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Induced inflammatory process in the sea urchin *Lytechinus variegatus*

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Abstract. *In vitro* and *in vivo* studies confirmed endocytic activity of free phagocytic amebocytes in *Lytechinus variegatus*. Amebocytes in the perivisceral coelom were labeled with injected ferritin, and ferritin-labeled amebocytes were found in the peristomial connective tissue only one hour after injection of India ink or yeast into this tissue. The presence of ferritin inside the amebocytes indicates that these cells migrated from the perivisceral coelom to the peristomial connective tissue. After 24 hours, particles of India ink or yeast were observed inside the ferritin-labeled amebocytes, indicating the amebocytes' ability to respond to an inflammatory stimulus. These were the only inflammatory cells found in *L. variegatus*, using the above mentioned stimuli and time spans.

Additional key words: Echinoidea, Echinodermata, amebocytes, coelomocytes, phagocytes

In the late 19th century, the Russian biologist Elie Metchnikoff (1854–1916) observed that some cells present in the perivisceral coelom and mesenchymal tissue of echinoderms were able to move and to internalize inert or even live particles. He observed this phenomenon in various taxa, although not in cephalochordates (Metchnikoff 1891; Silva et al. 1995, 1998), and developed the Phagocytic Theory: "I used the term phagocytosis to design the amoeboid cells able to capture and digest microorganisms and other elements. I called Phagocytic Theory the theory based on this defensive cell property" (Metchnikoff 1891). In the present study, the definition of inflammatory process used is also that of Metchnikoff: cellular migration followed by phagocytosis.

Phagocytosis by echinoderm coelomocytes was first observed in the perivisceral coelom of *Asterias rubens* by Durham in 1891 (in Smith 1981, p. 532), after injection of India ink or blue aniline. Phagocytic amebocytes were also found in the tube feet, gut wall, and other organs by Cuénot in 1891 (in Smith 1981, p. 532). Since then, numerous studies have reported many species of echinoderms taking up various substances, including bacteria, inert particles, foreign cells, and senescent cells (Cuénot 1948; Millott 1950; Boolootian & Giese 1958; Johnson & Beeson 1966; Johnson 1969c; Hobaus 1978). Among echinoderms, the uptake of particles seems to take place only by the amebocytes (Johnson 1969c; Bertheussen & Seljelid 1978; Smith 1981; Isaeva & Korenbaun 1990; Edds 1993; Plytycz & Seljelid 1993; Chia & Xing 1996; Xing et al. 1998), and only by the petaloid form of this cell type (Boolootian & Giese 1958; Johnson 1969a,c; Smith 1981). The amebocytes are also the only coelomocytes containing intranuclear iron bodies (Millott 1964; Burton 1966; Vevers 1967; Millott & Vevers 1968; Johnson 1969b; Hobaus 1978), which can be considered a typical feature of these cells.

Although phagocytosis by coelomocytes has been previously reported in sea urchins, the source of the amebocytes involved in the inflammatory process is still not clear and there are only a few references about natural occurrence of inflammatory process (Smith 1981; Chia & Xing 1996). For this reason, we were motivated to study and characterize the inflammatory cells found in the peristomial membrane tissue after injury and determine the kinetics of these cells.

Methods

Sea urchins (*Lytechinus variegatus* LAMARCK 1816) were collected along the northern coast of São Paulo state (23°49.530'S, 0.45°26.394'W). They were 140 ± 10 g in weight and contained 90 ± 10 ml perivisceral coelomic fluid. The animals were maintained in the laboratory in a 1000-liter aquarium (with running seawater passed through a biological filter; salinity 34‰; 23–25°C) and were fed algae once a week. For all procedures, the animals were anesthetized by immersion in 10% magnesium chloride dissolved in 34S sea-

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Table 1. Diameter and percentage of cell types from the perivisceral coelom of *Lytechinus variegatus,* expressed as mean ± standard deviation.

