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Engagement of the periesophageal ring during *Holothuria polii* response to erythrocyte injection

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SUMMARY

In *Holothuria polii*, the periesophageal ring is an important organ supplying spherule cells after stimulation with foreign material. In animals injected with formalinized sheep erythrocytes, in fact, a depletion of spherule cells is observed in the periesophageal ring, whereas in the connective tissue, in the external epithelium and around the antigen-injected site, small, transparent cells can be visualized. It is supposed that the latter are stem cells of spherule cells.

INTRODUCTION

The invertebrates, as the vertebrates, have humoral and cellular mechanisms of defense. The presence of humoral substances such as aglutinins, lysins, lectins and other compounds that exert antigenic effects confers to invertebrates an immune capacity (Parrinello *et al.*, 1976, 1979; Yeaton, 1981; Karp and Rheins, 1980; Reins *et al.*, 1980; Ey and Jenkin, 1982; Amirante and Basso, 1984; Parrinello and Arizza, 1988, 1989; Olafsen, 1986; Canicattì, 1989, 1990, 1991; Parrinello, 1991; Vasta, 1991; Arizza *et al.*, 1991, 1993; Cammarata *et al.*, 1993; Lotzovà, 1993; Cooper *et al.*, 1996; Vasquez *et al.*, 1996; Arizza *et al.*, 1997).

Besides the immune responses to introduced foreign material or injected particulate material, are mediated principally by coelomocytes as observed in sipuncula (Dybas, 1981); molluscs (Cheng and Galloway, 1970; Anderson, 1987), insect (Salt, 1970; Gotz and Vey, 1974; Nappi and Carton, 1986), tunicata (Anderson, 1971; Parrinello, 1981; Scofield and Nagashima, 1983; Parrinello *et al.*, 1984; Parrinello and Patricolo, 1984; Raftos *et al.*, 1987 a,b; Raftos and Cooper, 1991; De Leo, 1992).

Phagocytic activity was evidenced in sipuncula (Blanco *et al.*, 1997) and tunicates (Smith, 1970; Anderson, 1971; Wright, 1974, 1981; Fuke, 1979; Rowley, 1981; Sawada *et al.*, 1991; Ohtake *et al.*, 1994; Ballarin *et al.*, 1994; Parrinello and Cammarata, 1995).

Beck *et al.*, (1993) isolated interleukin-1- like molecules which regulate cellular activities and enhance phagocytosis during inflammation by tunicate and echinoderm cells.

A cytotoxic activity was evidenced in sipuncula cells (Boiledien and Valembois, 1977), molluscs (Leippe and Renwantz, 1988; Franceschi *et al.*, 1991), anellids (Porchet-Henneré *et al.*, 1992; Suzuki and Cooper, 1995), crayfish (Tyson and Jenkin, 1974; Soderhall *et al.*, 1985), tunicates (Fuke, 1980; Saito and Watanabe, 1982; Scofield and Nagashima, 1983; Raftos *et al.*, 1987b, 1991; Sabbadin *et al.*, 1992; Kelly *et al.*, 1992; Parrinello and Arizza, 1992; Parrinello *et al.*, 1993, 1995; Peddy and Smith, 1993, 1994; Nappi and Vass, 1993; Jackson

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et al., 1993; Arizza *et al.*, 1995; Cammarata *et al.*, 1995; Parrinello *et al.*, 1996; Lipari *et al.*, 1996), fishes (Cammarata *et al.*, 2000).

In spite of extensive research on immunological reactivity, especially using tunicates, little is known about the echinoderms; and even less about the Holothuroidea (Hyman,1955; Smith, 1981; Cooper, 1982; Shinn, 1983; Dybas and Frankboner, 1986 ; Xing *et al.*, 1998).

Recently genes expressed by activated coelomocytes were identified in echinoderms which encode proteins (SpC3 and SpBf) that are likely to be important in immune function (Smith *et al.*,1998; Al-Shariff *et al.*, 1998; Gross *et al.*,1999; Gross *et al.*, in press; Clow *et al.* in press.

These proteins constitute a simple complement cascade that act to opsonize foreign cells, particles and molecules for removal and destruction by the phagocytic coelomocytes. The identification of these proteins is important for understanding the evolution of the vertebrate immune system (Smith, 2000).

