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TECHNICAL REPORT

Immune-neuroendocrine biology of invertebrates: a collection of methods

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Abstract

In the last decade there has been a considerable increase of interest towards the elucidation of several aspects of invertebrate biology, including immunity and neuroendocrinology. However, due to the difficulties connected to the great variety of morphology and adaptations displayed by invertebrates, and also in consideration of the number of techniques that are applied in the various laboratories, research on invertebrates still suffers from hampering that have been substantially overcome in vertebrate models, especially in mammals. The aim of this Technical Report is to provide the reader a useful list of well-established morphological and morpho-functional protocols in order to facilitate the design and make more homogeneous the realization of experiments in the field of invertebrate immune-neuroendocrinology.

Key words: morphology; laboratory techniques; immunity; neuroendocrinology**Contents**

- Introduction
- Collection of the hemolymph/immunocytes
- Morphological methods
- Morpho-functional methods
- Quantitative methods
- Concluding remarks
- References

Introduction

In the last decade the literature in the field of the invertebrate immune-neuroendocrine biology has been widely increasing and important contributions have allowed a better understanding of the mechanisms and the strategies of defenses set up from the species of different taxa. However, many

problems remain unsolved in the field of comparative invertebrate immunology, such as the classification of circulating cells (Ribeiro and Bréhelin, 2006), the evolution of soluble mediators as the cytokines (Gerber *et al.*, 2007; Malagoli and Ottaviani, 2007), the presence of memory (Bréhelin and Roch, 2008) and the characterization of immune-related stem cells (Bachère *et al.*, 2004; Söderhall *et al.*, 2005) among others. In the insect *Drosophila melanogaster* (Lemaitre and Hoffmann, 2007) and the nematode *Caenorhabditis elegans* (Alper *et al.*, 2008) several steps have been taken towards a better comprehension of the molecular mechanisms of immune response, but comparative immunologists well know that two organisms, even if carefully and deeply characterized, cannot be considered as the paradigmatic representation of the million of species widespread along the protostomian and deuterostomian lineages of invertebrates.

Among comparative immunologists, it is commonly accepted that the difficulties in extending the results obtained in a given invertebrate species to others invertebrate taxa are not only the direct

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consequence of the intrinsic difficulties connected to the great variety of morphology and adaptations displayed, but also to the variety of techniques that are applied and that often lead to different results in the various laboratories (Hooper *et al.*, 2007). Indeed, the majority of the utilized techniques derive from those developed for investigations in vertebrate models and laboratories moving their first steps in the field of comparative immunology may encounter problems in adapting available protocols to their models. Remarkably, molecular biology approaches are giving an important contribution in supporting/clarifying the data collected by mean of morphological and functional investigations. Notwithstanding that, morphological and functional assays obviously remain the first-choice approaches to collect those evidences that could eventually direct future molecular biology-based experiments.

In this Technical Report we collected several well-established morphological and morpho-functional protocols used in the field of invertebrate immune-neuroendocrinology. This report has not the ambition to represent a complete guide for all the possible experiments that can be performed with invertebrate models. Our aim is rather to provide the reader a useful guide in order to facilitate and unify the design of future studies. Besides this, we also hope to offer a brief collection of methods that should be applied when studying invertebrate immune-neuroendocrinology. In particular, we will refer to protostomian and deuterostomian invertebrates, such as Mollusca (*Planorbarius corneus*, *Viviparus ater*, *Lymnaea stagnalis*, *Mytilus galloprovincialis* and *Mytilus edulis*), Annelida (*Eisenia foetida* and *Hirudo medicinalis*), Crustacea (*Astacus leptodactylus*, *Homarus americanus*, *Neprops norvegicus*, *Munida rugosa*, *Paguristes oculatus*, *Palaemon elegans* and *Squilla mantis*), Insecta (*Calliphora vomitoria* and *Galleria mellonella*, cell lines from *Estigmene acrea*, *Lymantria dispar* and *Mamestra brassicae*) and Tunicata (*Botryllus schlosseri*, *Ciona intestinalis*, *Phallusia mamillata*, *Styela plicata*).

Collection of the hemolymph

Mollusca

In gastropods the hemolymph is obtained by exerting slight pressure on the animal's foot and collected by a Pasteur pipette. In bivalves, e.g., *Mytilus spp.* the classical protocols indicate that hemolymph can be taken from the posterior adductor muscle using a 2 ml syringe (Ottaviani, 1983; Ottaviani *et al.*, 1997b). Recently, we have also proposed a protocol for *M. galloprovincialis* in which the hemolymph is collected from mussels directly by inserting a sterile syringe between the valves, just beneath the exit-point of the byssus. After insertion, hemolymph can be obtained by exerting a gentle aspiration. This method is useful when large amount of fluid is needed, but it is of extreme importance to check for the quality of the hemolymph immediately after the withdrawal, in order to ensure that neither the gonads nor the digestive gland have been touched or damaged by the needle (Malagoli *et al.*, 2007).

Annelida

H. medicinalis immune cells, which are entrapped in a thick connective tissue, can be isolated and cultured utilizing the injection of matrigel matrix gel (MG) supplemented with different growth factor/cytokines selected among those that play a major role in invertebrate/vertebrate wound healing (Grimaldi *et al.*, 2008). MG is an extract of the murine Engelbreth-Holm-Swarm (EHS) tumor grown in C57/b16 mice and produced as described by Kleinman (1986). It is rich in basement membrane components (laminin, collagen IV, nidogen and perlecan) and it is a thermo-sensitive material liquid at 4 °C which polymerizes when warmed to room temperature (RT). Leeches are injected with 300 µl of MG added either with 50 ng of monocyte chemoattractant protein-1 (MCP-1/CCL2, Pepro Tech, London, UK), with a pivotal role in the recruitment of monocytes and macrophages, or with 50 ng of vascular endothelial growth factor (VEGF, Pepro Tech), playing a pivotal role in the recruitment of hematopoietic and endothelial precursor cells. For *in vivo* analysis, anesthetized leeches are dissected and polymerized matrigel pellets are removed and histologically, ultrastructurally examined (Grimaldi *et al.*, 2008). For *in vitro* analysis, each matrigel pellet is minced in small pieces using sterilized razor blades and plated in wells of 60 µm in diameter in Dulbecco's modified Eagle's medium (DMEM, Celbio, Milan, Italy) modified by dilution (1:4) to reach iso-osmolarity and supplemented with 1 % glutamine and 10 % fetal bovine serum (Grimaldi *et al.*, 2008).

The coelomocyte collection from the earthworm *E. foetida* is performed putting the animals in a sterile plastic Petri dish in cold phosphate buffered saline (PBS-1) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 10 mM NaH₂PO₄, pH 7.3) diluted 1:1 with ethanol. After a few sec, the earthworms extrude coelomocytes vigorous through their dorsal pores. Then the coelomocytes are transferred to plastic tubes and washed by centrifugation in PBS-1 without alcohol at 500xg for 5 min (Cooper *et al.*, 1995).

Crustacea

Hemolymph sample is taken from the pericardic sinus of shrimps (*P. elegans*), hermit crabs (*P. oculatus*), lobster (*H. americanus*), Norway lobster (*N. norvegicus*), squat lobster (*M. rugosa*) crayfish (*A. leptodactylus*) and mantis shrimps (*S. mantis*). Blood samples are taken from the coxal sinus of crab. In the smallest species 50 µl of hemolymph is collected using a sterile 1 ml syringe fitted with a 25G needle, in the biggest animals up to 1 ml of hemolymph is obtained, depending on of the size of specimens (Lorenzon *et al.*, 1999, 2007, 2008; Giulianini *et al.*, 2007).

Insecta

In the fly *C. vomitoria* the hemolymph is taken from a small incision in the ptilinum at the front of the head. By softly squeezing of the abdomen and thorax, a drop of hemolymph is forced out of the incision and collected with a pipette (Franchini *et al.*, 1996). The hemolymph in the *G. mellonella* larvae is collected by piercing with a small needle in one of the first prolegs (Wittwer *et al.*, 1999).

Tunicata

In solitary ascidians the tunic is cleaned from epiphytes and sterilized with ethyl alcohol. In *S. plicata* incurrent siphon is incised and the exuding hemolymph is collected, while in *C. intestinalis* a syringe is inserted directly into the hearth and the hemolymph is collected. In both cases, hemolymph is put in sterile tubes containing a 5-fold excess of calcium/magnesium-free artificial sea water (ASW) (9 mM KCl; 0.15 M NaCl; 29 mM Na₂SO₄, NaHCO₃, pH 7.4) with 10 mM EDTA (ASW-EDTA) as anticoagulant (1:9 medium/hemolymph ratio), in ice. After centrifuging at 400xg for 10 min at 4 °C, the hemocytes are washed three times in sterile ASW-EDTA.

In the colonial ascidian *B. schlosseri*, blood is collected with a glass micropipette after puncturing, with a fine tungsten needle, the tunic marginal vessel of colonies previously rinsed in 0.38 % Na-citrate or 10 mM L-cysteine in filtered seawater (FSW), pH 7.5, to prevent hemocyte clotting. It is then centrifuged at 780xg for 10 min and the pellet is re-suspended in FSW at the final concentration of 6-8x10⁶ cells/ml. The cell concentration is evaluated with a Burker's hemocytometer. Sixty µl of hemocyte suspension are placed in the centre of culture chambers, made by gluing Teflon rings (15 mm internal diameter, 1 mm thick) on siliconized glass slides (Fig. 1a). Washed coverslips are laid over the Teflon rings, smeared with vaseline and gently pressed down to touch the drop of the cell suspension (Ballarin *et al.*, 1994). The culture slides

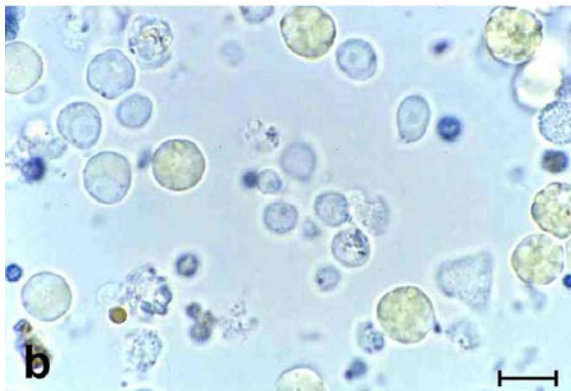
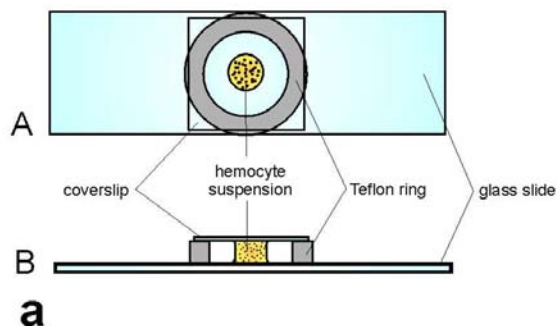


Fig. 1 Hemocyte culture. **a)** schematic drawing of a culture chamber with a drop of cell suspension in the center **A:** upper view; **B:** side view. **b)** living *B. schlosseri* hemocytes adherent to coverslips. Bar = 15 µm.

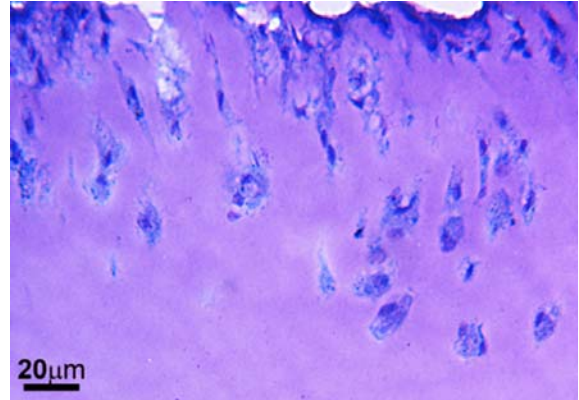


Fig. 2 Cryosection of matrigel supplemented with VEGF. The MG sponge is infiltrated by ovoidal and agranular hematopoietic precursor cells stained with May-Grünwald and Giemsa solution.

are kept upside-down for 30 min at RT to allow the cells to settle and adhere to the coverslips (Fig. 1b).

Morphological methods

Optical and electron microscopy-based methods for characterization of circulating hemocytes (immunocytes)

Mollusca

In freshwater snails and marine bivalves, the immunocytes are obtained in hemolymph drops on glass or by cytocentrifugation (Cytospin II, Shandon Instruments, UK) of hemolymph samples onto a slide at 500-1000 rpm for 5-10 min. They are observed by phase-contrast microscope and stained with or without fixation for morphological observations (Ottaviani, 1983, 1989; Ottaviani *et al.*, 1998b).

Annelida

May-Grünwald and Giemsa differential staining (Bio-Optica, Milan, Italy) and the vital dye acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indo-carbocyanine perchlorate (Dil-Ac-LDL) (Biomedical Technologies Inc., MA, USA) are a quick method to characterize leech immunocytes (Grimaldi *et al.*, 2008).

May-Grünwald and Giemsa procedure

In *in vivo* experiments MG implant is removed from the animal, embedded in polyfreeze tissue freezing medium (Polysciences, Eppelheim, Germany) and immediately frozen in liquid nitrogen. Cryosections (7 µm), obtained with a cryotome, are immerse in 100 % May-Grünwald for 4 min and then directly transferred to 4 % Giemsa (diluted in tap water) for 4 min. Slides are then washed in tap water, air dried, mounted with Eukitt mounting medium (Electron Microscopy Science, Washington, PA, USA) and subsequently observed under a light microscope (Fig. 2). In *in vivo* experiments immunocytes of leech, extracted from MG and plated in wells, are allowed to air dry onto slides and then they are stained as described above (Fig. 3).

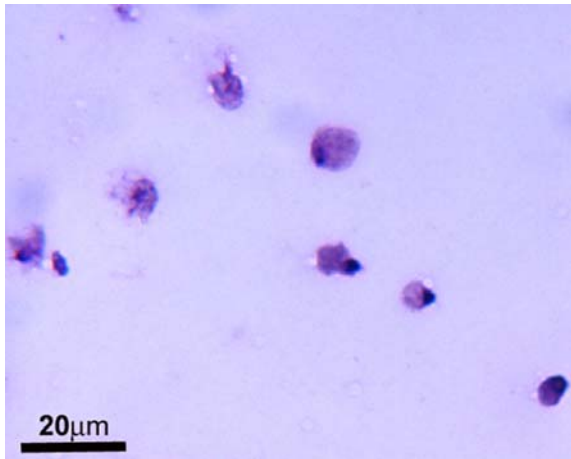


Fig. 3 May-Grünwald and Giemsa staining micrographs showing cultured hematopoietic precursor cells.