	Phagocytic amebocytes	Red spherule cells	Colorless spherule cells	Vibratile cells
Diameter (μm)	28.8 ± 5.5 (n = 23)	16.4 ± 4.5 (n = 24)	17.2 ± 4.6 (n = 18)	7.5 ± 0.9 (n = 14)
% of all cells	56 ± 16 (n = 31)	11 ± 5 (n = 31)	12 ± 5 (n = 31)	21 ± 8 (n = 31)

water (see Silva et al. 1995), which led to loss of the closing reflex of the Aristotle's lantern.

Counting and characterization of cells

Perivisceral coelomic fluid samples of 30 animals were collected through the peristomial membrane. The coelomocytes were classified according to Smith (1981), under an interference phase contrast microscope (Zeiss) with video camera (Classen), connected to an IBM-PC computer (board Iris-16). The cells were computer-scanned and a mean diameter estimated, using Sigma Scan Pro 3.0 software. The cells were also counted in a Neubauer chamber. Data are given as mean ± standard deviation.

Ferritin endocytosis *in vivo* and *in vitro*

In this study, samples were considered *in vivo* when taken from the perivisceral coelom and analyzed either immediately or after immediate fixation. Samples were considered *in vitro* when taken from the perivisceral coelom and observed and/or fixed only after at least 10 min.

For *in vivo* experiments, 0.2 ml ferritin (54 mg/ml, type I, Sigma) was injected into the perivisceral coelom of 30 sea urchins through the peristomial membrane, and samples were taken at 9 times (30 min, 1, 2, 4, 6, 8, 12, 24 h, and one week). At 30 min, a perivisceral coelomic fluid sample (0.2 ml) was taken from each of the 30 sea urchins, and the peristomial membranes of 3 animals were fixed. At each successive sampling time, a perivisceral coelomic fluid sample was taken from each of the remaining sea urchins, and again the peristomial membranes of 3 animals were fixed. The perivisceral coelomic fluid samples (n = 135) were deposited over slides, and kept in a humid chamber at 23–25°C. We used a modified McDowell fixative (McDowell & Trump 1976) prepared in 34S seawater instead of a phosphate buffer; the slides were fixed for 5 min (see Johnson 1969b) and stained by Perls' method to reveal the ferritin iron (Bancroft & Stevens 1982). Peristomial membranes (n = 27) were fixed for 48 h in McDowell saline, decalcified using 10% EDTA (in 34S seawater), dehydrated, and embedded in Historesin (LKB). Sections 2 μm thick were cut and stained with toluidine-fuchsin and Perls' method. For transmission electron microscopy (TEM), the membranes were fixed using 3% glutaraldehyde in 34S seawater, postfixed in 1% OsO_4, decalcified using 10% EDTA (in 34S seawater), dehydrated, and embedded in Spurr resin (Hayat 1981). Ultrathin sections were obtained (ultramicrotome 9LKB 500) and viewed (TEM-JEOL-JEM-100 CXII).

For *in vitro* experiments, 2 ml perivisceral coelomic fluid was collected from each of the same 30 sea urchins, just before the beginning of the *in vivo* experiment, and immediately mixed with 0.2 ml ferritin. Samples of this mixture were taken after 30 min, 1, 2, 4, 6, 8, and 12 h. No 24-h or one-week samples were taken, because the coelomocytes *in vitro* presented degenerative morphological changes. Fixation and staining were the same as for *in vivo* studies.

Table 2. Percentage of phagocytic amebocytes in the perivisceral coelom with ferritin after different experimental periods, expressed as mean ± standard deviation.

	% of amebocytes with ferritin	
Period (h)	*In vivo*	*In vitro*
0.5	4.4 ± 2.4	13.2 ± 7.0
1	11.4 ± 3.2	49.5 ± 5.9
2	33.5 ± 10.4	61.0 ± 9.2
4	69.1 ± 13.8	78.8 ± 9.1
6	100 ± 0	100 ± 0
12	100 ± 0	100 ± 0

Induced phagocytosis

The coelomic fluid of 10 animals was withdrawn and a yeast suspension (10 yeast cells/amebocyte) in seawater was added to it. Samples (0.2 ml) of this solution taken after 30 min, 1, 2, 4, and 6 h were mixed with fluorescent stains—0.1 ml fluorescein diacetate (2 μg/ml) and 0.1 ml ethyl bromide (50 μg/ml), both in seawater—for immediate observation under a fluorescence microscope (Nikon Optitphot-2, Hg lamp and B2A-560/UV-2A-510 filter).

