Aquatic Acute Toxicity Testing Using the Nematode *Caenorhabditis elegans*

Kazuhiro Ura,[a, c] Toshinori Kai,[a] Sachiko Sakata,[a, c] Taisen Iguchi,[b, c] and Koji Arizono*[, a, c]

[a]*Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3–1–100 Tsukide, Kumamoto 862–8502, Japan,* [b]*Center for Integrative Bioscience, Okazaki National Research Institutes, 38 Nishigonaka, Myodaiji, Okazaki, Aichi 444–8585, Japan, and* [c]*CREST, Japan Science and Technology Corporation, 4–1–8 Honmachi, Kawaguchi, Saitama 332–0012, Japan*

(Received July 30, 2002; Accepted August 9, 2002; Published online August 13, 2002)

To evaluate the toxicity of environmental chemicals to invertebrates, a static bioassay was developed in the laboratory using the *Caenorhabditis elegans* (*C. elegans*). First, reproducibility of this aquatic acute toxicity test system was confirmed. In order to estimate chemical toxicities in *C. elegans*, worms were subsequently exposed to eleven different xenobiotics. Mortality after 24 hr was adopted as the endpoint of toxicity. We found that benzo[*a*]pyrene, nonylphenol, benzophenone, bisphenol A and cadmium chloride affected viability of *C. elegans*. These data suggest that *C. elegans* is a suitable toxicity test organism for environmental xenobiotic chemicals, and that lethality can be used as a testing endpoint.

Key words —— invertebrate, acute toxicity test, *Caenorhabditis elegans*, nematode

INTRODUCTION

Caenorhabditis elegans (*C. elegans*) is a free-living nematode that lives mainly in the liquid phase of soils. It has been widely used as a test organism owing to its short life span and ease of culture. In addition, *C. elegans* is a simple multicellular organism whose genome has been fully sequenced,[1] making it a good model to test the effects of xenobiotic chemicals *in vivo*.

To date, a large number of toxicity test have been developed using *C. elegans*.[2–7] These tests are adopted as a bioassay system for ecological risk assessment and screening of drugs in pharmaceutics. Most studies have focused on the effects of metals or agricultural chemicals, but little is known about the relative toxic effects of other environmental chemicals on *C. elegans*.

The increasing exposure of the environment to an unimaginably large number of man-made chemicals has affected organisms in many ecosystems. Therefore, the need for bioassay systems, especially employing terrestrial invertebrate, is widely accepted by the OECD (Organization for Economic Cooperation and Development) and EPA (Environmental Protection Agency). Accordingly, we have been setting up several laboratory-scale bioassay systems to evaluate the impact of various chemicals on *C. elegans in vivo*. In general, determination of lethal concentrations, such as the median lethal concentrations (LC_{50}s), is recognized first steps for risk assessment of synthetic and natural chemicals. In this study, we use lethality of environmental xenobiotic chemicals as an endpoint in an aquatic acute toxicity testing system based on the soil nematode *C. elegans*, and we discuss its usefulness as a toxicity testing organism.

MATERIALS AND METHODS

Chemicals —— The following compounds were tested: dimethyl sulfoxide (DMSO) (solvent); 17β-estradiol (natural product); bisphenol A (raw material of resins); nonylphenol (raw material for oil-solvent phenol resins); benzo[*a*]pyrene (unintended product); aldicarb (insecticide); benzophenone (synthetic raw materials for medical products, perfume *etc.*); styrene (raw material for polystyrene); *trans*-1,2-diphenylcyclobutane (styrene dimmer); 2,4,6-triphenyl-1-hexene (styrene trimmer); ponasterone A (natural products); and cadmium chloride (metals).

Strain and Cultivation —— Wild type N2 strain

*To whom correspondence should be addressed: Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3–1–100 Tsukide, Kumamoto 862–8502, Japan. Tel. & Fax: +81-96-383-6062; E-mail: arizono@pu-kumamoto.ac.jp