Dil-Ac-LDL procedure

In *in vivo* experiments, injection of 10 μ l of 10 μ g/ml Dil-Ac-LDL in PBS-2 buffer (138 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) is performed at the level of the 80th superficial metamere of the leech, where the MG is subsequently inoculated. After 1 week the MG implant is removed from the animal and quickly frozen. Cryosections are mounted in Citifluor (Citifluor Ltd, London, UK) and observed on fluorescence microscope through a rhodamine filter set (excitation/emission filters 550/580 nm) to visualize the Dil (Fig. 4). In *in vitro* experiments, leech immunocytes are cultured in DMEM containing 10 μ g/ml Dil-Ac-LDL according to the manufacturer's suggestions and Tamaki *et al.* (2002). After 4 h at RT, cells are washed several times with probe-free medium and directly observed using an inverted-fluorescence microscope (Fig. 5). Nuclei are colored with 4'-6-diamidino-2-phenylindole (DAPI) (Sigma, St Louis, MO, USA) (excitation/emission filters 410/460 nm).

Ultrastructural procedures

MG pellets (see above) are fixed for 2 h in 0.1 M cacodylate buffer pH 7.2, containing 2 % glutaraldehyde. Specimens are then washed in the same buffer and postfixed for 2 h with 1 % osmic acid in cacodylate buffer, pH 7.2. After standard serial ethanol dehydration, specimens are embedded in an Epon-Araldite 812 mixture. Semithin sections (750 nm) are obtained with an ultratome, stained by conventional methods (crystal violet and basic fuchsin) according to Moore *et al.* (1960), and observed under a light microscope. Thin sections (80 nm) are stained by uranyl acetate and lead citrate and observed with a transmission electron microscope (TEM) (Fig. 6).

Crustacea

To characterize the different classes of blood cells in *A. leptodactylus*, by means of light and electron microscopy, 200 μ l of hemolymph (about 2×10^5 hemocytes counted by using a Bürker's

hemocytometer) are drawn into a sterile plastic 1 ml syringe filled with an equal volume of fixative (2.5 % glutaraldehyde, 1 % paraformaldehyde, 7.5 % saturated picric acid solution in 0.1 M cacodylate buffer, pH 7.4) and, after a fixation for a minimum of 10 min, the hemocytes are pelleted in 1 ml of fixative by 10,300xg centrifugation for 10 min at 20 °C. The pellets obtained are then washed in 0.1 M cacodylate buffer pH 7.4 and post-fixed in 1 % osmium tetroxide in the same buffer, serially dehydrated in ethanol and embedded, via propylene oxide, in Embed812/Araldite (Electron Microscopy Sciences, Fort Washington, PA, USA) or, without post-fixation, they are serially dehydrated in ethanol and embedded in LR-White (Sigma). For light microscopy, sections (2 μ m thick) are collected on slides, baked for 5 min at 80 °C and stained with 0.5 % toluidine blue in 0.1 % carbonate solution at pH 11.1 at the same temperature. For TEM, silver/gold-coloured sections are stained with uranyl acetate and lead citrate and observed with a TEM Philips EM 208.

For light microscopy, selected areas were observed with an Olympus BX50 microscope, and images are acquired with an Olympus DP11 photo camera at a resolution of 1712x1368 pixels. For TEM, negative plates are digitized with an Epson Photo Perfection scanner at 1200 dpi (optical resolution) and saved as a Tagged Image Format file. All measurements, statistical analyses and photocomposition are performed with Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA) (Giulianini *et al.*, 2007).

Insecta

For the morphological studies, hemolymph from several *C. vomitoria* flies are pooled to a volume of 50-100 μ l and cytocentrifuged on a slide using a cytocentrifuge Cytospin II (Shandon Instruments) running at 500 rpm for 5 min. Subsequently the immunocytes are stained with May-Grünwald and Giemsa (Franchini *et al.*, 1996).

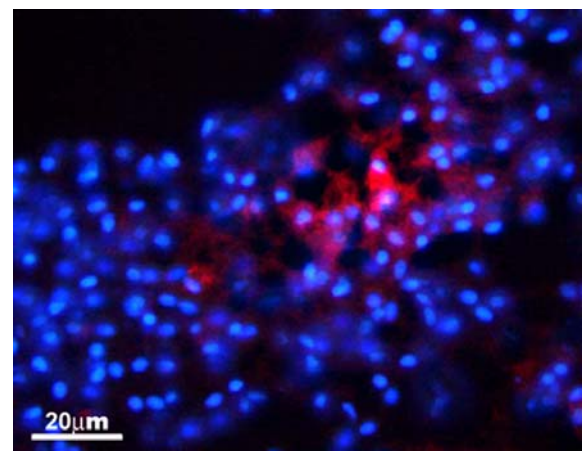


Fig. 4 Cryosection of matrigel supplemented with VEGF. The implanted MG is invaded by precursor cells and identified with Dil-Ac-LDL. Nuclei are stained with DAPI (in blue).

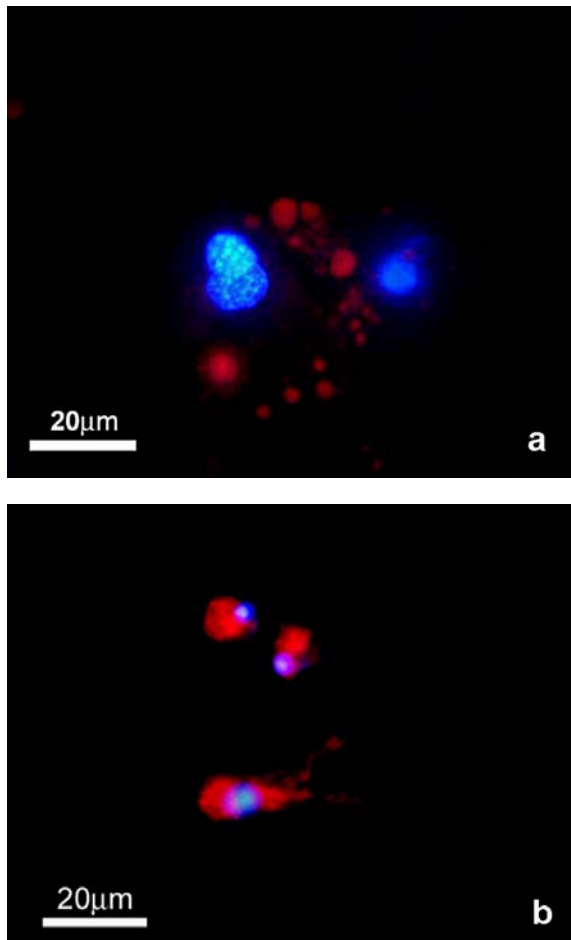


Fig. 5 Characterization of cultured immunocytes of leech by Dil-Ac-LDL uptake. In macrophages the fluorochrome-conjugated probe showed a spotted localization a), while a diffused cytoplasmic localization was evident in the hematopoietic precursor cells b). Nuclei are stained with DAPI (in blue).

Tunicata

In *B. schlosseri*, several morphological protocols have been set up for both fixed or living hemocytes. However, it is important to underline the possibility to obtain sub-populations from a pool of circulating hemocytes.

Blood harvested from large colonies (of about 1000 zooids) is centrifuged at 780xg for 10 min. Pelleted hemocytes are re-suspended in 1 ml of FSW (final concentration: 50×10^6 cells/ml) and layered over a Ficoll discontinuous gradient obtained by dissolving Ficoll 400 (Pharmacia, Canada) in FSW to final concentrations of 10, 14.5, 20 and 34 % (modified after Michibata *et al.*, 1987) and sequentially overlaying 2 ml of each solution into a 10 ml centrifuge tube. Tubes are centrifuged at 400xg for 10 min at 4 °C. Blood cell fractions are collected with a glass micropipette from the top of the tube, diluted in an equal volume of FSW, and centrifuged at 780xg for 10 min. Pelleted cells are re-suspended in 300 µl of FSW and the concentration of hemocytes is estimated with a Bürker's hemocytometer.

The fixation procedures for cytochemical staining of hemocytes (or hemocyte sub-population) is performed as follows. After adhesion of the hemocytes to the coverslips, the debris-containing FSW is discarded and cell monolayers are washed by dipping the coverslips (see above) repeatedly in a large volume (100 ml) of FSW. The best and simplest fixative mixture for cell morphology preservation is represented by a solution of 1 % glutaraldehyde (Fluka, Buchs SG, Switzerland) and 1 % saccharose in FSW at 4 °C for 30 min. Cells are then washed in PBS-3 (1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4 , 0.065 M Na_2HPO_4 , pH 7.2) or in the buffers used for the cytochemical assays reported below.

For *B. schlosseri* hemocyte identification and characterization, the following cytochemical staining methods can be used (Cima *et al.*, 2001; Ballarin and Cima, 2005):

Giemsa dye: Fixed hemocytes adhering to coverslips are stained for 10 min with a 10 % Giemsa (Fluka) aqueous solution and then washed in distilled water and mounted in Acquovitrex (Carlo Erba, Milan, Italy) on glass slides. With this dye, the nucleus appears blue and cytoplasm light blue or violet, due to metachromasia, under the light microscope.

Pappenheim's panoptical stain: Fixed hemocytes are stained for 3 min in May-Grünwald's dye (Fluka). After washing in distilled water they are stained for 10 min with 5 % Giemsa, washed again in distilled water and mounted in Acquovitrex (C. Erba) (Mazzi, 1977; Bancroft and Gamble, 2002). With this technique, basophilic granules appear blue, neutrophils brown and acidophils dark pink (Fig. 7a).

Ehrlich's triacid mixture: This mixture is composed of 12 parts of saturated Orange G aqueous solution, 8 parts of saturated acid fuchsin aqueous solution, 10 parts of saturated methyl green aqueous solution, 30 parts of distilled water, 18 parts of absolute ethanol and 5 parts of glycerin. Fixed hemocytes are stained with this mixture for 15 min, washed in distilled water and mounted. Basophilic granules appear light green, neutrophils violet and acidophils coppery red (Mazzi, 1977; Bancroft and Gamble, 2002).

Toluidine blue staining: Fixed hemocytes are stained for 10 s in a filtered aqueous solution of 0.5 % toluidine blue (Fluka) and 0.5 % sodium tetraborate, to reveal the presence of mucosubstances and glucosaminoglycans which show pink-violet metachromasia.

Neutral red dye: After adhesion of hemocytes to coverslips, the FSW of the culture chambers is substituted with 60 µl of 8 mg/ml neutral red solution (Merck, Darmstadt, Germany) in FSW. Living hemocytes are directly observed. This dye specifically stains acid compartments (e.g., lysosomes or acid vacuolar contents) of living cells (Mazzi, 1977; Bancroft and Gamble, 2002).

Sudan black for lipids: After adhesion of hemocytes, coverslips are dipped in 70 % ethanol for 30 s and stained with a saturated solution of Sudan black (Sigma) in 70 % ethanol for 15 min at 70 °C. Cells are then rinsed in 70 % ethanol and washed in distilled water (Mazzi, 1977; Bancroft and Gamble, 2002). Black spots reveal the presence of lipids (Fig. 7b).

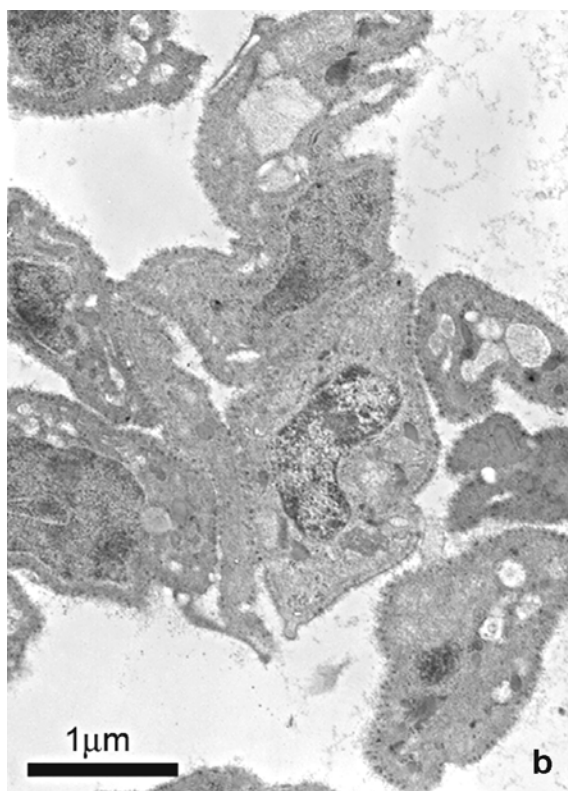
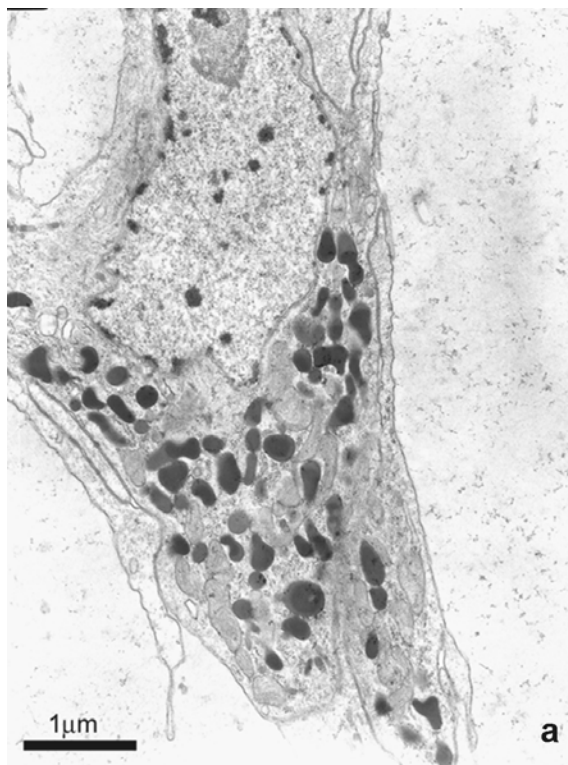


Fig. 6 TEM micrographs. Ultrastructural analysis of matrigel added with different cytokines. Monocyte chemoattractant protein-1 (MCP-1/CCL2) **a**) selectively recruit cells characterized by cytoplasm filled with granules of diverse sizes while vascular endothelial growth factor (VEGF) **(b)** recruits cells characterized by large nuclei with a scarce cytoplasm occupied by organelles and few small granules.

Periodic acid Schiff (PAS) reaction for polysaccharides: Fixed hemocytes are incubated in 1 % periodic acid for 10 min, rinsed in tap water and stained with Schiff's reagent for 30 min at 37 °C. Coverslips are then dipped in a solution of 0.6 % sodium metabisulfite in 0.02 M HCl for 6 min, washed in tap water for 10 min, and then rinsed in distilled water (Mazzi, 1977; Bancroft and Gamble, 2002). Positive sites appear primary red (Fig. 7c).

