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IMMUNOCHEMICAL DETECTION OF HUMAN LIVER CYTOCHROME P450 FORMS RELATED TO PHENOBARBITAL-INDUCIBLE FORMS IN THE MOUSE

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Abstract—Polyclonal antibodies generated to four distinct mouse liver phenobarbital-inducible cytochrome P450 isoforms were used to analyse related forms in human liver. N-terminal sequence analysis and biochemical properties of the P450s used as antigens suggest that they belong to P450 subfamilies IIB (P450PBI), IA (P450PBII), IIC (P450PBIII) and IIA (P450Coh). In immunoblot analysis, anti-P450PBII detected a single protein presumed to be P450IA2 in all the human livers tested. No proteins corresponding with P450IA1 could be detected. Anti-PBIII and anti-P450Coh antibodies each detected one band (54 and 48 kDa, respectively) in the liver samples. No bands were revealed by anti-P450PBI antibody. Protein dot-immunobinding analysis showed that P450s immunodetectable by anti-P450PBII, anti-P450PBIII and anti-P450Coh antibodies are expressed in human liver (range 9 to 69 pmol P450/mg protein). In immunoinhibition experiments the activity of 7-ethoxyresorufin *O*-deethylase (EROD) was blocked up to 90% by the anti-P450PBII antibody. Aryl hydrocarbon hydroxylase (AHH) was inhibited only by anti-P450PBIII, and coumarin 7-hydroxylase (COH) only by anti-P450Coh antibody. Testosterone hydroxylations in positions 6β, 7α, 15α and 16α were not affected significantly by any of the antibodies. These data suggest that the human liver P450IA2 is responsible for most of the elevated EROD activity, P450s in the IIC subfamily for constitutive AHH and P450s in the IIA subfamily for all of COH activity.

Numerous exogenous and endogenous compounds are metabolized by the microsomal cytochrome P450 system [1]. In mammals, the P450 gene superfamily consists of eight families. Until now, over 20 human P450 genes have been sequenced [2]. Sixteen P450 genes are known to be expressed in human tissues [3]. A major goal in human P450 research is to correlate individual variability in the levels of P450 isoforms with variable metabolism of drugs and carcinogens. Before this can be achieved the substrate preferences of the various human P450 forms must be clarified.

The assessment of human P450 isoforms involves two main lines of study: purification of human P450s and structural studies of their genes and employment of antibodies against heterologous P450s in search for related human P450 forms [3].

We have recently isolated and characterized four forms of phenobarbital-inducible marine P450 [4, 5]. P450Coh catalyses the 7-hydroxylation of coumarin, and it has proved to be closely related to the $P450_{15\alpha}$ catalysing testosterone hydroxylation in the 15α-position [6, 7]. From N-terminal amino acid analysis, the other forms purified from mouse liver, P450PBI, P450PBII and P450PBIII, appear related to rat forms P450b, P450d and P450f, respectively [5].

In this study, antibodies raised against these four P450 forms have been used to identify structurally related proteins in the human liver. In addition, the antibodies were used to delineate the contribution of each of these P450 forms to several human hepatic P450-mediated reactions.

MATERIALS AND METHODS

P450 nomenclature. An updated list of the sequenced P450 genes was published recently [2]. The new proposed nomenclature for P450 gene products will be used in this paper interchangeably with the trivial names of individual P450 forms.

Materials. The sources of the chemicals used are given in previous reports [5, 8].

Human liver specimens. Liver samples (N = 7, 2 females, 5 males) were obtained at laparotomy from patients undergoing surgery for either primary liver tumors or hepatic metastases. The mean age of the patients was 59 years (range 40 to 72). One of the patients was a smoker, one was an ex-smoker and the remaining five did not report any smoking. In each case the histology of the tumor and the surrounding liver tissue was determined. Only tissue outside the tumors was used for this study. One sample (number 4) was excluded from the study since it consisted mostly of tumor tissue. The use of surplus liver tissue was approved by the Ethics Committee of the Medical Faculty, University of Oulu.

