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Development of a Comprehensive Panel of Antibodies against the Major Xenobiotic Metabolising Forms of Cytochrome P450 in Humans

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ABSTRACT. Mono-specific antibodies against the human cytochrome P450 (P450) enzymes CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP4A11 and an antibody that binds to CYP2C8, CYP2C9 and CYP2C19 have been produced by immunising rabbits with synthetic peptides representing small regions of each of these P450 enzymes. The specificity of the antibodies was confirmed by immunoblotting using recombinant P450 enzymes and samples of human hepatic microsomal fraction. Each of the antibodies bound only to their respective target P450 enzyme(s). The relative intensity of immunoreactive bands was compared with a variety of P450 activities and correlations were found between CYP1A2 and phenacetin *O*-deethylase activity, CYP2A6 and coumarin 7-hydroxylase activity, CYP2C9 and tolbutamide 4-hydroxylase activity, CYP2C19 and S-mephenytoin 4-hydroxylase activity, CYP2D6 and debrisoquine 4-hydroxylase activity, CYP2E1 and chlorzoxazone 6-hydroxylase activity, CYP3A4 and midazolam 1′-hydroxylase activity, and CYP4A11 and lauric acid 12-hydroxylase activity. A proportion of the 30 liver samples examined lacked CYP2A6 (7%), CYP2C19 (10%) or CYP2D6 (13%), consistent with the polymorphic expression of these P450 enzymes in human liver. Although CYP3A5 was detected in most individuals (97%), expression was polymorphic with 20% containing substantially higher levels. CYP2B6 was expressed in 20% of the human liver samples, with one sample containing a particularly high level. No immunodetectable CYP1A1 or CYP1B1 was found, consistent with the low level of expression of these P450 enzymes in human liver. The results demonstrate the utility of the antipeptide approach for producing specific antibodies against human P450 enzymes, enabling a comprehensive panel of antibodies against human P450 enzymes to be produced. BIOCHEM PHARMACOL **56**;3:377–387, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cytochrome P450, anti-peptide antibodies, human, liver

The numerous P450† enzymes present in the endoplasmic reticulum of the liver and other organs catalyse the oxidation of a wide range of compounds, including drugs, steroids, vitamins, prostaglandins and leukotrienes [1]. In human liver, P450 enzymes can be constitutively expressed, may be subject to induction, and may be expressed polymorphically [2–4]. As a result, the level of individual P450 enzymes is often highly variable. This has important consequences for drug metabolism, toxicology and carcinogenesis.

The identification and quantification of P450 enzymes present in human tissues may be determined *in vitro* by measuring enzyme activity using appropriate substrates. Some compounds are metabolised selectively by particular P450 enzymes and this has led to their use as substrates for quantifying the activity of particular P450 enzymes. However, the broad substrate specificity of most P450 enzymes means that very few substrates are metabolised exclusively by a single P450 enzyme. Consequently, an unknown proportion of any P450-catalysed reaction is likely to be due to the activity of unidentified P450 enzymes.

One alterative is to measure the level of P450 apoprotein using antibodies. Ideally, such antibodies should bind to individual P450 enzymes. Polyclonal antibodies raised against purified P450 enzymes tend to cross-react with related P450 enzymes [5]. Although this problem can be overcome by immunoabsorption chromatography [6], the procedure depends on the availability of suitable antigens. On the other hand, monoclonal antibodies, which recognise a single epitope, often bind selectively to particular P450 enzymes, although some do bind to more than one form of P450 [5, 7]. Many monoclonal antibodies have been produced against purified P450 enzymes and have been used widely [7]. Most of these antibodies have been raised against rodent P450 enzymes, however, their use has not

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† *Abbreviations:* Fmoc, *N*-α-9-Fluorenylmethoxycarbonyl; KLH, keyhole limpet haemocyanin; and P450, cytochrome P450.

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TABLE 1. Regions of human P450 enzymes targeted by antipeptide antibodies

P450	Target sequence
Antibodies directed towards the C terminus	
CYP1A1	-Met-Gln-Leu-Arg-Ser
CYP1B1	-Lys-Glu-Thr-Cys-Gln
CYP2C8	-Phe-Ile-Pro-Val
CYP2C9	-Phe-Ile-Pro-Val
CYP2C19	-Phe-Ile-Pro-Val
CYP2D6	-Ala-Val-Pro-Arg
CYP2E1	-Val-Ile-Pro-Arg-Ser
CYP3A4	-Thr-Val-Ser-Gly-Ala
CYP3A5	-Thr-Leu-Ser-Gly-Glu
CYP4A11	-Asp-Lys-Asp-Gln-Leu
Antibodies directed towards internal sequences	
CYP1A2 (284–296)	-Thr-Gly-Ala-Leu-Phe-Lys-His-Ser-Lys-Lys-Gly-Pro-Arg-
CYP2A6 (268–279)	-Ile-Asp-Ser-Phe-Leu-Ile-Arg-Met-Gln-Glu-Glu-Glu-
CYP2B6* (265–276)	-Ile-Asp-Thr-Tyr-Leu-Leu-**Arg**-Met-Glu-Lys-Glu-Lys-

Antibodies were raised against either the C terminus or an internal sequence of the various P450 enzymes.
*Antibodies were raised against the sequence from rat CYP2B1/2 which varies from CYP2B6 only in Arg for His.

been restricted to the species from which the immunogen was purified. Hence, monoclonal antirat P450 antibodies have been shown to bind to other related P450 enzymes in a variety of species, including human [8–10]. In the past, this has been useful for the identification of related forms in other species [8]. Nevertheless, unexpected cross-reactions frequently occur with P450 enzymes from different subfamilies [10, 11] limiting their use for this purpose. Monoclonal antibodies produced from mice immunised with human P450 enzymes avoid these problems [12, 13]. Even so, the production and characterisation of monoclonal antibodies is time consuming and expensive.