Quinones: Living hemocytes are incubated in a 2 mM solution of 3-methyl-2-benzothiazolinone hydrazone chloride (MBTH) in FSW, containing 0.4 % dimethylformamide for 5 min, in the presence or in the absence (controls) of the PO inhibitor 10 mM Na-benzoate (Winder and Harris, 1991; Ballarin *et al.*, 1995). Positivity is revealed by a marked red color (Fig. 7d).

DOPA-containing proteins/quinoproteins: After the adhesion to coverslips, hemocytes are fixed as described above, washed in PBS-3 and incubated with a solution of 0.24 mM nitroblue tetrazolium (NB) (Sigma) in 2 M potassium glycinate buffer (150 g/l glycine and KOH 2 N to pH 10.0) and 20 mM sodium benzoate (Flückiger *et al.*, 1995). Cells are then washed in PBS-3 and coverslips mounted with Acquovitrex (C. Erba). Positive sites feature a dark blue color (Fig. 7e).

Lectin cytochemistry

Fixed hemocytes are incubated for 30 min in PBS-3 containing 5 % powdered milk, washed three times for 10 min in PBS-3 and incubated for 60 min in a 50 µg/ml lectin solution in PBS-3 containing 0.1 M CaCl₂. Both FITC-labeled and biotin-conjugated lectins can be used, e.g., *Ulex europaeus* agglutinin-I (UEA-I, specific for L-fucose), *Datura stramonium* lectin (DSL, specific for N-acetyl-β-D-glucosamine and N-acetyl-lactosamine), *Ricinus communis* agglutinin-I (RCA, recognizing β-D-galactosides), wheat germ agglutinin (WGA, specific for N-acetyl-β-D-glucosamine), *Helix pomatia* agglutinin (HPA, recognizing N-acetyl-α-D-galactosamine), *Arachis hypogaea* agglutinin (PNA, specific for galactosyl (β-1,3) N-acetyl-galactosamine), concanavalin A (ConA, recognising α-D-glucopyranosides and α-D-mannopyranosides), *Vicia villosa* agglutinin (VVA, specific for N-acetyl-D-galactosamine), *Narcissus pseudonarcissus* agglutinin (NPA, specific for α-D-mannosyl carbohydrate residues). Incubation with FITC-labeled lectins is followed by extensive washing in PBS-3, and coverslips are mounted with Fluorsave (Calbiochem, Darmstadt, Germany) on glass slides, and observed at a magnification of 1,250x with a fluorescence light microscope equipped with a filter block for FITC excitation (450-490 nm; emission at 525 nm). Conversely, after incubation with biotin-conjugated lectins, hemocytes are washed in PBS-3, incubated for 30 min in avidin-biotin-peroxidase complex (ABC) (Vector Lab., Burlingame, CA, USA) in PBS-3, washed in PBS-3, incubated for 30 min in 0.5 mg/ml 3-3' diaminobenzidine (DAB) (Sigma) in PBS-3 containing 0.04 % H₂O₂, mounted in Acquovitrex (C. Erba) and observed under microscope.

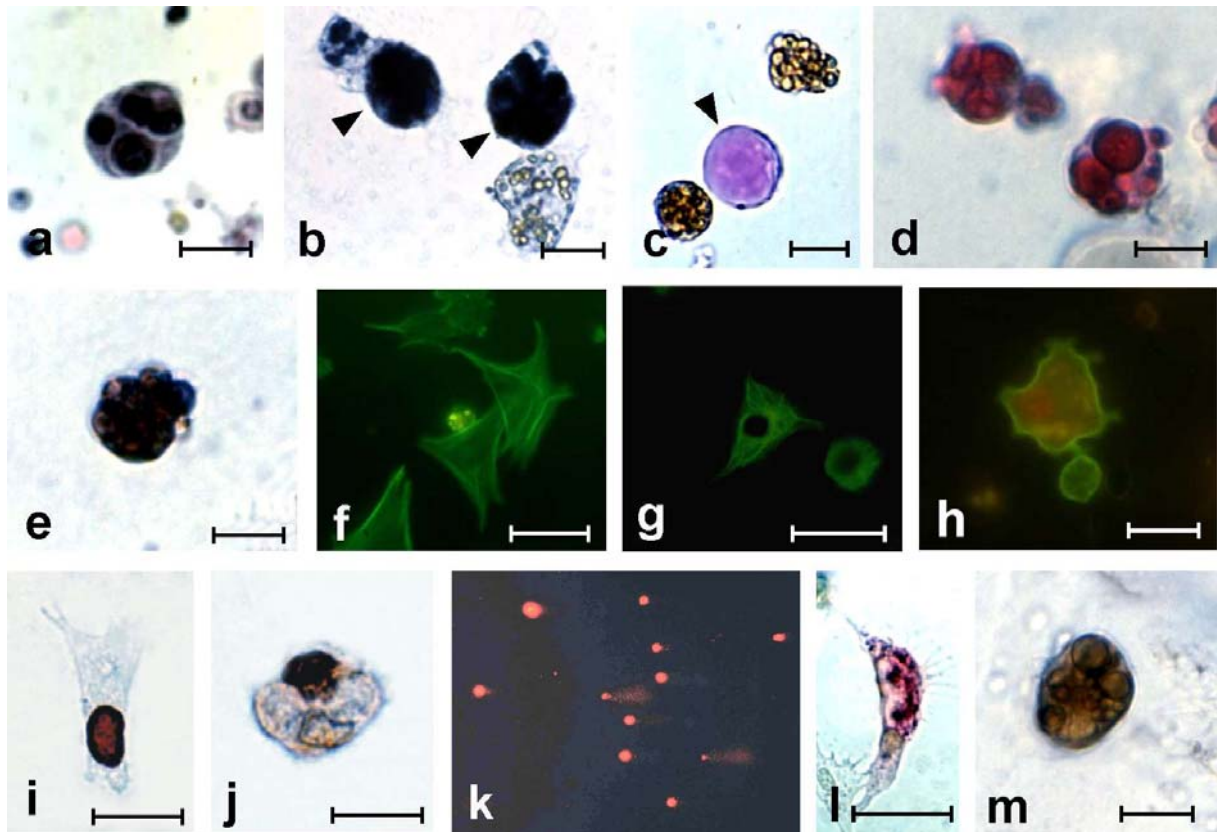


Fig. 7 *B. schlosseri* hemocytes. **a**: fixed macrophage-like cells (MLC) after Pappenheim's panoptical stain; **b**: living MLCs (arrowheads) stained with Sudan black for lipids; **c**: fixed MLC positive for PAS reaction; **d**: living morula cells treated with MBTH to reveal quinones; **e**: fixed morula cell showing positivity at the reaction for DOPA-containing proteins; **f**: actin cytoskeleton of fixed spreading phagocytes revealed with FITC-labeled phalloidin; **g**: tubulin cytoskeleton of fixed spreading phagocytes revealed, in immunofluorescence, with anti-tubulin antibodies; **h**: senescent living phagocyte with bleb after treatment with fluorescent Annexin-V; **i**, **j**: fixed hyaline amoebocytes (spreading phagocyte; **i**) and MLC (**j**) showing TUNEL positivity for DNA fragmentation; **k**: COMET assay showing DNA fragmentation in nuclei of senescent hemocytes; **l**: fixed hyaline amoebocyte showing positivity for β -glucuronidase; **m**: fixed morula cell positive for phenoloxidase. Bar = 10 μ m.

Assay for cytosolic Ca^{2+}

Sustained increases in cytosolic Ca^{2+} are revealed as dark-blue precipitates by Von Kossa's substitution method (Callis and Bone, 2002). Glutaraldehyde-fixed monolayers are immersed in 5 % silver nitrate and exposed to ultraviolet light for 5 min. Cells are then rinsed in distilled water, incubated for 2-4 min in a 5 % aqueous sodium thiosulfate solution, washed in distilled water, mounted in Acquivitrex (C. Erba), and observed under the light microscope.

TEM

Samples, represented by selected whole colonies or pellets of fixed hemocytes embedded in small pre-heated agar pieces, are fixed in a solution of 1.5 % glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, plus 1.6 % NaCl for 2 h at 4 °C. Specimens are then rinsed in cacodylate buffer containing 1.6 % NaCl, post-fixed in 1.5 % OsO_4 in cacodylate buffer, dehydrated and embedded in Epon. One- μ m-thick sections are stained with toluidine blue and observed with light microscope. Ultrathin sections, briefly stained with uranyl acetate and lead citrate, are examined under a TEM at 75 kV.

For enzymatic activity or antigen preservation, specimens are fixed by immersion in 4 % paraformaldehyde plus 0.1 % glutaraldehyde in saline buffer (SB) (0.2 M cacodylate buffer, pH 7.4, 1.7 % NaCl and 1 % saccharose) for 2 h at 4 °C, and then washed three times in SB without post-fixation in OsO_4 . In the case of detection of enzymatic activity, a pre-embedding method is usually employed (Cima *et al.*, 2002a). Specimens are pre-incubated in 0.1 % Triton-X in SB for 10 min and then incubated overnight at RT in the reaction mixtures reported in the "Cytoenzymatic assays" section. For each test, control specimens are prepared omitting the specific enzyme substrate. After incubation in the reaction and/or post-incubation mixture, specimens are post-fixed in 1 % OsO_4 in 0.2 M cacodylate buffer, pH 7.4, dehydrated, and embedded in Epon for sectioning. Ultrathin sections are treated as reported above.

In the case of immunocytochemistry, a post-embedding method is applied. Thin sections of fixed specimens, dehydrated and embedded in catalyzed LR White resin (Polysciences, Warrington, PA, USA) are collected on gold grids and processed for immunohistochemistry. After immersion in PBS-3

plus 10 % normal goat serum (Vector Lab., Burlingame, CA, USA) for 10 min at RT, the grids are incubated in the primary antibody diluted in PBS-3 according to the manufacturer's instructions overnight at 4 °C. They are then rinsed in PBS-3 and incubated in 10 nm gold-conjugated goat anti-rabbit IgG (British Biocell International, Cardiff, UK), diluted 1:50, 1:100 in PBS-3 for 1 h at RT. In controls, the primary antibody is omitted. Before observation with TEM, the grids are also stained with uranyl acetate and lead citrate (Fig. 8).

Scanning electron microscopy (SEM)

Living hemocytes are left to adhere to poly-L-lysine-coated coverslips. They are then fixed for 30 min at 4 °C in the same fixative mixture for TEM and post-fixed in 1 % OsO₄ in cacodylate buffer for 60 min. Dehydration through a graded ethanol series is followed by critical-point drying in liquid CO₂ with absolute acetone as transitional fluid. Cells are then sputtered with gold and observed with a SEM.

Immunocytochemical procedures Mollusca, annelida and insecta

Immunocytochemical assay is carried out on fixed or air-dried molluscan immunocytes in order to detect the presence of adrenocorticotropin hormone (ACTH) using a human ACTH polyclonal antibody (pAb). After hemolymph cyto centrifugation, the immunocytes are air-dried, immersed in absolute ethanol followed to passages in 95 % ethanol (3 min each). Endogenous peroxidase are inhibited by immersion in 0.3 % H₂O₂ in methanol for 15 min at RT, then the slides are immersed in running tap water for 10 min and rinsed in PBS-1 (3x5 min). Incubate in normal goat serum (1:5) (Vector Lab.) for 30 min at RT; incubate in primary rabbit anti-human ACTH (1-24) pAb (1:200) (Dakopatts, Denmark) overnight at 4 °C; wash in PBS-1 (3x10 min); incubate in secondary biotinylated anti-rabbit IgG (1:200) (Vector Lab.) for 30 min at RT; wash in PBS-1 (3x10 min); incubate in ABC (1:100) (Vector Lab.) in PBS-1 for 30 min at RT; wash in PBS-1 (3x10 min); incubate in a 0.025 % solution of DAB tetrahydrochloride (Sigma) in McIlvaine buffer at pH 5.5 containing 5 µl H₂O₂. Stop the reaction by washing in tap water for 5 min and then rinse in distilled water; counterstain nuclei with hematoxylin; dehydrate through a graded ethanol series: 70, 80, 95 and 100 % (3 min each); clear in xylene and mount in Eukitt (Bio-Optica, Milan, Italy). Controls of immunocytochemical reaction are performed using the same procedure omitting the primary antibody or by pre-absorbing the primary antibody overnight at 4 °C with the homologous antigen (in excess) (Ottaviani *et al.*, 1990).

The same immunocytochemical procedure is also applicable to the annelid *E. foetida* in studying the presence of cytokine- and POMC-derived peptide-like molecules in the coelomocytes as well as for the immunocytes of the insect *C. vomitoria* (Cooper *et al.*, 1995; Franchini *et al.*, 1996).

Crustacea

Immunocytochemistry of neuroendocrine organs

Immunocytochemistry is performed on thick and semithin sections of the eyestalk of the test species to validate the specificity and cross-reactivity

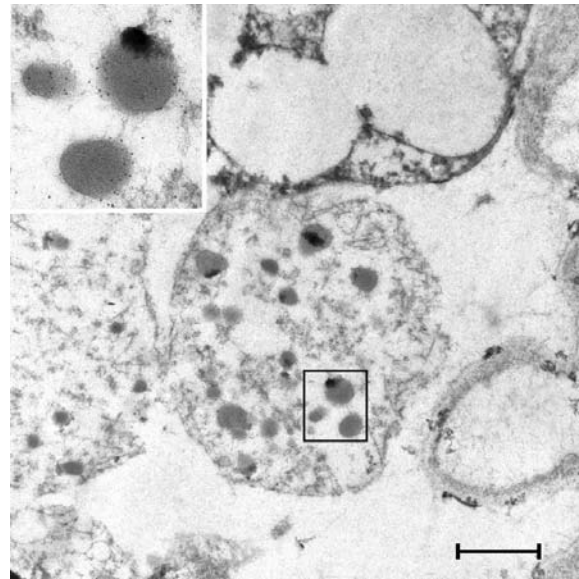


Fig. 8 Immunocytochemistry at TEM: phagocyte with granular content labeled with colloidal gold. Bar = 3 µm.

of the purified antibody anti-NenchH (anti-*N. norvegicus* crustacean Hyperglycaemic Hormone) (Giulianini *et al.*, 2002).