Microsomal fractions were prepared by standard differential centrifugation. In short, liver tissue was minced with scissors and homogenized in a glass

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homogenizer in 4 volumes of 0.1 M sodium–potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 10,000 *g* for 30 min and the obtained supernatant was centrifuged at 100,000 *g* for 1 hr. The microsomal pellet was washed and stored at −70° until used.

Purification of mouse liver P450s. P450s PBI, PBII and PBIII were isolated from phenobarbital-treated DBA/2N mouse liver as described in detail previously [5]. P450Coh was purified from pyrazole-treated DBA/2N mouse liver [4]. All these proteins were electrophoretically homogeneous and had specific P450 contents of 17.0, 11.2, 19.4 and 19.9 nmol/mg protein for P450s PBI, PBII, PBIII and Coh, respectively. Based on N-terminal sequence analysis and biochemical properties [5], P450s PBI and PBIII can be tentatively assigned as members of P450 subfamilies IIB and IIC, respectively. The N-terminal amino acid sequence of P450PBII shows 100% homology with mouse P_3450, and can thus be assigned as a member of the IA subfamily. P450Coh has been conclusively shown to be identical with type II $P450_{15\alpha}$, a member of subfamily IIA [6, 7].

Polyclonal antibodies were raised against the purified P450s in New Zealand White rabbits as described [5], purified by ammoniumsulphate precipitations and protein content was measured. The antibodies exhibited minor (<10%) cross-reactivity in dot-immunobinding analysis with the purified proteins, but not in Western immunoblot analysis. All the antibodies were checked with the three other heterologous isoforms.

Dot-immunobinding and immunoblot analysis. Dot-immunobinding analysis was done by a modification of Domin *et al.* [9]. Microsomal samples were diluted serially in Tris-buffered saline (TBS*) containing 0.1 mg/mL of bovine serum albumin as carrier protein. The samples were applied onto nitrocellulose filters and were serially treated with (i) a blocking solution (2% defatted powdered milk diluted in TBS), (ii) anti-P450 IgGs at 1:500–1:1000 dilutions, (iii) goat anti-rabbit IgG alkaline phosphatase conjugate (1:1000 dilution in the blocking solution) and (iv) 5-bromo-4-chloro-indolyl phosphate (0.8 mg/mL in 0.1 M $NaHCO_3$ and 1 mM $MgCl_2$, pH 9.1). Between each step the filters were washed extensively with TBS supplemented with 0.05% Tween-20. The intensities of the staining reactions were quantified with a Shimadzu CS-930 scanner. The microsomal content of each of the isozymes was determined from the linear part of the slope derived from serially diluted samples (N = 4) and standards. The limit of detection for different forms was between 0.2 and 0.8 pmol/mg protein. It is possible that quantitation of proteins in human liver microsomes is relative rather than absolute because slightly differing epitopes in mouse and human proteins may lead to differing staining responses.

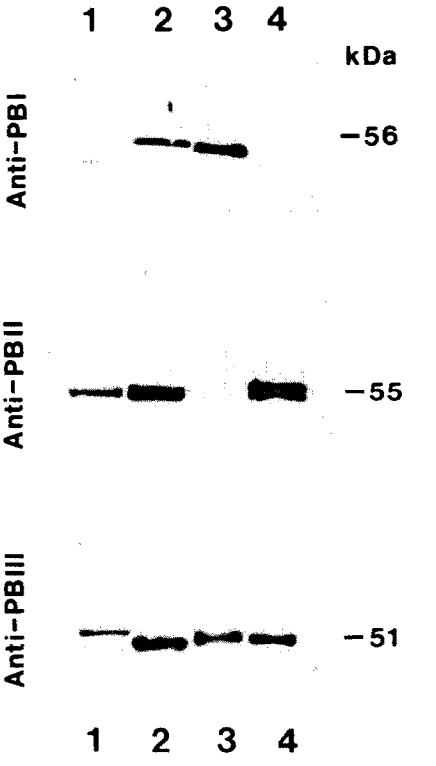

Fig. 1. Immunoblot analysis of human microsomes and reference samples. In each panel, the lanes contained: (1) microsomes (5 μg) from a single representative human liver; (2) the respective purified mouse liver P450; (3) microsomes (0.5 μg) from phenobarbital-treated rat liver; (4) microsomes (2.5 μg) from 3-methylcholanthrene-treated rat liver. The filters were probed with antibodies against P450PBI (top), P450PBII (middle), and P450PBIII (bottom). The minimum molecular masses of the purified mouse P450s are given in kilodaltons (kDa).

