Original article DOI: 10.1515/aiht-2016-67-2763

Assessment of genotoxicity of Lannate-90® and its plant and animal metabolites in human lymphocyte cultures

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[Received in December 2015; CrossChecked in December 2015; Accepted in May 2016]

This study evaluated direct and metabolic genotoxic effects caused by Lannate-90®, a methomyl-based formulation (90 % active ingredient), in human lymphocyte cultures using sister chromatid exchange assay (SCE). Two processes were used for the plant promutagens evaluation: *in vivo* activation, applying the insecticide systemically in plants for 4 h and subsequently adding plant metabolites containing extracts to lymphocyte cultures; and *in vitro* activation, where the insecticide was incubated with *Vicia faba* S10 mix plus human lymphocyte culture. Direct treatment with the insecticide significantly increased SCE frequency in human lymphocytes (250-750 mg L^{-1}), with cellular death observed at 1000 mg L^{-1} concentration. Using the extracts of *Vicia faba* treated with Lannate-90® to treat human lymphocytes, a dose-response relationship was observed. In lymphocyte cultures treated directly with the insecticide for 2 h, a negative response was obtained. When S10 mix was added, SCE frequency did not change significantly. Meanwhile, a mixture of S9 mammalian metabolic mix and Lannate-90® increased the SCE frequency, with an observed concentration-dependent response. Although Lannate-90® induced cellular death at the highest concentrations, it did not cause a delay in cell proliferation in any of the treatments, confirming its genotoxic action. This study is one of the first to evaluate and compare the direct effect of Lannate-90® in two bioassays, animal and vegetal, and the effect of plant and animal metabolism on its genotoxic potential.

KEY WORDS: *animal metabolism; carbamate insecticides; cellular proliferation kinetics; plant metabolism; replication index; sister chromatid exchange*

Despite the beneficial effects associated with the use of agrochemicals in agriculture and households, many of these products could be potentially hazardous because of their extensive use (1). Among various types of agrochemicals, large quantities of carbamates are particularly applied to different crops. These pesticides have produced inconclusive results in mutagenicity tests according to different cytogenetic end-points evaluated or the pesticide assessed (2).

Lannate-90® is a methyl carbamate insecticide in which the active ingredient is methomyl. Methomyl (S-methyl-N-(methylcarbamoyloxy)thioacetimidate; CAS 16752-77-5; Fig. 1) is an oxime carbamate insecticide that controls a broad spectrum of arthropods. This contact insecticide exerts rapid knockdown effects on insects and acts as an ovicide, larvicide and adulticide. Methomyl was first introduced by E.I. du Pont de Nemours in 1966 (3). In 1978, the US Environmental Protection Agency classified it as a restricted-use pesticide (4). Like other carbamates, its main action is through the inhibition of acetylcholinesterase (AChE) activity, resulting in nerve and/or tissue failure and possible cell death. Due to these findings, methomyl was classified as "highly hazardous" (class 1B) according to the WHO (5) classification.

In relation to genotoxicity, methomyl has been evaluated with different biological tests. Negative results have been observed in the majority of these tests, particularly in those detecting gene mutation, as in bacteria with or without metabolic activation (6-10), *Drosophila* (11-13), and cultured cells (6, 14). However, positive results have been reported in yeast (15). Additionally, methomyl induced micronuclei formation in mice (16), and elevated levels of sister chromatid exchange (SCE) and chromosomal aberrations (CA) in mice and cultured cells (17-20). As for plants, *Vicia faba* root tips also demonstrated elevated levels of CA (21, 22). *Drosophila* S2, HeLa, and HEK293 cells

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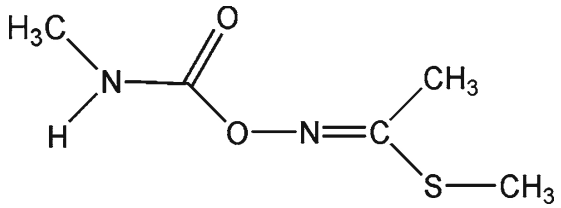

Figure 1 *Methomyl structure*

showed significantly higher DNA damage and apoptosis after treatment (3). Methomyl did not induce carcinogenic activity in rats (23) or dogs (24). Teratogenic effects were also not observed in pregnant rats or rabbits (24).

