Genotoxic Activity of Dichlorvos, Trichlorfon and Dichloroacetaldehyde

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Two structurally related organophosphorous insecticides, dichlorvos and trichlorfon, and their main metabolite dichloroacetaldehyde, were assayed for their ability to induce DNA repair, detected as unscheduled DNA synthesis in human epithelial-like cell (EUE) cultures *in vitro*. A dose-response relationship was found for treatment with the two pesticides, but negative results were obtained with dichloroacetaldehyde. Tests for induction of gene mutation, as ouabain resistance in Chinese hamster cells (V79), failed to show any mutagenic activity by these compounds.

1. Introduction

Because of its wide agricultural, industrial and domestic use, the organophosphorus insecticide dichlorvos has been the subject of many mutagenicity/carcinogenicity studies.[1] It has been shown to induce gene mutations in bacteria[2–4] and *Aspergillus*,[5] and somatic segregation in *Aspergillus* and yeast.[6,7] Contrasting results in cytogenetic tests have been reported using different mammalian cell cultures.[8–10] Negative results,[11,12] or doubtful results,[13,14] have been obtained in *Drosophila*. In-vivo studies in mammals[15–17] showed no genotoxic activity except for induction of sperm abnormalities in mice.[18] Furthermore, according to a recent evaluation by Reuber,[19] dichlorvos can be considered to be carcinogenic in both mice and rats.

Trichlorfon is known to be spontaneously converted to dichlorvos, both *in vitro* and *in vivo*.[20] It was found to induce gene mutation in bacteria,[4,13,21] mitotic recombination in yeast[21] and *Aspergillus*,[5] and dominant lethal mutations in mice.[22] Carcinogenic, teratogenic, hepatotoxic and hematotoxic activities were demonstrated in many rodent systems.[23,24]

Dichloroacetaldehyde, the common major metabolite of dichlorvos and trichlorofon, induced gene mutation in microorganisms,[25,26] and dominant lethal mutations in mice.[27]

Because of the limited data available on DNA repair and mutation induction in mammalian systems, an investigation was made of the ability of these compounds to induce unscheduled DNA synthesis (UDS) in human epithelial-like cells (EUE) and gene mutation in Chinese hamster cells (V79).

2. Experimental methods

2.1. Chemicals

Chemicals were purchased as follows: 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) from Koch-Light Laboratories Ltd; ethyl methanesulphonate (EMS) from K. & K. Laboratories, Plainview, New York, USA; 2,2-dichlorovinyldimethylphosphate (dichlorvos) and dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate (trichlorfon) from Chem. Service, West Chester; dichloroacetaldeyde from Fluka; ouabain from Sigma Chemical Company, St. Louis, Missouri, USA.

2.2. Cell cultures

The human epithelial-like cell line EUE, and the Chinese hamster cell line V79, were a gift from Dr A. Abbondandolo (Lab. Mutagenesi e Differenziamento, Pisa, Italy). The cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% foetal calf serum (Flow).

2.3. DNA repair assay: detection of unscheduled DNA synthesis (UDS)

The induction of UDS in EUE cells was detected as described by Benigni *et al.*[28] Briefly, cells were seeded on coverslips (12×12 mm) in 5 cm tissue culture dishes (Falcon) and treated when the monolayer was nearly confluent. The chemicals were dissolved in DMEM buffered with 20 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid (HEPES) (pH 7.4) and kept in contact with the cells for 1 h at 37°C, in single gas-tight containers to avoid any loss of chemical through evaporation. Then the cells were rinsed and incubated for 4 h in DMEM with 10% foetal calf serum and [^{3}H]thymidine at 5 μCi ml^{-1} (specific activity 60 Ci mmol^{-1}) (Radiochemical Centre, Amersham). UDS was detected by autoradiography.[28]

2.4. Mutagenic assay

Mutation induction, measured as resistance to ouabain (a specific inhibitor of Na^+/K^+-activated ATPase in cell membranes), was studied in V79 cells. The cells were plated 16 h before treatment at a concentration of 5×10^6 cells per 75 ml flask (Falcon), and were incubated overnight. The treatment was carried out in DMEM without serum and the incubation time, at 37°C, was 1 h for dichlorvos, trichlorfon and dichloroacetaldehyde, and 30 min for EMS. After treatment the cells were washed twice, trypsinised and replated for determination of cytotoxicity and of the period required for the expression of mutation. Cytotoxicity was determined by plating 100 cells per 60 mm petri dish and the colonies were scored after 7 days. After an expression period of 48 h, 1×10^6 cells per 100 mm dish were plated in a selective medium (1 mM ouabain).

