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Sipunculus nudus: Particulate components of the coelomic fluid and its relationship with brown bodies

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Sipunculus nudus: particulate components of the coelomic fluid and its relationship with brown bodies

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ABSTRACT

Corpuscular components of the coelomic fluid were observed in *Sipunculus nudus* using cytochemical and cytoenzymatic methods. In particular, granulocytes were studied and two different types, based on morphology, chemical composition, and function were distinguished. Cell aggregates, identified as stem cells of type II granulocytes, were also evidenced. Besides, empty vesicles, vesicle fragments, laminar structures and brown bodies were observed. Based on the observation of their structure and ultrastructure, brown bodies were attributed with the function of accumulating foreign material, as well as aged components typical of the coelomic fluid.

KEY WORDS: *Sipunculus nudus* - Coelomic fluid particulate material - Cellular ageing and brown bodies.

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INTRODUCTION

The phylum Sipuncula contains over 300 described ubiquitous marine species (Ghirardelli, 1995; Brusca & Brusca, 1996) which vary in size, but all possess a thick trunk and a more slender introvert with a mouth at its end surrounded by tentacles and hooks. Inside, beyond the mouth there are an oesophagus and a U-shaped coiled intestine with one or two loops and a dorsal anterior anus.

The musculature enclosing the body consists of outer circular smooth muscles under the derm and inner longitudinal muscles. The enclosed coelomic cavity is lined with a peritoneum of flattened cells, and is filled with coelomic fluid containing coelomocytes and gametes.

Attached to the surface of the oesophagus, one or two compensation sacs extend into the perivisceral coelom.

Extensive research has been done on sipunculan coelomocytes, which have been classified in as many as five categories: haemocytes, granulocytes, large multinuclear cells, ciliated urn cell complexes, and immature cells (Dybas, 1981a). Not all cell types are represented in every species. Several types of coelomocytes of the genus *Phascolosoma* (Andrews, 1890; Dawson, 1933; Volkonsky, 1933; Ohuye, 1938; Hyman, 1959; Towle, 1970; Dybas, 1975, 1976, 1981a, b) and of the genus *Golfingia* (Hérubel, 1908; Brown & Winterbottom, 1969; Ochi, 1975) were described. In *Sipunculus nudus*, coelomocytes were studied by Hérubel (1908), Volkonsky (1933), and (Dybas, 1981a). In this species, Hérubel (1908) described hématies, young and old amoebocytes and also enigmatic vesicles and motile and fixed urns.

Granulocytes appear to be the main phagocytic cells in *P. vulgare* (Dybas, 1981a), *S. nudus* (Cantacuzène, 1922; Volkonsky, 1933; Valembois & Boileudieu, 1980; Matozzo *et al.*, 2001), *Dendrostomum zostericolum* (Triplett *et al.*, 1958) and *Themiste petricola* (Blanco *et al.*, 1995). Moreover, several granulocytes act in humoral defence in *S. nudus* (Boiledieu & Valembois, 1977) or in other Sipunculida (Weinheimer *et al.*, 1970; Evans *et al.*, 1973). Dybas (1981b) in *P. agassizii* demonstrated that acid and alkaline phosphatases, peroxidase and lipase enzymes were present in the granulocytes.

Free-swimming ciliated urn cell complexes were observed for the first time in *S. nudus*. They originate from fixed urn cells protruding from the connective tissue (Selensky, 1908) or the epithelial lining of the coelom (Cantacuzène, 1928). Ciliated, transparent and pluricellular (Metalnikoff, 1900), the urn cell complexes have been described as consisting of two separate, firmly attached cells though able to move at the attachment. The anterior cell can be described as a transparent bubble with a flattened base and short neck. The posterior cell has a clear central secretory area (Bang & Bang, 1962). Ciliated urn cell complexes secrete a sticky mucus by which they can recognize and trap foreign cells, as described in *S. nudus* (Matozzo *et al.*, 2001). The foreign cells are then phagocytized and

lysed by other coelomocytes (Cantacuzène, 1922; Bang & Bang, 1962, 1965, 1974, 1975, 1976, 1980). The release of mucus from urn cell complexes is lectin-induced (Nicosia, 1979).

In *P. agassizii*, urn cell complexes do not secrete any mucus and both particles and cells are trapped in the current created by the beating cilia, and then phagocytized by both cupola cells and lobe cells (Dybas, 1976).

In some holothurians, echinoids and ophiuroids, coelomocyte aggregates, conventionally referred to as brown bodies (Hyman, 1955; Hetzel, 1965; Shinn, 1985; Dybas & Frankboner, 1986; Canicattì & D'Ancona, 1989; D'Ancona *et al.*, 1989), form around foreign materials (Smith, 1981). They represent an efficient structure related to the defence mechanism of the host. The formation of brown bodies has also been experimentally induced in *Holothuria polii*, following injection of formalinized sheep erythrocytes (Canicattì & D'Ancona, 1989; D'Ancona *et al.*, 1989). In both, natural and experimental cases, brown bodies consist of nodules constituted by amebocytes accumulated in a granular extracellular matrix containing spherula cells.

With the exception of urn cell complexes, little is known about the specific functional roles of the other coelomic cells in *S. nudus* Linnaeus, 1767. In the present study, we aim to present new data on the corpuscular components of the coelomic fluid, and attention has been focused on the relationship between components of the coelomic fluid and brown bodies.

MATERIALS AND METHODS

Adult specimens of *S. nudus* from the Vietnamese sand beaches were supplied and maintained in a minimum amount of seawater.

The cell containing coelomic fluid was obtained using a suction needle. A drop of diluted coelomic fluid was thinly spread on a glass microscope slide, and observed under interference microscopy. Unfixed smears were used for cytoenzymatic reactions; other smears were fixed in formalin vapours for 2 min according to Hetzel (1963), and used for cytochemical reactions. The coelomic fluid was diluted (1:1) with seawater.

Also the coelomic cells of animals were observed, fixed *in toto* for five days in Bouin, or, according to reactions, in acid-free 37% formaldehyde solution (Merck), embedded in paraffin and sectioned with a microtome.