In the coelomic fluid of *H. polii*, three major categories of cells have been characterized and described both morphologically and functionally: amebocytes, spherule cells and progenitor cells (Canicattì *et al.*, 1989; D'Ancona and Canicattì, 1990).

Amebocytes showed an intense phagocytic activity after antigenic stimulation (Canicattì and D'Ancona, 1989). These cells originate from the stone canal (D'Ancona, 1996) and combine to form nodules and brown bodies in the coelomic cavity (Canicattì et al, 1989) or in the Polian vesicle (D'Ancona *et al.*, 1989). The aggregation of spherule cells and nodules gives rise to brown bodies. Type III spherule cells, in particular, are responsible for the production of melanin-like pigments, whereas type I cells produce an extracellular granular matrix which is found in both naturally and experimentally induced brown bodies (Canicattì and D'Ancona, 1989).

The periesophageal ring, in *H. polii*, is in turn, the structure carrying the water system and is connected with the coelomic cavity, the Polian vesicles and the stone canal. Since the Polian vesicles and the coelomic cavity are engaged in the inflammatory response after sheep erytrocyte injection, we have investigated the engagement of the periesophageal ring in the mechanism of defense.

Therefore, we have observed in this structure the numerical change of spherula cells during the different experimental conditions and the results were compared with those deriving from non injected animals (controls).

MATERIALS AND METHODS

Specimens of *H. polii* were collected from the Gulf of Palermo and maintained in tanks with running sea water at 15°. Pieces of digestive apparatus, including the periesophageal ring, were isolated from five animals, fixed in Bouin's solution, embedded in paraffin and sectioned with a microtome. The 7µm seriated sections were stained using the histochemical methods described by Mazzi (1977) and Ganter-Jollès (1970), herewith listed, and already used for the classification of spherule cells (D'Ancona and Canicattì, 1990):

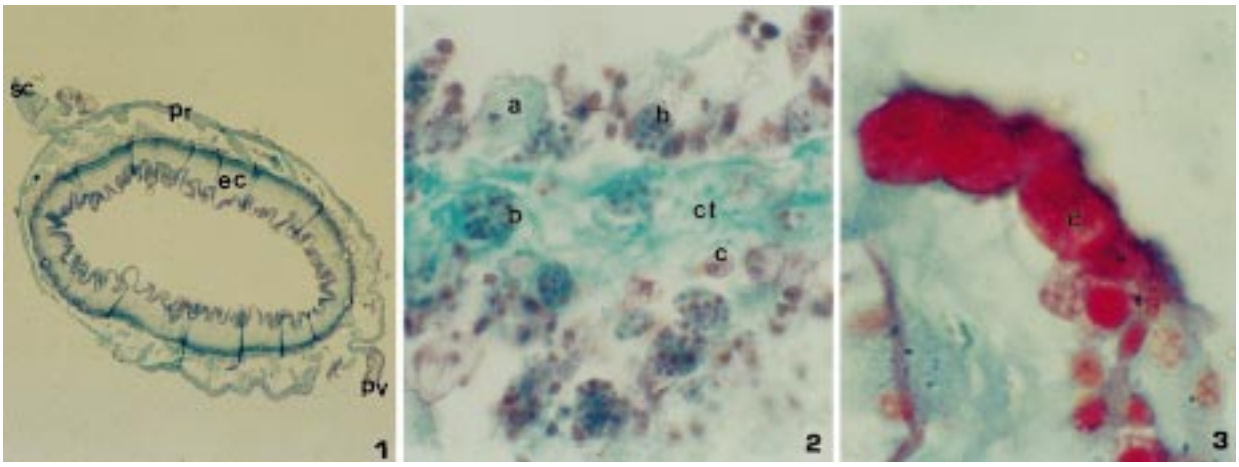
- Gomori triple staining technique, i.e. a specific stain for collagen which evidences type II spherule cells.
- Sevki reaction to evidence type I spherule cells.
- Azocarmine-blue aniline-orange G to evidence type III spherule cells.

Preparation of fSRBC

Periesophageal rings were also collected from specimens subjected to antigenic stimulation. Sheep erythrocytes (SRBCs) were used as particulate antigens. After washing with phosphate-buffered saline, pH 7.4 (PBS), a suspension of SRBCs was formalinized according to Csizmas's method (1960). The formalinized SRBCs (fSRBCs) were thoroughly washed in PBS and resuspended in the same buffer at a concentration of $6x10^8$ cell/ml. A single dose of 0.20 ml of the above antigen suspension was injected into the coelomic cavity of four animals. These were then autopsied 20, 24, 48, 58 and 96 hrs after the injection and their periesophageal rings subjected to the same procedures of non injected controls.

