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Cytotoxicity of Lindane and Paraquat to Human Hepatoma Cell Lines

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Cell culture systems are suitable for the study of cytotoxicity mediated by free radicals under defined conditions and for the understanding of cytoprotection and cellular adaptive responses. Various cell types may be used to assess cytotoxicity, ranging from animal or human cells in suspension or in support culture to transformed cells such as human hepatoma Hep G2 and Hep 3B cell lines. Several authors showed that Hep G2 cells can be useful in toxicology testing as target cells and means of endogenous biotransformation (Doostdar et al. 1991; Roe et al. 1993; Liu et al. 1994, Rueff et al 1996). Conversely, the metabolizing capacities of Hep 3B cells are lower (Castro et al. 1990; Descampiaux et al. 1996).

In order to confirm the potential use of Hep 3B and Hep G2 cells more particularly in the detection of free radical-induced toxicity, we chose to investigate, in these two cell lines, the cytotoxicity of two pesticides : lindane, on the one hand, which is an organochloride insecticide (1,2,3,4,5,6-hexachlorocyclohexane) with a complex metabolism following several pathways in mammalians, and paraquat, on the other hand, an ammonium quaternary herbicide (1,1'-dimethyl 4,4'-bipyridinium), which is only oxido-reduced in cells. Both are known to induce free radical toxicity. Moreover, lindane induces a dose-dependent decrease in reduced glutathione (GSH) content of Hep 3B cells. Conversely, glutathione disulphide (GSSG) level and glutathione transferases (GST) and superoxide dismutase (SOD) activities are enhanced linearly with pesticide concentrations (Descampiaux et al. 1996).

In this study, we assessed the cytotoxic effects of lindane and paraquat on viability, growth, lactate dehydrogenase (LDH) release, ATP and glutathione contents, enzymatic activities [SOD, catalase, glutathione peroxidases (GPx), glutathione reductase (G.red.), and glutathione transferases (GST)], and membrane fluidity of both cell lines.

MATERIALS AND METHODS

Hep 3B and Hep G2 cells were routinely grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

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Culture flasks were seeded with 10,000 Hep 3B cells/cm^2 and 50,000 Hep G2 cells /cm^2. Cell viability was assessed by Trypan blue exclusion. Protein content was determined according to the Bradford method (1976).
The cells in the culture medium were exposed for 24 hours to lindane or paraquat at concentrations ranging from 0.5 to 50 mg/L and from 0.5 to 100 mg/L, respectively.
Paraquat was dissolved in ultrapure water and lindane in dimethyl sulphoxide (DMSO). The concentration of DMSO in the culture medium did not exceed 0.1 %. Untreated and DMSO-treated (0.1%) cultures served as controls.
Determinations of LDH, ATP, GSH and GSSG levels, GST, G.red., GPx, SOD and catalase were performed as previously described (Descampiaux et al. 1996).
Membrane fluidity was determined by ESR (Varian E-109 spectrometer) using 5-doxyl stearic acid as a membrane marker, and the order parameter S was defined according to Bertini and Drago (1979). The formation of superoxide radical anions was assessed by chemiluminescence using lucigenin as the luminescent probe (Allen 1986, Peters et al. 1990).The content of MDA in cell pellet was performed following the method of Halliwell and Gutteridge (1989).

Results are expressed as mean ± standard deviation. Data were subjected to an analysis of variance (ANOVA), and when significant differences were found, comparisons between control and treated groups were made by using the Dunnett's t-test. The significance of these tests was noted by asterisks in the tables. In addition, when the ANOVA was significant, an analysis of variance regression was also carried out in order to prove the existence of a linear relationship between cellular responses and pesticide concentrations. The significance of this test was expressed by calculating F1 and F2 values, and the non-null slope was verified by Student's t-test (Miller, 1991).
In all cases, homocedasticity of variances was preliminarily checked by using Bartlett's test (Gad and Weil, 1989). When variances were not homogenous, we used the non-parametric Krusdall Wallis' test.