Cytochemical reactions

Cellular components and the particulate material were tested with cytochemical reactions as described by Mazzi (1977), Ganter & Jòlles (1969) and Bancroft *et al.* (1996):

- Haematoxylin-eosin.
- Gomori triple stain reaction which colours the nucleus with haematoxylin, and the cytoplasm with acid stain fast green.
- Periodic Acid Schiff (PAS) reaction for Neutral Mucopolysaccharides (MPS). Controls were subjected to acetylation and saponification.
- Stedman reaction with 1% Alcian blue in 3% acetic acid for acid MPS.
- Hale's method for acid MPS. These absorb the dialysed iron (1 vol dialysed iron, BDH, 1 vol. 2 M acetic acid). The iron is shown by potassium ferrocyanide (Prussian blue).
- 1% Alcian blue in 0.1 N HCl (pH 1) for sulphated MPS.
- 0.1% toluidine blue staining in ethanol at 30 °C for 7 min to evidence acid and sulphated MPS.
- Biebrich scarlet reaction (pH 9.5) for basic proteins. This material was fixed in Bouin only.
- Lendrum's method for fibrin (after Culling). The solutions used were celestin blue-haematoxylin for nuclei (dark-blue); picro orange and acid fuchsin solutions for collagen (blue) and fibrin (red).
- Mann-Dominici reaction, which colours the nucleus blue, the acidophilic cytoplasm pink and the acid MPS purple.
- Treatment of unfixed smears with Sudan black, a general stain for lipids according to Lison.
- Schmorl's reaction with ferric-ferricyanide solution, for melanin, lipofuscin and SH groups.
- Hueck's method with Nile blue sulphate and Lillie's method with Nile blue A to distinguish melanin from lipofuscins.
- Perls' reaction for ferric iron.

Immunohistochemistry

For immunohistochemistry animals with cutaneous wounds were used, fixed in Bouin solution, embebbed in paraffin and sectioned. The sections were incubated for 1 h at room temperature in the mouse primary monoclonal antibodies Euroclon IL4 or IL10, both diluted 1:100 in PBS (Phosphate-Buffered Saline: 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4, 0.065 M Na_2HPO_4, pH 7.4). Washed rapidly in PBS, they were then incubated for 30 min at room temperature in biotinylated goat anti-mouse immunoglobulins (Kit DAKO LSAB2). Washed in PBS, the sections were treated with streptavidin, peroxidase conjugated for 30 min at room temperature. After washing, sections were incubated for 15 min in the chromogen, ready to use, aminoetilcarbamate: this results in a red coloured precipitate at the antigen site. Animals without cutaneous wounds were used as control. Sections incubated in normal non immune rabbit serum, immunoglobulin fraction were also used as control.

Cytoenzymatic reactions

The enzymatic reactions employed were:

- Chloroacetate-esterase was demonstrated by the naphthol AS-D chloroacetate method with a substrate concentration of 0.1 mg/ml; 0.067 M phosphate buffer, pH 7.6; 10 min incubation (Burstone, 1958).
- To evidence the alkaline phosphatase activity, according to Fredricsson's modified method (Pearse Everson, 1978), smears were incubated at 4 °C for 20 min in 2% veronal-Na buffer (70 ml) pH 9.4 containing α-naphthyl-phosphoric acid (24.5 mg) and variamine blue B (70 mg).
- Acid phosphatase activity was demonstrated by the Gomori method. After fixation in cold formalin for 10 min, smears were incubated in 0.1 M tris-maleate buffer, pH 5.0, containing 1.25% Na β-glycerophosphate and 0.2% Pb nitrate. After an incubation period of 1 h at 37 °C, smears were washed with distilled water, treated with 0.5% ammonium sulfide and mounted in glycerin (Mazzi, 1977).
- Peroxidase activity (after Strauss) was demonstrated using the following incubation mixture for 5 min: saturated benzidine in 96% alcohol, 4 ml distilled water and 0.02% of 3% H_2O_2 (Mazzi, 1977).

Brown bodies

Formalinized sheep erythrocytes (fSRBC), according to Csizmas method (1960), were washed in PBS and resuspended in the same buffer at a concentration of 6×10^3 cell/ml. A single dose of 0.15-0.20 ml of this antigen suspension, injected into the coelomic cavity of *S. nudus*, in order to induce brown bodies formation, caused the death of the animals after about 24 h. Consequently, natural brown bodies were used. Brown bodies were collected from the coelomic fluid with a Pasteur pipette. They were washed several times by gentle centrifugation (200 *g*) before use. Some