RESULTS

The periesophageal ring of *H. polii* is a canal made of connective tissue, and is large, being about 30 µm in thickness (Fig. 1). By "proximal" is intended the extremity of the ring directed towards the mouth, "distal" being the opposite end. The cell distribution is summarized in Table I. In controls, spherule cells (type I, II and III) are found in both the proximal and the distal part of the periesophageal ring (Fig. 2), with no significant quantitative difference between the two parts. Type I and II cells are ubiquitous and more numerous than type III, which are



Figs. 1/3 - (1) Non injected specimens. Cross section of the esophageal canal (ec) with the periesophageal ring (pr). The figure shows also the stone canal (sc) and the Polian vesicle (pv). Magnification 18,8x. **(2)**Selected image of the periesophageal ring. The figure shows type I spherule cells (a), type II (b), type III (c) in the connective tissue (ct). Magnification 504x. **(3)**Selected image of type III spherule cells (c), some of which are degranulated. Magnification 1100x.

prevalently present in the proximal part (Table II).

Morphologically, type I and II spherule cells appear to be more transparent, small and disorganized than type III. Sometimes type II cells are reduced in number, with a small or defective inner protein core. Some type III spherule cells lack granules and show little affinity to the dye (Fig. 3). In the periesophageal ring, brown masses and brown bodies of variable size are also found; the same has already been observed in the coelomic cavity (Canicattì and D'Ancona, 1989) and in the Polian vesicle (D'Ancona *et al.*, 1989) of *H. polii*.

On account of the lack of significant differences found in controls between the average amounts of spherule cells in the proximal and in the distal part of the periesophageal ring, for the injected specimens only the times of antigenic stimulation are considered.

The results are summarized in Table III and in Graph 1. It is evident that type I and II spherule cells are reduced numerically over 48 hrs of stimulation. At the same time, the connective tissue becomes looser and small spherical cells (2-5 µm), some of which are active in mitosis, others in migration (Figs. 4, 8) are found among collagen fibres, particularly on their internal side. Immediately afterward, type I and II spherule cells duplicate and then remain unchanged until 96 hrs of stimulation. Also type III cells are reduced numerically and disappear 48 hrs after injection (Fig. 5). They reappear 58 and 96 hrs after injection but their amount is significantly lower than that found in controls (Figs. 1, 6, 7). After 20 hrs of stimulation, formalinized erythrocytes (antigens) surrounded by amoebocytes, by a few mature type I spherule cells and by numerous small spherical cells with metachromatic granules, can be observed in the space between the wall of the alimentary canal and the periesophageal ring (Fig. 9). Four hrs later, type I spherule cells produce a large amount of extracellular granular matrix (Fig. 10).

Table I
Mean and standard error of the proximal and distal part cells of the periesophageal ring of 5 non injected animals (controls)

Spherule cells	Number of spherule cells		
	Proximal part (**pp**)	Distal part (**dp**)	P
Type I	227.28±17.6	260.50±23.3	>0.5
Type II	169.14±24.5	223.43±39.4	>0.4
Type III	74.00±10.3	69.43±30	>0.9

Table II
Student' s (t) test between cell pairs in **pp** and **dp** of animals in Table I

Spherule cells	t(**pp**)	P	t(**dp**)	P
Type I - Type II	1.351	>0.2	0.509	>0.6
Type II- Type III	2.457	>0.05	2.268	>0.05
Type I - Type III	5.546	<0.05	3.823	<0.05

