Interactive Effects between Trichloroethylene and Pesticides at Metabolic and Genetic Level in Mice

P. Hrelia,1,3 F. Maffei,1 F. Vigagni,1 C. Fimognari,1 P. Flori,2 R. Stanzani,2 and G. Cantelli Forti1,3

1Dipartimento di Farmacologia; 2Dipartimento di Protezione e Valorizzazione Agroalimentare, Centro di Fitofarmacia, Università di Bologna, Bologna, Italy; 3Department of Preventive Medicine and Community Health, The University of Texas Medical Branch, Galveston, Texas

A combined cytogenetic urine metabolite analysis approach was used to assess potential interactive effects between Fenarimol (FN), a fungicide, and trichloroethylene (TRI), a halogenated solvent. FN was demonstrated to selectively induce P450-2B1 isofoms in different organs of treated mice. Since the rate of metabolism and the stereospecificity of metabolism are dependent on the types and amount of P450s available, FN might drastically alter the metabolic activation of a precarcinogen, such as TRI, and its toxicological consequences. Male CD1 mice were divided into untreated, vehicle control, and experimental groups. Animals of the latter groups were treated ip with 150 mg/kg bw FN in corn oil, 457 mg/kg bw TRI in corn oil, TRI plus FN separated by different time intervals. Bone marrow cells were harvested for determination of micronuclei (MN) frequencies in polychromatic erythrocytes (PCE). The presence of the known metabolite of TRI, trichloroethanol (TCE), was quantitated in collected urine by gas chromatography using an electron-capture detector. Linear regression analysis shows that MN frequency by TRI is correlated with TCE concentration in urine. Observed potentiation of genotoxicity of TRI by FN pretreatment (1 hr before TRI treatment) apparently reflects changes in the spectra of enzymes involved in TRI metabolism, and altered toxicokinetic, as witnessed by the 20% difference in TCE excretion from combined treated mice. However, no increased genetic or metabolic effects were observed when FN was administered 3 hr before TRI. No significant interactive effects were observed at a genetic level when FN was administered 1 hr and 3 hr after TRI whereas a 33 to 47% loss in TCE excretion was recorded. On the other hand, concomitant exposure to TRI and FN caused decreased TRI metabolic levels and decreased genetic effects by TRI through its metabolites probably due to an inactivation of microsomal enzymes critical for TRI metabolism. However, exposure to combined treatment at any time interval has an overall genotoxicity lower than that calculated by a simple additivity model.—Environ Health Perspect 102(Suppl 9):31–34 (1994)

Key words: chemical interactions, metabolism, micronuclei, clastogenicity, Fenarimol

Introduction

The increase in concern over the health hazard associated with the use of multiple pollutants is a growing awareness of the hazard associated with long-term exposure to some of these environmental carcinogens. Concurrent or sequential exposure to several xenobiotics may occur in air, water, and food. A realistic scenario is groundwater contamination related to hazardous waste disposal and agriculture activities.

Pesticides have properties invaluable to modern industrial society; however they can induce a wide array of health effects, ranging from myelotoxicity to cytogenetic changes and carcinogenic effects.

A preliminary study on the mutagenic/carcinogenic potential of pesticides indicated that several agents are toxic but poor initiating agents, as shown by negative or weak positive results at different genetic end points (1,2). On the other hand, biochemical and immunochemical studies showed that a number of pesticides induced specific P450 isoforms in different organs of rodents (3). This inductive capability may be responsible for pharmacokinetic interactions with other xenobiotics, and in particular, alter the metabolic activation of precarcinogens and their toxicological consequences.

This study was designed to evaluate if exposure to Fenarimol (FN), a fungicide, previously demonstrated to selectively induce P450-2B1 isoforms in liver, kidney, and lung of treated mice (3), may alter the rates of biotransformation and the toxic effects of a carcinogen such as trichloroethylene (TRI).

TRI is a versatile solvent with numerous commercial, industrial, household, and pharmacological applications. It is commonly found in waste disposal sites and is a frequent contaminant of both surface and groundwater (4). TRI is rapidly absorbed and metabolized extensively in a multistep process (5–7). Through an apparent oxide intermediate, TRI can form chloral hydrate, which undergoes oxidation to trichloroacetic acid. Alternatively, chloral hydrate can be metabolized to trichloroethanol (TCE), which undergoes phase II glucuronidation to produce TCE-glucuronide and accounts for approximately 94% of the total urinary metabolism in mice (7). Under certain conditions, the oxide intermediate can form dichloroacetic acid (6,7). Trichloroacetate, dichloroacetate and chloral hydrate are postulated as reactive metabolites of TRI responsible for induction of DNA damage (8).

