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Acute-Sublethal Copper Effects on Phagocytosis and Lysozyme Activity in the Earthworm *Amynthas hawayanus*

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Soil environments are often subject to chemical contamination, threatening the survival of resident biota. The development of invertebrate models for screening the toxicity of xenobiotics is of great interest for environmental risk assessment, due to the importance of such organisms in most terrestrial ecosystems. The immune system of earthworms has good potential for use in assessing the biological risk of terrestrial contaminants. Certain aspects of their immune responses are similar to those in higher organisms. In *Lumbricus terrestris, Eisenia foetida,* and *Eisenia hortensis,* lysozyme activity and phagocytosis have demonstrated good sensitivity as biomarkers for testing the immunotoxic impact of chemical contamination (Chen *et al.* 1991; Goven *et al.* 1994; Weeks and Svendsen 1996; Ville *et al,* 1995, 1997; Giggleman *et* al. 1998). Although biomarkers reflect a critical link between chemical exposure, internal dose, and health, other characteristics, such as physiological status and genetic factors influence the responses of lysozyme and phagocytosis to chemical exposure. Thus, it is necessary to validate, for each target species, the parameters that are indicative of dysfunction, and establish their specificity and sensitivity, as well as the appropriate methods of measurement.

Among annelids, *Amynthas hawayanus* appears to be very sensitive to long-term, sublethal, chemical exposure, limiting its use for chronic toxicity studies. However, it is amenable for short-term experiments. This species is commonly found in tropical soil ecosystems (Drachenberg 1992), and exposed to chemical contamination in agricultural areas treated with pesticides. Thus, this organism is an appropriate choice for the development of immune based-biomarkers for monitoring environmental quality. Nonetheless, its physiological responses to environmental stress have not been extensively examined. This study presents immunoassays for testing acute effects of metals on nonspecific immune functions of earthworm based on coelomic fluid lysozyme activity and oxidative antimicrobial activity of coelomic phagocytes. Phagocytic activity was measured by a reduction of nitroblue tetrazolium (NBT) dye using a modification of the previously standardized procedure for *L. terrestris* (Chen *et al.* 1991). Copper was used as the reference immunosuppressor and was applied by contact with a filter paper. This metal is an environmental immunotoxicant for vertebrates and invertebrates, affecting their ability to mount normal protective responses (Anderson *et* al. 1994; Goven *et al.* 1994; Mushiake *et al.* 1985; Nusetti *et al.* 1998; Dethloff and Bailey 1998).

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MATERIALS AND METHODS

Adult earthworms were maintained in a moist block of a soil/horse manure culture system at 25°C before experimental use. Twelve earthworms were individually exposed for 48 h to a nominal Cu^{+2} concentration of 0.75 µg cm^{-2} (from 1 mL of Cu $SO_4 5H_2O$ stock solution) applied to a 6-cm diameter Whatman (N° 1) filter paper disk in an 0.5 L glass jar. Each jar was covered with aluminum foil held in place with rubber bands and incubated in darkness at 25°C. Control earthworms (n=12) were prepared identically, but without exposure to copper. The jars were opened every 12 hours and earthworms were examined for morbidity and mortality; 100% survival was observed for the experimental treatment. Following the exposure period, copper content in the worm carcasses was determined by flame atomic absorption spectrophotometry using a Varian AA-20 Plus as previously described by Marcano *et al.* (1997). The copper exposure concentration represented 50% of the calculated 48 h median lethal concentration (LC_{50}: 1.50 µg $Cu^{+2} cm^2$, 95% confidence limits: 1.00-5.00 µg $Cu^{+2} cm^2$) estimated by an LC_{50} computer program developed by Stephan (1977). The copper concentration for the 48 h toxicity test was selected because it was found in preliminary experiments to be the highest Cu level ensuring maximal survival.

Coelomocytes from experimental and control earthworms were obtained by a non-invasive extrusion procedure previously developed for use in immunotoxicity studies (Eyambe *et al.* 1991). Briefly, individual worms were rinsed and their posterior portion was massaged to expel the gut contents and to prevent fecal contamination. Each worm was then placed in a 10-cm diameter Petri dish containing 3 mL of extrusion medium, consisting of 5% ethanol and 95% saline supplemented with 1.25 mg mL^{-1} disodium EDTA and 10 mg mL^{-1} of guaiacol glyceryl ether (Sigma Chemical Co., St. Louis, Mo., USA) and adjusted to pH 7.3 with NaOH. Earthworms were bathed in the extrusion fluid for a maximum of 3 min. The extruded cells were immediately transferred into a test tube containing 3 mL of calcium-free Lumbricus Buffered Saline Solution (LBSS), washed, and centrifuged at 250 x g three times, and resuspended in 1.0 mL LBSS at 4°C at a concentration of 1 x 10^6 cells mL^{-1}. LBSS is composed of 71.5 mM NaCL, 4.8 mM KCL, 3.8 mM $CaC1_2$, 1.1 mM $MgSO_4 7H_2O$, 0.4 mM KH_2PO_4, 0.3 $Na_2HPO_4 7H_2O$, 4.2 mM $NaHCO_3$ in distilled water with the pH adjusted to 7.3 with NaOH (Eyambe *et al.* 1991). Coelomocytes from each earthworm were processed separately for the phagocytosis assays.

