Biochemical Pharmacology, Vol. 37, No. 20, pp. 3889–3895, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

IMMUNOCHEMICAL AND CATALYTICAL STUDIES ON HEPATIC COUMARIN 7-HYDROXYLASE IN MAN, RAT, AND MOUSE

HANNU RAUNIO,*† TUULA SYNGELMÄ,* MARKKU PASANEN,* RISTO JUVONEN,‡ PAAVO HONKAKOSKI,‡ MATTI A. KAIRALUOMA,§ EERO SOTANIEMI,‖ MATTI A. LANG‡ and OLAVI PELKONEN*

* Department of Pharmacology, University of Oulu, § Department of Surgery, ‖ Department of Medicine (Clinical Research Unit), University of Oulu Central Hospital, 90220 Oulu, and ‡ Department of Pharmacology and Toxicology, University of Kuopio, POB 6, 70211 Kuopio, Finland

(*Received* 29 *December* 1987; *accepted* 28 *April* 1988)

Abstract—The cytochrome P-450-mediated coumarin 7-hydroxylase (COH) was studied in microsomal preparations from Wistar rat, DBA/2N mouse, and human liver. Human liver contained the highest constitutive COH activity of up to about 500 pmol/mg microsomal protein/min. The rat liver contained low levels of COH (about 3–5 pmol/mg protein/min) which could be demonstrated only with high substrate concentrations. Rabbit polyclonal antibody generated against P-450$_{Coh}$ (a P-450 isozyme purified from pyrazole-treated DBA/2N mouse liver showing high activity for coumarin 7-hydroxylation) inhibited COH activity by almost 100% in human liver microsomes and 86–99% in mouse liver microsomes. Also the deethylation of 7-ethoxycoumarin was inhibited somewhat by the antibody, whereas no inhibition was obtained in ethoxyresorufin *O*-deethylase and aryl hydrocarbon hydroxylase activities. None of these enzyme activities was affected by the antibody in the rat liver microsomes. In Ouchterlony immunodiffusion analysis precipitin lines were obtained with human, mouse and rat liver microsomes. Complex coalescence patterns were obtained suggesting full identity between human and pyrazole-treated mouse antigens, partial identity between mouse and rat antigens, and no identity between human and rat antigens. Western blot analysis with the anti-P-450$_{Coh}$ antibody revealed a distinct 48-kDa protein in all four human samples tested. A 50-kDa protein comigrating exactly with P-450$_{Coh}$ was observed in microsomes from PB and pyrazole-treated mouse liver microsomes. No distinct protein bands appeared in rat liver samples. These data suggest that despite slightly differing molecular masses, the human and mouse P-450s supporting COH are structurally conserved at their active centers. The corresponding rat P-450 appears to differ from that of mouse and man.

Coumarin occurs naturally at high concentrations in several plants. It is being used, although decreasingly, as a fixative and enhancer of odors in cosmetic preparations [1]. *In vivo* and *in vitro* studies have demonstrated a wide interspecies variation in the metabolic disposition of coumarin [2]. In man coumarin is excreted mainly as free or conjugated 7-hydroxyderivative, while in the rat the amount of excreted 7-hydroxycoumarin is less than 1% of the dose administered [3].

These differences are also reflected in the hepatic enzymes involved in the metabolism of coumarin. Human liver contains an active cytochrome P-450-linked coumarin 7-hydroxylase (COH)¶ system [4], whereas this activity in the rat liver is negligible or absent [5, 6]. In recent reports we have confirmed these differences and extended the analysis of the human liver COH [7]. In addition to humans, some inbred strains of mice are also known to have a high COH activity, most notably the DBA/2 strain [8–10].

Characterization of human xenobiotic-metabolizing enzymes is difficult because of limited availability of tissues and ethical considerations involved. Livers from renal transplant donors and from other surgical patients have been used in the purification and characterization of human P-450-linked enzymes [11–13]. Another kind of approach is to do comparative studies using antibodies raised against rodent P-450 enzymes. We [14] and others [15–17] have successfully employed monoclonal antibodies raised against specific rat P-450s in the characterization of the human P-450-mediated monooxygenase system.

In this study we have used a polyclonal antibody raised against purified murine hepatic P-450 associated with high COH activity to do interspecies comparisons. We show here by catalytic activity and immunochemical analyses that human and mouse liver coumarin 7-hydroxylating P-450s are immunologically closely related whereas the COH activity in rat liver differs from that of these two species.

† To whom all correspondence and requests for reprints should be addressed.

