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Wound Healing in Earthworms *Lumbricus terrestris:* A Cellular-Based Biomarker for Assessing Sublethal Chemical Toxicity

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Elsewhere, we describe several humoral and non-specific immunity-based biomarkers in earthworms for assessing sublethal immunotoxic risks to wildlife from exposure to terrestrial chemical contaminants and hazardous waste site (HWS) materials (e.g. Rodriguez et al. 1989; Chen et al. 1991; Goven et al. 1993a,b; Goven et al. 1994). These earthworm biomarkers are exposure / dose sensitive, repeatable and sufficiently analogous or homologous to those in vertebrates for use in predicting risks to wildlife (see overviews by Fitzpatrick et al. 1990; Venables et al. 1992; Goven et al. 1994, 1996). Our objective here is to present a cellular-based biomarker involving the inflammatory response, which is widespread among animals, and is associated with the wound-healing process in the integument of earthworms (*Lumbricus terrestris*). Specifically, we describe an experiment conducted to demonstrate the response sensitivity of wound healing in *L. terrestris* and its potential as a biomarker for use with HWS soils. We exposed earthworms to organic and inorganic chemicals (chlordane and cadmium, Cd^{++}) at different exposure concentrations and durations, and compared wound healing between worms exposed to chlordane-treated artificial soils with those exposed to Super-fund HWS soils contaminated with chlordane.

Development and use of wound healing in earthworms as a biomarker for assessing chemical toxicity follows seminal works by Cooper and his colleagues (e.g. Cooper 1968, 1969; Cooper and Roch 1984, 1986, 1992; Ville et al. 1995). Wound healing in earthworms is a continuing process from open to healed wound, with stages and activities characterized histologically and immunologically by Cooper and Roch (1986). It involves the inflammatory response and various cell types, including immunoactive macrophage-like coelomocytes. Although wound healing is complex and continuous, we have simplified the assay or endpoint to include only two conditions: Healed and non-healed. Basically, a three-sided patch of integument of *L. terrestris* is cut with surgical scissors, lifted and returned in place. The wound is observed daily until it is fully healed. To see effects of chemical exposure on wound healing, worms are first exposed in artificial soil (AS) (Greene et al. 1989) to various concentrations of chemicals or HWS

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materials, then removed, cleaned, wounded as described and observed for 5 d. Their response is then compared to healing in controls, which is usually 100% within 5 d.

MATERIALS AND METHODS

We held adult *Lumbricus terrestris,* purchased from Carolina Biological Supply (Burlington, NC), for 2 weeks prior to experimentation in moistened peat moss in an unlit environmental chamber at 10°C. We sprinkled commercial dry Gerber (Freemont, Ml) baby cereal on top of the peat moss as an ad libitum food source.

We exposed robust worms to technical chlordane and cadmium nitrate ($CdNO_3$) using the artificial soil (AS) exposure protocol adopted by the Environmental Protection Agency and described in Greene et al. (1989). Chlordane and $CdNO_3$ (hereafter Cd^{++}) were dissolved in acetone and distilled H_2O, respectively, and then mixed with AS by hand in zip-top plastic bags. Acetone was evaporated from AS under a hood before worms were introduced. Adult worms of equivalent masses (4-6g) were exposed to three sublethal concentrations, determined to be sublethal by range-finding tests, of chlordane (6.25, 12.5 and 25.0 µg/g AS dry mass) and Cd^{++} (100,200 and 300 µg/g AS dry mass) mixed with 400 g AS within 0.5 L glass jars with screw top lids for 10, 20 or 30 d at 10°C within an unlit environmental chamber. We used four replicate jars of 10 worms for each concentration (N = 40) at each of the three exposure durations. Controls were treated similarly, exposed to moistened AS treated with acetone (then evaporated) or water, but not chlordane or Cd^{++}.

To determine how well the wound-healing biomarker works on real-world materials, we exposed worms for 10, 20 and 30 d to a sublethal (5%) dilution, with AS, of soils from a Super-fund HWS in Massachusetts, contaminated with chlordane and described in Callahan et al. (1991). The 5% dilution represented a nominal 25 µg chlordane / g dry mass, corresponding to our highest sublethal technical chlordane concentration in AS. Exposures were conducted as described for the neat chemicals.

After exposure, worms were removed, cleaned with ice-cold distilled water, anesthetized in 5% ethanol (Cooper 1968) and wounded as follows. Under a dissecting microscope, we used small surgical scissors to make a three-sided cut in the integument on the dorsal surface 10 segments posterior to the clitellum. The resultant flap, several mm on each side, was folded back, excess fluid removed using a cotton swab and then replaced. All exposed and control worms were maintained separately in small clear plastic sandwich containers lined with moistened filter paper and held for 5 d at 10°C in the environmental chamber.

Wound healing in exposed and control groups was determined 5 d post wounding, because our preliminary work showed that robust non-exposed worms completely heal within that time. With aid of a dissecting microscope we judged wounds healed when the three-sided cut was completely closed and integument continuous. Non-healed wounds were those with at least one open gap in the cut. We made statistical comparisons among exposure, duration and control groups using Chi square contingency table analyses (Zar 1984) to determine if the wound-healing biomarker was significantly ($P < 0.05$) affected by (i.e. sensitive to) exposure concentration or exposure duration or both for chlordane, Cd^{++} and HWS soil.

