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In Vitro Earthworm *Lumbricus terrestris* Coelomocyte Assay for Use in Terrestrial Toxicity Identification Evaluation

S. W. Burch,[1] L. C. Fitzpatrick,[2] A. J. Goven,[2] B. J. Venables,[3] M. A. Giggleman[2]

[1] Chem-Aqua, 2727 Chemsearch Boulevard, Irving, TX 75015, USA
[2] Environmental Effects Research Group, Department of Biological Sciences, University of North Texas, Denton, TX 76203, USA
[3] TRAC Laboratories, 113 Cedar Street, Denton, TX 76201, USA

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Development of bioassays using invertebrate surrogates in place of vertebrates to assess risks of chemical contaminants to environmental and public health has been accelerated by increasing socioeconomic pressures to use less controversial and less costly organisms. Numerous invertebrate species have been used to evaluate risks, usually as whole organisms exposed in vivo to toxicants in the form of neat chemicals, chemical mixtures, solid matrices or their elutriates. Although whole-organism exposure may more closely simulate real-world conditions and subsequent biological responses, there are pressures to develop rapid and highly sensitive cell-based in vitro assays. We have used earthworms (*Lumbricus terrestris*), exposed as whole individuals, to develop a suite of biomarkers to assess risks of exposure to terrestrial contaminants and hazardous waste site (HWS) materials (Fitzpatrick et al. 1990; Venables et al. 1992; Goven et al. 1994, 1996). Of these biomarkers, phagocytosis by immunoactive coelomocytes and their viability is one of our most promising surrogate assays to assess immunotoxic risks to higher wildlife, including mammals. Since phagocytosis is phylogenetically conserved within the animal kingdom as a first line non-specific immune defense against microbial invaders, the earthworm phagocytic assay may be considered homologous to phagocytic assays using vertebrates. Suppression of phagocytosis portends a compromise of an animal's ability to sequester and kill microorganisms, reducing its resistance to infection.

Herein we describe an in vitro phagocytic and cell-viability assay that allows for direct coelomocyte exposure to small volumes and low concentrations of toxicants. Specifically, we report exposure response sensitivity of coelomocyte viability and phagocytosis to inorganic ($CuSO_4$) and organic (Aroclor® 1254, a polychlorinated biphenyl) chemicals that we have previously used as toxicants for standardizing whole-worm in vivo tests, and compare sensitivities between coelomocytes exposed in vitro and in vivo using lowest observable effects levels (LOEL's) of chemical exposure concentrations. Direct in vitro coelomocyte exposure provides a more sensitive complement to our in vivo exposure assay, capable of detecting

Correspondence to: L. C. Fitzpatrick

potential immunotoxicity of contaminants at very low concentrations and in very small volumes of material. A single coelomocyte biomarker having both in vivo and in vitro routes of exposure offers a wide range of sensitivity, and should be useful in toxicity identification evaluation (TIE) of HWS materials. As with aquatic TIE (EPA 1988), once HWS materials are shown to be toxic using whole-worm exposure assays, chemical fractionations are made to isolate specific contaminants responsible for the toxicity. Often these fractionations are in such small quantities that whole-worm exposures are not possible. Thus, microassays using coelomocytes exposed in vitro may be necessary. Since both in vivo and in vitro assays use the same cell types, results should be directly comparable.

MATERIALS AND METHODS

Adult *L. terrestris,* obtained from Ward's Natural Science (Rochester, NY), were maintained at ≤ five worms/liter of a 4:1 dry mass mixture of organic potting soil and Michigan peat, moistened with 200-220 ml distilled deionized water/kg within an environmental chamber set at 10°C, 65-70% humidity without light. Worms were held for at least 1 week before use and fed dry Gerber (Freemont, Ml) baby food, sprinkled on the surface of the mixture as needed.

