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Dog liver microsomal P450 enzyme-mediated toluene biotransformation

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1. We studied toluene metabolism in dog liver microsomes and the major metabolite was benzyl alcohol with *o*-and *p*-cresol as minor metabolites.

2. The enzyme kinetics of toluene biotransformation were examined by means of Lineweaver–Burk analyses. The Michaelis–Menten values differed among the three pathways, the order being; K_m, *o*-cresol > *p*-cresol > benzyl alcohol; V_{max}, benzyl alcohol > *o*-cresol > *p*-cresol; and Cl_{int}, benzyl alcohol > *p*-cresol > *o*-cresol.

3. The formation of benzyl alcohol, *o*- and *p*-cresol from toluene was substantially inhibited by the P4502E inhibitors such as DDC (diethyldithiocarbamate) and 4-methylpyrazole in all pathways, with IC_{50}'s in the range of 0·02–0·59 mM. The P4502B inhibitors, metyrapone and secobarbital also inhibited benzyl alcohol and *p*-cresol formation, whereas *o*-cresol was not inhibited by these latter compounds.

4. Anti-rat P4502E1 antibodies inhibited benzyl alcohol, *o*- and *p*-cresol formation from 26 to 30% 0·2 ml serum/mg microsomal protein. Furthermore, anti-rat P4502B1/2 antibody inhibited benzyl alcohol and *p*-cresol formation (47 and 44% respectively), but not that of *o*-cresol. Anti-rat P4502C11/6 antibody also inhibited benzyl alcohol and *p*-cresol formation 31 and 24% respectively in a similar manner to that by the anti-rat P4502B1/2 antibody.

5. These results suggested that the P4502B, 2C and 2E isozymes in dog liver contribute to the formation of benzyl alcohol and *p*-cresol from toluene, and 2E isozyme preferentially contributes to the formation of *o*-cresol.

Introduction

Toluene is an organic solvent with widespread industrial and commercial uses and it is also the most widely abused inhaled solvent (Press and Done 1967, Fishbein 1985). A number of investigations have shown that toluene produces several biochemical changes in the liver, central nervous system and other organs of human experimental animals (O'Brien *et al.* 1971, Rea *et al.* 1984, Rees *et al.* 1987). It is considered that the urinary metabolites of toluene, hippuric acid and *o*-cresol are very important indices for the biological monitoring of individuals exposed to toluene (Fishbein 1985).

The initial step of toluene metabolism is catalysed by the hepatic P450-dependent monooxygenase system to benzyl alcohol, *o*- and *p*-cresol (Dean 1978, Fishbein 1985). Benzyl alcohol is further oxidized to benzoic acid, and excreted in urine mainly as hippuric acid (Bakke and Scheline 1970, Woiwode *et al.* 1979). Aromatic oxidation to form cresols occurs through the formation of epoxides and accounts for

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< 1% of an absorbed dose of toluene (Jerina and Daly 1974); these metabolites are subsequently excreted in the urine as sulphate or glucuronide conjugates (Bakke and Scheline 1970, Woiwode *et al.* 1979).

Kinetic analyses have shown that phenobarbital and ethanol enhance toluene metabolism in the rat liver (Sato and Nakajima 1985, Wang and Nakajima 1991). Also, in an attempt to identify some of the P450 isozymes responsible for the metabolism of toluene in rat liver, Nakajima *et al.* (1991–1993) examined the inhibition of metabolism of this substrate using monoclonal antibodies to different P450 isozymes. 2C11/6 and 2B1/2 reportedly catalyse the aliphatic hydroxylation or toluene to benzyl alcohol. Aromatic hydroxylation is catalyzed by different isozymes, with *o*-cresol being produced by 1A1/2 and 2B1/2, whereas *p*-cresol is formed by 2B1/2.