When yeast cells (*Saccharomyces cerevisiae*) are labeled with these fluorescent stains and viewed under fluorescent light (λ, 380–420 nm) live cells radiate green light, whereas dead ones radiate red light (Cor-

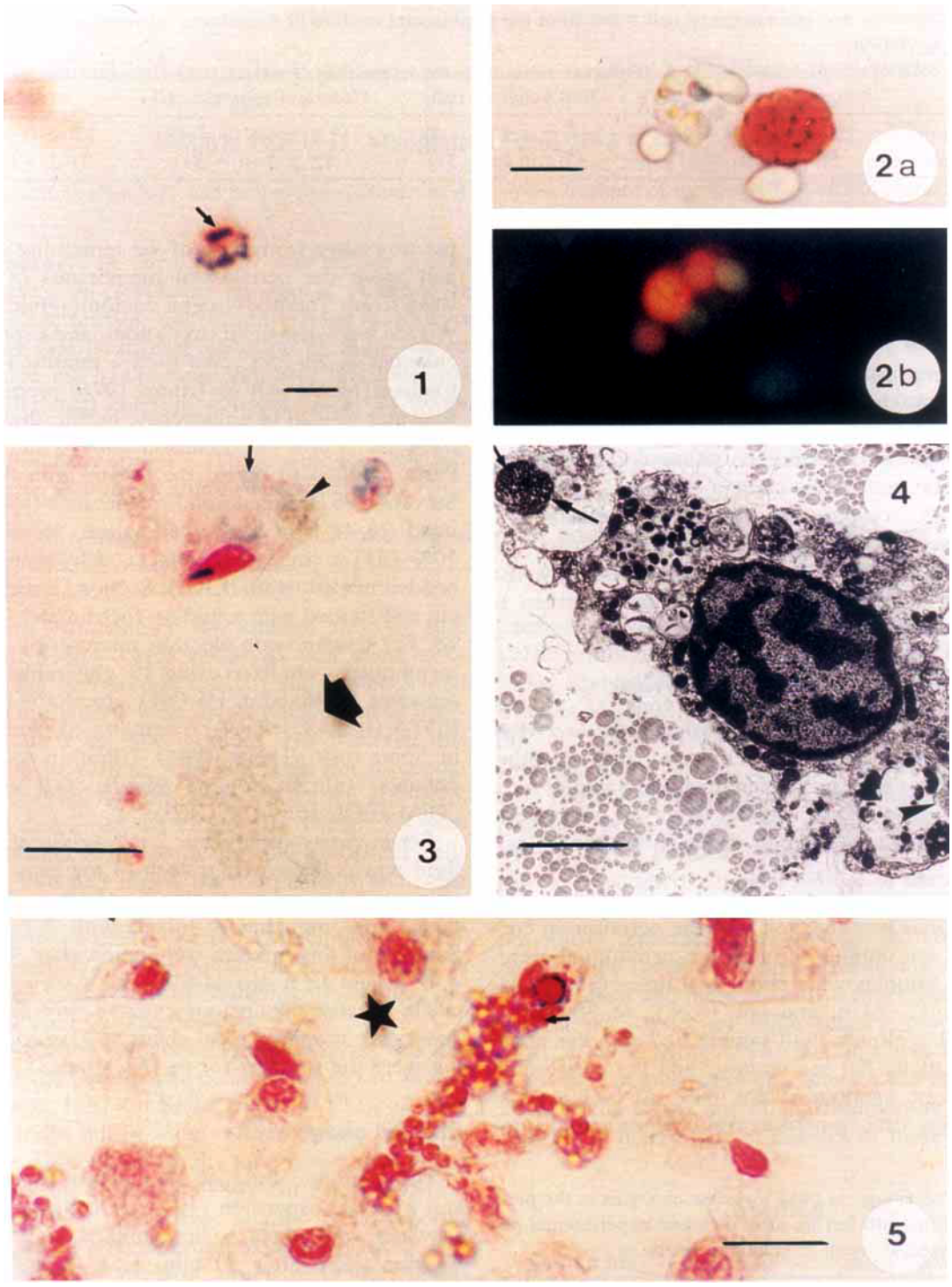

Fig. 1. Phagocytic amebocyte obtained from the perivisceral coelom 30 min after ferritin was injected into the coelom. The amebocyte presents cytoplasmic ferritin (irregular bluish granules) and an angular, intranuclear iron body (arrow) stained by Perls' method. Scale bar, 10 μm. Photomicrograph. **Fig. 2. a.** Phagocytic amoebocyte after 6 h of incubation with yeast solution, with phagocytized yeast cells, under conventional light microscopy. We also observed a red spherule cell. **b.** Cells in Fig. 2a under fluorescence microscopy, the dead yeast cells are red and the live yeast cells are green. Scale bar, 10 μm. Photomicrographs. **Fig. 3.** Section of the peristomial membrane collected from a sea urchin with intracoelomic ferritin inoculation 6 h before injection of intraperistomial India ink. The animal was maintained for more than 24 h before sacrifice. We observed an infiltrated phagocytic amoebocyte with intranuclear iron bodies, intracytoplasmatic ferritin (small arrow)

rea 1991). A sample of yeast in seawater was also subjected to the same experimental conditions, as a yeast viability control.

Induced cell migration

We injected 0.2 ml ferritin into the perivisceral coelom of 30 animals in order to mark the phagocytic amebocytes. After 6 h, 0.2 ml India ink was injected, as an irritant, into the connective tissue of the peristomial membrane. After 30 min, 1, 2, 4, 6, 12, 24, and 48 h, the animals were dissected and their peristomial membranes fixed and processed for light microscopy (Perls' method) and TEM. In this study, we considered India ink as being pinocytized (Rabinovitch 1995), despite a small number of authors who consider it to be phagocytized (Smith 1981; Plytycz & Józkwicz 1994).

Induced inflammatory process

We injected 0.2 ml ferritin into the perivisceral coelom of 10 animals in order to mark the amebocytes. After 6 h, 0.2 ml yeast suspension (7900 yeast cells/mm^3) was injected into the connective tissue of the peristomial membrane. After 24 h, the animals were dissected and their peristomial membranes were fixed and processed for light microscopy and TEM.