The use of synthetic peptides as immunogens offers a simple and effective means of producing antihuman P450 antibodies. In this approach, antibodies are raised against short synthetic peptides that represent small regions of a protein antigen [14]. Antibodies that bind specifically to P450 enzymes can be produced by targeting unique peptide sequences [15, 16]. Therefore, by consideration of the known sequences of human P450 enzymes [1], regions that are suitable as epitopes can be identified, the corresponding peptides synthesised, conjugated to carrier protein and antibodies raised. This is a relatively straightforward procedure, and as antipeptide antibodies usually bind equally to native and denatured protein they are particularly suited to immunoblotting studies [14].

Here the utility of the antipeptide antibody approach has been investigated by producing antibodies against all of the major human hepatic P450 enzymes in families 1, 2, 3 and 4. Antibodies were raised against peptides of 4–5 amino acid residues at the C terminus of P450 enzymes and peptides of 12–13 residues that occur as internal sequences. To assess the binding specificity of the antibodies immunoblotting of microsomal samples from human liver and recombinant human P450 enzymes was performed. The relative apoprotein levels in human liver samples were also compared with the activity of various P450-catalysed reactions.

MATERIALS AND METHODS

Materials

Fmoc amino acids linked to *p*-benzyloxybenzyl alcohol or Kieselguhr/polyamide resin, Fmoc-protected amino acid pentafluorophenyl esters and Fmoc-protected amino acid 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine esters were purchased from Novabiochem. HPLC columns were from Jones Chromatography and acetonitrile (grade S) was from Rathburn Chemicals. Sephadex G-15 and Sephadex G-25 were from Pharmacia. KLH was from Calbiochem. All SDS-polyacrylamide gel electrophoresis reagents were from National Diagnostics, except for prestained molecular weight standards, which were from Sigma. Goat antirabbit immunoglobulin conjugated to horseradish peroxidase was from ICN-Flow. All other chemicals were purchased from Sigma or Merck-BDH and were of analytical grade or the best equivalent.

Peptide Synthesis and Antibody Production

Peptides (Table 1) were synthesised using a Gem semi-automated peptide synthesiser (NovaBiochem). Fmoc solid-phase synthesis was carried out on either Kieselguhr or Polyhipe supports containing polydimethylacrylamide functionalised with ethylenediamine, norleucine and, the trifluoroacetic acid labile, 4-hydroxymethylphenoxyacetic acid group through which the C-terminal Fmoc-protected amino acid was coupled. For the purpose of coupling to carrier protein, KLH, a cysteine residue was added to the N terminus of each peptide. This allowed orientation-specific coupling of the peptide through its N terminus to KLH. Details of the method of synthesis have been published previously [17]. All peptides had the correct molecular weight by mass spectrometry. Antibodies were raised against peptide conjugates in New Zealand White rabbits as described previously [17].

The synthesis and conjugation of the peptide, Cys-Lys-

Glu-Thr-Cys-Gln, used to raise antibodies against the C terminus of CYP1B1, required some changes to the conditions used previously. To allow for the synthesis of a peptide with glutamine at the C terminus, an α-*t*-butyl ester of Fmoc-glutamic acid coupled through its side-chain carboxyl group to a *p*-methylbenzhydrylamide resin using a Rink Amide linkage was employed. On completion of the synthesis of the peptide chain, treatment of the solid-phase resin with trifluoroacetic acid (see below) resulted in the simultaneous release of the peptide and amidation of the side-chain carboxyl group of glutamic acid and thus formation of glutamine at the C terminus [18, 19]. To achieve specific coupling to carrier protein through the *N*-terminal cysteine, different side-chain protecting groups were used for each of the two cysteine residues in the peptide. The *N*-terminal cysteine was protected with a trityl group and the cysteine in the penultimate position from the C terminus was protected with a *t*-butylthio group. After deprotection and cleavage of the peptide from the solid-phase resin in 95% trifluoroacetic acid, 2.5% water and 2.5% triisopropylsilane for 2 hr, the *t*-butylthiocysteine remained protected whereas all other side-chain groups including the trityl group on the other cysteine were removed. This was confirmed by determination of the molecular weight of the product by thermospray mass spectroscopy. After coupling of the peptide to KLH, the t-butylthio group was removed by addition of 0.2 M 2-mercaptoethanol. The conjugate was then dialysed against 0.05 M sodium phosphate, 0.1 M sodium chloride, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4 to remove excess 2-mercaptoethanol and reaction products.

Human Liver

Histologically normal human liver samples from transplant donors were collected, processed, stored and the microsomal fraction prepared under the conditions described previously [20]. Local Ethics Committee approval and Coroner's permission were obtained for their use in these studies.

Recombinant P450 Enzymes

Samples of microsomal fraction prepared from human B-lymphoblastoid cell lines and insect cells transfected with plasmid vectors expressing human P450 enzymes and untransfected cells were purchased from Gentest Corporation. The specific content of P450 holoprotein determined spectrophotometrically in each of the preparations was 122 (CYP1A1), 133 (CYP1A2), 74 (CYP1B1), 132 (CYP2A6), 65 (CYP2B6), 590 (CYP2C8), 690 (CYP2C9), 385 (CYP2C19), 194 (CYP2D6-Met), 250 (CYP2D6-Val), 200 (CYP2E1), 60 (CYP3A4) and 244 (CYP3A5) pmol/mg protein. No P450 was detected in preparations from untransfected cells. Samples of yeast microsomal fraction containing recombinant CYP2D6-Val and CYP2D6-Met, specific contents 44 and 61 pmol/mg of protein, respectively, were a kind gift from Dr. Wynne Ellis (University of Sheffield, U.K.). It should be noted that in recombinant P450 enzyme preparations produced by heterologous expression the level of P450 holoprotein measured spectrophotometrically does not necessarily equate with the amount of P450 apoprotein detected by immunoblotting. As the quantity of apoprotein often exceeds that of the holoprotein, the values of P450 content may underestimate the amount of apoprotein in these preparations.