DISCUSSION

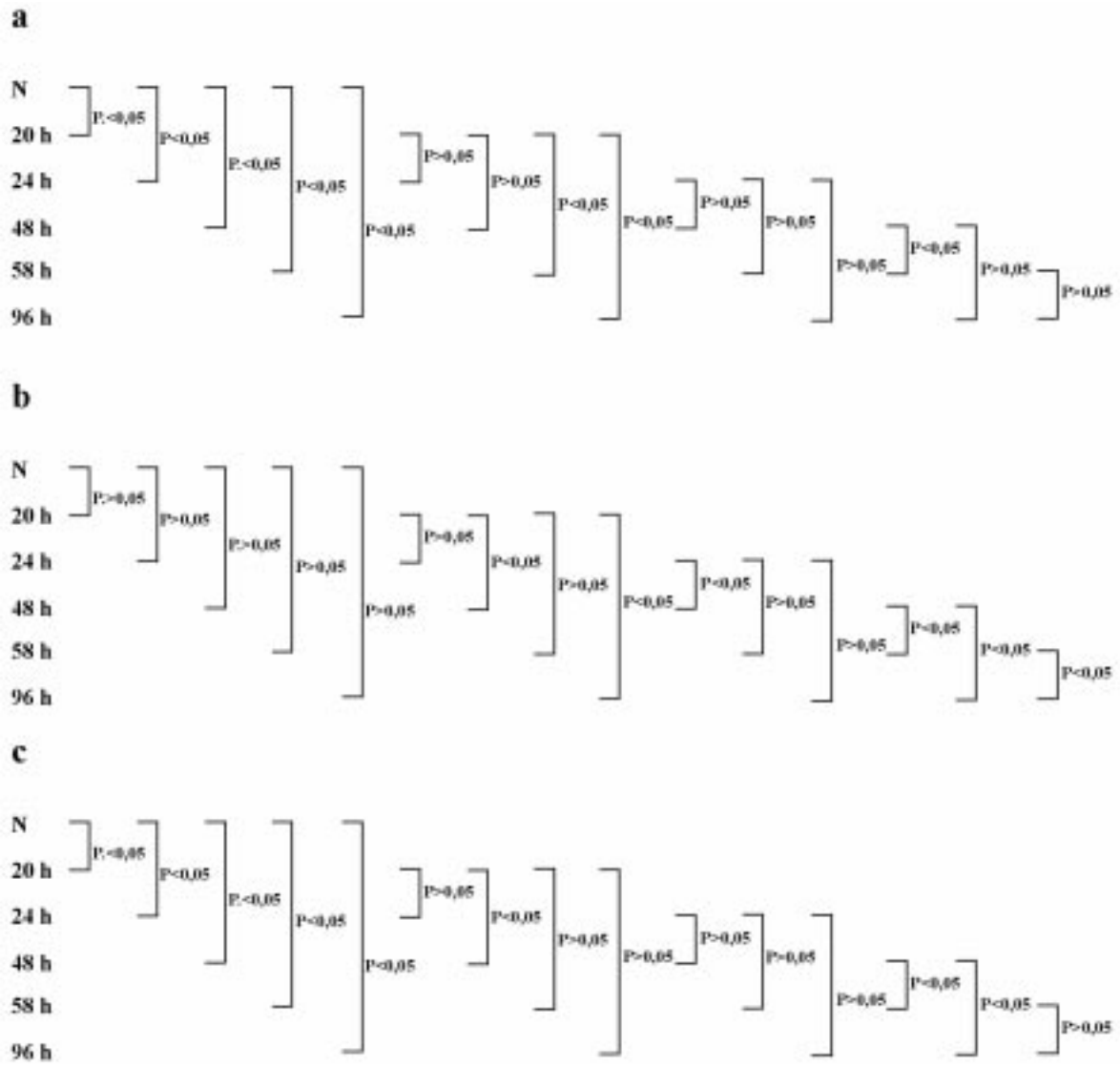
In controls, the difference between the average amount of spherule cell types found in the proximal and in the distal part of the periesophageal ring is not significant. Consequently, the distribution of spherule cells can be considered to be random. On the contrary, the comparison of each cell type against the two other types shows that between type I and II the difference is not significant, whereas between type II and III the difference is noticeable, and remarkable between type I and III. Therefore, in the proximal part of the periesophageal ring, both type I and II are equally distributed and more numerous than type III cells. In the same area of the injected animals, spherule cells are reduced in number. The above results are consistent with the processes occurring during the cellular immune response induced in *H. polii*. The cellular events taking place after injection of sheep erythrocytes into the coelomic cavity, are in fact various (Canicattì and D'Ancona, 1989) and include, throughout the 96 hrs of injection, the recruitment of a large number of amoebocytes from the stone canal (D'Ancona, 1996), in order to eliminate the injurious agents by phagocytosis. The amoebocytes cluster in a syncytial structure which tends to form nodular aggregates and brown bodies.

