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Original article

Characteristics of coelomocytes of the stubby earthworm, *Allolobophora chlorotica* (Sav.)

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Abstract

Coelomic fluid and cells (coelomocytes) were extruded by the electric shock (1 min, 4.5 V) from field-collected individuals of *Allolobophora chlorotica* (Sav.). Coelomocytes were subjected to bright field and fluorescence light as well as electron microscopy (transmission and scanning). Results of all methods combined, revealed two distinct populations of circulating cells in the coelomic fluid: (1) autofluorescent eleocytes/chloragocytes filled with numerous large granules (chloragosomes) containing fluorophores, mainly riboflavin; and (2) amoebocytes, which can be differentiated into: (a) hyaline amoebocytes, firmly spreading in form of crinoline-like membranes; (b) granular amoebocytes with numerous dark granules spreading in form of fine pseudopodia. Flow cytometry confirmed the presence of highly complex autofluorescent cells (eleocytes/chloragocytes) and two cohorts of not-autofluorescence much less complex amoebocytes.

Keywords: Earthworms; Coelomocytes; Eleocytes; Chloragocytes; Chloragosomes; Amoebocytes; *Allolobophora chlorotica*

1. Introduction

Earthworm coelomic fluid is part of the hydrostatic skeleton, it acts as communicator between the inner and the outer milieu, plays an important role in homoeostasis maintenance and contains an abundant population of immunocompetent cells, coelomocytes [6], consisting of two cell populations of different morphology and origin, namely amoebocytes (hyaline and granular) and eleocytes [7]. There are indications that amoebocytes derive from the mesenchymal lining of the coelom [7,10], but eleocytes (chloragocytes) differentiate from chloragogen cells that cover the coelomic surfaces of the alimentary tract and major blood vessels [2]. Amoebocytes recognise and eliminate foreign material, primarily by phagocytosis and encapsulation [6,8,18,22]. Moreover they are involved in clotting, wound healing [7], cytotoxicity, inflammation [6], graft rejection, granuloma formation [6] and coelomic fluid coagulation [6,7]. Eleocytes, also called chloragocytes, contain numerous spherical granules, the chloragosomes [e.g. 2,6,18,22]. They have the capacity to store endogenous materials such as glycogen and lipids, as well as pigments [2,6], including riboflavin [14]. Riboflavin-derived autofluorescence of earthworm eleocytes/chloragocytes was recorded so far in *Dendrobaena veneta* [19], *Allolobophora chlorotica*, *Dendrodrilus rubidus*, *Eisenia fetida*, and *Octolasion* sp. [5],

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whereas autofluorescent coelomocytes are very scarce in *Lumbricus* sp. and *Aporrectodea* sp. [5,20].

Eleocytes take part in ion and pH balance of the coelomic fluid, in some aspects of nutrition and excretion, but are also associated with immune defense [6]. They are involved in encapsulation and brown body formation. Secretions of eleocytes have bacteriostatic properties. Moreover they take part in detoxification of earthworm tissues and heavy metals accumulation [6,7]. Different types of stress (i.e. heat, pollution) lead to heat shock proteins expression in coelomocytes [11,16].

Coelomocytes morphology has been described for *Lumbricus terrestris* by Cooper and Stein [7] and Linthicum et al. [18], *Eisenia fetida* by Hamed et al. [10] and for *D. veneta* by Adamowicz [1]. Flow cytometry was applied for coelomocyte characteristics [8,9]. Previously we have shown that the number and composition of earthworm coelomocytes are species-specific. It changes during the annual cycle [15] and in response to various adverse factors, e.g. soil pollution [11].

A. chlorotica (green or yellow stubby worm) is widespread, found in a wide range of soil types, mainly in wet gardens, fields, pastures, forests, and in manure. This species is easily to recognise by its muddy green or yellow colour and sucker-like *tubercula pubertalis* [13]. It belongs to a group of species with an abundant population of riboflavin-containing autofluorescent eleocytes [5,14,20].

The objective of our present study was to analyse morphological characteristics of *A. chlorotica* coelomocytes.

2. Materials and methods

2.1. Animals

Sexually mature (clitellated) earthworms, *A. chlorotica* (Sav.) (0.29 ± 0.08 g) were field-collected from uncontaminated soil of the garden of the Institute of Zoology, Jagiellonian University in Krakow. They were transferred to the laboratory and maintained for at least 4 weeks in original soil with fallen leaves at a temperature of 22 °C (within the range of thermal optimum for this species) [17] and at a 12:12 h light/dark regime [15,17].

2.2. Coelomocyte retrieval

Coelomocytes were extruded via dorsal pores by electrical stimulation (1 min, 4.5 V) in extrusion fluid (0.9% NaCl solution supplemented with 2.5 mg/ml EDTA to prevent cell aggregation) [8,21], counted in a haemocytometer and adjusted to about 5×10^5 cells/ml after centrifugation.

2.3. Light microscopy analysis

Extruded coelomocytes were transferred to cytocentrifuge funnels connected to microslides. After centrifugation (5 min in 1900 rpm; Hettich Universal, Germany), cells were fixed in 2% formalin, stained with May–Grünwald–Giemsa (MGG method; [4]) and analysed by light microscopy (Jenamed 2, Carl Zeiss Jena). Cells areas were measured with microscope accessory camera (Bischke, Switzerland) and Multi-Scan 5.10.

2.4. Scanning electron microscopy (SEM) analysis

Coelomocytes spread on coverslips during a short incubation were fixed in 2.5% glutaraldehyde for 2 h at 4 °C, and routinely processed for scanning electron microscopy using a BS-300 SEM (Tesla, Bruno) [12].

2.5. Transmission electron microscopy (TEM) analysis

Cell-containing coelomic fluid was fixed in 2% glutaraldehyde in HBSS for 2 h and centrifuged at 2500 rpm for 2 min. The cell pellets were postfixed in 1% osmium tetroxide, ethanol dehydrated and embedded in Epon. Ultrathin sections were contrasted with 0.5% uranyl acetate and 2.5% lead citrate solutions and analysed in Tesla BS-500 electron microscope, as described elsewhere [3].

2.6. Flow cytometric analysis

Cell samples were analysed with a FACScalibur flow cytometer (BD Biosciences). During analytical experiments, 10,000 thresholded events per sample were collected, with side scatter (for cell complexity/granularity), forward scatter (for cell size or FL1 fluorescence) on histograms. The resulting files were analysed by using WinMDI 2.8 software.

3. Results and discussion

Several methods applied in the present work allowed to distinguish three prominent populations of cells freely floating in the coelomic fluid as illustrated in Fig. 1. These are eleocytes/chloragocytes (E) and