Immunoblotting

Immunoblotting was performed using between 2–75 μg of microsomal protein, as appropriate, under conditions described previously [17, 21]. In order to compare the relative immunoreactivity in thirty samples of human hepatic microsomal fraction, it was necessary to perform SDS-polyacrylamide gel electrophoresis using two separate gels for each immunoblot. To minimise variation in electrotransfer and subsequent development with antibodies, the central section of the resolving gels (each approximately 4 cm in length of 12-cm gels) were excised and placed adjacent to one another on a single nitrocellulose filter to allow simultaneous electrotransfer and development with antibodies. To assess variability, a pooled sample, produced by mixing several samples of human liver microsomal fraction containing detectable levels of each of the P450 enzymes, was applied to four separate wells. Blots were developed for immunoreactivity using the respective antiserum containing antibodies targeted against CYP1A1 (diluted 1:4000), CYP1B1 (diluted 1:1000), CYP1A2 (diluted 1:4000), CYP2A6 (diluted 1:1000), CYP2B6 (diluted 1:4000), CYP2C forms (diluted 1:4000), CYP2D6 (diluted 1:1000), CYP2E1 (diluted 1:4000), CYP3A4 (diluted 1:4000), CYP3A5 (diluted 1:4000), or CYP4A11 (diluted 1:1000). Antibody binding was detected using goat anti-rabbit IgG coupled to horseradish peroxidase (diluted 1:25,000) or protein G coupled peroxidase (12.5 ng/mL) and visualised using enhanced chemiluminescence, the result being recorded on Hyperfilm. The relative intensity of immunoreactive bands was determined by laser densitometry using an LKB UltroScan XL Enhanced Laser Densitometer (Pharmacia LKB Biotechnology, St. Albans, U.K.). The coefficient of variation of the intensity of the immunoreactive bands in the pooled samples was on average 12.7%.

Measurement of P450 Enzyme Activities

P450 activity was measured using eight different substrates, each of which is selectively metabolised by a particular human P450 enzyme. In each case, the assay procedure was similar. The specific conditions stated in Table 2 were such that enzyme activity was linear with respect to protein concentration and incubation time. Assay mixtures were incubated for 5 min at 37° in a shaking water bath before initiating the P450 reaction by addition of substrate, except for S-mephenytoin 4-hydroxylase activity where addition of

TABLE 2. Conditions for P450 activity determinations

Activity	Buffer and cofactors	Substrate concentration (μM)	Protein amount (μg)	Incubation time (min)	Incubation volume (ml)	Method of analysis	Reference
Phenacetin O-deethylase	75 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1.2 mM NADPH	4	200	15	1.00	GC-MS	[22]
Coumarin 7-hydroxylase	100 mM Tris-HCl, pH 7.25, 5 mM $MgCl_2$, 0.5 mM NADPH	100	100	10	0.50	Fluorescence	[21, 23]
Tolbutamide 4-hydroxylase	40 mM Tris-HCl, pH 7.4, 0.5 mM NADP, 3 mM glucose-6-phosphate, 2.5 units G6PDH	10	500	60	1.00	UV-HPLC	[24, 25]
S-mephenytoin 4-hydroxylase	70 mM potassium phosphate buffer, pH 7.4, 1.2 mM NADPH	200	200	60	1.00	UV-HPLC	[26]
Debrisoquine 4-hydroxylase	50 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 1.2 mM NADPH	1000	500	12	1.00	GC-MS	[27]
Chlorzoxazone 6-hydroxylase	50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5mM $MnCl_2$, 1 mM NADPH, 5 mM isocitric acid, 1.5 units isocitrate dehydrogenase	1000	60	15	0.12	UV-HPLC	[28]
Midazolam 1′-hydroxylase	10 mM phosphate, pH 7.4, 2 mM NADPH	60	500	10	1.00	UV-HPLC	[29]
Lauric acid 12-hydroxylase	50 mM Tris-HCl, pH 7.4, 1 mM NADPH	100	333	10	3.00	Radiometric-HPLC	[30]

NADPH was used to start the reaction. Reactions were terminated by addition of acid, base or organic solvent, as appropriate and the metabolite prepared as described in the publications cited in Table 2, except for S-mephenytoin 4-hydroxylase, where 4-hydroxymephenytoin was prepared by solid phase extraction after stopping the reaction by addition of 100 μL of 43% (v/v) phosphoric acid. C_{18} BondElut columns were washed twice with 2 mL of methanol, followed by two washes with 2 mL of MilliQ water, the reaction mixture added, the column washed twice with 2 mL of water and 4-hydroxymephenytoin eluted with 2 mL of methanol. The extract was dried under a stream of nitrogen gas, and then dissolved in 150 μL of water. Analysis by HPLC was performed by injecting 100 μL of extracted sample onto a 25 cm × 4.6 mm (internal diameter) Spherisorb ODS2 5-μm chromatography column. An isocratic mobile phase consisting of 0.5% (v/v) phosphoric acid:acetonitrile (70:30) was used at a flow rate of 1.3 mL/min and detection at 210 nm.