P450 enzymes are important in the metabolism of numerous endogenous compounds such as steroids and fatty acids, as well as a wide range of foreign chemicals such as drugs and environmental pollutants. The metabolism of foreign chemicals can frequently produce toxic metabolites, of which some have been implicated as agents that may be responsible for tumour initiation, promotion, and progression (Conney 1982). Many of the mammalian P450 isozymes have been purified and characterized (Nelson *et al.* 1993) and understanding species differences in the P450 isozymes that mediate metabolism is an integral part of toxicological research. Although toluene is a common organic solvent used in industries worldwide, very little is known about toluene metabolism except in the rat and mouse. This study was undertaken, therefore, to determine the possible roles of P450 isozymes and species differences in toluene metabolism using dog liver microsomes with various P450 inhibitors and anti-rat P450 antibodies.

Materials and methods

Materials

Toluene (99·5%), benzyl alcohol (99·0%), *o*-(99·0%) and *p*-cresol (99·0%) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Resorufin, 7-ethoxyresorufin, 7-pentoxyresorufin, 6β-hydroxytestosterone, diethyldithiocarbamate and troleandomycin were purchased from Sigma Chemical Co. (St Louis, MO, USA). 7-Methoxyresorufin and 7-benzyloxyresorufin were purchased from Molecular Probes, Inc. (Eugene, OR, USA). 7-Ethoxycoumarin, *p*-methylbenzyl alcohol, 12-hydroxylauric acid, metyrapone and 4-methylpyrazole were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). NADPH was obtained from Oriental Yeast Co., Ltd (Tokyo, Japan). SKF-525A was from Paesel GmbH (Frankfurt, Germany), and hexobarbital and secobarbital were from Tokyo Kasei Co. (Tokyo, Japan). Goat anti-rat P4501A1/2, 2C11/6, 2E1, 3A2/1 and 4A1 antibodies were purchased from Daiichi Pure Chemical Co., Ltd (Tokyo, Japan). Horseradish peroxidase anti-goat IgG was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). All other reagents and solvents were of the highest quality commercially available.

Dog liver microsomes

Female Beagle dogs (6 months old, 7·1–7·9 kg) were obtained from Hazleton Research Product Inc. (Cumberland, VA, USA). The dogs were fasted overnight, decapitated after anaesthesia (pentobarbital sodium 3·75 mg/kg, i.v.), and their livers immediately removed. To prepare the microsomal suspension, a 20% (w/v) liver homogenate in 25 mM Tris–HCl buffer (pH 7·6) containing 0·25 M sucrose, 25 mM KCl and 5 mM $MgCl_2$ was centrifuged at 9000*g* for 20 min, then the supernatant was further centrifuged at 105 000*g* for 60 min. The resulting microsomal pellets were resuspended with 25 mM Tris–HCl buffer (pH 7·6) containing 0·15 M KCl, and centrifuged at 105 000*g* for 60 min. The pellets were homogenized with 50 mM potassium phosphate buffer (pH 7·4) containing 10% glycerol and frozen at − 80°C until use. The P450 and protein levels of the microsomes were estimated spectrally as described by Omura and Sato (1964) and Lowry *et al.* (1951) respectively.

P450-dependent monooxygenase activities

The activities of 7-ethoxyresorufin *O*-deethylase, 7-methoxyresorufin *O*-demethylase, 7-pentoxyresorufin *O*-depentylase and 7-benzyloxyresorufin *O*-debenzylase were determined fluorometrically from

the amounts of resorufin produced (Pohl and Fouts 1980, Burke *et al.* 1985, Nerurkar *et al.* 1993). 7-Ethoxycoumarin *O*-deethylase was measured by means of the fluorometric determination of 7-hydroxycoumarin (Aitio 1978). Aminopyrine *N*-demethylase and erythromycin *N*-demethylase activities were determined by measuring the formaldehyde concentration according to the method of Nash (1953). Aniline 4-hydroxylase, testosterone 6β-hydroxylase and lauric acid 12-hydroxylase were measured by the formation of 4-aminophenol, 6β-hydroxytestosterone and 12-hydroxylauric acid respectively (Imai *et al.* 1966, Yoshihara *et al.* 1982, Aoyama and Sato 1988).

