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Cytogenetic effects of permethrin in cultured human lymphocytes

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The pyrethroid insecticide permethrin was tested for its ability to induce sister chromatid exchanges (SCE), micronuclei (MN) and structural chromosome aberrations (CA) in cultured human peripheral blood lymphocytes. Permethrin was tested in the range of 5–500 μg/ml in the absence and in the presence of a rat liver activation system (S9 mix). Small elevations in the SCE frequencies were found and even though statistically significant may have no biological meaning, the more so since there was no dose-effect relationship. Permethrin induced both MN and CA when it was evaluated in the absence of a metabolic activation system. Nevertheless, it cannot be said that S9 mix suppressed the activity in itself. The effect of permethrin seemed to be time of exposure dependent. Permethrin could be characterized as a S-phase independent agent with greater potential for inducing chromosomal damage than sister chromatid exchanges.

Introduction

Synthetic pyrethroid insecticides are analogues of the natural pyrethrins of botanical origin. The synthesis of biologically active, photostable pyrethroids has led to the rapid development of the use of some of them as insecticides (Elliot, 1976; Elliot *et al.*, 1978; Casida, 1980) due to their high activity against insects and relatively low mammalian toxicity (Papadopoulou-Mourkidou, 1983). Permethrin is a synthetic pyrethroid developed by Elliot *et al.* (1973). This compound has exhibited high light stability because of the lack of a photolabile methyl group and it has been shown to be an effective insecticide against several major insect species (Elliot *et al.*, 1978).

The carcinogenic potential of the pyrethroids has been discussed in the review of Litchfield (1985). Although high dose levels of permethrin caused an increased number of lung and liver tumours in mice in some studies, the compound was judged by the US EPA to be of very little or no hazard to humans. The tumours were non-malignant, and no oncogenic effects were noted in rats. Other pyrethroids tested were not carcinogenic (Bradbury and Coats, 1989).

Permethrin has been evaluated for gene mutation, primary DNA damage and chromosomal effects in different short-term tests.

Permethrin was reported to be non-mutagenic for gene mutation in *Salmonella typhimurium* (Moriya *et al.*, 1983; Pluijmen *et al.* 1984; Herrera and Laborda, 1988); *Escherichia coli* (Moriya *et al.*, 1983); V79 Chinese hamster cells (Pluijmen *et al.*, 1984) and *Drosophila melanogaster* (Gupta *et al.*, 1990).

Permethrin did not induce primary DNA damage measured as differential toxicity in *E.coli*, *Bacillus subtilis* and *S.typhimurium* (Miyamoto *et al.*, 1976).

Permethrin was evaluated for aneuploidy in *D.melanogaster* and the results were negative (Woodruff *et al.*, 1983).

In considering all of the relevant data, the IARC Working Group on the evaluation of carcinogenic risk to humans has included permethrin in the group 3, i.e. it is not classifiable as to its carcinogenicity to humans (IARC, 1991).

To provide additional genotoxicity data for permethrin, we report here our results on the effect of this compound in the induction of sister chromatid exchanges (SCE), micronuclei (MN) and structural chromosome aberrations (CA) in cultured human peripheral blood lymphocytes.

Materials and methods

Chemicals

Permethrin (CAS Registry no. 52645-53-1) (Figure 1), with a purity of 99.5%, was provided by Chem. Service, Inc. This compound was dissolved in dimethylsulphoxide (DMSO), supplied by Merck. The final DMSO concentration was 1% in both treated and control cultures. Permethrin was tested in the range of 5–500 μg/ml.

Lymphocyte cultures

Lymphocyte cultures were set up by adding 0.5 ml of heparinized whole blood from healthy volunteer donors, to 5 ml of chromosome medium (RPMI 1640, Flow) supplemented with 15% heat inactivated fetal calf serum (Flow), antibiotics and glutamine. Lymphocytes were stimulated with 2% phytohaemagglutinin (Flow). Two parallel whole-blood cultures for each test concentration were established in each assay. The number of leukocytes was determined in a hemocytometer (Improved Neubauer), each culture contained $2–4.5 \times 10^6$ leukocytes.

