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Insecticide Cytotoxicity and CYP1A1/2 Induction in Primary Human and Rat Hepatocyte Cultures

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Abstract—With the increasing demand for insecticide products, the question of their safety has become one of the serious world public health concerns. The capability of compounds belonging to the major insecticide families [such as chlorinated hydrocarbons (DDT), carbamates (carbaryl: CBR), organophosphorus compounds (malathion, tetrachlorvinfos: MAL, TCV), pyrethroids (cypermethrin: CPR) and benzoylurea (diflubenzuron: DFU)] in inducing CYP1A1 in rat and human hepatocyte cultures has been tested. Cells were treated during 3 days with six non-toxic increasing doses of insecticides and CYP1A1 expression was assessed by ethoxyresorufin *O*-deethylase (EROD) activity and by Northern blots. A strong and dose-dependent induction was observed with TCV and DFU, both in human (approx. five- and sevenfold over control, respectively) and in rat hepatocytes (approx. sevenfold). However, EROD induction and CYP1A1 mRNA levels were correlated for DFU but not for TCV, suggesting different regulation mechanisms for *CYP1A1* gene expression by the two compounds. CBR and CPR exerted less induction in both cell types (approx. 2.5-fold induction compared with approximately 16-fold for 3-methylcholanthrene), whereas DDT and MAL showed no action on human hepatocytes but decreased EROD activity in rat cells. Finally, cytotoxicity studies performed using the MTT and the neutral red tests demonstrated significant differences between insecticides. © *1997 Elsevier Science Ltd*

Abbreviations: CBR = carbaryl; CPR = cypermethrin; CYP = cytochrome *P*-450; DFU = diflubenzuron; DMSO = dimethyl sulfoxide; EROD = ethoxyresorufin *O*-deethylase; FCS = foetal calf serum; MAL = malathion; 3-MC = 3-methylcholanthrene; NR = neutral red; TCV = tetrachlorvinfos.

INTRODUCTION

Pesticides are widely present in our environment, resulting in constant exposure for humans, occurring through many routes (Hodgson and Levi, 1996; Thomas, 1996). Although the risks associated with high-level short-term (acute) exposure to these compounds are well documented in workers, there is great debate concerning their possible role in many chronic human health problems. Such problems include carcinogenesis, neurotoxicity and reproductive and developmental toxicity, but less is known about chronic effects of low-dose insecticide residues in food and drinking water on the liver. Only a few data are available specifically concerning about insecticide action on hepatic cytochrome *P*-450s (CYPs) (Ikeda *et al.*, 1991; Li *et al.*, 1995), an enzyme superfamily known to play an important role in xenobiotic detoxification and toxification processes (Denison and Whitlock, 1995; Gonzalez *et al.*, 1993).

Among all the CYP forms expressed in the liver, CYP1A1 is an ubiquitous polycyclic aromatic hydrocarbon (PAH)-responsive monooxygenase enzyme (Nebert, 1989; Okey *et al.*, 1994) that is involved in the activation of procarcinogen and promutagens into reactive genotoxic metabolites (Nebert, 1989). Like some other drug-metabolizing enzymes, its induction is governed by genetic polymorphisms (Nebert *et al.*, 1996). As CYP1A1 is not constitutively expressed (or only at low level) in most tissues, its induction is of clinical and toxicological relevance and has been proposed as an indicator of the potentially harmful effects of environmental samples (Kopponen *et al.*, 1994). This enzyme activation could be considered to be one of the most sensitive and subtle biochemical cellular responses, since it generally takes place at much lower doses of a given chemical than those known to cause lethal, or overly toxic, effects by the same chemical.

