Biochemical Pharmacology, Vol. 41, No. 3, pp. 395–404, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

MONOCLONAL ANTIBODY-DIRECTED CHARACTERIZATION OF CYTOCHROME P450 ISOZYMES RESPONSIBLE FOR TOLUENE METABOLISM IN RAT LIVER

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(*Received* 19 *July* 1990; *accepted* 24 *September* 1990)

Abstract—Monoclonal antibodies (MAbs) were used to study the contribution of cytochromes P450IA1/IA2, P450IIB1/IIB2, P450IIC11/IIC6 and P450IIE1 to toluene side-chain (benzyl alcohol, BA, formation) and ring (*o*- and *p*-cresol formation) oxidation in liver microsomes from fed, one-day fasted, and phenobarbital (PB)-, 3-methylcholanthrene (MC)- and ethanol-treated rats. All rats were fed synthetic liquid diets. MAb 1-7-1 against P450IA1/IA2 inhibited markedly *o*-cresol formation and slightly *p*-cresol formation but not BA formation only in microsomes from MC-treated rats. MAbs 2-66-3, 4-7-1 and 4-29-5 against P450IIB1/IIB2 strongly inhibited BA, *o*-cresol and *p*-cresol formation only in PB-induced microsomes. MAb 1-68-11 against P450IIC11/IIC6 inhibited BA formation at high toluene concentration in the following order: fed > fasted > ethanol = MC > PB, and ethanol ≥fed = fasted > MC > PB on the basis of the percentage and net amount inhibition, respectively. MAb 1-91-3 against P450IIE1 inhibited BA formation at low toluene concentration, but not at high concentration, in the following order: ethanol > fasted = fed > MC, and ethanol > fasted > fed > MC on the basis of percentage and net inhibition, respectively. MAbs 1-68-11 and 1-91-3 also inhibited *p*-cresol formation at high and low toluene concentrations, respectively. These results indicate that (i) both P450IIE1 and P450IIC11/IIC6 are constitutive isozymes mainly responsible for the formation of BA and *p*-cresol from toluene as low- and high-K_m isozymes, respectively; (ii) P450IIE1, but not P450IIC11/IIC6, is induced by one-day fasting and ethanol treatment; (iii) both P450IIE1 and P450IIC11/IIC6 are decreased by PB and MC treatments; (iv) P450IIE1 is inhibited by high concentration of toluene; (v) P450IIB1/IIB2 can contribute to the formation of BA, *o*- and *p*-cresol from toluene, while P450IAI/IA2 preferentially contributes to the formation of *o*-cresol.

Toluene is a common organic solvent currently in worldwide industrial usage. It is also found in the general environment possibly as a result of its use as a thinner for paints [1]. The population exposed to toluene is estimated to be large.

In a previous paper, we reported on the basis of kinetic analysis of toluene metabolism in rat liver microsomes that (i) at least two types of toluene side-chain oxidation exist in normal fed control rats and that an enzyme with low K_m was induced by ethanol treatment; and (ii) phenobarbital (PB¶) treatment induces toluene side-chain oxidase at a K_m similar to that in control rats [2]. However, little is known about the character of these low- and high-K_m isozymes and the PB-inducible isozymes concerned with toluene metabolism.

Cytochrome P450 is a key enzyme in the metabolism of many chemicals. Recently, monoclonal antibodies (MAbs) to different forms of cytochrome P450 have been developed [3–6] and been shown to be useful in determining the contribution of specific isozymes to the metabolism of specific xenobiotics [7, 8]. For example, cytochrome P450c,d (P450IA1 and IA2), P450b,e (P450IIB1 and IIB2) and P450h (P450IIC11) [9] contribute to benzene aromatic hydroxylation at low-affinity sites, while P450j (P450IIE1) acts at high-affinity sites [10, 11]. In this study we examined the involvement of these P450 isozymes in toluene side-chain or ring oxidation.

The contributions of cytochrome P450IA1/IA2, P450IIB1/IIB2, P450IIC11/IIC6 and P450IIE1 to toluene side-chain and ring oxidations were investigated in liver microsomes from control, one-day fasted, ethanol-, PB- and 3-methylcholanthrene (MC)-treated rats using the method of MAb-directed inhibition for reaction phenotyping.

