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The Biological Effects of Bifenthrin on T-cells and Neurons: A Comparison of Activity

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ABSTRACT

Pyrethroids are considered a less toxic class of pesticides and are therefore increasingly formulated for household use. In current study we compare the toxicity of the pyrethroid (bifenthrin) with the organochlorine (lindane) and the mitochondrial poison (rotenone) to mammalian T-cells and neurons. Cells were treated for twenty-four hours with various concentrations of pesticide and then assessed for morphology and measured for viability using trypan blue exclusion. Lindane and rotenone produced an LD_{50} of 500 μM and 500 nM, respectively, in T-cells and 5 μM and 250 nM, respectively, in neurons. Lindane and rotenone had a lowest observable effect level (LOEL) of 100 μM and 50 nM, respectively, in T-cells and 100 nM and 1 nM, respectively, in neurons. Significant toxicity was observed with lindane and rotenone, whereas bifenthrin did not reduce the viability of either cell type at concentrations as high as 1 mM. However, bifenthrin stimulated T-cell homotypic aggregation which is associated with cell activation. Further, bifenthrin inhibited the neurons from forming neurites. While these results support the claim that pyrethroids are less toxic than many other pesticides, they raise concerns that chronic exposure to pyrethroids could contribute to inflammation and hypersensitivity and also to developmental neurotoxicity and neurodegenerative diseases.

Key words: Pyrethroids, bifenthrin, neurons, T-cells, neurite, toxicity.

INTRODUCTION

Pyrethroid insecticides are a relatively new group of synthetic compounds derived from the natural compound known as pyrethrin, which is isolated from some of the *Chrysanthemum* flowering plants (Casida, 1980; Laskowski, 2002; Shafer *et al.,* 2005). Pyrethroid insecticides exert their effects by binding to and inhibiting insect voltage-sensitive sodium channels and consequently causing a prolonged channel current and lethal paralytic convulsion (Desi *et al.,* 1986; Soderlund and Bloomquist, 1989; Warmke *et al.,* 1997; Narahashi 2000; Tabarean and Narahashi, 2001; Vais *et al.*, 2001; Soderlund *et al.,* 2002; Shafer and Meyer, 2004; Shafer *et al.,* 2005). The selective toxicity of pyrethroids to insects compared to mammals has been well documented including one study in which *Xenopus oocytes* engineered to express *Drosophila melanogaster* sodium channels showed a 100-fold greater affinity for pyrethroid binding compared to sodium channels of rat brain (Soderlund and Bloomquist, 1989; Warmke *et al.,* 1997; Tabarean and Narahashi, 2001; Soderlund *et al.,* 2002; Shafer and Meyer, 2004). While pyrethroid toxicity is highly selective for insects, pyrethroid neurotoxicity has been observed in mammals including humans (Desi *et al.,* 1986; Trainer *et al.,* 1993; Narahashi, 2000; Vais *et al.,* 2001; Shafer *et al.,* 2005). Concerns for the effect of pyrethroids on the human immune system, as well as the developing and adult human nervous system, has grown as pyrethroids, such as bifenthrin and others, have become increasingly included in pesticide preparations marketed for household use.

With regard to the effect of pyrethroids on the immune system, cypermethrin reduces the humoral and cell-mediated response in rabbits to inoculation with *S. Typhi* (Desi *et al.*, 1986). Furthermore, chronic exposures to permethrin cause a decrease in human T-lymphocyte proliferation and a significant reduction in the production of IFN-γ and IL-4 which play an important role in T-cell and B-cell regulation, respectively (Diel *et al.,* 1998). Topical permethrin application also inhibits B-cell and macrophage activity in C57Bl/6N mice (Punareewattana *et al.*, 2001). Also, fenvalerate was shown to reduce the immune response of goats to *Brucella abortus* strain 19 vaccine (Singh and Jha, 1996).

