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Cell cooperation in coelomocyte cytotoxic activity of *Paracentrotus lividus* coelomocytes

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Abstract

The coelomic fluid from the sea urchin *Paracentrotus lividus* contains several coelomocyte types including amoebocytes and uncoloured spherulocytes involved in immune defences. In the present paper, we show a Ca^{2+}-dependent cytotoxic activity for the unfractionated coelomocytes assayed *in vitro*, with rabbit erythrocytes and the K562 tumour cell line. In a plaque-forming assay, whole coelomocyte preparations as well as density gradient separated coelomocyte populations revealed that cell populations enriched in uncoloured spherulocytes, exerted high cytotoxic activity by releasing lysins in the presence of amoebocytes. This cooperative effect could be dependent on soluble factors released by amoebocytes. With regard to this, we show that an enhanced cytotoxic activity was found by adding the supernatant from sonicated amoebocytes or hemocyte culture medium into spherulocyte preparations.

Keywords: Coelomocytes; Cytotoxicity; Coelomocyte cooperation; Echinoderms; Innate immunity; *Paracentrotus lividus*

1. Introduction

Echinoderms are invertebrates and include a number of species with key roles in the marine ecosystem (Menge et al., 1994). Many species, living in coastal and estuarine waters, are directly exposed to potentially pathogenic microorganisms and have developed defence responses mainly based on immunocytes and humoral factors contained in the coelomic fluid (Coffaro and Hinegardner, 1977; Smith, 1981; Chia and Xing, 1996; Smith et al., 1996; Pancer et al., 1999; Gross et al., 2000; Pancer, 2000; Kudriavtsev and Polevshchikov, 2004). The morpho-functional properties of the immunocytes are still controversial and their immune mechanisms are, in part, unknown.

In echinoids, four main coelomocyte types have been described: amoebocytes, vibratile cells, and red and uncoloured spherulocytes (Boolootian and Giese, 1958; Johnson, 1969; Karp and Coffaro, 1980; Smith, 1981). Amoebocytes and spherulocytes represent the main coelomocyte populations that seem to be responsible for a wide repertory of immunological functions: cellular recognition (Bertheussen, 1979), phagocytosis, ROI production (Johnson, 1969; Bertheussen, 1981a,b; Ito et al., 1992), cytotoxicity (Bertheussen, 1979; Lin et al., 2001), antibacterial activity (Gerardi et al., 1990; Plytycz and Seljelid, 1993; Stabili et al., 1996; Haug et al., 2002), inflammatory reactions (Canicattì and Miglietta, 1989; Smith et al., 1996), including C3-like expression after activation with LPS (Gross et al., 1999, 2000), prophenoloxidase activity (Smith and Söderhäll, 1991), capsule formation and graft reaction (Coffaro and Hinegardner, 1977; Jans et al., 1996).

In the sea urchin *Paracentrotus lividus*, a calcium-dependent cytolytic activity has been attributed to cytoplasmic granules separated through a Percoll density gradient from amoebocyte populations (Canicattì, 1991; Pagliara and Canicattì, 1993). Moreover, an innate antibacterial activity against *Vibrio alginolyticus* has been shown in coelomocyte lysates, and in cell-free coelomic fluid (Gerardi et al., 1990; Stabili et al., 1996).

In the present study, we show cytotoxic activity for *P. lividus* unfractionated coelomocytes against erythrocytes and the tumour K562 cell line and identify, after a density gradient

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separation, the cytotoxic coelomocyte population by using a plaque forming assay. Results reveal that uncoloured spherulocytes exerted cytotoxic activity by releasing lysins that can be enhanced as an effect of the cooperative presence of amoebocytes or their cytosolic factors.

2. Materials and methods

2.1. Animals and bleeding procedure

Sea urchins, collected in the Gulf of Palermo, were maintained at 15 °C in marine aquaria equipped with biological and physical filters and fed with commercial invertebrate food (Azoo, Taikong Corp. Taiwan).

