Genotoxic activity of the insecticide Nuvacron (Monocrotophos) detected by the micronucleus test in bone marrow erythrocytes of mice and in CHO cells

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ABSTRACT

The organophosphorus insecticide Nuvacron (Monocrotophos) is a very toxic agent widely utilized in Brazilian agriculture. To evaluate the clastogenic potential of this insecticide, *in vivo* and *in vitro* micronucleus (MN) assay experiments were carried out on Swiss mice and on Chinese hamster ovary (CHO) cells, respectively. Nuvacron administered at doses of 2.5 and 5.0 mg/kg induced a statistically significant increase in the frequencies of MN detected in polychromatic bone marrow erythrocytes from animals (six/group) treated ip 24 h before. Exponentially growing CHO cells were treated continuously (16 h) with Nuvacron diluted in water to final concentrations of 1, 10, 100, 200, and 400 μg/ml. Three experiments were carried out using the cytokinesis-block method and a total of 6000 binucleated cells were scored to determine MN frequencies. A statistically significant increase in the frequencies of MN was observed for the cells treated with 1 and 10 μg/ml Nuvacron. A marked decrease in cell proliferation rates was observed for CHO cultures treated with higher concentrations. These data demonstrate that Nuvacron has a genotoxic effect on both *in vivo* and *in vitro* mammalian test systems.

INTRODUCTION

Many different types of pesticides have been introduced on the market in order to increase the production of food-stuffs. In addition to the direct occupational exposure of workers during the formulation, manipulation and application of pesticides, the human population is also exposed to them indirectly due to the persistence of their residues in the soil and in animal and plant tissues (Grover and Malhi, 1985).

Several methods are available to determine the genotoxic effects of chemicals. The micronucleus (MN) test, described by Schmid (1975), can detect mutagenic substances in mammals by revealing compounds that cause chromosome breaks or interfere with the mitotic spindle. MN can also be detected in fibroblast cell cultures using the cytokinesis-block method with cytochalasin B (Cyt-B). This process gives rise to binucleated cells with individualized nuclei, clearly showing that these cells only underwent one mitotic division, thus permitting MN analysis in first cycle cells (Fenech and Morley, 1985).

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The mutagenic potential of organophosphorus pesticides has been investigated in many laboratories (Niraj and Patnaik, 1992) and there is a considerable body of evidence showing the ability of these insecticides to induce chromosome damage (Adhikari and Grover, 1988), sister chromatid exchange (Sobti *et al.* 1982) or point mutations (Moriya *et al.* 1983). Their genotoxic properties have been observed in plant systems in which the product induced several structural chromosome alterations in root tip cells (De Kergommeaux *et al.*, 1983; Devadas *et al.*, 1987).

Cytogenetic studies on Chinese hamster ovary (CHO) cells have indicated that Monocrotophos induces significant levels of chromosome exchanges (Lin *et al.*, 1987). Vaidya and Patankar (1982) have also observed altered levels of chromosome aberrations in human lymphocyte cultures. Shandhu *et al.* (1985) observed that Monocrotophos causes gene mutations in bacteria and DNA damage in human lung cells in culture. *In vivo* studies on chicken bone marrow cells and blood erythrocytes have also revealed the clastogenic action of this product (Bhunya and Jena, 1993).

The objective of the present study was to detect the genotoxic effects of Nuvacron (Monocrotophos) by applying the micronucleus test to bone marrow cells of mice and CHO cells in culture.

MATERIAL AND METHODS

Chemical agent

The organophosphorus insecticide Monocrotophos [(cis-2-methyl carbamoyl 1 methyl vinyl) dimethyl phosphate], commercially sold as Nuvacron-400, is manufactured by Ciba-Geigy Química S/A (Brazil). This product has been assigned to toxicological class I (highly toxic) and has the following structural formula:

$$CH_3 - O \diagdown P \diagup ^O_{O-C} \diagup ^{CH - CO - NH - CH_3}$$
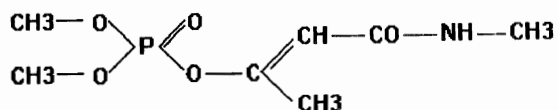
The product used in our experiments is commercially sold in agricultural goods stores. This pesticide contains 400 g/l of the active principle Monocrotophos in non-aqueous concentrated solution, pH 1.5. The acute oral LD_{50} dose for rats is 14-23 mg/kg and the dermal dose is 333 mg/kg (Ciba-Geigy technical guide).