Metabolic activation system

The supernatant of the post-mitochondrial liver fraction (S9) from Wistar male rats weighing ~200 g was used as the metabolic activation system. The liver S9 fraction was prepared according to the method described by Maron and Ames (1983). Arochlor 1254 was used for induction.

Immediately before the genotoxicity tests, S9 mix (0.5 ml/culture) was prepared, i.e. S9 (10%) with 4 mM NADP and 5 mM glucose-6-phosphate cofactors according to Maron and Ames (1983).

Cytogenetic methods

For the SCE assay the cultures were incubated at 37°C for 70 h, in the dark, and 24 h after the initiation of the cultures 15 μg/ml of 5-bromodeoxyuridine (BrdU) was added together with the different concentrations of permethrin assayed. At 1.5 h prior to harvesting, 1.5 μg/ml of colchicine (Sigma) was added (Preston *et al.*, 1987).

For the micronucleus assay the cultures were incubated at 37°C for 72 h, and 24 h after the initiation of the cultures the test compound was added and it was present in the culture medium until the harvest. For inducing binucleated cells,

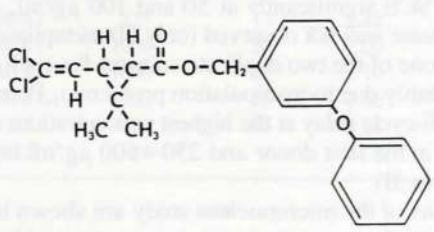

Fig. 1. Structure of permethrin.

cytochalasin B (final concentration 3 μg/ml) was added at 48 h following initiation of the cultures (Ramalho *et al.*, 1988).

For the CA assay the cultures were incubated at 37°C for 69 h, and 48 h after initiation the test compound was added. Two separate experiments were carried out. In one of them the compound was present in the culture medium until the harvest and in the other the compound was only present for 2 h. At 1.5 h prior to harvesting, 1.5 μg/ml of colchicine (Sigma) was added (Scott *et al.*, 1990).

The treatment with S9 mix, in the three assays, was relatively short in duration (2 h) and performed in serum-free medium 24 h after culture initiation for SCE and MN and 48 h after culture initiation for CA. Following the treatment, cells were washed with medium and re-established in fresh tissue culture medium with serum.

For SCE and CA cells were collected by centrifugation, resuspended in a pre-warmed hypotonic solution (KCl, 0.075 M) for 10 min and fixed in acetic acid: methanol (1:3 v/v). Cells were spread on slides, air dried and stained with Giemsa (CA) or fluorescence plus Giemsa (Perry and Wolf, 1974) (SCE).

For MN, after harvest, the cultures were treated with 5.28 μg/ml of potassium cyanide for 4 min in order to lyse erythrocytes (Maki-Paakkanen and Norppa, 1987) and cells were fixed in methanol:acetic acid (3:1 v/v). Cells were spread on slides, air dried and stained according to the May-Grünwald-Giemsa technique.

SCE, MN and CA analysis

For the SCE assay a total of 50 well spread metaphases in second division containing 46 ± 2 chromosomes were examined per treatment for each duplicate cultures of the donors (if possible) on coded slides in a blind study. The data were expressed as the mean number of SCE per cell ± SD. The two-tailed Student's *t*-test was used to compare SCE frequencies between the treated and control groups. The proliferative rate index (PRI) was calculated for each point according to the formula $PRI = [(M_1 + 2M_2 + 3M_3)/100]$ where M_1, M_2 and M_3 indicate those metaphases corresponding to first, second and third or subsequent divisions (Lamberti *et al.*, 1983).

For the MN assay 1000 binucleated cells for each treatment level in each donor were analysed (if possible) on coded slides in a blind study. The micronucleus data were tested statistically (Kastenbaum and Bowman, 1970). The percentage of binucleated cells with regard to the total cells, i.e. mononucleated and binucleated cells, was calculated in order to establish the toxic effect of permethrin. Taking the percentage of binucleated cells in the control as 100%, a test compound was considered toxic when it produced approximately a 10–20% binucleated cells.