Toluene metabolism

Toluene metabolism was determined measuring the rate of formation of benzyl alcohol, *o*- and *p*-cresol, according to the method of Wang and Nakajima (1991) with minor modifications. The standard incubation mixture contained dog liver microsomal proteins (0·2 mg), 1·0 mM NADPH and toluene (0·25–4·0 mM) in a final volume of 1·0 ml 50 mM phosphate buffer (pH 7·4). After an incubation at 37°C for 5 min, the reaction was started by adding NADPH. The mixture was incubated at 37°C for 20 min, and stopped with 0·1 ml 15% $ZnSO_4$ and saturated $Ba(OH)_2$. The assay internal standard (*p*-methylbenzyl alcohol) was added to the incubation mixture, which was then centrifuged at 2000*g* for 10 min. The supernatant (1·0 ml) was extracted with 4·0 ml diethylether, the organic phase separated from the aqueous phase and evaporated at 25°C. The metabolites were dissolved in 0·3 ml methanol then quantified using a isocratic hplc system consisting of a pump (Shimadzu LC-6AD), a UV detector (Shimadzu SPD-6A), an autosampler (Shimadzu SIL-6B). The standard curve samples were treated in the same manner as the incubation samples. The analytical conditions of hplc were as follows: column, reversed phase column (4·6 mm × 15 cm) packed with Inertsil ODS-80A (GL Science Inc., Tokyo, Japan); mobiles phase, 25% acetonitrile; flow rate, 0·8 ml/min: wavelength, 200 nm. Under these conditions, the retention times of benzyl alcohol, *o*-cresol, *p*-cresol and *p*-methylbenzyl alcohol were 5·4, 14·0, 12·4 and 9·6 min respectively. The recoveries of benzyl alcohol, *o*-cresol, *p*-cresol and *p*-methylbenzyl alcohol (internal standard) were 82, 84, 79 and 84% respectively.

Inhibition of toluene metabolism by various compounds

Twelve compounds (SKF-525A, 7,8-benzoflavone, caffeine, metyrapone, secobarbital, hexobarbital, tolbutamide, sparteine, DDC, 4-methylpyrazole, troleandomycin and lauric acid) were tested for their inhibitory effects on toluene metabolism. The chemicals were dissolved in methanol or dimethyl sulphoxide (final concentration in the reaction medium, 1·0% (v/v)), with methanol or dimethyl sulphoxide being used as the solvent control. The inhibitory effect of each chemical was determined at five concentrations (0·1, 0·2, 0·4, 0·8 and 1·6 mM). The final toluene concentration was set at 2·0 mM. The IC_{50}'s were determined graphically.

Inhibition of toluene metabolism by various anti-rat P450 antibodies

Goat anti-rat P4501A1/2, 2B1/2, 2C11/6, 2E1, 3A2/1 and 4A1 antibodies (0·2 ml serum/mg microsomal protein) were added to microsomes and incubated at room temperature for 30 min prior to starting metabolism assay at 37°C by adding buffer, toluene and NADPH. The control was preimmune goat serum instead of the antibodies. The final toluene concentration was 2·0 mM and metabolism determined as indicated above.

Immunoblot analysis

Dog or rat liver microsomal proteins were separated by 7·5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Laemmli 1970), and transferred to nitrocellulose membranes according to Towbin *et al.* (1979). Following an incubation in blocking solution (phosphate-buffered saline 3%, bovine serum albumin) for 16 h at room temperature, the nitrocellulose membrane was incubated with either anti-P4501A1/2, 2B1/2, 2C11/6, 2E1, 3A2/1 or 4A1 antibody. P450 antibodies bound to microsomal proteins were detected by incubating the membrane with anti-goat IgG-horse radish peroxidase conjugate, followed by a colorimetric determination with hydrogen peroxide and 4-chloro-1-naphthol.

Calculations

The Michaelis–Menten parameters (apparent K_m and V_{max}) for toluene metabolism were estimated by analysing Lineweaver–Burk plots using the software ENZYME KINETICS (Trinity Software, NH, USA). All reported values are mean ± SD of three determinations.

Results

P450 content and P450-dependent monooxygenase activities in dog liver microsomes

Table 1 indicates the total P450 content and that of 11 P450-dependent monooxygenase activities in dog liver microsomes. The P450 content, and the activities of 7-methoxyresorufin *O*-demethylase, 7-pentoxyresorufin *O*-depenty-

Table 1. P450 content and P550-mediated monooxygenase activities in dog liver microsomes.