Micronucleus test in bone marrow erythrocytes of mice

Male and female Swiss mice (*Mus musculus*) weighing approximately 30 g were maintained in polyethylene boxes with a wire lid of the type normally used in the laboratory, with food and water *ad libitum*. Experimental and control groups composed of six animals each were used. On the basis of the oral LD_{50} in rats we carried out pilot experiments for the determination of the concentrations to be used. The 10 mg/kg concentration proved to be highly toxic to the animals and the intraperitoneal LD_{50} was observed at a dose of 7.0 mg/kg. On the basis of these results, Nuvacron was diluted in distilled water and single doses of 1.25, 2.5 and 5.0 mg/kg body weight (70% of the LD_{50}) were administered to the animals intraperitoneally. Cyclophosphamide (Sigma) was used as a positive control at 8 mg/kg body weight, and distilled water was used as a negative control. The animals were sacrificed by ether inhalation 24 h after treatment.

Bone marrow cells for MN analysis were obtained by the technique of Schmid (1975). The slides were prepared with fine smears of the material and after 24 h the cells were fixed with absolute methanol for 10 min. The air-dried preparations were stained with Giemsa diluted in phosphate buffer (1:10) for 10 min. One thousand cells per animal were analyzed for the presence of MN in polychromatic erythrocytes (PCEs), for a total of 6000 cells per treatment. The frequencies of MN were determined as the number of MN per 1000 micronucleated PCEs.

Micronucleus test in CHO cells

CHO cells (CHO$_9$ line) were kindly provided by Prof. Dr. A.T. Natarajan (University of Leiden, Holland). The cells were grown in HAM F10 (Sigma) + DEM (Sigma) (1:1) supplemented with 10% fetal calf serum (Cultilab), 2.38 mg/ml Hepes (Sigma), plus 0.01 mg/ml streptomycin and 0.005 mg/ml penicillin. Cells were cultured at 37°C as a monolayer in 5 ml culture medium using disposable 25-cm^2 flasks (Corning). As a routine, the cells were subcultured every 72 h when they reached a state of semi-confluence. A solution of 0.2% trypsin plus 0.02% versene was used to detach the cells from the inner surface of the flask. In each experiment, 1×10^6 cells were plated onto each culture flask, and after 1 h the cells were treated with the insecticide. Based on the study by Lin *et al.* (1987), who employed concentrations of 800, 400, 200, 100 and 50 µg/ml to test the clastogenicity of Monocrotophos, by analysis of

chromosome aberrations in CHO cells, we performed pilot tests to determine the concentrations to be used. Since concentrations higher than 800 µg/ml proved to be highly cytotoxic, we established the concentrations of 1, 10, 100, 200 and 400 µg/ml culture medium as the test doses. The cells were incubated for 16 h at 37°C in the presence of 3 µg/ml Cyt-B in order to obtain binucleated cells, as with the cytokinesis-block method for human lymphocytes (Fenech and Morley, 1985). The antitumoral alkaloid ellipticine (EPC) (Sigma) was used as a positive control, at a final concentration of 3 µg/ml culture medium in 30-min pulse treatment. The preparations were fixed with methanol/acetic (3:1) and stained with Giemsa diluted in phosphate buffer for 10 min. Two thousand binucleate cells per culture were analyzed in each experiment, for a total of 6000 cells per treatment, and the presence of binucleated cells with micronuclei was recorded.

To verify whether Nuvacron can induce any effect on the kinetics of the cell cycle, we calculated the cell proliferation rate (CPR) by analyzing the frequencies of mono-, bi-, tri- and tetranucleated cells for different treatments, according to the following formula: CPR = (1 x frequency of mononucleated cells) + (2 x frequency of binucleated cells) + (3 x frequency of tri- or tetranucleated cells) (Hoffmann et al., 1994).

RESULTS

The results obtained in the *in vivo* test system are presented in Table I. It can be seen that the animals treated with an aqueous solution of Nuvacron at doses of 2.5 and 5.0 mg/kg body weight presented the highest frequencies of MN scored in PCE. The data for the experimental and negative control groups were analyzed statistically by the exact Fisher test, and the differences were shown to be significant ($P < 0.05$),

indicating clastogenic action of Nuvacron at high concentrations.