P450 Content

Total P450 content was estimated by carbon monoxide-difference spectroscopy as described previously [31].

RESULTS

Immunoblotting using recombinant P450 enzymes expressed in the microsomal fraction of lymphoblastoid cells or insect cells showed that the antibodies targeted against CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 bound only to their respective target antigens (Fig. 1). The CYP2C-targeted antibody bound to recombinant CYP2C8, CYP2C9 and CYP2C19, but to no other P450 enzyme (Fig. 1). The antibody targeted against the C terminus of CYP4A11 did not bind to any of these recombinant P450 enzymes (data not shown). The amount of each recombinant P450 loaded was in the range or exceeded the amount in samples of human liver microsomal fraction. Antibody specificity was maintained even when the amounts loaded greatly exceeded that present in human liver microsomal fraction (data not shown).

Antibodies targeted against CYP1A2, CYP2A6, CYP2B6, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP4A11 bound to single bands in samples of human hepatic microsomal fraction (Fig. 2). The anti-CYP2C antibody recognised three immunoreactive bands in samples of human hepatic microsomal fraction corresponding to CYP2C8, CYP2C9 and CYP2C19 (Fig. 2). Immunoreactive bands corresponding to CYP1A2, CYP2C8, CYP2C9, CYP2E1, CYP3A4 and CYP4A11 were detected in all 30 samples although one of the samples (number 16) contained a very low level of CYP2E1 and in another (number 13) the level of CYP4A11 was very low. CYP2A6 was detected in 28/30 samples, including samples 1, 25 and 26 where immunoreactivity was weak. No immunoreactivity was found in samples 18 and 19. CYP2D6 was detected in 26/30 samples, but was absent in samples 1, 13, 16 and 26. A clear assessment of the expression of CYP2C19 was difficult in five of the samples due to the relatively high levels of the closely migrating CYP2C8 band. For the purpose of quantification the level of CYP2C19 in these samples was recorded as zero. Also, it was necessary to increase the amount of antiserum used to develop the blot (to a dilution of 1:1000) to improve visualisation of CYP2C19 (data not shown). Nevertheless, CYP2C19 was certainly expressed in 22 of the samples and absent in 3 (samples 13, 16 and 26). Although CYP3A5 was expressed in most samples (29/30),

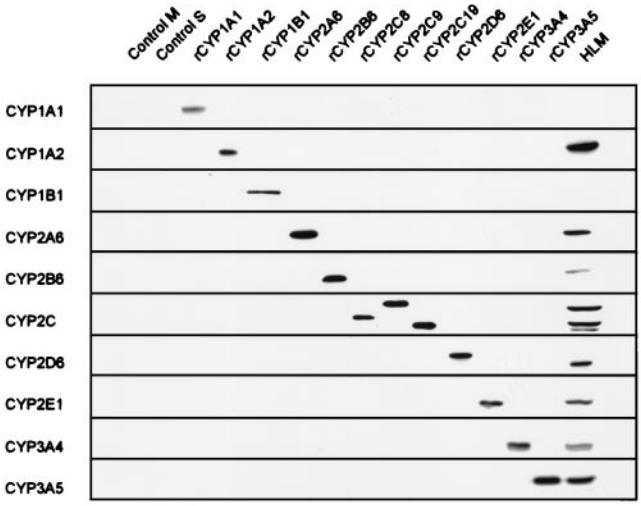

FIG. 1. Binding of antipeptide antibodies to recombinant P450 enzymes. The following amounts of microsomal protein from the samples indicated were applied to each lane: untransfected lymphoblastoid cells (control M) (10 μg), untransfected insect cells or "supersomes" (control S) (10 μg), transfected lymphoblastoid cells expressing recombinant CYP1A2 (rCYP1A2) (10 μg), recombinant CYP2A6 (rCYP2A6) (10 μg), recombinant CYP2B6 (rCYP2B6) (10 μg), recombinant CYP2D6-Val (rCYP2D6) (10 μg), recombinant CYP2E1 (rCYP2E1) (5 μg), or recombinant CYP3A4 (rCYP3A4) (10 μg), and transfected insect cells expressing recombinant CYP1A1 (rCYP1A1) (2 μg), recombinant CYP1B1 (rCYP1B1) (10 μg), recombinant CYP2C8 (rCYP2C8) (2 μg), recombinant CYP2C9 (rCYP2C9) (5 μg), recombinant CYP2C19 (rCYP2C19) (5 μg), or recombinant CYP3A5 (rCYP3A5) (5 μg). Immunoblotting of recombinant forms has been compared with 25 μg of human liver microsomal fraction (HLM) from a pooled sample, except for blots developed with antibodies targeted against CYP2C forms, CYP2D6 and CYP3A5 where liver samples numbered 8, 25 and 7, respectively, were used. Each blot was developed with an antipeptide antibody targeted against a different P450 enzyme (left column) under the conditions described in the Methods section. Only the central section of each immunoblot containing immunoreactive bands is shown.

6 clearly had particularly high levels (samples 7, 12, 19, 21, 22 and 27) (Fig. 2). CYP2B6 was found in only 6/30 samples (numbers 5, 6, 8, 12, 24 and 27), with one sample (number 5) containing a high level. In contrast, no immunoreactive bands were detected in any of the 30 liver samples analysed using the antibodies against CYP1A1 or CYP1B1 (Fig. 1) even when 75 μg of microsomal protein was used and the amount of antiserum used was increased to a dilution of 1:1000 (data not shown).