Paraffin immunocytochemistry

Eyestalks dissected from *N. norvegicus*, *A. leptodactylus*, *M. rugosa* and *S. mantis* were fixed in Bouin's solution for 24 h, serially dehydrated in ethanol and embedded in paraffin. Sections (7 µm thick) are collected on Superfrost Plus (Bio-Optica) slides and baked for 30 min at 50 °C. The slides are deparaffinized in xylene, hydrated, microwaved in 6 M urea at 800 W for 1 min, washed in ultra pure water and treated with tyramide amplification (NEN, TSA-Indirect kit, Zaventem, Belgium). Endogenous peroxidases were blocked with 1 % H₂O₂ in PBS (Sigma) for 15 min. Slides are incubated in 10 % normal goat serum in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl buffer containing 0.5 % TSA blocking reagent (TNB) for 1 h at RT. The sections are then incubated overnight at 4 °C with the primary antibody diluted 1:20,000 in TNB; concentrations of pre-immunization normal rabbit serum (NRS) as well as anti-GST (anti-glutathione-S-transferase) antibody are employed for parallel controls. After washing for 20 min in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl buffer containing 0.05 % Tween 20, they are incubate for 1 h in HRP-labelled goat anti-rabbit IgG (NEN) diluted 1:200 in TNB. Afterwards, the slides are incubated in biotinyltyramide working solution for 10 min and in streptavidin-HRP diluted 1:100 in TNB for 30 min at RT. Finally slides are developed in 50 mM Tris-HCl buffer, pH 7.5, containing 0.05 % DAB and 0.01 % H₂O₂ at dark for 20 min at 4 °C. Sections are dehydrated and subsequently mounted in Eukitt (Bio-Optica).

Resin immunocytochemistry

Eyestalks of *N. norvegicus* and *P. elegans* are fixed in 2 % glutaraldehyde in 0.1 M sodium cacodylate

and 0.4 M sucrose at pH 7.6 (and for *N. norvegicus* only post-fixed in 1 % OsO₄ in the same buffer), serially dehydrated in ethanol and embedded in LRWhite (Sigma). One- μ m-sections are collected on Superfrost Plus (Bio-Optica) slides and baked for 30 min at 80 °C. The slides are microwaved in 6 M urea at 800 W for 1 min, washed in ultra pure water, and only for *N. norvegicus*, deosmicated with 3 % H₂O₂ in PBS (Sigma) for 15 min. Slides are incubated in 10 % normal goat serum in 0.1 M Tris-HCl, pH 8.3, 0.15 M NaCl buffer (Tris buffered saline, TBS-1) containing 1 % bovine serum albumin and 0.1 % Tween 20 (TBB) for 30 min. Sections are then incubated overnight at 4 °C with the primary antibody diluted 1:400 in TBB; concentrations of pre-immunization normal rabbit serum are employed for parallel controls. After washing for 20 min in TBS-1, the sections are treated with goat anti-rabbit IgG labelled with 5 nm colloidal gold (British Biocell International) diluted 1:200 in TBB. Afterwards, the slides are washed in ultra pure water and developed with British Biocell silver-enhancing kit (British Biocell International) for 20 min at dark and mounted in Eukitt (Bio-Optica).

Fluorescence-based morphological methods *Mollusca*

Fluorescence-based methods have been applied in molluscs especially to document cytoskeletal rearrangements following the exposure to chemotactic factors/cytokines (Franchini and Ottaviani, 1994; Ottaviani *et al.*, 2000).

In the freshwater snail *V. ater*, ACTH is able to stimulate the motility of immunocytes. The withdrawn hemolymph plus ACTH are dropped onto a slide in which a small circle is delimited by a PVC adhesive strip. After 30 min of incubation in a humidified chamber, the supernatant is removed and the adhered immunocytes are left to air-dry for 30 min before the fixation in 1 % paraformaldehyde for 5 min at RT. After fixation, different procedures of permeabilization are followed on the basis of the target of the study. For the detection of actin and vinculin, the immunocyte membrane is permeabilized with an incubation in HEPES-Triton-X-100 for 5 min at RT, while for detection of microtubules, the permeabilization is achieved with a dehydration in cold absolute methanol (5 min at -20 °C), followed by cold acetone (5 min at -20 °C) and air drying. Specific reactions are finally performed by incubating immunocytes for 30-60 min at 37 °C in a humidified chamber with either 2.5 μ g/ml fluorescein isothiocyanate (FITC)-labeled phalloidin (a specific marker of F-actin) (Sigma), or with 1-10 μ g/ml mouse anti-vinculin mAb (Boehringer-Mannheim, Germany), or with rabbit anti-tubulin (Chemicon). Slides can be mounted with an ordinary aqueous mounting medium (e.g., 50 % PBS-1/glycerol) or with commercial anti-fading media. Observations are performed under a fluorescence microscope (Ex/Em wavelengths 495/519 nm).

In the bivalve *M. galloprovincialis*, the actin microfilament modifications in immunocytes exposed to 100 ng/ml human recombinant IL-8 (Pepro Tech) have been analyzed by using a similar method. Briefly, cell preparation is carried out as described above but fixation is performed with 4 % paraformaldehyde in PBS-1, pH 7.4, for 5 min at 4

°C. Permeabilization is still achieved with a HEPES-Triton X-100 solution (5 min at RT), then mussel immunocytes are incubated in a humidified chamber with 10 μ g/ml FITC-labeled phalloidin at 37 °C for 30 min. Finally, slides are again washed three times with PBS-1 and mounted in aqueous/anti-fading medium.

Insecta

Cytocentrifugation is the first-choice method for slide preparation when working with cell cultures growing in suspension. In the insect cell line IPLB-LdFB, derived from the larval fat body of the lepidopteran *L. dispar*, it has been observed that cytocentrifugation has to be performed at a speed as low as possible, i.e., 200 rpm for 1 min. This is important since high speed cytocentrifugations can alter the morphology as well as the integrity of the cells and may alter results when studying cytoskeletal elements (Malagoli *et al.*, 2006). The cytoskeletal organization of IPLB-LdFB cells has been evaluated by FITC-labelled phalloidin (Sigma). After cytocentrifugation, the cells are fixed for 10 min at RT in 4 % paraformaldehyde, washed in PBS-1 for 5 min and incubated for 30 min at 37 °C in 30 nM FITC-labelled phalloidin in presence of 0.3 % Triton X-100 in PBS-1. Subsequently, nuclei are stained for 5 min at RT with 10 μ M Hoechst 33342 and the cells are mounted in MOWIOL[®] (Calbiochem). It should be remarked that fixation is a fundamental step not only for the preservation of cell morphology, but because it allows a tighter adhesion of cells to the slide. A similar cell preparation has been also applied for TUNEL assay (useful for detecting the presence of apoptotic nuclei) (Malagoli *et al.*, 2006). Conversely, for the Hoechst 33342-propidium iodide (PI) staining, applied for the evaluation of cell membrane integrity, the cytocentrifugation is performed on unfixed cells after the incubation with Hoechst 33342 but before the incubation with PI. More in detail, IPLB-LdFB cells are stained for 10 min at 26 °C with 10 μ M Hoechst 33342 and subsequently cytocentrifuged. Slides are then submerged at 26 °C in a 10 μ M PI solution for 5 min and immediately observed (Malagoli *et al.*, 2005). The Ex/Em wavelengths for the cited fluorochromes are the following: 495/519 nm (FITC), 343/483 nm (Hoechst 33342), 536/617 nm (PI).

Tunicata

Some differences exist in fixation procedures for immunocytochemical protocols with respect to that described for characterization of circulating hemocytes of *B. schlosseri*. For antigen preservation in immunocytochemical assays, hemocytes are fixed in 4 % paraformaldehyde (Serva, Heidelberg, Germany) plus 0.1 % glutaraldehyde in saline buffer (SB) for 30 min at 4 °C, and then washed in SB.

Fixed hemocytes are washed in PBS-3 plus 1 % sucrose and permeabilized with 0.1 % Triton X-100 (Merck) in PBS-3 for 5 min. For the specific detection of F-actin, the monolayers are then incubated for 30 min at 25 °C in FITC-labeled phalloidin (Sigma), 1 μ g/ml in PBS-3. Lastly, the coverslips are rinsed in 0.1 M carbonate buffer, pH 9.5, to amplify the fluorescent signal, mounted with Fluorsave (Calbiochem) on glass slides, and

observed at a magnification of 1,250x with a fluorescence light microscope equipped with a filter block for FITC excitation (450–490 nm; emission at 525 nm) (Fig. 7f).

To observe microtubules, hemocytes are preincubated in PBS-3 containing 3 % powdered milk and 0.5 % fetal calf serum for 30 min to block aspecific interactions of antibodies. Coverslips are then incubated for 60 min in monoclonal anti- α -tubulin antibody (Sigma) (1:2,000 in PBS-3), washed in PBS-3 and incubated with 50 μ g/ml FITC-labeled goat anti-mouse IgG (Sigma) in PBS-3. Slides are then mounted with Fluorsave (Calbiochem), as described above, and observed under the fluorescence microscope (Fig. 7g).

In addition to the analysis of the cytoskeleton, fluorescence-based methods have also been successfully applied to analyze apoptotic process of *B. schlosseri* hemocytes. We have analyzed both early-apoptosis and late-apoptosis markers, even if the boundary between these two phases is obviously not always clearly distinguishable.

Phosphatidylserine exposure (early apoptosis): living cells are exposed for 60 min to xenobiotics and then incubated with FITC-coupled annexin-V (Annexin-V Fluos Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions, to detect the presence of phosphatidylserine in the outer leaflet of the plasma membrane of living cells, a marker of early apoptosis (Martin *et al.*, 1997). After 15 min, cells are observed under the fluorescence microscope, equipped with a filter block for FITC excitation, at the magnification of 1,250x. At least 300 cells/coverslip are counted to evaluate the apoptotic index, i.e., the percentage of fluorescent cells (Fig. 7h).

DNA fragmentation (late apoptosis): to evaluate the occurrence of DNA fragmentation, hemocytes, exposed for 60 min to xenobiotics, are fixed for 30 min at 4 °C in 4 % paraformaldehyde plus 0.1 % glutaraldehyde in SB rinsed in PBS-3 and incubated for 30 min in 0.3 % H₂O₂ as blocking solution. Then, cells are incubated in the permeabilization solution (0.1 % Triton X-100 in 0.1 % Na-citrate) for 2 min at 4 °C. Samples are then rinsed twice with PBS-3 and incubated in the TUNEL reaction mixture (Roche Diagnostics) for 60 min at 37 °C according to the manufacturer's instruction and immediately observed under the fluorescence microscope, equipped with a filter block for FITC excitation (Abrams, 1997). The reaction product can also be longer preserved when, after this step, hemocytes are incubated with peroxidase-conjugated anti-FITC antibodies, stained with 0.05 % DAB in PBS-3 containing 1.5 % H₂O₂, dehydrated, mounted with Eukitt (Bio-Optica) and observed under the light microscope. The percentage of cells with fluorescent or stained (brown) nuclei (Fig. 7i, j) is expressed as the DNA fragmentation index.

Comet assay: this test for DNA fragmentation is performed as described by Singh *et al.* (1988) and modified by Kamer and Rinkevich (2002). Ten μ l hemocyte suspension, containing 10⁴ cells are mixed with 90 μ l of a 0.5 % solution of melted agarose (low-melting-point type), cast on a microscope slide pre-coated with 0.5 % regular agarose and

followed, after solidification, by a layer of 0.5 % low-melting-point agarose. Slides are then immersed in lysis buffer (0.01 M Tris-HCl, 2.5 M NaCl, 0.1 M Na₂EDTA, 1 % Triton X-100, 10 % dimethylsulfoxide, pH 10) for 2 h at 4 °C. The slides are placed on a horizontal gel electrophoresis unit filled with chilled electrophoretic buffer (0.3 M NaOH, 1 mM Na₂EDTA) for 40 min, to denature DNA. After electrophoresis (20 min at 20 V), the slides are washed in 0.4 M Tris-HCl buffer (pH 7.5), blotted dry, stained with a 25 μ g/ml solution of ethidium bromide, and observed under a fluorescence light microscope, with an excitation filter of 590 nm. The frequency of apoptosis is estimated by counting the percentage of cells showing nuclei transformed in typical apoptotic comets (Fig. 7k) by counting at least 100 cells for each slide at 1,250x (Bacsó *et al.*, 2000).

Finally, a fluorescence-based immunocytochemical approach to identify stem hemocytes among a hemocyte population or subpopulation is here proposed.

After adhesion to the coverslips, hemocytes are fixed for 30 min at 4 °C in 4 % paraformaldehyde plus 0.1 % glutaraldehyde in SB. They are washed in PBS-3, permeabilized with 0.1 % Triton X-100 (Merck) for 5 min, immersed for 30 min in a PBS-3 solution containing 10 % normal goat serum (Vector Lab.) to block aspecific reactions, and then washed again in PBS-3 for 5 min. Cells are incubated in 50 μ g/ml of anti-CD34 (Calbiochem), anti-CD100 (Alexis Co, Lausen, CH) or SCF-R (Sigma) mAbs for 1 h at RT. CD34 is a highly glycosylated surface antigen of still unknown function, probably involved in cell adhesion (Holyoake and Alcorn, 1994); CD100 is also a transmembrane glycoprotein belonging to the IgG superfamily, expressed by the majority of hemopoietic cells (Hall *et al.*, 1996); SCF-R, receptor of stem cell growth factor (SCF), also known as c-Kit or CD117, seems to exert an essential role on vertebrate hemopoiesis (Broudy, 1997).

Hemocytes are then washed in PBS-3 for 5 min and incubated for 30 min in 10 μ g/ml fluoresceinated goat anti-mouse-Ig antibody (Sigma). Lastly, they are washed for 5 min in PBS-3 and mounted in Fluorsave (Calbiochem). Observations are carried out under a fluorescence light microscope equipped with a filter block for FITC excitation (Ex/Em wavelength 495/519 nm).

In situ hybridization

Mollusca and Insecta

The following method has been successfully applied in molluscs (Ottaviani *et al.*, 1998c) as well as in the insect cell line CRL-8003 (ATCC 6538) from the cabbage moth *M. brassicae* (Malagoli *et al.*, 2002b). In the procedure reported for the detection of the expression of ACTH receptor-like mRNA in molluscan immunocytes (Fig. 9) plasmid pBluescript II KS-Phagemid containing about 3,000 bp bovine ACTH receptor cDNA is used as probe (Raikhinstein *et al.*, 1994). The probe is labeled by incorporating digoxigenin (Dig)-labeled deoxyuridine triphosphate (Boehringer Mannheim) by random primer DNA-labeling, according to Feinberg and Vogelstein (1983). The reaction is stopped by adding 0.2 M EDTA, pH 8. The hybridization assay proceeds as follows: incubate unfixed and fixed immunocytes with PBS-1 plus glycine (0.7 %) and permeabilize with 0.3 % Triton

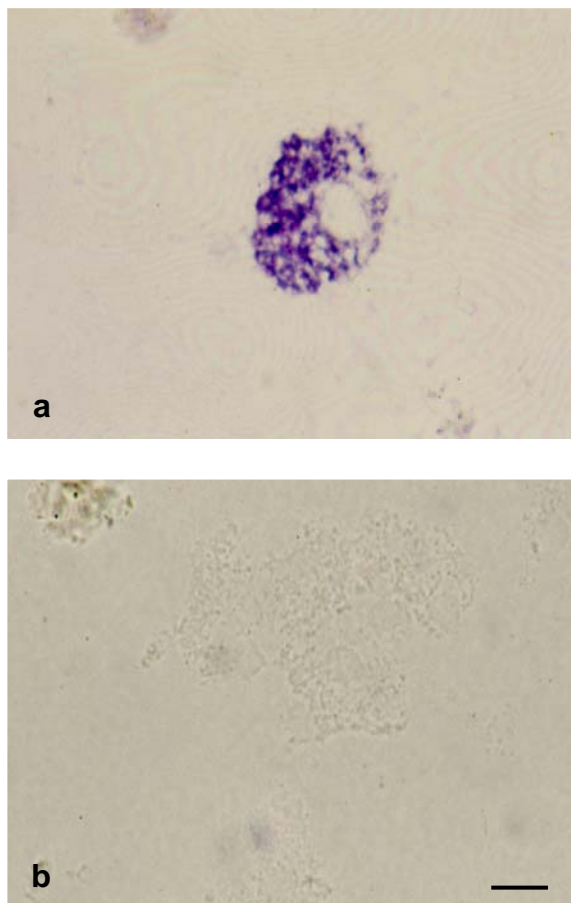


Fig. 9 *In situ* hybridization of the expression of ACTH receptor-like mRNA in *M. galloprovincialis* immunocyte (a). Negative control (b). Bar = 10 μ m.