Table II presents the total number of binucleated CHO cells with micronuclei observed in the different treatments with Nuvacron, compared to the control. The highest percentages of MN scored in binucleated cells were obtained at the lowest concentrations. When the data were submitted to the exact Fisher test, the cultures treated with 1.0 and 10 µg/ml Nuvacron presented significantly higher values ($P < 0.05$). At these concentration levels, the cell proliferation indices were constant but they showed reduction at concentrations higher than 10 µg/ml (Table III). In both assays, the chemicals used as positive controls were very efficient at inducing chromosome damage, agreeing with reports in the literature (Sakamoto-Hojo et al., 1988; Preston et al., 1987).

DISCUSSION

The genotoxicity of the organophosphorus insecticide Nuvacron (Monocrotophos) has been demonstrated in several organisms such as *Salmonella* (Shirasu et al., 1976), *E. coli* (Moriya et al., 1983), *Allium cepa* root-tip cells (Rao et al., 1987), rat bone marrow cells (Wang et al., 1987; Grover et al., 1988), CHO cells (Lin et al., 1987), human lymphocytes (Vaidya and Patankar, 1982), and chicken bone marrow cells (Bhunya and Jena, 1993). Monocrotophos was found to have a clastogenic action on all of these test systems, and also on mouse bone marrow cells and CHO cells in culture, as demonstrated in this study.

The micronucleus test is one of the most efficient methods to detect clastogenic and aneugenic compounds in the environment (Schmid, 1976; Savage, 1988). The evaluation of MN incidence is important because they have been correlated with the process of tumor initiation (Jayashree et al., 1994).

The *in vivo* test system showed a significant increase in the frequencies of MN at concentrations of 2.5 and 5 mg/kg body weight, compared to the negative control (Table I). These results diverge from those reported by Bhunya and Behera (1968), who observed significant variations for chromosomal aberrations and sperm shape abnormalities, but not for MN in mice treated *ip* with Nuvacron.

Table I - Frequencies of micronucleus (MN) in polychromatic erythrocytes (PCE) observed in Swiss mice treated *ip* with different doses of Nuvacron. The animals were sacrificed 24 h after treatment.

Treatment	Dose mg/kg body weight	Number of MN per 1000 PCE/animal Animal						Mean (%) MNPCE
		A	B	C	D	E	F	
Control (water)	0.0	3	3	2	0	3	3	(0.23)
Cyclophosphamide	8.0	17	11	12	11	12	9	(1.20)*
Nuvacron	1.25	3	2	3	2	1	2	(0.21)
Nuvacron	2.5	6	7	6	8	8	7	(0.70)*
Nuvacron	5.0	8	6	8	6	4	6	(0.63)*

*$P < 0.05$ compared to controls (exact Fisher test).

Table II - Frequencies of micronucleus (MN) in binucleated Chinese hamster ovary cells after treatment with different concentrations of Nuvacron (NV) in three experiments (E1, E2 and E3).

Treatment (µg/ml)		Number of binucleated cells with MN/2000 cells	% of cells with MN
Control	E1	8	
(0.0)	E2	6	0.35
	E3	7	
Ellipticine	E1	229	
(3.0)	E2	105	7.80*
	E3	134	
NV1	E1	13	
(1.0)	E2	13	0.60*
	E3	14	
NV2	E1	17	
(10)	E2	11	0.80*
	E3	20	
NV3	E1	11	
(100)	E2	13	0.53
	E3	8	
NV4	E1	10	
(200)	E2	10	0.43
	E3	6	
NV5	E1	9	
(400)	E2	7	0.41
	E3	9	

*$P < 0.05$ compared to controls (exact Fisher test).

Table III - Frequencies of mono-, bi-, tri- and tetranucleated cells and cell proliferation rates observed in Chinese hamster ovary cell cultures treated with different Nuvacron concentrations; one thousand cells were analyzed per treatment.

Treatment (µg/ml)	Frequency of			CPR
	Mononucleated cells	Binucleated cells	Tri- and tetranucleated cells	
0	0.11	0.82	0.07	1.96
1	0.10	0.81	0.09	1.99
10	0.10	0.83	0.07	1.97
100	0.26	0.68	0.05	1.77
200	0.29	0.61	0.06	1.69
400	0.43	0.50	0.07	1.64

CPR: Cell proliferation rate.