The coelomic fluid (CF) was withdrawn by inserting the needle of a syringe, preloaded with isosmotic anticoagulant solution (20 mM Tris, 0.5 M NaCl, 70 mM EDTA pH 7.5) (ISO–EDTA), into the peristomal membrane. After centrifugation (900 ×*g* for 10 min at 4 °C), the coelomocytes were washed two times in ISO–EDTA and resuspended at 5×10^6 cells mL^{-1} in ISO–EDTA. Coelomocyte number was calculated with a haemocytometer chamber, and dead cells were evaluated by using the eosin-y exclusion test (0.5% in ISO–EDTA).

2.2. Coelomocyte populations separated through a discontinuous density gradient

An Iodixinol (Optiprep; Nycomed Oslo, Norway) discontinuous density-gradient was performed as follows: 10, 20 and 30% (v/v) Iodixinol stock solutions in 0.5 M NaCl containing 10 mM EDTA were layered into a 15 mL centrifuge tube. Then, 4 mL of washed coelomocytes adjusted at 1.5×10^6 mL^{-1} were layered on the top of the gradient, and the gradient was centrifuged in a swing-out rotor (800 ×*g* for 30 min at 7 °C). The resulting cell populations on the top of 10, 20 and 30% Iodixinol were referred to as bands B1, B2, B3 respectively, whereas B4 contained the coelomocytes collected at the bottom. To calculate the percentage of each coelomocyte type, the cell bands were gently removed, washed two times with ISO–EDTA and identified according to Smith (1981). Finally, the separated fractions were suspended to obtain an appropriate cell number and used for subsequent assays.

2.3. Preparation of coelomocyte lysate supernatant (CLS)

Coelomocytes (10×10^6) were suspended in 1 mL of ISO containing 10 mM of Ca^{2+} (ISO–Ca^{2+}), sonicated (Sonifier Branson, model B-15 Danbury, CT USA) for 1 min at 0 °C (1 pulse per second, 70% duty cycle) and centrifuged at 27,000 ×*g* for 30 min at 4 °C to remove any precipitate. The supernatant was exhaustively dialyzed against ISO–Ca^{2+}, and sample aliquots were stored at −20 °C.

2.4. In vitro short-term coelomocyte culture

Coelomocytes were suspended (2×10^6 mL^{-1}) in MS (12 mM $CaCl_2{\cdot}6H_2O$, 11 mM KCl, 26 mM $MgCl_2{\cdot}6H_2O$, 43 mM Tris, 38 mM HCl, 0.4 M NaCl pH 8) and 1 mL aliquots were placed into the wells of a sterile flat-bottomed culture plate (Falcon Plastic). Cultures were maintained for 1 h at a constant temperature (10 °C). After incubation, the cell cultures were removed from the plate and centrifuged at 900 ×*g* for 10 min at 4 °C. The resulting supernatant was divided into aliquots and stored at −20 °C.

2.5. Target cells

Rabbit (RE) and sheep (SE) erythrocytes, provided by the "Istituto Zooprofilattico della Sicilia" (Palermo, Italy), were washed three times in phosphate buffered saline (PBS: NaCl 150 mM, KH_2PO_4 146 mM, Na_2HPO_4 0.8 mM, KCl 2.6 mM, $CaCl_2{\cdot}\ 2H_2O$ 0.9 mM, $MgCl_2{\cdot}\ 6H_2O$ 0.49 mM pH 7.4) by centrifuging at 400 ×*g* for 10 min at 4 °C and then suspended at 1% (v/v) in PBS.

The human erythromyeloid leukaemia-derived cell line K562, generously provided by the Department of Pharmaceutical Chemistry and Technology, University of Palermo, was maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% of heat inactivated foetal calf serum (Flow Laboratories, Irvine, Scotland), gentamycin and streptomycin (Boehringer Mannheim, Germany), at 37 °C in 5% CO_2. Short term cultures were fed biweekly in order to maintain a constant exponential cell growth.