	Related P450 subfamily	Level
P450 content[a]	—	0·74 ± 0·02
7-Ethoxyresorufin *O*-deethylase[b]	1A	0·23 ± 0·01
7-Ethoxycoumarin *O*-deethylase[b]	1A	1·52 ± 0·04
7-Methoxyresorufin *O*-demethylase[c]	1A	75·1 ± 6·6
7-Pentoxyresorufin *O*-depentylase[c]	2B	16·7 ± 1·6
7-Benzyloxyresorufin *O*-debenzylase[b]	2B	0·24 ± 0·03
Aminopyrin *N*-demethylase[b]	2D	4·88 ± 0·13
Aniline 4-hydroxylase[b]	2E	0·37 ± 0·01
Nitrosodimethylamine *N*-demethylase[b]	2E	1·20 ± 0·05
Testosterone 6β-hydroxylase[b]	3A	0·23 ± 0·01
Erythromycin *N*-demethylase[b]	3A	7·12 ± 0·41
Lauric acid 12-hydroxylase[b]	4A	0·67 ± 0·09

Experimental conditions are described in Materials and methods. Each value represents the mean ± SD of three determinations performed using pooled liver microsomes from three dogs.

[a] nmol/mg protein.

[b] nmol/min/mg protein.

[c] pmol/min/mg protein.

lase, aminopyrine *N*-demethylase, aniline 4-hydroxylase, nitrosodimethylamine *N*-demethylase and erythromycin *N*-demethylase in the dog were similar to those in the rat (Hanioka *et al.* 1995a, b). The activities of 7-ethoxyresorufin *O*-deethylase, 7-ethoxycoumarin *O*-dethylase and 7-benzyloxyresorufin *O*-debenzylase in the dog were from 3·3 to 4·8-fold higher than those in the rat, whereas the testosterone 6β-hydroxylase activity in dog was less than half that in the rat (Hanioka *et al.* 1995a, b)

Kinetic analysis of toluene metabolism

The formation of benzyl alcohol, *o*- and *p*-cresol was linear with respect to a 30-min incubation in the presence of 0·2 mg microsomal protein (data not shown). A 20-min incubation was therefore routinely used to ensure initial rate conditions. Toluene biotransformation by dog liver microsomes followed Michaelis–Menten kinetics as demonstrated by Lineweaver–Burk plots (for toluene concentrations between 0·25 and 4·0 mM) (figure 1). The apparent kinetic constants for the formation of benzyl alcohol, *o*- and *p*-cresol from toluene are shown in table 2. The K_m for *o*-cresol formation was greater than those for the formation of benzyl alcohol and *p*-cresol. The V_{max}'s were also different as follows: benzyl alcohol > *o*-cresol > *p*-cresol formation. The Cl_{int} (V_{max}/K_m) for benzyl alcohol formation was substantially greater than that for the formation of *o*- and *p*-cresol.

Inhibition of toluene biotransformation by various compounds

These assays were performed with a substrate concentration of 2·0 mM, and the final concentration of the tested compounds was set at 0·1–1·6 mM. Results are presented in table 3. The general inhibitor SKF-525A inhibited the formation of toluene metabolites by 26–64% at a concentration of 1·6 mM. The known inhibitors of P4502E, DDC and 4-methylpyrazole effectively inhibited the formation of benzyl alcohol, *o*- and *p*-cresol, with an IC_{50} < 0·6 mM. Metyrapone and secobarbital, regarded as inhibitors of P4502B-type reactions (Lunetta *et al.* 1989, Murray and Reidy 1990), also inhibited the formation of benzyl alcohol and *p*-cresel by 45–82% at 1·6 mM, whereas *o*-cresol formation was not affected by these compounds. In