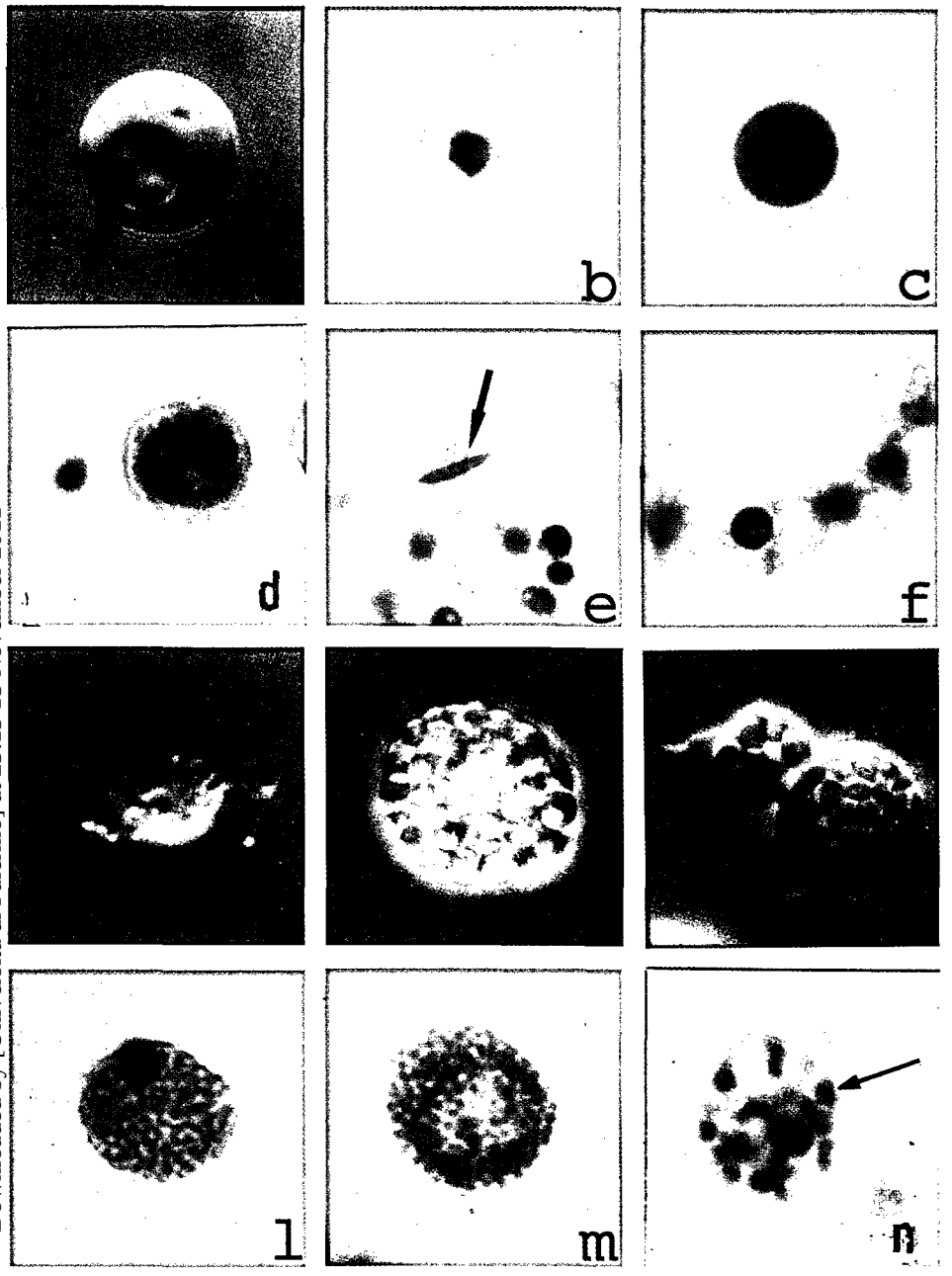

Fig. 1 - Morphology and cytochemical stains of haemerythrocytes and granulocytes. Haemerythrocytes: **a**, interference microscope observation; the arrow indicates the nucleus (×1680); **b**, haematoxylin-eosin stain (×1680); **c**, Lendrum's method (×1680); **d**, Perls' reaction (×1680); **e**, Gomori triple staining; the arrow indicates an irregular cell with elongated nucleus (×1120); **f**, Gomori triple staining; the haemerythrocytes are next to each other (×1400). Granulocytes: **g**, interference microscope image of type I granulocyte; thin cytoplasmatic protusions are also present near the granules (×1680); **h**, interference microscope image of type II granulocyte (×1680); **i**, type II granulocyte moving under the interference microscope; the arrow indicates a pseudopode in which some compartments are pushed by the cell (×1420); **l**, Mann-Dominici stain-positive type I granulocyte (×1680); **m**, Gomori triple stain-positive type I granulocyte (×1680); **n**, Gomori triple stain, type II granulocyte; the arrow indicates a compartment with a basophil granule (×1680).

were fixed in Bouin, embedded in paraffin, sectioned and stained (with haematoxylin-eosin, Perls, PAS, toluidine blue, Gomori triple stain, Hueck, Schmorl, Mann-Dominici) for observation under the light microscope. For electron microscopy observations, other brown bodies were fixed, for 90 min, in 2.5% glutaraldehyde in 0.4 M cacodylate buffer, pH 7.4, postfixed in 1% osmium tetroxide in cacodylate buffer, dehydrated in a graded alcohol series and embedded in Epon resin. Thin sections were cut and double stained with uranyl acetate and lead citrate.

RESULTS

A few cellular types, identifiable as haemerythrocytes, granulocytes, "signet-ring cells," and urn cells complexes, were found in the coelomic fluid of *S. nudus*. Besides, finely granular acidophilous material, mixed to empty vesicles, vesicle fragments, laminae of connective tissue, aggregates of staminal cells, and one to three brown bodies were observed in each animal.

Haemerythrocytes

Haemerythrocytes were very abundant (about 80% of total coelomocytes). Their size ranged from 10 to 12 μm and their shape was spherical with an evident nucleus (Fig. 1a). The haemerythrocytes were stained with haematoxylin-eosin (Fig. 1b) and the Lendrum reaction evidenced the presence of respiratory pigments, haemerythrin (Fig. 1c), whose iron content was shown by Perls' reaction, where Fe^{2+} turned into Fe^{3+} (Fig. 1d). Some nuclei showed a central fissure, and others were needle-shaped (Fig. 1e). In other cells, the cytoplasm

