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Defence Capacity Modulation of Human Hepatoma Cell Line Hep 3B by Three Pesticides

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Organophosphorous, carbamate and substituted urea compounds are widely used pesticides for controlling weeds, disease carrying vectors and agricultural pests.

The detection of aldicarb, an oxime carbamate nematicide in groundwater resources in Wisconsin and 15 other states nationwide (USEPA, 1988) and the detection of paraoxon, the major metabolite of parathion, an organophosphorous insecticide in cow's milk samples in Portugal (Lino and Noronha da Silveira, 1992) have prompted serious concern over the toxic effects of these compounds in animals and humans.

The aim of this study was to investigate the toxic effects of these two compounds on some enzymatic activities using a human hepatoma cell line, Hep 3B. A phenylurea herbicide, isoproturon, which does not seem very toxic but is known to induce several rat liver metabolic enzymes (Schoket and Vincze, 1985 ; 1986), was also studied in this work.

MATERIALS AND METHODS

Isoproturon, aldicarb and parathion were obtained from Interchim (Montluçon, France). Reduced glutathione (GSH), glutathione disulphide (GSSG), pyruvate, FAD, o-phtalaldehyde (OPT), dithiothreitol, cytochrome c from horse heart, Triton X 100, NADPH, 1 chloro-2,4-dinitrobenzene (CDNB) and NADH were purchased from Sigma Chemical (St Louis, MO USA). DMEM, foetal calf serum, compounds used for culture and buffers were purchased from GIBCO BRL (France). All other chemicals were of the best analytical quality grade.

Hep 3B cells were isolated by Aden *et al* (1979). They were

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routinely grown as a monolayer culture in Dulbecco's modifed Eagle's Medium (DMEM) supplemented with 10% foetal calf serum. Culture flasks were seeded in the ratio of 10 000 Hep 3B cells/cm^2. The cells were harvested using trypsin-EDTA solution (0.25%-0.1%), 48 hours after seeding.
Cell viability was assessed by Trypan-blue exclusion.
The cells were exposed over 24 hours, (that is from 24 to 48 hours of culture) at isoproturon concentrations ranging from 5 to 100 mg/L, parathion : from 5 to 50 mg/L ; and aldicarb from 0.01 to 50 mg/L in the culture medium.

Isoproturon and parathion were first dissolved in dimethyl sulfoxide (DMSO) The final concentration of DMSO in the culture medium was 0.1%. It was used as a solvent control for these two pesticides. Aldicarb was dissolved in ultra-pure water.

The determination of GSH and GSSG, glutathione reductase (G.Red.) activity and lactate dehydrogenase (LDH) release were completed as described previously (Herno *et al.*, 1992). Glutathione transferase (GST) activity was determined according to Habig *et al.* (1974) using the capacity of GST to conjugate GSH (20 m*M*) with CDNB (20 m*M*). The cell suspension was diluted in 50 m*M* Tris HCl buffer pH 6.5 to 2.10^6 cell/mL. The absorbance of the conjugate was measured at 340 nm, every 30 sec. for 10 min. The NADPH - cytochrome P-450 reductase activity measurement is based on the method of Phillips and Langdon (1962). The required cellular concentration was 2.5 10^6 cells/mL in 50 m*M* Tris HCl, EDTA 0.1 m*M* buffer pH 7.7. The reaction was started by the addition of NADPH (10 m*M*), cytochrome c (0.5 m*M*), KCN (1 m*M*) at 30°C. The absorbance at 550 nm was measured every 30 sec. for 10 min.

Mean values ± standard deviation (SD) are presented for each criterion. ANOVA and Dunnett's t-test were used to determine the significant level of differences between pesticide-treated samples and untreated samples. Homoscedasticity was checked using Bartlett's test. Linear regression was tested by ANOVA.

RESULTS AND DISCUSSION

The aim of this work was to determine the toxic effects of parathion, aldicarb and isoproturon, on Hep 3B human cells, which derived from a human hepatoma biopsy (Aden *et al.*, 1979).