Table 1. Detection of unscheduled DNA synthesis as measured by autoradiography

Compound	Concentration (mM)	Mean net no. of grains per nucleus (±s.e.)[a]	
Dichlorvos	0	3.58 (±0.51)	
	6.5	8.00 (±2.91)	P=0.015
	65	12.74 (±2.89)	P=0.001
	650	13.66 (±3.37)	P=0.002
Trichlorphon	0	3.58 (±0.51)	
	0.4	4.66 (±1.12)	
	4	3.96 (±1.16)	
	40	7.42 (±2.08)	P=0.047
	400	8.62 (±2.09)	P=0.015
	4000	11.62 (±2.66)	P=0.003
Dichloroacetaldehyde	0	2.72 (±1.32)	
	6	3.87 (±1.12)	
	60	1.93 (±0.43)	
	600	2.48 (±0.33)	
	6000	5.54 (±0.50)	P=0.025
MNNG[b]	0	3.86 (±1.80)	
	1	9.60 (±1.42)	P<0.001
	10	17.76 (±3.11)	P<0.001
	100	28.38 (±3.07)	P<0.001

[a] The P values refer to t tests, with an accepted $P\leq0.002$ significance level.

[b] 1-Methyl-3-nitro-1-nitrosoguanidine.

The medium (DMEM+10% foetal calf serum) containing the drug was not changed until the end of the experiment. The cultures were fixed with absolute methanol and stained with 10% Giemsa after 12 days. Colonies of more than 20 cells were counted.

3. Results

Both dichlorvos and trichlorfon induced UDS in EUE cells (Table 1) and the increase in UDS was statistically significant and dose-dependent. No significant change in the number of grains per nucleus was observed after treatment with dichloroacetaldehyde. The data shown in Table 1 refer to typical experiments, repeated three or more times.

No induction of ouabain-resistant mutations was detected in cultured V79 cells with the three tested compounds (Table 2). Mutation frequency was calculated per 10^6 survivors, taking into account the number of cells plated and plating efficiency. Reproducibility of the results was confirmed by repeating the experiments at least twice. Spontaneous mutation frequencies were in the range $0.09–0.6\times10^{-6}$ for resistance to ouabain. The number of induced resistant colonies was not influenced by the expression time, and the data shown in the tables are related to the optimum expression time for EMS (48 h), included every time as a reference mutagen.

Table 2. Induction of ouabain resistant mutations

Compound	Concentration (mM)	Cytotoxicity[a]	Mean number resistant colonies per plate	Mutation frequency[b]	Fisher[c] test
Dichlorvos	0	100.0	0.2	0.21	
	1.25	80.0	0.6	0.61	0.32
	2.50	86.0	0.2	0.19	0.72
	5.00	50.5	0.8	0.84	0.19
Trichloropon	0	100.0	0.1	0.09	
	0.04	100.0	0	<0.17	0.65
	0.40	100.0	0.3	0.29	0.31
	0.80	100.0	0	<0.20	0.68
Dichloroacetaldehyde	0	100.0	0.3	0.12	
	0.12	97.0	0.6	0.25	0.49
	0.60	93.0	0	<0.12	0.50
	1.20	84.0	1.0	0.36	0.31
Ethyl methanesulphate	0	100.0	0.2	0.21	
	20	73.0	39.0	38.41	<0.001

[a] Expressed as plating efficiency (% of cells that formed colonies).
[b] Expressed as the number of resistant colonies per 10^6 survivors.
[c] The accepted significance level is $P\leq0.01$.

4. Discussion

The DNA-damaging properties of dichlorvos and trichlorfon have been investigated by many authors. Induction of strand breakage was observed when calf thymus DNA,[29] *Escherichia Coli* or Chinese hamster cell cultures[30] were exposed to dichlorvos. DNA strand breakage induced by these agents was correlated with the extent of DNA alkylation. In fact, both pesticides were shown positive when tested for their alkylating capabilities with the 4-(4-nitrobenzyl)pyridine (NBP) colour test;[31] further analysis demonstrated that dichlorvos methylates the DNA of intact *Coli* and HeLa cells by an SN2 mechanism, very similar to that of methyl methanesulphonate, which also methylates preferably the N7 atom of guanine.[32] The attack by the alkylating agent on the DNA bases, followed by depurination, provides the substrate for the base excision repair pathway. The positive result found in the UDS test is the expression of the above mentioned step-by-step repair mechanism, and is in accord with data reported by other authors in lymphocytes and WI38 cells.[21,33]

Dichloroacetaldehyde gave a negative reaction with the NBP colour test,[31] suggesting that its mutagenicity is due to a mechanism of interaction with cellular macromolecules that is different from alkylation.[34] The failure of dichloroacetaldehyde to induce UDS in EUE cells is in agreement with previous reports; the interaction with the cellular targets may not be detectable as incorporation of radioactive nucleotides into repair patches.

No mutagenic effect in the form of ouabain-resistant mutations was detected in Chinese hamster cells. These data, as well as the published cytogenetic studies,[8–10, 17] indicate different responses to these agents in microorganisms and mammalian cells in culture. This discrepancy may be because mammalian cells are more sensitive than microbes to the toxic effects of dichlorvos and trichlorfon.[1]

In conclusion, the data presented here clearly show that both dichlorvos and trichlorfon are able to damage DNA and stimulate the repair processes in human cells, although no induction of gene mutation was shown in V79 cells.

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