With regard to the effect of pesticides on the nervous system, recent studies have raised concerns regarding the potential for long term exposure to pesticides to cause neurodegenerative diseases (Casida, 1980; Desi *et al.,* 1986; Soderlund and Bloomquist, 1989; Warmke *et al.,* 1997; Shafer and Meyer, 2004; Shafer *et al.,* 2005). For example, striatal dopaminergic pathways are targeted by permethrin and chlorpyrifos in the mouse (Soderlund *et al.*, 2002). Further, in rats, deltamethrin causes prolonged expression of c-*fos* and c-*jun* in the cerebral cortex (Tabarean and Narahashi, 2001). Also, permethrin causes neuronal cell death and cytoskeletal alterations in the cerebral cortex and the hippocampus, and Purkinje cell loss in the cerebellum (Abdel-Rahman *et al.,* 2001). With regard to developmental neurotoxicity in maternal systems, utero exposure of rats to deltamethrin results in increased hippocampal acetycholinesterase activity and decreased learning and

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memory in the 6 to 12 week postnatal period (Vais *et al.,* 2001).

Since the pyrethroid pesticides are increasingly included over-the-counter formulations for household uses, there is increasing concern that chronic low level exposure to pyrethroids may result in toxicity. In this study, we compare the toxicity of the pyrethroid, bifenthrin, to the toxicity of the organochlorine, lindane, and the mitochondrial poison, rotenone, on mammalian T-cells and neurons. In addition, to explore the frank toxicity of the pesticides, we investigate the sublethal effect of bifenthrin which suggests that this pesticide may have hidden immunotoxicities and neurotoxicities.

MATERIALS AND METHODS

Chemicals

Trypan blue was obtained from Sigma Chemical Co. Technical grade bifenthrin and lindane were obtained from Chem. Service Inc. Rotenone was purchased from LC Chemical Corp. Nerve growth factor (NGF) was purchased from Invitrogen. Lindane, bifenthrin, and rotenone were dissolved in dimethyl sulfoxide (DMSO).

Cells and culture condition

Rat PC12 cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) containing 7.5% fetal bovine serum (FBS), 7.5% horse inactivated serum, and 0.01% gentamycin, and incubated in a CO_2 incubator at 37°C. For use in experiments, cells were collected by agitation at approximately 70-80% confluence.

H9 and Jurkat human T-cell lines were obtained from the NIH AIDS Research & Reference Reagent Program and grown in suspension in RPMI 1640 medium (Biowhittaker) supplemented with 10% of fetal bovine serum (Fisher) and 0.01% gentamycin. The cells were incubated at 37°C in a CO_2 incubator.

A hemocytometer was used to determine cell numbers and cell viability was counted by trypan exclusion.

Trypan Blue viability assays

Cells (H9 and Jurkat and PC12) were harvested and seeded in 0.5 ml of medium in wells of a 24 well tissue culture cluster. PC12 cells were seeded into 16 mm diameter laminin-coated tissue cluster wells in medium containing 100 ng/ml of nerve growth factor (NGF) at 10,000 cells per well. H9 and Jurkat cells were seeded into 16 mm diameter untreated tissue cluster wells at 200,000 cells per well. The cells were treated in replicate wells immediately after seeding with concentrations of bifenthrin, lindane, and rotenone ranging from $1x10^{-10}$ M to $1x10^{-3}$ M. These concentrations were achieved by adding 1 µl to the test wells. The pesticides were dissolved in dimethylsulfoxide (DMSO) and stocks were prepared. Therefore, the control (no treatment wells) received 1 µl of DMSO (0.2%). After twenty-four hours of treatment, the cells were analyzed for morphology (see below) and harvested using a 1.0 ml micropipette. PC12 cells were then placed in a 15 ml conical tube and centrifuged at 1000 rpm for 10 minutes. After centrifugation, cells were resuspended in 100 µl of medium. The H9 and Jurkat cells were kept in the 0.5 ml of medium they were collected in. After then, 10 µl of the cell suspension was combined with 10 µl of 0.4 % trypan solution and viewed using a hemocytometer. The cells in 16 peripheral squares (conversion area for 1.0 ml) were examined for staining and the percent of the cells which excluded trypan was used to determine cell viability. For bifenthrin and lindane treatment of PC12 cells, experiments were conducted in duplicate wells with five trials. For rotenone treatment of PC12 cells and all experiments with T-cells, experiments were conducted in triplicate wells with two trials.