Parathion increased LDH release. Although the percentage of LDH

release with 30 mg/L is higher than with 50 mg/L, the statistical analysis reveals that this criterion is a linear function of the parathion concentration. This reflects an alteration in membrane permeability (Table 1). GSH was similarly dose-dependent on the pesticide concentration, but GSSG was not modified to any appreciable extent. This stability of GSSG could be explained by the enhanced activity of G. Red. Concurrently, GST activity showed dose-dependent stimulation, although a level seems discernable for concentrations over 25 mg/L. So parathion metabolites appear to be conjugated with GSH. These results go to confirming those of Hollingworth et *al* (1973) and Sultatos et *al* (1985) which showed that GSH could be conjuguated either directly with parathion or with its metabolites, one of which is paranitrophenol. Nevertheless, no GSH diminution was observed but rather an increase; perhaps a stimulation of the GSH synthesis enzymes (GSH synthetase and Glu-Cys synthetase) might occur (Meister and Anderson, 1983).
No effect of parathion on NADPH-Cyt P-450 reductase could be detected (Table 1). But all the same, the oxidation of parathion to paraoxon was demonstrated early (Gage, 1953) and this reaction is promoted by microsomal enzymes requiring NADPH and oxygen. The same enzyme system also produces diethylhydrogen phosphorothionate (Neal, 1967).

As for aldicarb, the LDH release decreased in a non-dependent relation. This diminution suggests that the pesticide may alter enzyme affinity or its activity (Table 2). This result is in accordance with Gill *et al.* (1990) who also found that aldicarb inhibited the LDH activity of fish liver (*Puntius conchonius* Ham.). GSH level appeared to be unchanged but GSSG was enhanced linearly as a function of aldicarb concentration according to ANOVA results. Therefore, in the same way as parathion, an increased synthesis of GSH could occur to compensate for the loss of GSH by oxidation to GSSG, all the more as G.Red. activity appeared not to be changed. GST activity was not stimulated : thus, conjugations with GSH were not enhanced. Low concentration (0.01 mg/L) of this pesticide stimulated NADPH-cyt P-450 reductase activity. Studies by Gillette *et al.* (1957) have presented evidence that NADPH-cyt P-450 reductase was involved in xenobiotic metabolism. Lu *et al.* (1969, 1970) demonstrated the necessity of this enzyme in monooxygenase activity. It can then be supposed that Hep 3B cells are capable of metabolizing aldicarb to oxidized metabolites such as aldicarb sulfoxide and perhaps sulfone. It was shown that aldicarb sulfoxide was as toxic as aldicarb (LD 50 rat = 0.9 mg/kg) (Jackson *et al.*, 1990).

Table 1. Effects of parathion on glutathione and enzymatic activities in Hep 3B cells. (means ± SD)

Parathion (mg/L)	LDH release (%) n = 3	GSH nmol/10^6cells n = 4	GSSG nmol/10^6cells n = 4	G. Red. mUI/10^6cells n = 3	GST mUI/10^6cells n = 3	Cyt.P-450 Red mUI/10^6 cells n = 3
Control	3.05 ± 0.61	2.50 ± 1.37	0.93 ± 1.00	4.07 ± 0.46	13.28 ± 2.03	0.40 ± 0.12
5	4.27 ± 1.84	3.04 ± 1.04	1.20 ± 1.11	3.72 ± 0.65	14.98 ± 3.58	0.41 ± 0.08
10	4.55 ± 1.25	2.40 ± 1.01	0.80 ± 0.35	6.00 ± 0.88**	16.64 ± 2.49	0.37 ± 0.14
20	4.76 ± 1.54	3.91 ± 0.82	1.42 ± 1.26	8.60 ± 0.80**	22.82 ± 5.89**	0.44 ± 0.14
30	5.50 ± 0.59**	4.63 ± 0.53 *	1.69 ± 0.86	7.23 ± 0.24**	27.92 ± 1.82**	0.40 ± 0.05
50	5.42 ± 0.52**	5.37 ± 0.73**	2.00 ± 1.57	7.36 ± 0.54**	26.22 ± 3.88**	0.45 ± 0.09
	r = 0.5607 F = 0.586	r = 0.7527 F = 0.800			r = 0.4574 F = 0.821	

* $p < 0.05$ ** $p < 0.01$ vs control

Table 2. Effects of aldicarb on glutathione and enzymatic activities in Hep 3B cells. (means ± SD)

