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The Function of Metallothionein in *Caenorhabditis elegans*: is Detoxification of Copper or of Cadmium More Important?

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Abstract: Much of the literature on metallothioneins (MT) focuses on their role in protecting against Cd toxicity. Here we compare the effects of Cu, Hg, Ag, Cr, Pb, Zn and Cd on survival, growth and reproduction of wild type and *mtl-2* deleted individuals of *Caenorhabditis elegans*. The largest difference was in the survival of animals exposed to Cu, where *mtl*-2 deleted worms were 100 times more sensitive. LC_{50} values for Pb did not differ and were between 1.4 and 5 times greater for wild type worms exposed to other metals. The two strains showed only small (less than 3-fold) differences in the effects of metals on reproduction and growth. After 72 hours of exposure, the LC_{50} for copper was 0.06 µM for *mtl-2* deleted worms. Cu concentrations in excess of this are regularly found in soil pore water, indicating that a major role of MT in wild populations of *C elegans* is the prevention of acute Cu toxicity. By contrast, effects of Cd on survival, reproduction and growth occur only at concentrations that are between 1400 and 80000 higher than those reported in soil pore water. This suggests that the reduction of Cd toxicity is not an important function of MT in *C. elegans*, even though MT gene expression is very responsive to Cd.

Key words: *C. elegans* · Metallothioneins · Toxicity and metals

INTRODUCTION

Metallothionein (MTs) is a family of low molecular weight (6-KDa), cysteine-rich, non-enzymatic, metal binding proteins. They are found in all animals, higher plants, eukaryotic microorganisms and many prokaryotes [1-5]. MTs are known for their extremely high metal and sulphur contents.

In *Caenorhabditis elegans*, there are two genes, *mtl-1* and *mtl*-2, which encodes the MTs of C. elegans. Both contain 75 and 63 amino acid residues, respectively and each binds 6 atoms of metals. The *C. elegans* MT resemble in most of its chemical and spectroscopic features the MTs of other animal phyla [6-10].

Metallothioneins (MT) have high affinity for Zn, Cd, Ag, Cu and Hg [11-13] and their synthesis is induced in organisms by metal exposure, chemicals, heat and other environmental contaminants, or stressors [14-17]. Their suggested roles include homeostasis of Zn and Cu, the detoxification of heavy metals, particularly Cd and scavenging of free radicals [18- 20]. However, the relative importance of these remains unclear. One research concluded that the function of MT was "to protect against cadmium toxicity" [21], but others are more equivocal, describing its function as "elusive" [22]. Also a research on MT genes in *C. Elegans* [23] argued that both metallothioneins in this species (*mtl-1* and *mtl-2*) play "a pivotal role" in detoxification of Cd. The authors based their conclusions on gene expression patterns in response to exposure to Cd, Zn and Cu and observations of increased effects of Cd on growth and reproduction in *mtl* knock-outs or individuals in which *mtl* genes were suppressed using RNAi. Increased expression of both genes occurred at 2.5 µM Cd, but no increase in expression in response to Cu or Zn was detected using semi-quantitative PCR. Suppression or knock-out of either *mtl* gene led to a five-fold reduction in brood size at 150 µM Cd, relative to animals not exposed to Cd,

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although at 30 µM Cd, the difference in brood size was only about 10%. Suppression or knockout of either gene increased the effects of 75 µM Cd on individual growth and knockout of mtl-2 had a small (<5%) but significant effect on generation time at 30 and 75 µM Cd. Because both *mtl* genes appeared unresponsive to Cu and Zn, the authors did not examine the effects of gene suppression on Cu or Zn toxicity [24, 25].

Although much of the literature on MT has focused on the toxicity of Cd, this metal is not very common and high environmental concentrations occur exclusively as a result of man's activities. Here we examine the effects of seven metals on survival, growth and reproduction in *C.elegans* and assess whether sensitivity of individuals is increased in a strain in which the mtl-2 gene has been knocked out.

MATERIALS AND METHODS

Nematode Culture: The bacteria stock (*Escherichia coli*, strain (OP50) and the wild type (N2) *C. elegans* used in this experiment were obtained from the Caenorhabditis Genetic Centre, University of Minnesota (Minneapolis, MN, USA), While the *C. elegans* strain VC128 (MT-deleted strain) was obtained from Jonathan Freedman (Duke University, North Carolina). *C. elegans* were propagated on K-agar plates, with bacteria (OP50) as food source Williams and Dusenbery [24]. Standard methods were used to harvest eggs and generate age-synchronized adult worms Williams and Dusenbery [24] but nematodes were killed with 10% NaOH because we found that this gave higher egg recoveries than the more commonly used solution of NaOCl.

