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Effects of Agrochemicals on the Immune Systems of Earthworms

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Earthworms are an ideal organism for use in the study of environmental contamination, both as an indicator species and a test species; they are ubiquitous and are also relatively easily maintained under laboratory conditions. Many laboratory tests are for risk assessment purposes and are aimed at determining the lethality of chemicals. However, sub-lethal effects, e.g. on reproduction or immune competence, can potentially have effects on population levels that are as significant as mortality and may also provide information on the effects of lower levels of environmental contamination. Methods have been developed for testing the sub-lethal effects of xenobiotics on earthworms; these include assessments of growth and functioning of nervous, immune and reproductive systems, e.g. Drewes and Lingamneni (1992) Kokta (1992) Goven *et al.* 1994 and Cikutovic (1993).

The use of the immune system as a sub-lethal biomarker for xenobiotics has been of increasing interest in recent years (Goven *et al.* 1988, 1993, 1994, Fitzpatrick *et al.* 1992). Any impairment of the immune system can lead to increased susceptibility to infection from numerous sources, with potentially lethal consequences. Although the immune system has increased in complexity throughout the course of animal evolution, certain aspects have been conserved phylogenetically (Goven *et al.* 1988); this facilitates the extrapolation of responses from lower to higher organisms. In earthworms coelomic leukocytes (coelomocytes) are predominantly responsible for immune responses. Coelomocytes can be obtained relatively easily, and non-destructively, by extrusion or by puncture of the coelomic cavity. A number of sub-lethal immune system tests have been developed using coelomocytes including: *in vitro* assessment of immune responses (Goven *et al.* 1993) lysozyme activity (Goven *et al.* 1994) and the use of nitroblue tetrazolium dye (NBT) reduction to assess the non-specific immunotoxicity of environmental xenobiotics (Chen 1991).

Immune system tests developed for earthworms have concentrated on the effects of hazardous waste site contaminants, such as PCBs and metals. There have been

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no data reported on the effects of agrochemicals. The effects of pesticides on earthworm populations are of importance due to the potential impact on soil fertility and drainage. The aim of this study was to investigate the effects of exposure of earthworms to a range of pesticides on coelomocyte activity (Goven *et al.* 1993). This should provide the basis for assessing the suitability of these methods for detecting sub-lethal effects following field applications of pesticides.

MATERIALS AND METHODS

Adult earthworms (*Eisenia veneta*) (Walker Organics, Consett, UK) were maintained in moistened Arther Bowers "Mulch and Mix" (100% organic) covered by a layer of rotting leaves. The earthworms were sustained on commercial flaked oat cereal (Ready Brek) with a natural summer temperature (mean 20°C) and daylight routine.

Two methods for extracting coelomocytes from the earthworms were compared in order to assess their effects on cell viability and immune function, and thus their suitability for subsequent tests. Coelomocytes were isolated from 10 earthworms by the non-invasive extrusion method devised by Eyambe *et al.* (1991) and from 10 earthworms by the invasive puncture method (Lockwood 1963). The percentage viability of the extruded cells was calculated following staining with 0.4% Trypan Blue in LBSS (Lumbricus balanced salt solution, Goven *et al.* 1993). The immune function of the extruded cells was then assessed using the immunological assays developed by Goven *et al.* (1993). Coelomocytes were incubated with rabbit erythrocytes in LBSS for 24 hours at 20°C. One hundred coelomocytes were examined in an haemocytometer. The number of coelomocytes showing no activity; phagocytosis of the erythrocytes; formation of rosettes of erythrocytes around coelomocytes in a single layer (erythrocyte rosettes, ER); or in 2 or more layers (secretory rosettes, SR) (Goven *et al.* 1993) were recorded. The total immune activity (TIA) was determined as the sum of the cells showing SR, ER and phagocytosis.