Besides the concentration of insecticides and time period in which cells and their genetic material are exposed to the insecticide action (3), genotoxic and carcinogenic risks of insecticides also depend on the complexity of insecticide compounds, since sometimes they can stimulate a toxic effect with their presence or during/after biotransformation in a plant/animal organism. Also, the metabolites of pesticides produced by plants and animals can act as promutagens, which is why it is necessary to evaluate not only the chemical itself but also the plant/animal metabolites produced after exposure to insecticides in order to determine their genotoxic risks to human health. When working on human lymphocyte cultures, it is necessary to accompany these with S9 rat liver enzymatic fraction in order to obtain data on those metabolites that would normally be found after biotransformation of a chemical in the human body. In case of plant exposure, *Vicia faba* is considered to be a good plant system for mutagenic studies since it can metabolise the studied pesticide and form metabolically active compounds. As such, it is a sensitive and effective plant to study the effects of pesticide metabolites on genome stability measured by the SCE assay either in the plant or on other cell lines with promutagen extracts (25-27).

Although the genotoxic effect of methomyl alone is rather known, genotoxicity of Lannate-90® as a commercial methomyl-based formulation needs to be explained in more detail, since it is one of the carbamate pesticides most widely used in Mexico due to its broad spectrum of action in all three stages of pest development.

One of the assays commonly used not only as an indicator of genotoxic effects in cells but also as a biomarker of exposure and repair is the SCE assay. SCE involves reciprocal exchanges of chromatid segments that occur at low levels in untreated cells, but their frequency increases substantially following exposure to DNA-damaging agents. SCEs are primarily formed through homologous recombination, an error-free type of DNA repair. Due to the differential labelling of SCEs, achieved by the incorporation of base analog 5′-bromodeoxyuridine (BrdU) in a cell culture during semiconservative DNA replication, these exchanges can be visualised in second-division metaphase cells whose further division was stopped by spindle inhibitor (e.g. colchicine) in order to accumulate cells in that phase. SCE can be measured both in mammals and non-mammalian systems. When cells are exposed to a test substance, it is recommended to use appropriate metabolic activation systems together with the test, since metabolites can cause a higher frequency of SCE (28).

The aim of this study was to examine a possible genotoxic effect of Lannate-90® (measured as SCE frequency) caused directly to human lymphocyte cultures. Moreover, we wanted to examine the influence of the *in vivo* and *in vitro* transformation metabolites of Lannate-90® retrieved from the treated *Vicia faba* plant in a lymphocyte culture. Likewise, plant and animal metabolites were induced *in vitro* by S10 mix and S9 metabolic lymphocyte activation to estimate their influence on genotoxic effects, cell-cycle progression and proliferation measured as a proportion of the first (M_1), second (M_2), and third (M_3) metaphases as well as the replication index ratio (RI).

MATERIALS AND METHODS

Chemicals

RPMI medium 1640 with L-glutamine and phytohemagglutinin were purchased from Gibco, of Mexico; Lannate-90®-WD (methomyl CAS number 16752-77-5) was provided by Dupont of Mexico. The following chemicals were purchased from Sigma Chemical, St. Louis MO, USA: mitomycin C (MMC, CAS number 50-07-7), cyclophosphamide (CAS number 6055-19-2), bromodeoxyuridine (BrdU, CAS number 59-14-3), Giemsa (CAS number 51811-82), ethanol (purity 99.2 %, CAS number 6415-5), methanol (purity 99.8 %, CAS number 6756-1), acetic acid (purity 99.7 %, CAS number 5323-26-45), sodium phosphate monobasic (NaH_2PO_4, CAS number 7558-80-7), sodium phosphate dibasic (Na_2HPO_4, CAS number 7758-79-4) and sodium chloride (NaCl, CAS number 7647-14-5). Colchicine (CAS number 64-86-8) and potassium chloride (KCl, CAS number 222425) were purchased from Merck of Mexico.

Direct treatments, without the metabolic activation of human lymphocyte cultures

Whole blood samples were used separately in different experiments. Samples were taken from three healthy donors, male non-smokers and non-drinkers, with an average age of 30 years. Donors did not use any medications in the last 6 months before sampling, and did not undergo any radiation/diagnostic treatment in the same period. This study was performed in accordance with the principles stated in the Declaration of Helsinki. All participants were acquainted with the content of the study, accepted the idea of taking part in it, and they subsequently signed an informed consent form.

Since the SCE baseline frequencies among donors were not significantly different, the influence of inter-individual

variability was negligible in response to the mitogens, culture conditions or blood samples themselves, as suggested by Morgan and Crossen (29), and Speit et al. (30).