2.6. Coelomocyte cytotoxic activity (CCA) assay

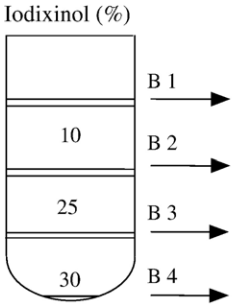
The CCA was examined according to Parrinello et al. (1996). Briefly, 1×10^6 cells of coelomocyte (effector cells, E) suspended in 200 μL of ISO–Ca^{2+}, were mixed with 8×10^6 freshly prepared erythrocytes (target cells, T) in 200 μL of ISO–Ca^{2+}. The reaction mixture was incubated at 20 °C for 30 min. After centrifugation (400 ×*g* for 10 min at 4 °C), the amount of the released haemoglobin present in the supernatant was estimated by reading the absorbance at 541 nm. The hemolysis degree was registered according to the formula:

$$\text{Hemolysis degree} = \frac{(\text{measured release} - \text{spontaneous release})}{\text{complete release} - \text{spontaneous release}} \times 100$$

Complete haemoglobin release was obtained by preparing an erythrocyte suspension in distilled water at room temperature. A control erythrocyte suspension was prepared in the same medium of reaction mixture and incubated under the same experimental conditions and the haemoglobin release was measured (spontaneous release).

Coelomocyte mortality in ISO–Ca^{2+}, under our experimental conditions, was more than 5% inferior, and spontaneous haemoglobin release from untreated erythrocytes never exceeded 5% of the total release (100% erythrolysis).

A cytotoxicity assay against the tumour cell line K562 was performed in standard flat bottom microplates (Nunc, Kamstrups, Denmark) using a cytotoxic detection kit (Boehringer, Germany) based on the evaluation of lactate dehydrogenase (LDH) released from lysed target cells (Korzeniewski and



	Cell types (%)			
	Amoebocytes	Vibratile cells	Uncoloured spherulocytes	Red cells
B 1	89.2 ± 9.2	7.5 ± 1.5	2.6 ± 1.1	0.0 ± 0.0
B 2	8.2 ± 2.1	84.3 ± 1.5	3.9 ± 6.3	3.1 ± 1.6
B 3	1.5 ± 0.3	6.2 ± 1.6	90.2 ± 5.6	5.5 ± 1.4
B 4	1.1 ± 1.5	2.0 ± 0.8	3.3 ± 2.1	91.4 ± 11.2

Fig. 1. A typical separation of *Paracentrotus lividus* coelomocytes through an Iodixinol discontinuous gradient. For each separated band the percentage ± SD of every cellular type is shown; values were obtained from three independent separations.

Callewaert, 1983). The target cells were washed and suspended in ISO–Ca^{2+} at a concentration of 1×10^5 cells mL^{-1}. All tests were performed in triplicate with 1×10^4 target cells $well^{-1}$ and with appropriate effector-to-target ratios (1×10^4–2.5×10^5) in a total volume of 200 μL.

Plates were centrifuged for 1 min at 100 ×*g* and incubated for 2 h at 18 °C. The plates were centrifuged for 5 min at 400 ×*g*, and 100 μL supernatant was used to detect the LDH activity by registering the absorbance at 490 nm (microplate reader, Uniskan I, Labsystems). The spontaneous release from coelomocytes and target cells, and the complete release from target cells treated with 1% Triton X-100 were evaluated. These values were subtracted from the degree of target cell lysis, and percent of lysis was calculated according to the equation:

$$\text{Lysis}\,(\%) = \frac{(\text{measured release} - \text{T spontaneous release}) - \text{E spontaneous release}}{\text{complete release} - \text{T spontaneous release}} \times 100$$

where T = target cell and E = effector cell.

2.7. Plaque forming cell (PFC) assay

A PFC assay was performed as described by Cunningham and Szenberg (1968) with slight modifications. Briefly, 50 μL of coelomocyte suspension in ISO–Ca^{2+} (1×10^6 mL^{-1}) was mixed with 50 μL of a 5% (v/v) RE suspension (4×10^7 mL^{-1}) in ISO–Ca^{2+}. The reaction mixture was gently loaded in a chamber formed by a thin strip of adhesive tape inserted between the borders of a coverslip and a microscope slide. After a 15 min incubation at 20 °C, the cell mixture was examined under a phase contrast microscope at 10× magnification. In ten distinct experiments, plaque number was recorded in 10 microscopical field/slide (5×10^4 coelomocytes in total). Unfractionated and density separated coelomocytes were assayed.