Since the rate-limiting step in the overall metabolism of TRI is the step from TRI to chloral hydrate, which is catalyzed by cytochrome P450, changes in TRI metabolism and toxicity produced by FN may result from differences in the catalytic properties of the enzymes induced by FN.
A combined cytogenetic and toxicokinetic study was performed by using reliable biomarkers to assess interaction at genetic level (as quantified by the frequency of micronucleated polychromatic erythrocytes in mice) and metabolic level (in terms of urinary excretion of TCE) between FN and TRI. Results obtained contributed to a mechanistic interpretation of these interactions and provide information on their implication to human health.

Materials and Methods

Chemicals

Reagent grade FN (CAS no. 60168-88-9) was purchased from Lab Service Analytic (Bologna, Italy); TRI was from Janssen (Beerse, Belgium). TCE, α-Cellulose, Celullose Type 50, Acridine Orange, and β-glucoronidase Type M-3 were purchased from Sigma Co (St. Louis, MO); fetal calf serum (FCS) and HBSS solution were supplied by GIBCO-BRL (UK).

Animals and Treatment

Adult male Swiss albino CD1 mice (30 ± 2 g) were purchased from Nossan (Correzzano, Italy). They were housed in the animal facility which had 12 hr day–night cycles and temperature and humidity controls. Purchased animals were allowed a 1 week acclimation period before beginning treatment.

FN and TRI were dissolved in corn oil immediately before administration to mice ip with a volume of 0.01 ml/g bw treatment.

Groups of five mice were assigned to group as negative control, solvent control, and exposure (457 mg/kg bw TRI and 150 mg/kg bw FN). To investigate the interactive effects between TRI and FN, FN was administered to mice 3 hr and 1 hr before and at 0 hr, 1 hr, and 3 hr after TRI administration. Metabolic study was also conducted with these groups and compared to the group exposed to TRI alone.

Urine Collection and Metabolite Analysis

Different treated groups were housed separately in clean metabolic cages containing food and drinking water. The urine from each group of five mice was collected for 24 hr in glass tubes. The exact volume of urine collected from each group was measured, then every urine sample was centrifuged at 1500 rpm for 10 min. The presence of the known principal metabolite of TRI, TCE, was quantitated in collected urine by gas chromatography using an electron-capture detector (GC-ECD) (9).

TCE was excreted with the urine in the form of its glucuronide and, before gas chromatography determination, it was hydrolyzed enzymatically. Therefore, 0.1 ml of urine was pipetted into glass ampules and brought to a volume of 1.0 ml with distilled water followed by the addition of 1.0 ml acetate buffer (0.5 M, pH 4.5) containing β-glucuronidase. The ampules were allowed to react at 37°C for 18 hr. After cooling, the solution was appropriately diluted with acetone.

Gas chromatography operating conditions for TCE determination were: gas chromatograph 6000 Model Vega (Carlo Erba, Milan, Italy) equipped with a 63Ni electron-capture detector and integrator SP 4270 Model Vega; stainless steel column (30 m length, 0.32 mm diameter); carrier gas: helium (30 m/sec); column: fused silica on RTX 200 (0.5 μ); temperature: injection part 240°C, detector 310°C; analysis program: 40°C for 6 min, then from 40°C to 150°C (15°C/min).

Micronuclei (MN) Analysis

Mice were killed 30 hr after treatment for the single-treatment groups and after the first treatment for the combined-treatment groups. In order to study the frequency of MN in mouse bone marrow, the femoral cells were flushed out with FCS; the cellular suspension was added on a column of about 2.0 cm height of cellulose Type 50 and α-cellulose and washed out with HBSS solution with a drop speed of 15 drops/min, according to Selig et al. (10).

The cells collected were sedimented by centrifugation (5 min, 1800 rpm) and after resuspension of pellet in FCS prepared on microscopical slides. After air drying, the cells were fixed in methanol for 15 min and then dipped in fresh phosphate buffer (0.66% w/v potassium phosphate monobasic and 0.32% w/v sodium phosphate dibasic, pH 6.4—6.5). Then they were stained in a solution of Acridine Orange (AO) (12.5 mg AO/100 ml buffer) for 60 sec (11). The slides were then allowed to stand in buffer for 10 min, mounted using the phosphate buffer, and observed for the presence of MN in polychromatic erythrocytes (PCE). The data were summarized as the mean number of micronucleated PCE for 1000 PCE.