Metal Stock Solutions: All the chemicals used in this study were analytical reagent grades purchased from Sigma-Aldrich Chemicals (Poole, UK), VWR (Lutterworth, UK) and Fisher Scientific (Loughborough, UK). The metals used were chosen because of their prevalence in the environment and availability of toxicological data from *C. elegans* and other organisms. Stock solutions of $CuCl_2.2H_2O$, $ZnSO4.7H_2O$, $CdCl_2$, $PbCl_2$, $AgNO_3$, $HgCl_2$, $K_2Cr_2O_7$ were used to make test solution. The range of norminal concentrations used was 0.005 to 40 mg/L Cu, 0.5 to 200 mg/L Cd, 0.05 to 100 mg/L, Pb and Zn. Inductively coupled plasma optical emission spectrometry measurement of actual metal concentrations were made in number of test solutions covering the whole concentration range of each metal. Actual concentrations were between 90 and 95% of nominal concentrations, so nominal concentrations are used throughout the results.

Acute and Chronic Aquatic Toxicity Testing: The assay methods were based on Williams and Dusenbery [24]. All dilutions of metal stock solutions were made in K-medium. A 3-day old bacteria culture (cultured in LB broth) of volume equal to the volume of the K-medium required for dilution of the stock solution was centrifuged, washed twice an the pellet added to diluent as the food source of the nematodes. OP50 concentration in each test sample was standardized by measuring the optical density at 700 nm, using a spectrophotometer. Each test sample consisted of several concentrations in triplicates. A control test without metal was run concurrently. A 1.0 ml aliquot of each test solution was put into Falcon tissue culture plates (with covers). Each culture plate contained 24 test wells. The space above the solution level in the test wells was enough for good aeration, which was necessary for the survival of the organisms. Six adult nematodes (3 days old) were transferred from K-agar plates into each of the wells, using nematode picks sterilized by passing it over a flame. They were observed under the microscope to ascertain the number of nematodes transferred. The set up was left in an air-conditioned room maintained at 23°C. Following exposure, the wells were observed under dissecting microscope to determine survival at 24 hours intervals for 72 hours. At each time intervals, death was ascertained by probing with the nematode pick. Any nematode that did not respond to the probing was assumed dead. The number of dead and live nematodes in each well was recorded at each time interval. Testing was repeated at least five times. The average number of dead and live animals was calculated and LC_{50} values were calculated using Probit analysis in SPSS (SPSS Inc. Chicago). Other statistical parameters such as mean ±SD and men standard errors were calculated where necessary. The sensitivity of the two strains of *C. elegans* (N2 and VC128) being tested was compared using Relative Potentials.

Reproduction Measurement: Reproduction was carried out over a period of 72 hours at 24 hour intervals in tissue culture plates. The test procedures were in accordance with Middendorf and Dusenbury [20] and Dhawan *et al*. [6] Hoss and Haitzer[10] and Anderson *et al.* [1]. Two gravid age synchronized adult nematodes were transferred from K- agar plates into wells maintained at 20-23°C incubator. Control tests without metals were run concurrently. Reproduction was quantified at 24 hours intervals for 72 hours by counting all the nematodes in solution under a light microscope after killing and eosin staining at 60°C for one hour.

Reproduction was expressed as a percentage of the control value. EC50 values were calculated by linear interpolation, as before Norberg-King [21].

Growth Measurement: The method adopted was as reported by Ibiam and Grant [13]. Stock solutions were prepared as already described. Age synchronised nematodes were generated as described above. After 20 to 24 hours of exposure, the adult nematodes were removed by micropipetting, leaving their offspring to grow in the medium. Each experiment was set up in triplicates. All liquid cultures were shallow to allow for sufficient aeration and were left in air-conditioned room maintained at 20 to 23°C. At 24 hour intervals teat well was sacrificed. A solution of 100 ML OF 0.02% eosin solution (VVR) was dropped in each of the wells and incubated at 60°C for one hour. This treatment resulted in straight, very visible, easily measurable dead worms. Length of at least five randomly chosen dead worms was measured for each time interval, using a stage micrometer with graticule calibrated eyepiece and the average length recorded. Concentrations in which the juvenile did not survive were discarded and the test repeated with lower concentrations. The average juvenile length from repeated tests was calculated and the dose response relationship plotted for the intervals. Tests in which the control died were discarded and repeated.