The effects of direct exposure of the earthworms to various chemicals on the immune function of their coelomocytes were investigated. The pesticides and PCB (Table 1) were dissolved in acetone at the required concentrations. The concentrations used (Table 1) were: the highest dose that the earthworms survived (following a range finding test based around the rat LD50), or equivalent to the soil surface residues following field application (mg/cm^2 soil) in cases where these data were available for uses in the UK. The limited availability of pesticide toxicity data for earthworms prevented the use of LC50 data in the selection of appropriate dose levels. In cases where changes were observed at high doses, lower doses were also tested. Soda glass specimen tubes (30ml) were lined with a 7cm x 7cm piece of Whatman filter paper and lml of pesticide or PCB in acetone was evenly pipetted onto the filter paper (10 per treatment). Control tubes (10) were also treated with lml acetone. All acetone was allowed to evaporate to dryness before

Table 1. Chemicals and treatment levels used in earthworm exposure studies. The pesticide concentrations used are expressed as mg/ml acetone and compared to the rat LD50 (mg/kg) (for a 2g earthworm) and surface residues following application at the field rate (mg/cm^2 soil).

Compound	Type	Cone mg/ml	% Rat acute oral LD50	% Application rate
PCB (Arochlor 1254)	Industrial pollutant	0.49	-	-
Prochloraz	Conazole fungicide	0.21	6.5	117
Propiconazole	Conazole fungicide	0.3	9.9	470
Captan	Phthalimide fungicide	0.95	5.2*	1600
Pirimicarb	Carbamate insecticide	0.13	44	147
Pirimicarb	Carbamate insecticide	0.013	4.4	14
Dimethoate	Organophosphorus (OP) insecticide	0.21	36	84
Dimethoate	OP insecticide	0.021	3.6	8
Disulfoton	OP insecticide	0.01	125	-
Disulfoton	OP insecticide	0.001	12.5	-
Pirimiphos methyl	OP insecticide	0.5	12.1	113
Pirimiphos methyl	OP insecticide	0.05	1.21	11
Paraquat	Bipyridyl herbicide	0.35	116	78
Chlorpropham	Carbamate herbicide	1.23	12.3	769
Chlorpropham	Carbamate herbicide	0.16	1.6	100
Prometon	1,3,5-Triazine herbicide	0.75	12.5*	15
Prometon	1,3,5-Triazine herbicide	0.09	1.5	1.8
Tri-allate	Thiocarbamate herbicide	2.68	100	279

* - limited by solubility in acetone

1ml of distilled water was applied to the filter paper. An individual worm was then placed in each tube and the top was sealed with a plastic stopper with air holes. The tubes were maintained on their sides, in darkness, at 20°C±1°C for 5 days. The coelomocytes were extracted using the invasive puncture method (Lockwood 1963) viability was assessed using Trypan Blue and the cells were incubated with rabbit erythrocytes for 24 hours at 20°C. The immune function of the coelomocytes was then assessed as above. All results were analysed using Students t-test.

RESULTS AND DISCUSSION

Significantly higher cell viability ($p<0.001$) was observed in cells obtained by invasive puncture (98.9±1.1%) when compared with the non-invasive extrusion method (67.5±5.7%). This difference was probably due to the presence of 5% ethanol, an irritant, and 10mg/ml of the mucolytic agent guaiacol glycerol ether in the extrusion medium. However, there was no noticeable improvement in viability

when the alcohol content was reduced to 2%. Eyambe *et al.* (1991) reported no differential effect on the immune system of *E. foetida* with the two extrusion methods. However, this does appear to be species dependent as Eyambe *et al.* (1991) reported a slightly lower viability in cells obtained by the invasive extrusion method (89.4±0.57%) than the non-invasive method (94.2±0.40%) for *Lumbricus terrestris.*

The immune activity of the coelomocytes was expressed as the number of the individual components (phagocytic cells, ER and SR) and as the total number of cells showing these immune functions (TIA) within the 100 cells counted. There was a small but significant reduction ($p<0.05$) in the ER formation with the non-invasive extrusion method when compared to the invasive puncture method. However, there was no significant difference between extrusion methods in either SR formation or the TIA of the coelomocytes. The invasive puncture method was therefore used for subsequent experiments.