To determine in-vitro exposure-response sensitivity of coelomocyte phagocytosis and viability to our reference inorganic (Cu^{++}) and organic (Aroclor® 1254) toxicants, we collected coelomocytes (see below) from robust worms and exposed them within replicate centrifuge tubes at 1×10^6 cells per ml to 10 different concentrations for each toxicant. After exposure for 24 h (see below), percent coelomocyte viability and phagocytosis were determined (see below) for each exposure replicate. Phagocytosis and viability data for exposed and control groups were tested for normality, then a one-way ANOVA and Tukey's Multiple Comparisons test (Zar 1984) were used to compare between and among experimental and control groups for each chemical. The strength of relationship between exposure concentration of each chemical and coelomocyte viability and phagocytosis (i.e. exposure-response) was tested using linear regression analysis (Zar 1984). All statistical analyses were done using SAS® (1985) Software.

Coelomocytes were obtained by shocking each worm intermittently for 30-60 sec using two 18 gauge electrodes attached to a 300 mAmp universal transformer set at 7.5 v DC. The mild shocks stimulated muscular contraction, that resulted in extrusion of mucus that contained coelomocytes through body wall pores. Prior to cell collection, fecal matter was expelled by massaging the posterior one-third of the worm toward its anus, then the worm was cleaned by rinsing in 0.85% w/v NaCl ice water. The clean worm was placed into a glass Petri dish containing 6 ml of 4°C Hanks Balanced Salt Solution (HBSS; H1387 Sigma, St. Louis, MO), adjusted to 150 mOsm,

and containing 1% w/v guaiacol glyceryl ether (G5627, Sigma) and 0.02% w/v EDTA (E478; Fisher, Fair Lawn, NJ). Guaiacol glyceryl ether, a mucolytic agent, and EDTA increase collection efficiency by freeing cells from co-extruded mucus (Eyambe et al. 1991) and by reducing cell clumping, respectively. However, EDTA cannot be used when cells are exposed to toxicants such as Cu^{++} that can be chelated. After extrusion, Petri dish contents were poured into centrifuge tubes maintained in crushed ice. Tubes were then centrifuged for 6-8 min at 150 x g at 4°C, the supernatant decanted and coelomocytes resuspended in 6 ml 4°C HBSS supplemented with 0.35 g/l $NaHCO_3$.

Coelomocyte viability was determined by the Trypan Blue exclusion method (Kirk et al. 1975). Samples of 100 µl HBSS containing resuspended coelomocytes were taken from each centrifuge tube, mixed with 100 µl 0.04% Trypan Blue dye (T8154; Sigma) and placed on a hemacytometer. Viability was determined as the number of live cells in the first 100 counted, and expressed as percent live. Phagocytosis was determined similarly by counting the number of coelomocytes containing at least one phagocytosed yeast (Saccharomyces cerevisiae; Fleishmann's® bakery yeast, Specialty Brands, San Francisco, CA) cell. Pooled coelomocytes, adjusted to 1 x 10^6 cells/ml 10°C HBSS, were centrifuged as described and resuspended in 1 ml 10°C HBSS containing 1 x 10^7 inactivated yeast cells stained with Congo Red (C6767; Sigma). After the coelomocyte-yeast mixture was incubated at 10°C for 24 h on an orbital shaker, undiluted replicate samples were examined on a hemacytometer and the mean percent phagocytosing coelomocytes determined.

After exposure for 24 h to 10 Cu^{++} dilutions (1 - 1 x 10^6 µg Cu^{++} in 1.0 l HBSS from a stock $CuSO_4$ solution (C495-500; Fisher) of 1.0 mg Cu^{++}/ml HBSS, coelomocytes at 1 x 10^6/ ml were centrifuged and resuspended in HBSS, and then viability and phagocytosis were determined as described. Controls were exposed to HBSS without $CuSO_4$. Coelomocytes, exposed at 10°C for 24 h to 10 Aroclor® 1254 dilutions (1 x 10^{-3} - 1 x 10^2 µg /l HBSS) were similarly centrifuged, resuspended, and viability and phagocytosis determined. Aroclor® 1254 (F115; Chem Service, West Chester, PA) was diluted from a 100 µg ml methanol stock solution. The concentration of the carrier, methanol, was tested by exposure and shown not to affect coelomocyte viability or phagocytosis. Controls were exposed to the carrier, but without Aroclor® 1254. Viability and phagocytosis for exposed groups are expressed herein as percent of the coelomocytes counted.