Primary cultures of hepatocytes have been demonstrated to provide relevant information in this matter, as they represent a metabolically competent cellular system, in which hepatic toxicity (Fautrel *et al.*, 1993) and induction (Daujat *et al.*, 1990) of xenobiotics can be investigated simultaneously. Furthermore, the use of human cells offers

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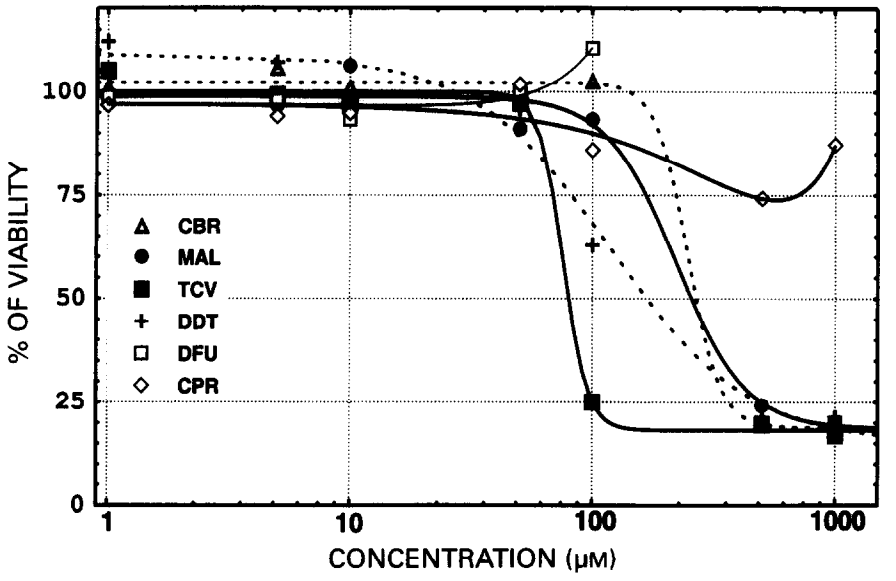

Fig. 1. *In vitro* cytotoxicity of insecticides determined by the neutral red test, after 48 hr exposure. Results are representative of experiments using human hepatocytes (mean of five replicates).

considerable scientific advantages because examples of failures in the extrapolation of animal toxicological data (*in vivo* or *in vitro*) to humans are frequent (de Sousa *et al.*, 1995; Rahmani *et al.*, 1993).

The aim here was primarily to investigate whether various compounds belonging to the major insecticide families, such as DDT (a chlorinated hydrocarbon), malathion (MAL) and tetrachlorvinfos (TCV) (two organophosphorus compounds), carbaryl (CBR; a carbamate), cypermethrin (CPR; a pyrethroid) and diflubenzuron (DFU; a benzoylurea) can induce or inhibit CYP1A1 expression at the protein activity and transcription levels. Effects were examined both in humans and in rats in primary cultures of hepatocytes.

The data presented demonstrate that many structurally different insecticides, such as TCV, DFU, CBR and CPR significantly induce ethoxyresorufin *O*-deethylase (EROD) activity and/or CYP1A1 mRNAs, at concentrations below their respective toxicity. Therefore, as consumers are daily exposed, even at very low concentrations, to insecticide residues in food, potential acute and chronic adverse effects of these compounds to human health through the liver should be seriously considered.