MATERIALS AND METHODS

Animals. Male Wistar rats (6 weeks of age) were obtained from Nippon SLC Inc. (Shizuoka, Japan). All rats were housed in an air-conditioned room (20 ± 2°) with a 12-hr light–dark cycle and maintained on pelleted feed (Nippon Clea, CE-2, Tokyo) and

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¶ Abbreviations: MAb, monoclonal antibody; PB, phenobarbital; MC, 3-methylcholanthrene; BA, benzyl alcohol.

water *ad lib.* until they reached the age of 8 weeks. They were divided into five groups: control, fasted, and PB-, MC- and ethanol-treated groups. Liquid diet was used in this experiment because of easy changing of its constituents. The first four groups received a nutritionally adequate liquid diet (basal diet) prepared according to the method of DeCarli and Lieber [12] with a slight modification [13]. The basal diet contained 15.0 g of sucrose, 3.66 g of casein (sodium salt), 51 mg of L-cysteine, 31 mg of DL-methionine, 2.5 g of oil (olive/corn = 4/1), 265 mg of ethyl linoleate, 509 mg of vitamin mixture, 1.0 g of mineral mixture, 25 mg DL-α-tocopherol and 265 mg carrageenan in a volume of 100 mL (1 kcal/mL). The fasted group was deprived of food the day before decapitation; the PB-treated group was given PB at 80 mg/kg per day for 4 days and MC-treated group MC at 20 mg/kg per day for 4 days intraperitoneally. Rats in the ethanol-treated group received a diet prepared by adding 2.5 g ethanol per 100 mL of basal diet in which the sucrose content had been reduced to 5.0 g/100 mL and the oil content increased to 5.0 g/100 mL, so that the ethanol and basal diets were isocaloric. These liquid diets were given once a day, 80 mL/rat, at 4:00 p.m. for 3 weeks as the only source of food and water.

Isolation of liver microsomes. All rats were decapitated at 10:00 a.m. and the livers removed. Microsomes were isolated by differential centrifugation at 105,000 *g* for 60 min. Microsomal pellets were washed once in 1.15% KCl solution and subsequently suspended in distilled water. After gassing with N_2, microsomes were stored at −85° at a concentration of 20 mg/mL.

Enzymes and protein assays. Toluene metabolism was determined by measuring the rate of formation of benzyl alcohol (BA), *o*- and *p*-cresol, according to the method of Wang and Nakajima [2], with a slight modification of the reaction mixture. Measurement was performed using 200 μg microsomal protein/0.5 mL of reaction mixture containing a final concentration of 1.0 mM NADP, 20 mM glucose 6-phosphate, 50.0 mM magnesium chloride, 2 I.U. glucose 6-phosphate dehydrogenase, 50 mM potassium phosphate buffer (pH 7.4) and toluene. The reaction was initiated with the addition of substrate and the reaction vials were placed in a thermoregulated shaking water bath (37°). After 10 min incubation, 0.1 mL of 15% $ZnSO_4$ and saturated $Ba(OH)_2$ solution were each added to stop the reaction. The mixture was centrifuged at 1800 *g* for 15 min, and 20 mL of the supernatant was injected into a high-performance liquid chromatograph (HPLC, Hitachi L-6000) with UV-detector (Hitachi L-4200) and chromato-integrator (Hitachi E-2500). The analytical condition of HPLC was as follows: column, 4.0 mm diameter × 250 mm stainless column packed with Unisil C_{18} (Gasukurokogyo, Inc. Tokyo); mobile phase, 30% acetonitrile solution at a flow speed of 1.0 mL/min; wavelength, 200 nm. Under these conditions, the amounts of BA, *o*- and *p*-cresol formed were confirmed to increase linearly with respect to incubation time for at least 10 min and to microsomal protein until 0.90 mg. Microsomal protein and cytochrome P450 contents were measured by the method of Lowry *et al.* [14] and Omura and Sato [15], respectively.

Monoclonal antibody inhibition. MAbs were produced at the US National Cancer Institute, Laboratory of Molecular Carcinogenesis using a modification of the method of Koehler and Milstein [16]. In the present study, six MAbs that had been shown to be specific towards different cytochromes P450s were used as follows: clone 1-7-1 [3] to MC-inducible P450c (P450IA1), which cross-reacted with P450d (P450IA2), clones 2-66-3 and 4-29-5 [4] to PB-inducible P450b (P450IIB1), which cross-reacted with P450e (P450IIB2), clone 4-7-1 to P450IIB1, which cross-reacted with P450IIB2 and P450 3(P450IIA1), clone 1-91-3 [5] to ethanol-inducible P450j (P450IIE1) and clone 1-68-11 [6] to P450h (P450IIC11), which cross-reacted with P450 PB-1 (P450IIC6), and is active in the metabolism of steroid hormones [17–19]. As a control monoclonal antibody, MAb (Hy-Hel) against chicken lysozyme was used to determine any non-specific reaction. MAbs were added to pooled microsomes and buffer at room temperature 30 min prior to starting the toluene metabolism assay at 37° by adding NADPH-generating system and substrate (toluene). In preliminary experiments, maximal inhibition by each MAb was obtained below a MAb protein/microsomal protein ratio of 1.0 for assays of toluene metabolism.

