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Induced cell proliferation in putative haematopoietic tissues of the sea star, *Asterias rubens* (L.)

Kristina Holm[1], Samuel Dupont[2], Helen Sköld[2], Anna Stenius[2], Michael Thorndyke[3] and Bodil Hernroth[3,*]

[1]Department of Zoology, University of Gothenburg, Box 463, SE-405 30 Göteborg, Sweden, [2]Institute of Marine Ecology, Kristineberg 566, SE-450 34 Fiskebäckskil, Sweden and [3]The Royal Swedish Academy of Sciences, Kristineberg 566, SE-450 34 Fiskebäckskil, Sweden

*Author for correspondence (e-mail: bodil.hernroth@marecol.gu.se)

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SUMMARY

The coelomic fluid of the echinoderm *Asterias rubens* possesses large populations of circulating coelomocytes. This study aimed to expand the knowledge about the haematopoietic sources of these cells. Injection of the immune-stimulating molecules lipopolysaccharide (LPS) and concanavalin A (ConA) resulted in an increase in coelomocytes. To investigate if these molecules induce cell proliferation in putative haematopoietic tissues (HPTs), short-term exposure of the substitute nucleotide 5-bromo-2′-deoxyuridine (BrdU) was conducted. Immunohistochemical analysis, using fluorescein-labelled antibodies to trace BrdU, showed pronounced cell division in the coelomic epithelium and axial organ. In the pyloric caeca, not considered as an HPT, proliferation was not detected. BrdU labelling of monolayers of cells obtained by collagenase treatment of coelomic epithelium, axial organ and Tiedemann body revealed induced cell proliferation in response to both LPS and ConA while proliferation of pyloric caeca and circulating coelomocytes remained sparse. By using confocal microscopy it was observed that both the morphology and functional behaviour of cells released from explants of coelomic epithelium showed high similarity to those of circulating phagocytes. It was concluded that the increased coelomocyte numbers observed in response to LPS and ConA were reflected in an induced cell proliferation in coelomic epithelium, axial organ and Tiedemann body, which reinforces the idea that these organs are HPTs and the sources of coelomocyte renewal.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/16/2551/DC1

Key words: echinoderm, sea star, haematopoietic tissue, cell proliferation, coelomocyte, mitogenic factor.

INTRODUCTION

The echinoderms are, with a few exceptions for brackish water, exclusively marine. They are among the most abundant of all benthic animals and may constitute more than 90% of the benthic biomass in the deep sea. Most species exhibit a remarkable plasticity as adults and are capable of extensive regeneration of large parts of their bodies (Hyman, 1955) following traumatic loss or damage through predation, trawling activities, strong currents, etc. In some areas, more than 80% of individuals show signs of injury (Sköld and Rosenberg, 1996). The ability to heal and reconstitute lost arms contributes to their importance in the food web of many habitats.

The coelomic fluid of the common sea star, *Asterias rubens*, possesses large populations of circulating coelomocytes, the mediators of the immune response. Following injury, the coelomocytes take part in wound healing by migrating to the damaged site to prevent bleeding by clotting (Smith, 1981) and interact in modulation of the extracellular matrix (Tanney et al., 1998). The morphological and biochemical characterization of coelomocytes is, however, still not completely understood, and no reliable, specific cell markers are available. Four sub-populations – phagocytes (previously called amoebocytes), vibratile cells (with flagellum), morula cells (also called haemocytes) and slow-moving cells with an irregular shape (recently called amoebocytes) – have been described (Smith, 1981; Pinsino et al., 2007) but the nomenclature has still not been standardized. Phagocytes are the pre-dominating cell type, comprising 80–95% of total coelomocyte counts. These cells can rapidly convert to petaloid and filopodial stages able to form networks when attached to glass slides (Pinsino et al., 2007). Knowledge of the recruitment of the coelomocytes is poor, and the haematopoietic tissue (HPT) of *A. rubens* has still not been clearly identified. The coelomic epithelium, lining the dorsal part of the coelomic cavity throughout the animal, has been suggested as the most probable source of the coelomocytes in echinoderms (Muñoz-Chapuli et al., 2005). However, the axial organ (Leclerc et al., 1987) that comprises the junction of the perivisceral coelom and the haemal system, as well as the Tiedemann bodies (Kaneshiro and Karp, 1980), small paired organs located as pockets on the peristomal ring, have also been suggested as HPTs. There is an additional possibility of direct self-replication of circulating coelomocytes. Regeneration studies conducted on asteroids also point out that morphallactic processes and cell cycle activities are intense in the coelomic epithelium (Carnevali, 2006).

In mammals and fish, there is good evidence that the lymphocytes that proliferate in response to lipopolysaccharide (LPS) are B lymphocytes, and those that respond to the plant lectin concanavalin A (ConA) are T lymphocytes (Sizemore et al., 1984). In invertebrates, which lack the lymphoid cell line, addition of ConA to primary cell cultures of haemocytes has shown effects on cell attachment and cell proliferation (Lebel et al., 1996), and LPS has been used successfully for stimulating proliferation of HPT cells (Van de Braak et al., 2002). Such self-renewal is a process by which haematopoietic stem cells generate daughter cells *via* division and

is necessary for retaining a pool of stem cells able to differentiate into immune-active circulating coelomocytes. Here, we aimed to expand the knowledge of the source of circulating coelomocytes in *A. rubens*. We investigated mitogenic effects of the well-known immune-stimulating compounds LPS and ConA on the putative sources of the coelomocytes: the coelomic epithelium, the axial organ and the Tiedemann body; the pyloric caeca, which is not considered an HPT, was used as a negative control. In addition, mitogenic-induced self-replication of circulating coelomocytes was also investigated. The overall hypothesis was that increased total numbers of circulating coelomocytes would be reflected in an increased cell proliferation in the presumed HPTs and that cells released from such tissues have morphological and functional similarities to circulating coelomocytes.