Two experiments were carried out for each treatment using 3 mL of RPMI medium 1640 with L-glutamine and 0.2 mL of phytohemagglutinin added to 0.5 mL of a healthy donor's blood in a culture flask.

The cultures were incubated at 37 °C for 72 h. After 24 h, BrdU was added to the culture medium at a final concentration of 5 μg mL^{-1}. In the experiments without metabolic activation, the cultures were directly exposed to 50, 100, 250, 500, 750, and 1000 mg L^{-1} of Lannate-90®.

Mitomycin C (400 ng mL^{-1}) was used as a positive control. Lannate-90® was dissolved in sterilised distilled water. The negative control was handled under the same experimental conditions, but lymphocytes were treated with the same amount of distilled water. Preliminary experiments were used to identify optimal concentrations of Lannate-90® that did not cause cellular death.

The cultures were incubated for additional 48 h at 37 °C, and 0.1 mL of colchicine (5 μM) was applied to the cultures 2 h before harvesting. Metaphase cells were harvested by centrifugation, treated with 0.075 M KCl for 20 min, and fixed in methanol-acetic acid (3:1).

Slides were stained using the fluorescence-plus-Giemsa technique (31). In addition to the study of SCE, a BrdU differential staining technique was used to estimate the effect of Lannate-90® on cell replication. According to the OECD guideline, it is enough to analyse 25 well-spread metaphases per culture and treatment.

For the evaluation of cytokinetics, the proportion of the M_1, M_2, and M_3 metaphases was obtained from 100 consecutive mitotic cells for each treatment, and the RI was calculated as follows: $RI = 1M_1 + 2M_2 + 3M_3/100$ (32). To avoid bias, the slides were scored blindly.

Treatments with metabolic activation

In vivo *activation. Treatment of human lymphocyte cultures with extracts of* Vicia faba *roots exposed to Lannate-90®*

Vicia faba (var. minor) seeds were germinated between two cotton layers soaked in tap water. When primary roots reached a length of 4-6 cm, they were immersed in Lannate-90® at 500, 1000, 1500, 2000, and 2500 mg L^{-1} in a water solution at 20 °C in the dark. The concentrations were chosen based on preliminary experiments that examined the phytotoxicity of the insecticide. The positive control roots were exposed to 3600 mg L^{-1} of ethanol (0.1 M) for 4 h at 20 °C. Ethanol is a promutagen in *Vicia faba* and increases the SCE frequency (27, 33-35). The negative control was handled under the same experimental conditions, but the roots of *Vicia faba* were immersed in distilled water alone to obtain the S10 fraction.

After the treatment, the roots were rinsed three times with distilled water and cut at approximately 2 cm from the primary root tips. The roots were macerated and homogenised at 4 °C in 0.1 M sodium-phosphate buffer, pH 7.4. The ratio of the buffer solution in millilitres (2.0-2.5) to the fresh weight of root cuttings in grams (2.0-2.5) was 1:1 (26). Homogenised roots were centrifuged for 15 min at 10000 x *g* at 4 °C. The supernatant was sterilised using Millipore filters (0.45 μm pore size) and immediately used to treat human lymphocytes in culture. The total protein concentration in these extracts was determined using the Bio-Rad method (35). Protein concentration was fairly constant from one experiment to the other, with values between 5.0-5.9 μg μL^{-1}.

In vitro *activation. Treatment using* in vitro *promutagen activation by* Vicia faba *S10 mix*

Lymphocytes cultured for 48 h were exposed to Lannate-90® at 500, 1000, 1500, 2000, and 2500 mg L^{-1} (concentrations chosen in preliminary experiments) for 2 h in the dark at 37 °C with and without the S10 mix (cultures were stationary for one hour and were then gently shaken for another hour). After the treatment, the cells were rinsed twice in 0.9 % sodium chloride and incubated for 24 h in RPMI medium containing BrdU at a final concentration of 5 μg mL^{-1}. Colchicine (0.1 mL, 5 μM) was added 70 h after the start of the culture, and the same harvesting and staining procedures were performed as described above.

The metabolic activation system with the S10 mixture was prepared from the microsomal S10 fraction at a 1:9 ratio (v/v) with following compounds: 8 mM $MgCl_2$, 3.3 mM KCl, 5 M glucose-6 phosphate, 4 mM NADP and NAD, and 0.1 M Na_2HPO_4-NaH_2PO_4 at pH 7.4. The 48 h cultures were incubated for 2 h at 37 °C with 500 μL of the activation system and several concentrations of the insecticide. 3600 mg L^{-1} ethanol (0.1 M) was used as a positive control (26-27, 33, 35).