The effects of chemical exposure on the total immune activity (TIA) of earthworms' coelomocytes is shown in Figure 1. PCB, an industrial pollutant, was used as a positive control as it has been reported to reduce the immune function of earthworms (Goven *et al.* 1993). In this study exposure of the earthworms to the PCB reduced the TIA of exposed worms by about 65% ($p<0.005$).

The TIA was significantly reduced following exposure of the earthworms to organophosphorus and carbamate insecticides (Figure 1). Exposure to disulfoton significantly reduced the TIA (0.01mg/ml, $p<0.005$ and 0.001mg/ml, $p<0.05$) and the reduction at the higher dose was significantly ($p<0.005$) greater than at the lower dose. The TIA was significantly reduced ($p<0.005$) at 0,21mg/ml of dimethoate (84% of the application rate), but not at the lower dose. The TIA was also significantly reduced following exposure to 0.5 and 0.05 mg/ml pirimiphos methyl ($p<0.05$ and $p<0.005$ respectively). However the difference in the reduction of immune activity between the two concentrations was not significant. At 0.13 mg/ml pirimicarb (1.44 times the application rate) the TIA was significantly reduced ($p<0.05$) but there was no significant reduction at the lower dose.

Pruett (1992) suggested that the immunotoxicity of organophosphorus pesticides observed in vertebrates may result from direct action on the cells or from excessive cholinergic stimulation, thus affecting lymphocyte or macrophage function. However, the depression in immunity may also be an indirect effect as a response to stress (Pruett 1992). The results of this study show effects on the immune function of earthworms by anticholinesterase compounds of diverse structure at levels equivalent to the application rate. It is therefore probable that effects would also be detected following exposure to a wider range of organophosphorus and carbamate compounds at similar levels.

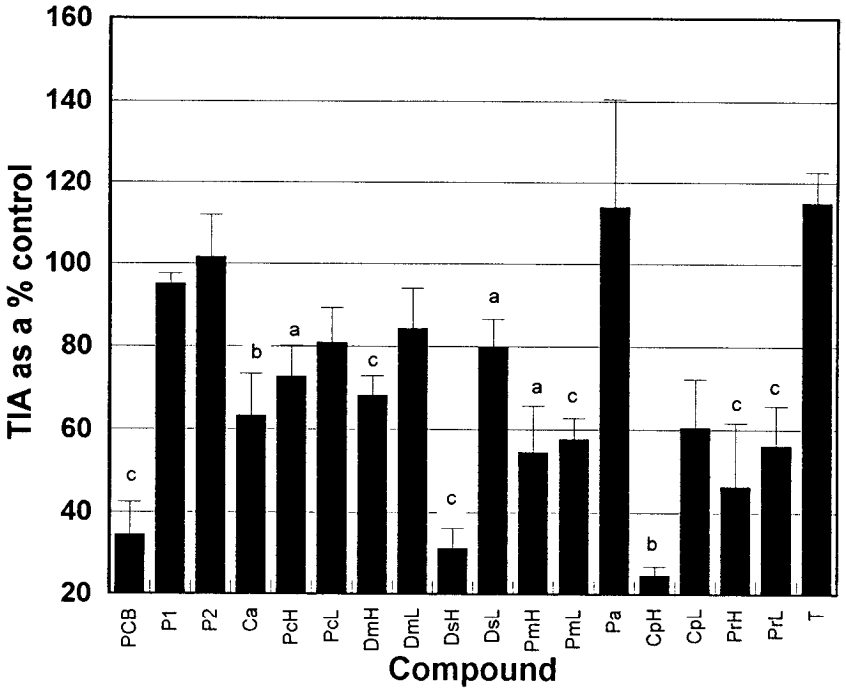

Figure 1. Effects of exposure to to a range of compounds on total immune activity (TIA) in earthworm coelomocytes. Standard error is shown, each pesticide was compared with a concurrent control (Students t-test) a- $p<0.05$, b- $p<0.01$, c- $p<0.005$ (Students t-test). H-high dose, L -low dose (see table 1). Ca - captan, P1 - prochloraz, P2 - propiconazole, Pc - pirimicarb, Dm - dimethoate, DS - disulfoton, Pm - pirimiphos methyl, Pa - paraquat, Cp - chlorpropham, Pr - prometon, T - tri-allate.