2.8. Statistical analysis

Each experiment was performed in triplicate. The values were the mean of three assays ± SD. Significance was determined by using the Student's *t* test and differences were considered significant at $P < 0.05$.

2.9. Protein content determination

Protein content was evaluated according to Bradford (1976).

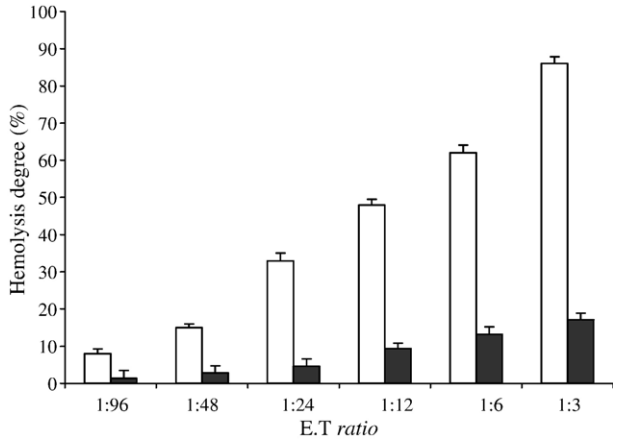


Fig. 2. *Paracentrotus lividus* coelomocytes cytotoxic activity against rabbit □ or sheep ■ erythrocytes. Data are the means ± SD of three separated experiments, each of which was performed in triplicate.

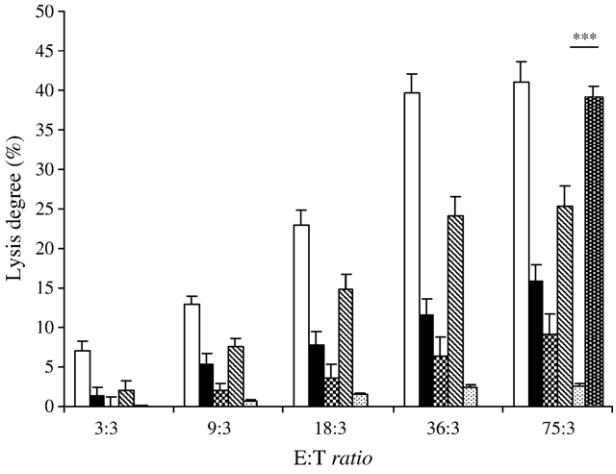


Fig. 3. Anti K562 cytotoxic activity of unseparated and density gradient separated *Paracentrotus lividus* coelomocytes. Unfractionated coelomocytes □, B1 ■, B2 ▩, B3 ▧ and B4 ▨. B1 + B3 ▩. Data are expressed as mean value ± SD of three distinct experiments, each of them performed in triplicate.

2.10. Chemicals

Unless otherwise reported, all chemicals were purchased from Sigma, St. Louis, USA.

3. Results

3.1. Coelomocyte populations

Preliminary observations revealed that coelomic fluid of the examined sea urchins contained amoebocytes (~76%), vibratile cells (~12%), uncoloured spherulocytes (~4%) and red cells (~8%).

In a discontinuous Iodixinol gradient, coelomocytes (6×10^6) were partitioned into 4 distinct bands (B1, B2, B3, B4) placed, respectively, from the highest gradient layer to the lowest one (Fig. 1).

As shown in Fig. 1, each band did not contain pure coelomocyte types, whereas a significant enrichment in distinct coelomocyte populations could be found. B1 was mainly composed with amoebocytes (89.2%), and B2 with vibratile cells (84.3%), B3 contained 90.2% uncoloured spherulocytes and B4 91.4% red cells and cellular debris. In the figure, the coelomocyte types identified in each band are listed.

3.2. Cytotoxic activity of the unfractionated coelomocytes

Coelomocytes lysed RE and K562 tumour cells in the presence of 10 mM Ca^{2+}, whereas 10 mM EDTA in the reaction medium significantly decreased ($P<0.01$) the cytotoxic activity.

As shown in Fig. 2, the assay with rabbit erythrocytes revealed that the hemolytic degree increased depending on the E:T ratio, and reached the maximum value (86%) at a 1:3 ratio. Sheep erythrocytes were less sensitive to cytotoxic coelomocytes, and a low value of hemolysis degree (17%) was found in the same experimental conditions. Consequently, RE were used in the following assays.