Aldicarb (mg/L)	LDH release (%) n = 3	GSH nmol/10^6cells n = 6	GSSG nmol/10^6cells n = 6	G.Red. mUI/10^6 cells n = 3	GST mUI/10^6 cells n = 3	Cyt.P-450 Red mUI/10^6cells n = 3
Control	3.15 ± 0.31	1.74 ± 0.15	0.55 ± 0.14	3.38 ± 0.77	21.62 ± 6.68	0.40 ± 0.10
0.01	2.30 ± 0.36 *	1.99 ± 0.56	0.57 ± 0.13	3.78 ± 0.62	25.71 ± 1.32	0.63 ± 0.15 *
0.1	2.41 ± 0.33 *	1.86 ± 0.43	0.60 ± 0.14	3.60 ± 0.32	25.28 ± 0.99	0.56 ± 0.12
1	2.39 ± 0.23 *	1.90 ± 0.70	0.64 ± 0.23	3.86 ± 0.29	24.15 ± 4.87	0.54 ± 0.11
10	2.39 ± 0.13 *	1.96 ± 0.53	0.78 ± 0.13	2.67 ± 0.86	25.94 ± 4.68	0.46 ± 0.08
50	2.43 ± 0.38 *	1.85 ± 0.47	0.84 ± 0.23	3.28 ± 0.52	25.68 ± 4.35	0.49 ± 0.11
			r=0.4701 F=1.007			

* $p < 0.05$ vs control

Table 3. Effects of isoproturon on glutathione and enzymatic activities in Hep 3B cells. (means ± SD)

Isoproturon (mg/L)	LDH release (%) n = 6	GSH nmol/10^6 cells n = 4	GSSG nmol/10^6 cells n = 4	G. Red. mUI/10^6 cells n = 3	GST mUI/10^6 cells n = 4	Cyt.P-450 Red mUI/10^6 cells n = 3
Control	3.30 ± 0.41	3.20 ± 0.43	0.52 ± 0.26	3.53 ± 0.97	16.21 ± 3.77	0.44 ± 0.12
5	3.41 ± 0.82	3.37 ± 1.34	0.51 ± 0.31	2.31 ± 0.46 *	15.52 ± 4.07	0.49 ± 0.14
25	3.61 ± 0.33	2.91 ± 1.29	0.39 ± 0.17	2.27 ± 0.52	17.49 ± 3.08	0.52 ± 0.04
50	3.94 ± 0.56	2.80 ±1.24	0.41± 0.31	2.49 ± 0.35	17.06 ± 6.04	0.51 ± 0.15
100	3.28 ± 0.95	1.64 ± 0.53	0.41 ± 0.22	2.21 ± 0.19 *	18.23 ± 7.56	0.62 ± 0.09
		r = - 0.4390 F = 0.136				r = 0.5476 F = 0.464

* $p < 0.05$ ** $p < 0.01$ vs control

For isoproturon, no modified LDH release was observed (Table 3). The herbicide induced a dose-dependent decrease in GSH, no change in GSSG content but an inhibition of G. Red activity. In addition, GST activity was not significantly increased, although Schoket and Vincze (1985) found activity increased by 175 mg/kg for isoproturon in rats *in vivo*. This difference is almost certainly due to higher hepatic metabolism *in vivo* than in cell cultures *in vitro*. It seemed unlikely that GSH was used to form mercapturic conjugates because GST activity was not modified. It would be useful to identify isoproturon metabolites to ensure this hypothesis. In this way, the GSH decrease in treated samples could be explained by a higher degradation of GSH than in the control, and/or lower turnover. Isoproturon led to a dose-dependent increase in NADPH-cyt P-450 reductase activity, Hep 3B cells could therefore play a role in metabolizing this pesticide. Schoket et Vincze (1985) also observed that isoproturon was an inducer of this enzyme in rat liver *in vivo*. Furthermore, Limbosch (1983) studied the metabolism of benzo[a]pyrene and aldrin through 5 human and rat hepatoma cell lines and demonstrated that Hep 3B cells were capable of metabolizing these two procarcinogens into active compounds. Moreover, this cell line retains different cytochrome P-450 isoenzymes : cyt. P-450 IA1, IA2 and IIIA3 (Fukuda *et al.*, 1992).

The results of the present study and those of Limbosch (1983) and Fukuda *et al.*(1992), indicate that Hep 3B cells constitute a promising toxicological tool for their defence capacities and their metabolic potential which should be investigated more exhaustively.

The enzyme activities studied in Hep 3B cells reflect cellular disturbances noted under the effect of parathion, aldicarb and isoproturon. Apart from the results recorded with parathion, statistically meaningful modifications are few and not very significant, probably because of the lower toxicity of these pesticides compared to their predecessors. These variations should be related to the forming of metabolites and their analysis in cell media would guide their interpretation. These effects could be observed over a longer period, after 6 to 12-day exposure at very low concentrations (what would be done in the next work), to make the cells more sensitive to these pesticides and evaluate the effect of traces rather than high concentrations; such evaluation would be more closely comparable to the conditions found in our aquatic environment.

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