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Phenotypic and functional characterization of earthworm coelomocyte subsets: Linking light scatter-based cell typing and imaging of the sorted populations

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ABSTRACT

Flow cytometry is a common approach to study invertebrate immune cells including earthworm coelomocytes. However, the link between light-scatter- and microscopy-based phenotyping remains obscured. Here we show, by means of light scatter-based cell sorting, both subpopulations (amoebocytes and eleocytes) can be physically isolated with good sort efficiency and purity confirmed by downstream morphological and cytochemical applications. Immunocytochemical analysis using anti-EFCC monoclonal antibodies combined with phalloidin staining has revealed antigenically distinct, sorted subsets. Screening of lectin binding capacity indicated wheat germ agglutinin (WGA) as the strongest reactor to amoebocytes. This is further evidenced by WGA inhibition assays that suggest high abundance of N-acetyl-D-glucosamine in amoebocytes. Post-sort phagocytosis assays confirmed the functional differences between amoebocytes and eleocytes, with the former being in favor of bacterial engulfment. This study has proved successful in linking flow cytometry and microscopy analysis and provides further experimental evidence of phenotypic and functional heterogeneity in earthworm coelomocyte subsets.

1. Introduction

Innate immune responses in earthworms are maintained by humoral and cellular components. Earthworm coelomocytes are free-circulating immune cells in the coelomic cavity (Bilej et al., 2010; Cooper et al., 2002). In the late 19th and 20th century, coelomocytes were typified first based on light-microscopic observations (Liebmann, 1942). Since then, the nomenclature of the distinct coelomocyte subtypes was rather diffuse based on the applied morphological terminology analogous to vertebrate leukocytes (Jamieson, 1981; Valembois et al., 1985).

In general, earthworm coelomocytes can be divided into amoebocyte and eleocyte subgroups. Amoebocytes are characterized by the relatively large eccentric nucleus and numerous pseudopodia. Using various hematological staining methods and/or based on light- and electron microscopic evaluations amoebocytes can be further classified (Stein et al., 1977; Linthicum et al., 1977). The described subgroups may represent various maturation stages of coelomocytes. In the case of eleocytes the picture is less complicated since these cells represent a more homogeneous group.

These immunocytes have a mesodermal origin (Engelmann et al., 2005; Vetvicka and Sima, 2009), but so far properly defined hematopoietic organs have not been identified in earthworms (Fischer, 1993). Although not confirmed, some believe

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coelomocytes are rather loosely produced by the epithelial lining of the coelomic cavity adjacent to the gut and longitudinal muscle layers (Jamieson, 1981). It is likely that the various subpopulations of coelomocytes have different maturation sites (Liebmann, 1942; Jamieson, 1981). However there is no clear consensus whether the various morphological subtypes are separate lineages or transient cells in the course of maturation.

In contrast, eleocytes (or free-floating chloragocytes) are originated from the chloragogenous tissue covering the alimentary canal (Homa et al., 2013). Eleocytes are considered as a final maturation stage of sessile chloragocytes. This hypothesis is supported mainly by morphological observations of certain intracellular structures (chloragosomes) of eleocytes/chloragocytes (Prentø, 1986). Sometimes these structures are referred to as modified, tertiary lysosomes (Molnár et al., 2012; Varute and More, 1972).

Flow cytometry is a common approach to study the two major subpopulations of earthworm coelomocytes (amoebocytes and eleocytes) as these cells can be separately gated according to their light scatter profiles (corresponding to their size and granularity). Using this approach, several authors have provided valuable data about the different functional aspects (e.g. cytotoxicity, phagocytosis, proliferation, and ecotoxicology) of earthworm coelomocytes (Cossarizza et al., 1996; Fuller-Espie et al., 2008; Homa et al., 2013; Vernile et al., 2007). However, the link between the light scatter-based two subpopulations and microscopy-based phenotyping remains obscured, with no clear consensus on the cell types encompassed in the coelomocyte populations. Here we show, by means of light scatter-based cell sorting, the two major subpopulations can be physically isolated with downstream morphological, cytochemical and functional analysis of sorted populations. Our results confirm the previously documented phenotypic features of amoebocytes and eleocytes, with the first example of performing post-sort functional assays in earthworm coelomocytes.

2. Materials and methods

2.1. Earthworm husbandry

Breeding stocks of *Eisenia andrei* earthworms were maintained at room temperature and fed with manure complemented soil. Prior to coelomocyte harvesting earthworms were placed onto moist tissue paper for overnight depuration.