RESULTS AND DISCUSSION :

Previous studies in our laboratory (Descampiaux et al. 1996; 1997) demonstrated that lindane is toxic to Hep 3B cells. Hep 3B cells exposed to lindane (Table 1) had an increased LDH release, but there was no effect on viability ($\overline{x}$ = 96.1 ± 0.6%) and ATP level ($\overline{x}$ = 51.2 ± 17.1 nmoles/mg prot.). Lindane decreased growth in the 25 and 50 mg/L pesticide concentration groups. In addition to the non detected GPx activity and the induction of SOD activity previously observed (Descampiaux et al. 1996), a lindane dose-dependent response of MDA was observed in Hep 3B cells (Table 1). Moreover, cells exposed to 50 mg/L lindane concentration exhibited an increase in chemiluminescence signal after seven minutes, which indicated free radical production; SOD supplementation in reactive medium inhibited this response (Figure 1). A significant ($p<0.01$) decrease in the order parameter of 5-doxyl stearic acid (observed by ESR)

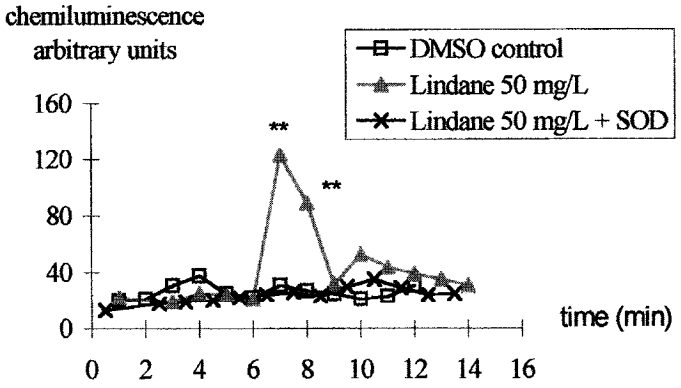

Figure 1. Measure of chemiluminescence signal on Hep 3B cells in the presence of 50 mg/L lindane (n=3).
** p<0.01 vs DMSO control (Student test).

Table 1. Effect of 24-hr lindane exposure on growth, LDH, glutathione contents (Descampiaux et al, 1996), and MDA in Hep 3B cells.

Samples	Growth % n = 14	LDH release % n = 4	GSH nmol./mg prot n = 3	GSSG nmol./mg prot n = 3	MDA nmol./10^6 cells n = 3
Controls					
DMEM	---	4.0 ± 0.7	29.7 ± 4.3	2.7 ± 0.3	0.020 ± 0.001
DMSO	100.0 ± 0.0	3.9 ± 0.1	24.0 ± 3.7	3.1 ± 2.9	0.022 ± 0.001
Lindane					
0.5 mg/L	101.0 ± 13.7	4.0 ± 0.1	18.6 ± 0.3	4.4 ± 0.6	0.016 ± 0.001
5 mg/L	98.0 ± 19.4	* 4.3 ± 0.1	17.9 ± 6.1	4.5 ± 1.1	0.018 ± 0.004
25 mg/L	** 72.2 ± 12.4	** 12.4 ± 0.2	17.1 ± 8.4	5.2 ± 2.3	0.028 ± 0.004
50 mg/L	** 58.2 ± 10.6	** 10.3 ± 0.6	7.92 ± 3.6	6.6 ± 0.1	0.035 ± 0.005
ANOVA regression					
F1	p < 0.05	p < 0.05	p < 0.01	p < 0.05	p < 0.05
F2	p < 0.05	NS	p < 0.01	p < 0.05	p < 0.05
r	p < 0.05	NS	p < 0.01	p < 0.01	p < 0.05

* p < 0.05, ** p < 0.01 vs DMSO control NS : non significant

indicated an increase in cell, membrane fluidity after 24-hr lindane exposure (50 mg/L). Values of this order parameter for DMSO and 50 mg/L lindane samples were 0.285 ± 0.007 and 0.267 ± 0.006, respectively.

The same parameters were studied in Hep G2 cells. Lindane induced an increase in LDH release, a decrease in GPx activity and GSH content, with no significant change in the total glutathione level (the GSH/GSH+GSSG ratio varied from 95.6 % for control cells to 73.4 % for cells exposed to 50 mg/L lindane) (Table 2). Viability ($\bar{x}$ = 93.3 ± 1.3 %), growth ($\bar{x}$ = 89.2 ± 6.7 %), ATP levels ($\bar{x}$ = 32.8 ± 4.9 nmol / mg prot.), MDA content ($\bar{x}$ = 0.050 ± 0.008 nmol / 10^6 cells),

Table 2. Effect of 24 hr lindane exposure on LDH release, glutathione contents and GPx activity in Hep G2 cells.

