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RESTORATION OF COELOMOCYTES IN THE EARTHWORM *DENDROBAENA VENETA*

MAŁGORZATA KLIMEK*, JERZY KRUK** AND BARBARA PŁYTYCZ*[1]

** Department of Evolutionary Immunology, Institute of Zoology, Jagiellonian University, Gronostajowa 9, 30-387 Cracow, Poland*
*** Faculty of Biochemistry, Biophysics, and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Cracow, Poland*

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We have previously shown that *Dendrobaena veneta* belongs to the earthworm species which possesses two main cohorts of coelomocytes, namely amoebocytes and autofluorescent eleocytes, the latter storing moderate amounts of riboflavin. The aim of the present experiments performed at 17°C was to follow the restoration of amoebocytes and eleocytes, as well as riboflavin stores in eleocytes after experimental extrusion of coelomocyte-containing coelomic fluid through the dorsal pores induced by electrostimulation (4.5V, 1 min). The analyses were conducted using a combination of cell counts, flow cytometric detection of eleocytes, and spectrofluorimetric measurements of riboflavin in coelomocyte lysates. It has been found that the depleted coelomocyte system recovers slowly. The number of amoebocytes reaches the level characteristic of that in undisturbed worms in 4 weeks, while the number of elecytes is still below the control level 6 weeks after extrusion. The amount of riboflavin stored in recovering chloragocyte-derived eleocytes is higher than that in mature eleocytes for at least 4 weeks.

Key words: Earthworms, coelomocytes, amoebocytes, eleocytes, riboflavin

INTRODUCTION

In natural and semi-natural conditions *Dendrobaena veneta* inhabits dung and compost heaps. It is also cultured on municipal sludge (LOEHR et al., 1985; LOFTS-HOLMIN, 1986) or paper sludge (FAYOLLE et al., 1997) for the fishing bait market and/or vermicomposting (for a review see MORGAN, 2010; YADAV and GARG, 2011). Composting species, like *D. veneta* and *Eisenia sp.,* consuming a wide range of organic wastes are often exposed to high diversity of microorganisms; thus, their immune system is strongly challenged by microbial antigens and various sorts of pollutants and has to be very efficient to cope with them successfully. Therefore it is worthwhile to explore the mechanisms of immunity of earthworm species adapted to such extreme antigenic challenges.

Earthworm immunity comprises cellular and humoral defense mechanisms, recently reviewed

[1] e-mail: barbara.plytycz@uj.edu.pl

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by Bilej et al. (2011). Like other annelid invertebrates, earthworms possess the metameric coelomic cavity filled with coelomic fluid containing various antimicrobial factors like lysozyme and antimicrobial peptides and free wandering cells named coelomocytes. The earthworm coelomocytes consist of amoebocytes, being classical immunocytes (according to Ottaviani's nomenclature, 2011), plus a species-specific portion of eleocytes, being detached chloragocytes derived from the chloragogen tissue that surrounds the intestine. The chloragocytes/eleocytes, but not amoebocytes, exhibit autofluorescence, which predisposes them to flow cytometric analysis (Cholewa et al., 2006). This autofluorescence is restricted to chloragosomal vesticles (Plytycz et al., 2007) and comes from – among other sources – riboflavin (Koziol et al., 2006; Cygal et al., 2007). Riboflavin (vitamin B2) plays an important role in immunity of animals (e.g. Powers, 2003; Verdrengh and Tarkowski, 2005) and plants (Dong and Beer, 2000; Asai et al., 2010), and it is responsible for quorum sensing in bacteria (Rajamani et al., 2008; Atkinson and Williams, 2009). The amount of riboflavin stored in eleocytes is species-specific (Plytycz et al. 2006) and changes in a species-specific manner in response to various edaphic factors (Plytycz et al., 2011; Plytycz & Morgan, 2011).

Coelomocyte-containing coelomic fluid can be expelled through dorsal pores during convulsive body movements of worms stressed by various mechanical stimuli (e.g. predators) or chemical factors. The presence of dorsal pores is commonly exploited for the non-invasive quantitative retrieval of coelomocyte-containing coelomic fluid of worms stimulated by alcohol (Cooper et al., 1995), ultrasounds (Hendawi et al., 2004) or mild electric current (Roch et al., 1979), the latter procedure being applied also in the present studies.