X-100 in PBS-1 for 15 min at RT; wash with PBS-1 and with a mixture containing 2x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7) for 10 min; incubate with pre-hybridization mixture (4x SSC), 40 % formamide and 1x Denhardt's solution for 1 h at RT; hybridization is performed by adding of a denaturated Dig-labeled ACTH receptor cDNA probe (50 μ g/ μ l) to the pre-hybridization mixture overnight at 37 °C; rinse once in 2x SSC for 1 h at RT, once in 1x SSC for 30 min at RT, once 0.5x SSC for 30 min at 37 °C and once 0.5x SSC for 30 min at RT; incubate in a blocking solution [2 % normal sheep serum, 0.3 % Triton X-100 in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5)] for 30 min at RT; incubate with sheep anti-Dig Fab fragments conjugate to alkaline phosphatase diluted 1:500 in buffer 1 (Boehringer Mannheim) for 1 h at RT; wash twice in buffer 1 for 5 min and twice in buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) for 2 min at RT. The alkaline phosphatase activity is revealed by incubation in the following medium at RT: 45 μ l nitro blue tetrazolium (75 mg/ml in dimethylformamide), 35 μ l 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt (50 mg/ml) (Boehringer Mannheim), 10 ml buffer 3, and 2.4 mg levamisole (Sigma). The slides are mounted in glycerol diluted 50 % in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8). Controls are performed by mock-hybridizing slides without probe.

Morpho-functional methods

Mollusca

Cell shape changes

According to Manske and Bade (1994) cell motility is defined as cellular movements that do not result in translocation of the affected cell, while cell migration (chemotaxis) is a cell translocation over a clearly measurable distance as a consequence of the chemotactic activity exerted by a chemoattractant.

Cell shape changes can be easily evaluated by mean of a computer-assisted image analysis performed on digital pictures. When this procedure was proposed (Schön *et al.*, 1991) some specific tools were required. Nowadays, the majority of laboratories has at least one digital camera installed on the microscope, while freeware softwares (e.g., ImageJ 1.32j, Wayne Rasband, National Institute of Health, USA, <http://rsb.info.nih.gov/ij/>) can be utilized to calculate the shape factor (SF), i.e., the circularity, of the cells. Two are the true limiting factors in the application of this technique. The first is represented by the necessity to collect a reasonable amount of hemolymph (minimum 300 μ l). The second is that the hemocytes have to remain functionally unaltered onto the slide for at least 30 min, after withdrawal. The hemolymph of *M. galloprovincialis* satisfies both of these requirements, thus it has been successfully utilized for cell shape analyses. The technique is based on the assumption that an inactive immunocyte has a round shape while, upon activation, it assumes a more elongated (ameboid) form. Briefly, the SF is calculated as: $AC/AT = [LT/LC]^2$, where AT is the area of an hypothetical circle with the same perimeter of the cell under measurement, LT is the perimeter of an hypothetical circle with the same area of the cell under measurement, and AC and LC are the actual area and perimeter, respectively, of the cell under measurement. The lower the SF value, the greater the perimeter of the cell with respect to its area, meaning that more the shape is ameboid. It descends that once a software able to calculate actual perimeter and area is available, it is possible to obtain the SF by relatively simple calculations.

In cell shape change experiments, the hemolymph is collected from the mussel and 100 μ l are spread onto the glass slide into a vaseline/rubber ring. The aim of the ring is to prevent the hemolymph from dropping out from the slide. A glass coverslip is then put over the ring, paying attention not to cover a small portion of the ring itself, so that it is possible to have access to the spread hemolymph in order to add molecules of interest, e.g., chemoattractants or signaling transduction pathway inhibitors (Ottaviani *et al.*, 1997b; Malagoli *et al.*, 2003). After 3-5 min for allowing immunocyte adherence, the operator has to select the cells (usually 5 for slide) that will be followed during the experiment. The photographs of these cells are taken at regular intervals (usually 5 min), and exposure to the light should be minimized. Once the last photograph has been taken, it is strongly recommended to immediately perform the following controls. First, the cells that have been chosen must display a morphology similar to that of the majority of the other cells of the slide. If, for

example, the chosen cells are the only ones active in all the slide, the experiment should be discarded. Second, in order to exclude any effect connected to the permanence of the slide under the microscope, it is important to immediately verify if a slide that was treated identically to that used for cell shape analysis but was not kept under the microscope, presents cells with a morphology almost overlapping to the first one. Obviously, control slides in which the chemoattractant and/or the inhibitors are absent have also to be prepared and this explains why a relatively high amount of hemolymph is required for this method.

The electronic images of the experiments can now be utilized for the actual evaluation of the SF, by following the edge of the cells with the pointer of the mouse and asking the software to directly evaluate the circularity or, as an alternative, the perimeter and the area of the chosen cells for each time point.

Chemotaxis

The migration assay refers to the effect of human cytokines on molluscan immunocytes (Ottaviani *et al.*, 1995b). The collected hemolymph is centrifuged at 200xg for 15 min and the pellet resuspended in 1 ml of snail saline solution (SSS) ($\text{Na}^+ = 41.15 \text{ mM}$; $\text{K}^+ = 0.54 \text{ mM}$; $\text{Ca}^{2+} = 3.55 \text{ mM}$; $\text{Mg}^{2+} = 2.61 \text{ mM}$; pH 7.5; osmolarity = $109 \pm 5 \text{ mOs}$) (Ottaviani, 1983) containing 0.1 % bovine serum albumin (BSA) (Sigma). The test is carried out using 48-well microchemotaxis chambers (Nucleopore, Pleasanton, CA, USA), in which the upper and lower compartments are separated by a 5 μm pore, polycarbonate polyvinylpyrrolidone-free filter allowing the cell to migrate actively through the pores. Fifty μl of immunocyte suspension is placed in the upper compartment, while in the lower one are placed different cytokines [concentrations: interleukin-1 α (500, 50, 5 pg/ml), tumor necrosis factor (TNF)- α (100, 10, 1 U/ml)]. After 90 min incubation at 37 °C, the migrated immunocytes adhering to the distal part of the filter are fixed, stained, identified microscopically and counted using light microscopy (magnification 40x). Data are expressed as migrate cells/field. Controls are obtained measuring the immunocyte migration in the presence of SSS.

Phagocytic tests

Mollusca

A classical *in vitro* bacterial phagocytosis is reported in Ottaviani *et al.* (1995b). One hundred μl of freshwater snail hemolymph, are placed on a slide in the form of a ring by applying an adhesive PVC strip, in which two holes (\varnothing 10 mm) are performed. The experiment is carried out by mixing the hemolymph with *Staphylococcus aureus* (10^6 - 10^8 bacteria/ml) in presence or in absence of different stimulators. The incubation is performed in humidified chamber and the phagocytic activity was tested after 40 min. The phagocytized bacteria is determined by immunocytochemical procedure (Ottaviani, 1990) using a rabbit anti-*S. aureus* polyclonal antibody. The number of phagocytized bacteria is performed counting under the light

microscopy (magnification 1,000x) 20 random immunocytes for each preparation.

Recently a different approach has been developed for the phagocytic test in the mussel *M. galloprovincialis* (Malagoli *et al.*, 2007). In this case, instead of bacteria, fluorescent micro-beads (diameter 1 μm) are used to assess phagocytic activity of immunocytes. After withdrawal, 100 μl of hemolymph (about 10^4 cells) are incubated at dark with 0.1 % v/v of 1 μm diameter green-fluorescent microspheres (FluoSpheres®, Molecular Probes, OR, USA) in a 1.5 ml plastic tube and kept in gentle rotation for 30 min at RT. After incubation, immunocytes are placed on microscope slides and there left for a further 5 min to allow their adhesion. The number of phagocytic immunocytes out of 100 and the number of phagocytized microspheres from each phagocytic immunocyte are counted under a fluorescence microscope with the filter commonly used for FITC (Ex/Em wavelengths 495/519 nm). Negative control, i.e., the discrimination between effectively phagocytized and non-specifically bound micro-beads, are performed by incubating the immunocytes with 1 % sodium azide alone for 5 min before the addition of the micro-beads. In this way it is possible to set a baseline percentage of phagocytic immunocytes that have to be taken into consideration when performing statistic calculations.

Insecta

The *in vitro* bacterial phagocytosis is performed in 1.5 ml plastic tube, by adding *S. aureus* (10^8 bacteria/ml) to hemolymph collected from adult flies of *C. vomitoria*. The phagocytosis assay is determined after 30 min. The samples are cytocentrifuged, and the immunocytes are stained with 0.5 % toluidine blue. After staining, the cell phagocytic activity is recorded under the microscope (Franchini *et al.*, 1996).

Cytotoxicity

The natural cytotoxicity is performed on freshwater molluscan immunocytes (Franceschi *et al.*, 1991) in a short term (4 h) ^{51}Cr release test following the classical protocol for human studies using the K562 cell line as a target (Lozzio and Lozzio, 1975; Grimm and Bonavida, 1979). Target cells (T), are labeled overnight with $\text{Na}_2^{51}\text{CrO}_4$ solution (Amersham Int., Amersham, UK) in complete medium. The effector (E)/T ratio varies from 100:1 to 6:1. The percentage of specific cytotoxicity is calculated according to the following formula:

$$\% \text{ s.c.} = \frac{\text{exp. rel.} - \text{spontaneous rel.}}{\text{max. rel.} - \text{spontaneous rel.}} \times 100$$

s. c. = specific cytotoxicity
exp. rel. = experimental release
max. rel. = maximum release

Enzyme activity

Acid phosphatase activity has been examined in molluscan immunocytes (Franchini and Ottaviani, 1990). The activity is performed on fixed (1.5 % glutaraldehyde in 20 mM cacodylate buffer, pH 7.5)

and unfixed cytocentrifuged immunocytes. Slides are incubated for 2 h at 37 °C in Barka and Anderson media (1962). After incubation, cells are treated with 1 % ammonium sulfate for 1 min and covered with Acquovitrex (C. Erba).

Flow cytometry

The cytofluorimetric analysis has been applied to molluscan hemolymph in order to detect the different cell populations on the basis of physical characteristics, such as diameter, size, density, etc allowing to distinguish them on the basis of forward and side light scatters. A further phenotype characterization of immunocytes has been carried out using various antibodies, such as anti-human-ACTH pAb and mAb, anti-N-acetylmuramic acid pAb and different mouse anti-human mAbs recognizing different molecules (Ottaviani and Montagnani, 1989; Franceschi *et al.*, 1991; Ottaviani *et al.*, 1991). Procedure using rabbit anti-human-ACTH pAb and mouse anti-human ACTH mAb: 400 µl of hemolymph are divided into two parts. One is incubated with 10 µl of anti-ACTH pAb (Dakopatts) or with mouse anti-ACTH mAb (Chemetron, Milan, Italy) for 20 min at 4 °C, while the second is taken as a negative control. The stained samples are then washed with cold PBS-1, incubated with 10 µl FITC-labeled goat anti-rabbit IgG (Dakopatts) or with 10 µl phycoerythrin (PE)-labeled goat anti-mouse IgG (Becton-Dickinson, San José, CA, USA), washed with cold PBS-1 and analyzed with a flow cytometry (Becton-Dickinson). Negative controls are prepared according to the above procedure, but omitting the anti-ACTH pAb or anti-ACTH mAb. Samples are finally analyzed by using state of the art flow cytometry, taking into account the requirements for a precise setting of the fluorescence intensity and of the compensation, should one use two or more fluorochrome-conjugated antibodies to stain cells.

Tunicata

Trypan blue exclusion assay

After cell adhesion, primary cultures of *B. schlosseri* hemocytes are exposed for 60 min to FSW containing xenobiotics at various concentrations. Living hemocytes are then incubated for 5 min in a solution of 0.25 % trypan blue in FSW and observed under the light microscope at 1,250x. The percentage of stained hemocytes is estimated on a total count of at least 300 cells, and the concentration of xenobiotics able to cause mortality to 50 % of the hemocytes (LC₅₀) is calculated according to the probit method (SPSS 11.0, SPSS Corp., Chicago, IL, USA).

Adhesion assay

Since the capability of *B. schlosseri* hemocytes to adhere is fundamental for their role in immunity, adhesion assay can be usefully applied to acquire information on the effects of xenobiotics on this species. Hemocytes are left to adhere for 60 min on clean coverslips in the presence of xenobiotics, at various concentrations. Slides coated with 50 µg/ml poly-L-lysine are used as reference controls (100 % adhesion). After glutaraldehyde-fixation and staining for 5 min in 10 % Giemsa solution, coverslips are mounted in Acquovitrex (C. Erba) and observed. In order to evaluate the ability of hemocytes to adhere to glass in the presence of xenobiotics, the total

number of hemocytes in 10 optic fields at 1,250x is counted and expressed as the adhesion index, i.e., the ratio, expressed as percentage between the total hemocyte count in experimental slides and the cell count in similar conditions in poly-L-lysine-coated slides.

Cell spreading assay

After 60 min-exposure to biocides at various concentrations, *B. schlosseri* hemocyte monolayers are fixed with glutaraldehyde, stained with Giemsa solution and mounted in Acquovitrex (C. Erba). Their morphology is then observed under the light microscope at 1,250x, and the cell-spreading index, i.e., the percentage of hemocytes with ameboid shape, is estimated after counting at least 300 cells per coverslip (Cima and Ballarin, 1999). In addition, computer-assisted image analysis (Casting Image NT, HP Laboratories, Palo Alto, CA, USA) is performed to evaluate the SF, as defined above for cell shape change evaluation in molluscs. Lower shape factors indicate larger perimeters with respect to the areas and, therefore, an increased ameboid shape.