Samples	LDH release % n = 4	GSH nmol/mg prot n = 3	GSSG nmol/mg prot n = 3	GPx UI/mg prot. n = 3
Controls				
DMEM	3.97 ± 0.35	79.3 ± 11.2	3.6 ± 2.2	0.29 ± 0.04
DMSO	3.55 ± 0.46	68.1 ± 17.1	5.8 ± 4.2	0.27 ± 0.01
Lindane				
0.5 mg/L	** 4.83 ± 1.11	* 41.3 ± 5.0	* 16.3 ± 5.3	0.29 ± 0.01
5 mg/L	** 4.57 ± 0.55	65.3 ± 18.0	** 22.5 ± 0.3	0.27 ± 0.04
25 mg/L	** 5.18 ± 1.07	* 47.7 ± 8.1	22.2 ± 15.6	0.25 ± 0.03
50 mg/L	** 6.65 ± 1.39	* 41.5 ± 10.4	14.8 ± 10.5	0.25 ± 0.03
ANOVA regression				
F1	$p < 0.05$	NS	Krusdall Wallis	Krusdall Wallis
F2	$p < 0.05$	NS	$p < 0.05$	$p < 0.05$
r	$p < 0.05$	NS		

* $p < 0.05$ ** $p < 0.01$ vs DMSO controls NS : non significant

GST activity ($\bar{x}$ = 66.0 ± 13.5 UI/mg prot.), G.red. activity ($\bar{x}$ = 0.249 ± 0.019 UI/mg prot.), SOD activity ($\bar{x}$ = 3.0 ± 0.3 UI/mg prot.), and catalase activity ($\bar{x}$= 23.4 ± 3.3 UI/mg prot.) were not significantly modified by lindane ($p>0.05$). Moreover, no superoxide anion radical production was observed in the presence of lindane, the chemiluminescence signal presenting a similar response for DMSO control and lindane samples (between 15 and 25 chemiluminescence arbitrary units). Some lindane effects are common to both cell lines, but are more marked in Hep 3B cells. Cellular membrane alterations, revealed by LDH leakage and fluidification of cellular membranes, were particularly prominent for Hep 3B cells. Furthermore, in this cell line, the chemiluminescence assay indirectly indicated the presence in the extracellular medium of superoxide radicals which normally are unable to go through the membrane : in this case, the latter may be altered.

The decrease in GSH may occur before or after the propagation of lipid peroxidation. As Comporti et al (1991) argued in studying hepatocellular damage produced by GSH-depleting agents, it is likely that electrophilic intermediates produced during the metabolization of lindane react with GSH leading to GSH depletion. When the depletion reaches a certain threshold value, lipid peroxidation can be observed. In our study, lipid peroxidation could be detected only in Hep 3B cells in which lindane induced an important decrease in GSH (from 30 to 8 nmoles/mg protein) compared to Hep G2 cells (from 80 to 42 nmoles/mg protein). Moreover, in the Hep G2 cell line, the initial level of GSH was markedly higher and less decreased after lindane exposure.

Changes in levels of some biochemical compounds, especially those implicating glycine metabolism (methionine, choline, betaine), suggest that some metabolic

pathways are affected by lindane (Descampiaux et al. 1997).
In conclusion, most responses to lindane in these two cell lines pertained to free radical-induced toxicity (antioxidant enzymes, production of MDA and superoxide anions). It is not surprising that Hep G2 cells which have GSH level and antioxidant enzymatic activities markedly superior to those of Hep 3B cells appeared less sensitive to this pesticide (Descampiaux et al. 1996). Moreover, lindane metabolism is complex and five pathways involving numerous metabolites have been described (Hayes and Laws, 1991). Lindane and its metabolites can undergo hydroxylation, dehydrogenation, hydrodechlorination or transdehydrochlorination. These reactions are followed by glucuronosyl-, sulpho- or mercapto- conjugations. Hep 3B cells may metabolize lindane by conjugation with glutathione, as suggested by enhanced GST activity and decreased GSH. Junqueira et al (1986) involved this metabolic pathway in superoxide ion production. This fact is in accordance with SOD activation and the chemiluminescence results which were noted.
These phenomena were not observed in Hep G2 cells. It can be supposed that enzymatic activities which are more marked in these cells (phase I activities and phase II enzymes such as UDP glucuronosyl- and sulpho-transferase) (Castro et al. 1990; Doostdar et al. 1991; Roe et al. 1993; Liu et al. 1994) promote a more complete lindane metabolism and thus a detoxication of this pesticide, implicating more metabolites with final glucuronosyl- and sulpho- conjugations. Conversely, Hep 3B cells appeared unable to exhibit such detoxication because of their lower enzymatic potential.