Dendroabena veneta belongs to the species with a high number of eleocytes storing moderate amounts of riboflavin which is also accumulated in chloragocytes of the chloragogen tissue (Mazur et al., 2011; Plytycz and Morgan 2011). We have previously shown that restoration of the coelomocyte number of *D. veneta* after experimental extrusion is a long-lasting temperature-dependent process (Olchawa et al. 2003). The aim of the present research is to continue these investigations focusing on restoration of riboflavin-storing eleocytes.

MATERIALS AND METHODS

Earthworms

Adult *Dendrobaena veneta* (Oligochaeta; Lumbricidae), purchased from the commercial supplier (EKARGO, Słupsk), were reared in commercial soil (PPUH BIOVITA, Tenczynek) under controlled laboratory conditions (17°C; 12:12 LD). The worms were kept in plastic boxes with perforated lids and the moisture level was checked weekly. The worms were fed *ad libitum* a mixed diet comprised of dried/boiled nettle (*Urtica dioica*) and dandelion (*Taraxacum officinale*) leaves, boiled/dried tea leaves, and powdered commercial mouse pellets. The experiments were performed in May-June.

Restoration of the coelomocyte system

At the start of the six-week experiments, 30 individuals with similar body weight (1.27+0.23 g) were put into 5 separate boxes with fresh soil covered with perforated lids, forming 5 experimental groups, 6 worms per group. The worms were subjected to electrostimulation for coelomocyte extrusion, at various intervals of time before the end of the experiments. Thus, they had various time periods for recovery of their coelomocyte systems: 6 weeks (6W), 4 weeks (4W), 2 weeks (2W), and 1 day (1D). After 6 weeks from the start of the experiments all these animals, their body weight reaching 1.66+0.33 g, were once again subjected to electrostimulation to assess the recovery of the coelomocyte system, while the intact animals from the last box were electrostimulated for the first time and served as the control group with no time to recover (0D) (Fig. 1).

Coelomocyte extrusion

The earthworms were stimulated for 1 minute with an electrical current (4.5V) to expel coelomic fluid with suspended coelomocytes through the dorsal pores. Briefly, the weighed earthworms were individually placed in Petri dishes containing 3 mL of extrusion fluid (phosphate-buffered saline, PBS, supplemented with 2.5 g/L ethylenediamine tetra-acetic acid, EDTA; Sigma-Aldrich); 2mL samples of the extruded coelomocyte suspensions

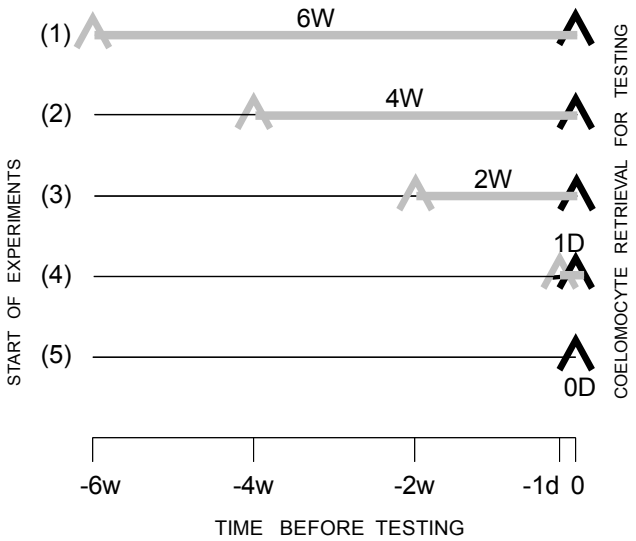

Fig. 1. Scheme for experiments on restoration of coelomocytes of *Dendrobaena veneta* depleted by extrusion induced by electrostimulation. At the start of the experiments 30 worms with similar body weight were divided into 5 experimental groups (6 worms per group) and put into fresh soil samples. The worms from groups 1-4 were stimulated for coelomocyte extrusion at selected time points (grey marks), i.e. 6 weeks (-6w), 4 weeks (-4w), 2 weeks (-2w) or 1 day (-1d) before the end of the experiments at time 0, while group 5 served as the control. At the end of the experiments (time 0) coelomocytes from all the five groups of worms were extruded by electrostimulation (black marks) and comparisons were made between the control worms stimulated for the first time at time 0 (0D, control) and those restoring their immune systems for 6 weeks (6W), 4 weeks (4W), 2 weeks (2W), or 1 day (1D) (thick lines).

were used for spectrofluorimetric analysis, while 1 mL was fixed in 2% formalin (Sigma) and used for cell counts in haemocytometer and for flow cytometry.