Coelomocyte counts from each worm were made using a Neubauer hemocytometer following standard procedures (Eyambe *et al.* 1991) and were expressed as the mean ± SD per mL. Cell viability was examined by staining with 0.4 % Trypan blue (Sigma Chemical Co.) in LBSS and reported as the percentage of live cells at counting. Cell-free coelomic fluid was collected for measuring lysozyme activity by the method of McHenery *et al.* (1979). The coelomic fluid from individual earthworms (controls and experimentals) whose guts had been emptied was obtained by inserting a Pasteur pipette through a small incision on the abdominal surface directly behind the clitelum. Prior to this treatment, the organisms were cold anesthetized in a Petri dish on an ice block and rinsed with cold distilled water. The

collected fluid was transferred to a 3-mL plastic tube, where it was diluted with an equal volume of potassium phosphate buffer, pH 6.2. Afterward, the tubes were centrifuged at 200 x g for 10 min at 25°C. The supernatant was tested for lysozyme activity.

The phagocytic activity of coelomocytes was recorded by a modified NBT assay for leukocytes from earthworms, mice, and humans (Chen *et al.* 1991). Thus, 10^5 cells in 0.1 mL of coelomocyte suspension in LBSS and 0.1mL of non-viable bacterial extract (Stimulant, Sigma Chemical Co., St. Louis, Mo.) were mixed in 3 mL plastic tube, and incubated and gently shaken for 15 min at 25°C. Afterwards, 0.1 mL NBT (2 mg mL^{-1} LBSS) was added to the mixture and then incubated for 24 h at 16°C. Then, 0.2 mL of 0.1% hexadecytrimethyl ammonium bromide (CTMAB, Sigma, Co.) and 0.4 mL 100 mM potassium phosphate buffer, pH 6.2 were added to the tube, and incubated for an additional 3 h at 25°C in order to rupture cell membranes. The content of the tubes were centrifuged at 200 x g for 15 min and the pellet containing reduced NBT was dissolved in 1.0 mL dimethyl sulfoxide (DMSO, Sigma Chemical, Co.). The optical density (OD) of the extract was determined at 510 nm using a Beckman spectrophotometer. MTT formazan (Sigma Chemical, Co.) was used to establish a calibration curve with a detection limit of 0.1 µg mL^{-1}. Control assays were conducted for a reagent blank and for endogenous reduction of NBT. The DMSO extraction efficiency was over 95%, using MTT as the reference standard. The addition of 100 units of superoxide dimutase, from bovine erythrocytes (Sigma Chemical Co.) to the incubation mixture was used to test the specificity of NBT reduction.

Lysozyme activity was determined by the method of McHenery *et al.* (1979) using 40 µL of coelomic fluid dispensed into 5-mm diameter wells in 1% agarose in 5-cm diameter Petri dishes. The agarose contained 100 mM phosphate buffer pH 6.2 and *Micrococcus lysodeikticus* (0.6 mg freeze-dried cells mL^{-1}). After incubation for 48 h at 27°C the diameter of zone of lysis was measured and lysozyme concentration was determined by reference to a calibration curve established with hen egg-white lysozyme (HEL Sigma Chemical Co.). The results were calculated by the following regression:

$$\text{HEL-equivalent } (\mu g \ rnL^{-1}) = \text{antilog10 } \{a + b \text{ (diameter, mm)}\}$$

The phagocytic and lysozyme activities of the experimental and control groups were compared using a Student's t test (Sokal and Rohlf 1979).

RESULTS AND DISCUSSION

The body copper concentration increased from 7.9 ± 1.6 in controls to 67.0 ± 19.4 µg Cu g^{-1} dry mass in copper-exposed worms. Such copper accumulation resulted in marked effects on the earthworm's immune system. The mean values for the total count and viability of coelomocytes changed significantly between copper-exposed and non-exposed worms (Table 1). The decrease in total count and viability of coelomocytes caused by copper exposure suggests a stress that could compromise the

organism's immunocompentence. The mechanisms causing this decrease are not understood.

Table 1. Total counts and viability of coelomocytes from Cu-exposed and control earthworms. Results are expressed as the mean ± SD (n=12)

Exposed groups	Total cell count mL^{-1}	Cell viability (%)
Non-exposed worms	$2.8 \times 10^6 \pm 1.3 \times 10^6$	79.8 ± 7.2
Cu-exposed worms (0.75 µg Cu^{2+} cm^{-2})	$1.7 \times 10^6 \pm 0.7 \times 10^6$	70.4 ± 9.6
Statistical significance:	$t = 2.15$ ($p<0.05$)	$t = 2.46$ ($p<0.05$)

The tests for detecting endogenous and non-specific reduction of NBT to formazan were negative, confirming that coelomocytes produced the oxygen anion ($O°_2$) during phagocytosis. Copper-exposed earthworms showed significantly lower ($p< 0.001$) phagocytic activity than controls, and the coelomic iysozyme activity increased approximately 2-fold after copper exposure (Table 2).