MATERIALS AND METHODS

Animal collection and handling

Specimens of *Asterias rubens* L. were collected by scuba divers from approximately 5–15 m depth at the mouth of the Gullmar Fjord, situated on the Swedish west coast. The sea stars were brought to Sven Lovén Centre for Marine Sciences (Kristineberg, situated by the fjord) and maintained in tanks supplied with running, natural seawater of ambient temperature and salinity (~33‰). The study was carried out during two periods: one from mid-February to mid-April, with animals collected in November; and one during May–June, with animals collected in late March. Once per week, animals were fed with blue mussels (*Mytilus edulis*). All sea stars used for the study were about 10 cm across, from one arm tip to the most distant arm tip.

Total coelomocyte counts (TCC)

This study was performed during May–June. *A. rubens* were pre-treated by injection with 100 µl of either LPS (1 mg ml^{-1}; Sigma L-7261, Sigma-Aldrich, Stockholm, Sweden), ConA (25 µg ml^{1}; Sigma C-0412), dissolved in sterile filtered calcium- and magnesium-free saline buffer (CMFSS; 435 mmol l^{-1} NaCl, 10.7 mmol l^{-1} KCl, 27 mmol l^{-1} Na_2SO_4, 16.6 mmol l^{-1} $C_6H_{12}O_6$, 12 mmol l^{-1} Hepes; pH 7.4) or CMFSS (control-group), 4 and 24 h prior to collection of coelomic fluid. By cutting the outermost edge of an arm, the coelomic fluid was collected in an equal amount of anticoagulant buffer (CMFSS with 5 mmol l^{-1} EGTA) on ice. Cell counts were performed either by counting formalin-fixed cells in a Bürker chamber (BT, Brand, Wertheim, Germany) or by analysis in a NucleoCounter (ChemoMetec A/S, Allerod, Denmark). Prior to the study, the two methods were compared to verify that equivalent data were obtained. The cell counter operates by loading 40 µl of lysed cells (lysis buffer provided by the manufacturer) into a disposal NucleoCounter cassette, precoated with the nuclei staining dye propidium iodide, which is recorded by a camera. The image is analysed by NucleoView software (ChemoMetec A/S). The measured volume is approximately 1.5 µl and the result is presented as cell counts per ml.

Cell proliferation (recorded with BrdU technique)

Cell proliferation was investigated both in tissue sections [immunohistochemistry (IHC); February–April] and in monolayers of cells [immunocytochemistry (ICC) May–June)] by using the substituted nucleotide 5-bromo-2′-deoxyuridine (Sigma B 5002) as a tracer for cell division (Gratzner, 1982). Pilot studies were performed to optimise the IHC technique in terms of fixation, permeabilisation, blocking, antibody incubation, secondary antibodies, etc., and the following protocols were finalised.

Immunohistochemistry on tissue sections (IHC)

A. rubens were pre-injected with either 125 µl of ConA [150 µg ml^{-1}; immersed in filtered seawater (FSW; 0.2 µm)], to stimulate mitosis, or 125 µl FSW (controls) 1 h prior to injection with 32.5 mmol l^{-1} BrdU (10 µl g^{-1} wet mass). After 4 h incubation in running seawater, the sea stars were anesthetized in $MgCl_2$ for 5–20 min and then dissected. Axial organ, coelomic epithelium and pyloric caeca were dissected and fixed for 1.5–2 h in 4% paraformaldehyde (PFA) made up in FSW. The tissue was dehydrated and paraffin embedded using standard methods. Tissue sections of 4–6 µm on SuperFrost®Plus slides (Menzel GmbH & Co. KG, Braunschweig, Germany) were de-waxed, and after rehydration the sections were nuclease treated (0.5%; cell proliferation kit RPN202; Amersham Biosciences, Amersham, UK) for permeabilising the tissue and then blocked with 5% normal goat serum (NGS) for 1 h. Thereafter, the sections of coelomic epithelium were incubated for 1 h and the other tissues incubated for 2 h with anti-BrdU (raised in mouse; provided in the kit) diluted 1:100 with nuclease and 1% NGS. Incubation for 1.5 h with the secondary antibody, Texas Red conjugated anti-mouse IgG (TI-2000; Vector Laboratories, Burlingame, CA, USA), diluted 1:100 in phosphate-buffered saline (PBS), was performed before mounting the sections with Hydromount (HS-106; In vitro Sweden AB, Stockholm, Sweden). Between each step in the protocol, the slides were rinsed several times with PBS. The pilot study established that there were problems with making good sections of the Tiedemann body and thus this tissue was not included in the IHC analysis.