In addition, the ratio between the PCE and normochromatic erythrocytes (NCE) were also determined.

Statistical Analysis

The data were evaluated by the chi-square test to compare the MN frequency between treated groups (12). Linear regression analysis was calculated to determine the relationship between the level of bone marrow MN and the changes in the levels of TCE in urine.

Results

Induction of Micronuclei and Effects on Cell Proliferation

As detectable changes in activity of P450-linked enzymes were recorded only in animals treated with doses of FN ranging from 150 to 300 mg/kg bw (3), 150 mg/kg FN (25% DL50) was chosen as experimental dose.

Data from the preliminary time-course study on the clastogenic effects of TRI in bone marrow cells of mice showed that TRI induces the major MN frequency at 30 hr after treatment (Table 1). For this reason 30 hr was chosen as the experimental time. Very low MN frequencies were observed in untreated and vehicle-treated control mice (2.00 and 2.40 MN/1000 PCE, respectively) (Table 2).

From the data of the combined treatments reported in Table 2, mice treated with FN and 3 hr later with TRI showed an induced frequency of 8.77 MN/1000 PCE similar to the frequency of 9.00 MN/1000 PCE caused by TRI alone. However, the groups exposed to FN and 1 hr later to TRI, showed a highly significant increase (p<0.01) in the frequency of MN in PCE with respect to TRI-exposed group. Simultaneous exposure to FN and TRI induced a marked decrease (p<0.05) in the MN frequency with respect to TRI exposure, and significantly reduced the PCE/NCE ratio. These results may be due to the toxicity of simultaneous administration of the compounds in mice, which led to a depression of the erythropoiesis. Increasing the interval between TRI and FN from 1 hr to 3 hr led to slight but not significant enhancement effects, the frequencies being from 10.80 to 11.20.

Table 1. Time-course study on the clastogenic effect of trichloroethylene in bone marrow cells of mice.6

| Hours after treatment | PCE with MN/1000 cells | PCE/NCE |
|----------------------|------------------------|---------|
| FN                   | 24                     | 5.00 ± 0.71 | 0.95 ± 0.01 |
| TRI                  | 30                     | 9.60 ± 0.68 | 0.86 ± 0.02 |
| FN+TRI               | 48                     | 6.00 ± 0.71 | 0.80 ± 0.02 |

Abbreviations: PCE, polychromatic erythrocytes; MN, micronuclei; NCE, normochromatic erythrocytes. Mice were administered a single dose of trichloroethylene (457 mg/kg) ip. There were five mice per group and 1000 PCE were examined per mouse. Data indicate means ± S.E.M.
MN/1000 PCE, respectively. These combined treatments did not affect the PCE/NCE ratio, and their values were comparable to control.

Mice treated with FN showed a significant increase (p<0.01) in MN in PCE over the control (Table 2). On the other hand the increases after combined treatments with TRI at any time intervals were less than the expected value (MN = 18.2/1000) based on the simple sum of the effects of the individual compounds.

**Urine Metabolite Analysis**

The principal breakdown product of TRI in urine, TCE, in mice was measured in relation to the different exposures. After the combined treatments, when FN was administered 3 hr before TRI, TCE content did not show any appreciable change with respect to the excretion in urine of mice treated with TRI alone. However, reducing the interval between FN and TRI from 3 to 1 hr, there was an alteration in the excretion of TCE, as witnessed by the 20% difference in TCE quantity (Figure 1) recorded in group exposed to combined treatment compared to treatment with TRI alone.

On the other hand, groups treated with TRI first, followed 0, 1, and 3 hr later with FN demonstrated a decrease in TCE quantity, being the 49, 53, and 67% of control (TRI-alone treated mice), respectively.

**Discussion**

In addition to genotoxicity assays, biochemical studies are recommended to be used to obtain information that is useful in the evaluation of the mechanism of action of a chemical and risk identification (3). In fact, changes in the spectra of enzymes involved in carcinogen metabolism may dramatically alter pharmacokinetic and toxicological consequences of a precarcinogen (9).

It is demonstrated that TRI requires metabolic activation in order to express its genotoxic activity in vivo (8). At least three isoenzymes of P450 appear to be involved in activation of TRI and, in particular, the P450-2B1 linked enzymes and the P450-2E1 class (13).