Assay for reduced glutathione (GSH) content

After treatment with xenobiotics, *B. schlosseri* hemocyte monolayers are washed in FSW, stained for 10 min at 37 °C in 40 mM chlorobimane (a fluorescent dye specific for GSH with λ_{max} of 461 nm) (Sigma) solution in FSW, obtained from a 20 mM stock solution in 95 % ethyl alcohol, and then rinsed in FSW (Cookson *et al.*, 1998). Living cells are immediately observed under a fluorescence microscope equipped with an ultraviolet light filter block (Ex/Em 270-380/460 nm), at a magnification of 1,250x. Positive sites appear fluorescent blue. The percentage of stained cells is then determined and expressed as the GSH index.

Morula cell (MC) degranulation assay

Fifty µl of *B. schlosseri* hemocyte suspension (15×10^6 cells/ml) are placed in the center of the culture chambers prepared as already described and left to adhere to washed coverslips for 30 min. After discarding the FSW, hemocytes are then incubated for 60 min at RT with 50 ml of autologous or heterologous blood plasma or with a suspension of *Escherichia coli* (200×10^6 cells/ml), *S. aureus* (50×10^6 cells/ml), *Bacillus clausii* spores (200×10^6 spores/ml) or ordinary baker's yeast (*Saccharomyces cerevisiae*) (50×10^6 cells/ml) in FSW, and washed in FSW (Ballarin *et al.*, 1998, 2005). The morphology of living MCs is then observed under a phase contrast microscope at a magnification of 1,200x (Fig. 10).

Chemotactic assay

Migration of *B. schlosseri* hemocytes is analyzed using a 24-well chemotaxis chamber (Transwell, Costar, Corning, NY, USA) with the lower and upper wells separated by 8-µm polycarbonate filters. The upper wells are filled with 100 µl of hemocyte suspension (5×10^5 cells) in FSW and 500 µl of FSW are added to the lower wells. Solutions in upper and lower wells are allowed to equilibrate for 15 min, and then 20 µl of the lower solution are substituted with 20 µl of the putative chemoattractant at the concentration of 2 mg/ml

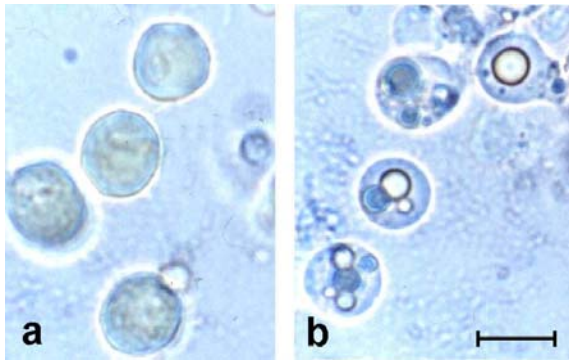


Fig. 10 Living morula cells incubated in the absence (a) or in the presence (b) of heterologous blood plasma. In this last case cells have degranulated and have released their vacuolar content. Bar = 15 µm.

(FSW in controls) (modified according to Raftos *et al.*, 1998). Cells are allowed to migrate for 2 h at 18 °C. After incubation, the lower surface of the filters are flushed extensively with FSW, in order to detach adhering hemocytes and collect all the cells which have passed through the membrane. Hemocytes in the lower wells are then collected in a 1.5 ml vial and centrifuged at 780xg for 10 min.

The mean number of cells in 10 random optical fields, at a magnification of 1,250x, is used to define the migration stimulation index, i.e., the ratio between the mean number of migrated cells per field in experimental series and the mean number of migrated cells in controls.

Phagocytosis assay

After adhesion, *B. schlosseri* hemocytes are incubated with 60 µl of a suspension in FSW of various test particles (Ballarin *et al.*, 1994) represented by living or autoclaved (15 min at 120 °C) cells of *S. cerevisiae*, sheep erythrocytes, zymosan (Sigma), latex beads (1 and 3 µm diameter) (Sigma), fluorescent *E. coli* cells (Phagotest kit, Orpegen, Heidelberg, Germany). The number of test particles is adjusted to a particle:hemocyte ratio of 10:1 after counting with a Bürker's hemocytometer. The best results are obtained with yeast suspensions in FSW since this type of target particles is both extensively engulfed by phagocytes and easily recognizable in phagosomes. Cultures are kept upside-down for 5-120 min at 25 °C and the uningested yeast is then removed by dipping repeatedly the coverslips in a large volume of FSW. Viability, assessed by the trypan blue exclusion assay, exceeds 95 % after 2 h of incubation. When fluorescent test particles are used, uningested particles are quenched by quick immersion of coverslips in a solution of 2 mg/ml trypan blue and 2 mg/ml crystal violet in 0.02 M citrate buffer, pH 4.4 containing 33 mg/ml NaCl. Hemocyte monolayers are then fixed in 1 % glutaraldehyde and 1 % sucrose in FSW for 30 min at 4 °C and stained with 5 % Giemsa for 5 min. The coverslips are mounted on glass slides with Aquovitrex (C. Erba) or Fluorsave (Calbiochem), the

latter being used for assays with fluorescent test particles. At least 300 hemocytes are observed per slide under microscope, in ten optical fields, at the magnification of 1,250x, and, the phagocytic index, i.e., the percentage of hemocytes with ingested cells, is evaluated. The phagocytic index increases progressively with time reaching a plateau of 10-15 % after an incubation time of 60 min, which represents the optimum incubation time for this assay. In other experiments, at the end of the incubation time, the medium is collected and centrifuged to obtain a "conditioned" supernatant, which is used to detect enzymatic activity, reactive oxygen species, opsonins and cytokines released in the incubation medium (Cima *et al.*, 1996) and to study its effects on other phagocytosis assays (Menin *et al.*, 2005). Finally, the phagocytosis assay can be also used for evaluating the immunosuppressant effects of xenobiotics added at various concentrations to the incubation medium containing the target particles.

Enzyme activity

Assays are carried out on glutaraldehyde-fixed hemocytes, previously exposed or not to xenobiotics. These methods can be applied either to investigations aimed at hemocyte characterization or to the study of xenobiotic effects on hemocyte functions. In controls, the specific enzyme substrate is omitted. After incubation in the reaction mixtures, monolayers are thoroughly washed in distilled water, coverslips mounted in Acquovitrex (C. Erba), and at least 300 cells per slide are counted to determine the fraction of positive hemocytes, expressed as the enzymatic index.

Acid phosphatase

This is a lysosomal enzyme used as a marker of phagocytes. Fixed hemocytes are washed in 0.1 M sodium acetate buffer, pH 5.2, for 10 min and incubated for 3 h at 37 °C in the reaction mixture made by 10 mg naphthol AS-BI phosphate (Sigma) in 400 µl dimethylformamide (DMF), 400 µl solution A (4 % new fuchsin (Sigma), 2 % HCl in distilled water), 400 µl of a 4 % aqueous solution of NaNO₂ solution B and 20 ml of 0.1 M sodium acetate buffer. Positive sites appear red (Lojda *et al.*, 1979).

Alkaline phosphatase

After fixation, hemocytes are washed in Tris-HCl buffer 0.1 M, pH 9, for 10 min and incubated for 2 h at 37 °C in a reaction mixture similar to that used for acid phosphatase assay, but containing 20 ml of Tris-HCl buffer instead of sodium acetate buffer (Burstone, 1962). Hemocytes are then washed in Tris-HCl buffer for 10 min and mounted. Positive sites stain red.

β-glucuronidase

It is another lysosomal hydrolytic enzyme, marker of phagocytes. Fixed hemocytes, washed in 0.1 M sodium acetate buffer, pH 5.2, for 10 min, are incubated for 3 h at 37 °C in the reaction mixture made by 4 mg naphthol AS-BI β-glucuronide (Sigma) dissolved in 250 µl DMF, 400 µl solution A, 400 µl of aqueous solution of 4 % NaNO₂ and 20 ml of sodium acetate buffer (Lojda *et al.*, 1979). Positive sites stain red (Fig. 71).

5'-nucleotidase

Fixed hemocytes are washed for 10 min in Tris-maleate buffer 0.2 M, pH 7.2 and incubated for 2 h at 37 °C in the following reaction mixture: 20 mg adenosine-5'-monophosphate (AMP) (Sigma), 22 ml distilled water, 20 ml Tris-maleate buffer, 3 ml 2 % PbNO₃, 5 ml 2.5 % MgSO₄ (Wachstein and Meisel, 1957). Hemocytes are then washed twice with distilled water and with ammonium sulfide solution (21 %) (Fluka), diluted 1:100 in distilled water, for 2 min. They are then washed again with distilled water and mounted. Positive sites stain black.

Acid esterase

Fixed hemocytes are washed for 10 min in phosphate-citric acid buffer 0.1 M, pH 5.5, and incubated for 16 h at 4 °C in the following reaction mixture: 3 mg naphthol acetate (Sigma) dissolved in 500 µl acetone, 250 µl solution A, 250 µl solution B, 19 ml phosphate-citric acid buffer (Lojda, 1977). Hemocytes are then washed with distilled water and mounted. Positive sites stain pinkish-brown.

Chloroacetyl esterase

Hemocytes, washed in PBS-3 for 10 min, are incubated for 1 h at RT in the following reaction mixture: 6 mg naphthol chloroacetate (Sigma) dissolved in 1 ml DMF and added to 19 ml PBS-3 containing 20 mg Fast Blue B (Moloney *et al.*, 1960). Hemocytes are washed in PBS-3 for 10 min and mounted. Positive sites stain blue.

Non-specific esterases

Non-specific esterases are reference hydrolytic enzymes, typical of phagocyte lysosomes. This assay was previously used to label the phagocytic line of *B. schlosseri* hemocytes (Ballarin and Cima, 2005) in acute toxicity assays with antifouling xenobiotics. Hemocytes are washed in PBS-3 and incubated for 3 h at 4 °C in 100 ml of PBS-3 containing 2 ml of 1 % 1-naphthyl acetate (Sigma) as a substrate, previously dissolved in 1 ml acetone, 1 ml of hexazonium-p-rosaniline [500 µl of a solution of 0.4 g new fuchsin (C. Erba) in 7.2 % HCl added to 500 µl of a 4 % solution of NaNO₂ in distilled water] (Davis *et al.*, 1959). Positive sites stain red.

Cytochrome-c-oxidase

It is an enzyme of the mitochondrial respiratory chain, and its activity, related to mitochondrial functionality, is required for ATP production. Cells are washed in 0.1 M Na-acetate buffer, pH 5.5, for 10 min, and incubated for 4 h at 37 °C in a solution of 0.2 % DAB in 1 % MnCl₂ containing 0.01 % H₂O₂ (Novikoff and Goldfischer, 1969). Positive sites stain brown.

Ca²⁺-ATPase

It is involved in the sequestration of Ca²⁺ ions in intracellular stores. Hemocytes are incubated for 20 min at 37 °C in 0.1 M Na-barbital buffer, pH 9.0, containing 3 mM ATP, 3.25 mM 2,4-dinitrophenol (DNP) and 0.1 M anhydrous CaCl₂. Cells are then repeatedly washed in 1 % anhydrous CaCl₂, further rinsed in 1 % Co(NO₃)₂, immersed in 0.5 % ammonium sulfide for 1 min, and washed in distilled water (Chayen *et al.*, 1969). Positive sites appear black.

Arylsulfatase

Hemocytes are washed in sodium acetate buffer 0.1 M, pH 5.2, for 10 min and incubated for 2 h at 37 °C in the following reaction mixture: 0.16 g p-nitrocatecholsulfate (Sigma), 4 ml distilled water, 12 ml 0.1 M sodium acetate buffer, pH 5.5, 4 ml 8 % PbNO₃ (Goldfischer, 1965). After incubation, hemocytes are washed with distilled water and then with 21 % ammonium sulfide solution (Fluka) diluted 1:100 in distilled water, for 2 min. Finally, they are washed with distilled water and mounted. Positive sites stain brownish-black.

Peroxidase

After fixation, hemocytes are washed in PBS-3 for 10 min and incubated for 2 h at 37 °C in the following reaction mixture (Graham and Karnovsky, 1966): 0.5 mg/ml DAB in distilled water containing 0.02 % H₂O₂. Hemocytes are then washed in distilled water and mounted. Positive sites stain brown.

Phenoloxidase

Phenoloxidase is an oxidative enzyme with a cytotoxic activity involved in defense reactions in *B. schlosseri*, where it can be used as a marker for the cytotoxic line of blood cells. After fixation, hemocytes are incubated for 1 h in saturated dihydroxy-L-phenylalanine (L-DOPA) solution in PBS-3 (Hose *et al.*, 1987), washed in distilled water, and mounted in Acquovitrex (C. Erba). Positive cells stain blackish-brown (Fig. 7m).

Assay for lysozyme

Hemolymph is collected from the colonial marginal vessel with a fine micropipette and centrifuged at 780xg for 10 min. The supernatant, corresponding to the cell-free blood plasma, is immediately used for the lysozyme assay. Pellets are resuspended in distilled water, sonicated for 5 min at 0 °C with a Braun Labsonic U sonifier at 50 % duty cycles, and centrifuged at 12,000xg for 15 min at 4 °C. Supernatant, corresponding to the hemocyte lysate, is collected and immediately used for the lysozyme assay. One percent of methylene blue 0.13 % in distilled water is added to a 0.25 % suspension of *Micrococcus lysodeikticus* (Sigma) in 0.1 M phosphate/citrate buffer, pH 5.8, and incubated for 30 min at 25 °C to stain cells. Seven ml of the above suspension are then added to 50 ml of 1.5 % melted agarose in 0.1 M phosphate/citrate buffer, pH 5.8, and 5 ml of the solution are poured into Petri dishes. When the agarose solidifies, 5-mm wells are made and filled with 50 µl of hemocyte lysate. Lysis diameter are observed after overnight incubation at 37 °C and compared with those obtained using known dilutions of lysozyme (5 and 10 mg/ml) from chicken egg white (Sigma) in 0.1 M phosphate/citrate buffer, pH 5.8 (Fig. 11a). Protein concentration in hemocyte lysate and blood plasma is determined according to Bradford (1976) using BSA as standard.