Assessment of cell morphology

Immediately prior to harvesting, the cells for trypan exclusion, the bifenthrin, lindane, and rotenone were assessed for morphology using an inverted microscope and photographed at 400X magnification. The formation of homotypic aggregations were determined and scored as follows: no aggregates (-), 1-100 cells/aggregate (+), 101-200 cells/aggregate (++), 201-300 cells/aggregate (+++), and 301+ cells/ aggregate (++++). For neurite formation, cells were examined for the presence of neurites using an inverted microscope. Cells were considered to be positive for neurites only if the cells had neurites twice the cell diameter or greater. To determine percent neurite outgrowth, three fields of each well was assessed at 200X magnification. The number for each well was averaged. Neurite outgrowth was then scored as follows: 95-100% with neuritis (++++), 75-94% with neurites (+++), 50–74% with neurites (++), 10-49% with neurites (+), and less than 10% with neurites (-).

RESULTS

Pesticide toxicity to T-cells and neurons

Lindane demonstrated toxicity to both T-cells and neurons by significantly reducing trypan exclusion in these cells (Table 1). For both T-cell lines, H9 and Jurkat, the dose at which 50% of the cells were judged to be dead (LD_{50}) for lindane, was 500 µM. A significantly greater toxicity was witnessed in the neurons which displayed a lindane LD_{50} of 5 µM. The greater sensitivity of the neurons to lindane was also reflected in the lowest observable effect level (LOEL) which was 100 µM to the T-cell lines and 100 nM to the neurons. In order to achieve the no observable effect level (NOEL) for toxicity, the lindane had to be diluted to 10 mM for T-cells and 1 nM for neurons. Rotenone was more highly toxic to all cell types compared to

Table (1): A comparison of the toxic effect of bifenthrin, lindane and rotenone on T-cells and neurons[1].

Cell Type	Bifenthrin			Lindane			Rotenone		
	NOEL*	LOEL*	LD_{50}	NOEL	LOEL	LD_{50}	NOEL	LOEL	LD_{50}
T-Cells									
H9		10μM	>1mM	10μM	100μM	500μM	5nM	50nM	500nM
Jurkat	1μM	10μM	>1mM	10μM	100μM	500μM	N.D.	N.D.	N.D.
Neurons									
PC12	100nM	1μM	>1mM	1nM	100nM	5μM	0.1nM	1nM	250nM

[1]The human H9 and Jurkat T-cell lines and the rat neuronal PC12 cell line were subjected to treatment with the indicated pesticides, at concentrations ranging from 0.1 nM to 1.0 mM, for twenty-four hours and subsequently assayed for viability using trypan blue exclusion. LD_{50} is the dose of pesticides at which 50% of the cells died, (NOEL) is the dose at which there was no observable effect, and (LOEL) represents the lowest observable effect.

* Bifenthrin effect on T-cell and PC12 cell morphology represents the level of biological activity not the level of toxicity.

lindane (Table 1). LD_{50} of rotenone to the H9 T-cells was 500nM and for PC12 cells, the rotenone LD_{50} was 250 nM. The LOEL for rotenone was 50 nM in H9 cells and 1nM in neurons. The greater toxicity of rotenone to neurons was reflected again in the rotenone NOEL for H9 T-cells which was 5 nM, while the rotenone NOEL for neurons was 0.1 nM. Compared to lindane and rotenone, bifenthrin was extraordinarily nontoxic. Indeed, even at 1 mM, a reduction in trypan exclusion for all cell types was not observed. While Table (1) does show a NOEL and LOEL, these data refer to concentrations at which biological activities were observed and not frank toxicity.

Biological activities of bifenthrin at sublethal doses

While toxicity, as judged by trypan exclusion, was not observed with bifenthrin, bifenthrin was observed to significantly affect the morphology of both the T-cells and neurons (Table 1). Both H9 and Jurkat cells formed homotypic aggregations characteristic of T-cell activation when treated with bifenthrin (Fig. 1). This effect could be observed at concentrations as low as 10 μM in both cell types with a NOEL of 1 μM. The size of the T-cell aggregates was dose dependant with aggregates of H9 cells larger than 300 cells/ aggregate at 1 mM bifenthrin (Table 2). At the LOEL of 10 μM, H9 aggregates ranged 100-200 cell/aggregate in size (Tables 1&2).