Results

Count and characterization of coelomocytes

Four types of coelomocytes were found in the sea urchin *Lytechinus variegatus*: phagocytic amebocytes, red spherule cells, colorless spherule cells, and vibratile cells (Table 1). The total number of coelomocytes varied substantially between animals: 1685 ± 989 coelomocytes/mm^3 (n = 31 counts).

The amebocytes were observed in filopodial and petaloid forms as well as an intermediate form; *in vitro* they progressively acquired a filopodial form. As previously found (Smith 1981), these cells present wide cytoplasmic projections in all directions, are colorless, exhibit various sizes and forms, and are slightly acidophilic. Estimates of diameter did not include all the cytoplasmic projections. Iron intranuclear bodies were found only in amebocytes, thus serving as a distinguishing factor of these cells.

Ferritin endocytosis *in vivo* and *in vitro*

All forms of amebocytes phagocytized ferritin that was injected into the perivisceral coelom (Fig. 1), and the percentage of amebocytes containing ferritin increased with time, both *in vivo* and *in vitro* (Table 2). In the *in vivo* experiment, ferritin was still found inside the amebocytes after one week. Ferritin appeared as round cytoplasmic inclusions of various sizes, differing from the intranuclear crystalloids, which were angular and deep blue (Fig. 1).

In light microscopy of the peristomial membrane, we were unable to detect any amebocytes in the connective tissue through Perls' method, which stains the intranuclear iron crystalloids. Furthermore, no ferritin was observed in the epithelium or connective tissues of the peristomial membrane, in any of the experimental time periods of the *in vivo* ferritin endocytosis studies.

Induced phagocytosis

The amebocytes phagocytized and killed the yeast cells *in vitro*. Under fluorescence microscopy, the amebocytes with phagocytized yeast cells radiated green light after 30 min. After 6 h, the free yeast cells that remained radiated green light, whereas 75% of those phagocytized (1 to 5 yeast cells per amebocyte) radiated red light (Fig. 2a,b), indicating that they were dead.