Immunocytochemistry on cell monolayers (ICC)

One group of sea stars was pre-challenged with ConA (N=6), as described above, 1 h prior to the injection with BrdU and then incubated for another 3 h in BrdU. Another group of 12 sea stars was challenged with 100 µl of LPS (1 mg ml^{-1}) and then incubated for 1, 13 and 21 h (N=4) prior to the 3 h of BrdU incubation. The same numbers of animals were used as control groups and injected at the same time points with CMFSS (which was also used for dissolving ConA and LPS). For baseline determination of proliferation, an additional group of non-injected sea stars was used (N=4). Coelomic fluid (N=4) was collected to perform monolayers of coelomocytes. The axial organ, coelomic epithelium, Tiedemann body and pyloric caeca were dissected and then prepared in accordance with the study of Söderhäll et al. (Söderhäll et al., 2003). Briefly, the tissues were transferred to 600 µl of 0.1% collagenase Type I and 0.1% collagenase Type IV (dissolved in CMFSS). After approximately 1 h incubation at room temperature (RT), the tissue was gently crushed and removed before the released cells were centrifuged (2500 ***g***, 5 min, RT) and washed twice in 500 µl CMFSS. To prepare the cell monolayers, the cell suspension was mixed with $CaCl_2$ (final concentration 40 mmol l^{-1}) on SuperFrost®Plus slides and left for 45 min to settle. The cells were fixed with 70% ethanol in 50 mmol l^{-1} glycine (USB Corporation, Cleveland, OH, USA) for 5 min. Permeabilising of cells was done by incubation in 2 mol l^{-1} HCl with 0.2 mg ml^{-1} pepsin (Merck, Hohenbrunn, Germany) for 30 min at 30°C and then neutralised in 0.1 mol l^{-1} Na-borate (pH 8.5) for 5 min at RT. Bovine serum albumin (0.5% BSA; Intergen 3305-60, New York, NY, USA) in PBS–Tween (0.05% Tween-20; Merck) was used as a blocking medium for 1 h in a humid chamber at RT before incubation for 30 min with anti-BrdU-FITC (347583; BD Bioscience, San Jose, CA, USA) diluted 1:10 in blocking medium. To enhance the FITC labelling, the slides were incubated in the same way with a secondary anti-mouse IgG-FITC (Sigma F3008),

diluted 1:100 in blocking medium, before being mounted in Vectashield with propidium iodide (H-1300; Vector Laboratories). Each step of the procedure was followed by rinsing the slides several times in PBS–Tween.

Background controls for both sections of tissue and monolayers of cells were performed by using tissue sections/cell monolayers from sea stars without BrdU treatment and with and without the secondary antibody. The slides were examined using a Leica DMRBE fluorescent microscope (Wetzlar, Germany) equipped with 20, 40 and 100× objectives. Filter N2.I-green was used for visualising Texas Red and propidium iodide, and filter I3-blue for FITC. The ICC results were calculated from at least 300 cells per slide, with the exception of the pyloric caeca, where approximately 120 cells per slide were calculated due to improper adherence of the cells to the glass.

Cell viability assay

In order to investigate the influence of LPS and ConA on the viability of the cells, a colourimetric method based on enzymatic reduction of tetrazolium (MTS) and phenylmetasulfazone (PMS) to formazan (CellTiter 96® aQuenous Non-Radioactive Cell Proliferation Assay G5421; Promega Corporation, Madison, WI, USA) was used. Coelomocytes of untreated sea stars (N=6) were diluted to approximately 2×10^6 cells ml^{-1} in homologous coelomic fluid. 50 µl aliquots of the cell suspensions were incubated *in vitro* in 96-well microplates (five replicates) for 16 h at RT either with LPS (0.5 and 5 µg), ConA (0.25 and 2.5 µg) dispersed in physiological saline buffer (PSB; 20 mmol l^{-1} Hepes, 436 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 53 mmol l^{-1} $MgSO_4$) or PSB (control) to a final volume of 100 µl before adding 20 µl of the substrate. After another 2 h of incubation, the formazan product was measured (490 nm) in a microplate reader (Labsystems iEMS Reader MF; Helsinki, Finland). The survival index (SI%) of LPS- and ConA-exposed cells was calculated in relation to cells incubated with only PSB.

Protein composition

For comparison of organ protein patterns, coelomocytes, coelomic epithelium, Tiedemann bodies, axial organ and pyloric caeca were collected from *A. rubens* (N=2). The tissues were lysed in 1:2 (w/w) ice-cold RIPA lysis buffer [50 mmol l^{-1} Na_2HPO_4, 150 mmol l^{-1} NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 2 mmol l^{-1} EDTA, including 1 mmol l^{-1} Pefablock (Hernebring et al., 2006) (Roche Biochemicals Inc., Indianapolis, IN, USA) as protease inhibitor]. The coelomocytes were separated from the coelomic fluid by spinning at 1500 ***g*** for 10 min, and the pellet was resuspended in RIPA lysis buffer as above. Tissues and cells were homogenised in the buffer on ice using a plastic homogeniser and centrifuged (10,000 ***g***, 10 min, 4°C). Aliquots of the supernatants were stored at –80°C. Protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). The samples were boiled in NuPage LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) and separated by gel electrophoresis using NuPage 12% Bis-Tris precasted gels and NuPage MOPS running buffer (Invitrogen). Equal amounts of proteins were added to each lane (6 µg). The SeeBlue Plus2 Pre-stained Standard (Invitrogen) was used as a molecular mass marker. The gel was post-stained with PageBlue Protein Staining Solution (Fermentas, Vilnius, Lithuania).

Microscopic studies on cells released from explants of coelomic epithelium

In order to investigate if the coelomic epithelium releases mature coelomocytes, the morphology and putative phagocytic behaviour of cells migrating from dissected explants were recorded using a Leica SP5 confocal laser scanning microscope (CSLM). Explants of coelomic epithelium were transferred to either 4 ml cell-free coelomic fluid, obtained by separating cells from coelomic fluid by centrifugation (2900 ***g***, 10 min, 4°C) followed by filtration (0.2 µm) or to FSW in chambers (diameter 34 mm). Both the cells that migrated from the tissue and the coelomocytes collected from the coelomic fluid were exposed to FITC-labelled yeast, *Saccharomyces cerevisiae*, and the behaviour of the cells was compared. The FITC-labelling was performed in accordance with the procedure described by Anderson and Mora (Anderson and Mora, 1995). Cell activity was assessed by time-lapse video recording (1 image per second for up to 40 min) using CSLM. The procedure was repeated with cells collected from different individuals (N=5) and at different cell concentrations.