Bifenthrin also failed to demonstrate a reduction in trypan exclusion to PC12 cells. However, bifenthrin treated PC12 cells failed to extend neurites compared to what is normally seen in untreated controls (Fig. 1). The ability of bifenthrin to inhibit neurite outgrowth in the absence of frank toxicity was observed at concentrations as low as 1 μM and a NOEL of 100 nM (Table 1). Further more, bifenthrin-mediated inhibition of neurite outgrowth was dose dependant, with approximately a 10% reduction in neurite outgrowth at the LOEL of 1 μM (Tables 1&2). At 10 μM and 100 μM bifenthrin, neurite outgrowth was inhibited by over 25% and 50%, respectively, and greater than 90% inhibition of neurite formation at 1 mM (Table 2).

Lindane and rotenone did not cause morphological changes in any of the cell types that were not associated with overt cell toxicity.

Table (2): Sublethal biological effects of bifenthrin on H9 T-cells and PC12 neurite cells.

Concentration of Bifentherin (Molar)	H9 T-cell Aggregation	PC12 Neurite Formation
10^{-10}	-	++++
10^{-9}	-	++++
10^{-8}	-	++++
10^{-7}	-	++++
10^{-6}	-	+++
10^{-5}	++	++
10^{-4}	+++	+
10^{-3}	++++	-

The formation of homotypic aggregates, after 24 hourstreatment, were assessed and scored as described in materials and methods.

DISCUSSION

Due to high toxicity, organochlorines, organophosphates, and carbamates are being more sparingly used in over-the-counter pesticide preparations and being replaced by synthetic pyrethroids, including bifenthrin, in part due to their greater selective toxicity to insects (Soderlund and Bloomquist, 1989; Trainer *et al.,* 1993; Narahashi, 2000; Tabarean and Narahashi, 2001; Vais *et al.,* 2001; Sanborn *et al.,* 2002; Soderlund *et al.,* 2002; Stok *et al*., 2004). This study confirms that bifenthrin has a low toxicity by failing to demonstrate frank toxicity at all tested concentrations. In other studies, however the pyrethroid (deltamethrin) has been shown to induce apoptosis in cultured cerebral cortical neurons