Statistics. Analysis of variance was performed. When there was significant difference among groups, means were tested by Student's *t*-test. The 0.05 level of probability was the criterion of significance.

RESULTS

Toluene metabolism

Two toluene concentrations (0.2 and 5.0 mM) were used to explore the differences in catalytic activities between low-K_m (0.13–0.17 mM) and high-K_m (0.60–2.98 mM) toluene-metabolizing enzymes [2] (Table 1). With the low concentration of toluene, neither *o*- nor *p*-cresol was found in microsomes from control and fasted rats, and *p*-cresol was not found in microsomes from MC-treated rats. BA was produced preferentially in all microsomes and represented 85–100% of all toluene metabolites. One-day fasting and PB and ethanol treatments increased the formation of BA from toluene by 2.0-, 7.1- and 5.8-fold, respectively, but MC treatment had no effect. Both ethanol and PB treatments increased the formation of *o*- and *p*-cresol from toluene; more *p*-cresol was formed after ethanol treatment than after PB treatment, but no difference in the amount of *o*-cresol formed was seen. MC treatment also increased *o*-cresol formation, and MC was the strongest inducer of the formation of *o*-cresol when expressed as ratio of *o*-cresol formation to total metabolite formation.

At the high concentration of toluene, BA was still the main metabolite in all microsomes, although the ratios to total metabolites decreased slightly (80–99%) except in microsomes from ethanol-treated rats. The rate of BA formation at this concentration was higher in all microsomes than at the low concentration of toluene. In contrast to the data with the low concentration of toluene, one-day

Table 1. Effects of different treatments on the metabolic activity of toluene

Treatment	nmol/mg protein/min			
	Benzyl alcohol	*o*-Cresol	*p*-Cresol	Total
Toluene 0.2 mM*				
Fed	0.65 ± 0.28 (100)	ND	ND	0.65 ± 0.28 (100)
Fasted	1.28 ± 0.14 (100)†	ND	ND	1.28 ± 0.14 (100)†
PB	4.64 ± 0.47 (94.9)†	0.10 ± 0.01 (2.0)†	0.15 ± 0.03 (3.1)†	4.89 ± 0.50 (100)†
MC	0.39 ± 0.13 (84.8)	0.07 ± 0.02 (15.2)†	ND	0.46 ± 0.13 (100)
Ethanol	3.75 ± 0.95 (89.0)†	0.07 ± 0.01 (1.7)†	0.39 ± 0.05 (9.3)†	4.21 ± 0.93 (100)†
Toluene 5.0 mM*				
Fed	6.02 ± 1.50 (98.5)	0.04 ± 0.02 (0.7)	0.05 ± 0.01 (0.8)	6.11 ± 1.52 (100)
Fasted	6.49 ± 0.88 (98.3)	0.05 ± 0.01 (0.8)	0.07 ± 0.02 (0.9)	6.60 ± 0.89 (100)
PB	29.14 ± 9.15 (90.3)†	1.34 ± 0.39 (4.2)†	1.81 ± 0.62 (5.5)†	32.28 ± 9.63 (100)†
MC	4.11 ± 0.63 (79.7)	0.86 ± 0.04 (17.7)†	0.12 ± 0.02 (2.5)†	5.16 ± 0.81 (100)
Ethanol	8.83 ± 1.08 (93.4)†	0.08 ± 0.01 (0.8)	0.55 ± 0.12 (5.8)†	9.46 ± 1.09 (100)†

Hepatic microsomal protein (mg/g liver) and cytochrome P450 (nmol/mg protein) contents: control, 22.5 ± 3.5, 0.61 ± 0.08; fasted, 23.1 ± 2.6, 0.72 ± 0.05; PB, 31.8 ± 3.6, 1.24 ± 0.10; MC, 23.4 ± 3.1, 1.17 ± 0.05; ethanol, 34.3 ± 1.3, 0.96 ± 0.06 (see Ref. 11).

Each figure represents the mean ± SD for five rats.

Figures in parentheses show the ratio of each metabolite to the total metabolites.

ND, not detectable.

* Toluene concentration used in enzyme assays. The maximum percentage conversion of substrate in the experiments was 9.8% at 0.2 mM toluene and 2.6% at 5.0 mM toluene.