Although the electrophoretic migration of all of the recombinant forms of P450 was similar to the immunoreactive bands in hepatic microsomal fraction it was not always identical (Fig. 1). For example, recombinant CYP2D6 (CYP2D6-Val) expressed in lymphoblastoid cells or in insect cells migrated more slowly than CYP2D6 in hepatic microsomal fraction (Fig. 3). However, CYP2D6 expressed in yeast migrated identically to CYP2D6 in hepatic microsomal fraction (Fig. 3). The electrophoretic migration of the CYP2D6-Met variant expressed in yeast and lymphoblastoid cells was the same as CYP2D6-Val (Fig. 3).

The relative intensity of immunoreactive bands was compared with a variety of P450 activities. Highly significant correlations ($r > 0.70$; $P < 0.001$) were found between CYP1A2 and phenacetin O-deethylase activity, CYP2A6 and coumarin 7-hydroxylase activity, CYP2C9 and tolbutamide 4-hydroxylase activity, CYP2C19 and S-mephenytoin 4-hydroxylase activity, CYP2D6 and debrisoquine 4-hydroxylase activity, CYP2E1 and chlorzoxazone 6-hydroxylase activity, and CYP3A4 and midazolam 1′-hydroxylase activity (Table 3). Slightly lower correlations ($r = 0.63–0.66$, $P < 0.001$) were found between CYP4A11 and lauric acid 12-hydroxylase activity, CYP2C8 and lauric acid 12-hydroxylase activity, and CYP3A4 and coumarin 7-hydroxylase (Table 3).

There was little correspondence between immunoreactive P450 levels, except that CYP3A4 correlated with both CYP3A5 and CYP2A6, and CYP2C8 correlated with CYP2C9 (Table 4).

Amongst enzyme activities, coumarin 7-hydroxylase activity correlated with midazolam 1′-hydroxylase activity and lauric acid 12-hydroxylase correlated with phenacetin O-deethylase and S-mephenytoin 4-hydroxylase activities (Table 5).

The P450 content of the samples was compared with immunoreactive P450 levels and monooxygenase activity. It was found that P450 content correlated with the levels of CYP2A6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4, and with coumarin 7-hydroxylase, tolbutamide 4-hydroxylase, S-mephenytoin 4-hydroxylase, midazolam 1′-hydroxylase, and lauric acid 12-hydroxylase activities (Tables 4 and 5).

DISCUSSION

Antipeptide antibodies have been successfully produced against all of the major xenobiotic metabolising forms of P450 from families 1, 2, 3 and 4 expressed in human liver. The specificity of the antibodies was confirmed by immunoblotting using recombinant P450 enzymes and samples of human hepatic microsomal fraction and by comparison with monooxygenase activity measurements using a variety of P450 selective substrates. Antibodies monospecific for CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP4A11 were produced by targeting a unique region of each apoprotein. For CYP2C8, CYP2C9 and CYP2C19 a single antibody was produced against a common sequence and this bound to all three forms. In most cases, antibodies were directed towards the C terminus as previous work has shown that such antibodies bind strongly and specifically to such epitopes [32]. The method is highly reliable and requires the synthesis of only short peptides [32]. Thus, antibodies were raised against the C termini of CYP1A1, CYP1B1, CYP2C forms, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP4A11.