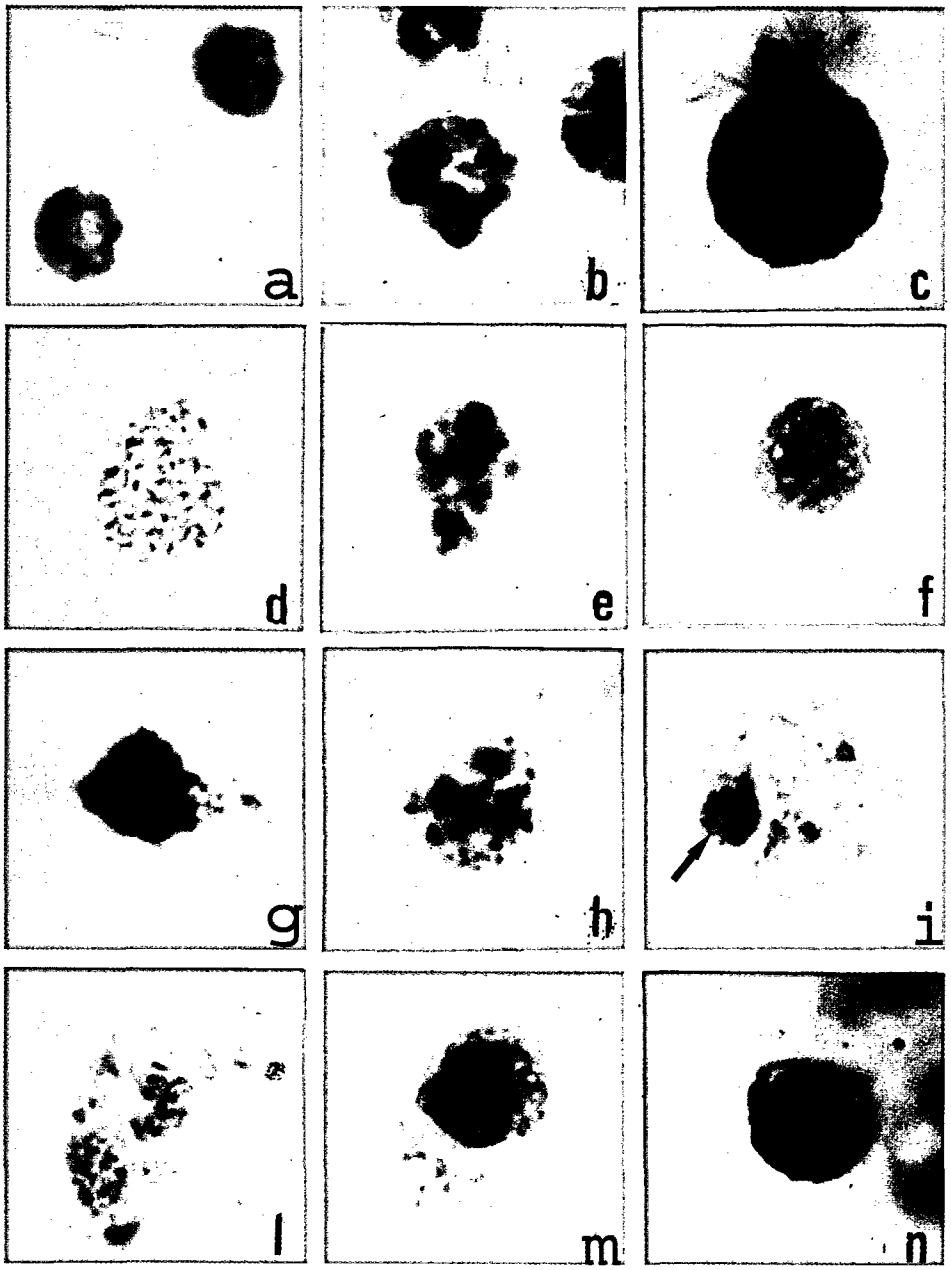

Fig. 2 - **a**, PAS-positive type I granulocytes (×1300); **b**, Alcian-PAS reaction, type II granulocytes; the granulocytes show Alcian-positive compartments (green) and PAS-positive compartments (red) (×1680); **c**, toluidine blue-positive type II granulocyte (×1470); **d**, peroxidase-positive type I granulocyte (×1680); **e**, peroxidase-positive type II granulocyte (×1680); **f**, Chloroacetate esterase-positive type I granulocyte (×1680); **g**, chloroacetate esterase-positive type II granulocyte (×1680); **h**, alkaline phosphatase-positive type I granulocyte (×1680); **i**, acid phosphatase-positive type II granulocyte; the arrow indicates the engulfed material (×1680); **l**, acid phosphatase-positive granulocyte type I (×1680); **m**, weak positivity to anti IL-10 Abs in type I granulocyte (×1680); **n**, strong positivity to anti IL-4 Abs in type I granulocyte (×1680).

protruded and the external membrane was flattened. Some haemerythrocytes had lost their spherical shape and appeared one next to the other (Fig. 1f).

Granulocytes

Granulocytes showed numerous granules masking the cytoplasm and the nucleus. Under the interference microscope, two different granulocytes types (I and II) could be identified. Type I granulocytes often showed thin cytoplasmatic filaments, oriented in all directions, with adhering granules (Fig. 1g). Type II granulocytes (Fig. 1h), while moving could extrude a single bulge into which large compartments were pushed (Fig. 1i). In physiological conditions, the mature type I granulocyte (about 5-7% of total coelomocytes) was 8.5 μm in size, with a spherical nucleus and the cytoplasm filled with many small granules which were demonstrated to be acidophilous by Mann-Dominici staining (Fig. 1l), haematoxylin-eosin or Gomori triple staining (Fig. 1m). Type II granulocytes (about 8% of total coelomocytes) were 12 μm in size and showed green compartments with a red round granule (Fig. 1n). The results of the cytochemical reactions of the granulocytes are summarized in Table I.

It was evident that type I and II granulocytes did not have the same chemical composition. Neutral MPS were stained after PAS reaction in the cytoplasm of type I granulocytes (Fig. 2a) and in some compartments of type II granulocytes (Fig. 2b). In the latter, the acid MPS reacted positively with Gomori triple stain, Hale and Alcian