RESULTS AND DISCUSSION

Viability and phagocytosis, determined after extruded coelomocytes were exposed for 24 h to 10 Cu^{++} and 10 Aroclor® 1254 concentrations, then incubated for 24 h with yeast cells in toxicant- free HBSS, are expressed as

percentages of the first 100 cells counted in Tables 1 and 2, respectively. Compared to controls, significant effects ($p<0.05$; Tukey's test) on viability and phagocytosis are evident at and above the lowest concentration we used for Cu^{++} (1.0 µg/l), except for viability at 2.0 µg Cu^{++}/l; an anomaly in the data we cannot explain biologically. Since the lowest observable effects levels (LOEL) were at our lowest concentration, the absolute sensitivity of the in vitro viability and phagocytosis assays is probably below 1.0 µg Cu^{++}/l. For coelomocytes exposed to Aroclor® 1254, LOEL for viability and phagocytosis were 1.0 and 1 x 10^{-2} µg/l, respectively. The anomaly at 10 µg/l for viability may have resulted from the small sample size. Phagocytosis was completely suppressed and viability nearly so at 100 µg/l. Based on LOEL, phagocytosis appears to be 200 times more sensitive to Aroclor® 1254 than is coelomocyte viability.

The apparent greater sensitivity of phagocytosis than viability to Aroclor® 1254 accords with work on in vivo exposure of intact *L. terrestris,* that shows phagocytosis is a more sensitive and stable biomarker than is coelomocyte viability (e.g. Goven et al. 1988, 1994; Giggleman 1997). In Table 3 we compare phagocytosis data from this study with whole-worm exposure data. Because coelomocytes were exposed directly in vitro to Cu^{++} and Aroclor® 1254 in HBSS, we believe the best comparison with in vivo exposure is to use chemical body burdens, expressed per wet mass of exposed worms from which coelomocytes were extruded for assay. Thus, we express Cu^{++} and Aroclor® 1254 per unit wet (live) mass for direct comparison with in vitro exposure using liquid HBSS exposure media as parts per million (ppm = µg/g tissue and µg/ml HBSS, respectively). Comparison of the lowest exposure concentration of Cu^{++} significantly suppressing phagocytosis, expressed for tissue and HBSS exposure media in Table 3, suggests that the apparent sensitivity of the in vitro phagocytosis assay is at least 10.7 x 10^{3} times greater than the in vivo exposure assay. For Aroclor® 1254, the sensitivity of the in vitro assay is 8.94 x 10^{6} greater than the in vivo assay.

Because Aroclor® 1254 is more likely to be sequestered in live worms than Cu^{++}, actual in vivo coelomocyte exposure to it within coelomic fluid may have been less than suggested by the whole worm body burdens. Without analyzing target tissue for actual tissue (coelomocytes here) dose residues, whole body burdens are probably the best estimates for exposure-based assessment. Clearly, actual chemical body burdens should be more reliable in ranking chemical toxicity and in comparing susceptibility of species to chemicals than using environmental (e.g. soil) exposure concentrations. Elsewhere (Fitzpatrick et al. 1992), we have shown this to be true when comparing lethal toxicity of Aroclor® 1254 in *L. terrestris* and *Eisenia fetida* using LC_{50}s based on soil exposure concentrations and LD_{50}s based on actual body burden concentrations: LC_{50}s indicate that *E. fetida* is 10 times more sensitive to Aroclor® 1254 than *L. terrestris,* but LD_{50}s show it to be four times more resistant. Although other workers have studied immune

Table 1. Mean (± se) phagocytosis of yeast cells by *L. terrestris* coelomocytes and their viability after 24-h in vitro exposure to 10 Cu^{++} concentrations in HBSS, expressed as percent of the first 100 cells counted. Asterisks show significant (P<0.05) differences from controls.