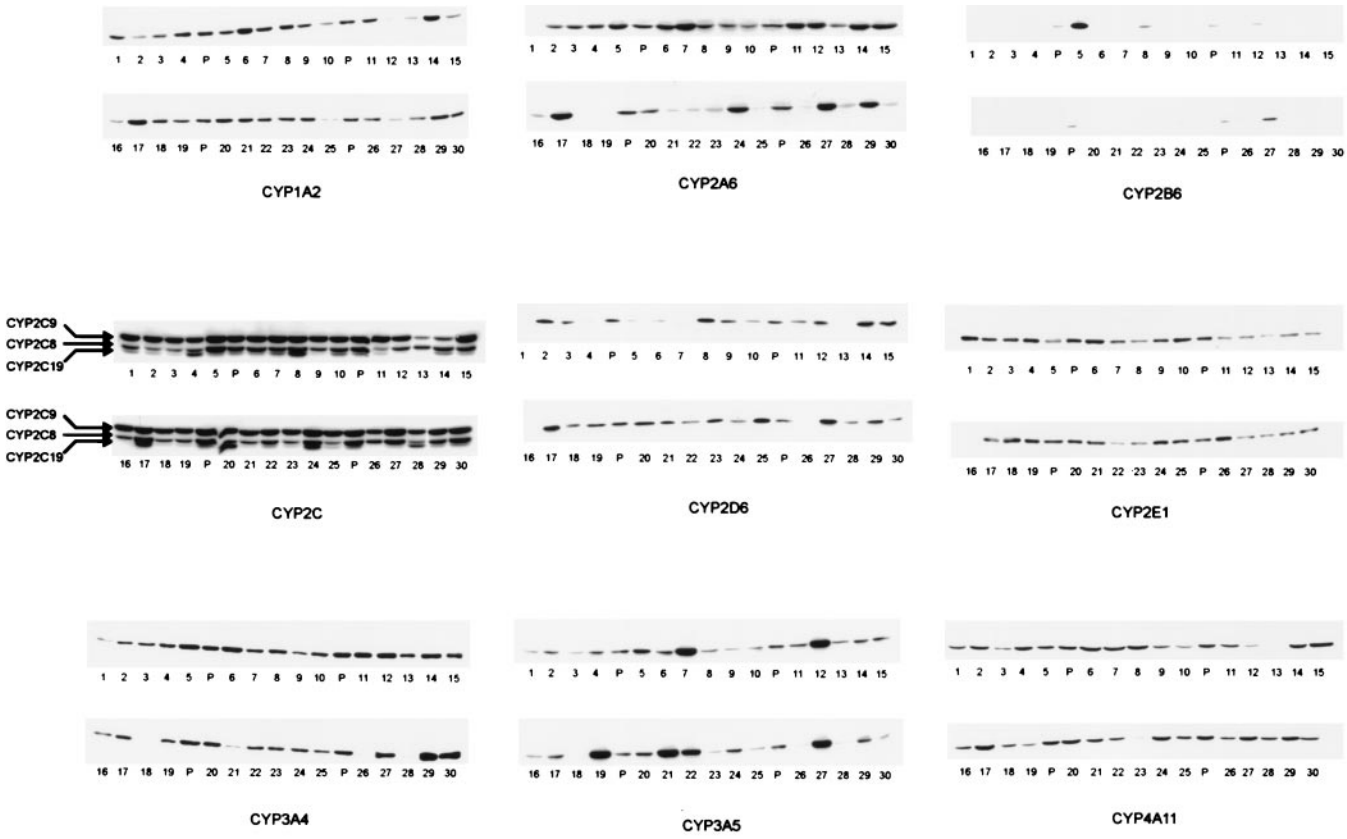

FIG. 2. Relative levels of various P450 enzymes in human liver determined using targeted antibodies. Samples of human liver microsomal fraction from thirty individuals and a pooled sample (P) were subjected to immunoblotting and developed with each of the antibodies against human P450. The amount of microsomal protein applied varied depending on the target antigen and was 20 μg for CYP1A2, 75 μg for CYP2A6, 20 μg for CYP2B6, 25 μg for CYP2C forms, 25 μg for CYP2D6, 10 μg for CYP2E1, 10 μg for CYP3A4, 25 μg for CYP3A5, and 25 μg for CYP4A11. Each immunoblot was developed with an antipeptide antibody targeted against one of the P450 enzymes under the conditions described in the Methods section.

The C termini of CYP1A1, CYP1B1, CYP2D6, CYP2E1 and CYP4A11 are unique to the respective P450 enzyme and do not resemble the C terminus of any other human P450 enzyme. Consequently, antibodies raised against these sites bound specifically to the target P450 enzyme in each case. Although the C-terminal region of CYP3A4 and CYP3A5 are quite similar (Table 1), they are clearly sufficiently different that antibodies raised against these peptides bound selectively to the respective target antigen. This is because binding of such antibodies to peptide and protein antigens is highly dependent on the C-terminal residue and small alterations in the structure of the antigen in this region cause a large reduction in antibody binding [32–34]. On the other hand, the C termini of CYP2A6 and CYP2B6 are very similar, i.e. -Ser-Phe-Leu-Pro-Arg and -Arg-Phe-Leu-Pro-Arg, respectively, the only difference being at the N terminus of the peptides. It was found that an antibody raised against the C terminus of CYP2A6 [32] bound to both CYP2A6 and CYP2B6 and these forms also migrate closely in immunoblotting (data not shown). To overcome this problem, antibodies to CYP2A6 and CYP2B6 were produced by targeting another region of these proteins. We had previously raised an antibody against rat CYP2B1/2 by targeting residues 265–276 of these P450 enzymes [35] and, although there is a single amino acid difference between rat CYP2B1/2 and human CYP2B6 in this region (Table 1), this antibody recognised CYP2B6 but not CYP2A6 (Fig. 1). Therefore, an antibody

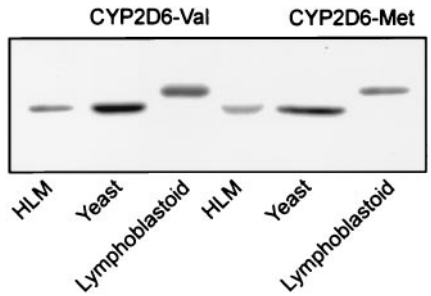

FIG. 3. Comparison of the electrophoretic mobility of recombinant CYP2D6 expressed in yeast and lymphoblastoid cells. Immunoblotting was performed using a pooled sample of human liver microsomal fraction (10 μg) (HLM), microsomal fraction containing CYP2D6-Val expressed in yeast (25 μg) and lymphoblastoid cells (10 μg) and CYP2D6-Met expressed in yeast (25 μg) and lymphoblastoid cells (10 μg). The immunoblot was developed with an antipeptide antibody targeted against CYP2D6 under the conditions described in the Methods section. Only the central section of the immunoblot containing immunoreactive bands is shown.

TABLE 3. Comparison of immunoreactivity with measurements of P450 activity

	CYP1A2	CYP2A6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4	CYP3A5	CYP4A11
Phenacetin O-deethylase	0.85*	0.12	0.40	0.09	0.34	0.08	0.30	0.29	0.20	0.41
Coumarin 7-hydroxylase	0.21	0.97*	0.52	0.53	0.28	0.26	−0.27	0.66*	0.51	0.46
Tolbutamide 4-hydroxylase	0.09	0.26	0.37	0.78*	0.31	0.22	0.13	0.23	0.18	0.37
S-Mephenytoin 4-hydroxylase	0.47	0.32	0.53	0.37	0.89*	0.15	0.15	0.39	0.23	0.51
Debrisoquine 4-hydroxylase	0.02	0.02	0.02	0.00	−0.06	0.80*	−0.26	0.19	0.18	0.15
Chlorzoxazone 6-hydroxylase	0.21	−0.29	0.05	−0.11	−0.09	−0.17	0.71*	−0.19	0.08	−0.05
Midazolam 1′-hydroxylase	0.21	0.50	0.50	0.45	0.33	0.11	−0.09	0.80*	0.52	0.14
Lauric acid 12-hydroxylase	0.53	0.31	0.66*	0.41	0.43	0.06	0.19	0.29	0.33	0.63*

The values shown are Spearman Rank correlation coefficients *$P < 0.001$. Activity or relative apoprotein levels were determined for each of 30 samples, except that debrisoquine 4-hydroxylase and chlorzoxazone 6-hydroxylase activities were only measured in 29 and 25 samples, respectively.