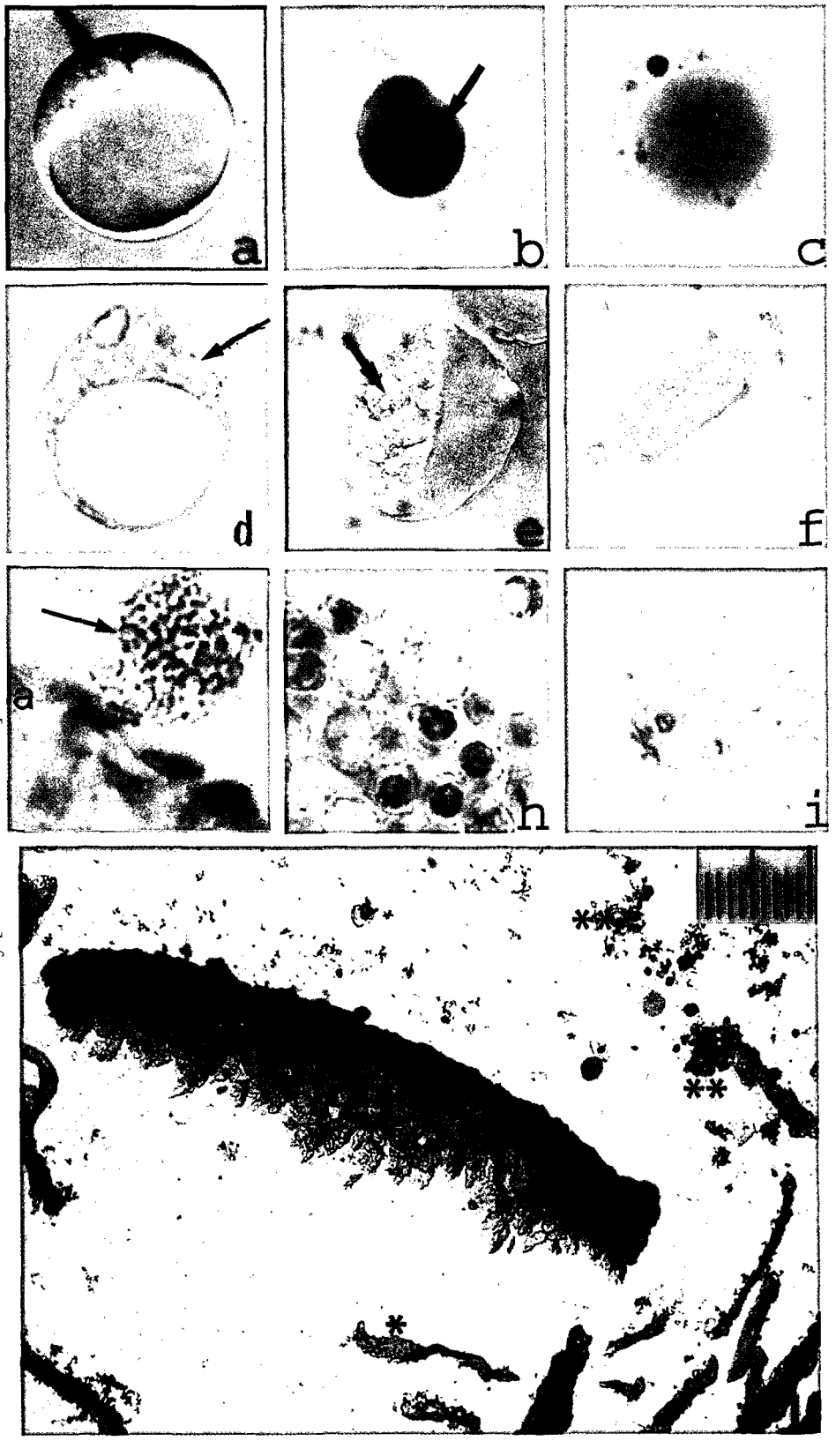

Fig. 3 - **a**, interference microscope image of a signet-ring cell; the arrow indicates the nucleus in the thicker cytoplasm; in the central side of the cell an apparent large cavity can be observed (×1260); **b**, signet-ring cell after Gomori triple stain; central side of the cell contains abundant material (arrow) (×1680); **c**, toluidine blue-positive signet-ring cell; small metachromatic spots are evident in the cytoplasm (×1510); **d**, positive chloroacetate esterase peripheral side of signet-ring cell; the arrow indicates numerous compartments like those of type II granulocytes (×1680); **e**, interference microscope observation of urna cell complexes; the arrow indicates the adhering granulocytes (×520); **f**, chloroacetate reaction-positive urna cell complexes on the concave side (×1680); **g**, detail of a cell aggregate stained with Mann-Dominici; the arrow indicates a type I granulocyte undergoing degranulation; its acidophilic material (a) moves around each aggregate cell (×1680); **h**, aggregate of putative stem cells stained with toluidine blue; each cell presents a variable number of metachromatic granules; in insert: an almost mature granulocyte, of about 6 µm, found near the cell aggregate (×840); **i**, Gomori triple stain-positive empty vesicle (×320); **l**, some particulate components present in the coelomatic cavity: Gomori triple stain-positive (cl) connective lamina; *, filaments; **, type I granulocyte undergoing degranulation, ***, material degranulated by type I granulocytes; in the lower side of connective lamina, it is possible to see nucleated colourless cells with compartments; insert 100 µm.

reactions (Fig. 2b), the acid sulphated MPS to toluidine blue (Fig. 2c) and Alcian reaction (pH 1). Type I granulocytes proved negative with the toluidine blue reaction.

Few acid polysaccharides were evidenced in type I granulocytes. In both cell types, granules contained basic proteins and a small amount of SH-proteins. Fibrinoid material and lipofuscin were hardly present in type I granulocytes, whereas in type II ones iron and lipids were found.