The growth of Hep 3B cells was affected by paraquat. The growth inhibition observed was a linear function of pesticide concentration, the IC 50% being 93 mg/L. LDH release was also enhanced linearly with concentration (Table 3). Conversely, paraquat had no significant effect on viability $\bar{y}$ (x = 93.7 ± 1.4%, n=13), ATP ($\bar{x}$ = 27.8 ± 2.0 nmol/mg prot.), GSH and GSSG contents ($\bar{x}$ = 46.2 ± 10.7 nmol/mg prot. and $\bar{x}$ = 4.1 ± 1.4 nmol/mg prot., respectively), GST ($\bar{x}$ = (0.254 ±_ 0.025 UI/mg prot.), G.red. ($\bar{x}$ = 0.113 ± 0.011 UI/mg prot.), SOD ($\bar{x}$ = 0.77 ± 0.01 UI/mg prot.) and catalase ($\bar{x}$ = 15.8 ± 1.2 UI/mg prot.) activities after 24 hr exposure. A previous ^{1}H NMR study on paraquat exposed cells (Descampiaux et al. 1997), showed that peaks characteristic to paraquat were not detectable in the NMR spectrum of cells. A hydrochloric acid hydrolysis was necessary to establish the presence of paraquat in cells. It was suggested that the pesticide can interact with cellular compounds. Thus, this herbicide may become unavailable to exhibit its toxicity on the other cellular activities measured in the present study, due to covalent binding to cellular macromolecules.

Almost all the cellular parameters in Hep G2 cells were modified by paraquat. Glutathione and linked-enzymes seem to play a role in paraquat toxicity : this pesticide induced a decrease in GSH levels and GPx activity and an increase in GSSG content and G.Red and GST activities (Table 4). GSH oxidation to GSSG, by a pathway different from the one implicating GPx activity, increases with paraquat concentration. Kojima et al (1992) suggested that the decrease in GPx

Table 3. Effect of 24 hr paraquat exposure on growth and LDH release in Hep 3B cells.

Paraquat (mg/L)	Growth n = 13 %	LDH n = 5 %
0	100.0 ± 0.0	6.5 ± 1.7
0.5	92.9 ± 17.0	7.7 ± 2.5
5	** 79.6 ± 10.0	7.9 ± 0.4
25	** 76.7 ± 8.5	9.2 ± 1.4
50	** 61.5 ± 8.3	* 11.9 ± 3.6
100	** 51.4 ± 10.9	* 11.3 ± 2.2
ANOVA regression		
F1	p < 0.01	p < 0.05
F2	p < 0.01	p < 0.05
r	p < 0.01	p < 0.05

* p < 0.05 ** p < 0.01 vs 0 NS : non significant

Table 4. Effect of 24 hr paraquat exposure on glutathione contents and linked enzymes in Hep G2 cells.

Paraquat (mg/L)	GSH nmol/mg prot n = 3	GSSG nmol/mg prot n = 3	GST UI/mg prot n = 3	G.Red. UI/mg prot n = 3	GPx UI/mg prot n = 3
0	80.2 ± 8.2	7.3 ± 3.5	0.112 ± 0.022	0.216 ± 0.018	0.29 ± 0.03
0.5	78.4 ± 4.5	8.8 ± 3.3	0.095 ± 0.002	0.272 ± 0.008	*0.28 ± 0.02
5	74.3 ± 7.5	10.2 ± 2.2	0.083 ± 0.004	0.230 ± 0.024	**0.23 ± 0.02
25	63.2 ± 8.1	20.1 ± 5.9	0.092 ± 0.006	*0.410 ± 0.084	**0.20 ± 0.33
50	54.5 ± 7.1	27.8 ± 6.6	0.135 ± 0.042	**0.476 ± 0.020	**0.19 ± 0.01
100	48.1 ± 14.1	30.4 ± 5.3	** 0.180 ± 0.009	**0.750 ± 0.148	**0.17 ± 0.01
ANOVA regression					
F1	p < 0.01	p < 0.05	NS	p < 0.05	p < 0.01
F2	p < 0.01	p < 0.05	NS	NS	p < 0.01
r	p < 0.01	p < 0.05	NS	NS	p < 0.01

