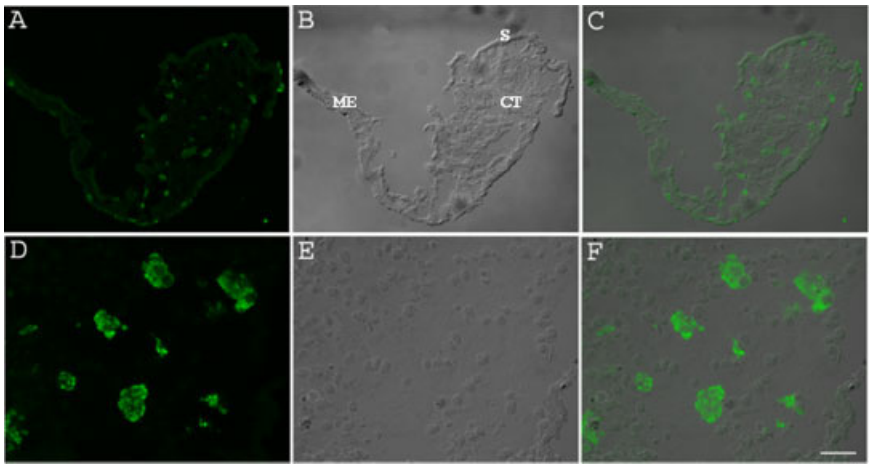
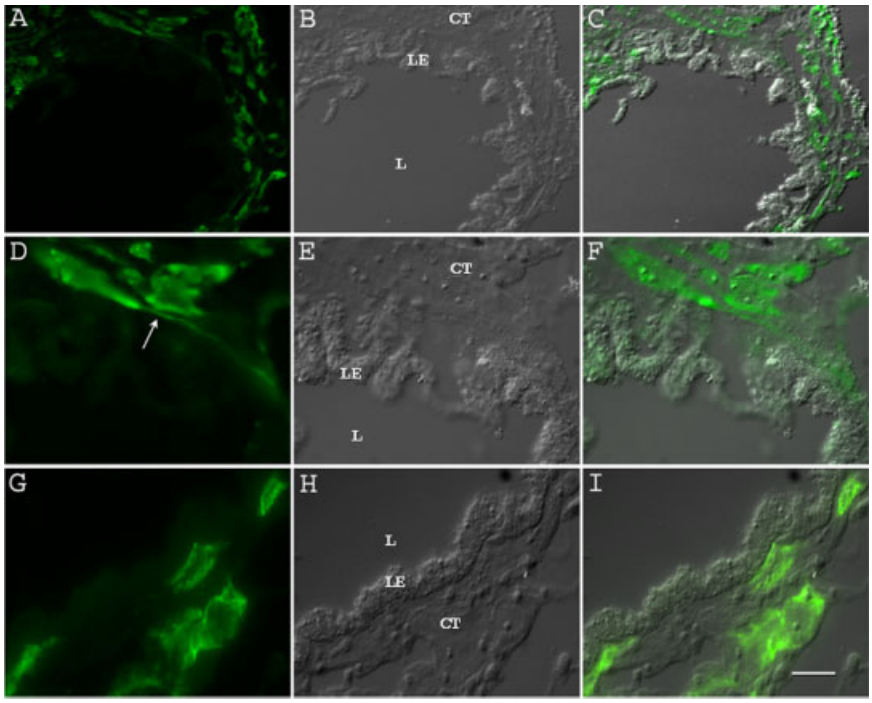


Fig. 6.