¶ Abbreviations used: AHH, aryl hydrocarbon hydroxylase; COH, coumarin 7-hydroxylase; ECDE, 7-ethoxycoumarin *O*-deethylase; ERDE, 7-ethoxyresorufin *O*-deethylase; MC, 3-methylcholanthrene; P-450$_{Coh}$, purified cytochrome P-450 highly active in the 7-hydroxylation of coumarin; PB, phenobarbital; TBS, Tris-buffered saline.

MATERIALS AND METHODS

Chemicals. Coumarin and 7-ethoxycoumarin were purchased from Aldrich (Beerse, Belgium). Benzo(*a*)pyrene, 7-hydroxycoumarin, and 7-ethoxyresorufin were from Sigma Chemical Co. (St. Louis, MO). Biotinylated Protein A and biotinylated streptavidin-horseradish peroxidase complex were from Amersham (Buckinghamshire, U.K.). 4-Chloro-1-naphthol was obtained from Bio-Rad (Richmond, CA).

Liver preparations. Human liver wedge biopsy samples (about 1–10 g) were taken during laparotomy from four patients with either hepatomas or cholestatic disease. The liver samples were histologically normal-looking. However, the extent to which the disease process affects P-450 enzyme activities is not fully known [18]. Parts of the biopsies were used for this study on approval by the Ethical Committee of the Faculty of Medicine, University of Oulu. Groups of four adult male DBA/2N mice and Wistar rats were given single daily i.p. injections of 3-methylcholanthrene (MC, 25 mg/kg), suspended in corn oil, or pyrazole (200 mg/kg, dissolved in physiological saline), both for three consecutive days. Phenobarbital (PB) was administered in drinking water (500 mg/l) for 7 days. Control animals received corn oil as single daily i.p. injections for three days. The animals were killed by decapitation 24 hr after the last treatment, and liver microsomal fractions were prepared by standard differential centrifugation [19]. For COH assays, aliquots of the individual samples were pooled. The other enzymes were assayed from the individual samples.

Preparation of the antibody. Pyrazole-inducible P-450 associated with high COH activity (designated as P-450$_{Coh}$) was purified to apparent electrophoretic homogeneity from livers of DBA/2N mice as reported elsewhere [19], and antiserum against it was raised in rabbits. Of several monooxygenase activities tested, the antibody inhibits significantly only the activities of COH and ECDE (Järvinen *et al.*, unpublished). The antibody does not cross-react with purified ethanol and pyrazole-inducible rat liver P-450 (P-450$_j$) and its associated monooxygenase activities (Honkakoski *et al.*, unpublished). Ammonium sulphate-precipitated fractions of the antiserum containing approximately 13 mg of IgG/ml were used in this study.

Enzyme assays. COH activity was determined according to Aitio [20] as described in detail earlier [19]. In studies on the effect of substrate concentration on COH activity, 0.1–10 mM coumarin was used as the substrate. In subsequent experiments, 0.1 mM coumarin was used as the substrate with mouse and human liver preparations and 10 mM coumarin was used with rat liver preparations. Aryl hydrocarbon hydroxylase (AHH) activity was measured according to Nebert and Gelboin [21] and 7-ethoxycoumarin *O*-deethylase (ECDE) was determined as described by Greenlee and Poland [22]. Ethoxyresorufin *O*-deethylase (ERDE) activity was assayed according to Burke *et al.* [23]. The substrate concentrations were 80 μM for AHH, 500 μM for ECDE and 1 μM for ERDE assays. Protein determinations were done according to Bradford [24].

Inhibition of enzyme activities by the antibody. The antibody was added to the incubation mixture of the enzyme assays at concentrations ranging from 1:1 to 5:1 (IgG protein:microsomal protein) 2 min before initiation of the appropriate reactions. Preimmune serum controls were always assayed in parallel with the antibody.

Immunodiffusion analysis. Immunodiffusions were carried out essentially as originally described by Ouchterlony [25] in 1% agarose gels. The microsomal fractions were solubilized in 1% sodium cholate and 0.2% Emulgen 911 (which were also used in the gels), and 0.3 mg of microsomal protein were applied to the wells. An equal amount of IgG protein (0.3 mg) was used in centre wells. The gels were incubated at 4° for 48 hr.

Protein electrophoresis and Western blots. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was done by the method of Laemmli [26]. Proteins were transferred onto nitrocellulose sheets according to Towbin *et al.* [27]. For P-450$_{Coh}$ detection, the nitrocellulose sheets were first blocked with 3% (w/v) gelatin in TBS (50 mM Tris–200 mM NaCl, pH 7.4) for 1 hr at 37°. The sheets were subsequently serially reacted with (a) anti-P-450$_{Coh}$ antibody at 1:200 dilution in TBS, (b) biotinylated Protein A (1:400 dilution), and (c) streptavidin-biotinylated horseradish peroxidase complex (1:400 in TBS). Between each step the sheets were washed 3 × 10 min in TBS–0.05% Tween-20. The sheets were developed with 4-chloro-1-naphthol and hydrogen peroxide as substrates.