The nodules are surrounded by type I and II spherule cells which produce an extracellular granular matrix, constituted of acid and neutral mucopolysaccarides and hydrolytic enzymes, having a cementing action (D'Ancona and Canicattì, 1990). Brown bodies are also surrounded by type III spherule cells, whose positive Schmorl's reaction suggests the presence of melanin (Canicattì and D'Ancona 1989).

H. polii is a benthic animal (Tortonese, 1965) subjected to a long list of commensals and parasites (Jangoux, 1987 a, b, c). Therefore, the discovery of brown masses and brown bodies in the periesophageal ring of the injected specimens proves the necessity of the animal to react readily to the attack of likely natural antigens, taking place through the mouth. Moreover, the morphological alterations and reduced affinity to the dye found, in controls, in the spherule cells of the periesophageal ring, support the hypothesis of their contribution to the local making of brown bodies. As a consequence, the periesophageal ring acts as a supplier of spherule cells and is engaged in the immune reaction. However, what is the origin of the new spherule cells which, 58 hrs after injection, begin to repopulate the periesophageal ring? Ohuye (1938) proposed that in various invertebrates, including two holothurian species, lymphocytes represent primitive pluripotent cells from which all the other coelomocyte types are derived. Endean (1958) indicated that in *H. leucospilata*, amoebocytes originate from the

Table III
Mean and standard error of spherule cells after different times of antigen stimulation. Four specimens were used for each stimulus time

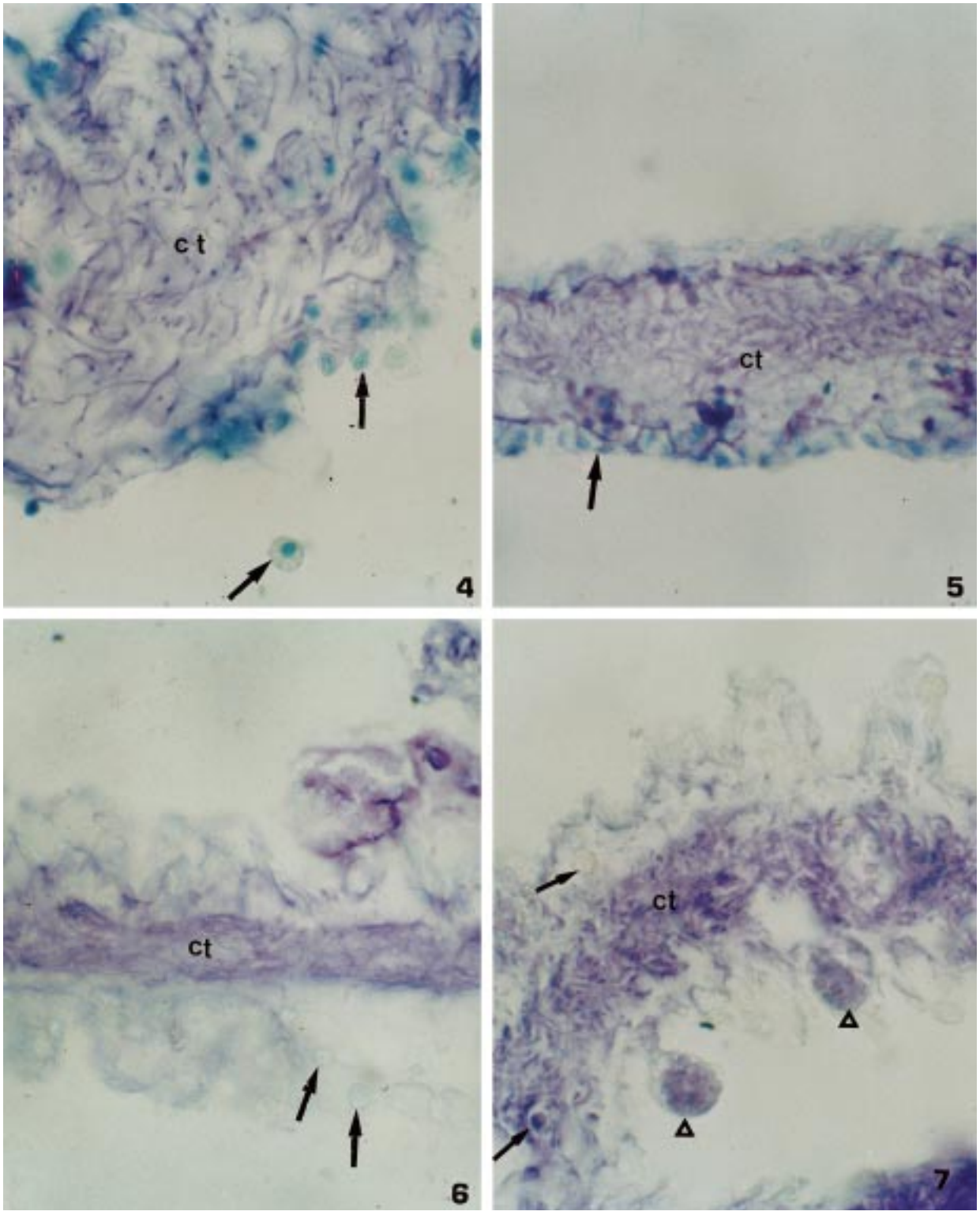
Hours of antigenic stimulation	Number of spherule cells		
	Type I	Type II	Type III
20	49.0 ± 3.4	104.0 ± 8.6	3.75 ± 0.50
24	71.0 ± 10.7	125.0 ± 10.8	5.75 ± 2.05
48	59.0 ± 5.6	69.0 ± 4.3	0
58	96.2 ± 8.2	136.7 ± 17.0	2.4
96	80.5 ± 2.3	168.7 ± 23.7	17.2 ± 5.50