Western immunoblot analyses were done as reported previously [8].

Enzyme assays and immunoinhibition analysis. The activities of 7-ethoxyresorufin *O*-deethylase (EROD) and 7-pentoxyresorufin *O*-deethylase (PROD) were measured with the end-point method of Burke *et al.* [10] using 1 μM 7-ethoxyresorufin and 2 μM 7-pentoxyresorufin as substrates. The activities of 7-ethoxycoumarin *O*-deethylase (ECOD) and coumarin 7-hydroxylase (COH) were measured according to Aitio [11] with 0.1 mM substrate concentrations. The activity of aryl hydrocarbon hydroxylase (AHH) was determined by the method of Nebert and Gelboin [12] with 70 μM benzo(*a*)pyrene as substrate. Testosterone hydroxylase activities were determined as described by Waxman *et al.* [13]. Testosterone concentration was 100 μM and the limit of detection of metabolites about 5–10 pmol/min/mg protein. The Bradford method [14] was used for protein determinations.

Immunoinhibition assays were done by first determining the amount of antibody needed for maximal inhibition of the key monooxygenase reactions in a few specimens and then applying this dose in all incubations. Identical amounts of preimmune IgG were always used as controls in the assays.

* Abbreviations used: AHH, aryl hydrocarbon (benzo(*a*)pyrene) hydroxylase; COH, coumarin 7-hydroxylase; ECOD, 7-ethoxycoumarin *O*-deethylase; EROD, 7-ethoxyresorufin *O*-deethylase; PROD, 7-pentoxyresorufin *O*-deethylase; TBS, Tris-buffered saline.

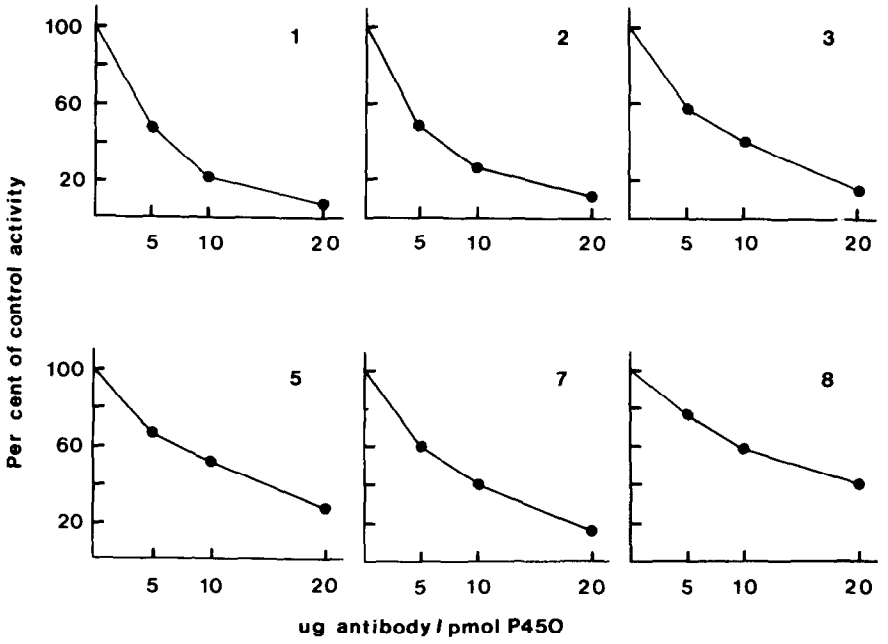

Fig. 2. Inhibition of EROD activity by anti-P450PBII antibody; 5–20 μg IgG/pmol P450 was used. Experiments with six individual human liver microsomes are shown. The liver specimen number is shown in each panel.