For the CA assay a total of 50 or 100 well spread metaphases containing 46 ± 2 chromosomes were examined per treatment for each donor on coded slides in a blind study. All the aberration data were recorded according to the Savage's description (1975). Gaps (achromatid lesions) were recorded to analyse the data excluding and including gaps. Nevertheless, gaps should not be included in the analysis of CA incidence, since their cytogenetic significance is not known (Swierenga *et al.*, 1991). A chi-square test was used to compare CA frequencies between the treated and control groups.

Results

The effect of permethrin on the induction of SCE in human lymphocytes is summarized in Tables I and II. The SCE values in controls were similar in the absence of S9 mix for the two donors (Table I), whereas in the presence of S9 mix there was a slight difference between assays carried out with different donors (Table II). The results in the absence of S9 mix (Table I) indicated that 50 μg/ml of permethrin increased significantly the number of SCE in the first donor; similar increases were observed in the second donor at the highest concentration, 100 μg/ml, in only one of the two cultures. Cells exposed to several concentrations of permethrin in the presence of S9 mix (Table II) did not show any statistically significant increase in SCE values in the first donor; in the second donor permethrin increased the number of SCE significantly at 50 and 100 μg/ml, but a dose-related increase was not observed (only 50 metaphases in second division in one of the two duplicate cultures for each donor were found, probably due to manipulation problems). Permethrin also caused a cell-cycle delay at the highest concentrations assayed,i.e. 100 μg/ml in the first donor and 250–500 μg/ml in the second donor (Table II).

The results of the micronucleus study are shown in Tables III and IV. Initially, a preliminary assay was conducted in the absence of S9 mix, with three concentrations of permethrin, 10, 25 and 50 μg/ml. The increase of micronuclei was statistically significant at all concentrations. The effect of permethrin seemed

Table I. Induction of SCE and cell cycle delay by permethrin in human lymphocytes in the absence of S9 mix

Donor	Concentration (μg/ml)	No. metaphases scored	SCE/cell ± SD	PRI[a]
1	0	50	6.26 ± 2.67	2.10
1	5	50	5.74 ± 2.10	2.11
1	10	50	6.70 ± 2.63	2.09
1	25	50	6.68 ± 2.47	2.01
1	50	50	7.80 ± 3.34‡	1.85
1	0	50	6.06 ± 2.25	2.19
1	5	50	5.58 ± 1.82	1.95
1	10	50	6.02 ± 2.16	1.96
1	25	50	6.92 ± 2.28	1.93
1	50	50	8.32 ± 3.79‡‡	1.85
2	0	50	6.32 ± 2.04	2.30
2	25	50	6.40 ± 2.15	2.78
2	50	50	7.18 ± 2.40	1.97
2	75	50	7.14 ± 2.64	2.02
2	100	50	7.36 ± 2.75‡	2.00
2	0	50	6.80 ± 2.15	2.13
2	50	50	7.00 ± 2.73	2.05
2	75	50	7.82 ± 3.01	1.96
2	100	19	7.63 ± 2.16	1.96

[a]PRI was calculated as: (M1 + 2M2 + 3M3)/100, where M1 is the per cent value of cells in the first, M2 in the second and M3 in the third and higher divisions respectively (Lamberti *et al.*, 1983).
‡$P < 0.05$ and ‡‡$P < 0.01$ (Student-test two-tailed for SCE).

Table II. Induction of SCE and cell cycle delay by permethrin in human lymphocytes in the presence of S9 mix

Donor	Concentration (μg/ml)	No. metaphases scored	SCE/cell ± SD	PRI[a]
1	0	50	6.67 ± 2.07	2.08
1	5	50	6.62 ± 2.15	2.11
1	10	50	7.34 ± 2.36	2.03
1	25	50	7.14 ± 2.73	2.17
1	50	33	6.90 ± 2.91	1.82
1	100	6	9.70 ± 2.91	1.37
1	0	28	6.00 ± 1.73	2.08
1	5	11	6.09 ± 1.00	2.00
1	10	31	6.39 ± 2.57	1.98
1	25	12	6.08 ± 2.02	1.86
2	0	50	4.94 ± 2.26	2.45
2	50	50	6.40 ± 2.13‡‡	2.15
2	75	50	6.20 ± 2.30	1.55
2	100	50	6.34 ± 3.41‡	1.96
2	250	10	7.50 ± 2.61	1.43
2	500	2	10.50 ± 3.50	1.25
2	0	50	4.90 ± 1.90	2.40
2	50	38	5.89 ± 2.01‡	2.13
2	75	9	6.11 ± 2.72	1.80
2	100	40	6.41 ± 2.82‡‡	1.80