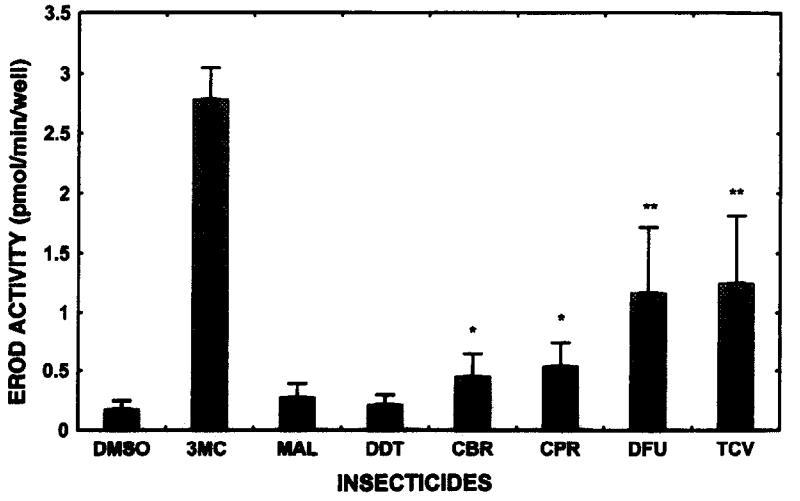

Fig. 2. Maximal induction effect of the insecticides on rat hepatocytes by reference to 3-MC. EROD activity was determined as described in Materials and Methods. Results are the mean of four separate experiments in triplicate. Chemical concentrations: 3-MC (0.5 μM), MAL (50 μM), DDT (10 μM), CBR (100 μM), CPR (100 μM), DFU (100 μM), TCV (75 μM). Asterisks indicate significant differences from control (*$P < 0.05$; **$P < 0.001$; one-way ANOVA and Newman–Keul's *post hoc* test.

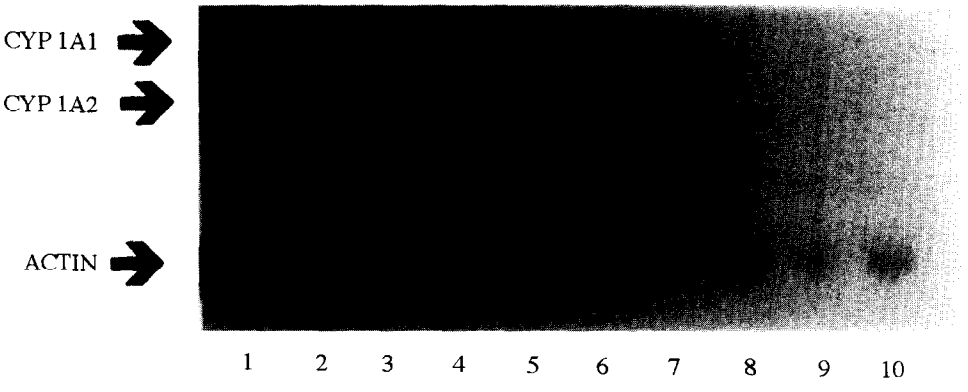

Plate 1. Induction of CYP1A1/2 mRNA in cultures of human hepatocytes. Cells were treated with either DMSO or various concentrations of chemicals. 10 μg total RNA was analysed and CYP1A1/2 was revealed with a radiolabelled CYP1A1 probe as described in Materials and Methods. Chemical concentrations: (lane 1) 2 μM 3-MC; (lanes 2, 3, 4) 10, 25, 50 μM DFU; (lanes 5, 6) 5, 10 μM DDT; (lanes 7, 8, 9) 10, 5, 25 μM TCV; (lane 10) DMSO.