RESULTS

Effect of substrate concentration on COH

Figure 1 illustrates the dependency of COH activity on the substrate concentration in DBA/2N and C57BL/6 mice and Wistar rats. Raising the substrate concentration from 0.1 to 10 mM did not markedly affect COH activity in either mouse strain. With substrate concentrations above 5 mM, some COH activity could also be demonstrated in rat liver microsomes (Fig. 1). The reaction product formed with rat liver microsomes co-chromatographed with authentic 7-hydroxycoumarin in thin layer chromatography and had identical fluorescence properties

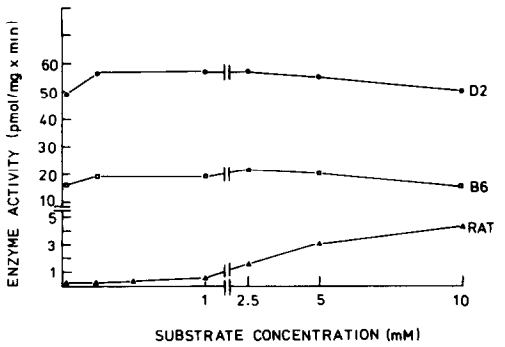

Fig. 1. The effect of substrate concentration on the reaction velocity of rat and mouse hepatic microsomal COH. D2 denotes DBA/2 mice and B6 denotes C57BL/6 mice.

with it (data not shown). In the Lineweaver–Burk analysis, the rat liver COH exhibited a K_m value of about 10 mM and a V_{max} value of 5 pmol/mg microsomal protein/min. Human and DBA/2N mouse K_m values are approximately 2 μM (data not shown). Experiments with several monooxygenase inhibitors such as alpha-naphthoflavone, SKF 525A, metyrapone and aniline, all shown to be inhibitory with human liver COH [7], demonstrated that the same compounds also inhibit the rat liver enzyme (data not shown).

Immunoinhibition of monooxygenase activities by anti-P-450$_{Coh}$ antibody

Four human liver microsomal preparations were assayed for COH, ECDE, ERDE and AHH activities. The COH activities in these preparations varied between 150 and 491 pmol/mg microsomal protein/min. Anti-P-450$_{Coh}$ antibody very efficiently inhibited human liver COH activity to a level of 12% or less of the basal activity in each preparation when equal amounts of IgG and microsomal protein were added to the incubation mixture (Fig. 2). With higher concentrations of the antibody (5:1) the COH activity was inhibited 98–99% in each case. Preimmune serum caused either a slight inhibition (patient 1), or activation (patient 3) of COH activity with high antibody:microsomal protein concentrations.

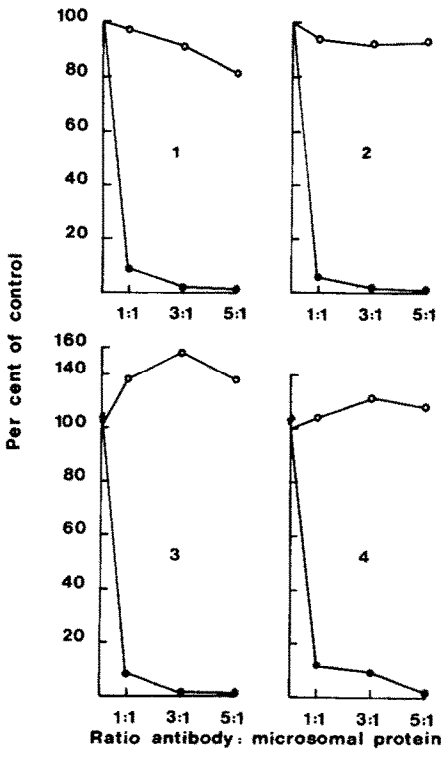

Fig. 2. Inhibition of human COH by anti-P-450$_{Coh}$ antibody. The activity of COH was assayed in the presence of the antibody at concentrations ranging from 1:1 (IgG protein: microsomal protein) to 5:1 (●—●). Preimmune serum was used at same concentrations (○—○). Basal activity was determined in assays without the antibody. Numbers 1–4 denote individual patients.

Table 1. Inhibition by anti-P-450$_{Coh}$ antibody of human liver monooxygenase activities

Enzyme	Antibody	Preimmune serum
	Per cent of activity	
COH	1 ± 1*	107 ± 24
ECDE	58 ± 14	96 ± 2
ERDE	98 ± 4	95 ± 8
AHH	111 ± 6	118 ± 12

A 5:1 ratio of antibody or preimmune serum: microsomal protein was used.