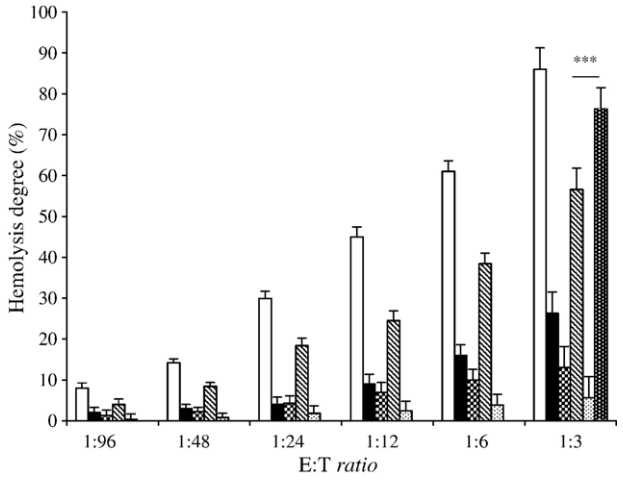


Fig. 4. Anti rabbit erythrocyte cytotoxic activity of unseparated and density gradient separated *Paracentrotus lividus* coelomocytes. Unfractionated coelomocytes □, B1 ■, B2 ▩, B3 ▧ and B4 ▨. B1+B3 ▩. Data are expressed as mean value±SD of three separated experiments, each of them performed in triplicate.

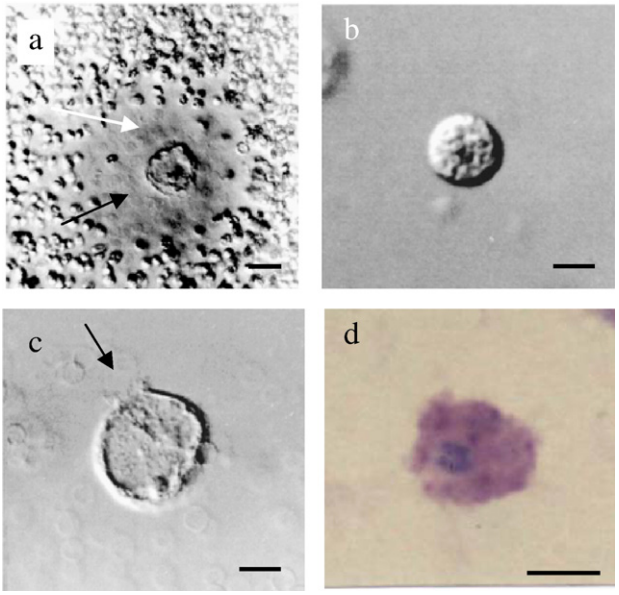


Fig. 5. Plaque forming assay of *Paracentrotus lividus* coelomocytes against rabbit erythrocytes. a, b, c: Nomarski interferential contrast microscope bar=5 μm; d: uncoloured spherulocyte stained with toluidine blue. Bar=5 μm. a: lysis plaques. White arrow: erythrocyte ghosts; black arrow: uncolored spherulocyte. c: Uncoloured spherulocyte in the centre of lysis plaque; arrow: membrane profile discontinuance.

Similar results were found when the K562 tumour cell line was used as a target. Fig. 3 shows that the CCA unfractionated coelomocytes against the tumour cells was dependent upon the E:T ratio. The maximum CCA value (41%) was found at a 36:3 ratio.

3.3. Cytotoxic activity of the separated coelomocyte populations

To identify anti-RE effector coelomocytes, enriched coelomocyte populations from B1, B2, B3 and B4 were assayed

Table 1
Number of plaque forming cell (PFC) referred to the number of *Paracentrotus lividus* amoebocyte and uncoloured spherulocyte calculated in unseparated and density gradient separated coelomocytes