In their study, each animal received two doses of the product, with a 24-h interval, and the bone marrow cells were fixed 6 h after the last treatment.

According to Cole *et al.* (1981), induction of MN in mice coincides with the kinetics of the erythropoietic system (22-24 h). Several studies have shown that maximum expression of MN in bone marrow cells occurs when the material is collected 24 h after treatment. This seems to be the ideal time for the clastogen to reach the target cells (erythrocytes) and cause the appearance of MN (Jenssen and Ramel, 1978; Krishna *et al.*, 1991; Jagetia and Ganapathi, 1994; Hayashi *et al.*, 1994).

In the present study, the fact that the animals treated with 5 mg/kg dose presented lower frequencies of MN than the animals treated with 2.5 mg/kg can be explained by the high cytotoxicity induced at the highest concentration.

Our results also indicate the clastogenic potential of Monocrotophos for CHO cells. Cultures treated with low concentrations (1 and 10 µg/ml) showed significantly increased frequencies of MN compared to the negative control. In the pilot experiments performed, the insecticide proved to be highly cytotoxic to CHO cells at the concentration of 800 µg/ml, causing a high rate of cell death. This high cytotoxicity may also have been responsible for the lower frequencies of MN in binucleated cells observed in the treatment with the concentrations of 100, 200 and 400 µg/ml. In these treatments, the frequencies of cells in the first cycle were higher and there was a reduction in cell proliferation rate compared to the control, suggesting that the insecticide interfered with the kinetics of the cell cycle, causing a delay in the cycle progression. This cytotoxic effect of the insecticide justifies the lower frequencies of MN observed at high concentrations. This result confirms those reported by Sobti *et al.* (1982), who stated that several organophosphorus insecticides, among them Azodrin (which also has Monocrotophos as the active principle), can disturb the kinetics of the cell cycle in human lymphocytes cultured *in vitro*.

The mechanism of action of Monocrotophos at the molecular level is not fully clear. Attention is being focused upon electrophilic reactivity as the fundamental cause of the toxicity of many cytotoxic compounds. DNA alkylation might be one of the reasons for the production of chromosomal aberrations by the organophosphates (Jayashree *et al.*, 1994). The common structural element of these chemicals, i.e., P-O-C with phosphorus and carbon as electrophilic sites, offers the key for understanding the reactions of the organophosphates with nucleophiles. In general, the phosphate group is a good substrate for the nucleophilic attack that causes DNA phosphorylation, inducing chromosome damage (Klopman *et al.*, 1985).

We conclude that, besides the well-known genotoxicity of Monocrotophos that has been demonstrated since twenty years ago (Niraj and Patnaik, 1992), our results confirm the ability of the product to interact with the genetic material of mammalian cells *in vivo* and *in vitro*, causing chromosome damage.

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RESUMO

O inseticida organofosforado Nuvacron (Monocrotophos) é um produto muito tóxico e amplamente utilizado na agricultura brasileira. Para avaliar o potencial clastogênico desse inseticida, foi utilizado o teste do micronúcleo (MN) *in vivo* em camundongos Swiss e *in vitro* em células CHO (células de ovário de hamster chinês). O Nuvacron administrado nas concentrações de 2,5 e 5,0 mg/kg induziu um aumento estatisticamente significativo nas freqüências de MNs em eritrócitos policromáticos de medula óssea dos animais tratados intraperitonealmente 24 h antes. Esses experimentos foram realizados utilizando seis animais por tratamento. Células CHO em crescimento exponencial receberam tratamento contínuo (16 h) com Nuvacron diluído em água destilada nas concentrações finais de 1, 10, 100, 200 e 400 µg/ml. Foram realizados três experimentos utilizando o método de bloqueio da citocinese pela citocalasina-B e um total de 6000 células binucleadas foram analisadas para a determinação das freqüências de MNs. Um aumento estatisticamente significativo nas freqüências de MNs foi observado nas células tratadas com 1 e 10 µg/ml de Nuvacron. Verificou-se também uma queda nos índices de proliferação celular nas culturas de CHO tratadas com as concentrações mais elevadas, mostrando um efeito citotóxico nesses níveis de concentração, o que justifica a ausência de indução de MNs. Dessa forma podemos concluir que o inseticida Nuvacron apresentou efeito genotóxico nos dois sistemas-teste *in vivo* e *in vitro*.

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