Type I and type II granulocytes reacted positively

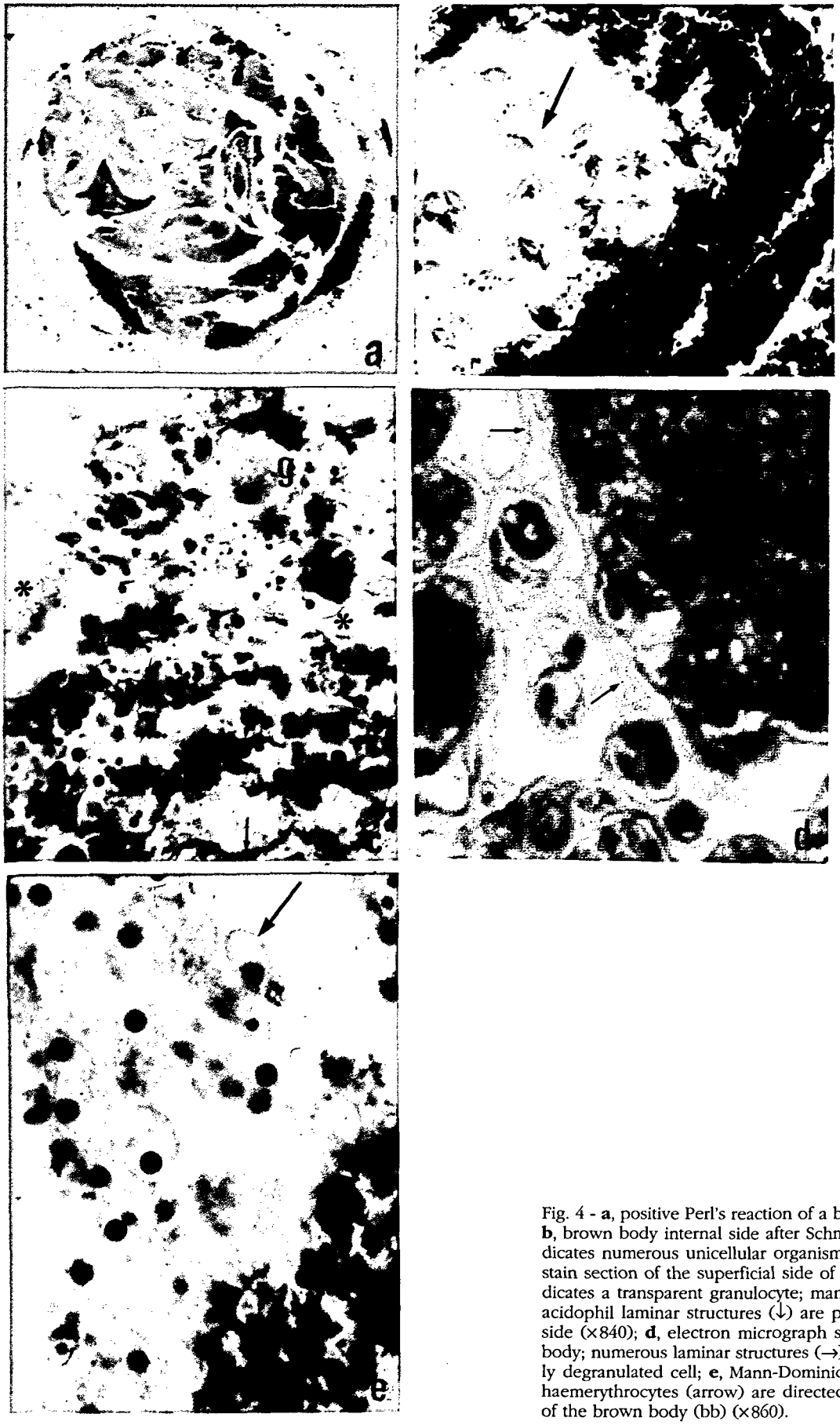

Fig. 4 - **a**, positive Perl's reaction of a brown body section (×108); **b**, brown body internal side after Schmorl reaction; the arrow indicates numerous unicellular organisms (×420); **c**, Gomori triple stain section of the superficial side of a brown body; The (g) indicates a transparent granulocyte; many filiform basophil (*) and acidophil laminar structures (↓) are present toward the internal side (×840); **d**, electron micrograph selected image of a brown body; numerous laminar structures (→) are present near a partially degranulated cell; **e**, Mann-Dominici staining; numerous aged haemerythrocytes (arrow) are directed towards the cortical side of the brown body (bb) (×860).

TABLE I - *Cytochemical reactions of type I and type II coelomocytes. Strength of reactions was rated on a + scale: ---, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.*

Cytochemical reactions	Type I	Type II
Eosin	+++	---
PAS	+++	++
Alcian 1%	+	++
Hale	+	++
Alcian 1% pH 1	---	++
Blue toluidine	---	+++
Ninidrin-Schiff	+++	+++
Biebrich	+++	+++
Lendrum	+	---
Sudan black	---	+++
Hueck	++	---
Lillie	+	---
Schmorl	++	---
Perls	---	+++
IL-4	+++	---
IL-10	+	---
Chloroacetate-Esterase	++	+++
Alkaline phosphatase	++	+
Acid phosphatase	++	++
Peroxidase	++	+++

with peroxidase (Fig. 2d, e) chloroacetate-esterase (Fig. 2f, g), and both alkaline (Fig. 2h) and acid phosphatases (Fig. 2i, l), though with variable intensity. However, only type II granulocytes contained engulfed material (Fig. 2i).

Type I granulocytes were positive, only in wound repairing animals, to anti-IL-4 and anti-IL-10 Abs, with a higher intensity to anti IL-4 Abs (Fig. 2m, n).

After Gomori triple stain, also granulocytes devoid of, or with only a few granules could be distinguished in the coelomic fluid, even *in situ*, *in toto* fixed specimens: type I granulocytes were transparent, while type II one were green and with a little cytoplasm. Besides basic proteins, acid sulphated MPS and chloroacetate esterase were reduced. Some granulocytes showed compartments positive to Perls' reaction only in the peripheral zone.

Signet-ring cells

We have named signet-ring cells (10 µm in diameter) some rare cells (about 1% of all coelomocytes) because of their peculiar appearance especially when examined under the interference microscope (Fig. 3a) or after Gomori triple stain (Fig. 3b). They consisted of a large central vacuole surrounded by a thin cytoplasm layer, thicker around the nucleus. The central part of the cell contained sulphated acid MPS after toluidine blue staining. Small metachromatic spots were also present in the perinuclear cytoplasm (Fig. 3c). The entire cell reacted positively to both alkaline and acid phosphatase and chloroacetate esterase (Fig. 3d). Near the nucleus it was possible to recognize some cytoplasmatic compartments.

Urna cell complexes

The urna cell complexes (about 4-7% of total circulating corpuscles) were 50 to 200 µm in diameter. Under the light microscope, a convex, smooth surface could be seen and a discoidal opening with a rim of moving cilia was present on the opposite side. Urna cell complexes under the interference microscope showed adhering granulocytes and particulate material (Fig. 3e); they stained with toluidine blue and reacted positively with chloroacetate esterase (Fig. 3f).

Cellular aggregates

These were observed both in the isolated cell fluid and in the coelomic cavity of *in toto* fixed animals. The largest cellular aggregates (about 100 µm) consisted of small cells (5 µm) cemented by acidophilous material after Mann-Dominici staining. In just one instance, an acidophilous type I granulocyte adhered to the cellular aggregate (Fig. 3g). Other aggregates consisting of 20-50 cells, after blue toluidine staining showed cells with a basophil nucleus and a cytoplasm with a variable number of metachromatic granules (Fig. 3h). The insert shows an almost mature granulocyte, about 5 µm in size, found near a cellular aggregate.

Empty vesicles and laminar structures

On average, two empty vesicles, about 700 × 500 µm in size, were found inside each coelomic cavity. The external layer was positive to fast green reaction, presumably owing to its high acid MPS content (Fig. 3i). Laminae of connective tissue (Fig. 3l) dispersed in the coelomic fluid or adhering to the inner muscular tissue, supported colourless cells with compartments.

Abundant fragments (about 2-20 µm thick) were mixed with finely granular acidophilous material and partially or totally degranulated granulocytes.

Brown bodies

Brown bodies, two or three per animal, were observed in the coelomic fluid. They were spherical or ovoid structures, brown in appearance, whose size varied from 0.1 to 4 mm. Smaller brown bodies showed a dense and homogeneous structure. The largest showed a cortical part with numerous circularly arranged parallel layers next to compact, frequently vacuolar material filled with different inclusions.

External layers contained type I and type II granulocytes, frequently misshaped, containing little cytoplasm and a large quantity of granulated material. In the internal part of brown bodies, there were numerous type I granulocytes rich in lipofuscin, lipids and melanin. A large amount of iron (Fig. 4a), acid MPS, many of which sulphated, neutral MPS, lipids, and lipofuscin were located in the middle of cells. Different inclusions such as unicellular organism (Fig. 4b) and small quartz crystals were also present. Filiform basophilus material and numerous laminar structures, rich in acid MPS were seen

among material of different nature, under both light (Fig. 4c) and electron microscopy (Fig. 4d).

In some histological sections, haemerythrocytes that were partially destroyed or changed into flattened vesicles were observed devoid of the nucleus arranged in parallel lines along the brown body surface (Fig. 4e).

DISCUSSION

In *S. nudus*, we recognised two different types of granulocytes (type I and type II) on the basis of their morphology and chemical composition. Observed under the light microscope, type I granulocytes do not contain any foreign material and do not show any phagocytic capability, even though they do have lytic enzymes. Only *in vivo* and using the interference microscope could long thin extensions releasing granules be seen. Since large amounts of finely granular acidophil material in the coelomic cavity could be seen, we suppose that type I granulocytes perform their function outside the cell by releasing their granules near the material to be digested or on which to act, causing different effects.