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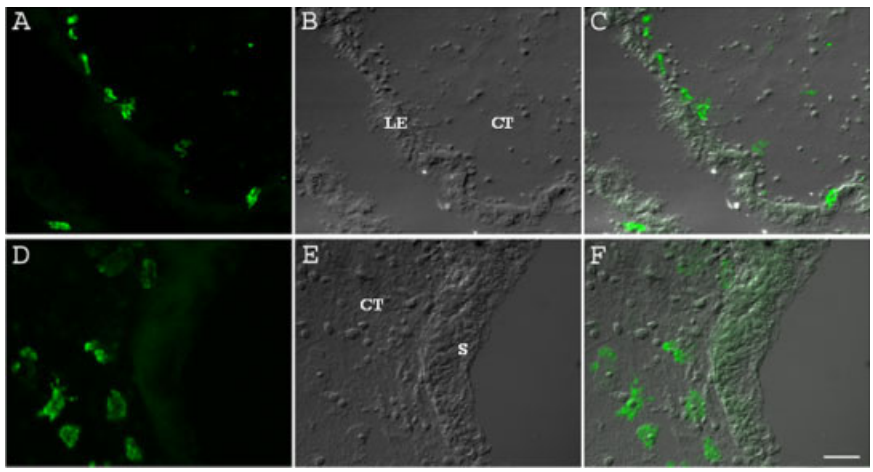

Fig. 8.

tain mucopolysachharides, which are responsible for the cell tinctorial properties. This finding is consistent with findings at the electron microscope level, showing some vesicles to contain an electron dense core, made probably of proteins, and an outer shell, made of sulfated acid mucopolysaccharides (Fontaine and Lambert, 1977). However, the negative results obtained with dyes that should have labeled at least the better known glucosaminoglucans, as well as hyaluronic acid, suggest that the cellular content might be an unusual group of mucopolysaccharides or some type of protein. The content is clearly not collagen or fibronectin, because previous findings from our group do not show collagen or fibronectin associated with spherulocytes during the extracellular

Fig. 6. Temporal changes in spherulocytes in the regenerating intestine. **A–C:** Low magnification of a cross-section from a 7-day regenerating intestine shows Sph2 immunoreactive spherulocytes within the blastema-like structure (A,D), in phase contrast (B,E) and an overlay (C,F). **D–F:** A higher magnification of a different tissue section shows that spherulocytes still have a rounded morphology and are distributed within the connective tissue (CT) of the regenerating structure. S, serosa; Me, mesentery. Scale bar = 250 μm in A–C, 25 μm in D–F.

Fig. 7. Temporal changes in spherulocytes in the regenerating intestine, 14 days postevisceration. **A–I:** Cross-sections of the 14-day regenerating intestine shows Sph1-immune (A,D) and Sph2-immune (G) labeled cells, in phase contrast (B,E,H) and an overlay (C,F,I) at low (A–C) and high (D–I) magnifications. During the second week of regeneration, Sph-immunoreactive spherulocytes undergo a dramatic change in morphology. Cells extend within the connective tissue and the immunoreactivity appears to become distributed throughout the whole cell, and in some cases even outside the cell boundaries. In some cases, the cells can be seen to extend along the basement membrane that separates the connective tissue (CT) from the recently formed luminal epithelium (LE) (arrow in D). L, lumen. Scale bar = 120 μm in A–C, 25 μm in D–F, 15 μm in G–I.

Fig. 8. **A–F:** Temporal changes in spherulocytes in the regenerating intestine at the third week (A–C) and fourth week (D–F) postevisceration. Cross-sections of the 21- and 28-day regenerating intestine shows the morphology and localization of Sph2-immune labeled cells (A,D), in phase contrast (B,E) and an overlay (C,F). Cells at this stage have mostly recovered their spherical or oval morphology and are once again distributed, in lesser numbers, close to the luminal epithelium (LE, A–C) and serosa (S, D–F) of the regenerated intestine. Scale bar = 15 μm in A–C, 25 μm in D–F.

remodeling that accompanies intestinal regeneration (Quiñones et al., 2002). This result agrees with findings by Byrne (1986) stating that the spherule-containing cells do not appear to be involved directly in collagen biosynthesis but may produce molecules that participate in fibrogenesis.