Table 1. Dot-immunobinding analysis of human liver microsomes

Liver sample No.	P450PBI	P450PBII (pmol/mg protein)	P450BPIII	P450Coh
1	2	24	53	38
2	3	25	69	28
3	2	14	63	46
5	4	10	67	39
6	2	9	52	44
7	2	25	38	25
8	2	16	32	25

The analysis was done as described in Materials and Methods.

Statistics. Correlation coefficients were calculated with linear regression (least-square) analysis.

RESULTS

Immunoblots of human liver microsomes from seven patients were developed with antibodies against the purified P450s. As controls, the purified mouse liver P450s and liver microsomes from 3-methylcholanthrene and phenobarbital-treated rats were analysed on the same blots (Fig. 1).

As expected, anti-P450PBI antibody recognized a single 52kDa band (presumably the P450b+e complex) in phenobarbital-treated rat liver microsomes. No distinct bands were observed in human microsomes. Anti-P450PBII readily recognized two proteins (most likely P450c and P450d) in 3-methylcholanthrene-pretreated rat liver microsomes. A single band migrating similarly with purified P450PBII (55 kDa) was detected by anti-P450PBII in all the human microsomes tested. Anti-P450PBIII antibody reacted with a single approximately 52-kDa protein in 3-methylcholanthrene- and phenobarbital-treated rat liver microsomes. A protein with a M_r of about 54 kDa was detected by anti-P450PBIII in all the human liver microsomes (Fig. 1). Anti-P450Coh antibody detected a 48-kDa protein in the human livers (shown in Ref. 15).

In dot-immunobinding analysis, the antibodies against mouse P450 forms PBI, PBII, PBIII and Coh reacted with human liver microsomes in a dose-dependent fashion. Quantitation of the staining showed that there is less than 4 pmol/mg of P450(s) recognized by anti-P450PBI antibody, whereas P450(s) recognized by antibodies against P450PBIII and P450Coh are abundantly present in human liver microsomes (Table 1). The amount of P450s reacting with anti-P450PBII varies between 9 and 25 pmol/mg protein in the seven liver samples tested (Table 1).

Table 2. Immunoinhibition of human liver monooxygenase activities by anti-P450 antibodies

Substrate	Liver specimen number						
	1	2	3	5	6	7	8
EROD							
Activity	9.31*	3.47	2.26	1.53	1.29	4.94	1.04
+ anti-PBI	39†	64	83	76	97	80	90
+ anti-PBII	8	11	15	27	26	16	41
+ anti-PBIII	86	83	92	87	126	97	108
+ anti-Coh	103	92	100	101	118	104	105
+ preimmune serum	101	97	108	114	128	105	105
ECOD							
Activity	0.44	0.27	0.44	0.27	0.23	0.40	0.23
+ anti-PBI	85	152	101	114	124	97	185
+ anti-PBII	47	58	88	74	94	55	84
+ anti-PBIII	108	100	85	98	97	104	110
+ anti-Coh	82	69	40	42	49	66	37
+ preimmune serum	108	108	105	106	101	101	118
AHH							
Activity	0.15	0.09	0.14	0.18	0.10	0.17	0.13
+ anti-PBI	128	159	128	88	145	150	115
+ anti-PBII	117	143	125	129	129	147	ND
+ anti-PBIII	61	51	57	28	71	55	38
+ anti-Coh	221	181	180	123	190	168	160
+ preimmune serum	159	153	128	115	141	167	170
COH							
Activity	0.81	0.76	1.83	1.18	1.38	1.30	1.46
+ anti-PBI	125	110	113	103	111	93	109
+ anti-PBII	119	119	102	101	116	109	101
+ anti-PBIII	124	132	96	99	112	107	114
+ anti-Coh	2	2	9	0	1	1	1
+ preimmune serum	85	109	89	101	72	80	87

* The activities are in nmol/nmol P450/min.
† The numbers denote per cent of control activity.
ND, not determined.