µg Cu^{++}/l Exposure	Replicate N	Relative Coelomocyte Viability	Relative Coelomocyte Phagocytosis
0 (controls)	54	62.4 ± 1.59	19.9 ± 0.47
1	24	54.2 ± 1.14 *	16.6 ± 0.51 *
2	12	59.9 ± 1.65	15.9 ± 1.56 *
4	54	50.2 ± 0.76 *	9.99 ± 0.32 *
6	12	48.0 ± 1.31 *	6.22 ± 0.58 *
8	12	51.1 ± 1.67 *	9.25 ± 0.47 *
10	54	49.7 ± 0.83 *	7.17 ± 0.25 *
20	12	26.6 ± 1.24 *	4.30 ± 0.53 *
100	12	40.8 ± 1.30 *	5.13 ± 0.44 *
1000	12	35.5 ± 1.82 *	4.52 ± 0.36 *
$1x10^6$	12	0	0

Table 2. Mean (± se) phagocytosis of yeast cells by *L. terrestris* coelomocytes and their viability after 24-h in vitro exposure to 10 Aroclor® 1254 concentrations in HBSS, expressed as percent of the first 100 cells counted. Asterisk the same as in Table 1.

µg Aroclor® 1254 /l Exposure	Replicate N	Relative Coelomocyte Viability	Relative Coelomocyte Phagocytosis
0 (controls)	16	67.8 ± 1.90	20.1 ± 0.91
1×10^{-3}	20	69.6 ± 1.30	16.7 ± 1.08
5×10^{-3}	8	63.5 ± 3.13	17.6 ± 1.95
1×10^{-2}	24	65.9 ± 1.04	13.8 ± 1.10 *
1.25×10^{-2}	16	60.9 ± 1.51	8.12 ± 0.64 *
2.5×10^{-2}	16	60.0 ± 1.77	8.22 ± 1.11 *
5×10^{-2}	12	58.1 ± 1.43	4.52 ± 0.56 *
1×10^{-1}	8	65.0 ± 1.98	4.75 ± 1.17 *
1	12	52.5 ± 4.84 *	7.08 ± 1.53 *
10	4	70.4 ± 1.94	2.09 ± 0.27 *
100	8	0.69 ± 0.52 *	0

suppression in earthworms, including the excellent study on Aroclor® 1254 effects on phagocytosis by Ville et al. (1995), none provide actual body burden concentrations that allow for direct comparison with our work. Also, for Aroclors in weathered soils, bioassays such as our phagocytosis assay must be run with specific congeners, not pure, fresh standards.

Table 3. Comparative sensitivity of the *L. terrestris* phagocytosis assay between in vitro and whole-worm in vivo exposures to Cu^{++} and Aroclor® in terms of lowest observable effects levels (LOEL), and expressed as ppm (µg/ml) HBSS exposure media and ppm (µg/g) wet earthworm tissue mass.

Chemical	Exposure In vitro (LOEL)	In vivo (LOEL)
Cu^{++}	1.0×10^{-3} [a]	10.7 [b]
Aroclor® 1254	1.0×10^{-5} [a]	89.4 [b]
		15.8[c]
		20.0[d]

a = This study
b = Giggleman 1997
c = Goven et al. 1988
d = Goven et al. 1994

The strength of relationship between in vivo exposure to Cu^{++} and Aroclor® 1254, and response in terms of reduced coelomocyte viability and phagocytosis is shown by linear regression analysis in Table 4. Both viability and phagocytosis were significantly affected by increasing concentrations of both toxicants, with coefficients of determination (r^2) showing that between 46 and 84 percent of the variation in viability and 61 to 72 percent of the variation in phagocytosis are significantly (p c 0.05) explained by exposure to the two toxicants.

Table 4. Linear regression analysis of viability and phagocytosis in *L. terrestris* coelomocytes after 24-h in vitro exposure to 10 Cu^{++} and 10 Aroclor® 1254 concentrations in HBSS.

Chemical	Assay	Regression model	p	r^2
Cu^{++}	Viability	= 56.0 - 9.06 log conc	0.0002	0.84
	Phagocytosis	= 11.6 - 2.29 log conc	0.007	0.61
Aroclor® 1254				
	Viability	= 47.7 - 8.88 log conc	0.030	0.46
	Phagocytosis	= 4.99 - 3.25 log conc	0.002	0.72

Based on our results, the in vitro assay shows potential as a highly sensitive microassay for use in toxicity assessment of chemicals at very low concentrations. This would be of great value in TIE's of HWS soils, where chemicals in various fractionations or extractions may be in very low volumes or concentrations or both.

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