C. elegans was used in this study. Worms were maintained and handled basically as described.[8]

Acute Toxicity Test —— An acute toxicity test was performed according to a procedure of Donkin and Williams.[9] Because of the possible age influence on toxicity, age-synchronous populations were used in this study. A nematode growth medium (NGM) plate was inoculated with a single worm. After 4–5 days of incubation at 20°C, mixed-stage worm populations were dominated by one-day-old larvae. All worms were gently washed off the plates with K-medium (32 mM KCl, 51 mM NaCl),[3] and age-synchronous populations (1-day-old larvae) isolated into a glass centrifuge tube using Sephadex G-25. Collected worms were allowed to settle by gravity. The supernatant was removed, and then the worms rinsed with K-medium to remove bacteria completely. Ten worms each were dispensed into 24-well tissue culture plates containing 0.5 ml of K-medium and variable amounts of xenobiotics per well. All treatments were done in triplicate. No feed was given during the test. Worms were exposed for 24 hr at 20°C, and the number of dead and/or live worms was determined by the absence of touch-provoked movement when probed with a platinum wire under dissecting microscope (Nikon, ECLIPSE, TS100, Japan). The median lethal concentration (LC_{50}) was calculated using the PROBIT method.

RESULTS AND DISCUSSION

During the last several decades the global environment has been polluted by an astonishing number of natural and xenobiotic compounds. There is growing concern about these chemical compounds may affect several physiological system, development, growth, reproduction and behavior of all organisms in the ecosystem. In the current study, we used an invertebrate bioassay to evaluate the toxicity of environmental chemicals on the free-living soil nematode *C. elegans*.

In our previous study, aquatic acute toxicity tests on *C. elegans* were conducted in a standard culture medium (S-basal) using 96-well tissue culture plates.[10] Although an easy test system, we found that the worms could not be stirred easily, and that test metal sometimes precipitated in the medium. S-basal therefore appeared unsuitable as a vehicle for testing metals, prompting the use of K-medium as the medium for metals in aquatic tests, as recommended by Donkin and Williams.[9] The test methodology was thus improved to allow evaluation of a range of chemicals, including metal compounds.

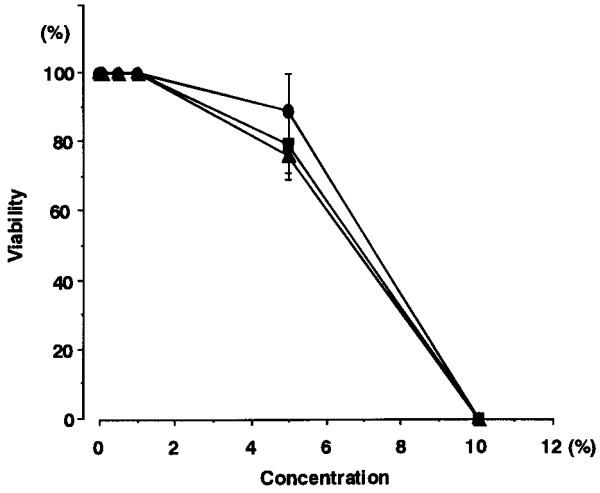

Fig. 1. Concentration-Response Plots, Showing the Viability of *C. elegans* after 24 hr of Exposure to DMSO

Plotted are the means ± standard error ($n = 30$) for three different tests, 1st (●), 2nd (■) and 3rd (▲).

Since many environmental chemicals are insoluble in water, it can be difficult to carry out toxicity tests without using organic solvents. DMSO is a common organic solvent and could be used as a toxicant vehicle in toxicity test.[11,12] Although there are a few reports concerning the effects of DMSO on fecundity,[13,14] its impacts on viability of *C. elegans* have not been documented. We determined the maximum "safe" concentration of DMSO as a first, preliminary step, because we used this solvent as a toxicant vehicle in our study. The changes in survival rates after exposure to DMSO in three different trials performed on different dates is shown in Fig. 1. Increasing concentrations of DMSO had a marked effect on viability of exposed worms for 24 hr. There is no mortality, *i.e.*, 100% survival, at 0 (K-medium only), 0.1, 0.5, and 1.0% DMSO. However, the worms showed approximately 80% survival in 5%, and no survival in 10% DMSO after 24 hr exposure. The findings obtained in our test system were highly reproducible, as a significant difference between the three test trials could not be demonstrated. Based on these results, the use of DMSO in final concentrations at or below 1.0% therefore appears appropriate for 24 hr-acute toxicity tests using *C. elegans*.