The contribution of the P450 isozymes recognized by the antibodies to "diagnostic" catalytic activities was studied by enzyme immunoinhibition. Initial studies with doses up to 20 μg IgG/pmol P450 indicated that human liver EROD activity is substantially inhibited by the anti-P450PBII antibody (Fig. 2).

Table 2 lists the effects of the antibodies on monooxygenase activities in the human liver samples. Anti-P450PBII antibody inhibited EROD activity by 59–92%, the inhibition being greatest in livers with high EROD activity. Also anti-P450PBI antibody clearly inhibited EROD activity in the high-activity liver samples indicating that both antibodies may react with P450s mediating EROD. The other antibodies and preimmune serum caused no marked effects. The activity of ECOD was also variably inhibited by anti-P450PBII antibody (6–53%), and also anti-P450Coh antibody decreased this activity (18–63%). The only antibody capable of inhibiting AHH activity was anti-P450PBIII (29–72% decrease). The other antibodies appeared to stimulate AHH activity. COH activity was efficiently blocked by anti-P450Coh antibody (98–100% inhibition), while all other antibodies failed to appreciably affect this activity. The activity of 7-pentoxyresorufin *O*-deethylase (PROD) was negligible in all the livers tested (data not shown).

Table 3. Testosterone hydroxylation by human liver microsomes

Sample No.	Testosterone metabolite formation			
	15α	16α	7α	6β
1	0.01	<0.01	0.01	0.60
2	0.01	0.01	0.02	0.87
3	0.01	<0.01	0.02	0.37
5	0.01	<0.01	0.01	0.14
6	0.02	0.01	0.03	0.98
7	0.01	0.01	0.02	0.64
8	0.01	0.01	0.02	0.55

The values are given in nmoles metabolite formed/nmol P450/min.

The testosterone hydroxylation profile of the liver samples is shown in Table 3. The major position of testosterone hydroxylation catalysed by human liver microsomes was 6β, while hydroxylations at positions 7α, 15α and 16α occurred at a low rate. The effect of all the antibodies on testosterone hydroxylation was tested, and none of the hydroxylation pathways were found to be significantly affected (data not shown).

Linear regression analysis showed that there exists a good correlation between the activity of EROD and the amount of immunodetectable P450PBII ($r = 0.72$, $P < 0.01$). No other significant correlations could be found between enzyme activities and P450 concentrations. Despite the ability of anti-P450Coh antibody to eliminate almost 100% of COH activity in all the liver samples tested, the correlation between COH activities and the content of immunodetectable P450Coh was rather poor ($r = 0.33$) in this material. The reason for this poor correlation may be immunological cross-reactivity, i.e. that the human liver contains several P450 isoforms recognized by the anti-Coh antibody.

DISCUSSION

In this study, antibodies to murine liver phenobarbital-indicible P450 isoforms were used to characterize related forms in the human liver. In this type of study it is important to note the possibility of cross-reactivity of murine antibodies in dot-blotting with several closely related isozymes of human liver. A minor cross-reactivity with distinct purified murine isozymes does not guarantee that cross-reactivity of a greater degree could not occur in the human samples. However, with this reservation in mind, the results corroborate several earlier findings and provide new data on substrate specificities of human hepatic P450s.

We were unable to detect in immunoblot analysis P450s in human liver microsomes with the anti-P450PBI antibody. The minor staining occurring in dot-immunobinding analysis with this antibody may reflect cross-reactivity with other P450s. In addition, the activity of PROD, which is considered diagnostic of phenobarbital-inducible P450s [16], was extremely low in all the liver samples tested. Indirect evidence suggests that human liver may contain phenobarbital-inducible P450s [17]. Polyclonal antibodies against phenobarbital-induced rat liver P450s recognize proteins also in human liver microsomes [18, 19]. Studies with cDNA clones isolated from human liver libraries with rat IIB cDNA probes [20] suggest that at least two IIB genes exist in humans. Despite some attempts [21], no proteins unequivocally belonging to the IIB subfamily have yet been purified from human tissues, and the enzymatic specificities of the putative human IIB P450s are unknown.