In vitro *activation. Treatment using* in vitro *promutagen activation mammalian S9 mix*

The commercial form (Mol Tox, from Molecular Toxicology Inc USA) of the rat liver microsomal S9 fraction was prepared according to Ames et al. (36) and Frantz and Malling (37). The S9 mixture was prepared with 1 mL of S9 extract and NADPH generator system containing sterile cofactors of 0.2 mL (0.4 M) $MgCl_2$, 5 mL of pH 7.4 phosphate buffer (0.2 M Na_2HPO_4 and NaH_2PO_4), 3.35 mL of distilled water, 0.4 mL of NADP (0.1 M) and 0.05 mL of glucose-6-phosphate (1 M); all of the reagents were freshly prepared. Similarly to direct treatments, BrdU at a final concentration of 5 μg mL^{-1} was added after 24 h, and 500 μL of the S9 mixture and Lannate-90® were added 48 h later and cultured for 2 h (cultures were stationary for one hour and were then gently shaken for another hour). After each treatment, cell cultures were washed twice with 0.9 %

sodium chloride and incubated for 24 h with RPMI medium containing 100 µL of BrdU. The positive control was 50 µL (40 µM) cyclophosphamide (CP) with S9, and the negative control was 50 µL CP (40 µM) without S9.

Statistical analysis

To quantify the SCE frequencies, twenty five metaphase cells at the second division were scored for each concentration. Two experiments were performed, and the results obtained were compared using the Student's *t*-test. SCEs were statistically analysed using the ANOVA assay to determine significant differences among the treated groups. When a significant F value ($p<0.0001$) was found, a Newman-Keuls multiple comparison test was applied to identify groups with significant differences at $p<0.001$ when compared with controls. A chi squared (χ^2) test was used for RI. The parts of decomposed χ^2 were used to compare the values of M_1, M_2, and M_3.

RESULTS

Direct treatments without the metabolic activation of human lymphocyte cultures

Table 1 shows the averages of two experiments in which Lannate-90® was added directly to human lymphocyte cultures. Low concentrations of the insecticide did not induce significant differences between the SCE frequencies and the negative control. Meanwhile, a concentration-dependent relationship was observed starting at 250 mg L^{-1} and up to 750 mg mL^{-1}; 1000 mg L^{-1} caused cell death. Cell kinetics and the RI are also listed in Table 1, no significant differences were found.

As expected, significant differences were observed in the SCE frequency, cell kinetics and RI with the positive control MMC (Table 1).

Treatments with metabolic activation

In vivo *activation. Treatment of human lymphocyte cultures with extracts of* Vicia faba *roots exposed to Lannate-90®*

The extracts from untreated roots (negative control) did not increase the SCE frequency (Table 2).

When ethanol was added directly to lymphocyte cultures, the SCE frequency was not significantly affected (negative control); however, significant differences were obtained with root extracts treated with the same concentration of ethanol (positive control).

The *in vivo* activation by S10 *Vicia faba* is shown in Table 2. When root extracts were first treated for 4 h with the insecticide, and were then applied to lymphocyte cultures for 48 h, an increase in the SCE frequency was observed for all concentrations of Lannate-90® metabolites.

The transformed Lannate-90®, contained in the *Vicia faba* root extracts that were applied to lymphocyte cultures, did not produce significant effects on either cell kinetics or RI (Table 2).

In vitro *activation. Lannate-90® treatment with* in vitro *promutagen activation by mammalian S9 mix and* Vicia faba *S10 mix*

As shown in Table 3, when Lannate-90® was applied directly to lymphocyte cultures for 2 h, non-significantly different SCE frequencies compared to control values were obtained. Meanwhile, when the S9 metabolic fraction was added, a positive concentration-dependent relationship was observed. However, non-significantly different higher SCE frequencies were also obtained when the S10 metabolic

Table 1 *Sister chromatid exchanges induction and effects on cell kinetics (M_1, M_2 and M_3 cells) and replication index (RI) by direct treatments of Lannate-90® in human lymphocyte cultures*[a]

		$\bar{X}$±S.E.	M_1	M_2	M_3	% RI[b]
Negative control		4.79±0.35	26	38	36	2.10
MMC (400 ng mL^{-1}) (positive control)		15.36±0.65*	51	31	18**	1.67**
Lymphocyte cultures directly treated with Lannate-90® (mg L^{-1})	50	6.36±0.34	31	46	23	1.92
	100	6.45±0.35	25	33	42	2.17
	250	8.94±0.53*	24	37	39	2.15
	500	11.18±0.54*	20	39	41	2.21
	750	13.44±0.74*	25	41	34	2.09
	1000	Cellular death[c]				