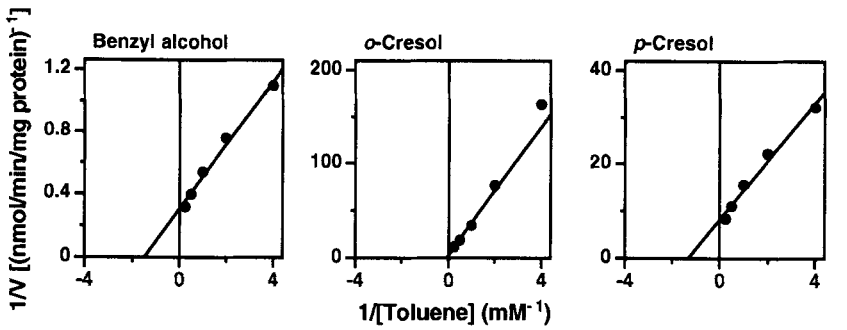

Figure 1. Lineweaver–Burk plots for the formation of benzyl alcohol, *o*- and *p*-cresol by dog liver microsomes. Each point represents the mean of three determinations performed using pooled liver microsomes from three dogs.

Table 2. Estimated Michaelis–Menten parameters for toluene biotransformation by dog liver microsomes.

	Formation		
	Benzyl alcohol	*o*-Cresol	*p*-Cresol
K_m (mM)	0·95 ± 0·05	6·89 ± 0·81	1·19 ± 0·08
V_{max} (nmol/min/mg protein)	3·81 ± 0·10	0·23 ± 0·01	0·15 ± 0·01
Cl_{int} (μl/min/mg protein)	4·01	0·03	0·13

Experimental conditions are described in Materials and methods. K_m and V_{max} are the mean ± SD of three determinations performed using pooled liver microsomes from three dogs analyzed by Lineweaver–Burk plots.

Table 3. Effects of various compounds on toluene biotransformation.

	Related P450 subfamily	IC_{50} (mM)		
		Benzyl alcohol	*o*-Cresol	*p*-Cresol
SKF-525A	General	0·15 ± 0·02	> 1·60	> 1·60
7,8-Benzoflavone	1A	> 1·60	> 1·60	> 1·60
Caffeine	1A	> 1·60	1·00 ± 0·18	> 1·60
Metyrapone	2B	0·04 ± 0·01	> 1·60	0·12 ± 0·02
Secobarbital	2B	1·43 ± 0·13	> 1·60	> 1·60
Hexobarbital	2C	> 1·60	> 1·60	> 1·60
Tolbutamide	2C	> 1·60	> 1·60	> 1·60
Sparteine	2D	> 1·60	> 1·60	> 1·60
DDC	2E	0·35 ± 0·04	0·39 ± 0·03	0·59 ± 0·08
4-Methylpyrazole	2E	0·06 ± 0·01	0·28 ± 0·032	0·02 ± 0·01
Troleandomycin	3A	> 1·60	> 1·60	> 1·60
Lauric acid	4A	1·06 ± 0·16	> 1·60	> 1·60

Experimental conditions are described in Materials and methods. Each value represents the mean ± SD of three determinations performed using pooled liver microsomes from three dogs.

contrast, the inhibition of *o*-cresol formation by caffeine and sparteine was greater than that of benzyl alcohol or *p*-cresol. As much as 22–61% inhibition of toluene biotransformation was also evident with a high lauric acid concentration. There was little or no inhibition of toluene biotransformation by 7,8-benzoflavone, hexobarbital, tolbutamide and troleandomycin, with IC_{50}'s were all > 1·6 mM.