* p < 0.05 ** p< 0.01 vs 0 NS : non significant

Table 5. Effect of 24 hr paraquat exposure on SOD and catalase activities and on viability, growth, LDH release, and ATP in Hep G2 cells.

Paraquat (mg/L)	SOD UI/mg prot n = 3	Catalase UI/mg prot n = 3	Viability % n = 16	Growth % n = 14	LDH % release n = 3	ATP mg/mg prot. n = 3
0	2.5 ± 0.4	22.6 ± 4.3	97.2 ± 4.7	100 ± 0.0	4.9 ± 0.3	43.7 ± 9.4
0.5	2.8 ± 0.5	21.6 ± 4.5	90.2 ± 3.8	** 92.9 ± 17.0	5.9 ± 0.7	39.2 ± 10.2
5	2.9 ± 0.6	21.1 ± 3.4	91.4 ± 3.4	** 79.6 ± 10.0	6.0 ± 0.5	39.2 ± 5.4
25	3.1 ± 0.3	20.8 ± 0.9	90.2 ± 4.2	** 76.7 ± 8.5	6.7 ± 0.7	31.7 ± 5.6
50	3.4 ± 0.8	24.4 ± 3.3	*86.8 ± 4.9	** 61.5 ± 8.3	7.3 ± 0.2	26.1 ± 13.2
100	3.5 ± 0.5	30.1 ± 3.8	**76.5 ± 6.3	** 51.4 ± 10.9	10.8 ± 0.5	**10.7 ± 6.3
ANOVA regression						
F1	p < 0.05	NS	p < 0.05	p < 0.05	p < 0.05	p < 0.05
F2	p < 0.05	NS	p < 0.05	NS	NS	p < 0.05
r	p < 0.05	NS	p < 0.05	NS	NS	p < 0.05

* p < 0.05 ** p< 0.01 vs 0 NS : non significant

activity was due to GSH depletion. G.Red activity, although induced, was insufficient to restore the GSH pool from GSSG.

The induction of SOD activity, the variation of glutathione contents and enzymes in relation to paraquat and the alteration of intracellular ATP levels seem to confirm a free radical-induced toxicity involving superoxide anion production which has been described by several authors (Bus and Gibson 1976; Llopis et al. 1992; Hirai et al. 1992). Catalase activity only slightly increased at the two highest concentrations tested and was unable to detoxify H_2O_2 formed from superoxide ion dismutation. Total GPx was not modified by paraquat. However, a decrease in cellular viability and growth and a dose-dependent increase in LDH release were observed in response to paraquat, indicating membrane alteration (Table 5).

Concerning paraquat, it is relatively easy to understand its effects on the different enzymatic activities. The differing results between the two cell lines can be explained mainly by the higher NADPH cytochrome P-450 reductase activity level in Hep G2 cells (Hep 3B cells : 6.0 ± 0.7 UI/mg prot; Hep G2 cells : 36.1 ± 6.1 UI/mg prot.). This activity could increase the oxido-reductive metabolism of paraquat leading to a more massive production of superoxide radicals than in Hep 3B cells. Furthermore, the presence of interactions between paraquat and other cellular macromolecules is certainly more important in Hep 3B cells. It could be interesting to study thoroughly the intracellular fate of paraquat by performing cellular fractionation in order to better understand the reasons for the Hep 3B cell resistance.

This study demonstrates the advantage of using hepatic cells with metabolizing capacities compared to other cell lines. It permits thereby, in function of the presence or not of possible metabolites, to assess more validly the consequences for humans of an exposure to these environmental toxic compounds.

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