Statistics

One-way analysis of variance (ANOVA) was performed to investigate LPS and ConA effect on TCC data. The homogeneity and power of data were tested before entering the ANOVA and the significance was set to P=0.05. t-test was used to compare the ICC data of the percentage of BrdU-incorporated cells from sea stars pre-treated with LPS and ConA and to compare the survival index of cells after *in vitro* exposure to LPS and ConA. Results are means ± s.e.m. The analyses were performed using Sigma Stat (version 3.5; Jandel Scientific software, San Rafael, CA, USA).

RESULTS

Total coelomocyte counts

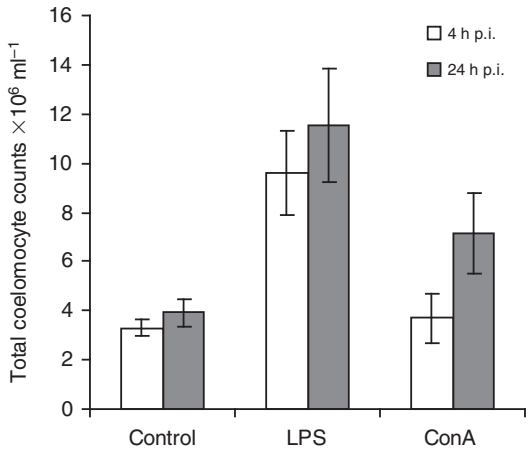
At 4 h post-injection (p.i.), the TCC of LPS-treated sea stars was significantly higher ($9.6\pm3.4\times10^6$ ml^{-1}) than that of the ConA-treated sea stars ($3.7\pm3.4\times10^6$ ml^{-1}; P=0.006) as well as of the control group ($3.3\pm0.7\times10^6$ ml^{-1}; P=0.004; Fig. 1). At 24 h p.i., the TCC of the LPS-treated group ($11.5\pm7.8\times10^6$ ml^{-1}) was still significantly higher than that of the control group ($3.9\pm2.1\times10^6$ ml^{-1}; P<0.003) but not compared with the ConA-treated group ($7.2\pm4.1\times10^6$ ml^{-1}; P=0.492). Here, ConA treatment showed an increase compared with the control group, although this was not statistically significant (P=0.068).

IHC and ICC analysis after *in vivo* exposure to mitogenic factors

The IHC analysis showed that BrdU was incorporated in the tissue sections of the axial organ and coelomic epithelium in response to ConA but not in the pyloric caeca (Fig. 2). In the control group, pre-treated with FSW, the incorporation was negligible.

The ICC analysis showed that ConA significantly induced cell proliferation in coelomic epithelium (P=0.002) and in the axial organ (P<0.001) but not in the Tiedemann body (P=0.120), pyloric caeca (P=0.590) and circulating coelomocytes (P=0.496) (Fig. 3). When LPS was used to follow the proliferation response at different time intervals (Fig. 4A), proliferation was enhanced after 4 h, significantly so in the Tiedemann body (P<0.001) and in the axial organ (P=0.002). In coelomic epithelium, the numbers of dividing cells were slightly but not statistically significantly higher than the control group (P=0.069). Sixteen hours post-LPS injection, proliferation decreased, while the coelomic epithelium, axial organ and Tiedemann body of control animals, which were injected with CMFSS, showed a slight increase in cell proliferation, which was even more obvious after 24 h. By 24 h it had increased by approximately 4% (Fig. 4B). At that time, the LPS-stimulated animals showed proliferation rates similar to the basal value,

A



B

	After 4 h		After 24 h	
	Diff. of mean	*P*	Diff. of mean	*P*
LPS *vs* Control	6.30	0.004	8.95	0.003
LPS *vs* ConA	5.90	0.006	3.11	0.492
ConA *vs* Control	0.40	0.813	5.84	0.068

Fig. 1. (A) Mean numbers (± s.e.m.) of total coelomocyte counts (10^6 ml^{-1}) of *A. rubens* determined 4 h (*N*=8) and 24 h (*N*=10) post-injection (p.i.) of either 100 µl CMFSS buffer (Control), lipopolysaccharide (LPS) or concanavalin A (ConA). (B) A comparison between the different treatments showed significant differences between LPS and Control, both at 4 and 24 h, and between LPS and ConA at 4 h p.i.

1.6±0.3%, which was the mean value recorded in the different organs of animals not pre-challenged before the BrdU-injection. The proliferation of cells from the pyloric caeca and the circulating coelomocytes remained at a low level, both in LPS- and CMFSS-stimulated cells, throughout the experiment (Fig. 4).

Cell viability test

The estimation of the viability, calculated in relation to the controls, for coelomocytes exposed to LPS and ConA *in vitro* showed a great individual variability. There was no noteworthy difference in survival index (SI%) between the different concentrations of the mitogenic compounds nor were the mean values of pooled data (obtained for the two different concentrations) significantly different (LPS, 95±20%; ConA, 122±46%; *N*=6).

Protein composition comparisons

Gel-electrophoretic separation of proteins extracted from coelomic epithelia, axial organ, coelomocytes, pyloric caeca and Tiedemann bodies (Fig. 5) showed similar protein composition patterns between coelomocytes, coelomic epithelia and Tiedemann bodies. The protein pattern of the axial organ was also similar to that of the coelomocytes but some of the protein bands in the size range of 97–110 kDa showed higher density. The pyloric caeca showed a different protein pattern compared with coelomocytes.