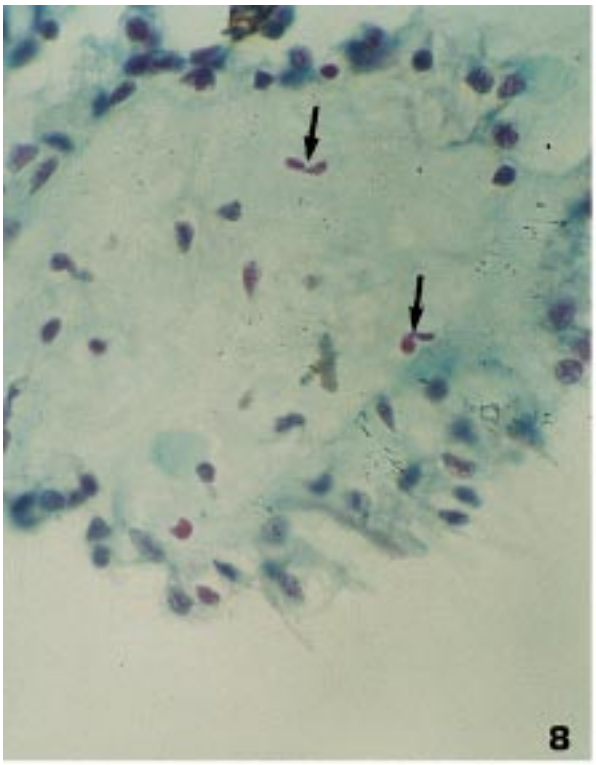
Graph 1 – Probability that numerical differences between controls and stimulated cells are random as calculated by Student's (t). a= type I spherule cells; b= type II; c= type III. N= uninjected animals (controls).

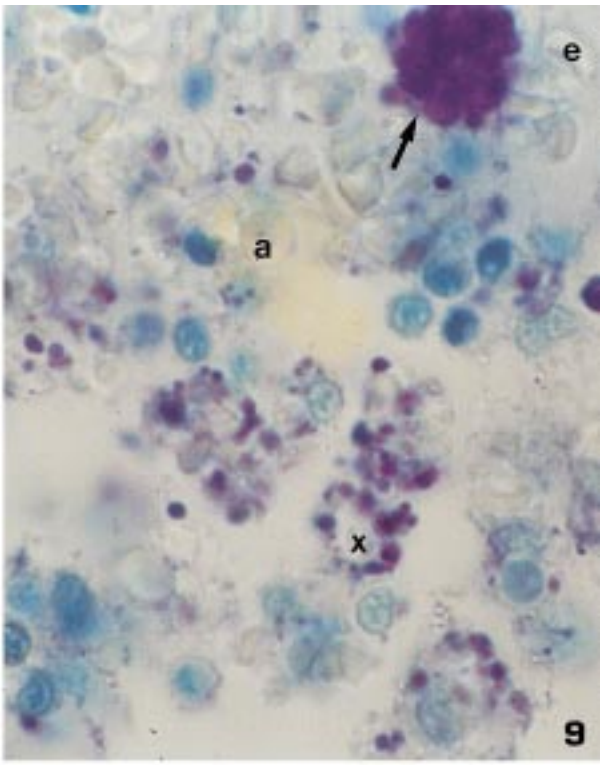
lining epithelium of respiratory trees, migrate into the coelomic fluid and then differentiate into spherule cells. Observations by Hetzel (1965) indicate that at least some of the lymphocytes may differentiate from mesenchymal connective tissue cells located in the walls of the hemal vessels, and that lymphocyte-like cells of the hemal vessels possibly differentiate into spherule cells.