[a] *n=50 metaphase cells in two experiments.*
[b] *Replication index, n=100 consecutive metaphases.*
[c] *Stimulated cells were not observed.*
**Significant differences among controls and each treated group were obtained by analysis of variance F=57.83,* p *value is <0.0001, and therefore a Newman-Keuls multiple comparison test was applied* p*<0.001.*
***Significant with* χ^2, p*<0.05*

fraction was added. Significant differences in the SCE frequency were observed in the positive controls, which were cyclophosphamide (CP) addition to the S9 mammalian metabolic fraction and ethanol addition to the S10 *Vicia faba* metabolic fraction. Again, no significant effect was seen in either cell kinetics or RI changes, whether after direct treatment or treatment together with S9 or S10 mix.

DISCUSSION

Carbamates are part of a large group of synthetic pesticides generally used in agriculture as insecticides, fungicides, herbicides, nematicides or sprout inhibitors (39). Many carbamates cause genotoxicity (3). Up to now, their genotoxicity, including DNA damage, micronucleus (MN) frequency, elevated levels of CA, SCE, and effects on plant systems and mammalian cells have been evaluated under *in vitro* and *in vivo* conditions (20, 22, 40, 41).

On the other hand, diverse studies about genotoxic and/or cytotoxic effects obtained with pure and commercial formulations of diverse pesticides indicate that commercial formulations may contain additional unsafe xenobiotics supporting the importance of evaluating not only the active principle but also the commercial formulation, which in fact constitutes the real hazard from agrochemicals (42-51).

Methomyl is a carbamate insecticide that has shown genotoxic effects under *in vitro* and *in vivo* conditions; it has induced elevated MN frequency in human blood lymphocytes (52) and CA in agricultural (53) and greenhouse exposed workers (54). Its commercial formulation, Lannate®, has also demonstrated genotoxicity in previous studies. Lannate-20® induced a significant increase in the number of sex-linked recessive lethal (55) and CA in mice germ cells including polyploidy and sex univalent (56). Lannate-25® had a higher clastogenic activity than pure methomyl *in vivo* (16) and *in vitro* (18). With Lannate-90®, elevated levels of CA and SCE were found in *Vicia faba* root tips (22, 40).

Based on all these studies, genotoxicity of Lannate-90® insecticide has already been demonstrated, but not much is known about its metabolic compounds and their influence on plant/animal organisms. The aim of this study was to examine the influence of Lannate-90®, a formulated carbamate insecticide (90 % methomyl), on a possible genotoxic effect (measured as SCE frequency) caused in human lymphocyte cultures at a wide range of concentrations, chosen on the basis of no cell-toxicity induction. Although the evaluated concentrations of Lannate-90® in this study are expected to be rare in the environment, perhaps only observed when specific events occur (e.g. direct application), we cannot rule out the possibility that organisms and occupationally exposed human workers could be exposed accidentally to such high concentrations. We also need to point out that plants are exposed to those concentrations, and in Mexico the registered uses of methomyl based on labels or label translations provided by the manufacturer go up to 1500-12000 mg L^{-1} for wheat, barley, maize, sorghum, alfalfa, pea, asparagus, potato, onion, cauliflower, broccoli, beans, lettuce, pepper, cotton, cucumber, cabbage, peanut, apple, grape, citrus, melon, watermelon, strawberry, tomato (57). We also need to underscore that methomyl at these concentrations is not used only in Mexico, but it is registered for use as a pesticide to control a large variety of chewing and sucking insects on a wide range of crops in many countries.

Table 2 *Sister chromatid exchanges induction and effects on cell kinetics (M_1, M_2 and M_3 cells) and replication index (RI) by Lannate-90® with* in vivo *metabolic activation by* Vicia faba *in human lymphocyte cultures*[a]

		X̄±S.E.	M_1	M_2	M_3	% RI[b]
Negative control		4.79±0.35	26	38	36	2.10
Lymphocyte cultures + *V. faba* extracts, untreated (negative control)		4.57±0.35	20	37	43	2.23
Lymphocyte cultures + ethanol 3600 mg L^{-1} without metabolic activation (negative control)		4.35±0.21	28	39	38	2.20
Lymphocyte cultures + *V. faba* extracts from the treatment with ethanol 3600 mg L^{-1} (positive control)		8.96±0.53*	17	37	46	2.29
Lymphocyte cultures + *V faba* extracts from the treatment with Lannate-90® (mg L^{-1})	500	7.54±0.42*	20	28	52	2.32
	1000	7.59±0.51*	22	30	53	2.41
	1500	7.88±0.35*	23	30	47	2.24
	2000	8.38±0.52*	20	27	53	2.33
	2500	9.29±0.56*	25	27	48	2.39
	3000	Cellular death[c]				