The yeast cells in seawater solution (yeast viability control) radiated green light from the initial experimental period of 30 min until the 6-hour mark, under fluorescence microscopy. The viability was always higher than 98%.

Induced cell migration

One hour after injection of India ink into the peristomial membrane of the animals, amebocytes were observed by light microscopy in the connective tissue of the peristomial membrane. These cells, when stained using Perls' method, presented blue cytoplas-

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and India ink (arrowhead); and we also observed India ink free within the peristomial membrane tissues (large arrow). Stained by Perls' method. Scale bar, 10 μm. Photomicrograph. **Fig. 4.** Section of the peristomial membrane from a sea urchin with intracoelomic ferritin and intraperistomial India ink (24 h). A pseudopodial formation surrounds India ink particles (arrows). A vesicle containing ferritin can also be seen (arrowheads). Scale bar, 2 μm. TEM. **Fig. 5.** Section of the peristomial membrane from an animal 24 h after injection of yeast solution into the connective tissue of the peristomial membrane. The phagocytic amebocyte with intracytoplasmic yeast cells (arrow) can be seen in the connective tissue (star). Stained by toluidine-fuchsin method. Scale bar, 10 μm. Photomicrograph.

mic granules (ferritin) and, in some cells, angular intranuclear bodies stained deep blue. Amebocytes were predominantly observed infiltrated within the connective tissue of the peristomial membrane contiguous to the epithelium that separates the peristomial membrane from the peripharyngeal coelom. After 2 to 24 h, amebocytes were increasingly found deeper within the connective tissue of the peristomial membrane.

All amebocytes found in the peristomial membrane tissue contained ferritin during all experimental periods (from 1 to 48 h). Only after 24 to 48 h were both ferritin and darkened cytoplasm observed, indicating the presence of India ink (Fig. 3). In TEM, a vesicle containing ferritin showed an electron-dense core, surrounded by a less electron-dense material (Fig. 4). India ink presented an irregular crystalloid aspect at an ultrastructural level (Fig. 4). Some of these cells also presented angular intranuclear bodies.

Induced inflammatory process

Amebocytes were observed in histological preparations 24 h after the injection of yeast solution into the connective tissue of the peristomial membrane. When stained by Perls' method, these cells revealed a cytoplasm containing blue granules, indicating the presence of ferritin as well as intranuclear iron bodies. Amebocytes with phagocytized yeast were also observed (Fig. 5).

Discussion

The types and percentage variation among the different types of coelomocytes, the great variation in their total number, the finding that the phagocytic amebocyte was the most abundant coelomocyte, and that it appeared in different forms, all confirm previous findings in the literature on echinoids (Johnson 1969a; Bertheussen & Seljelid 1978; Smith 1981; Isaeva & Korenbaun 1990; Edds 1993; Chia & Xing 1996). The transformation of the amebocytes from petaloid to filopodial form, occurring progressively *in vitro,* could be triggered by their contact with glass (Bertheussen & Seljelid 1978; Hobaus 1978; Smith 1981; Isaeva & Korenbaun 1990; Edds 1993; Chia & Xing 1996). The finding that only the amebocytes displayed took up both ferritin and India ink suggests the capacity of this cell type to recognize foreign bodies.

After one week, the amebocytes showed no morphological alterations, suggesting that ferritin does not dramatically change their biology in the time period and dose range studied. Moreover, ferritin was endocytized only by amebocytes and not by other coelomocytes, and was easily identified in both light microscopy and TEM (see also Bancroft & Stevens 1982). For these three reasons, ferritin appears to be a good marker for free amebocytes in the coelomic cavity.