Microscopic studies on cells released from explants of coelomic epithelium

It was possible to visualize the release of cells from the dissected coelomic epithelium. A large proportion of the cells showed morphological similarities to the phagocytes of the coelomic fluid (Fig. 6). Cells attached to the bottom of the chamber showed petaloid and filopodial extendings that were often withdrawn or rearranged (Movies 1 and 2 in supplementary material). Cells were able to form dense clusters that rapidly could be dissolved and could also be found as syncytic formations. When interacting with the yeast cells, phagocytosis was observed for both coelomocytes and cells released from the coelomic epithelium. The density of coelomic cells seemed to determine their ability for network formation. This was also the case for coelomocytes (Fig. 7; Movies 3 and 4 in supplementary material). When coelomic fluid was undiluted, the network formation occurred almost immediately (Movie 5 in supplementary material), but when diluted to a density similar to what we found in chambers with the explants, the network became sparser. Cells from explants dispersed in coelomic fluid did not show any significantly higher activity compared with those dispersed in FSW.

DISCUSSION

The accumulated results of this study in terms of mitotic response to LPS and ConA, as well as the cellular behaviour, do support a haematopoietic role of the coelomic epithelium of *A. rubens*. The mitotic response in the axial organ and Tiedemann body also implies that these organs are haematopoietic. By contrast, such a mitotic response was not found in pyloric caeca, used as a negative control in the study. When comparing the protein composition of the organs,

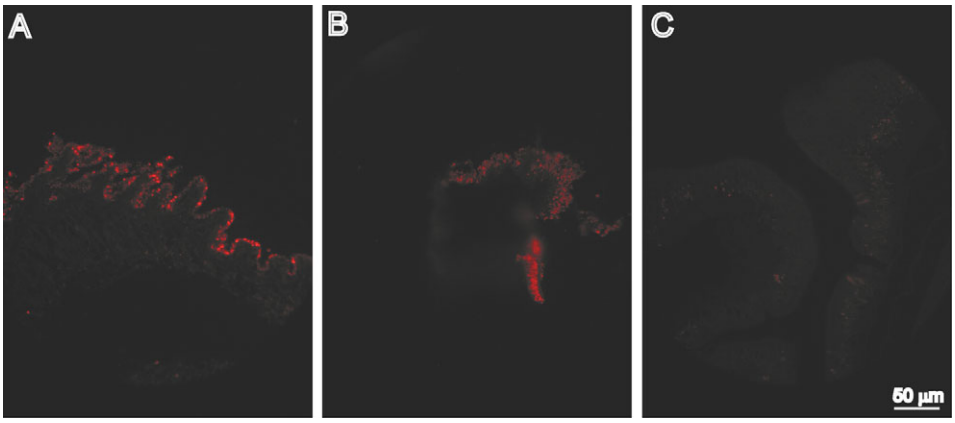


Fig. 2. Images showing Texas Red labelled anti-BrdU in tissue sections of (A) coelomic epithelium and (B) axial organ, indicating that cell proliferation occurred during the 4 h of BrdU incubation, as described in Materials and methods. (C) Corresponding tissue section of pyloric caeca (negative control).

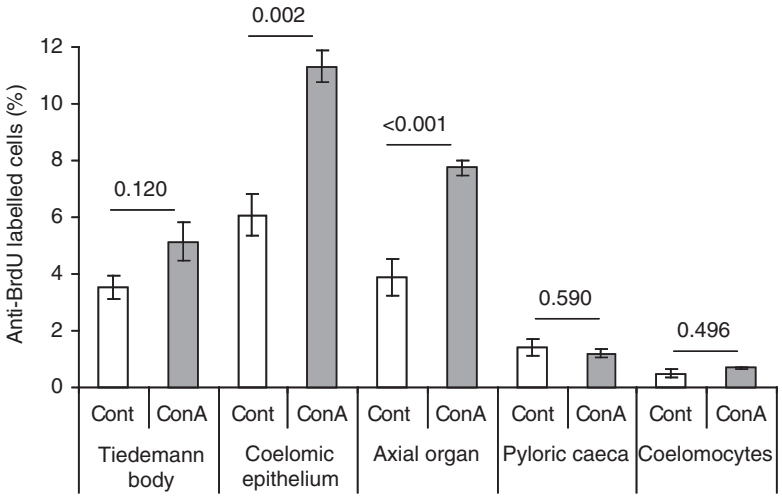
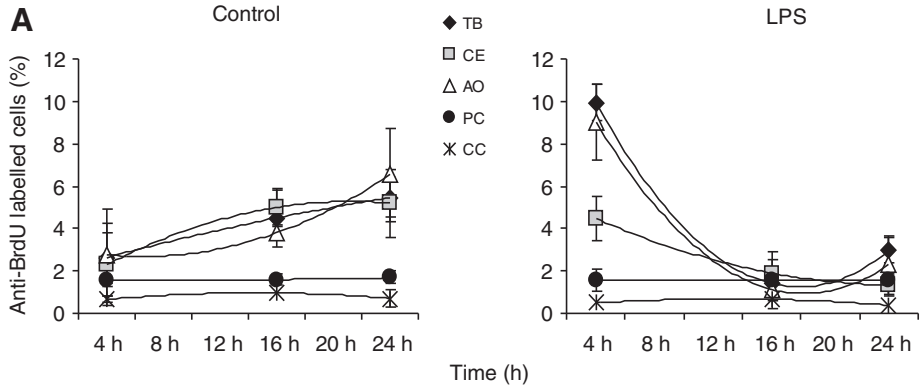