Coelomocytes (E:T ratio 1:3)	N. PFC[1]	Amoebocytes ($\times10^4$)	Uncoloured spherulocytes ($\times10^4$)
Unfractionated	62±3.4	3.8±0.1	0.25±0.04
B1	0	4.5±0.1	0.13±0.05
B2	0	0.41±0.1	0.19±0.1
B3	5±1.0***	0.07±0.01	4.5±0.3
B4	0	0.05±0.07	0.16±0.1
B1+B3	63±1.5	2.3±1.2	2.3±3.3
B3+B1 cells culture supernatant	30±2.3***	0.07±0.01[2]	4.5±0.3[3]
B3+B1 cells sonicate	39±2.5***	0.07±0.01[2]	4.5±0.3[3]

[1]Mean values±SD of 10 experiments; 5×10^4 mL^{-1} coelomocytes were counted.
[2]Amoebocytes in B3.
[3]Uncoloured spherulocytes in B3.
B3: ***$P<0.001$ compared with Unfractionated.
B3+B1: ***$P<0.001$ compared with B3.
B3+B1 sonicate: ***$P<0.001$ compared with B3.

(Fig. 4). Similar to the whole coelomocytes, the CCA of the separated coelomocyte bands was dependent upon the E:T ratio (1:3), even if they showed a lower activity level. Coelomocytes from B1 (89.2% amoebocytes) and B3 (90.2% uncoloured spherulocytes) displayed the highest cytotoxic activity (26.5% and 56.6% respectively); B2, mainly enriched with vibratile cells (84.3%), caused 13% hemolysis, whereas pigment cells mainly contained (91.4%) in B4 showed 5.6% CCA.

When coelomocyte populations from B1 were mixed with those from B3, the CCA reached the same level (78% hemolysis degree) displayed by the unfractionated coelomocytes (Fig. 4).

The coelomocyte activity against tumour cells was about 25% by assaying cell populations from B3, whereas 15% was the level of CCA exerted by the cells from B1. In Fig. 3, the level of CCA of B1+B3 resulted in more than 34%.

3.4. Identification of plaque forming cells with rabbit erythrocytes (PFC)

The unfractionated coelomocytes co-incubated with rabbit erythrocytes in a Cunningam–Snezeberg chamber generated plaques of lysis (Fig. 5). The reaction was very fast, occurring within 10 min of incubation time, and only one uncoloured spherulocyte was found in the centre of each plaque of lysis (Fig. 5a). This effector cell presented membrane profile discontinuance, and erythrocyte ghosts were observed nearby (Fig. 5b).

To support these findings, enriched coelomocyte populations were assayed. In Table 1, the number of PFCs observed, using coelomocytes from each separated band are reported. Only uncoloured spherulocytes enriched (~90%) in B3 gave rise to a negligible number of PFCs. However, their ability to generate plaques was restored (63 PFCs) when 2.5×10^4 coelomocytes from B1 (containing 2.3×10^4 amoebocytes), were added into the reaction mixture 2.3×10^4 containing B3-uncoloured spherulocytes (Table 1).

A significant increase in PFC number (30 PFC) was also found when the medium from B1 coelomocytes cultured for 1 h (5×10^6 cells, 89% amoebocytes) was added to the B3 suspension (Table 1). A similar result was obtained by adding 50 μL of B1 culture supernatant (1.0 μg±0.03 protein content) or 50 μL B1 CLS (1.0 μg±0.25 protein content) to the B3 suspension.

4. Discussion

According to Smith (1981), the coelomic fluid of echinoids contains four different coelomocyte types including (approximately) 76% amoebocytes, 8% pigmented or red cells, 4% uncoloured spherulocytes, and 12% vibratile cells. Among them, amoebocytes and uncoloured spherulocytes have been reported to be involved in immune functions (Gross et al., 1999). In the present paper we examine similar properties of *P. lividus* coelomocyte cytotoxic activity and show that uncoloured spherulocytes are the effector cells. The cytotoxic activity of unfractionated coelomocytes presented a certain range of specificity and required effector–target cellular contacts as revealed by RE lysis (maximum Hb release, 85% hemolysis degree) when the ratio of 1 effector and 3 target cells was performed. On the contrary, this proportion increased when K562 tumour cells were used, and 12 effector cells were required to obtain the highest cell lysis, suggesting that more cellular contacts are needed to affect tumour cells.