Significant changes in the P450-2B1-dependent oxidases were observed after single and repeated treatment with FN from the dose of 150 mg/kg (3). The time-course induction following FN showed that the maximum induction with FN was apparent in animals after 72 hr of treatment (three daily injections). This was confirmed by immunohistochemical analysis (3). Lower doses of FN did not cause any detectable changes in the activity of P450 species.

Our working hypothesis was that the fungicide FN, by inducing P450 isoenzymes similar to those responsible for TRI metabolism (P450-2B1) may alter the rates of TRI biotransformation and its clastogenic activity in mice. The importance of metabolism in toxic response by TRI was demonstrated by others in rodents pretreated with different inducers (13–15).

Our genotoxicity and toxicokinetic studies show that FN can affect the metabolism of TRI. In fact, the relationship between genetic damage by TRI as quantitated by MN frequency, and metabolism of TRI evaluated in terms of urinary excretion of TCE and investigated by comparing the time-response curves, shows that the shape of the curve for MN resembles that for TCE excretion (Figure 1). Furthermore, a linear correlation is shown (r = 0.76) between the induction of MN and urinary levels of TCE recorded within 24 hr.

The increased urinary excretion of TCE and the significant increases in MN produced by TRI after 1 hr pretreatment with FN apparently suggest not only that TRI metabolites are necessary for DNA damage to occur, but also that FN can induce biochemical pathways involving the active metabolites of TRI and contribute to the induction of MN by TRI. Similar results were found with phenobarbital, which increased the extent of liver injury (13) and in vivo strand breaks in DNA (9) following exposure to TRI, through its metabolites ethanol (16), 3-methylcholanthrene (14), and other inducers of the hepatic mixed function oxidase system (13,15). However, no increased effects by TRI were observed when FN pretreatment was extended to 3 hr.

Unlike pretreatment with FN, simultaneous exposure to FN and TRI results in a suppression of TRI's metabolism, as evidenced by decreased TRI metabolite levels in the urine of treated mice, and decreased genetic effects as compared to animals treated with TRI alone. This phenomenon may be due to an inactivation of the microsomal enzymes that are responsible for TRI metabolism, since both TRI and FN are substrates of the same P450 isoenzymes. More work is needed to elucidate the biochemical basis of this interaction. Suppression of TRI's metabolism and reduced TRI metabolite levels in urine were also demonstrated with exposure to

---

Table 2. Frequency of polychromatic erythrocytes (PCE) with micronuclei (MN) in mice exposed to trichloroethylene (TRI) and Fenamidol (FN).

| Exposure | PCE with MN/1000 cells | PCE/NCE |
|----------|------------------------|---------|
| Negative control | 2.00 ± 0.45 | 0.80 ± 0.01 |
| Solvent control | 2.40 ± 0.24 | 0.80 ± 0.01 |
| Positive control | 9.00 ± 0.89 | 0.78 ± 0.02 |
| TRI (150 mg/kg) | 9.20 ± 1.05 | 0.77 ± 0.02 |
| FN + TRI (3 hr apart) | 8.77 ± 1.02 | 0.75 ± 0.07 |
| FN + TRI (1 hr apart) | 16.40 ± 2.01 | 0.75 ± 0.06 |
| TRI + FN (0 hr) | 5.60 ± 0.51 | 0.63 ± 0.02 |
| TRI + FN (1 hr) | 10.80 ± 1.39 | 0.87 ± 0.06 |
| TRI + FN (3 hr) | 11.20 ± 1.39 | 0.77 ± 0.01 |

Each exposure group contains five mice and 1000 PCE were examined for each animal. The data are the mean ± S.E.M. *NCE, normochromatic erythrocyte.

| Significantly different from the control group (p<0.01, chi-square test). |
| Significantly different from the TRI-exposed group (p<0.01, chi-square test). |
| Significantly different from TRI-exposed group (p<0.01, chi-square test). |

---

**Figure 1.** Comparison of responses in mice exposed to Fenamidol (FN) and trichloroethylene (TRI). Time-dependent responses of MN induction and cumulative excretion of trichloroethanol (TCE) in urine (expressed as mg/kg bw) are plotted with TRI alone effects normalized to 1. A linear correlation (r = 0.76) was found between the frequency of MN and TCE levels recorded within 24 hr.
TRI and other solvents in dry cleaning workers (17). However, a direct interaction between the clastogenic effects of the two compounds can not be excluded.