† Significant by different ($P < 0.05$) from the fed rat group.

fasting did not increase the rate of BA formation. PB and ethanol treatments increased the rate, and the difference in BA formation rate between the low and high concentration of toluene was greater in PB-treated microsomes than in ethanol-treated ones. MC treatment did not increase the rate of BA formation either at high or low concentrations of toluene.

o-Cresol formation was found in all microsomes at the high concentration, and the formation rate was increased more markedly by PB than by MC. The ratio with total metabolites, however, was reversed; the ratio was larger in MC-treated microsomes than in PB-treated ones.

p-Cresol formation was induced by PB, MC and ethanol treatments, but not by one-day fasting; the induction occurred in the following order: PB > ethanol > MC.

Inhibition of toluene metabolism by MAbs

MAb 1-7-1 did not inhibit the formation of BA from toluene in any microsomes at either low or high toluene concentrations (Table 2). MAbs 2-66-3, 4-7-1 and 4-29-5 inhibited BA formation only in microsomes from PB-treated rats, at both low and high concentrations of toluene. The percentage inhibition, (difference between activity with Hy-Hel and with each MAb)/activity with Hy-Hel × 100, was almost the same with the two concentrations, while the actual inhibition (difference between activity with Hy-Hel and with each MAb), the MAb-sensitive toluene side-chain oxidase activity, was larger with the high concentration of toluene (9.90–11.75 nmol/mg protein/min) than with the low concentration (2.10–2.18 nmol/mg protein/min). There was no difference in the degree of inhibition by the three MAbs, although inhibition of MAb 4.29-5 was slightly less. MAb 1-68-11 inhibited BA formation only in microsomes from control rats at the low concentration of toluene, while it inhibited BA formation in all microsomes except in PB-induced microsomes at the high concentration. At the high concentration of toluene, the percentage inhibition increased in the following order: control > fasted > ethanol = MC > PB. The actual inhibitions, however, were as follows: ethanol ≥ control = fasted > PB = MC. In contrast, MAb 1-91-3 inhibited BA formation in all microsomes, except those from PB-treated animals, only at the low toluene concentration. The strongest inhibition was found in microsomes from ethanol-treated rats, followed by those from fed and fasted rats and those from MC-treated rats. The actual inhibition by this MAb was as follows: ethanol > fasted > fed > MC.

MAb 1-7-1 and MAbs 2-66-3, 4-7-1 and 4-29-5 inhibited *o*-cresol formation from toluene in microsomes from MC- and PB-treated rats at both low and high concentrations of toluene, respectively. MAb 1-68-11 slightly inhibited the formation at the high concentration in microsomes from fasted and ethanol-treated rats. However, MAb 1-91-3 did not affect the formation in microsomes at either concentration (Table 3).

MAb 1-7-1 slightly inhibited *p*-cresol formation from toluene only in MC-treated microsomes at the high concentration of toluene. MAbs 2-66-3, 4-7-1 and 4-29-5 strongly inhibited the formation at both concentration of toluene in microsomes from PB-treated rats. MAb 1-68-11 inhibited *p*-cresol formation slightly in microsomes from control and ethanol-treated rats. MAb 1-91-3 inhibited the formation in ethanol-treated microsomes at the low

Table 2. Inhibition of benzyl alcohol formation from toluene by six MAbs in rat liver

Treatment	MAbs (nomenclature of P450)*						
	Hy-Hel	1-7-1 (P450IA1/2)	2-66-3 (P450IIB1/2)	4-7-1 (P450IIB1/2)	4-29-5 (P450IIB1/2)	1-68-11 (P450IIC11/6)	1-91-3 (P450IIE1)
Toluene 0.20 mM†							
Fed	0.93 (100)	0.99 (106)	1.10 (118)	1.09 (117)	0.97 (104)	0.76 (82)	0.62 (67)
Fasted	1.98 (100)	2.16 (109)	1.88 (95)	2.00 (101)	1.88 (95)	1.73 (87)	1.29 (65)
PB	3.39 (100)	3.39 (100)	1.29 (38)	1.19 (35)	1.12 (33)	3.25 (96)	3.46 (102)
MC	0.71 (100)	0.66 (93)	0.80 (113)	0.79 (111)	0.81 (114)	0.64 (90)	0.64 (90)
Ethanol	3.35 (100)	3.89 (116)	3.18 (95)	3.28 (98)	3.12 (93)	3.18 (95)	1.17 (35)
Toluene 5.00 mM†							
Fed	4.86 (100)	5.58 (115)	4.86 (100)	5.15 (106)	5.20 (107)	1.02 (21)	5.25 (108)
Fasted	5.36 (100)	5.47 (102)	6.49 (121)	5.90 (110)	4.72 (88)	1.61 (30)	5.47 (102)
PB	16.78 (100)	17.42 (103)	5.97 (36)	6.88 (41)	11.53 (69)	15.03 (90)	17.25 (103)
MC	4.71 (100)	4.34 (92)	4.86 (103)	5.12 (109)	5.03 (107)	2.41 (51)	4.83 (103)
Ethanol	7.99 (100)	8.41 (105)	7.93 (99)	8.06 (101)	8.34 (104)	3.92 (49)	8.12 (102)

Each figure represents the average remaining activity (nmol/mg protein) of duplicate determinations performed with pooled microsomes.
Figures in parentheses show the percentages of remaining activity expressed as: (activity with MAbs/activity with Hy-Hel) × 100.