Fig. 3. The percentage of cells (± s.e.m.) from cell monolayers of Tiedemann body, coelomic epithelium, axial organ, pyloric caeca and coelomocytes that show FITC-labelled anti-BrdU 4 h post injection of either CMFSS buffer (Cont) or ConA (*N*=6). The numbers of labelled cells indicate the rate of cell division during 3 h of BrdU incubation, as described in Materials and methods. *P*-values show significant increases of proliferation in coelomic epithelium and axial organ compared with controls.

it was obvious that coelomic epithelium, axial organ and Tiedemann body showed high similarity to coelomocytes while the protein composition of the pyloric caeca was considerably different. Together with the observation that these cells were less adhesive to glass slides, it further supports the assumption that the pyloric caeca is a non-haematopoietic organ.

When challenged with LPS, the numbers of circulating coelomocytes were significantly elevated. Within four hours, the cell numbers were almost threefold those of the control group and remained at this high level after 24 h. Coteur at al. found that the total coelomocyte counts of *A. rubens* also showed a rather fast response to the Gram-positive bacteria *Micrococcus luteus* (Coteur at al., 2002). With the bacterial dose they used, the cell numbers increased threefold within 12 h. The time lapse between proliferation and release of differentiated coelomocytes is not known and we can only speculate that the high numbers also found after 24 h could be the result of the increased proliferation already shown after 4 h. Obviously, the rapid recruitment in the present study could not be explained by self-replication of coelomocytes, as indicated by the low proliferation of these cells in response to LPS and ConA. Circulating coelomocytes might be required from sources in the tissue such as from lymphoid-like organs, which does not contradict delivery by HPTs. The axial organ has been described as such a source that rapidly releases cells into the circulatory system in response to injury (Millott, 1969). However, the enhancement of cell proliferation that was observed in the axial organ, coelomic epithelium and Tiedemann body in response to mitotic compounds suggests that these organs are not only storage sites but also sites of renewal of coelomocytes. In, for example, the haematopoietic tissue of crustaceans, it has been described that the distribution of the different stages of haemocyte development is not homogenous (Chaga et al., 1995; Johansson et al., 2000). Cells are found densely packed in lobules, where the non-differentiated cells are located only in the apical parts. Here, the proliferation is more pronounced than among the more differentiated cells located in a more distal position of the HPT. Such delivery sites of HPTs may explain the rapid enhancement in circulating coelomocyte numbers of *A. rubens*, but more detailed studies are needed to prove this.



B

	Tiedemann body		Coelomic epithelium		Axial organ		Pyloric caeca		Coelomocyte	
	Diff. of mean	*P*	Diff. of mean	*P*	Diff. of mean	*P*	Diff. of mean	*P*	Diff. of mean	*P*
LPS 4 h *vs* Cont 4 h	7.31	<0.001	2.19	0.069	6.25	<0.01	0.63	0.8	0.16	0.33
LPS 4 h *vs* LPS 24 h	–7.0	<0.001	–3.14	<0.001	–6.75	0.001	0.04	0.8	0.11	0.58
Cont 4 h *vs* Cont 24 h	2.81	0.055	2.92	<0.01	3.76	<0.01	0.78	0.4	0.06	0.7

Fig. 4 (A) The percentage of cells (± s.e.m.) from cell monolayers of Tiedemann body (TB), coelomic epithelium (CE), axial organ (AO), pyloric caeca (PC) and coelomocytes (CC) that show FITC-labelled anti-BrdU during a time course (4, 16 and 24 h) post-injection of LPS or CMFSS buffer (*N*=4). The numbers of labelled cells indicate the rate of cell division during 3 h of BrdU incubation, as described in Materials and methods. Proliferation in pyloric caeca and coelomocytes was not affected, but in the Tiedemann body, coelomic epithelium and axial organ, proliferation had already increased at 4 h. The corresponding organs of the animals pre-treated with CMFSS (control) also showed an increase in proliferation but the response came later. (B) Table presenting the differences between the mean values of proliferation and the *P*-values when comparing the initial proliferation of the different organs of LPS-treated and Control animals and between the results obtained at 4 and 24 h p.i.

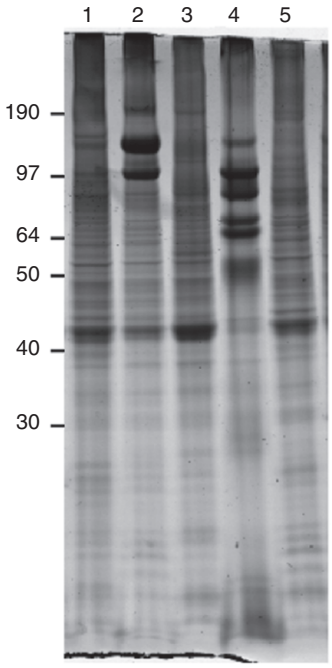


Fig. 5. A comparison of the protein composition between different organs, revealed by gel electrophoretic separation. Proteins were extracted from coelomic epithelia (lane 1), axial organ (lane 2), coelomocytes (lane 3), pyloric caeca (lane 4) and Tiedemann body (lane 5). Note the similar protein composition pattern between coelomocytes, coelomic epithelia and Tiedemann body; the axial organ showed a similar pattern but a higher density of some protein bands. Pyloric caeca showed the most different protein pattern compared with coelomocytes. Protein molecular masses, as approximated from markers, are shown in kDa on the left.