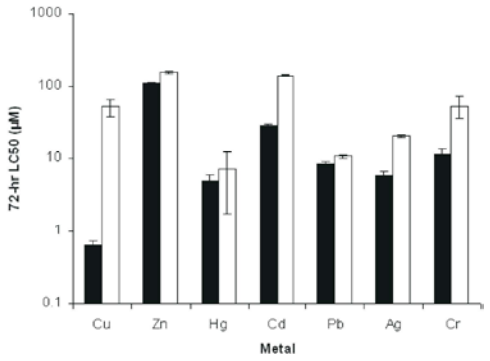

Fig. 1a

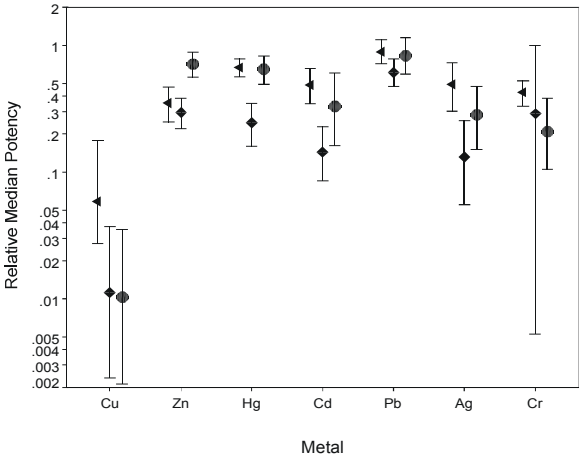

Fig. 1b

Fig. 1: Effect of metals on survival of two strains of *C.elegans*: a) 72-hour LC_{50} values for N2 (open bars) and VC128 (solid bars)

Fig. 1b): Relative median potency for ☯ 24 hour LC_{50}; ♦48 hour LC_{50} and •72 hour LC_{50}. Error bars are 95% confidence intervals, calculated using probit analysis in SPSS, version 12 (SPSS Inc., Chicago). A value less than 1.0 (Figure 1b) indicates that the VC128 strain is more sensitive to the metal than the N2 strain. Both strains of *C. elegans* were obtained from the *Caenorhabditis* Genetics Centre, Minnesota. Animals were cultured at 23°C on NGM Agar plates and toxicity tests carried out in K-medium, as described elsewhere. Individuals were considered to be dead if they did not respond to mechanical stimulation. Toxicity values reported here will not be directly comparable with those reported by Swain *et al*. (2004) as their tests were carried out on NGM plates or in M9 medium, where metal availability will be reduced by formation of insoluble phosphates

RESULTS AND DISCUSSIONS

Of the metals tested, mercury was the most toxic to *C. elegans*, as indicated by 72 hour survival (Figures 1a and 1b) and Cu had the most potent effect on growth and survival (Figures 2 and 3). Cd was of rather low toxicity. By far the greatest difference between strains is for sensitivity of survival rates to Cu. LC_{50} of mtl-2 deleted (VC 128 strain) animals was nearly 20 times higher after 24 hours and about 100 times greater after 48 and 72 hours. There was no significant difference between strains in sensitivity to Pb and for the other metals the difference in sensitivity varied from 1.4 to 5 times.

Reproduction and individual growth were more sensitive toxicity endpoints than acute toxicity. 72-hour EC_{50} values are shown in Figure 3. The two strains differ significantly in their EC_{50} values for growth for all metals except Cd and differ in their EC_{50} values for reproduction for Hg, Cd and Pb. However, the magnitude of these differences were small, with a maximum concentration ratio of 2.8 for growth (Zn) and a maximum of 1.6 for reproduction (Cd).

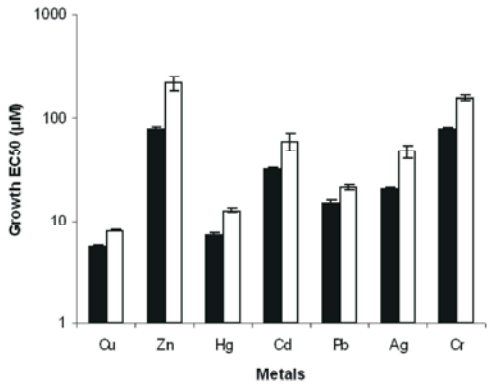

Fig. 2: 72-hour EC_{50} values for individual growth of N2 and VC128 strains of *Caenorhabditis elegans* after exposure to seven metals. Error bars are ± 1 standard error. Lengths of at least 5 individuals in each metal concentration were measured after heat killing, as described elsewhere[12]. Other details as Figure 1

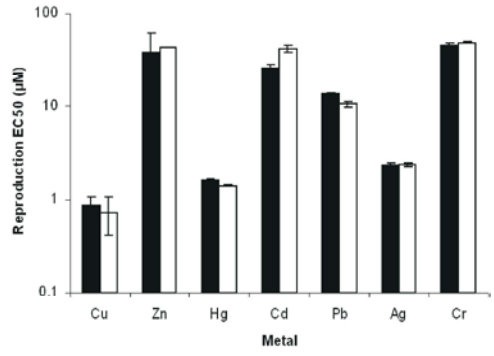

Fig. 3: 72-hour EC_{50} values for reproduction of N2 and VC128 strains of *Caenorhabditis elegans* after exposure to seven metals. Details of graph as for Figure 3. Culture methods are described elsewhere [12] and methods to quantify reproduction followed previously published methods [13; 14; 15].