Spherulocyte Role During Echinoderm Regeneration

Our results suggest that the spherulocytes are releasing their vesicular contents within the regenerating intestine. In fact, the spread of the immunoreactivity throughout the cell body suggests that vesicles fuse with one another before or at the same time their content is being released to the extracellular space. One possibility is that the cells are involved in ECM deposition. The first indication of such a role is that the cells are localized within the connective tissue of various organs. This localization is similar to what was observed by Byrne (1986) in *Eupentacta quinquesemita*, where cells with variable staining properties and/or electron densities were predominantly associated with the connective tissue of the body wall. Second, the temporal increase in cell number coincides with the regeneration stages where there is an increase in the size of the regenerating structure and new ECM is deposited (Quiñones et al., 2002). Once again, the events closely follow those reported by Byrne (1986) in *E. quinquesemita*, where spherule-containing cells were found to degranulate and their vesicular contents (which have similar ultrastructural properties to those of the ECM) appeared to disperse and mix with the ECM. These findings are also consistent with earlier reports correlating the role of spherule-containing cells with that of fibroblasts in vertebrates, in terms of their involvement in the synthesis and deposition of connective tissue (Endean, 1966).

However, other alternate roles are possible. For example, spherule-containing cells have been associated with wound healing (Menton and Eisen, 1973). These investigators show an increase in the number of spherule-containing cells within minutes after wounding and an accumulation in the wound area 2 days after, and they propose that the cells are involved in the production of connective tissue and ground substance. This finding contradicts a previous report by Cowden (1968) showing no particular role in wound healing. However, it is possible that the latter investigator was only detecting a subpopulation of spherule-containing cells that is different from those studied by other researchers. In fact, recent results from our laboratory also show that the number of immunoreactive spherule cells increase during wound healing of *H. glaberrima* body wall (San Miguel and García-Arrarás, manuscript in preparation).

It is interesting that the peak in spherule cell numbers within the regenerating intestine correlates with the formation of the intestinal lumen. We have previously shown that proliferation of luminal epithelial cells from the cloaca and esophagus, and migration into the intestinal primordium are the main events during lumen formation and that these events take place during the second week of regeneration (García-Arrarás et al., 1998; Quiñones et al., 2002). Therefore, it might be possible that the content released from the spherules is somehow regulating the luminal epithelium proliferation and migration. Moreover, the formation of the lumen brings on an immunological challenge to the animal with the presence of the intestinal flora. Thus the possibility that spherulocytes are playing a defense role or preventive role associated with the immunological system cannot be discarded at present.

It has not escaped our attention that spherulocytes are morphologically very similar to vertebrate mast cells. Mast cells are well known mediators of injury- or infection-associated inflammation; however, recently additional functions for these cells have been suggested. In this respect, reports that suggest a role of mast cells in ECM-related processes during wound healing and fibrosis (Noli and Miolo, 2001; Puxeddu et al., 2003; Maurer et al., 2003) are of particular interest. Specifically, a role of mast cells in the aggregation of collagen fibers (Iba et al., 2004) or collagen lattice contraction (Moyer et al., 2004) has been investigated. Therefore, our findings open up the possibility that spherulocytes in echinoderms, like mast cells in vertebrates, might play an important role in the processes of wound healing and regeneration.

EXPERIMENTAL PROCEDURES

Animals

Specimens of *Holothuria glaberrima* were obtained from the north and west coasts of Puerto Rico and kept in indoor aquaria in the laboratory. Evisceration was induced by injecting 3–5 ml of 0.35 M KCl into the coelomic cavity. Animals were dissected on various days post evisceration. Further details of animal maintenance and dissection have been given previously (García-Arrarás et al., 1998, 1999).

Immunohistochemistry

Monoclonal antibodies were obtained from fusions of spleens from mice immunized using various antigens. The immunogens used were as follows: for Sphl and Sph4, homogenates of intestinal tissue (see García-Arrarás et al., 1998); Sph2, an acid-extracted and semipurified holothurian collagen fraction (see Quiñones et al., 2002); and for Sph3, a Holothurian homeobox peptide (Méndez et al., 2000) coupled to bovine serum albumen. However, there is no apparent correlation between the immunogens used and the presence of monoclonal antibodies against this cell type. We followed the protocol of Harlow and Lane (1988) to produce monoclonal antibodies. In brief, the spleens of immunized animals were dissected and spleen cells were fused using the stirring method with a spleen-myeloma cell (SP20) ratio of 2:1. Wells exhibiting good hybridoma growth were assayed by using tissue culture supernatant as the source of primary antibodies for immunohistochemistry on holothurian intestine tissue sections. Cells in wells showing interesting immunoreactive patterns were subcloned by limiting dilutions and stored.