Immunoinhibition of toluene biotransformation by anti-rat 450 antibodies

Several antibodies raised against rat P450 isozymes were examined for inhibition of toluene biotransformation catalysed by dog liver microsomes (figure 2). Furthermore, to confirm immunoreactivity with anti-rat P450 antibodies in dog liver microsomes, we performed Western blots (figure 3). Anti-rat P4502B1/2 antibody immunoreacted with dog liver microsomes. The anti-rat P4502B1/2 antibody (0·2 ml serum/microsomal protein) inhibited the formation of benzyl alcohol and *p*-cresol to the extent of 47 and 44% respectively. However, there was no inhibitory effect of anti-rat P4502B1/2 antibody on the formation of *o*-cresol. Although the mobility of the band in dog liver microsomes that immunoreacted with anti-rat P4502E1 antibody differed in that of rat liver microsomes, the formation of benzyl alcohol, *o*-cresol and *p*-cresol were inhibited by 26–30% by anti-rat P4502E1 (0·2 ml serum/microsomal protein) antibody. Anti-rat P4502C11/6 antibody also inhibited the formation of benzyl alcohol and *p*-cresol to the extent of 24 and 16% respectively. However, the immunoreactivity in dog liver microsomes in Western blots was weaker than that in rat liver microsomes. Other antibodies including anti-rat P4501A1/2, 3A2/1 and 4A1 did not inhibit toluene biotransformation

Discussion

It has been reported that toluene is predominantly metabobilised by rat liver microsomes to benzyl alcohol, and *o*- and *p*-cresol, generated by side-chain and aromatic hydroxylation respectively (Dean 1978, Fishbein 1984). Nakajima *et al.* (1991–1993) have characterized the rat P450 isozymes that catalyse toluene metabolism *in vitro* using monoclonal P450 antibodies. In the current study, the biotransformation of toluene in dog liver microsomes was investigated using various P450 inhibitors and anti-rat P450 antibodies.

When toluene was incubated with dog liver microsomes *in vitro*, benzyl alcohol was the major metabolite, whereas *o*- and *p*-cresol were minor metabolites and *m*-cresol; was undetectable at any substrate concentration. These results agreed with data reported for the rat (Kaubisch *et al.* 1972, Wang and Nakajima 1991). The formation of metabolites in the dog followed Michaelis–Menten kinetics when analysed by Lineweaver–Burk plots. The K_m of the isozyme that catalysed the production of *o*-cresol was greater than those of benzyl alcohol and *p*-cresol, and the V_{max}'s of *o*- and *p*-cresol are significantly lower than that of benzyl alcohol. Therefore, we considered that different P450 isozymes in the dog liver catalyse the metabolism of toluene to benzyl alcohol, *o*- and *p*-cresol. The K_m's of the formation for *o*- and *p*-cresol are reportedly similar, although the V_{max}'s for *o*- and *p*-cresol formation are lower than that of benzyl alcohol in the control rat (Wang and Nakajima 1991). This species difference of K_m for toluene biotransformation may depend on the complement of P450 isozymes and their affinities for toluene.

Several chemicals and have been studied by various investigators as inhibitor probes for specific mammalian P450 isozymes (Murray and Reidy 1990, Guengerich and Shimada 1991, Wrighton *et al.* 1993, Newton *et al.* 1995). SHKF-525A is regarded as a general inhibitor of several P450 subfamilies (Murray and Reidy 1990, Jönsson *et al.* 1995). Table 3 shows that SKF-525A inhibited the formation of benzyls alcohol, *o*- and *p*-cresol from toluene *in vitro*, suggesting that toluene biotransformation is catalysed by the P450-dependent monooxygenase system. Also, DDC and 4-methylpyrazole, as 2E inhibitors (Guengerich *et al.* 1991, Newton *et al.* 1995), caused significant inhibition of toluene biotransformation in all pathways.