TABLE 4. Comparison of relative apoprotein levels of P450 enzymes and total P450 content in human liver microsomal fractions

	CYP2A6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4	CYP3A5	CYP4A11	P450 content
CYP1A2	0.24	0.27	−0.03	0.52	0.05	0.32	0.26	0.13	0.47	0.42
CYP2A6	—	0.50	0.48	0.29	0.24	−0.27	0.64*	0.46	0.46	0.64*
CYP2C8	—	—	0.61*	0.40	−0.02	−0.05	0.48	0.38	0.45	0.74*
CYP2C9	—	—	—	0.30	0.17	−0.11	0.37	0.24	0.40	0.68*
CYP2C19	—	—	—	—	0.12	0.13	0.28	0.04	0.54	0.55*
CYP2D6	—	—	—	—	—	−0.20	0.10	0.12	0.43	0.18
CYP2E1	—	—	—	—	—	—	−0.22	−0.11	0.01	0.15
CYP3A4	—	—	—	—	—	—	—	0.56*	0.23	0.66*
CYP3A5	—	—	—	—	—	—	—	—	0.24	0.41
CYP4A11	—	—	—	—	—	—	—	—	—	0.51

The values shown are Spearman Rank correlation coefficients, *$P < 0.001$, N = 30.

TABLE 5. Correlations between monooxygenase activities and total P450 content in human liver microsomal fractions

	Coumarin 7-hydroxylase	Tolbutamide 4-hydroxylase	S-Mephenytoin 4-hydroxylase	Debrisoquine 4-hydroxylase	Chlorzoxazone 6-hydroxylase	Midazolam 1′-hydroxylase	Lauric acid 12-hydroxylase	P450 content
Phenacetin O-deethylase	0.11	0.25	0.46	0.16	0.34	0.34	0.66*	0.51
Coumarin 7-hydroxylase	—	0.39	0.37	0.09	−0.22	0.55*	0.31	0.69*
Tolbutamide 4-hydroxylase	—	—	0.42	0.19	0.10	0.31	0.38	0.64*
S-Mephenytoin 4-hydroxylase	—	—	—	0.09	0.14	0.44	0.55*	0.68*
Debrisoquine 4-hydroxylase	—	—	—	—	0.03	0.16	−0.01	0.13
Chlorzoxazone 6-hydroxylase	—	—	—	—	—	−0.12	0.22	0.11
Midazolam 1′-hydroxylase	—	—	—	—	—	—	0.39	0.75*
Lauric acid 12-hydroxylase	—	—	—	—	—	—	—	0.57*

The values shown are Spearman Rank correlation coefficients, *$P < 0.001$. Activity and P450 content were determined for each of 30 samples, except that debrisoquine 4-hydroxylase and chlorzoxazone 6-hydroxylase activities were only measured in 29 and 25 samples, respectively.

was targeted against the equivalent region in CYP2A6. The corresponding peptides (Table 1) vary in 6/13 residues and the resultant antibody bound specifically to CYP2A6.

The C termini of all of the known human P450 enzymes in the CYP2C subfamily are identical, hence the antibody directed against the C terminus of the human CYP2C enzymes recognised CYP2C8, CYP2C9 and CYP2C19. However, these P450 enzymes can be separated from one another by the SDS-polyacrylamide gel electrophoresis step of the immunoblotting procedure. Hence, although this antibody cannot discriminate between these P450 enzymes, it is possible to determine the levels of each of these P450 enzymes in individuals. Indeed, as the epitope for the antibody is identical in these forms it is possible to compare the relative expression of the CYP2C forms. The results show that in most individuals expression is in the order CYP2C9 > CYP2C8 > CYP2C19, although there is considerable variation in the relative levels of expression between individuals. A polyclonal anti-P450 antibody raised against a purified CYP2C enzyme that binds to all forms would not be appropriate for this purpose, as the epitopes would not necessarily be identical and such an antibody is likely to bind with different affinities to the three forms. The antipeptide antibody would also be expected to bind to CYP2C18, which has the same C-terminal sequence as the other CYP2C forms. CYP2C18 migrates between CYP2C8 and CYP2C19 [36], however, human hepatic microsomal fraction contains little or no CYP2C18 [37]. No attempt was made in this study to produce specific antibodies against each of the CYP2C forms, however, this should be possible by targeting other regions of the apoprotein as discussed above. Richardson *et al.* [37] have produced a specific anti-CYP2C18 antibody in this way.

For CYP1A2 an antibody was produced by targeting a region that occurs at residues 284–296 [38]. Based on a comparison of the secondary structure of P450 enzymes, this region occurs between the G and I-helices of CYP1A2 [39] and is equivalent to the regions targeted in CYP2A6 and CYP2B6 to produce specific antibodies against these forms.