* See Ref. 9. The coefficient of variation (%) in the immunoinhibition was ≤14.5%

† Toluene concentration used in enzyme assays.

Table 3. Inhibition of *o*-cresol formation from toluene by six MAbs in rat liver

Treatment	MAbs (nomenclature of P450)*						
	Hy-Hel	1-7-1 (P450IA1/2)	2-66-3 (P450IIB1/2)	4-7-1 (P450IIB1/2)	4-29-5 (P450IIB1/2)	1-68-11 (P450IIC11/6)	1-91-3 (P450IIE1)
Toluene 0.2 mM†							
Fed	ND	ND	ND	ND	ND	ND	ND
Fasted	ND	ND	ND	ND	ND	ND	ND
PB	0.29 (100)	0.28 (97)	0.14 (48)	0.14 (48)	0.16 (55)	0.35 (121)	0.32 (110)
MC	0.27 (100)	0.14 (52)	0.26 (96)	0.27 (100)	0.30 (111)	0.30 (111)	0.32 (119)
Ethanol	ND	ND	ND	ND	ND	ND	ND
Toluene 5.0 mM†							
Fed	0.08 (100)	0.08 (100)	0.08 (100)	0.08 (100)	0.08 (100)	0.07 (88)	0.09 (113)
Fasted	0.08 (100)	0.08 (100)	0.08 (100)	0.09 (113)	0.09 (113)	0.06 (75)	0.08 (100)
PB	1.49 (100)	1.63 (110)	0.54 (36)	0.26 (17)	0.56 (38)	1.83 (123)	1.69 (113)
MC	1.73 (100)	0.76 (44)	1.92 (111)	1.96 (113)	2.01 (116)	1.89 (109)	1.77 (102)
Ethanol	0.21 (100)	0.22 (105)	0.21 (100)	0.20 (95)	0.19 (90)	0.18 (86)	0.21 (100)

Each figure represents the average remaining activity (nmol/mg protein/min) of duplicate determinations performed with pooled microsomes.
Figures in parentheses show the percentage of remaining activity expressed as: (activity with MAb/activity with Hy-Hel) × 100.
ND, not detectable.
The coefficient of variation (%) in the immunoinhibition was ≤13.6%.
*† See footnote * and † in Table 2.

Table 4. Inhibition of *p*-cresol formation from toluene by six MAbs in rat liver

Treatment	MAb (nomenclature of P450)*						
	Hy-Hel	1-7-1 (P450IA1/2)	2-66-3 (P450IIB1/2)	4-7-1 (P450IIB1/2)	4-29-5 (P450IIB1/2)	1-68-11 (P450IIC11/6)	1-91-3 (P450IIE1)
Toluene 0.2 mM†							
Fed	ND	ND	ND	ND	ND	ND	ND
Fasted	ND	ND	ND	ND	ND	ND	ND
PB	0.35 (100)	0.35 (100)	0.08 (23)	0.08 (23)	0.09 (26)	0.35 (100)	0.35 (100)
MC	ND	ND	ND	ND	ND	ND	ND
Ethanol	0.33 (100)	0.33 (100)	0.33 (100)	0.33 (100)	0.33 (100)	0.33 (100)	0.21 (64)
Toluene 5.0 mM†							
Fed	0.10 (100)	0.10 (100)	0.11 (110)	0.10 (100)	0.09 (90)	0.08 (80)	0.09 (90)
Fasted	0.10 (100)	0.09 (90)	0.09 (90)	0.10 (100)	0.10 (100)	0.09 (90)	0.10 (100)
PB	1.88 (100)	1.98 (105)	0.40 (21)	0.17 (9)	0.30 (16)	2.27 (121)	2.04 (109)
MC	0.33 (100)	0.23 (70)	0.29 (88)	0.34 (103)	0.32 (97)	0.29 (88)	0.30 (91)
Ethanol	0.53 (100)	0.53 (100)	0.66 (125)	0.53 (100)	0.53 (100)	0.43 (81)	0.53 (100)

Each figure represents the average remaining activity (nmol/mg protein/min) of duplicate determinations performed with pooled microsomes.
Figures in parentheses show the percentage of remaining activity expressed as: (activity with MAb/activity with Hy-Hel) × 100.
ND, not detectable.
The coefficeint of variation (%) in the immunoinhibition was ≤13.8%.
*† See footnote * and † in Table 2.