[a] *n=50 metaphase cells in two experiments.*
[b] *Replication index, n=100 consecutive metaphases.*
[c] *Stimulated cells were not observed.*
**Significant differences among controls and each treated group were obtained by analysis of variance F=19.84,* p *value is <0.0001, and therefore a Newman-Keuls multiple comparison test was applied* p*<0.001.*

Table 3 *Sister chromatid exchanges induction and effects on cell kinetics (M_1, M_2 and M_3 cells) and replication index (RI) by Lannate-90® without and with* in vitro *animal and plant metabolic activation in human lymphocyte cultures*[a]

Concentration	Without metabolic activation			With S9 animal metabolic activation			With S10 plant metabolic activation		
	SCE/ Metaphase X̄±S.E.	Metaphases M_1 M_2 M_3	RI[b]	SCE / metaphase X̄±S.E.	Metaphases M_1 M_2 M_3	RI[b]	SCE/metaphase X̄±S.E.	Metaphases M_1 M_2 M_3	RI[b]
Control	4.98±0.30	42 39 19	1.77	5.86±0.34	41 38 21	1.80	4.84±0.31	48 38 14	1.66
Cyclophosphamide	6.59±0.34	43 39 18	1.75	41.19±2.14*	40 40 20	1.80	7.40±0.34	50 33 17	1.67
Ethanol	6.01±0.35	47 39 14	1.67	7.96±0.47	48 37 15	1.67	9.10±0.25*	44 35 17	1.77
Lannate-90® (mg L^{-1}) 500	6.94±0.44	42 42 16	1.74	8.41±0.54	40 33 27	1.87	7.16±0.38	46 32 22	1.76
1000	7.20±0.48	39 36 25	1.86	9.82±0.40	47 39 14	1.67	6.94±0.38	50 34 16	1.66
1500	6.67±0.34	40 38 22	1.82	13.78±0.39*	45 36 19	1.74	5.46±0.29	46 32 22	1.76
2000	6.50±0.39	43 37 20	1.77	14.07±0.98*	46 36 19	1.73	5.96±0.28	46 30 24	1.78
2500	8.52±0.37	43 38 19	1.75	16.75±2.21*	49 31 20	1.71	6.78±0.81	50 36 14	1.64

[a]*n=50 metaphase cells in two experiments;* [b]*Replication index, n=100 consecutive metaphases*

**Significant differences among controls and each treated group were obtained by analysis of variance; F=94.37,* p *value is <0.0001, and therefore a Newman-Keuls multiple comparison test was applied* $p<0.001$; **Significant with χ^2, $p<0.05$

Another interest of the study was to examine the behaviour of the *in vivo*-obtained plant metabolites of Lannate-90® retrieved from the treated *Vicia faba* plant towards lymphocytes and of the *in vitro*-induced plant and animal metabolites in respect of the same end point: human lymphocytes using S10 mix from *Vicia faba* and S9 fraction for metabolic activation. The objective was to estimate their influence on genotoxic effects, cell-cycle progression and proliferation measured as a proportion of the M_1, M_2, and M_3 metaphases as well as the RI ratio.

When meristematic cells of *Vicia faba* were treated with Lannate-90®, chromatid type aberrations were observed (22). Regarding the type of aberrations produced, Lannate-90® can be considered an S-dependent agent that produces clastogenic effects mainly due to its alkylating groups (22). This is in agreement with the findings of Gómez-Arroyo *et al*. (58) who concluded that the carbamate herbicide molinate in *Vicia faba* is an S-dependent agent. Since SCE is an S-dependent event (59, 60), this type of agent is expected to be an efficient SCE inducer. This fact was confirmed in other studies since treatments of meristematic root cells of *Vicia faba* with Lannate-90® significantly increased SCE frequency, with a concentration-dependent effect (40).