Flow cytometric measurement and analysis

Samples of coelomocytes were analysed with a FACScalibur flow cytometer (BD Biosciences). During analytical experiments, 10000 thresholded events per worm sample were collected and analysed on the basis of their forward scatter (FS) (for cell size) and sideward scatter (SS) (cell complexity) properties. Fluorescence FL1-H (emission 530 nm; excitation 488 nm) was recorded. The resulting files were analysed for percentages of autofluorescent granulated eleocytes using WinMDI 2.8 software (Joe Trotter, http://facs.scripps.edu), by producing dot plots of cell size versus FL1 autofluorescence.

Spectrofluorimetry and analysis

Spectrofluorometric measurements were performed on 2 mL coelomocyte-suspension lysates (lysed with 2% Triton; Sigma-Aldrich) using a Perkin-Elmer LS50B spectrofluorimeter. Emission spectra of riboflavin were recorded in the 380-680 nm range (lambda at 370 nm), while excitation spectra were recorded in the 300-500 nm range (lambda at 525 nm). The spectrofluorimetric signatures of unbound riboflavin were characterised by two maxima (at 370 nm and 450 nm) in the excitation spectrum, and a maximum at 525 nm in the emission spectrum. Arbitrary units (AU) of fluorescence were recorded using Microsoft Excel v. 97. The amount of riboflavin in the sample was proportional to the maximum at 525 nm in the emission spectrum.

Statistical analysis

Coelomocyte-connected parameters were calculated using Microsoft Excel version 97. The results concerning restoration of coelomocyte systems were expressed as means +/- standard errors. Differences between the means were determined by ANOVA (STATGRAPHICS Plus 5.0), with the level of significance established at $P<0.05$.

RESULTS

As illustrated in Fig. 2, full restoration of the number of *D. veneta* coelomocytes after experimental depletion by extrelectrostimulation is a long-lasting process (Fig. 2a,b,c). Under the conditions of the present experiments, the number of coelomocytes (CN), including both amoebocytes (AN) and eleocytes (EN), is statistically significantly lower compared with the respective control (black bars), both after one-day (1D) and two-week (2W) recovery periods. After the 4-week recovery period (4W) the total number of coelomocytes did not differ significantly from the number of coelomocytes in worms after the shorter (1D and 2W) and longer (6W) periods after extrusion (Fig. 2a). Neverthe-