The coelomic epithelium, which covers the dorsal part of the entire coelomic cavity, showed a somewhat lower proliferative activity in response to LPS compared with that in the Tiedemann body and in the axial organ, but, given its large size, its contribution should be significant. It might also be that the maximal proliferation rate of the coelomic epithelium had already been passed at the first sampling occasion. Previous studies have shown that the proliferation rate of cells released from HPT of the Norway lobster, *Nephrops norvegicus*, was approximately 10% when determined in lobsters not exposed to any pre-challenge (Hernroth et al., 2004). In the tissues of *A. rubens* examined, the corresponding rate was no more than approximately 1.6%, and only after mitogenic stimuli was a level similar to the basic level of lobsters attained. The HPT of lobsters has been described as restricted to the dorsal membrane that covers the stomach (Chaga et al., 1995). Thus, the multiple sources of coelomocytes suggested in *A. rubens* and the relatively large area of coelomic epithelium might explain the observed difference.

The pattern recognition proteins of invertebrates are able to bind specific but widespread types of molecules (Medzhitov and Janeway, 1997). Proteins of the family known as the C-type lectins are implicated as the main players in carbohydrate recognition. These include LPS-binding proteins, peptidoglycan-binding protein and β-1,3-glucan recognition protein, which are considered to be constitutively expressed as free-floating molecules or as membrane receptors (Nappi and Ottaviani, 2000). When non-self molecules are recognized, the cellular responses are mediated by phagocytic coelomocytes, which, besides phagocytic activities (Santiago-Cardona et al., 2003), also generate increased levels of cytotoxic compounds and cytokine-like molecules, as described in the sea urchin *Arabica punctulata* (Lin et al., 2001). These humoral compounds give rise to proteolytic cascades that initiate opsonisation, agglutination, melanisation and the activation of antimicrobial peptides (Ratcliffe et al., 1984; Beck et al., 1993) as well as proliferation of haematopoietic cells (van de Braak et al., 2002). In the present study, the challenge with ConA gave a smaller increase in coelomocyte counts compared with stimulation by LPS. However, there was enhanced cell proliferation in response to ConA, observed when examining both anti-BrdU labelling on tissue sections and cell monolayers of the presumed HPTs, which classifies ConA as mitogenic.

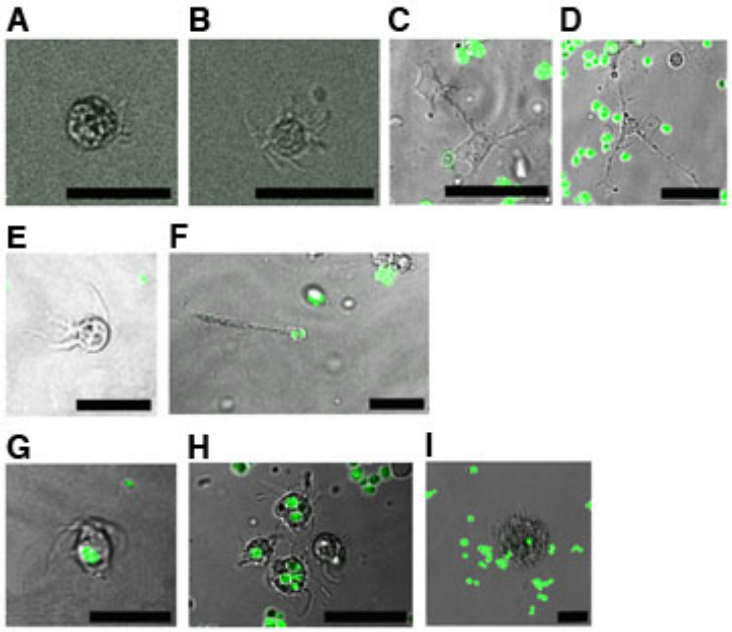


Fig. 6. Confocal microscope pictures showing coelomocytes and cells from coelomic epithelium explants of *A. rubens* and FITC-labelled yeast (green). (A–D) Different shapes of cells released by coelomic epithelium explant. (E,F) Different shapes of phagocytes in the coelomic fluid. Cells in B show high similarity to the phagocytes shown in E, and D shows high similarity to F. (G,H) Evidence of phagocytosis (G shows cells from the explant; H shows a cell from coelomic fluid). (I) Cluster of phagocytes at high density. Scale bars, 25 μm. (Supplementary material includes movies on phagocytic behaviour of cells from coelomocytes and from coelomic epithelium.)