Bolognesi *et al*. (16) have observed that *in vivo* methomyl treatments caused indirect damage of DNA through the formation of active oxygen species. The same authors observed a dose-dependent increase in DNA adducts with Lannate-25® commercial formulation, while negative results were obtained with pure methomyl. The induction of OH^8dG by methomyl suggests that DNA oxidative damage could account for the induction of single strand breaks and implies that this insecticide can act indirectly through the formation of hydroxyl radicals, responsible for DNA strand breaks (61). The formation of OH^8dG is considered to be a relevant factor for DNA damage and potential genotoxic and carcinogenic effects due to reactive oxygen species (62). The negative results generally obtained with gene mutation assays, together with the positive results from the test of chromosome damage, as in this study, are also consistent with an indirect mode of action of Lannate-90®.

Bonatti *et al*. (18), found a dose-related increase in MN frequency with both methomyl and Lannate-25®. However, a higher proportion of MN was induced by Lannate-25, indicating a relatively stronger clastogenic activity of the technical product. A major difference between the pure and the technical product was that Lannate-25®, but not methomyl, was able to induce DNA damage in the alkaline elution assay and oxidative damage in the HPLC-EC assay.

Direct treatments with Lannate-90® in lymphocyte cultures for 48 h induced a significant increase in SCE frequency starting at 250 mg L^{-1}, and a concentration-dependent response was observed. These results are in agreement with the data obtained with Lannate-25® (18) in a study on CA in lymphocyte cultures where authors

observed a dose-dependent increase of chromatid-type and total aberrations. However, our results were not in agreement with the SCE results from that study since we have found a significant increase only at the highest concentrations tested.

No significant differences were found in cell cycle kinetics and the RI in cells treated with Lannate-90®. Our results were in agreement with those of Wei *et al*. (19) who observed that the rate of cell proliferation in CHO cells treated with methomyl was not significantly altered.

When the extracts obtained from the *Vicia faba* roots treated for 4 h with different concentrations of the insecticide were applied to lymphocyte cultures, a positive response was obtained in all cases. The products gained from the *in vivo* transformation by *Vicia faba* of Lannate-90® applied to the lymphocyte cultures were capable of increasing the SCE frequency, which means that this compound acted directly and indirectly. Additionally, the toxicity diminished with plant metabolism, indicating the possibility that some detoxification mechanisms were involved. Direct treatment with 1000 mg L^{-1} of Lannate-90® induced cell death, but much higher concentrations (3000 mg L^{-1}) of the insecticide were required to cause cell death when cells were treated with *Vicia faba* extracts. This is in agreement with Gómez-Arroyo *et al*. (63) and Cortés-Eslava et al. (64).

Comparing both plant activation systems, *in vivo* and *in vitro*, significant SCE frequencies were only observed under *in vivo* conditions, and these differences could be related to the exposure time of lymphocytes and/or to active metabolites. In the *in vivo* activation experiments, the treatments lasted for 48 h, while the exposure lasted only for 2 h for the *in vitro* activation. The latter time period may not have been sufficient for the cells to metabolise the insecticides or this may have been due to the enzyme inactivation because direct contact of the insecticides with the S10 fraction could have inhibited the insecticides' metabolism.

In plant metabolism, peroxidases are among the most important enzymes involved in the oxidative transformation of xenobiotics (65). Peroxidases catalyse two categories of oxidative reactions in plant cells; the peroxidative reaction requiring H_2O_2 and the oxidative reaction using molecular oxygen (66). Calderón-Segura *et al*. (34) described the phytotoxic effect of the thiocarbamic herbicide butylate in *Vicia faba*.

Furthermore, 2 h of treatment with Lannate-90® and the mammalian S9 fraction activated the insecticide. A significant concentration-dependent relationship of SCE frequencies was obtained at concentrations starting at 1500 mg L^{-1}, but cell cycle kinetics and the RI were not affected. Direct treatment for 2 h showed no significant results, and cell kinetics and the RI were not altered.

The effects induced by a commercial formulation of any pesticide cannot account for the active component of the mixture alone and it has been suggested that a genotoxic agent(s) might be present in the technical formulate (18). The possibility of oxidative damage as a causative factor for genotoxicity of Lannate-90® has some support in the experimental evidence of the enzyme-inhibiting activities of different methyl carbamate pesticides. Enzymes involved in the defence against harmful oxygen species, such as superoxide dismutase, catalase, and glutathione transferase, are inhibited by these pesticides (67).