Investigation into the genotoxicity of FN are suggestive of clastogenic effects in vivo (3,18). The effect of exposure to two agents capable of producing the same genotoxic effect (induction of micronucleated erythrocytes in mice) may lead to additive or synergistic effects. Combined administration of TRI and FN to mice does not result in additive or synergistic effects in induction of genetic damage, but rather to an antagonistic effect. In fact, the observed increases in micronucleated erythrocytes in our time-course study were lower than the expected value based on the summation of the effects of the two toxicants.

In conclusion, this study pointed out that even if FN treatment enhances TRI metabolism towards the production of more genotoxic metabolite(s), the exposure to the mixture of FN and TRI does not increase clastogenesis in vivo. Inductive status has usually been associated with increased mutagenic/carcinogenic risk, even if this view has been demonstrated to be too restrictive (9). Caution should be exercised, therefore, in making broad generalizations concerning the effects of microsomal enzyme inducers on the toxicity of xenobiotics and the double-edged-sword nature of this phenomenon must be considered case by case.

REFERENCES

1. Hrelia P, Morotti M, Scotti M, Vigagni F, Paolini M, Perocco P, Cantelli Forti G. Genotoxic risk associated with pesticides: evidences on short-term tests. Pharmacol Res 22:93–94 (1990).
2. Hrelia P, Vigagni F, Morotti M, Cantelli Forti G. Genotoxic effects of pesticides and their value in the prediction of risk to humans. Pharmacol Toxicol 69 (2):18 (1991).
3. Cantelli Forti G, Paolini M, Hrelia P. Multi-end point procedure to evaluate risk from pesticides. Environ Health Perspect (in press).
4. Westrick JJ, Mello JW, Thomas RF. The ground water supply survey. J Am Water Works Assoc 76:52–59 (1984).
5. Ikeda M, Miyake Y, Ogata M, Ohmori S. Metabolism of trichloroethylene. Biochem Pharmacol 29:983–2992 (1980).
6. Bogen KT. Pharmacokinetics for regulatory risk analysis: the case of trichloroethylene. Regul Toxicol Pharmacol 8:447–466 (1988).
7. Dekant W, Metzler M, Henschler D. Novel metabolites of trichloroethylene through dechlorination reactions in rats, mice and humans. Biochem Pharmacol 33:2021–2027 (1984).
8. Nelson MA, Bull RJ. Induction of strand breaks in DNA by trichloroethylene and metabolites in rat and mouse liver in vivo. Toxicol Appl Pharmacol 94:45–54 (1988).
9. Kimmerle G, Eben A. Metabolism, excretion, and toxicity of trichloroethylene after inhalation. 1. Experimental exposure on rats. Arch Toxicol 30:115–126 (1973).
10. Selig, Schlegelmilch R, Wolf HU. Development of a method to increase the proportion of polychromatized erythrocytes from mouse bone marrow for more rapid evaluation of the micronucleus assay. Comparison with the conventional method with respect to micronucleus frequency and time required for preparation and evaluation. Mutat Res 234:23–30 (1990).
11. Tinwell H, Ashby J. Comparison of acridine orange and giemsa stains in several mouse bone marrow micronucleus assays–including a triple dose study. Mutagenesis.4:476–81 (1989).
12. Tallarida RJ, Murray RB. Manual of pharmacologic calculation with computer programs. New York:Springer-Verlag, 1981.
13. Nakajima T, Wang RS, Murayama N, Sato A. Three forms of trichloroethylene metabolizing enzymes in rat liver induced by ethanol, phenobarbital, and 3-methylcholanthrene. Toxicol Appl Pharmacol 102:546–552 (1990).
14. Carlson GP. Enhancement of the hepatotoxicity of trichloroethylene by inducers of drug metabolism. Res Commun Chem Pathol Pharmacol 7:637–640 (1974).
15. Molson MT, Reynolds ES, Szabo S. Enhancement of the metabolism and hepatotoxicity of trichloroethylene and perchloroethylene. Biochem Pharmacol 26:365–375 (1977).
16. Saito A, Nakajima T, Koyama Y. Dose-related effects of a single dose of ethanol on the metabolism in rat liver of some aromatic and chlorinated hydrocarbons. Toxicol Appl Pharmacol 60:8–15 (1981).
17. Inoue O, Seiji K, Kawai, T. Relationship between vapor exposure and urinary metabolite excretion among workers exposed to trichloroethylene. Am J Ind Med 15:103–110 (1989).
18. Pesticide Fact Sheet "Fenamilo". Toxicol Chem No 207AA. Environmental Protection Agency, 17 June 1985.