Table 2. Immunotoxicity response to copper in *A. hawayanus.* Results are expressed as mean ± SD (n=12)

Exposed group	Phagocytic activity (µg formazan mL^{-1})	Lysozyme activity (µg mL^{-1})
Non-exposed worms	4.3 ± 1.0	5.04 ± 4.6
Cu-exposed worms (0.75 µg Cu^{2+} cm^{-2})	2.4 ± 0.7	12.7 ± 6.5
Statistical significance:	$t = 7.22$ ($p<0.001$)	$t = 3.50$ ($p<0.001$)

The inhibition of phagocytosis by copper uptake might reflect a weakened ability of the coelomocytes to engulf antigens. This could be due to alterations of cellular structures which could impair the interactions with antigens at the cell surface, or to a decreased production of agglutinant factors (opsonins). Changes in phenotypes of the phagocytic cell population could also be responsible for this impaired response.

Alternatively, copper exposure could perturb the biochemical mechanisms which underlie the phagocytic activity. Phagocytosis is normally activated by antigenic stimuli, such as bacteria, responding with the production of the superoxide anion ($O°_2$) and other reactive intermediates to destroy invasive agents. This process is sensitive to heavy metal contamination in vertebrates and invertebrates (Elaser *et al.* 1986; Anderson *et al.* 1992 and 1994; Chen *et al.* 1991; Goven *et al.* 1994). Chen *et al.* (1991) reported a lowered *in vitro* NBT dye reduction, measured as OD 515 nm, by bacterial phagocytosing coelomcytes from the earthworm *L. terrestris* sublethally

exposed to increasing concentrations of refuse-derived fly ash (RDFF: commercial soil mixtures). Tissue levels of Cu and Pb increased with RDFF concentration, and resulted in suppression of NBT dye reduction at maximal Cu body uptake 13.10 ± 1.44 µg g^{-1} dry mass. Sublethal inhibitory effects of Cu on *in vitro* phagocytosis have been observed for the polychaete *E. complanata* (Nusetti *et al.* 1998). These observations suggest that non-specific cellular immune responses of annelids may provide adequate sensitivity for toxicity analysis of heavy metals in terrestrial and aquatic ecosystems.

The presence of lysozyme in the coelomic fluid of control *A. hawayanus* may protect the organism from bacteria in its environment and control its natural symbiotic flora. This also appears to be the role of lysozyme in the coelomic fluid and hemolymph of other invertebrates (Perin and Jolles 1972; McHenery *et al.* 1979; Hirigoyemberry *et al.* 1990; Hawking *et al.* 1993; Marcano *et al.* 1997). Interestingly, the inhibition of phagocytosis by Cu-exposure was associated with an increased concentration of lysozyme in *A. hawayanus.* This may represent a compensatory immune response to facilitate survival when the chemical stressors reduced cell-mediated immune defenses. Similar responses have been described for other species of earthworms exposed to PCBs (Ville *et al.* 1995). Sublethal exposure to PBCs inhibited coelomocyte phagocytosis and wound healing, but increased antibacterial activity. Furthermore, in the fish *Oncorhynchus mykiss,* lysozyme activity rose while phagocytosis decreased after 5-d exposure to distinct concentrations of Hg, Cd, and Zn (Sanchez-Dardon *et al.* 1996). Conversely, we observed that *E. foetida* exhibited a significant reduction in both phagocytosis and lysozyme after 5-d sublethal Cu-exposure (1.0 µg Cu^{2+} cm^{-2}) (unpublished data, 17th Annual Meeting of the Society of Environment, Toxicology and Chemical, 1996, Washington, D.C.). Chen and Roderick (1974) found that exposure to Pb and Cu decreased lysozyme activity in the hemolymph of the bivalve *Mya arenaria.* Furthermore, 5-d sublethal Cu-exposure lowered the coelomic lysozyme concentration in *L. terrestris* (Goven *et al.* 1994). Ville *et al.* (1997) reported differential immunomodulatory effects of the pesticides carbaryl and 2,4-dicholorophenoxyl acetic acid on the humoral and cellular defense of *E. foetida andrei* in 48 h exposure experiments.

In conclusion, sublethal suppressive effects of Cu on the cell-mediated immune system have been well established for annelids, and also appear to occur in vertebrates. Lysozyme activity shows different responses to chemical contamination in different species, perhaps reflecting distinct physiological and biochemical mechanisms which control the normal body burden and toxicity of xenobiotics. Our work suggests that the earthworm A. *hawayanus* is a sensitive organism for studying the acute sublethal toxicity of potential chemical contaminants in soil ecosystems. Moreover, the spectrophotometric protocol herein presented for measuring phagocytosis by NBT dye reduction should contribute to our ability to assess toxicological risks of chemicals in a wide variety of species. It is a simple and rapid test for phagocytosis, a phylogenetically conserved response.

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