To investigate the applicability of our test system, we tested eleven chemicals, *i.e.*, 17β-estradiol, bisphenol A, nonylphenol, benzo[*a*]pyrene, aldicarb, benzophenone, styrene monomer, styrene dimmer,

Table 1. Influence of Xenobiotic Chemicals on Viability of *C. elegans*

Compound	24 hr-LC_{50} (mg/l)	
17β-estradiol	> 1000	—
Bisphenol A	324.7	(301.1–349.5)
Nonylphenol	7.2	(6.7–7.7)
Benzo[*a*]pyrene	0.05	(0.047–0.053)
Benzophenone	56.8	(52.1–62.1)
Aldicarb	> 40	—
Styrene monomer	> 20	—
trans-1, 2-diphenylcyclobutane	> 20	—
2, 4, 6-triphenyl-1-hexene	> 20	—
Ponasterone A	> 10	—
Cadmium chloride	277.2	(252.8–303.9)

Values in parenthesis mean 95% confidence limits.

styrene trimmer, ponasterone A and cadmium chloride. Table 1 summarizes the results of the acute toxicity tests with *C. elegans* using these eleven different xenobiotics. Among the chemicals tested, benzo[*a*]pyrene, nonylphenol, benzophenone, bisphenol A and cadmium chloride proved potentially lethal for *C. elegans*. *C. elegans* has previously been used in a bioassay to determine the relative toxic effects of metals in aquatic tests. Donkin and Williams[9] reported 24 hr-LC_{50} values of 112–1124, 6.4–63.5, 2.1–207, and 2–20 mg/l for Cd, Cu, Pb, and Hg, respectively, for exposed worm larvae in K-medium free of bacteria. In our study, the 24 hr-LC_{50} values of worms were found to be in a comparable or lower range (benzo[*a*]pyrene, nonylphenol, benzophenone, bisphenol A and cadmium chloride), supporting the notion that it is possible to use *C. elegans* for acute toxicity testing of various environmental chemicals. At present, a popular terrestrial invertebrate test organism for ecological risk assessment by the OECD and EPA is the earthworm *Eisenia fetida* (*E. fetida*).[15] However, this organism needs 14-days for acute toxicity test. Furthermore, Peredney and Williams[16] reported similar sensitivity of *C. elegans* and *E. fetida* in response to metals. Given the ease of culturing and the short duration of testing, *C. elegans* is more convenient than the earthworm. Moreover, *C. elegans* is among the best characterized invertebrates, with a wealth of biological information available. These points also supported that *C. elegans* is a suitable terrestrial invertebrate organism for toxicity testing.

Traunspurger *et al.*,[17] for instance, reported that the percentage of gravid worms is a much more sensitive toxicity endpoint in a sediment bioassay. Dhawan *et al.*[18] similarly indicated that behavioral changes are much more sensitive as a diagnostic tool than death for assessing toxicological effects of metals on *C. elegans*. More recently, Kohra *et al.*[19] demonstrated that bisphenol A suppress feeding behavior at 0.1 (0.023 mg/l) and 10 μM (2.3 mg/l) in *C. elegans* on agar plates. In order to estimate the toxicological effects of the eleven different xenobiotic chemicals used in this study, other endpoints, such as growth, reproduction or movement, may therefore be required.

Acknowledgements The authors thank Dr. Mark Lokman, University of Otago, for critical reading of the manuscript. Thanks are also due to Yayoi Nomura and Sawako Yamaguchi, Prefectural University of Kumamoto, for their technical assistance. This research was supported in part by a research grant from Core Research for Evolutional Science of Japan Science and Technology Corporation (Japan), and UK-Japan Research Cooperation on Endocrine Disrupting Chemicals, Ministry of the Environment of Japan.

REFERENCES

1) Consotrium (The *C. elegeans* Sequencing Consortium) (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science*, **282**, 2012–2018.
2) Ohba, K. and Ishibashi, N. (1984) A nematode, *Caenorhabditis elegans*, as test organism for nematicide evaluation. *J. Pesticide Science*, **9**, 91–96.
3) Williams, P. L. and Dusenbery, D. B. (1990) Aquatic toxicology testing using the nematode, *Caenorhabditis elegans*. *Environ. Toxicol. Chem.*, **9**, 1285–1290.
4) Donkin, S. G. and Dusenbery, D. B. (1993) Soil toxicity test using the nematode *Caenorhabditis elegans* and an effective method of recovery. *Arch. Environ. Contam. Toxicol.*, **25**, 145–151.
5) Hoss, S., Haitzer, M., Traunsurger, W. and Steinberg, C. E. W. (1999) Growth and fertility of *Caenorhabditis elegans* (nematode) in unpolluted freshwater sediments: response to particle size distribution and organic content. *Environ. Toxicol. Chem.*, **18**, 2921–2925.
6) Mori, T., Mohamed, A. S. A., Sato, M. and Yamasaki, T. (2000) Ellagitannin toxicity in the free-living soil-inhabiting nematode, *Caenorhabditis elegans*. *J. Pesticide Science*, **25**, 405–409.