The percentage of phagocytic amebocytes in the perivisceral coelom with ferritin, at sampling times up to 2 h, was greater in the *in vitro* experiments than *in vivo* (Table 2). This difference could reflect the much greater ferritin concentration in the *in vitro* experiments, or be due to slow spreading through the perivisceral coelom, which is large compared to the small volumes used in the *in vitro* experiments. Moreover, attachment to the glass slides and consequent cellular aggregation may enhance endocytosis, as Yui & Bayne (1983) suggested for bacterial clearance in the sea urchin *Strongylocentrotus purpuratus.*

Our results on induced phagocytosis demonstrate that the amebocytes are able to recognize the yeast cells as non-self and continuously phagocytize and, at least *in vitro,* kill them. This finding is relevant because it suggests that amebocytes are also capable of participating in an inflammatory process and killing foreign cells in various tissues. The killing ability of *in vitro* amebocytes suggests that these cells may play a role in resistance to infection. The mechanisms involved in the yeast-cell death probably include the fusion of the phagocytized material with the cytoplasmic granules present in the amebocytes (Chia & Xing 1996; Gross et al. 1999).

In those animals that received an injection of India ink to the peristomial membrane 6 h after the injection of ferritin into the perivisceral coelom, amebocytes were later found in the peristomial membrane. The presence of ferritin in these cells indicates that they migrated from the perivisceral coelom, as no ferritin was present in the peristomial membrane. The India ink apparently triggered an active migration of amebocytes from the perivisceral coelom into the peristomial membrane. The mechanism that enables the amebocytes to recognize India ink as foreign material, as well as the factors responsible for cell migration, require further study.

After the injection of yeast into the peristomial membrane, cellular migration of amebocytes followed by phagocytosis of yeast occurred—the first report of an induced inflammatory process in the peristomial membrane of a sea urchin. Other published studies describe the presence of red spherule cells, in addition to amebocytes, during abnormal epithelial growth in *Strongylocentrotus franciscanus* (Johnson & Chapman 1970), during development of the spotted gonad disease in *Strongylocentrotus intermedius* (Shimuzu 1994), in the inflammatory-like reaction of individuals experimentally infested with the bald-sea-urchin disease (Maes & Jangoux 1984), and in the disease causing mass mortality of sea urchins (*Strongylocentrotus*

droebachiensis) in Nova Scotia (Jones et al. 1985). These findings, however, all involve infections associated with inflammatory processes in other tissues and for longer experimental periods. The absence of spherule cells in the present study suggests either that the amebocyte is the first coelomocyte cell type to migrate to the injury site during the inflammatory process, perhaps followed by the red spherule cell in longer experimental time spans, or that in this tissue and with this noninfective stimulus, it is actually the only cell type to migrate. A follow-up to the present study, using longer time spans and different types of stimuli, might illuminate the roles of the various cell types.

The inflammation concept postulated by Metchnikoff (1891), "cell migration followed by phagocytosis," was fully applicable to this study, with other usual definitions being best applied to mammals and other animals with closed circulatory systems. Based on Metchnikoff's concept, an inflammatory process can be characterized in *Lytechinus variegatus,* since the amebocytes migrated from the coelomic cavity, infiltrated through the connective tissue, reached the lesion site of the peristomial membrane and, once there, phagocytized yeast cells.

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References

Bancroft JD & Stevens A 1982. Theory and Practice of Histological Techniques. Churchill Livingstone, London. 662 pp.

Bertheussen K & Seljelid R 1978. Echinoid phagocytes in vitro. Exp. Cell. Res. 111: 401–412.

Boolootian RA & Giese AC 1958. Coelomic corpuscles of echinoderms. Biol. Bull. 115: 53–63.

Burton MM 1966. Echinoid coelomic cells. Nature 211: 1095–1096.

Chia F-S & Xing J 1996. Echinoderm coelomocytes, a review. Zool. Stud. 35(4): 231–254.

Correa B 1991. Fluorescent viability test of fungi cells. Appl. Fluorescence Technol. 3: 8–10.

Cuénot L 1948. Anatomie, éthologie, et systématique des échinodermes. In: Traité de Zoologie: Échinodermes, Stomocordes, Procordes, vol. 2. Grassé P, ed., Masson, Paris.

Edds KT 1993. Cell biology of echinoid coelomocytes. 1. Diversity and characterization of cell types. J. Invertebr. Pathol. 61: 173–178.

Gross PS, Al-Sharif WZ, Clow LA, & Smith LC 1999. Echinoderm immunity and evolution of the complement system. Dev. Comp. Immunol. 23(4–5): 429–442.