* Each figure is the mean ± SD of 4 individuals.

Of the other monooxygenase activities tested, ECDE was inhibited to a variable extent by the antibody. As listed in Table 1, the mean inhibition of human liver ECDE activity by the antibody at a 5:1 concentration was 42%, whereas ERDE and AHH activities were not affected.

The effects of PB, pyrazole and MC on DBA/2N mouse liver monooxygenase activities are listed in Table 2. Also in the mouse liver microsomal preparations, COH activity was efficiently inhibited by the antibody. As shown in Fig. 3, the COH activity was reduced by the antibody to less than 10% in control, PB-treated and pyrazole-treated mouse microsomes. In MC-treated mice, the inhibition was approximately 85% with all three antibody concentrations tested.

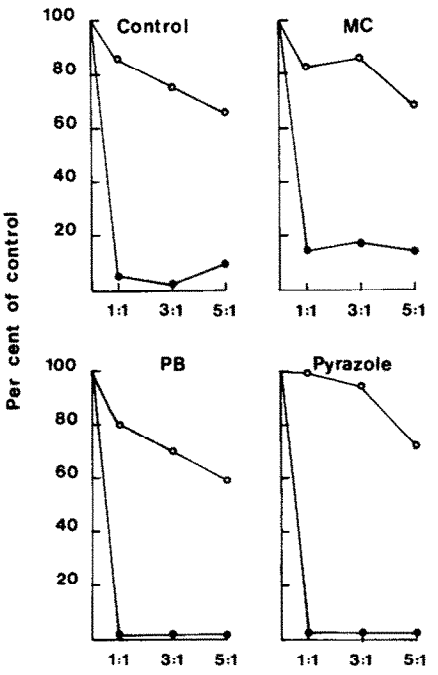

Fig. 3. Inhibition of mouse COH by anti-P-450$_{Coh}$ antibody. The experiment was done with microsomal samples pooled from four individual animals as in Fig. 2. Solid circles denote anti-COH antibody and open circles preimmune serum.

Table 2. Effect of inducers on monooxygenase activities in DBA/2N mouse liver

Treatment	COH	ECDE	ERDE	AHH
Control	0.03*	0.28 ± 0.05	0.69 ± 0.14	0.20 ± 0.05
PB	0.38	4.02 ± 0.38	3.34 ± 0.68	1.04 ± 0.08
Pyrazole	0.65	1.47 ± 0.65	0.13 ± 0.03	0.06 ± 0.03
MC	0.01	0.43 ± 0.11	1.02 ± 0.43	0.27 ± 0.08

All enzyme activities are given in nmoles/mg microsmal protein/min.
* COH activities were assayed from pooled liver microsomal preparations. The others are means ±SD of 4–6 individual preparations.

Table 3. Inhibition by anti-P-450$_{Coh}$ antibody of mouse liver monooxygenase activities

Enzyme	Treatment	Antibody	Preimmune serum
		Per cent of activity	
COH	Control	10*	66
	PB	1	58
	Pyrazole	6	52
	MC	14	68
ECDE	Control	71 ± 14†	97 ± 3
	PB	56 ± 4	94 ± 3
	Pyrazole	13 ± 3	93 ± 9
	MC	83 ± 3	92 ± 3
ERDE	Control	86 ± 5	100 ± 3
	PB	90 ± 19	87 ± 12
	Pyrazole	83 ± 3	92 ± 3
	MC	104 ± 3	107 ± 1
AHH	Control	106 ± 8	112 ± 9
	PB	126 ± 14	109 ± 8
	Pyrazole	199 ± 39	194 ± 37
	MC	129 ± 9	131 ± 13

A 5:1 ratio of antibody or preimmune serum: microsomal protein was used.
* Figures for COH are duplicate measurements of pooled samples from four mice.
† Figures for ECDE, ERDE and AHH are means ± SD of four individual mice.

As with the human preparations, mouse liver ECDE activity was also blocked by the antibody (Table 3). The inhibition was dependent on the pretreatment with pyrazole-treated mice showing the greatest response (an average of 87% inhibition). ERDE and AHH activities were not markedly affected by the antibody.

Since the constitutive COH activity in rat liver microsomes was too low for meaningful inhibition studies with the antibody (Table 4), only PB-pretreated rat liver microsomes were tested for their response to the antibody. No inhibition of COH activity was observed with any antibody concentrations used. A summary of the monooxygenase activities tested is given in Table 5. In addition to COH, also the other activities, ECDE, ERDE, and AHH, did not respond to the antibody.