[a]PRI was calculated as: (M1 + 2M2 + 3M3)/100, where M1 is the per cent value of cells in the first, M2 in the second and M3 in the third and higher divisions respectively (Lamberti *et al.*, 1983).
‡$P < 0.05$ and ‡‡$P < 0.01$ (Student-test two-tailed for SCE).

to be dose-dependent (Table III). According to the results obtained at the preliminary assay, another assay was performed including the previous concentrations together with 75 and 100 μg/ml using two healthy donors. The increase of micronuclei was statistically significant in cultures treated with 50, 75 and 100 μg/ml in both donors (Table IV). In the presence of S9 mix, the concentrations 25, 50,75, 100 and 200 μg/ml were assayed with the two donors. The increase of micronuclei, observed in all cases, was not statistically significant (Table IV). The percentage of binucleated cells decreased proportionally to the increase of permethrin concentration. The toxic effect of permethrin at concentrations of 100 μg/ml (without S9 mix) and at 100 and 200 μg/ml (with S9 mix) was observed.

The effect of permethrin on the induction of CA is shown in Table V and Figure 2. The results in the absence of S9 mix indicated that the increases observed in the chromosome aberrations frequency were statistically significant in the range of 75−150 μg/ml, when permethrin was present in the culture medium during 21 h; similar increases were observed in both donors. Permethrin was cytotoxic at 200 μg/ml. When permethrin was only present for 2 h in the culture medium, the increases observed in the chromosome aberrations frequency were statistically significant in the range of 150−200 μg/ml. In the presence of S9 mix, the increase observed in the CA frequency at 150 μg/ml was not statistically significant. Aberrations induced by permethrin were mainly chromosome-type aberrations (Figure 3).

Table III. Induction of micronuclei by permethrin in cultured human lymphocytes (preliminary assay)

Donor 0	Concentration (μg/ml)	Binucleates scored	Micronuclei (MN)		
			Total	% MN	Sign.[b]
	0[a]	1500	21	1.4	
	10	1000	24	2.4	$P < 0.05$
	25	1000	26	2.6	$P < 0.05$
	50	1000	30	3.0	$P < 0.01$

[a]Concentration 0 = Untreated control
[b]Significance according to the Kastenbaum−Bowman test.

Discussion

Permethrin has been found to be non-mutagenic in bacteria and V79 Chinese hamster cells (Moriya *et al.*, 1983; Pluijmen *et al.*, 1984; Herrera and Laborda, 1988). It did not induce mutations or aneuploidy in *D.melanogaster* (Gupta *et al.*, 1990; Woodruff *et al.*, 1983). It did not induce primary DNA damage measured as differential toxicity in bacteria (Miyamoto, 1976).

The present study, however, revealed a variety of cytogenetic effects of permethrin in cultured human lymphocytes.

Permethrin in the absence of S9 mix slightly increased the number of SCE in both donors at the highest concentration assayed but in one of the two donors only in one culture. In the presence of S9 mix increases in SCE values were observed only in one of the two donors and these increases in the frequency of SCE were not dose-related. Thus, these elevations in the SCE frequencies even though statistically significant may have no biological meaning because a compound is considered genotoxic only when the induced effect is reproducible and dose-related.

Permethrin increased the occurrence of MN over controls when it was assayed in the absence of a metabolic activation system. Nevertheless, it cannot be said that S9 mix suppressed the activity of permethrin. In the assays performed without S9 mix, the exposure was longer than in those performed with S9 mix in which the treatment was only 2 h.