Hemagglutinating assay

Rabbit erythrocytes are washed three times by centrifugation at 500xg for 10 min in Tris-buffered saline (TBS-2) (Tris-HCl 50 mM, NaCl 150 mM, pH 7.4) and incubated for 30 min at 37 °C in 0.1 mg/ml

trypsin in TBS-2 (Ballarin *et al.*, 1999). They are then washed again and resuspended in TBS-2 containing 0.2 % gelatin to get a 1 % solution (V:V). Fifty μ l of *B. schlosseri* blood plasma are serially diluted two-fold with TBS-2 in the wells of U-bottomed microtiter plates and an equal volume of 1 % rabbit erythrocyte suspension is added in each well. FSW is used in controls. TBS-2 containing 5 mM EGTA is also used to assess the Ca^{2+} -dependence of the reaction. In another experimental series blood plasma is incubated for 30 min with 1 % rabbit erythrocyte suspension in order to control the specificity of the interaction. The suspension is then centrifuged at 780xg for 10 min and the rabbit erythrocyte-absorbed supernatant is collected and used in the hemagglutination assay. Plates are gently shaken, incubated for 1 h at 37 °C and then kept at 4 °C. The hemagglutination titre (HT), i.e., the reciprocal of the highest dilution giving positive hemagglutination is then evaluated (Fig. 11b).

Agglutination activity is also evaluated *in vitro* by adding 50 μ l of a 1 % trypsinized rabbit erythrocyte suspension in FSW to culture chambers. After 30 min of incubation at 25 °C, adhesion of erythrocytes is observable under the light microscope as rosette formation around some hemocyte types.

In the solitary ascidian *C. intestinalis* erythrocytes from different species (i.e., rabbit, sheep, human ABO group) were washed three times with TBS-2, 0.1 % (w:V) pig gelatin, suspended at 1 % in TBS-2 with or without 5 mM CaCl_2 pH 7.4, 0.1 % gelatin, and used in a microtitre plate hemagglutination assay (HA) in which 25 μ l of serially diluted sample were mixed with an equal volume of erythrocyte suspension (Fig. 12). Serial dilutions (2-fold) of the TBS-2-dialyzed and diluted serum (1:10) are performed with TBS-2 containing 0.1 % gelatin. The HT is evaluated after 1 h incubation (from 18 to 37 °C). Also for *C. intestinalis* to increase the erythrocyte sensitivity to the hemagglutination assay, trypsin-treated erythrocytes are prepared by resuspending an erythrocyte pellet (obtained from 1.0 ml blood) in 6 ml TBS-2 containing 300 μ g trypsin (stock solution prepared in 10 mM HCl) (Parrinello and Canicatti, 1982). The reaction mixture is incubated at 37 °C for 15 min. The trypsin-treated erythrocytes are washed with TBS-2 and resuspended (1 %) in TBS-2.

Phenoloxidase (PO) activity

PO activity is measured according to Winder and Harris (1991) using the reagent 3-methyl-2-benzo-thiazolinone hydrazone (MBTH) which reacts directly with dopaquinones. This method has the advantage of measuring the direct product of dihydroxyphenyl-L-alanine (L-DOPA) oxidation since not all dopaquinones are converted to dopachromes which are measured when L-DOPA only is used as substrate. Briefly, 20 μ l of hemolysate were incubated with 490 μ l of PBS-3, 290 μ l of 20.7 mM MBTH in PBS-3 containing 4 % of DMF and 200 μ l of L-DOPA-saturated PBS-3. The reaction is read spectrophotometrically at 494 nm, each for 5 min.

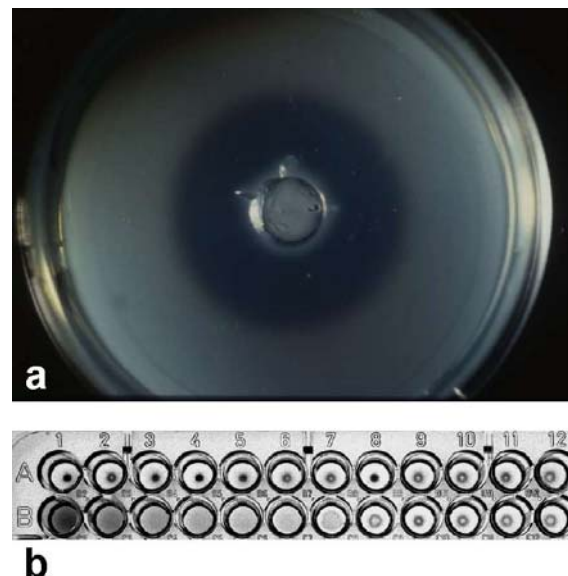


Fig. 11 a: lysis plaque of *Micrococcus lysodeikticus* in agarose due to lysozyme activity; **b:** hemagglutination activity of blood plasma: absence and presence of agglutination in row A (control; FSW) and row B (hemagglutination titer = 128), respectively.

Plaque-forming cell assay (PFC)

A PFC assay using tunicate hemocyte effectors has been originally described by Cunningham and Szenberg (1968) for the human B cell/sheep red blood cells and subsequently modified for tunicate hemocytes (Parrinello *et al.*, 1996; Cammarata *et al.*, 1997). Fifty μ l of hemocytes suspension (1×10^5 /ml) in marine solution (MS) (12 mM CaCl_2 , 11 mM KCl, 26 mM MgCl_2 , 45 mM Tris, 38 mM HCl, 0.45 M NaCl, pH 7.4) are mixed with 50 μ l of suspension of 5 % rabbit erythrocytes in MS. The reaction mixture is rapidly layered into the slide chamber by capillary action. The chamber has been constructed by placing three thin strips of double-stick tape placed between the borders and in the centre of a coverslip and another glass coverslip is then suspended onto the three pieces of tape forming a double chamber. After 15 min of incubation at 20 °C the cell mixture is examined under a phase contrast microscope. Each slide chamber can hold just under 0.1 ml on either side of the tape (0.2 ml per slide) (Fig. 13).

Determination of physical and chemical characteristics of lectins

To examine divalent cation requirements for lectin binding, CaCl_2 or MgCl_2 is added in the TBS-2 medium to obtain 5-10 mM final concentration. EDTA (1-10 mM) or EGTA (1-10 mM) are used to examine the effect of Ca^{2+} / Mg^{2+} depletion on the hemoagglutinating activity.

To examine the thermal stability of the protein, samples are incubated at different temperatures from 18 to 90 °C for 20 min and cooled down for 10 min on ice before the HA. Susceptibility of the HA to freeze-thaw is examined by carrying out the HA on samples maintained at -20 °C for 2 months and thawed at RT.

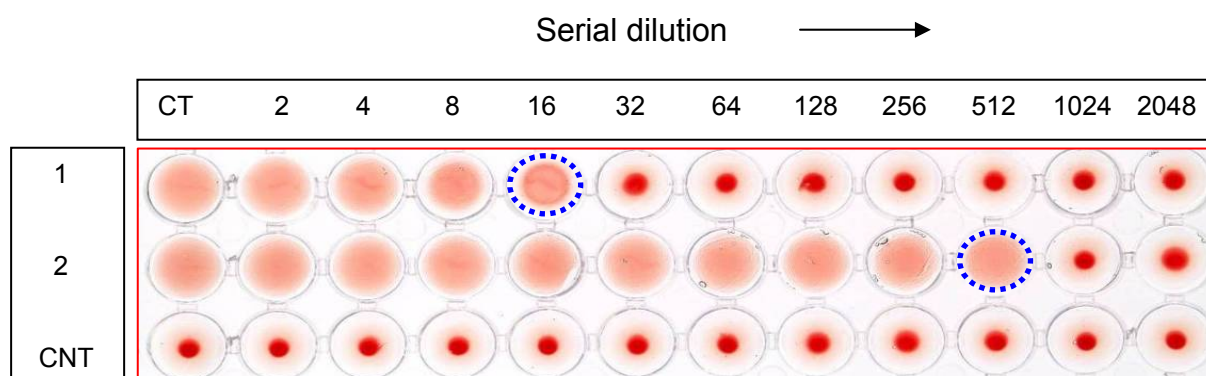


Fig. 12 Determination of hemoagglutinating titer. Each well of the microtitre plate contained 25 μ l of sample 1 and 2; 25 μ l 1 % suspension of rabbit erythrocyte. The agglutinated erythrocytes form a carpet that cover completely the well, the last effective dilutions were indicated in blue (hemagglutination titer: sample 1=16; sample 2=512). In absence of agglutinin the cell form a button in the well. CNT= control in which sample has been substitute with TBS-2.

Carbohydrate specificity and chromatographic resin selection of lectins

Hemagglutinating activity is assayed with rabbit erythrocytes in the presence of saccharides as potential inhibitors of lectin binding. When the lectin activity has been detected in the hemolymph or extract of interest, the second step is to identify those carbohydrate ligands for which the lectin show the highest affinity for matrix selection in order to allow the lectin enrichments by affinity chromatography. Inhibition experiments are carried out using decreasing concentrations (starting from 200 mM in TBS-2) of a large variety of monosaccharides that should include all four types of Mäkelä's classification (1957) plus sialic acid (i.e., L-fucose, D-arabinose D-galactose, D-glucose, D-mannose, N-acetyl-galactosamine, and N-acetyl-glucosamine), oligosaccharides (i.e., maltose, lactose, D-raffinose, cellobiose), mannan and glycoprotein. The lectin solution is incubated with each one of the sugars at different concentrations for 60 min and the rabbit erythrocytes are then added. The agglutination titer in the presence of sugars are compared to the control where the sugars are substituted with TBS-2.

The selected saccharide are coupled to the column support matrices by activation with cyanogen bromide or divinylsulfone. Often other purification steps are required for an optimal lectin purity (i.e., size exclusion, ion exchange, hydrophobic chromatography).

This additional protein purification step is an empirical process employing a large number of experiments, which often represent a limit especially for planning integrated project research. To overcome this limitation a plate- or tube-based experiments could be used avoiding the preparation of numerous packed columns, the use of large volume of sample and test on chromatography systems. This parallel screening can facilitate the selection of media and development of optimal binding and elution conditions. For this test the media slurry can be incubated with the same volume of sample (i.e., hemolymph or extract of interest) and, after 1 h, centrifuged at 400xg for 5 min to remove not binding proteins. The binding

activity can be detected analyzing the unbound proteins by electrophoresis or by centrifugation with the specific gel elution buffer for bound proteins recover.

Quantitative methods

Mollusca

Free amino acids (FAA)

The FAA has been evaluated in molluscan hemolymph (Ottaviani, 1984). The hemolymph is centrifuged at 3,000xg for 10 min and 100 μ l of the supernatant are utilized to determine the FAA using the Amino Acid Analyzer Liquimat III (Kontron, Zurich, Switzerland).

Radio-immuno assay (RIA) test

The following procedure has been utilized to quantify ACTH-like molecules in the molluscan serum and immunocytes (Ottaviani *et al.*, 1990). After hemolymph collection and the subsequently centrifugation at 600xg for 10 min, the serum is stored at -20 °C until use; the pellet must be resuspended in PBS-1, homogenized for 30 s and centrifuged at 1,500xg for 20 min at 4 °C before storing. The serum and pellet are freeze-dried, redissolved in PBS-1 and assayed via commercial RIA kits for ACTH.

High performance liquid chromatography (HPLC)

The HPLC assay has been used in the determination of the biogenic amines (BA) in molluscan serum and immunocytes (Ottaviani *et al.*, 1992, 1993, 1994, 1995a, 1997a, 1998a). After centrifugation of the hemolymph, the BA are detected by HPLC in the serum and pellet (immunocytes). The BA are extracted from the serum by using a Clin-Rep-Catecholamine kit (RECIPE Pharma Vertriebs GmbH and Co KG, München, Germany) and the extracted sample is analyzed with a rapid and sample isocratic simultaneous determination of norepinephrine, epinephrine and dopamine. Monoamine peaks are identified by comparing their retention times in the serum extracts with those of a standard solution. Each BA is quantified using the internal standard

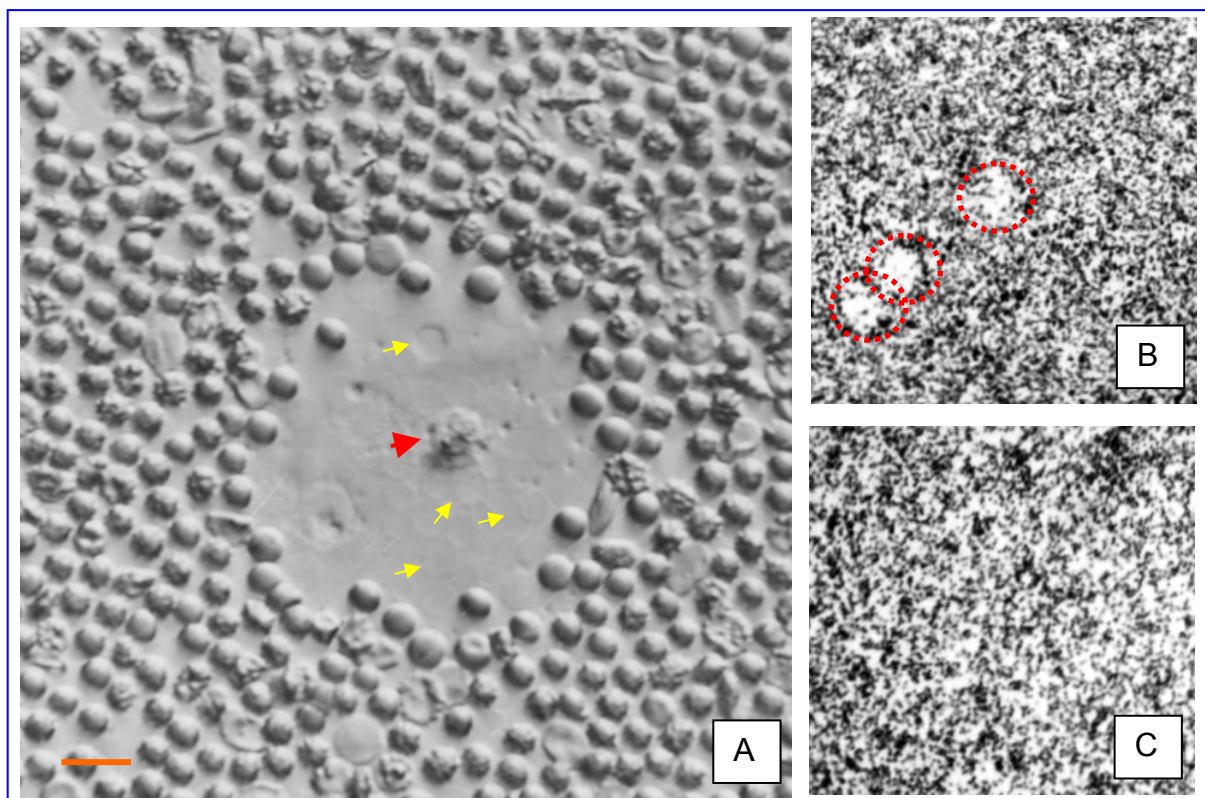


Figure 13 Plaque forming cell assay. **A:** Plaque of lysis observed by phase-contrast microscope. Red arrowhead = Plaque forming hemocytes capable of secreting lytic factors against rabbit erythrocytes; Yellow arrows = erythrocyte ghosts; Bar = 15 μ m. **B:** low magnification observation, three plaques are marked in red; **C:** control of erythrocytes.