Previous studies on the immunological activity of coelomocytes, reported that calcium-dependent cytolytic activity towards rabbit erythrocytes could be attributed to the content of amoebocyte granules (Canicattì, 1991; Pagliara and Canicattì, 1993). In this paper we show by a plaque-forming cell assay that uncoloured spherulocytes are responsible for a calcium-dependent cytotoxic activity against erythrocytes and the K562 tumour cell line. Moreover, this effector coelomocyte showed membrane profile discontinuance, suggesting that the activated cell could release cytosolic and/or granular contents. Although the coelomic fluid of the examined sea urchins contained about 4% uncoloured spherulocytes, the coelomocyte preparation performed by collecting coelomocytes from a large volume of coelomic fluid, up to 5×10^6 mL^{-1} cells, represented a first step of enrichment in cell type populations.

To examine the role of coelomocytes with regards to cytotoxic activity, an Iodixinol discontinuous density gradient method was used. The coelomocyte populations were enriched as follows: 89.2% amoebocytes in B1, 84.3% vibratile cells in B2, 90.2% uncoloured spherulocytes in B3, and 91.4% red cells in B4. The cytotoxic activity against both erythrocytes and tumour cells was mainly expressed by the fraction (B3), enriched in uncoloured spherulocytes, but also containing 1.5% amoebocytes. A very low cytotoxic activity displayed by the amoebocytes mainly enriched in B1 could be attributed to the presence of 2.6% uncoloured spherulocytes. A low activity was also observed when the coelomocytes from B2 enriched with 84.3% vibratile cells, 3.9% uncoloured spherulocytes and 8.2% amoebocytes were assayed. The same effect was found when K562 cells were the targets in reaction mixtures in which B1 and B3 were mixed.

These results support the cytotoxic role of uncoloured spherulocytes, and suggested that amoebocytes could contribute in displaying cytotoxic activity. With respect to this, a cooperative effect between uncoloured spherulocytes and amoebocytes was revealed by mixing amoebocytes from B1 with uncoloured spherulocytes mainly enriched in B3 that restored a high cytotoxic activity, reaching the level observed for the unfractionated coelomocytes.

Plaque forming assays supported that coelomocyte cytotoxic activity against erythrocytes could be exerted by lysins presumably released by uncoloured spherulocyte subpopulations following E–T contacts. In this respect, uncoloured spherulocytes contained in B1 probably are different than those enriched in B3 and are separated into two distinct Iodixinol gradient bands. Apparently, only the spherulocytes from B3 were cytotoxic, indicating that distinct subpopulations could be separated on the basis of their morpho-functional properties.

The PFC assay also showed that the cooperative effect of amoebocytes could be dependent on soluble factors released by these cells into the reaction mixture. Few PFCs (n. 5 PFCs in 5×10^4 coelomocytes) were obtained by assaying B3 (90.2% spherulocytes but only 1.5% amoebocytes), whereas PFCs were significantly more numerous (63 PFCs in 5×10^4 coelomocytes) when amoebocytes from B1 were mixed with coelomocytes from B3. Among the spherulocytes from B3, cytotoxic subpopulations could be distinguished. In fact, when B1 and B3 were mixed (2.3×10^4 amoebocytes and 2.3×10^4 uncoloured spherulocytes respectively), the amount of PFCs was 12 times higher than that observed by assaying B3, whereas it did not exceed the value found by assaying the unfractionated coelomocytes containing a low number (0.25×10^4) of spherulocytes. Presumably, the cooperative interaction could reside in a spherulocyte activation response due to factors released by the amoebocytes. In fact, the number of PFCs was enhanced (30 PFCs in 5×10^4 coelomocytes) by adding the medium from 1h-B1 coelomocytes cultures to B3 uncoloured spherulocytes. Since a similar result was obtained by assaying coelomocyte lysate supernatant from B1, we hypothesize that soluble factors released from activated amoebocytes, which are phagocytes (Beck and Habicht, 1986, 1996), could in turn activate uncoloured spherulocytes to release soluble cytotoxic lysins. A similar mechanism could be suggested for the cytoxic activity of uncoloured spherulocytes against a K562 cell line.

Research is in progress to characterise the soluble factors involved in the *P. lividus* coelomocyte cytotoxic reaction.

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