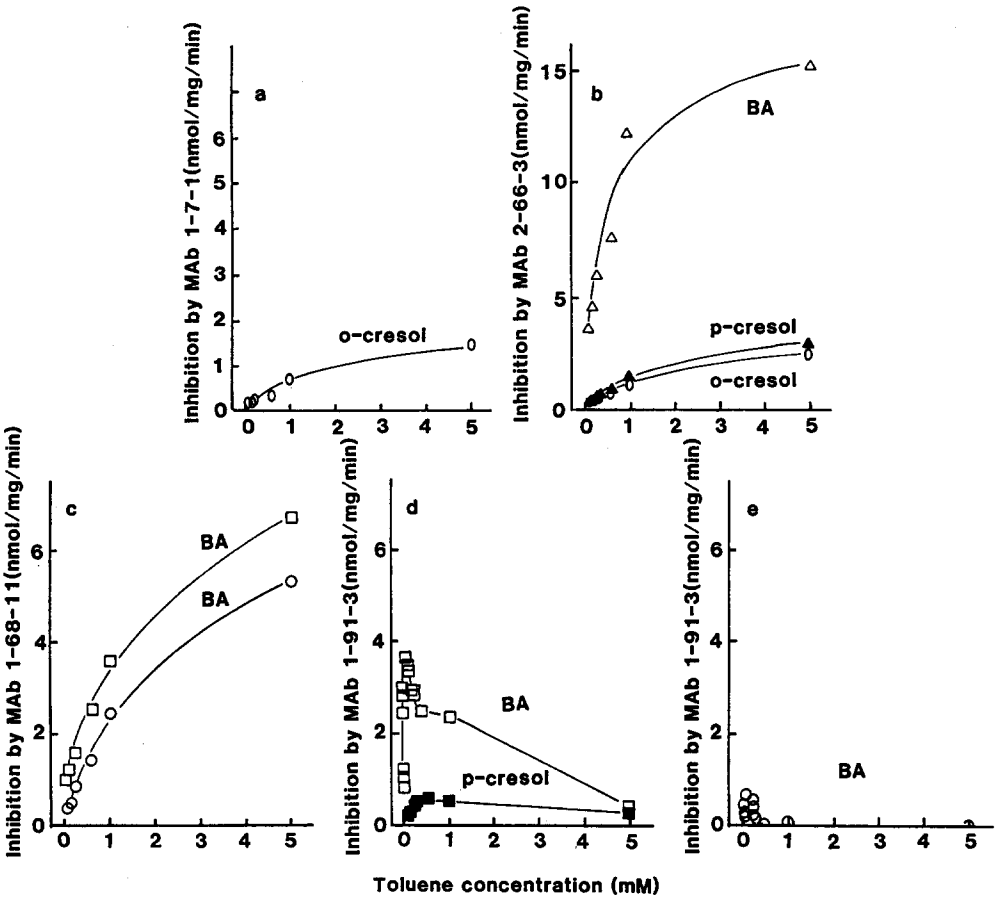

Fig. 1. Actual inhibition of toluene side-chain and ring hydroxylation by monoclonal antibodies (MAbs) 1-7-1, 2-66-3, 1-68-11 and 1-91-3. Each symbol represents the mean of duplicate assays. (a) Actual inhibition of *o*-cresol formation in liver microsomes from 3-methylcholanthrene-treated rats by MAb 1-7-1; (b) actual inhibition of the formation of benzyl alcohol (BA), *o*- and *p*-cresol in liver microsomes from phenobarbital-treated rats by MAb 2-66-3; (c) actual inhibition of BA formation in liver microsomes from control (○) and ethanol-treated rats (□) by MAb 1-68-11; (d) actual inhibition of BA and *p*-cresol formation in liver microsomes from ethanol-treated rats by MAb 1-91-3; and (e) actual inhibition of BA formation in liver microsomes from control rats by MAb 1-91-3.

concentration of toluene but not at the high concentration (Table 4).

Kinetics of inhibition of toluene metabolism by MAbs

The inhibition of toluene metabolism by MAbs was analysed at different toluene concentrations. The actual inhibition (nmol/mg protein/min) was calculated and plotted against the respective concentration of toluene (Fig. 1). The K_m and V_{max} of the inhibitable activity by each MAb were calculated from double-reciprocal plots, i.e. Lineweaver–Burk plots, using the least-squares method. The actual inhibition of *o*-cresol formation from toluene by MAb 1-7-1 in MC-treated microsomes increased with increasing toluene concentration. The K_m and V_{max} were 1.46 mM and 1.10 nmol/mg protein/min, respectively.