Permethrin increased the frequency of CA in the absence of S9 mix. When permethrin was present in the culture medium

Table IV. Effect of permethrin in the *in vitro* micronucleus assay using human lymphocytes

a) Without S9 mix:

Concentration (μg/ml)	Donor 1					Donor 2				
	Binucleates (BN)		Micronuclei (MN)			Binucleates (BN)		Micronuclei (MN)		
	% BN[d]	BN scored	Total	% MN	Sign.[b]	% BN[d]	BN scored	Total	% MN	Sign.[b]
0	48 (100%)	1000	15	1.50		53.5 (100%)	1250	17	1.36	
10	45 (94%)	1000	19	1.90	n.s.	45.5 (85%)	1000	12	1.20	n.s.
25	33 (69%)	1000	24	2.40	n.s.	36.5 (68%)	1250	20	1.60	n.s.
50	20 (42%)	1000	38	3.80	$P < 0.01$	31.5 (59%)	1500	52	3.47	$P < 0.01$
75	11 (23%)	1000	37	3.70	$P < 0.01$	17 (32%)	1000	48	4.80	$P < 0.01$
100[c]	7 (12.5%)	880	38	4.30	$P < 0.01$	14.5 (27%)	835	43	5.15	$P < 0.01$

b) With S9 mix:

Concentration (μg/ml)	Donor 1					Donor 2				
	Binucleates (BN)		Micronuclei (MN)			Binucleates (BN)		Micronuclei (MN)		
	% BN[d]	BN scored	Total	% MN	Sign.[b]	% BN[d]	BN scored	Total	% MN	Sign.[b]
0[a]	39 (100%)	1000	17	1.70		59 (100%)	875	13	1.48	
25	26 (67%)	1000	10	1.00	n.s.	39 (66%)	1000	20	2.00	n.s.
50	19 (49%)	1000	15	1.50	n.s.	27 (46%)	1000	17	1.70	n.s.
75	13 (33%)	1000	21	2.10	n.s.	18 (30.5%)	1000	15	1.50	n.s.
100[c]	10 (26%)	1000	19	1.90	n.s.	9 (15%)	263	6	2.28	n.s.
200[c]	4 (10%)	50	0	0	n.s.	4.5 (8%)	84	2	2.38	n.s.

[a]Concentration 0 = Control treated with 50 μl DMSO.
[b]Significance according to the Kastenbaum−Bowman test.
[c]It was observed a decrease in the number of total cells, i.e. mononucleates and binucleates.
[d]Taking into account the percentage of binucleates in the control as 100%, the number in brackets is the percentage of binucleates with respect to the control.

Table V. Induction of chromosome aberrations in cultured human lymphocytes treated with permethrin

Permethrin (μg/ml)	donor	% S9	treatment hours	No. of cells scored	Gaps	Chromatid-type[a]			Chromosome-type[a]			Total aberrations	
						B	E	T	B	E	T	(−gaps)	(+gaps)
0	1	0	21	100	4	1	0	1	1	0	1	2	6
50	1	0	21	100	4	1	0	1	2	1	4	5	9
75	1	0	21	100	8	0	0	0	14	0	14	14**	22**
100	1	0	21	100	21	6	1	8	11	2	15	23**	44***
0	2	0	21	50	3	0	0	0	2	0	2	2	5
50	2	0	21	50	3	2	0	2	3	0	3	5	8
75	2	0	21	50	2	1	2	5	0	2	4	9*	11
100	2	0	21	50	6	0	1	2	6	4	14	16**	22**
150	2	0	21	50	9	3	1	5	2	3	8	13**	22**
200	2	0	21	0									
0	2	0	2	50	4	0	0	0	2	0	2	2	6
50	2	0	2	50	3	1	0	1	0	0	0	1	4
75	2	0	2	44	1	1	0	1	0	0	0	1	2
100	2	0	2	50	1	0	0	0	2	0	2	2	4
150	2	0	2	50	1	3	2	7	0	1	2	9*	10
200	2	0	2	50	4	2	4	10	1	4	9	13**	15*
0	2	10	2	50	3	2	1	4	1	0	1	5	8
50	2	10	2	50	4	1	0	1	1	0	1	2	6
75	2	10	2	50	1	1	0	1	2	0	2	3	4
100	2	10	2	50	5	0	1	2	1	0	1	3	8
150	2	10	2	50	8	2	0	2	3	2	7	9	17

[a]Chromosome aberrations: B, breaks; E, exchanges; T, total. We calculated the total of aberrations by assuming that one exchange implies two breaks.
$*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (χ^2 test).