Conversely, type II granulocytes showed evident signs of phagocytosis. This is demonstrated by the occurrence, in this cell type, of particulate material, iron and lipids. The presence of lytic enzymes demonstrates their involvement in intracellular digestion.

We believe that, in some cases, type II granulocytes that have engulfed and digested large amounts of organic material then turn into signet-ring cells. This hypothesis is confirmed by the presence of compartments containing chloroacetate esterase or iron in some maturing signet-ring cells. It is probable that signet-ring cells function as a reserve of organic material.

Several filiform acidophil structures represented the final stage of the haemerythrocytes. In our opinion, after carrying out their function, they stretch, reduce the amount of cytoplasm, become flattened and join each other forming chains and then layers of various thickness and length. After dispersing their nuclei, they move close to the brown body.

Most of the laminae present inside the coelomic cavity, however, derive from fragmentation of the empty vesicles, whose origin is unknown. The enzymes of type I granulocytes could break the empty vesicles into small fragments which will constitute part of the brown bodies.

The material degranulated by type I granulocytes also distributes along the peritoneal membrane, adhering to the inner muscles of the coelomic cavity and causing detachment of the membrane together with pieces of connective tissue. The surface of this tissue is covered by colourless cells about 7 μm in size and, on one side (in the fragment found inside the coelomic cavity) by smaller cells of increasing size in a bottom to top direction. It thus seems that a cell maturity gradient exists from the peritoneal membrane toward the coelomic cavity.

These cells could be the stem cells of hyalinocytes observed in *P. vulgare* by Volkonsky (1933), and in *S. nudus* by Valembois & Boiledieu (1980) and by Matozzo *et al.* (2001).

In the observed aggregates the presence of cells of higher colour is evident in the centre and other cells with differentiated metachromatic granules at the periphery. We believe these to be clusters of type II granulocytes at various differentiation stages. This hypothesis is supported by the different cell dimensions and the variable amounts of granules found. Moreover, the colourless cells located in the cluster centre could be hyalinocytes undergoing differentiation.

Type I granulocytes expressed molecules recognized by anti IL-4 and IL-10 Abs in wound repairing animals. The role played by these molecules is not entirely clear, and ongoing research aims to determine the involvement of type I granulocytes in wound healing in *S. nudus*. We suppose that these molecules have also a function in the aggregation and maturation of germinal cells. In fact, we observed that substances released from type I granulocytes are distributed around the single cells of a cellular aggregate. Our results on granulocytes do not exactly match those by Matozzo *et al.* (2001). We believe that the different cell morphology, as evidenced by numerous cytochemical reactions, and above all the different working mechanism of the lytic enzymes present inside the granules, are to be related to the environment in which coelomocytes live. Inside the animal, i.e., in physiological conditions, cells continuously undergo stimuli of various nature, both exogenous and endogenous, which cells from a culture definitely do not.

In the coelomic cavity, brown bodies are structures associated with the defence mechanism of the host (Hyman, 1955; Hetzel, 1965; Dybas & Frankboner, 1986; Canicattì & D'Ancona, 1989; Canicattì *et al.*, 1989; D'Ancona *et al.*, 1989) and are histologically constituted by phagocyte cells and invading pathogens and parasites, or injected particulate matter. In *S. nudus*, materials such as unicellular organism or crystal fragments penetrate into the coelomic cavity. Consequently, the granulocytes with their hydrolases digest the organic foreign material or form a cemented matrix using an acidophilic substance. Numerous Schmorl's positive type I granulocytes are present in brown bodies. These cells are probably responsible for the production of melanin-like pigments that give the brown bodies their characteristic colour, as in the *H. polii* brown bodies (Canicattì *et al.*, 1989).

Partially modified type II granulocytes are also present in brown bodies. After taking part in the degradation process of material no longer useful for the organism, they constitute part of the brown body structure probably also due to cell ageing. To summarize, major components of the brown body structure are the filiform acidophilous structures, the laminar membranes rich in acid MPS and finely granular acidophil material. The structures are made up of a haemerythrocyte residual component (cell membranes and a part of haemerythrine, as evidenced by Perl's reaction); laminar membranes are probable fragments of empty vesicles and

above all connective components of the peritoneal wall (green in colour owing to the acid MPS); the acidophilic materials probably derive from degranulation of type I granulocytes whose function is probably that of cementing the various parts of the brown body.

All this allows us to state that brown bodies in *S. nudus,* unlike what is observed in other invertebrates, are associated with defence mechanisms, thus isolating either foreign material, such as unicellular organism and crystal fragments, or accumulating aged coelomic material before elimination.

REFERENCES

Andrews E., 1890 - Anatomy of *Sipunculus gouldi.* Stud. Biol. Lab. Johns Hopkins Univ., 4: 389-430.

Bancroft J. D., Stevens A., Turner D. R., 1996 - Theory and practice of histological techniques, Churchill Livingstone, New York.

Bang F. B., Bang B. G., 1962 - Studies on sipunculid blood: Immunologic properties of coelomic fluid and morphology of "urn cell". Cah. Biol. Mar., 3: 363-374.

Bang B. G., Bang F. B., 1965 - Mucus hypersecretion in a normally isolated non-innervated cell. Cah. Biol. Mar., 6: 257-264.

Bang B. G., Bang F. B., 1974 - Invertebrate model for study of macromolecular regulators of mucus secretion. Lancet, 2: 1292-1294.

Bang B. G., Bang F. B., 1975 - Cell recognition by mucus secreted by urn cell of *Sipunculus nudus.* Nature, 253: 634-635.

Bang B. G., Bang F. B., 1976 - The mucous secretory apparatus of the free urn cell of *Sipunculus nudus.* Cah. Biol. Mar., 17: 423-432.

Bang B. G., Bang F. B., 1980 - The urn cell complex of *Sipunculus nudus*: a model for study of mucus-stimulating substances. Biol. Bull., 159: 571-581.

Blanco G. A. C., Alvarez E., Amor A., Hajos S., 1995 - Phagocytosis of yeast by coelomocytes of the sipunculan worm *Themiste petricola*: opsonization by plasma components. J. Invertebr. Pathol., 66: 39-45.

Boiledieu D., Valembois P., 1977 - Natural cytotoxic activity of sipunculid leukocytes on allogenic and xenogenic erythrocytes. Dev. Comp. Immunol., 1: 207-216.

Brown A. C., Winterbottom R., 1969 - The fate of thorium dioxide injected into the coelom of the sipunculid *Golfingia capensis.* J. Invertebr. Pathol., 13: 229-234.