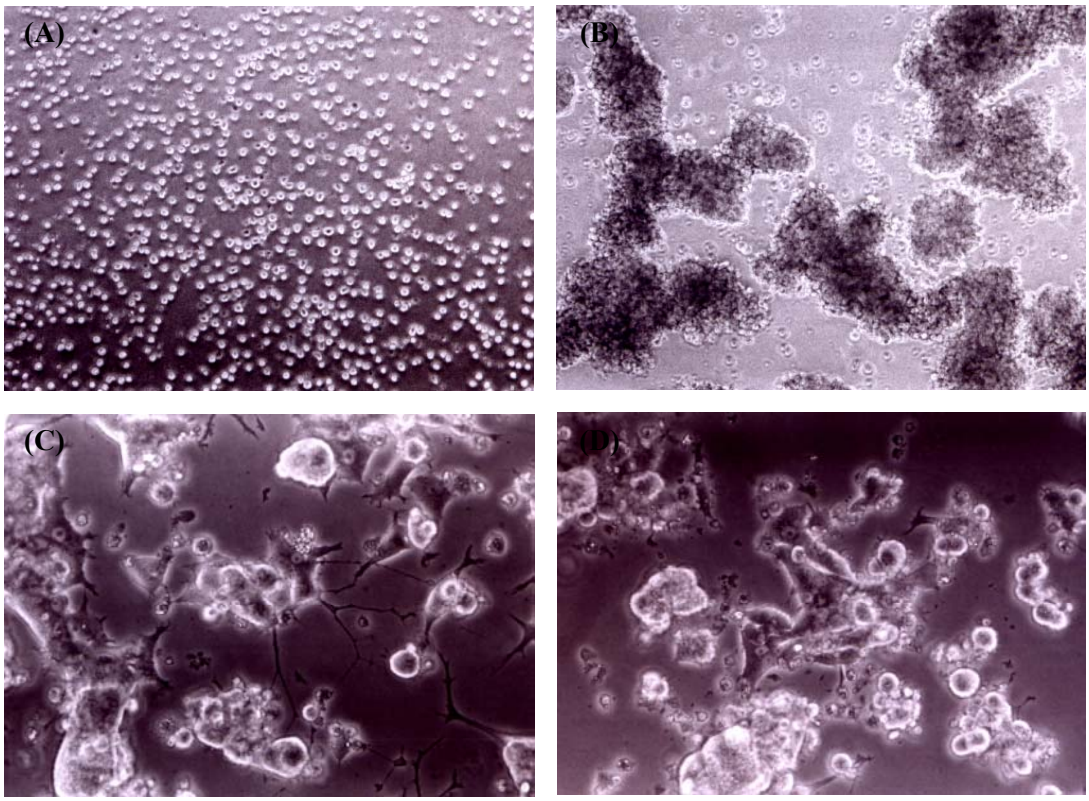

Figure (1): Effect of bifenthrin on T-cells and neurite outgrowth. The human T-cell line and H9 cells, were cultured for twenty-four hours in the absence (A) or presence, at 100 μM of bifenthrin (B). PC12 cells were cultured for twenty-four hours in the absence (C) or presence, at 100 μM bifenthrin (D). Magnification was 400X.

(Wu *et al.,* 2003) and to alter neurotransmitter release in primary hippocampal neurons (Grosse *et al.,* 2002). Additional evidence for pyrethroid toxicity includes histolological analysis of the brains of rats that were subjected to daily dermal exposure to permethrin which showed neuronal degeneration in the cerebral cortex, the dentate gyrus, and the hippocampus (Abdel-Rahman *et al.,* 2001). However, consistent with our results, other studies have also shown that pyrethroids can have deleterious effects on the nervous system in the absence of frank toxicity. For example, the pyrethroid permethrin, at nontoxic doses, represses Ca^{2+} induction of brain-derived neurotropic factor and c-*fos* gene expression in cultures of murine cerebellar granule cells (Imamura *et al.,* 2000).

For neurotoxicity, there is evidence and concern for pesticide immunotoxicities. Pesticides such as DDT, TCDD and PCB are immunotoxicants that cause a wide variety of immune dysfunction in animals and humans (Kannan *et al.,* 2000). However, pyrethroids have been shown to exhibit both immunosuppressive and immunopotentiative effects. For example, deltamethrin stimulates the immune system by increasing the number of B-lymphocytes in the spleen and enhancing the activity of natural killer cells (Madesen *et al.,* 1996). Other pyrethroids, such as permethrin and allethrin, also inhibit proliferation of PHA and concanavalin-A stimulated lymphocytes (Diel *et al.,* 1998; Diel *et al.,* 2003). Pyrethroids have also been shown to reduce T-cell and basophile interferon-gamma and interleukin-4 production and reduce basophile histamine release (Diel *et al.*, 1999a; Diel *et al.,* 1999b). Although these studies suggest that pyrethroids may reduce the ability of the immune system to engage in the inflammatory response, we found that bifenthrin activate T-cell homotypic aggregation. Therefore, pyrethroids may lead to immune dysfunction by artificially activating the immune system and leading to inappropriate inflammation.

In this study, a lethal immunotoxic and neurotoxic level of the pyrethroid, bifenthrin, could not be established which supports the claims that the pyrethroid bifenthrin is less toxic than many of the other pesticides available. However, our results showed that bifenthrin could inappropriately activate cells of the immune system as well as inhibit normal neurite formation in the absence of frank toxicity. Therefore, increas the availability of bifenthrin in common over-the-counter pesticide preparations marketed for household use raises the concerns that long term

exposure to bifenthrin could lead to inflammations and hypersensitivity as well as deleterious and chronic effects on the nervous system.

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