2.2. Extrusion of coelomocytes

Coelomocytes were collected as described earlier (Engelmann et al., 2004) and cell numbers were evaluated by the trypan-blue exclusion method.

2.3. Flow cytometry and cell sorting

Single coelomocyte suspensions isolated from earthworms were sorted according to their forward scatter/side scatter (FSC/SSC) characteristics that reflect the differences in cell size and granularity. Collected coelomocytes were kept in *Lumbricus* balanced salt solution (LBSS) (Engelmann et al., 2005) complemented with 1% fetal bovine serum (FBS, Biowest, Nuaillé, France) and 5 mM EDTA (Sigma–Aldrich, Hungary) to avoid cell aggregation. Sorting of amoebocyte and eleocyte subpopulations was performed by FACSAria III (BD Biosciences) cell sorter. Sorted coelomocytes were collected in RPMI-1640 medium supplemented with 1% FBS and 1% penicillin and streptomycin for better survival. Data acquisition and analysis of sorting were executed by FACS DiVa Software. Pre-sort and post-sort viability was monitored by 7-Aminoactinomycin D (7-AAD, Biotium) using a FACSCalibur flow cytometer (BD Biosciences). The collected flow cytometry data were analyzed with CellQuest (BD Biosciences), FCS Express (De Novo Software, Los Angeles CA, USA) and FlowJo (Ashland, OR, USA) softwares.

2.4. Scanning electron microscopy

Sorted amoebocytes and eleocytes (10^5/ml) were deposited onto silicon wafers. After 1 h the cells were fixed in 2.5% glutaraldehyde for 30 min, and subsequently postfixed with 1% osmium tetroxide for 30 min. Following rounds of gentle washings with PBS, the cells were gradually dehydrated by increasing ethanol concentrations before incubation in 100% bis(trimethylsilyl)amine (hexamethyldisilazane or HMDS) for 3 min and air-dried completely. The samples were then sputtered with gold (nominal 30 nm coating) and imaged by scanning electron microscopy (NovaSEM 600 FEI company, the Netherlands).

2.5. Transmission electron microscopy

Sorted amoebocytes and eleocytes were fixed in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde for electron microscopic post-embedding morphological observations. The fixative was dissolved in 0.1 M phosphate buffer (pH 7.4) and the specimens were incubated for 2 h at room temperature. Samples were post-fixed with 1% osmium tetroxide for 1 h at 4 °C. For post-embedding structural observations, the cells were dehydrated and embedded into epoxy resin (Durcupan ACM, Sigma–Aldrich). Serial ultrathin (60–70 nm) sections were cut with a Reichert ultramicrotome. Grids were then washed in drops of distilled water, counterstained in uranyl acetate and lead citrate. Observation and documentation were carried out with a JEOL-1200 (JEOL USA, Inc, Peabody, MA, USA) transmission electron microscope.

2.6. Cytochemistry

Sorted coelomocyte subpopulations (80 μl of 5×10^5/ml) were spread onto glass slides using Cytospin 3 (SHANDON, ThermoScientific, Waltham, MA, USA) apparatus. Haematoxylin-eosin and May-Grünwald-Giemsa stainings were performed following standard protocols. For visualization of polysaccharides the cytospin preparations were incubated in 1% periodic acid for 10 min followed by 10 min of incubation in Schiff's reagent.

For lipid visualization the samples were fixed in 10% formalin, and were stained with Oil Red O solution (Sigma-Aldrich) for 10 min. Subsequently, 60% isopropanol was applied for differentiation of the specimens. Following the different staining procedures haematoxylin counterstaining was performed.

2.7. Enzyme cytochemistry

For acid phosphatase activity the cytospin samples were fixed in formalin and incubated for 3 h with the reaction mixture. The reaction mixture constituted of 10 mg naphthol AS-BI phosphate (Sigma–Aldrich) in dimethylformamide (DMF), a solution of 4% pararosaniline, 2% aqueous HCl and an aqueous solution of 4% $NaNO_2$ (Sigma–Aldrich). After the incubation coelomocytes were washed in sodium acetate buffer and counterstained with haematoxylin.

Alkaline phosphatase activity was monitored with the mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Biotium) in substrate buffer (100 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) for 20 min. NBT and BCIP were dissolved in 70% and 100% dimethylformamide, respectively. Nuclear