The conazole fungicides propiconazole and prochloraz caused no significant reduction in the TIA of earthworm coelomocytes even at levels as high as 5 times the application rate. Captan, a phthalimide fungicide, caused a significant reduction ($p<0.01$) in the TIA at 0.95 mg/ml.

Exposure to the bipyridyl herbicide paraquat and the thiocarbamate herbicide tri-allate did not result in any significant changes in TIA activity. However, the TIA was significantly reduced ($p<0.01$) following exposure to the higher dose (approx 7 x application rate), but not the lower dose (application rate), of the carbamate herbicide chlorpropham. Prometon, a triazine herbicide, caused significant reductions ($p<0.005$ at the higher and lower doses) of immune activity but the reductions at the two different concentrations were not significantly different.

Although the TIA of the coelomocytes was reduced in several cases, there were no significant effects on the proportions of the individual components (SR, ER, phagocytosis). Each type of immune function was equally affected by the

compounds used. Therefore, for these classes of compounds, the assay can be reliably simplified to counting the proportion of the 100 cells counted which show any type of immune activity. However if the assay is being used to assess new classes of chemicals then the immune types should be assessed separately to establish any differential effects which may be masked by the total activity.

No prediction of the immunotoxicity of pesticides to earthworms can be made from the acute LD50 for rats or mode of action alone. At 5-10% of the rat LD50 neither the conazole tungicides prochloraz and propiconazole (rat LD50 1500-1600mg/kg) showed immunotoxic activity but the phthalimide fungicide, captan (rat LD50 9000mg/kg) did. At approx. 10% rat LD50 the herbicide prometon (triazine, rat LD50 3000mg/kg) showed an immunotoxic effect but chlorpropham (carbamate, rat LD50 5000-7000mg/kg) and paraquat (bipyridyl, rat LD50 150 mg/kg)) showed no response. As discussed above, all the anti-cholinesterase insecticides showed an immunotoxic effect at 30-40% of the rat LD50 (rat LD50s 4-147 mg/kg). Further testing is therefore needed to establish reliable QSAR to show which types of compounds have an immunotoxic action.

To predict chemicals with immunosuppressive potential further information is required on their mode of action. A range of additional methods for assessing immune function are available and a tiered approach is probably most appropriate. The first tier should comprise assays that are quick, simple and cheap to assess the scale of immunosuppression and the types of cells affected. The immunological assays investigated here have the potential to be first tier tests. The second tier of tests, which should be specific and sensitive, would then be used to identify the components of the immune system which have been affected, e.g. the potential of coelomocytes to oxidatively kill phagocytised micro-organisms (Chen 1991). Assays developed in mammals also have the potential to be developed for invertebrates, e.g. lymphocyte blastogenesis, cytotoxic t-cell (leukocyte) activity and plaque forming cell assays (Weeks *et al*. 1992).

The method used in this study provides rapid cost-effective screening for the presence of immunosuppressive chemicals. Impairment of the immune system may increase susceptibility to infection. However, there have been few reports of investigations of the impact of immunosuppression on the ability of the earthworm to respond to fungal, nematode or microbial attack. Pizl (1985) reported that earthworms from triazine-herbicide treated orchards were infected with moncystid gregarine parasites. Further laboratory studies confirmed that treatment with the herbicide increased the susceptibility of earthworms to infection by the parasites (Pizl 1985). Immunosuppression may also reflect the ability of the organism to withstand further environmental stresses. In order to predict population responses to chemicals from the results of laboratory studies further work is required. This work should be directed at 1) linking reductions in immune response with a physiological effect (ie. disease-related) on the earthworm and 2) the extrapolation of effects on the individual to effects at the population level.

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