The actual inhibition of formation of BA, *o*-, and *p*-cresol from toluene by MAb 2-66-3 in PB-induced microsomes also increased with increasing toluene concentrations. The K_m and V_{max} were 0.55 mM and 16.78 nmol/mg protein/min for BA formation, 5.67 mM and 4.59 nmol/mg protein/min for *o*-cresol formation, and 5.32 mM and 7.52 nmol/mg protein/min for *p*-cresol formation.

Similarly, the actual inhibition of BA formation by MAb 1-68-11 in microsomes from control and ethanol-treated rats increased with increasing toluene concentrations; the K_m and V_{max} were 1.81 mM and 6.67 nmol/mg protein/min in control microsomes and 1.75 mM and 9.80 nmol/mg protein/min in microsomes of ethanol-treated rats. The actual inhibition of *p*-cresol formation from toluene in ethanol-treated microsomes by MAb 1-68-11 was higher at the high concentration of toluene, and the K_m and V_{max} were 4.96 mM and 1.15 nmol/mg protein/min, respectively.

In contrast, the actual inhibition of BA formation from toluene by MAb 1-91-3 in control and ethanol-treated microsomes was maximal at 0.10–0.20 mM toluene. With increasing toluene concentration, the inhibition decreased. The actual inhibition of *p*-cresol formation by MAb 1-91-3 in ethanol-treated

Table 5. K_m and V_{max} of cytochrome P450 isozymes for toluene metabolism estimated by MAb inhibition studies

		Benzyl alcohol formation		
		P450IIB1/2	P450IIC11/6	P450IIE1
Control	K_m	ND	1.81	0.14
	V_{max}	ND	6.67	3.29
Ethanol	K_m	ND	1.75	0.19
	V_{max}	ND	9.80	12.30
PB	K_m	0.55	ND	ND
	V_{max}	16.88	ND	ND
		o-Cresol formation		
		P450IA1/2		P450IIB1/2
PB	K_m	ND		5.67
	V_{max}	ND		4.59
MC	K_m	1.46		ND
	V_{max}	1.10		ND
		p-Cresol formation		
		P450IIB1/2	P450IIC11/6	P450IIE1
Ethanol	K_m	ND	4.96	1.02
	V_{max}	ND	1.15	2.26
PB	K_m	5.32	ND	ND
	V_{max}	7.52	ND	ND

K_m in mM; V_{max} in nmol/mg protein/min.
ND, not determined.

microsomes reached a maximum at 0.30–0.50 mM toluene and decreased slightly with increasing concentrations. The K_m and V_{max} for the contribution of P450IIE1 to the formation of BA and *p*-cresol were calculated using the results obtained from 0.013 to 0.110 mM of toluene, and were 0.19 mM and 12.3 nmol/mg protein/min for BA formation and 1.02 mM and 2.26 nmol/mg protein/min for *p*-cresol formation in ethanol-treated microsomes, and 0.14 mM and 3.29 nmol/mg protein/min for BA formation in control microsomes.

DISCUSSION

The K_m and V_{max} of the P450 isozymes responsible for each step of toluene metabolism into BA, *o*-cresol and *p*-cresol, as determined by MAb-mediated analysis, are summarized in Table 5.

Cytochromes P450IIB1/IIB2, P450IIC11/IIC6 and P450IIE1 but not P450IA1/IA2 contributed to toluene side-chain oxidation (BA formation). These three isozymes had different K_m values towards this reaction: P450IIE1 had a low K_m, P450IIC11/IIC6 a high K_m and P450IIB1/IIB2 a medium K_m, although it cannot be denied that the kinetics of cross-reacting isozymes may differ from each other. Ethanol treatment clearly induced P450IIE1, but had little effect on P450IIC11/IIC6. In a previous paper [2], we reported using different microsomes from this study that two isozymes responsible for toluene side-chain oxidation, with different K_m values (0.13–0.17 and 0.60–0.87 mM), exist in control and ethanol-treated microsomes, and the low-K_m one can be induced by ethanol treatment. This low-K_m isozyme may be identical to P450IIE1, and the other, with a high K_m, may be P450IIC11/IIC6.

The V_{max} of P450IIB1/IIB2 was higher than that of P450IIC11/IIC6, which may corroborate the result of Waxman [17, 20] that the catalytic activity of toluene side-chain hydroxylation by purified P450 PB-4 (P450IIB1) is twice that of purified P450 2c (P450IIC11) and 4-fold that of P450 PB-1 (P450IIC6).