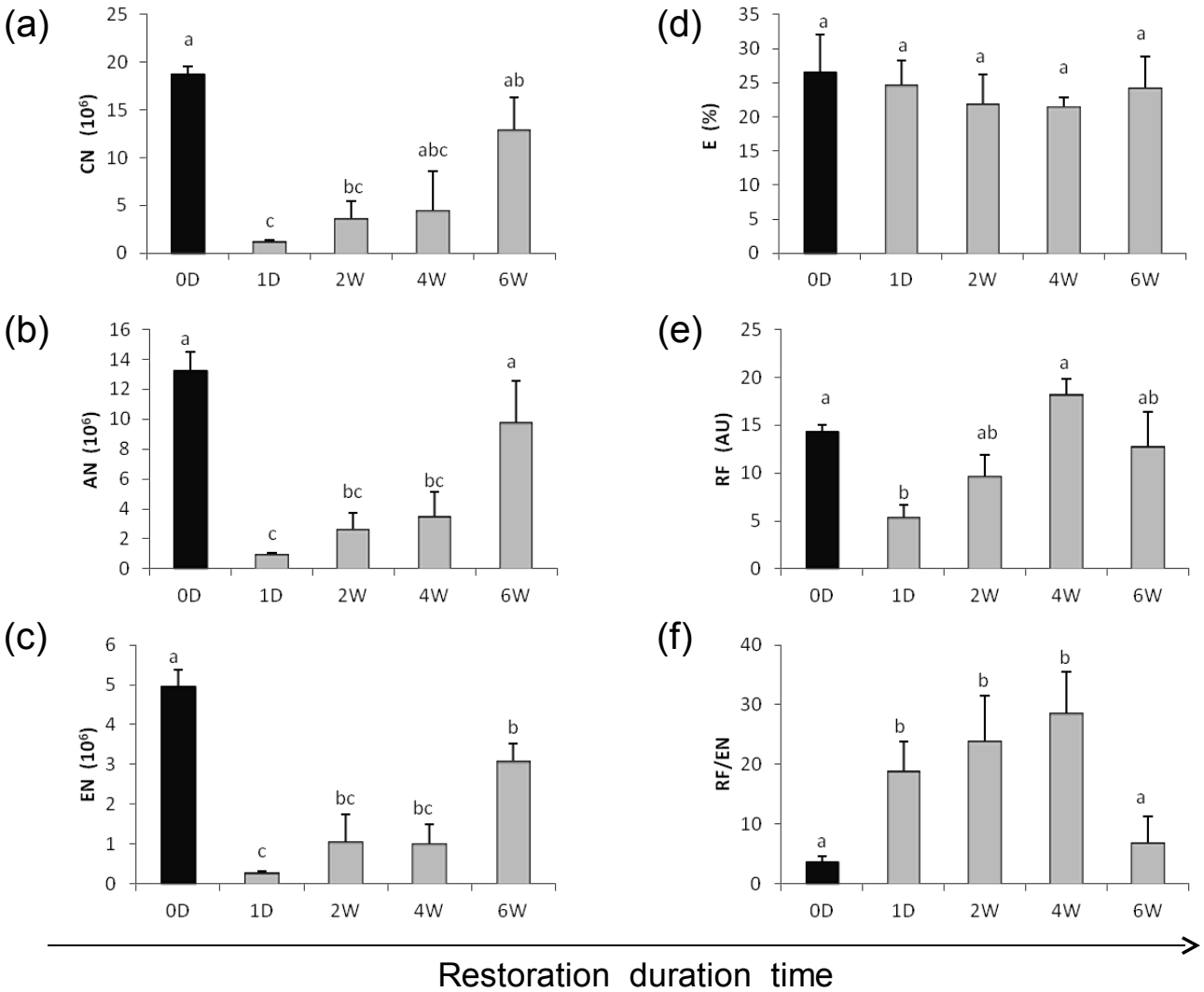

Fig. 2. Kinetics of restoration of the coelomocyte system in *Dendrobaena veneta.* Comparisons of *D. veneta* coelomocytes retrieved from the intact control worms electrostimulated for the first time (black bars, 0D) with those retrieved at the selected time intervals after experimental depletion by previous electrostimulation (grey bars) performed 1 day (1D), 2 weeks (2W), 4 weeks (4W), or 6 weeks (6W) earlier. (a) number of colomocytes (CN); (b) number of amoebocytes (AN); (c) number of eleocytes (EN); (d) percentage of autofluorescent granular cells, the eleocytes (E, %), established by flow cytometry; (e) total riboflavin content (RF) in arbitrary units (AU) established by spectrofluorimetry; (f) riboflavin content per eleocyte (RF/EN). The data are presented as means ±SE; n = 6 worms per group. Means not sharing the same small letter at error bars are significantly different according to ANOVA.

less, after the 4-week recovery period the number of amoebocytes (AN, Fig. 2b) and eleocytes (EN, Fig. 2c) was still lower as compared with the respective intact worms. It was the 6-week recovery period (6W) that was necessary for electrostimulated experimental worms to restore the number of coelomocytes (CN, Fig. 2a), including amoebocytes (AN, Fig. 2b), to the levels similar to those characteristic of their intact counterparts (black bars in Figs 2a and 2b, respectively); however, the number of eleocytes was still significantly reduced (Fig. 2c).

In contrast, the percentages of autofluorescent eleocytes established by flow cytometry were relatively stable and similar for the worms recovering the eleocyte system after electrostimulation and the intact control worms (Fig. 2d). The amount of riboflavin in coelomocyte lysates was statistically significantly diminished only 1 day after expulsion (Fig. 2e) and reached the level similar to that in

the control worms already one week later, despite of the long-term significant depletion of the number of riboflavin-storing eleocytes (compare with Fig. 2c). It can be explained by the fact that the amount of riboflavin per eleocyte (RF/EN, Fig. 2f) was significantly increased after electrostimulation-induced coelomocyte expulsion, and decreased till the low level characteristic of the intact worm eleocytes not sooner than 5 – 6 weeks of the 6-week recovery period (Fig. 2f).