Immunochemical analyses

In Ouchterlony double-diffusion experiments clear precipitin lines were obtained with all four human liver preparations (Fig. 4A). Under these conditions, one major and another minor precipitin line were formed with control and MC-treated DBA/2N mouse liver microsomes, whereas only one line was seen using PB and pyrazole-treated mouse liver microsomes (Fig. 4B). Also the rat microsomes yielded precipitin lines with the anti-P-450$_{Coh}$ antibody (Fig. 4C). Coalescence patterns suggesting partial identity between mouse and rat anti-P-450$_{Coh}$-detectable antigen, no identity between human and rat antigens, and full identity between pyrazole-treated mouse and human antigens were obtained (Fig. 4D). No identity was apparent between control mouse and human antigens.

The immunological relatedness between human and mouse enzymes was finally verified by Western blot analysis. The anti-P-450$_{Coh}$ antibody revealed a distinct protein band in a human liver microsomal preparation which migrates slightly faster (minimum M_r 48 kDa) than the purified P-450$_{Coh}$ (minimum M_r 50 kDa) (Fig. 5). The nature of the faint heavier band (approximately 55 kDa) also detected by the antibody is not known. Figure 6 illustrates the detection by the antibody of the respective antigen in

Table 4. Effect of inducers on monooxygenase activities in Wistar rat liver

Treatment	COH	ECDE	ERDE	AHH
Control	0.003*	0.36 ± 0.06	0.85 ± 0.07	0.51 ± 0.06
PB	0.032	1.20 ± 0.19	1.93 ± 0.07	0.69 ± 0.13
Pyrazole	0.007	0.54 ± 0.13	1.08 ± 0.36	0.30 ± 0.13
MC	0.003	2.77 ± 0.39	7.07 ± 0.09	1.08 ± 0.08

All activities are given in nmoles/mig protein/min.
* COH activity was assayed from pooled preparations. The other activities are the means ± SD of 4–6 individual assays.

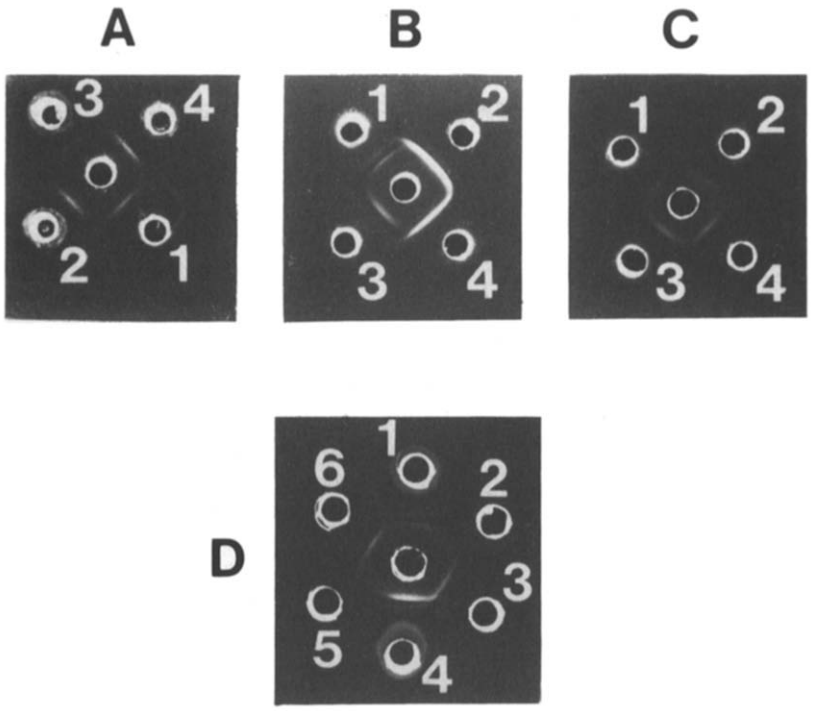

Fig. 4. Ouchterlony immunodiffusion analysis of human, mouse and rat liver microsomal preparations. The center wells contained 0.3 mg anti-P-450$_{Coh}$ antibody. (A) Human liver microsomes from patients 1–4. (B) Liver microsomes from control (1), PB-treated (2), MC-treated (3), and pyrazole-treated (4) DBA/2 mice. (C) Microsomes from control (1), PB-treated (2), MC-treated (3), and pyrazole-treated Wistar rats (4). (D) Microsomes from control DBA/2N mouse (1), human liver (2 and 5), control rat (3 and 6), and pyrazole-treated mouse (4). 0.3 mg of microsomal protein was used in each case.

human, mouse, and rat liver microsomal preparations. All four human samples showed distinct bands the relative intensities of which correlated with the actual COH catalytic activities (Fig. 6, upper panel). As expected, microsomes from PB- and pyrazole pretreated DBA/2N mice showed intense bands comigrating exactly with purified P-450$_{Coh}$ (Fig. 6, middle panel). Weak uniform diffuse staining in the 47–55 kDa region was visible in rat preparations (Fig. 6, bottom panel).