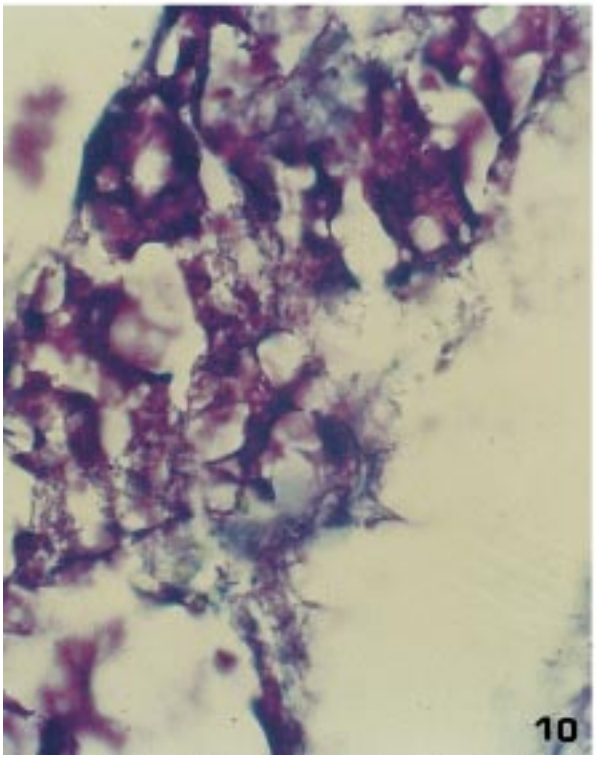
We suppose that the small spherical cells observed in the connective tissue 20 hrs after injection are stem cells of spherule cells. This hypothesis is confirmed by their mitotic activity and by the concurrent presence of metachromatic, not yet mature, granules, and mature spherule cells, both in the periesophageal ring and around the antigen. The majority of small spherical cells are found in organs showing inflammatory responsiveness, such as the Polian vesicle and the coelomic cavity (D'Ancona *et al.*, 1989; Canicattì and D'Ancona, 1989). Since mitotic figures were not observed in any of the circulating spherule cells and amoebocytes, it is likely that the periesophaeal ring acts also as a hemopoietic organ of spherule cells. The latter, at times of heavy antigenic stress, are prematurely released but are able to ripe out of the centre of origin.



Figs. 4-7 - Injected specimens. Cross sections of the perioesophageal ring coloured with toluidine blue. Magnification 800x. Twenty hrs after fSRBC injection **(4)**, the connective tissue (ct) is very loose and devoid of mature cells; it shows small colourless spherule cells in migration (→). Forty-eight **(5)** and fifty-eight **(6)** hrs after injection, the connective tissue is less and less loose and presents small cells on the margins (→). Ninety-six **(7)** hrs after injection the structure of the connective tissue is compact with not yet mature (→) cells and some mature (Δ) ones.







Figs. 8-10 - Injected specimens. **(8)**Cells undergoing mitosis (→) in the connective tissue (ct) of the periesophageal ring coloured with Gomori triple stain. Magnification 800x. **(9)**Cellular aspect of the immune response as visible in the space between the wall of the alimentary canal and the periesophageal ring. Type I spherule cells (sph. I) are almost all immature (X) and do not show any metachromatic granules. Toluidine blue. e = erythrocytes; a = amebocytes. Magnification 1600x. **(10)**The above response visualized four hrs later. Abundant metachromatic material is secreted by mature type spherule cells. Toluidine blue. Magnification 1600x.

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