The immunohistochemical techniques used have been described before (García-Arrarás et al., 1998, 1999). Briefly, tissues were fixed in either picric-acid formaldehyde (Zamboni) or 4% paraformaldehyde. After rinsing and embedding in Op-

timal Cutting Temperature (OCT, Tissue Tek) mounting medium, tissues were sectioned (10–20 μm) in a cryostat. Sections were treated with primary antibodies overnight at room temperature followed by the appropriate secondary antibody for 1 hr at room temperature. In cases where double labeling was performed, the two primary antibodies were added together and later the two secondary antibodies were added together (see García-Arrarás, 1993). To label nuclei, some sections were immersed in a bath of Hoescht (1 μM) during 5 min, after rinsing off the primary antibodies. Additional experiments were done by incubating sections with 4 μg/ml of biotinylated hyaluronic acid binding protein (b-HABP, from nasal cartilage, Calbiochem) for 1 hr before adding 20 μg/ml fluorescein (DTAF) -conjugated streptavidin (Jackson ImmunoResearch). All sections were observed either on a Leitz Laborlux fluorescent microscope with N2, I2/3, and D filters or on a Nikon Eclipse E600 fluorescent microscope with FITC, R/DII, and DAPI filters. Measurements were made with the help of an optical micrometer.

Histological Techniques

Tissue sections were submitted to the protocol described for staining cryostat sections with various stains, including toluidene blue, thionine, Alcian blue pH 2 and pH 3.4, Alcian green pH 4, Alcian yellow pH 5, and periodic acid–Schiff (Presnell and Schreibman, 1997). The staining was also done on tissue sections that had already been processed for immunohistochemistry. In brief, the sections were rinsed in PBS for 5–10 min, and then immersed on the dye solution for 2–10 min. They were then rinsed in PBS, mounted in buffered glycerol phosphate and viewed under the microscope.

Western Blots

Western blot techniques were similar to those used previously (Murray and García-Arrarás, 2004). In brief, homogenates were prepared from the intestinal tissue of *H. glaberrima* specimens using a Polytron (Brinkmann Instruments) and two different extraction buffers: (1) phosphate buffered saline (PBS) with protease inhibitors (Calbiochem) or (2) and a phenol/chloroform method (TriReagent, Molecular Research Center). Protein concentration was measured using Coomassie Plus protein assay (Pierce). From each sample, a volume equivalent between 1 and 50 μg of the soluble protein fraction were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Biotinylated broad range standards with avidin–horseradish peroxidase were used for molecular weight determination (Bio-Rad). Gels were equilibrated in Towbin transfer buffer with 15% methanol and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Co.). After transfer, membranes were blocked with 5% nonfat milk and rinsed in PBS with 0.1% Tween 20 (pH 7.4). Membranes were individually incubated with the Sph antibody supernatants (Sph2, -3, and -4) and detected using ECL chemiluminescent (Amersham Pharmacia).

Cell Counts

The number of cells labeled by the monoclonal antibodies was quantified by counting the number of immunoreactive cells and the number of Hoescht-labeled nuclei in an area of 280 mm^2 (the microscope field of view using the ×40 objective). Hoescht is a nuclear dye that labels all nuclei, thus providing the number of total cells within the field of view so as to obtain the percentage of total cells labeled by the monoclonal antibodies. Fields of view were chosen at random, and the area they covered was limited to the connective tissue.

In the mesentery, cells were also counted in each microscope field of view (1.4 mm mesenteric length) by moving sequentially from the junction with the regenerating structure to 4.2 mm toward the body wall. Immunoreactive cell numbers and percentages of cells (by comparing the number with the number of Hoescht-labeled nuclei) were obtained at each stage of regeneration.

Cell counts were done in at least three fields of view from each animal and at least in three different animals at each stage of regeneration. Students *t*-test and analyses of variance were used for statistical analyses.

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