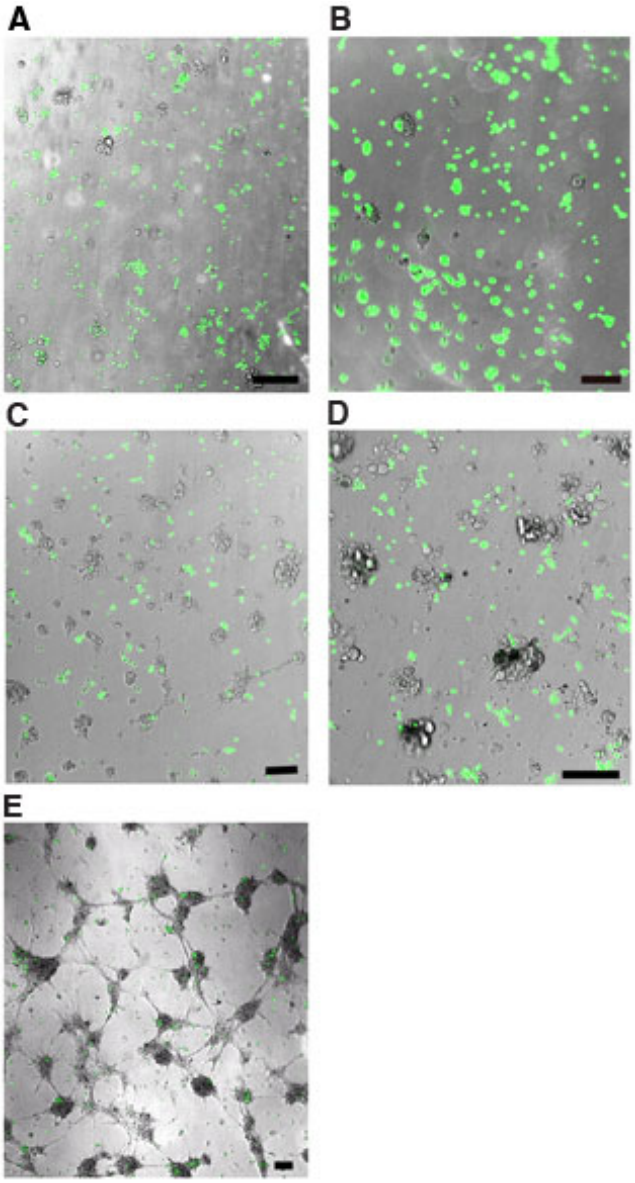


Fig. 7. Confocal microscope pictures of coelomocytes from coelomic fluid and cells from coelomic epithelium explants of *A. rubens* taken after 30 min incubation with FITC-labelled yeast (green). (A,C,E) Coelomocytes in different dilutions in filtered seawater (FSW) (A, low; C, medium; E, high density). High density is the cell density of undiluted coelomic fluid. (B,D) Cells from coelomic epithelium (explant in FSW). In A and B there is no clear network formation; in C and D there is evidence of network formation; in E there is clear network formation. Scale bars, 50 μm.

Previously, it has been recorded, both by measuring [^{3}H]thymidine uptake and by flow cytometric analysis able to detect the cell cycle phases S, G2 and M, that LPS induces proliferation of circulating haemocytes in the shrimp *Penaeus japonicus* (Sequeria et al., 1996). Certainly, the ability for self-replication of circulating haemocytes/coelomocytes differs between species. In agreement with our data for *A. rubens*, the haemocytes of the black tiger shrimp, *Penaeus mondon*, scarcely divided in response to LPS (Van de Braak et al., 2002). Although we cannot completely exclude the possibility of self-replication of the circulating coelomocytes of *A. rubens*, we can conclude that such a phenomenon seems of minor significance in comparison with what was recorded in the putative HPTs. Furthermore, the cell viability assay quantifies the increase in mitochondrial dehydrogenase activity due to blastogenesis and has been generally accepted to measure cell viability and proliferation (Mosmann, 1983; Roehem et al., 1991). In agreement with a study on circulating haemocytes of the blue mussel, *Mytilus edulis* (Hernroth, 2003), LPS did not affect the formazan production of circulating coelomocytes of *A. rubens*, indicating that it did not induce proliferation of these cells. It was noteworthy that neither the endotoxin LPS nor the plant lectin ConA showed cytotoxicity at these concentrations.

The balance between self-renewal and differentiation of stem cells is essential to maintain homeostasis, and loss of this balance may lead to uncontrolled cell growth or pre-maturation (Zhang and Li, 2005). Already after 16 h, the proliferation rate had returned to the basal levels of <2% (present study). Not only did the non-self molecules LPS and ConA effect cell proliferation in the putative HPTs, when sea stars were injected with the buffer solution this gave, in agreement with the study on the black tiger shrimp (Van de Braak et al., 2002), a lower and slower response compared with that of LPS, but still a notable increase of approximately 4% in the putative HPT organs after 24 h. However, neither in the pyloric caeca nor in coelomocytes were there such responses to the buffer solution.

In the absence of appropriate stem cell markers, high-resolution microscopy was performed to compare the morphology and behaviour of circulating coelomocytes and cells released from explants of coelomic epithelium. Evidently, many of the cells migrating from the coelomic epithelium showed morphological similarities to the phagocytes of the coelomic fluid. Similar to the phagocytes, the released cells were able to form petaloid and filopodial extendings that rapidly could be withdrawn or rearranged. The cells attached to the glass and, when encountering yeast cells, engulfment was observed. At an appropriate density, the cells were also able to form net constructions to trap the yeast, in the same way as observed for the circulating coelomocytes. In a published review (Muñoz-Chápuli et al., 2005), it was described that when carbon particles were injected into the coelom of adult sea stars, a delamination process of the mesothelial cells formed phagocytic cells (Bossche and Jangoux, 1976). Likewise, our results indicate that the coelomic epithelium constitutes a site for renewal of phagocytes. However, cells were seemingly released without the delaminating process.

It can be concluded that the non-self molecules induced proliferation of cells in the coelomic epithelium and increased the numbers of circulating coelomocytes. The rapid response in cell numbers indicates that coelomocytes could be released from storage in lymphoid-like organs but also that HPTs deliver differentiated cells, as indicated by the release of phagocyte-like cells from the epithelium explants. At a similar density, cells released from coelomic epithelium and free coelomocytes act in the same way, and both were able to phagocytose yeast cells. The microscopic method that was used seems promising and raises the possibility of extended studies, including explants from the axial organ and Tiedemann body. The increase in numbers of circulating coelomocytes in response to non-self molecules was certainly reflected in the proliferative responsiveness also in these organs, which reinforces the presumption of them being progenitor tissues for circulating coelomocytes.

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