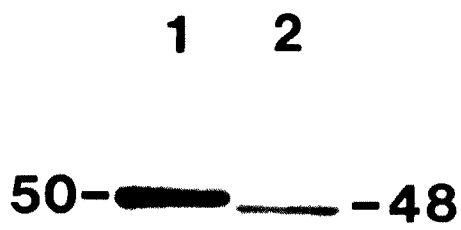

Fig. 5. Western blot with purified P-450$_{Coh}$ (lane 1, 50 ng) and human liver microsomes (lane 2, 5 μg). The proteins were visualized with anti-P-450$_{Coh}$ as described in Materials and Methods. The minimum M_rs are given in kilodalton (kDa).

DISCUSSION

In a previous report we showed that the human liver COH is more active than that of any other species studied to date [7]. Especially the rat has

Table 5. Inhibition by anti-P-450$_{Coh}$ of rat liver monooxygenase activities

Enzyme	Treatment	Antibody Per cent of activity	Preimmune serum Per cent of activity
COH*	PB	96†	100
ECDE	Control	93 ± 7‡	96 ± 4
	PB	90 ± 6	91 ± 10
	Pyrazole	98 ± 1	97 ± 4
	MC	94 ± 5	93 ± 2
ERDE	Control	92 ± 3	107 ± 4
	PB	98 ± 3	107 ± 7
	Pyrazole	99 ± 4	99 ± 3
	MC	101 ± 2	101 ± 1
AHH	Control	123 ± 9	117 ± 16
	PB	113 ± 12	101 ± 15
	Pyrazole	112 ± 4	104 ± 8
	MC	93 ± 5	84 ± 7

* COH activity was high enough for inhibition studies only with microsomes obtained from PB-treated rats.

† The figure is the result of a duplicate assay of a microsomal sample pooled from four rats.

‡ ECDE, ERDE and AHH figures are means ± SD of four individual rats.

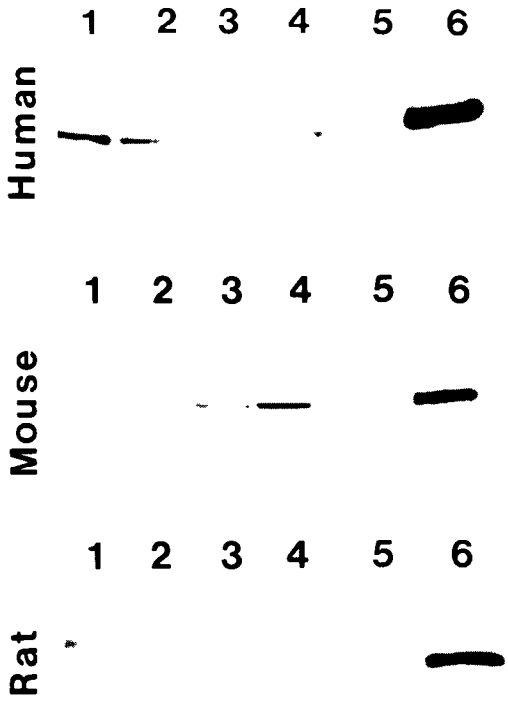

Fig. 6. Western blots with anti-P-450$_{Coh}$ antibody in human, mouse and rat liver microsomal preparations. 5 μg of liver microsomes from patients 1–4 were applied (upper panel, lanes 1–4, respectively). Specific COH activities were 491, 294, 150 and 285 pmol/mg microsomes/min in patients 1, 2, 3 and 4, respectively. 5 μg of liver microsomes from control (lane 1), MC (lane 2), PB (lane 3), and pyrazole-treated (lane 4) mice (middle panel) and rats (bottom panel) were applied. Lane 6 contained 50 ng of purified P-450$_{Coh}$ in each panel.

negligible COH activity when assayed at low substrate concentrations [5–8].

In the present study we show, however, that low but detectable COH activity actually exists in rat liver microsomes, but it can be demonstrated only with very high substrate concentrations of up to 10 mM. Although the Michaelis–Menten constant for the rat liver enzyme is considerably higher than that of the human or mouse enzyme, the rat COH is inhibited by the same inhibitors as the mouse and human enzyme [7, 28, this work], thus demonstrating similar properties. One possibility could be that the rat liver COH is in evolutionary terms an analogous P-450 form with a mutation affecting substrate binding. The present results, however, strongly suggest that this is not the case.