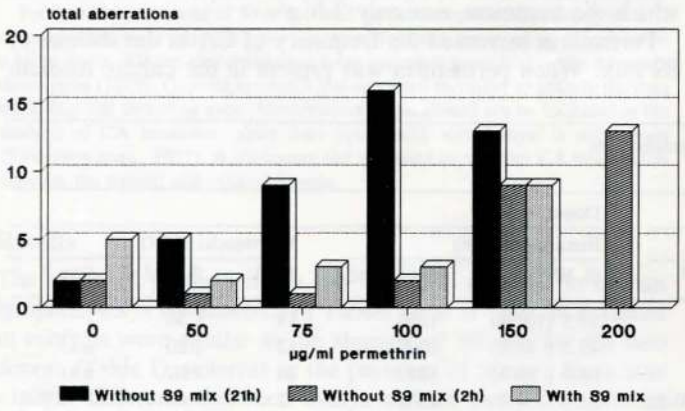

Fig. 2. Chromosome aberrations induced by permethrin on cultured human lymphocytes from donor 2 (50 metaphases/treatment were recorded).

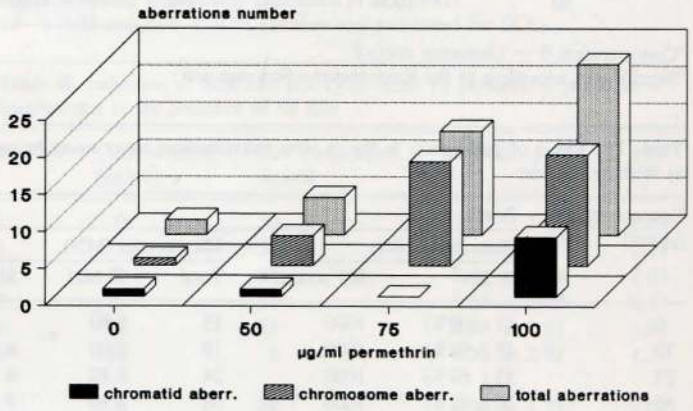

Fig. 3. Chromosome aberrations induced by permethrin on cultured human lymphocytes exposed during 21 h (donor 1) (100 metaphases/treatment were recorded).

for 21 h increases in the frequency of CA were observed at lower concentrations (75−150 μg/ml) than when it was present for only 2 h (150−200 μg/ml). The effect of permethrin seemed to be time of exposure dependent. In the presence of S9 mix the only increase observed in the frequency of CA at 150 μg/ml was not statistically significant. The activity of permethrin seemed to be decreased more by the reduction of the time-exposure than by the presence of S9 mix.

Aberrations formed in unreplicated G_1 chromosomes will be duplicated during the S-phase and appear as chromosome-type aberrations during the subsequent mitosis. Aberrations formed after DNA replication, i.e. during or after the S-phase, involve only one of the chromatids and will be recorded as chromatid aberrations. In this study, aberrations induced by permethrin were mainly chromosome-type aberrations. Thus, permethrin could be characterized as a S-phase-independent agent. All clastogens giving rise to lesions during the G_1- and S-phases, lead to the formation of micronuclei in the subsequent cell cycle. Permethrin was also positive in the micronucleus assay. Thus, permethrin was an agent with greater potential for inducing chromosomal damage than sister chromatid exchanges.

A linear relationship has been established between frequencies of SCE and gene mutation in V79 and CHO cells, and in human lymphocytes, for a limited number of chemical mutagens and gene loci (Carrano and Thompson, 1982). These results suggest a similarity between gene mutation and SCE formation with regard to the types of lesions involved, or the processing of the lesions (IARC, 1986). We have found that permethrin did not induce SCE in cultured human lymphocytes and it also was

reported not to induce gene mutation in bacteria, V79 Chinese hamster cells and *D.melanogaster*. Both endpoints, gene mutation and SCE, are S-phase-dependent (IARC, 1986).

Because of the limited data about the cytogenetic effect of permethrin and the positive results reported here with this compound in the CA and MN assays using cultured human lymphocytes, more studies are needed to confirm the clastogenic effect of permethrin. More data from CA assays in cultured human lymphocytes and so much better data from assays *in vivo*. These studies must consider the influence of two factors, the metabolic activation system or biotransformation and the duration of the treatment on the induction of the clastogenic effect.

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