DISCUSSION

Pioneer experiments on the restoration of coelomocytes experimentally extruded by worm immersion in 5% ethanol solution were performed on *Lumbricus terrestris* by Eyambe et al. (1991). The results revealed that in adult worms kept at 10°C the initial number and characteristics of coelomocytes, called in their paper the leukocytes, was achieved not sooner than 6 weeks after extrusion, while at shorter time intervals after extrusion the total number of coelomocytes was significantly lowered. The authors concluded that after initial extrusion, sequential leukocyte collection without affecting total and differential cell counts was possible at intervals of 6 weeks (Eyambe et al. 1991).

Lumbricus terrestris belongs to the earthworm species with a small contribution of chloragocyte-derived eleocytes among coelomocytes freely floating in the coelomic cavity which contains almost exclusively amoebocytes (Cholewa et al. 2006; Plytycz et al. 2006). In contrast, *Dendrobaena veneta* used for the present investigations belongs to lumbricid worms with coelomocytes consisting of amoebocytes plus an abundant population of eleocytes (Cholewa et al. 2006), the latter exhibiting distinct autofluorescence (Plytycz et al. 2006) derived from moderate amounts of riboflavin accompanied by another unidentified fluorophore (Cygal et al. 2007). In one of previous experiments *D. veneta* coelomocytes were sequentially 8 times extruded day by day by electric shock without adverse effects on worm viability, which was followed by cell proliferation (Homa et al. 2008).

The kinetics of restoration of *D. veneta* coelomocytes was preliminarily described by Olchawa et al. (2003). It turned out that this process was temperature-dependent. At 22°C the number of coelomocytes reached the level characteristic of undisturbed control worms as soon as 4 weeks after expulsion, while in worms maintained at 10°C only half the number of control cells, even 6 weeks after expulsion, was observed.

In other studies on *D. veneta* maintained at 16°C the coelomocyte system was compared between undisturbed worms and their counterparts subjected to coelomocyte expulsion 7 weeks earlier (Polanek et al. 2011). It turned out that the total number of coelomocytes, including amoebocytes, was similar for the control and experimental worms, while the percentage and the number of eleocytes were significantly lowered in the experimental worms. Such delayed restoration of the eleocyte system was observed both in worms fed ad libitum a mixed diet and in those deprived of external food supply. The 7-week food deprivation inhibited worm body weight gain without adverse effects on the number and composition of coelomocytes. The main novelty of the work of Polanek et al. (2011) consisted in the comparisons made between the level of riboflavin in undisturbed worms and in those tested 7 weeks after coelomocyte expulsion. In the experimental worms fed ad libitum there was only a slight diminution of riboflavin in worms restoring the coelomocyte system, both the total riboflavin content and that adjusted per body weight (RF/BW), while the amount of riboflavin adjusted per eleocyte number (RF/EN) was slightly increased. These tendencies were much stronger in worms deprived of external food supply, which was statistically significant for total riboflavin content in the coelomocyte lysates. This suggests that the newly formed eleocytes freshly detached from the chloragogen tissue accumulate more riboflavin per cell than the old eleocytes which have inhabited the coelomic fluid for a long time as freely floating cells.

The latter result has prompted us to repeat the studies on kinetics of restoration of *D. veneta* coelomocytes, which has been described in the present paper. As illustrated in Fig. 2, during the experiments performed at 17°C, i.e. the temperature intermediate between the temperatures used previously by Olchawa et al. (2003), the restoration of coelomocytes was faster than it was at 10°C, but slower than at 22°C. The number of amoebocytes reached the control level within 6 weeks, while the number of eleocytes was still below the control level, the result corresponding to

that obtained during 7-week studies performed by Polanek et al. (2011). In contrast to the latter, the percentages of eleocytes were relatively stable during the present 6-week experiments. The amount of riboflavin was significantly diminished only one day after coelomocyte extrusion but it reached the control level already after 2 weeks, which may be explained by increased riboflavin accumulation in newly formed eleocytes. The latter result is fully consistent with our previous assumption (Polanek et al. 2011). Studies on riboflavin trafficking within earthworm bodies are necessary to explain this phenomenon in detail.

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