RESULTS AND DISCUSSION

Results of the 5-d wound-healing experiment are expressed as percent healed according to concentration and duration of exposure (Table 1). The wound-healing biomarker appeared to be response-sensitive to both concentration and duration of exposure. Both concentration and duration of exposure to chlordane significantly affected wound healing (Chi-square = 128.68, $P < 0.001$ and 11.34, $P < 0.005$, respectively). Worms showed reduced wound healing at all concentrations and durations of exposure to chlordane compared to controls. Except at 6.25 µg chlordane / g AS, the percentage of completely healed wounds decreased with exposure duration. At each exposure duration, wound healing decreased with exposure concentration. Suppression of healing by exposure to chlordane accords with reports on reduction of wound healing in earthworms exposed to another chlorinated organic, the polychlorinated biphenyl (PCB) Aroclor 1254® (Cooper and Roch 1992; Ville et al. 1995). These workers postulate that PCB interferes with wound healing by affecting membranes of macrophages and other cells that function in the healing process. Organics such as PCB, pentachlorophenol (PCP) and chlordane suppress phagocytosis in earthworm coelomocytes, probably by affecting their cell membranes (Goven et al. 1993b; Ville et al. 1995; Giggleman 1997; Giggleman et al. 1998). Lindane, another chlorinated pesticide, is reported to reduce RNA synthesis in mammals (Lewis and Adams 1985; Thomas and Faith 1985), which could also interfere with tissue repair during wound healing. Cikutovic et al. (1993) report that both chlordane and Cd^{++}, at the same concentrations we used here, interfere with spermatogenesis in *L. terrestris,* presumably by affecting cell division, which is important in the healing process.

Wound healing in earthworms exposed to 5% dilution of HWS soil was lower than in controls and decreased with exposure duration (Chi-square = 110.45, $P < 0.001$ and 8.53, $P < 0.025$, respectively). Effects on wound healing were similar between exposure to 25 µg chlordane / g dry mass in HWS soil and AS (Chi-square = 0.0181, $P > 0.05$). Soil from the same HWS at the same

dilution also suppresses phagocytosis and agglutinin production in *L. terrestris* coelomocytes (Venables et al. 1992).

Table 1. Percent of earthworms *Lumbricus terrestris* having complete healing 5 d post wounding after 10, 20 and 30-d exposure in artificial soil (AS) to three sublethal concentrations (µg /g dry mass AS) of chlordane and three of Cd^{++}, and a sublethal dilution (5%) of Superfund hazardous waste site (HWS) soil contaminated with chlordane. Sample size is 40 for each group.

Chemical	Exposure Duration		
	10	20	30
Chlordane			
6.25	81.2	75.0	80.9
12.5	66.7	53.8	38.1
25.0	61.0	26.1	21.4
Control	100	100	100
Cadmium			
100	92.6	86.2	81.2
200	92.9	80.0	75.5
300	50.0	42.9	34.4
Control	100	100	100
HWS*	56.0	40.9	13.0
Control	100	100	100

*Chlordane ≈ 25 µg / g HWS soil dry mass

The wound-healing biomarker appeared to be response-sensitive only to Cd^{++} concentration. Exposure concentration of Cd^{++}, but not duration had significant effects on wound healing (Chi-square = 126.46, $P < 0.001$ and 5.94, $P > 0.05$, respectively). Compared to controls, all Cd^{++} exposure groups showed reduced wound healing. Although we did not determine the lowest observable effects levels (LOEL), chlordane reduced wound healing at exposure concentrations much lower than Cd^{++}. Heavy metals, such as Cd^{++}, may affect enzyme activity necessary for wound healing. We have shown that another heavy metal, Cu^{++}, interferes with an enzymatic pathway in coelomocytes leading to production of superoxide (O_2^-), responsible for killing phagocytosed microorganisms in many animal species (Chen et al. 1991). Cd^{++} exposure is also reported to increase infections (Koller and Vos 1981; Lawrence 1985), inhibit RNA and DNA synthesis (Daum et al. 1993),

and ATP utilization (Graham et al. 1975), in mammals. Giggleman (1997) reports that Cd^{++} suppresses phagocytosis in *L. terrestris* coelomocytes.

Although the exact mechanisms by which chlordane and Cd^{++} suppress wound healing in the earthworm integument are not known, effects on cell membranes, cell division, energy production / use, RNA / DNA synthesis and enzymatic pathways should be sufficient to interfere with the complex processes required to heal damaged tissue.

Suppression of the healing process portends pathological effects in wildlife exposed to environmental toxicants if they are wounded during natural activities (e.g. non-lethal predator-prey, ectoparasite-host encounters, territorial defense, competitive events during courtship). Healing of lacerations, punctures and abrasions of the integument or digestive tract may be compromised, resulting in microbial infection or parasite infestations. Injured animals may be even more susceptible to pathogens if the chemicals that suppress the healing process also affect immunoactive cells responsible for phagocytizing and killing microorganisms. Thus, as a biomarker, earthworm wound-healing shows potential for use in assessing sublethal risks of chemical exposure to wildlife. It appears to be sufficiently response-sensitive to organics and heavy metals, in both concentration and duration of exposure, and to complex real-world materials for use in assessing risks of exposure in the laboratory using the AS exposure protocol and perhaps in situ on HWS's.

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