7) Anderson, G. L., Boyd, W. A. and Williams, P. L. (2001) Assessment of sublethal endpoints for toxicity testing with the nematode *Caenorhabditis elegans*. *Environ. Toxicol. Chem.*, **20**, 833–838.
8) Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics*, **77**, 71–94.
9) Donkin, S. G. and Williams, P. L. (1995) Influence of developmental stage, salts and food presence on various and endpoints using *Caenorhabditis elegans* for aquatic toxicity testing. *Environ. Toxicol. Chem.*, **14**, 2139–2147.
10) Arizono, K., Ura, K., Tominaga, N., Kai, T., Kohara, Y. and Iguchi, T. (2002) *C. elegans* as a tool for environmental toxicology. In *Toxicogenomics* (Inoue, T. and Pennie, W. D., Eds), Springer Press, Tokyo, pp. 129–134.
11) Layman, D. L. (1987) Growth inhibitory effects of dimethyl sulfoxide and dimethyl sulfone on vascular smooth muscle and endothelial cells in vitro. *In Vitro Cell Dev. Biol.*, **23**, 422–428.
12) Menzel, R., Bogaert, T. and Achazi, R. (2001) A systematic gene expression screen of *Caenorhabditis elegans* cytochrome P450 genes reveals CYP35 as strongly xenobiotic inducible. *Arch. Biochem. Biophys.*, **395**, 158–168.
13) Goldstein, P. and Magnao, L. (1988) Effects of dimethyl sulphoxide on early gametogenesis in *Caenorhabditis elegans*: ultrastructural aberrations and loss of synaptonemal complexes from pachytene nuclei. *Cytobios.*, **56**, 45–57.
14) Goldstein, P., Magnano, L. and Rojo, J. (1992) Effects of dimethyl sulfone ($DMSO_2$) on early gametogenesis in *Caenorhabditis elegans*: ultrastructural aberrations and loss of synaptonemal complexes from pachytene nuclei. *Reprod. Toxicol.*, **6**, 149–159.
15) Ingersoll, C. G., Hutchinson, T., Crane, M., Dodson, S., DeWitt, T., Gies, A., Huet, M.-C., McKenney, C. L., Jr., Oberdorster, E., Pascoe, D., Versteeg, D. J. and Warwick, O. (1999) Laboratory toxicity tests for evaluating potential effects of endocrine-disrupting compounds. In *Endocrine Disruption In Invertebrates: Endocrinology, Testing, and Assessment* (DeFur, P. L., Crane, M., Ingersoll, C. and Tattersfield, L., Eds), Society of Environmental Toxicology and Chemistry (SETAC), U.S.A., pp. 107–198.
16) Peredney, C. L. and Williams, P. L. (2000) Utility of *Caenorhabditis elegans* for assessing heavy metal contamination in artificial soil. *Arch. Environ. Contam. Toxicol.*, **39**, 113–118.
17) Traunspurger, W., Haitzer, M., Hoss, S., Beier, S., Ahlf, W. and Steinberg, C. (1997) Ecotoxicological assessment of aquatic sediments with *Caenorhabditis elegans* (nematode)-A method for testing in liquid medium and whole sediment samples. *Environ. Toxicol. Chem.*, **16**, 245–250.
18) Dhawan, R., Dusenbery, D. B. and Williams, P. L. (2000) A comparison of metal-induced lethality and behavioral responses in the nematode *Caenorhabditis elegans*. *Environ. Toxicol. Chem.*, **19**, 3061–3067.
19) Kohra, S., Kuwahara, K., Takao, Y., Ishibashi, Y., Lee, H. C., Arizono, K. and Tominaga, N. (2002) Effects of bisphenol A on the feeding behavior of *Caenorhabditis elegans*. *J. Health Science*, **48**, 93–95.