Cytochromes P450IIB1/IIB2 and P450IA1/IA2 contributed to toluene ring oxidation at the *o*-position (*o*-cresol formation). The contribution of P450IIE1 and P450IIC11/IIC6 to this step seemed to be negligible, although the latter isozyme may make a slight contribution (Table 3).

Cytochrome P450IIB1/IIB2, P450IIC11/IIC6 and P450IIE1 contributed to toluene ring oxidation at the *p*-position (*p*-cresol formation). The contribution of P450IA1/IA2, however, was small (Table 4). Like the side-chain oxidation, the K_m value of P450IIE1 in this ring oxidation was lower than that of P450IIC11/IIC6. We have found previously that two toluene ring oxidases at the *p*-position, with different K_m values (0.47 and 1.31 mM), existed in ethanol treated microsomes [2]. P450IIE1 and P450IIC11/IIC6 may correspond to these low- and high-K_m isozymes, respectively, although the K_m values were different from those obtained in the MAb study reported here.

The K_m values of P450IIB1/IIB2, P450IIC11/IIC6 and P450IIE1 were higher for ring oxidation than for side-chain oxidation, indicating that these isozymes may attack the toluene side-chain more easily than the ring positions. In contrast, the P450IA1/IA2 may preferentially attack the ring position (Tables 2–4).

Cytochromes P450IIC11/IIC6 and P450IIE1 are constitutive isozymes [5, 6] with different inducibilities and affinities for toluene. These P450s contributed 50% of the total tissue activity of toluene metabolism in control rats measured at a low concentration of toluene (Table 2). At a high concentration, P450IIC11/IIC6 contributed 80% of the total tissue activity. The relative contribution of P450IIE1 was much less at the high concentration of toluene. This conclusion is supported by the fact that one-day fasting, a potent inducer of P450IIE1 [10, 11], had no effect on toluene side-chain oxidation at the high concentration of toluene but stimulated the oxidation at the low concentration. These results indicate that (i) P450IIE1 is a low K_m isozyme for toluene metabolism and is induced by one-day fasting and ethanol treatment, which is consistent with our previous reports [10, 11]; (ii) P450IIC11/IIC6 is a high-K_m isozyme for toluene metabolism and makes a greater contribution than P450IIE1, especially at high substrate concentrations, but is not induced by these treatments (Table 2 and Fig. 1); and (iii) P450IIE1, but not P450IIC11/IIC6, is inhibited by the high concentration of toluene. It is of interest that both P450IIC11/IIC6 and P450IIE1 were decreased by PB and MC treatments (Table 2), in good agreement with the report of Waxman *et al.* [19], although P450IIC6 is known to be induced by PB treatment.

Cytochrome P450IA1/IA2, P450IIB1/IIB2, P450IIC11/IIC6 and P450IIE1 are known to contribute to benzene aromatic hydroxylation [9, 10]. P450IIE1 and P450IIC11/IIC6 contributed to toluene metabolism in the same manner as in benzene metabolism, where P450IIE1 was a low-K_m isozyme and P450IIC11/IIC6 a high-K_m isozyme. The inhibition of benzene metabolism by MAbs to these P450s, however, was less strong than that of toluene: the inhibited activities of benzene and toluene metabolism by MAbs to P450IIE1 and P450IIC11/IIC6 in control microsomes were as follows: 0.23 and 0.31 nmol/mg protein/min by MAb to P450IIE1, 0.15 and 3.84 nmol/mg protein/min by MAb to P450IIC11/IIC6, respectively. These differences in the contributions of P450IIE1 and P450IIC11/IIC6 to benzene and toluene metabolism may explain the results reported previously that, in a mixture of benzene and toluene, they inhibit each other's metabolism competitively, and benzene metabolism is more readily suppressed by toluene than toluene by benzene [21].

In conclusion, P450IIC11/IIC6 and P450IIE1 are primary cytochrome P450 isozymes contributing mainly to toluene side-chain oxidation, and to a slight extent to toluene ring oxidation of the *p*-position, at low- and high-affinity sites, respectively. At low concentrations of toluene, however, about 50% of the metabolism in intact rats remains unaccounted for by the isozymes studied here. P450IIB1/IIB2 in PB-induced rats contributes to side-chain oxidation, and also to ring oxidations at the *o*- and *p*-position, although not all activity is inhibitable by the MAb studied. P450IA1/IA2 in MC-treated rats contributes mainly to toluene ring oxidation of the *o*-position. Isozymes other than P450IA1/IA2 inducible by MC may also be involved in the metabolism of toluene.

Acknowledgement—This work was supported in part by a research grant (No. 02807059) from the Japan Ministry of Education, Science and Culture.

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