Hayat MT 1981. Fixation for Electron Microscopy. Academic Press, London. 437 pp.

Hobaus E 1978. Studies on phagocytes of regular sea urchins (Echinoidea, Echinodermata). 1. The occurrence of iron containing bodies within the nuclei of phagocytes. Zool. Anz. 200: 31–40.

Isaeva VV & Korenbaun ES 1990. Defense functions of coelomocytes and immunity of echinoderms. Sov. J. Marine Biol. 15(6): 353–363.

Johnson PT 1969a. The coelomic elements of sea urchins (*Strongylocentrotus*). I. The normal coelomocytes, their morphology and dynamics in hanging drop. J. Invertebr. Pathol. 13: 25–41.

——— 1969b. The coelomic elements of sea urchins (*Strongylocentrotus*). II. Cytochemistry of the coelomocytes. Histochemie 17: 213–231.

——— 1969c. The coelomic elements of sea urchins (*Strongylocentrotus*). III. In vitro reaction to bacteria. J. Invertebr. Pathol. 13: 42–62.

Johnson PT & Beeson RJ 1966. In vitro studies on *Patiria miniata* (Brandt) coelomocytes, with remarks on revolving cysts. Life Sci. 5: 1641–1666.

Johnson PT & Chapman FA 1970. Abnormal epithelial growth in sea urchin spines (*Strongylocentrotus franciscanus*). J. Invertebr. Pathol. 16: 116–122.

Jones GM, Hebda AJ, Scheibling RE, & Miller RJ 1985. Histopathlogy of the disease causing mass mortality of sea urchins (*Strongylocentrotus droebachiensis*) in Nova Scotia. J. Invertebr. Pathol. 45: 260–271.

Maes P & Jangoux M 1984. The bald-sea-urchin disease: a biopathological approach. Helgol. Meeresunters. 37: 217–224.

McDowell EM & Trump BF 1976. Histologic fixatives suitable for diagnostic light and electron microscopy. Arch. Pathol. Lab. Med. 100: 405–414.

Metchnikoff E 1891. Lectures on the comparative pathology of inflammation delivered at Pasteur Institute in 1891. Dover, New York [republished in 1968]. 224 pp.

Millott N 1950. Integumentary pigmentation and the coelomic fluid of *Thyone briareus* (Lesueur). Biol. Bull. 99: 343–344.

——— 1964. Pigmentary system of *Diadema antillarum* Philippi. Nature 203: 206–207.

Millott N & Vevers HG 1968. The morphology and histochemistry of the echinoid axial organ. Phil. Trans. R. Soc. London 253: 201–230.

Plytycz B & Józkowicz A 1994. Differential effects of temperature on macrophages of ectothermic vertebrates. J. Leukoc. Biol. 56: 729–732.

Plytycz B & Seljelid R 1993. Bacterial clearance by the sea urchin *Strongylocentrotus droebachiensis.* Dev. Comp. Immunol. 17: 283–289.

Rabinovitch M 1995. Phagocytosis. Trends Cell Biol. 5: 85–88.

Shimuzu M 1994. Histopathological investigation of the

spotted gonad disease in the sea urchin *Strongylocentrotus intermedius*. J. Invertebr. Pathol. 63: 182–187.

Silva JRMC, Mendes EG, & Mariano M 1995. Wound repair in the amphioxus (*Branchiostoma platae*), an animal deprived of inflammatory phagocytes. J. Invertebr. Pathol. 65: 147–151.

——— 1998. Regeneration in the amphioxus (*Branchiostoma platae*). Zool. Anz. 237: 107–111.

Smith VJ 1981. The echinoderms. In: Invertebrate Blood Cells, vol. 2. Ratcliffe NA & Rowley AF, eds., pp. 513–562. Academic Press, London.

Vevers HG 1967. The histochemistry of the echinoid axial organ. Symp. Zool. Soc. London 20: 65–74.

Xing J, Leung M-F, & Chia F-S 1998. Quantitative analysis of phagocytosis by amebocytes of a sea cucumber, *Holothuria leucospilota*. Invertebr. Biol. 117(1): 67–74.