Brusca R. C., Brusca G. J., 1996 - Invertebrati. Zanichelli, Bologna.

Burstone M. S., 1958 - The relationship between fixation and techniques for the histochemical localization of hydrolytic enzymes. J. Histochem. Cytochem., 6: 322-339.

Canicattì C., D'Ancona G., 1989 - Cellular aspects of *Holothuria polii* immune response. J. Invertebr. Pathol., 53: 152-158.

Canicattì C., D'Ancona G., Farina-Lipari E., 1989 - The coelomocytes of *Holothuria polii* (Echinodermata). I. Light and electron microscopy. Boll. Zool., 56: 29-36.

Cantacuzène J., 1922 - Sur le rôle agglutinant des urnes chez *Sipunculus nudus.* C. R. Seances Soc. Biol. Paris, 87: 259-263.

Cantacuzène J., 1928 - Recherches sur les réactions d'immunité chez les Invertebrates. Arch. Roum. Pathol. Exp. Microb., 1: 7-80.

Csizmas L., 1960 - Preparation of formalinizes erythrocytes. Proc. Soc. Biol. N.Y., 103: 103-157.

D'Ancona G., Rizzuto L., Canicattì C., 1989 - Engagement of Polian vesicles during *Holothuria polii* response to erytrocyte injection. J. Invertebr. Pathol., 54: 300-305.

Dawson A. B., 1933 - Supravital studies on the colored corpuscles of several marine Invertebrata. Biol. Bull., 64: 233-242.

Dybas L., 1975 - Cell within a cell or a circulating cell pair. Nature, 257: 790-791.

Dybas L., 1976 - A light and electron microscopic study of the ciliated urn of *Phascolosoma agassizii* (Sipuncula). Cell Tissue Res., 169: 67-75.

Dybas L., 1981a - Sipunculans and echiuroids. *In*: N. A. Ratcliffe & A. F. Rowley (eds), Invertebrate blood cells, vol. I. Academic Press, London, pp. 161-188

Dybas L., 1981b - Cellular defense reactions of *Phascolosoma agassizii* a Sipunculan worm: Phagocytosis by granulocytes. Biol. Bull., 161: 104-114.

Dybas L., Frankboner P. V. 1986 - Holoturian survival strategies: mechanism for the maintenance of a bacteriostatic environment in the coelomic cavity of the sea cucumber *Parasticopus californicus.* Dev. Comp. Immunol., 10: 311-330.

Evans E. E., Cushing J. E., Evans L. M., 1973 - Comparative immunology: sipunculid bactericidal responses. Infect. Immun., 8: 355-359.

Ganter P., Jollès G., 1969 - Histochimie normale et pathologique. Gauthier-Villars, Paris.

Ghirardelli E., 1995 - Phylum sipunculi (Sipuncula). *In*: B. Baccetti *et al.* (eds), Zoologia, vol. 2. Grasso, Bologna, pp. 477-486

Hérubel M. A., 1908 - Recherches sur les sipunculides. Mem. Soc. Zool. France, 20: 107-418.

Hetzel H. R., 1963 - Studies on holothurian coelomocytes. I. A survey of coelomocyte types. Biol. Bull., 125: 289-301.

Hetzel H. R., 1965 - Studies on holothurian coelomocytes. II. The origin of coelomocytes and the formation of brown bodies. Biol. Bull., 128: 102-111.

Hyman L. H., 1955 - The invertebrates. Vol. IV: Echinodermata. McGraw-Hill Book Co., New-York.

Hyman L. H., 1959 - The invertebrates: smaller coelomate groups. McGraw Hill, New York.

Matozzo V., Perin L., Cima F., Ballarin L., 2001 - Phagocytic and enzymatic activities of cells and urn cell complexes in the coelomic fluid of the marine worm *Sipunculus nudus* (Sipunculida). Ital. J. Zool., 68: 273-280.

Mazzi V., 1977 - Manuale di tecniche istologiche e istochimiche. Piccin Editore, Padova.

Metalnikoff S., 1900 - *Sipunculus nudus.* Z. Wiss. Zool., 68: 261-323.

Nicosia S., 1979 - Lectin-induced mucus release in the urn complex of the marine invertebrate *Sipunculus nudus* (Limnaeus). Science, 206: 698-700.

Ochi O., 1975 - Cell types in the coelomic fluid of *Phascolosoma agassizii. In*: M. E. Rice & M. Torodovic (eds), Proc. int. symp. biol. Sipuncula and Echiurida, vol. I. Naucno Delo, Beograd, pp. 219-227.

Ohuye T., 1938 - On the coelomic corpuscles in the body fluid of some invertebrates. XII. Sci. Rep. Tohoku Univ. (4 Ser. Biol.), 31: 359-379.

Pearse Everson A. G., 1978 - Trattato di Istochimica. Piccin Editore, Padova.

Selensky W., 1908 - Untersuchungen über die Urnen der Sipunculiden. Zool. Anz., 32: 329-336.

Shinn G. L., 1985 - Reproduction of *Anoplodium himanae,* a turbellarian flatworm (Neorhabdocoela, Umagillidae) inhabiting the coelom of sea cucumber; production of egg capsules, and escape of infective stages without evisceration of the host. Biol. Bull., 169: 182-198.

Smith U. Z., 1981 - The Echinoderms. *In*: N. A. Ratcliffe & A. F. Rowley (eds), Invertebrate blood cells, vol. 2. Academic Press, London, pp. 513-562.

Towle A., 1970 - Cell types in the coelomic fluid of *Phascolosoma agassizii. In*: M. E. Rice & M. Torodovic (eds), Proc. int. symp. biol. Sipuncula and Echiura, vol. I. Naucno Delo, Beograd, pp. 211-218.

Triplett E. L., Cushing J. E. Durall G. L., 1958 - Observations on some immune reactions of the Sipunculid worm *Dendrostomum zostericolum.* Am. Nat., 92: 287-293.

Valembois P., Boiledieu D., 1980 - Fine structure and function of haemerythrocytes and leucocytes of *Sipunculus nudus.* J. Morphol., 163: 69-77.

Volkonsky M., 1933 - Digestion intracellulaire et accumulation des colorants acids. Etude cytologique des cellules sanguines des sipunculides. Biol. Bull., 67: 135-199.

Weinheimer P. F., Acton R. T., Cushing J. E., Evans E. E., 1970 - Reactions of sipunculid coelomic fluid with eryhrocytes. Life Sci., 9: 145-152.