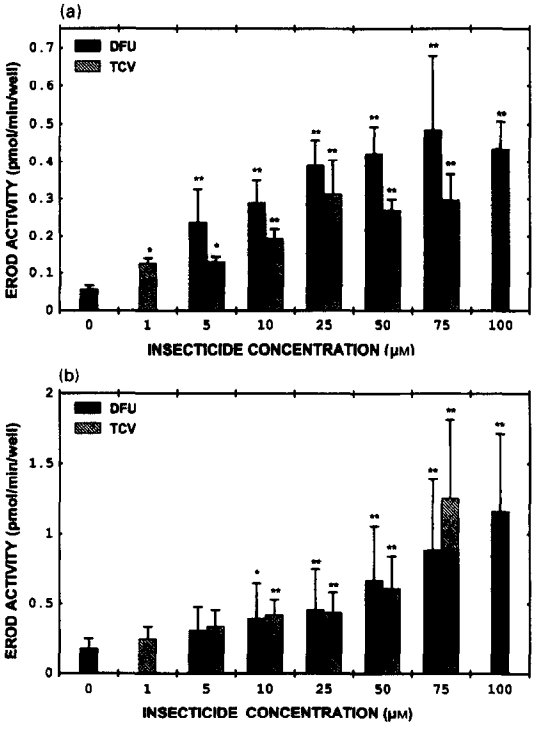

Fig. 3. Dose-dependent increase of EROD activity in human (a) and rat (b) hepatocytes. Cells were treated with DMSO (0.5%), or with various concentrations of DFU or TCV. EROD activity was monitored by kinetic appearance of resorufin at 37°C. Results are the mean of three (human cells) or four (rat cells) separate experiments in triplicate. Asterisks indicate significant differences from control ($*P < 0.05$; $**P < 0.001$; one-way ANOVA and Newman–Keul's *post hoc* test.

MATERIALS AND METHODS

Chemicals

Williams' E medium, penicillin-streptomycin, L-glutamine, amino acids and foetal calf serum (FCS) were from Eurobio (France). Dimethyl sulfoxide (DMSO), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β-NADP and 3-methycholanthrene (3-MC) were from Sigma (France). 7-Ethoxyresorufin and resorufin were from Boehringer (Germany). Pesticides were from Ets Cluzeau (France).

Cell culture and treatment

Human hepatocytes were obtained from liver biopsies resected from secondary tumours, by the classical two-step collagenase perfusion technique (Fabre *et al.*, 1988). Rat hepatocytes were isolated, as previously described (Berry and Friend, 1969), from male Sprague–Dawley rats weighing 220–240 g. Freshly isolated cells were resuspended in Williams' medium containing 10% FCS and supplemented with penicillin (50 U/ml), streptomycin (50 mg/ml) and insulin (0.1 U/ml). Hepatocytes were seeded in collagen type I-coated dishes. Plates containing hepatocytes were incubated for 4 hr at 37°C under a humidified 5% CO_2 atmosphere. The medium was then renewed with the same initial medium without FCS but supplemented with hydrocortisone hemisuccinate (1 μM), and containing increasing concentrations of the different pesticides. Cells were seeded on 100-mm diameter plates for Northern blot analysis, or 96-well microtitre plates for EROD. Then, insecticides or 3-MC, dissolved in DMSO (final concentration 0.5%) were added to the cultures for 72 hr, with a change of medium every 24 hr.

Cytotoxicity test

The cytotoxic effects of the insecticides on human and rat hepatocytes were assessed after 48 hr of exposure, by the neutral red (NR) assay and MTT test, carried out as previously described (Fautrel *et al.*, 1991).

EROD activity assay

The determination of the EROD enzyme activities in hepatocytes cultured for 3 days with the test compounds was performed according to Reiners *et al.* (1990) and Donato *et al.* (1993), with slight modifications. Briefly, after treatment, the medium was discarded and the 96-multiwell plates were frozen at − 80°C. After thawing, 200 μl of buffer per well, containing glucose-6-phosphate (3 mM), NADP (0.5 mM), dicoumarol (10 μM), ethoxyresorufin (2 μM) and glucose-6-phosphate dehydrogenase (0.1 U/ml), were added. The EROD activity was measured at 37°C by spectrofluorimetry (λ_{em} = 600 nm, λ_{ex} = 535 nm) by following the kinetics of appearance of resorufin from ethoxyresorufin.

mRNA analysis

Total RNA was isolated from the cell culture by the acidic phenol extraction procedure (Chomczynski and Sacchi, 1987). 20 μg RNA were size-fractionated on a 0.9% agarose gel containing 10% formaldehyde and transferred to a nitrocellulose membrane. Hybridization was performed with 480 pb cDNA insert of human CYP1A1 mRNA corresponding to nucleotides + 310 to + 790 and labelled using ^{32}P Biolabs kit.