Clear evidence that COH activities in rat and man (and mouse) are distinct at least concerning the active sites comes from the present experiments with the anti-P-450$_{Coh}$ antibody. The human COH activity was invariably inhibited 98–99% by the antibody at the antibody:microsomal protein ratio of 5:1, whereas the rat PB-induced COH level was not appreciably affected. Analogously with the human enzyme, DBA/2N mouse liver COH was also inhibited more than 85% regardless of the pretreatment.

Conclusions from the inhibition experiments with the antibody are that (1) coumarin 7-hydroxylating P-450s, or at least epitopes at or near the enzyme active site, are immunologically quite similar in human and mouse liver, and (2) although there is marked variation in COH activity in human liver microsomal preparations from different individuals, the response to immunological inhibition is uniform. The immunological relatedness between human and mouse enzymes was further assessed by immunochemical techniques. In Ouchterlony immunodiffusion analysis, all human, mouse and rat microsomes gave prominent immunoprecipitation bands with the anti-P-450$_{Coh}$ antibody. The precipitation patterns suggested almost full identity between mouse and rat antigens and no identity between rat and human antigens.

Western immunoblot analysis yielded more insight into the immunochemical properties of the enzyme. In all four human preparations anti-P-450$_{Coh}$ antibody recognized a distinct protein species with a molecular mass of 48 kDa. The relative intensity of this band correlated with the catalytic activity of COH in each preparation. In the mouse microsomes, staining was seen with control, BP and pyrazole-treated mouse samples. The possibility that the microsomal proteins recognized by the antibody are coincidentally related to P-450$_{Coh}$ is practically excluded by the findings that (1) the antibody efficiently inhibits COH catalytic activity without interfering with two other P-450-mediated activities (ERDE and AHH) and that (2) the anti-P-450$_{Coh}$-reactive protein in mouse microsomes comigrates exactly with purified P-450$_{Coh}$. In the rat microsomal preparations, weak diffuse staining with no prominent band was seen, suggesting that the precipitin line obtained in Ouchterlony immunodiffusion analysis is due to the presence of other anti-P-450$_{Coh}$-detectable epitopes besides the coumarin 7-hydroxylating protein in rat liver microsomes.

COH belongs to the P-450IIB subfamily of the P-450 gene product superfamily (see Ref. 29). To delineate the effect of the anti-P-450$_{Coh}$ antibody on catalytic activities associated with other subfamilies, we examined the response to the antibody of ECDE and AHH, which are partly, and ERDE, which is fully associated with the P-450IA subfamily (see Ref. 30). The results show that of these three activities only ECDE is inhibited, most notably in pyrazole-treated mice (87%). This is consistent with our previous studies showing that reconstituted P-450$_{Coh}$ also catalyzes 7-ethoxycoumarin *O*-deethylation in addition to coumarin 7-hydroxylation (Järvinen *et al.*, unpublished) and with other studies showing that the deethylation of 7-ethoxycoumarin is mediated by several P-450 isozymes in both P-450IA and P-450IIB families [22].

In conclusion, the present data show that the human and mouse P-450 isozymes catalyzing the 7-hydroxylation of coumarin are immunologically similar at their active centers although they have slightly differing molecular masses (48 kDa in the human vs 50 kDA in the mouse). On the other hand, the rat appears to lack a specific coumarin 7-

hydroxylating protein, and any COH activity detected in the rat may be a side-reaction by other P-450 isozymes principally involved in the oxygenation of other substrates.

Acknowledgements—The expert technical assistance of Ms Ritva Tauriainen is gratefully acknowledged. This study was financially supported by the Academy of Finland Council for Medical Research (Research Contract no. 04/320).