(3,4-dihydroxybenzylamine, DHBA) method with a correction factor. The pellet is washed with SSS, homogenized with a Fisher Sonic dismembrator Model 300 (GlobalSpec Inc., Troy, NY, USA), centrifuged and the supernatant analyzed by direct injection into the chromatographic system. Identification and calculation of the BA are performed by directly comparison areas of a standard calibration curve with those of the samples. The HPLC system used consisted of a Isocratic LC Pump (mod. 250 Perkin Elmer, Waltham, MA, USA) equipped with a degasser (ERC-3512-Erma), an automatic injector (Rheodyne 7125, 100 μ l loop), an electrochemical detector (mod. 460 Waters) and a Data System PE Nelson (mod. 1020 Perkin Elmer).

Spectrofluorimetry

This method has been utilized to detect the neutral endopeptidase-24.11 (NEP)-like activity in molluscan immunocytes (Ottaviani and Caselgrandi, 1997; Caselgrandi *et al.*, 2000). The collected hemolymph is centrifuged and the NEP-like activity of the pellet is measured according to the Deschodt-Lanckman *et al.* (1990) procedure. A maximum number of 700×10^3 immunocytes/ml have to be used for a correct evaluation since above this value enzymatic activity falls quickly in relation to a given number of cells. NEP cleaves the substrate (Suc-Ala-Ala-Phe-AMC) (Sigma) to Phe-AMC. A fluorescent spectrometer (Perkin Elmer, mod. 204)

at an excitation wavelength of 440 nm is utilized to detect the fluorescent product, free AMC. The calibration curve is measured with pig NEP enzyme.

Crustacea

ELISA

To quantify the variation of cHH (crustacean Hyperglycaemic Hormone) level in the eyestalks and hemolymph of *P. elegans* ELISA test using an antibody against recombinant cHH has been performed (Lorenzon *et al.*, 2004). In this method a group of 10 *P. elegans* is used for eyestalk ablation; animals are anaesthetized for 1 min on ice before ablation. The eyestalk was quickly frozen and the pigmented eyecup dissected. Eyestalk homogenate is prepared from 20 eyestalks homogenized in 2 ml cold PBS (Sigma) pH 8.0 and then centrifuged for 1 h at 930xg at 4 °C and the pellet is discarded. Homogenates are quickly deep frozen at -20 °C and stored until required for study. Hemolymph is withdrawn from groups of 10 animals, centrifuged for 1 min at 10,300xg at 4 °C and the supernatant is then stored at -20 °C. The standards are represented by a known concentration [from 1 to 0.001 μ g in 100 μ l of PBS (Sigma)] of 6xHis-NenCHHwt (*N. norvegicus* cHH wild type) recombinant protein (Mettulio *et al.*, 2004). One hundred μ l of the samples are loaded onto a 96 microwells plate (Costar) and incubated in duplicate overnight at 4 °C. The content of the wells is discarded and the wells are washed 4 times with

250 µl of PBS (Sigma) + 0.1 % Tween20, pH 7.4 (PBS-T); then filled with 100 µl of 3 % BSA (Sigma) solution in PBS (Sigma) pH 7.4 plus 5 % fetal calf serum (Sigma) and left for 2 h at RT. The content is discarded and the plates are again washed 4 times as described above. One hundred µl of 1 µg/ml biotinylated anti-NerCHH (anti *N. norvegicus* cHH; Giulianini *et al.*, 2002) antibody is then added to each well and the plate is incubated for 3 h at 36 °C. After removal of the biotinylated antibody, plates are washed extensively with PBS-T, followed by the addition of 100 µl of streptavidin-peroxidase solution (Sigma) diluted 1:5,000 and incubated for 1 h at RT. The plates are once again washed 4 times with PBS-T and developed with 2,2'-azino-bis-3-ethyl-benzthiazoline-6-sulphonic acid solution (Sigma) in darkness for 1 h at RT (100 µl per well). The absorbance is measured in a multiwell plate reader (Anthos 2020 version 1.1, Krefeld, Germany) at 405 nm.

Tunicata

Spectrophotometric assay for detoxifying enzymes Glutathione S-transferase (GST)

This enzyme plays a multiple role in xenobiotic metabolism, catalyzing the conjugation reactions of reduced glutathione (GSH) and electrophilic xenobiotics. GST acts as a mediator of xenobiotic detoxification and it has recently been successfully used as a biomarker, since it appears to be important in oxidative stress protection (Cima *et al.*, 2002b). GST activity is determined according to the method of Habig *et al.* (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma) as substrate ($\lambda = 340$ nm, $\epsilon = 9.6$ mM⁻¹ cm⁻¹) on hemolysates obtained after sonication at 4 °C at 50 % duty cycles for 5 min of hemocytes previously exposed *in vitro* to xenobiotics, then centrifuged and resuspended in 20 mM Tris-HCl buffer, pH 7.5. The reaction is performed in a final volume of 1 ml containing 160 µl hemolysate or FSW in controls (blank), 700 µl of 100 mM K₃PO₄, pH 7.0, 70 µl of 20 mM CDNB dissolved in absolute ethanol, 70 µl of 20 mM GSH (Sigma). Absorbances are read at 10 s intervals for 2 min, and enzyme activities, measured as decrease of substrate, are expressed as nmol/min/mg protein (mean \pm SD) with the following formula:

$$\text{Enz. Act.} = \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}) \times \text{final volume}}{1000 \times \epsilon \times \text{mg protein} \times \text{ml substrate}}$$

Enz. Act. = enzyme activity
Abs = absorbance

Glutathione peroxidase (GPX)

It is an antioxidant enzyme which protects from the effects of reactive oxygen species through reduction of hydrogen peroxide (or organic peroxides) to water (or alcohols), respectively, requiring GSH consumption. It also protects phagocytes from the deleterious effects of reactive oxygen species produced during the respiratory burst. Its activity is measured by the consumption of NADPH monitored at 340 nm ($\epsilon = 6.22$ mM⁻¹ cm⁻¹) using H₂O₂ or cumene hydroperoxide as substrates, for the Se-dependent or the sum of Se-dependent

and Se-independent forms, respectively. The rate of blank reaction is subtracted from the total rate (Gunzler and Flohe, 1985). The reaction is performed in a final volume of 1 ml containing 500 µl of 0.1 M phosphate buffer, pH 7.0, 1 mM EDTA, 0.1 % NaHCO₃, 100 µl of 2.4 U/ml glutathione reductase (Sigma), 100 µl of 10 mM GSH, 100 µl hemolysate. The reaction is initiated by the addition of 100 µl of 0.03 % H₂O₂ in distilled water or 100 µl of 25 mM cumene hydroperoxide (Sigma) in ethyl alcohol, respectively. Absorbances are read at 10 s intervals for 2 min, and enzyme activities is expressed as nmol/min/mg protein (mean \pm SD) and it is calculated with the formula reported above. Protein concentration is measured according to Bradford (1976) using BSA as standard.

Spectrophotometric assay for caspases

Whole blood from large colonies of *B. schlosseri* (≥ 10 systems) that have been previously blotted dry is collected and centrifuged at 780xg for 10 min. Supernatants are discarded and the pellets resuspended in 100 µl of the lysis buffer from colorimetric activity assay kits for caspase-3 and -8 (Chemicon). After a 10 min incubation, samples are centrifuged at 10,000xg for 10 min. Supernatants, referred to as hemocyte lysates, are collected and their protein content evaluated according to Bradford (1976). In the wells of a 96-well microtiter plate, 50 µl hemocyte lysate are incubated for 1 h at 37 °C with 10 µl of the specific colorimetric substrates N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline or N-acetyl-Ile-Glu-Thr-Asp-p-nitroaniline, for caspase-3 and -8, respectively, according to the manufacturer's instructions. The release of p-nitroaniline is measured at 405 nm in a microplate reader. A p-nitroaniline reference curve is obtained by serial dilution of a 10 mM standard solution in dimethylsulfoxide. One unit of caspase activity is defined as the amount of enzyme able to cleave 1 nmole substrate per hour at 37 °C. The results are expressed as specific activities (U/mg protein).

Spectrophotometric assay for lysozyme activity

Lysozyme activity is quantified in cell lysate. In this case, hemolymph is collected from blood vessels without previous dipping in Na-citrate, placed in 1.5 ml plastic tubes, and centrifuged at 780xg for 10 min. Hemocytes are then incubated at 20 °C for 60 min in FSW and then centrifuged at 780xg for 10 min, re-suspended in 1 ml of distilled water, sonicated at 0 °C for 2 min at 50 % duty cycles, and centrifuged at 12,000xg for 15 min at 4 °C. Supernatant, corresponding to cell lysate, is collected for the lysozyme assay. Fifty µl of cell lysate, from both controls and treated hemocytes, are added to 950 µl of a 0.15 % suspension of *M. lysodeikticus* (Sigma) in 66 mM phosphate buffer, pH 6.2, and the decrease in absorbance (DA)/min is continuously recorded at 450 nm for 5 min at 20 °C, according to Santarém *et al.* (1994). Standard solutions containing 1, 2.5, 5 and 10 µg/ml lysozyme in phosphate buffer are prepared from crystalline hen egg white lysozyme (Sigma). The average decrease in absorbance/min is determined for each enzyme solution and a standard curve of enzyme concentration versus DA/min is drawn. One unit of lysozyme is defined as the amount of cell lysate equivalent to 1 µg of lysozyme, in the conditions

described above. Results are expressed as μg lysozyme/mg protein. Protein concentration in cell lysate is quantified according to Bradford (1976).

Assays for detection of reactive oxygen species *Superoxide anion*

In cytochemical assay, modified after Song and Hsieh (1994), yeast cells prepared for phagocytosis assay are resuspended in 0.3 % nitroblue tetrazolium (Sigma) in FSW. Sixty μl of this suspension are added to each culture chamber. After incubation for 60 min, hemocyte monolayers are fixed, washed and mounted as previously described for the phagocytosis assay. Positive sites stain blue. Yeast is omitted in negative controls, while 150 U superoxide dismutase (Sigma) are added to the incubation medium as a control of specificity. The superoxide anion production is evaluated as percentage of hemocytes containing dark blue spots of precipitated formazan. In spectrophotometric assay, after incubation with 0.3 % nitroblue tetrazolium the hemocyte monolayers are washed and dipped in absolute methanol for 2 min before being air-dried. Eighty μl of a solution of 2 M KOH and dimethylsulfoxide (ratio 6:7) are added to the monolayers to dissolve formazan precipitates and recovered after 5 min. The absorbance at 620 nm is then read with a microplate reader.

Hydrogen peroxide

Collected hemocytes are centrifuged at 780xg for 15 min and resuspended in a freshly prepared hydrogen peroxide reaction mixture [200 μl of 1 % phenol red and 200 μl of 200 U/mg peroxidase (grade II, Roche) in 9.6 ml of FSW] (Pick, 1986) containing yeast. At the end of the incubation period, suspensions are centrifuged at 780xg for 15 min, 100 μl of 1 N NaOH are added to the supernatants and their absorbance at 620 nm is read with a microplate reader.

Hypochlorite ions

Washed hemocytes are resuspended in 1.87 % taurine (Sigma) and yeast in FSW. After 60 min, 0.5 μl of a 50 mg/ml solution of catalase (Sigma) are added to stop the reaction and suspensions are centrifuged at 780xg for 15 min. Twenty mM KI is added to the supernatants and absorbance at 350 nm is read with a spectrophotometer (Gressier *et al.*, 1994). The thiol-containing antioxidant sodium 2-mercaptoethane sulfonate (MESNA; Sigma) is added to the incubation medium at a concentration of 10 mM as a control for specificity.

Nitrite ions

Washed hemocytes are resuspended in yeast-containing FSW and, at the end of the incubation period, they are centrifuged at 780xg for 15 min and 100 μl of supernatants are incubated for 10 min with 100 μl of Griess reagent [equal volumes of 0.1 % naphthylethylenediamine (Sigma) in distilled water and 1 % sulfanilamide (Sigma) in 5 % H_3PO_4]. The absorbance at 550 nm is then read with a microplate reader (Shen *et al.*, 1994). A precalibrated standard curve, with NaNO_2 as standard,

is used to calculate nitrite concentrations. Yeast is omitted in controls.

Protein purification and analysis

Protein analysis by mean of electrophoresis and immunoblotting is usually applied in invertebrate models following protocols that are very similar to those realized in mammals. For instance, the common SDS-PAGE method (Laemmli, 1970) followed by the blotting procedure (Towbin, *et al.*, 1979) has been successfully applied in Mollusca (Malagoli *et al.*, 2003), Insecta (Malagoli *et al.*, 2002a) and Tunicata (Gasparini *et al.*, 2008). Therefore, we report here only a method that has been applied to purify specific lectins from *B. schlosseri* hemolymph, since from protein purification onwards, the methods used do not significantly differ from invertebrate and mammalian models.

Affinity chromatography for β -galactoside-recognizing lectins

Various pools of 10-20 large (500-800) zooid colonies of *B. schlosseri* are homogenized in a homogenizer in 10 ml of a solution of 10 mM Na-benzoate (Sigma) in FSW, containing 0.1 mg/ml pepstatin and 1 mg/ml leupeptin (Sigma). The homogenate is centrifuged at 2,000xg for 10 min at 4 °C and the supernatant is collected (average protein concentration: 2.5 mg/ml). Affinity chromatography is carried out on acid-treated Sepharose CL-6B (GE Healthcare, Chalfont St. Giles, UK), previously equilibrated with PBS-3 according to Parrinello and Canicatti (1982). The column is washed with 1 M NaCl, and absorbed proteins are eluted with a solution of 0.2 M D-galactose (Sigma) in 1 M NaCl. The flow rate is kept constant at 20 ml/h, and 2 ml fractions are collected, the absorbance of which is read at 280 nm. Fractions corresponding to single absorbance peaks are pooled, dialyzed overnight against distilled water, vacuum-dried in a vacuum concentrator, and stored at -20 °C. Protein content is evaluated according to Bradford (1976).

Concluding remarks

The majority of procedures developed for invertebrates and here reported find their counterpart in vertebrates, even if modifications to the canonic vertebrate protocols are needed. However, on the whole it emerges an important observation, i.e., the conservation in invertebrate and vertebrate species of the molecules involved in the immune-neuroendocrine interactions. This allows, for instance, that antibodies raised against components of vertebrate molecules can in most cases be used also for evidencing invertebrate molecules. However, nowadays, the immunoreactivity alone cannot be considered a sufficient indication to prove the existence of homologies between vertebrate and invertebrate molecules. The fundamental results collected by mean of careful morphological observations, as well as functional assays, should be integrated with molecular-biology and/or bioinformatics approaches whenever possible.

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