Statistical analysis

All enzymatic results were evaluated using a one-way analysis of variance for control and insecticide-treated hepatocytes. The statistical significance was calculated using Newman–Keuls' *post hoc* test for multiple mean comparisons against the control set. Cytoxicity data were standardized as the percentage of control cell viability. The function: optical density = $[(a - d)/(1 + (conc/c)^b] + d$, was then fitted with non-linear estimation using a quasi-Newton algorithm.

RESULTS AND DISCUSSION

Cytotoxicity studies of the insecticides were performed on rat and human hepatocytes by using well defined protocols that were developed in the framework of a multicentre study. In this study, *in vitro* data were well correlated with *in vivo* data. Two different endpoints were used — the MTT reduction (data not shown) and NR tests. As shown in Fig. 1, which depicts the comparative cytotoxicity curves for the various insecticides, significant differences were observed between the compounds tested. Indeed, the IC_{50} values ranged from 80 μM for TCV to 250 μM for CBR, whereas DFU and CPR were devoid of any cytotoxic effects in human hepatocytes. Similar results were obtained in hepatocytes of rat origin. However, it should be pointed out that owing to the poor solubility of some of the insecticides in the culture medium, their IC_{50} values may be underestimated. Nevertheless, these experiments allowed us to select the optimal concentrations to be tested for the induction studies.

The ability of the six insecticides in inducing EROD activity was tested in human and rat hepatocytes, at various non-cytotoxic concentrations. Basal EROD values for human ($n = 3$) and rat cells ($n = 4$) were 0.057 ± 0.01 and 0.175 ± 0.071 (pmol/min/well), respectively. Figure 2 shows the maximal induction effect of the insecticides on rat hepatocyte primary cultures, by reference to the positive control 3-MC. DFU and TCV are the more potent inducers (approx. five- to sevenfold over control, $P < 0.001$), followed by CBR and CPR (approx. 2.5 to threefold, $P < 0.05$). DDT and MAL exerted no significant effect, whereas 3-MC (0.5 μM) provoked a 16-fold induction.

Figure 3(a,b) illustrates the dose-dependent effect of the most active insecticides (DFU and TCV) in human and rat hepatocytes respectively. Although, for both insecticides, the induction seems more important in rat (from approx. 0.36 pmol/min/well at 10 μM to 1.25 pmol/min/well at 75 μM), than in human cells (from approx. 0.193 pmol/min/well at 10 μM to 0.484 pmol/min/well at 75 μM), the inducing factor over control is in the same range. However, some interspecies variability could be observed: thus, in rat hepatocytes (Fig. 3b), TCV and DFU dose-dependently induced EROD activity in a similar way and the effect began to be significant at 10 μM for both compounds. In contrast, DFU appeared to be a more potent inducer than TCV in human cells (Fig. 3a), with a significant effect beginning at lower concentrations. These results could be explained either by specific metabolic pathways of these two xenobiotics in rat or in human cells, or different regulation mechanisms for *CYP1A1* gene expression by the two compounds.

The increased expression of CYP1A1 protein was ascertained by the Northern blotting technique, in rat (data not shown) and human cells (Plate 1), which demonstrated a dose-dependent increased accumulation of mRNA encoding for this enzyme. CYP1A2 transcripts were also detected, as already observed by Schuetz *et al.* (1995). Surprisingly, the effect at the mRNA level was obtained only for DFU, as cell exposure to TCV failed to induce CYP1A1/2 expression at the level of transcription. This observation may be explained by a more rapid degradation of these mRNAs following TCV treatment, or different regulation mechanisms for *CYP1A1* gene expression by the two compounds.

In general, although insecticide concentrations in the human diet are very low, these hydrophobic molecules are known to be extensively accumulated in adipose tissues, and may then be liberated in the systemic circulation. Therefore, our results suggest that care should be taken because of possible risk stemming from the exposure to insecticides, given the well-established toxicological and clinical importance of CYP1A1 induction.

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