Deletion of the mtl-2 gene produces some increase in acute sensitivity to Cd, Zn, Hg, Ag and Cr at higher metal concentrations, but by far the greatest effect was on acute sensitivity to Cu. In Table 1, 72-hour LC_{50} values for the two strains of *C.elegans* are compared with the median pore water concentrations of 20 Dutch soil samples (Janssen *et al*., 1997). 72 hour LC_{50} values of wild type nematodes are higher than median pore water concentrations for all five metals for which data on pore water concentrations were available. However, the great increase in sensitivity of MT deleted worms to Cu means that median pore water concentrations (0.69 µM) are slightly higher than the 72 hour LC_{50} for VC 128 animals (0.62 µM). Thus, acute copper toxicity to MT-deleted worms occurs at environmentally realistic concentrations. By contrast, the other metals would only be expected to have adverse effects on *C. elegans* at grossly polluted field sites. The difference is greatest for Cd, there the toxicity endpoints for the two strains occur at concentrations that are between 14 000 and 80 000 higher than median soil concentrations.

This suggests that the primary role of MT is to protect against Cu toxicity and that in natural conditions it does not play an important role in detoxifying Cd. However, if one were examining MT gene expression patterns alone, one would reach the opposite conclusion. mtl-2 expression in *C. elegans* is very responsive to Cd, with increased mtl-2 expression detectable at Cd concentrations of 0.04 µM [13]. However, Cd toxicity was low and survival rates of the MT deleted strain is only 5 times more sensitive to Cd than the wild type. mtl-2 is crucial for the survival of *C. elegans* at low Cu concentrations, but at these same concentrations mtl-2 expression is undetectable using an *in-vivo* (LacZ) reporter gene. In our work, mtl-2 gene expression is only detectable at 80 µM Cu (Ibiam and Grant, submitted) and using a GFP reporter, Swain *et al*. [23]found detectable expression at 100 µM Cu or more. Presumably there is some increased expression of mtl-2 at the Cu concentrations where the presence of a MT gene reduces acute sensitivity, but because Cu concentrations are low, only a relatively small quantity of MT is necessary to detoxify the small quantities of Cu present and this low level expression is not detectable using a reporter gene based system. So, the responsiveness of gene expression to a novel, or rarely encountered stressor need not indicate the importance of that gene and may reflect an entirely non-adaptive response of gene regulation mechanisms. "Cd-responsive isoforms" of MT Dallinger *et al*. [5] need not play an important role in regulating Cd toxicity. Evolutionary pressure will favour increased efficiency in allocation of metabolic resources and will, if possible, lead to the suppression a molecular response to a commonly encountered stressor if that response does not reduce the negative impacts of that stress. Changes in expression of a gene will then reflect either damage by a stressor or a homeostatic response to it. But patterns of gene expression in response to novel toxins have not been subject to the same evolutionary pressures. They may, therefore, be the accidental result of interaction between the toxin and gene regulation mechanisms that normally respond to other chemicals.

Table 1: A comparison between 72-hour LC50s of the two strains of *C.elegans* and the median pore water concentrations of the same metals in 20 Dutch soils

Metal	Strain N2 LC_{50} (µM)	Strain VC128 LC_{50} (µM)	Dutch soil median pore water concentration (µM) Janssen *et al*., (1997)
Cu	52.5	0.62	0.69
Cd	142.7	28.2	0.0018
Hg	7.2	5.0	No data
Pb	10.6	8.5	.048
Zn	159.0	110.5	3.1
Ag	20.6	5.8	No data
Cr	53.7	11.7	0.096

Toxicogenomic studies should ideally be complemented by studies in which both chronic and acute responses to toxins are measured after prevention of expression of a gene by using knockout strains or RNA-mediated interference (RNAi) Kamath, *et al*. [19] and Ashrafi, *et al*. [2].

This study presents strong evidence that the primary role of mtl-2 gene in *C. elegans* is to protect against Cu toxicity and it may not play an important role in detoxifying Cd.. Preventing expression of a gene by using knock-out strain or RNAi-mediated interference gives useful information on which genes are essential for normal survival and development. This process has been used to systematically screen the C. elegans of genome for genes necessary for storage of fat Ashrafi *et al*. [2] Kamath *et al*. [19]. However, the approach used in this study goes one step beyond this by examining whether a gene is essential for normal response to particular stressors. The study indicates that MT-deleted worms show greater chronic toxicity to metals, especially Cu.

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