REFERENCES

1. International Agency for Research on Cancer, *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemical to Man*, Vol. 10, *Some Naturally Occurring Substances*, p. 113. IARC, Lyon, 1976.
2. Cohen AJ, Critical review of the toxicology of coumarin with special reference to interspecies differences in metabolism and hepatotoxic response and their significance to man. *Fd Cosmet Toxicol* **17**: 277–289, 1979.
3. Kaighen M and Williams RT, The metabolism of (3-^{14}C)-coumarin. *J Med Pharm Chem* **3**: 25–43, 1961.
4. Kapitulnik J, Poppers PJ and Conney AH, Comparative metabolism of benzo(*a*)pyrene and drugs in human liver. *Clin Pharmacol Ther* **21**: 166–176, 1977.
5. Creaven PJ, Parke DV and Williams RT, A spectrofluorimetric study of the 7-hydroxylation of coumarin by liver microsomes. *Biochem J* **96**: 390–398, 1965.
6. Gibbs PA, Janakidevi K and Feuer G, Metabolism of coumarin and 4-methylcoumarin by rat liver microsomes. *Can J Biochem Physiol* **49**: 177, 1971.
7. Pelkonen O, Sotaniemi EA and Ahokas JT, Coumain 7-hydroxylase activity in human liver microsomes. Properties of the enzyme and interspecies comparisons. *Br J Clin Pharmac* **19**: 59–66, 1985.
8. Wood AW and Conney AH, Genetic variation in coumarin hydroxylase activity in the mouse (mus musculus). *Science* **185**: 612–614, 1974.
9. Lush IE and Andrews KM, Genetic varition between mice and their metabolism of coumarin and its derivatives. *Genet Res (Cambridge)* **31**: 177–186, 1978.
10. Juvonen R, Kaipainen P, Hänninen O and Lang M, Coumarin 7-hydroxylase in inbred strains of mice: comparision with other microsomal monooxygenase activities. *Arch Toxicol* Suppl 9: 367–370, 1986.
11. Boobis AR, Brodie MJ, Kahn GC, Fletcher DR, Saunders JH and Davies DS, Monooxygenase activity of human liver in microsomal fractions of needle biopsy specimens. *Br J Clin Pharmacol* **9**: 11–19, 1980.
12. Beaune P, Dansette P, Flinois JP, Columelli S, Mansuy D and Leroux JP, Partiapurifiati of human liver cytochrome P450. *Biochem Biophys Res Commun* **88**: 826–832, 1979.
13. Wang PP, Beaune P, Kaminsky LS, Dannan GA, Kadlubar FF, Larrey D and Guengerich FP, Purification and characterization of six cytochrome P-450 isozymes from human liver microsomes. *Biochemistry* **22**: 5375–5383, 1983.
14. Pelkonen O, Pasanen M, Kuha H, Gachalyi B, Kairaluoma M, Sotaniemi EA, Park SS, Friedman FK and Gelboin HV, The effect of cigarette smoking on 7-ethoxyresorufin O-deethylase and other monooxygenase activities in human liver: analyses with monoclonal antibodies. *Br J Clin Pharmacol* **22**: 125–134, 1986.
15. Fujino T, Park SS, West D and Gelboin HV, Phenotyping of cytochromes P-450 in human tissues with monoclonal antibodies. *Proc Natl Acad Sci USA* **79**: 3682–3686, 1982.
16. Sesardic D, Boobis AR, McQuade J, Baker S, Lock EA, Elcombe CR, Robson RT, Hayward C and Davies DS, Inter-relatedness of some isoenzymes of cytochrome P-450 from rat, rabbit and human, determined with monoclonal antibodies. *Biochem J* **236**: 569–577, 1986.
17. Wolff T, Distlerath LM, Worthington MT and Guengerich FP, Human liver debisoquine 4-hydroxylase: test for specificity toward various monooxygenase substrates and model of the active site. *Arch Toxicol* **60**: 89–90, 1987.
18. Sotaniemi EA, Pelkonen O, Mokka RE, Huttunen R and Viljakainen E, Impairment of drug metabolism in patients with liver cancer. *Eur J Clin Invest* **7**: 269–274, 1977.
19. Juvonen R, Shkumatov V and Lang M, Purification and characterization of a liver microsomal P-450 isoenzyme with a high affinity and metabolic capacity for coumarin from pyrazole treated D2 mice. *Eur J Biochem* **171**: 205–211, 1988.
20. Aitio A, A simple and sensitive assay of 7-ethoxycoumarin deethylation. *Anal Biochem* **85**: 488–491, 1978.
21. Nebert DW and Gelboin HV, Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. *J Biol Chem* **243**: 6242–6249, 1968.
22. Greenlee WF and Poland A, An improved assay of 7-ethoxycoumarin O-deethylase activity: induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Pharmacol Exp Ther* **205**: 596–605, 1978.
23. Burke MD, Prough RA and Mayer RT, Characteristics of a microsomal cytochrome P-448-mediated reaction ethoxyresorufin *O*-deethylation. *Drug Metab Disp* **5**: 1–8, 1978.
24. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
25. Ouchterlony Ö, Antigen–antibody reactions in gels. *Acta Pathol Microbiol Scand* **26**: 507–513, 1949.
26. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227**: 680, 1970.
27. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
28. Wood AW, Genetic regulation of coumarin hydroxylase activity in mice. Biochemical characterization of the enzyme from two inbred strains and their F1 hybrid. *J Biol Chem* **254**: 5641–5646, 1979.
29. Nerbert DW, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato R and Waterman MR, The P450 gene superfamily; recommended nomenclature. *DNA* **6**: 1–11, 1987.
30. Åström A and DePierre JW, Rat-liver microsomal cytochrome P-450: purification, characterization, multiplicity and induction. *Biochim Biophys Acta* **853**: 1–28, 1986.