Finally, our results highlight that rounded knowledge of the toxic effect/s of the active ingredient of a pesticide is not enough in biomonitoring studies and agrochemical/s' toxic effect/s should be evaluated according to its/their commercial formulation available on the market. Furthermore, the deleterious effect/s of the excipient/s present within the commercial formulation should neither be discarded nor underestimated. The components of pesticide formulations used as wettable powders usually consist of the carrier, mineral clays, surface active agents, and other ingredients such as stabilisers and dyers. The presence of surface-active agents could increase the genotoxic activity of the active ingredient, favouring the amount of active metabolites at critical cellular targets (16). Metabolic transformation has been shown to have a great impact on pesticide genotoxicity. Therefore, pesticide constituents and metabolites should also be investigated for genotoxic effects in further studies on active ingredients.

CONCLUSIONS

Many carbamate pesticides produce genotoxic effects and long-term exposure of humans to this chemical could produce genetic damage. This study indicates Lannate-90®'s ability to induce genotoxic damage by increasing SCE frequency in human lymphocytes in direct treatments. This highlights the environmental risk of this commercial formulation. Our observations were in agreement with the classification proposed by the WHO for a potential deleterious effect of methomyl.

This study is one of the first to evaluate and compare not only the direct effect of Lannate-90® but also the effect of plant and animal drug metabolism on its genotoxic potential.

The results also confirmed that Lannate-90® is able to exert genotoxic and/or cytotoxic effects on human lymphocytes in a culture, with or without the presence of microsomal metabolic S9 or S10 fractions during cultivation. Thus, the non-consistent results reported so far by different research groups about the deleterious effects of methomyl could be attributed to the ability to convert this type of pesticide into its carbamate derivatives by different cellular systems employed. Then, it could be assumed that the deleterious effect induced by this insecticide on these bioassays is committed to the pesticide itself or to any metabolite/s or any other sub-products generated during the treatment period.

Finally, the study clearly demonstrated that Lannate-90® exerts both genotoxicity and cytotoxicity in human lymphocytes. The hypothesis that coformulants and impurities may play a role in technical grade methomyl-induced genotoxicity should be taken into account.

Acknowledgements

The authors would like to thank Ana Rosa Flores Márquez for technical assistance, Pietro Villalobos for editing the tables, Claudio Amescua assistance in editing, and Rafael A. Valencia-Sánchez for language editing.

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Procjena genotoksičnosti insekticida Lannate-90® i njegovih biljnih i životinjskih metabolita u kulturi ljudskih limfocita

Korištenjem testa izmjena sestrinskih kromatida (eng. *Sister Chromatide Exchange Assay – SCE*) u kulturama ljudskih limfocita ispitivani su izravni i metabolički genotoksični učinci insekticida Lannate-90®, formulacije koja se temelji na metomilu (90 % aktivni sastojak). Za procjenu biljnih promutagena provedena su dva postupka: *in vivo* aktivacija, kod koje se insekticid četiri sata sustavno primjenjivao na biljci, a potom su kulturama limfocita dodani biljni metaboliti s ekstraktom, i aktivacija *in vitro*, kod koje je insekticid inkubiran mješavinom S10 biljke *Vicia faba* i kulturom ljudskih limfocita. Izravno tretiranje insekticidom značajno je povećalo učestalost SCE-a u ljudskim limfocitima (250-750 mg L^{-1}), a stanična smrt uočena je pri koncentraciji od 1000 mg L^{-1}. Nakon tretiranja ljudskih limfocita ekstraktima biljke *Vicia faba* koji su tretirani insekticidom Lannate-90®, primijećen je odnos između doze i učinka. Kod kultura limfocita koje su dva sata bile izravno tretirane insekticidom primijećen je negativan odgovor. Kada je dodana S10 mješavina za metaboličku aktivaciju, učestalost SCE-a nije se značajnije promijenila. Naspram tomu, metabolička mješavina S9 za kultivirane stanice sisavaca i Lannate-90® povećali su učestalost SCE-a, uz zamijećen koncentracijski ovisan odgovor. Premda je Lannate-90® inducirao staničnu smrt pri najvišim koncentracijama, nije uzrokovao zastoj stanične proliferacije ni u jednom postupku, čime se potvrđuje njegovo genotoksično djelovanje. Ovo je ispitivanje među prvima kojim se procjenjivao i uspoređivao izravan učinak insekticida Lannate-90® u dvama biološkim testovima, životinjskom i biljnom, te učinak biljnog i životinjskog metabolizma na njegov genotoksični potencijal.

KLJUČNE RIJEČI: *izmjena sestrinskih kromatida; karbamatni insekticidi; kinetika stanične diobe; metabolizam biljaka; metabolizam životinja; replikacijski indeks*