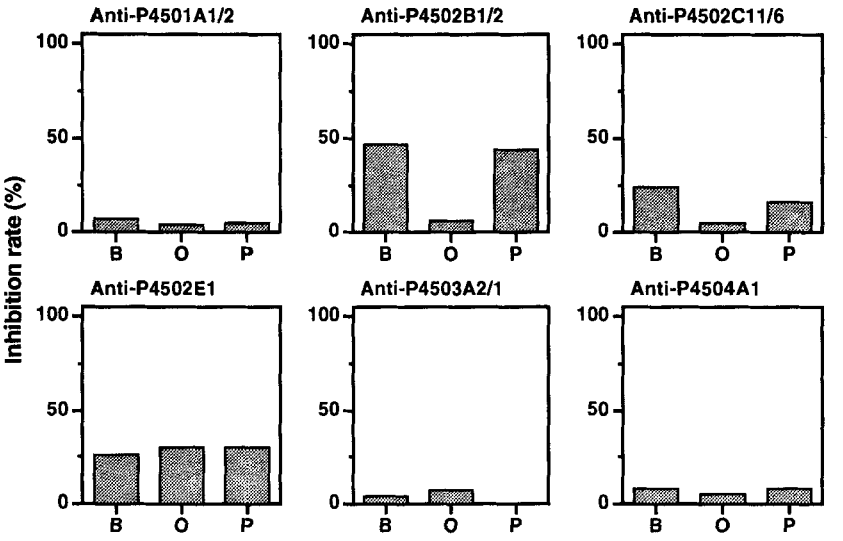

Figure 2. Effects of various anti-rat P450 antibodies on the formation of benzyl alcohol (B), *o*- (O), and *p*-cresol (P) by dog liver microsomes. Experimental conditions are described in Materials and methods. Activities are expressed as percentages of the control activity. Each bar represents the mean of three determinations performed using pooled liver microsomes from three dogs.

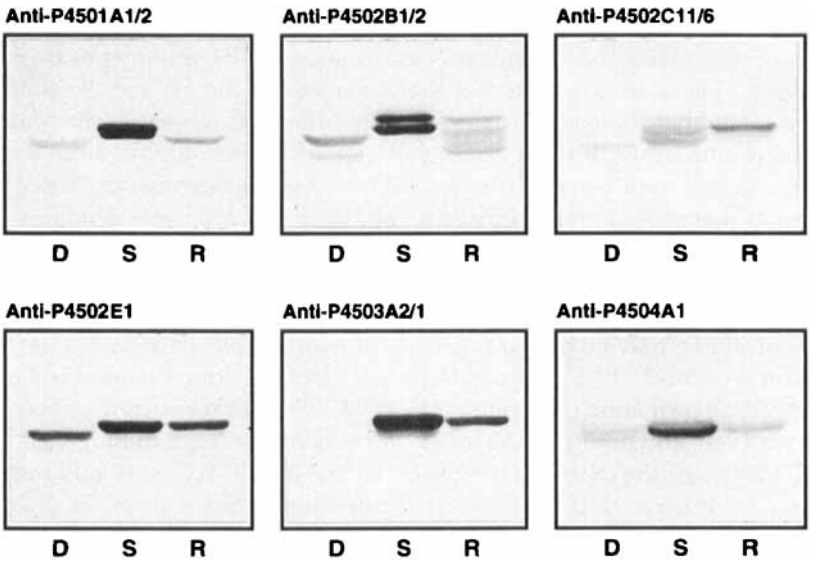

Figure 3. Western blots of dog or rat liver microsomes using anti-rat P4501A1/2, 2B1.2, 2C11/6, 2E1, A2/1 and 4504A1. Samples in the panels are: lanes D, dog; S, standard; and R, rat. Lane S represent rat treated with *B*-naphthlofavone (25 mg/kg for 4 days, i.p.) to evaluate anti-rat P4501A1, phenobarbital (80 mg/kg for 4 days, i.p.) for 2B1/2 and 2C11/6, isoniazid (80 mg/kg for 4 days, i.p.) for 2E1, pregnenolone 16α-carbonitrile (60 mg/kg for 4 days, i.p.) for 3A2/1 and clofibrate (200 mg/kg for 4 days, i.p.) for 4A1. Microsomal protein levels are: lanes D and R; 24·0, 2·0, 12·0, 8·0, 6·0 and 16·0 μg, and lane S; 2·0, 2·0, 12·0, 8·0, 6·0 and 16·0 μg for anti-rat 1A1/2, 2B1/2, 2C11/6, 2E1, 3A2/1 and 4A1 respectively.