Anti-P450PBII antibody readily reacted with what are presumed to be P450 forms c and d (IA1 and IA2) in rat liver and detected also a form related to P450IA2 in all the human livers tested. cDNA analyses have shown that human cells contain genes for both members in the IA subfamily [22–24]. Studies using epitope-specific monoclonal antibodies against rat P450c and P450d have shown that a P450 structurally and immunochemically related to P450d is invariably present in human liver [25]. In recent studies with immunological and chemical probes, P450IA2 has been shown to be expressed and inducible in the human liver whereas P450IA1 is present mainly in extrahepatic tissues [26, 27]. A P450 closely resembling P450d has also been purified from human liver [28]. The inhibition of EROD activity by the anti-PBI antibody may indicate that the human protein contains an epitope (or epitopes) which is recognized by the anti-PBI antibody and which is not present in the murine enzyme.

Previous studies with monoclonal antibody 1-7-1 generated against rat liver P450 forms c and d [29] showed that in the human liver, EROD activity is blocked by from 36% [30] to 60% [31] by this antibody while AHH, ECOD and COH are not affected. None of these activities are affected by the monoclonal antibody 2-66-3 generated against rat liver P550b+e. The immunoinhibition data obtained in the present study agree well with the results described above. It now appears obvious that in the human liver P450IA2 is responsible for most of constitutive EROD activity. Elevated activities may also be supported by other isoforms.

Anti-P450PBIII antibody markedly inhibited AHH activity in human liver microsomes as it does in the mouse [5]. Meehan *et al.* [32] reported recently that the mouse P450IIC family is closely associated with constitutive AHH activity. Recent studies have shown that at least three proteins are found in the human IIC subfamily [3]. Immunochemical studies such as those described here are not capable of differentiating between very close members of the P450 subfamily, so the identity of the form catalysing AHH activity remains uncertain. However, the present finding has direct implications on interpreting findings linking AHH activity to certain types of human cancers [33]. We have shown that the degree of inhibition of AHH activity by monoclonal antibody 1-7-1 correlates poorly with the inhibition of benzo(*a*)pyrene diolepoxide–DNA adduct formation by human liver microsomes [34]. This could now be explained by the inability of monoclonal antibody 1-7-1 to block P450s in the IIC subfamily which appears to contribute to a major part of human liver constitutive AHH activity but not to formation of ultimate carcinogenic metabolites of benzo(*a*)pyrene.

The inability of antibodies against P450 forms in subfamilies IA and IIB to affect human liver ECOD activity has been reported earlier [30, 31]. A likely explanation to this is provided in this study: constitutive ECOD activity is blocked most efficiently by anti-P450Coh antibody, suggesting that P450Coh is responsible for a significant part of human liver constitutive ECOD activity. Elevated activities seem to be mediated mostly by P450s in the IA subfamily, most likely P450IA2.

Previous studies have shown that in the mouse, testosterone 15α-hydroxylase and COH activities are mediated by a pair of closely related gene products, Type I and Type II $P450_{15\alpha}$ [6, 7, 35]. Based on the present studies the relationship between Type I and Type II $P450_{15\alpha}$ appears to be different in human and mouse livers. First, testosterone 15α-hydroxylase activity is very low in the human liver compared with the mouse liver, and human liver COH activities greatly exceed the constitutive levels in mice [15]. Second, anti-Coh antibody does not inhibit any of the four studied testosterone hydroxylations, including the hydroxylation at position 15α. The poor correlation between COH activity and immunodetectable P450Coh isoform observed here suggests

that the human liver may contain several immunologically indistinguishable P450 gene products having differing substrate specificity, as proposed recently by Lindberg and Negishi [36].

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