It has been reported that metyrapone and secobarbital are inhibitors of 2B-type reactions in rat and human liver microsomes (Lunetta *et al.* 1989, Murray and Reidy 1990). In our current study, metyrapone and secobarbital inhibited the formation of benzyl alcohol and *p*-cresol, but not that of *o*-cresol. Caffeine (a selective inhibitor for 1A) (Jönsson *et al.* 1995), sparteine (a selective inhibitor for 2D) (Larrey *et al.* 1984, Guengerich *et al.* 1986), hexobarbital (a selective inhibitor for 2C) (Knodell *et al.* 1989, Nedelcheva and Gut 1994) and lauric acid (a substrate for 4A) (Gibson *et al.* 1982, Tamburini *et al.* 1984, Sharma *et al.* 1989) inhibited the formation of benzyl alcohol, *o*- and *p*-cresol only at a high concentrations. However, 7,8-benzoflavone, tolbutamide or troleandomycin, which are inhibitors for 1A, 2C or 3A (Tassaneeyakul *et al.* 1993, Nedelcheva and Gut 1994, Jönsson *et al.* 1995, Newton *et al.* 1995) hardly affected toluene biotransformation in dog liver microsomes. These observations suggest that the metabolism to benzyl alcohol and *p*-cresol is catalysed by 2B and 2E isozymes, whereas the *o*-cresol formation is catalysed by the 2E isozyme in the dog liver.

To confirm that 2B and 2E are the isozymes involved in toluene biotransformation by dog liver microsomes, we performed immunoinhibition studies using various anti-rat P450 antibodies. The formation of benzyl alcohol and *p*-cresol were inhibited by anti-2B1/2 and 2E1 antibodies, whereas the formation of *o*-cresol was inhibited by anti-rat 2E1 antibody, but not inhibited by anti-rat 2B1/2 antibody. These results agree with those of the chemical inhibition described above. Nakajima *et al.* (1991–1993) reported that anti-rat 2B1/2 antibody inhibit benzyl alcohol, *o*- and *p*-cresol formations in the phenobarbital-induced rat, but not in the control rat. Furthermore, they also reported that benzyl alcohol and *p*-cresol formed by anti-rat 2E1 was inhibited only in the ethanol-induced rat at a low substrate concentration, and that *o*-cresol formation was not affected by anti-rat 2E1 antibody in any induced microsomes. These results indicated that isozymes of the 2B and 2E subfamilies contribute to toluene biotransformation in dog and rat. However, in the control dog liver used in this study, anti-rat 2B1/2 and 2E1 antibodies inhibited the formation of benzyl alcohol, *o*- or *p*-cresol. Figure 3 shows that the isozyme levels in dog liver microsomes that immunoreacted to anti-rat 2B1/2 and 2E1 were similar or higher than those of rat liver microsomes. Because the antibodies were raised against rat P450 isozymes, the protein levels of 2B and 2E subfamilies in dog liver microsomes may be higher than they appear. Therefore, the species difference in toluene biotransformation may be due to the levels of constitutive 2B and 2E isozymes. In the control rat, anti-2C11/6 antibody reportedly inhibits benzyl alcohol and *o*-cresol, but not *p*-cresol formation (Nakajima *et al.* 1992, 1993). In the current study, anti-rat 2C11/6 antibody also inhibited the formation of benzyl alcohol and *p*-cresol, but not *o*-cresol. Although the extent of inhibition by anti-rat 2C11/6 antibody were lower than those by anti-rat 2B1/2 antibody, the immunoinhibition profile was similar to that of the anti-rat 2B1/2 antibody. This indicated that 2C isozyme(s) in the dog liver also catalyse toluene biotransformation. Nakajima *et al.* (1991) reported that 1A1/2 preferentially contributes to the formation of *o*-cresol in rat liver microsomes. However, anti-rat 1A1/2 antibody as well as anti-rat 3A2/1 and 4A1 antibodies did not inhibit toluene biotransformation in dog liver microsomes (figure 2), suggesting that 1A, 3A and 4A subfamily isozymes do not significantly contribute to toluene metabolism in the dog liver.

In conclusion, our data demonstrated the involvement of dog liver 2B, 2C or 2E isozymes in toluene metabolism, differing from that of other animal species (Wang

and Nakajima 1991, Nakajima *et al.* 1991–1993). To evaluate the risk of toluene exposure, it is important to understand differences in the metabolism between species. Therefore, we consider that our results of kinetic analyses, chemical inhibition and immunoinhibition studies provided herein is important relating to toluene toxicity.

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