Interestingly, the migration of recombinant forms of P450 was not always identical with the same form expressed in human liver microsomal fraction. Recombinant CYP2D6 expressed in lymphoblastoid cells migrated slightly more slowly than human liver microsomal CYP2D6. In contrast, the migration of CYP2D6 expressed in yeast was indistinguishable from that of human liver microsomal CYP2D6. The reason for the aberrant migration of CYP2D6 derived from lymphoblastoid cells is not known. It may be the result of an error in the sequence of the CYP2D6 cDNA used in the expression systems (Dr. C. Crespi, personal communication), but it is not due to variation in the amino acid expressed at position 374, as both CYP2D6-Val and CYP2D6-Met migrate identically in both yeast and lymphoblastoid cells, respectively. This problem was less apparent with most of the other recombinant enzymes, e.g. CYP2C8, CYP2C9 and CYP2C19 expressed in insect cells all migrated similarly to the respective P450 enzymes in human liver microsomal fraction.

The binding of antibodies to their target antigens was further confirmed by comparing the relative levels of immunoreactivity with measurement of enzyme activity using a variety of selective substrates for reactions catalysed by these forms of P450. Previous studies have shown that, in humans, high affinity phenacetin O-deethylase activity is selectively catalysed by CYP1A2 [40], coumarin 7-hydroxylase by CYP2A6 [41], tolbutamide 4-hydroxylase by CYP2C9 [42, 43], *S*-mephenytoin 4-hydroxylase by CYP2C19 [44, 45], debrisoquine 4-hydroxylase by CYP2D6 [46], chlorzoxazone 6-hydroxylase by CYP2E1 [28], midazolam 1′-hydroxylase by CYP3A4 [47], and lauric acid 12-hydroxylase by CYP4A11 [48]. In agreement with this, a high degree of correlation was found between each of the apoprotein levels and the respective P450 enzyme activities. Unexpectedly, there was also a correlation between CYP3A4 and coumarin 7-hydroxylase. Consistent with this was the association between CYP3A4 and CYP2A6 levels, and midazolam 1′-hydroxylase and coumarin 7-hydroxylase activities in the group of 30 liver samples. Such a relationship between the expression of CYP2A6 and CYP3A4 has not been found in previous studies [49, 50] and may, therefore, be coincidental. A correlation between CYP2C8 and lauric acid 12-hydroxylase activity was also found, however, it has been shown that CYP2C8 expressed in yeast is unable to catalyse this reaction [51]. The lack of any other correlations is consistent with the use of substrates that are catalysed preferentially by one form of P450. Hence, although CYP1A2 and CYP3A4 can catalyse chlorzoxazone 6-hydroxylase [52, 53] their contribution to this reaction in human liver microsomal fraction under the conditions used here is relatively minor in comparison to catalysis by CYP2E1. Correspondingly, no correlation between chlorzoxazone 6-hydroxylase activity and either CYP1A2 or CYP3A4 was found in agreement with previous studies [53, 54].

Of the antibodies examined in the present study, none were found to be potent inhibitors of P450 activity ([38] and data not shown). However, we have been able to produce a pro-inhibitory antibody against human CYP1A2 using the antipeptide approach [55].

A surprisingly large proportion of the apoprotein levels and their corresponding monooxygenase activities correlated with spectrally determined P450 content. Although it might be expected that some of the more abundant P450 enzymes should correlate, i.e. CYP3A4 and CYP2C9, the amount of CYP2A6 [50] and CYP2C19 (this study) is low and would not. Nevertheless, a similar relationship between P450 content and several monooxygenase activities in human liver has also been described in a previous study [50]. This relationship and the general tendency towards positive correlations may explain the apparent association between lauric acid 12-hydroxylase activity and both phenacetin O-deethylase and *S*-mephenytoin 4-hydroxylase activities. The absence of correlations between the corre-

sponding apoprotein levels supports the assertion that there is no relationship between these P450 enzymes.

A proportion of the liver samples examined lacked CYP2A6 (7%), CYP2C19 (10%), and CYP2D6 (13%) and this is consistent with the polymorphic expression of these P450 enzymes in human liver [4, 36, 56]. Although various polymorphisms of the CYP2C9 gene have been described, these are all due to point mutations that affect enzyme activity but not protein expression [57, 58] consistent with the identification of this P450 in all samples examined.

The expression of CYP3A5 is also thought to be polymorphic, although there is some disagreement about the incidence of individuals lacking expression of the enzyme. Whereas Wrighton *et al.* [59] have reported that only 29% of individuals express CYP3A5, more recently, Jounaidi *et al.* [60] detected CYP3A5 apoprotein in 74% of individuals and CYP3A5 mRNA in all of their samples. The present results indicate that most individuals express CYP3A5 (97%) and also show that some (20%) express a substantially higher level. This large variation probably explains the previous difference in the estimation of the incidence of CYP3A5 expression. The antibody raised against the C terminus of CYP3A5 showed no reactivity with CYP3A4 and binding to any CYP3A7 that may be present in human liver is also excluded as the C terminus of CYP3A7 is identical to that of CYP3A4.

It is not clear if CYP2B6 is polymorphically expressed. Mimura *et al.* [61] found expression in 24% of human liver samples, similar to the incidence found here (20%). Among their samples, Mimura *et al.* [61] found that 4/50 (8%) contained a relatively high level of immunoreactivity and we found 1/30 (3%). It is possible that elevated CYP2B6 may be as a result of induction, although further studies will be needed to determine this. Nevertheless, the expression of CYP2B6 in normal individuals does appear to be polymorphic, with little or no expression in approximately 75–80% of the population.

CYP1A2, CYP2E1 and CYP3A4 were detected in all samples. Previous results have shown that expression of CYP1A2 and CYP3A4 is highly variable, but in neither case is this related to polymorphic expression [50, 62, 63]. CYP2E1 polymorphisms have been described that may affect transcriptional activity [64] or, more rarely, enzyme activity [65], but none result in a lack of enzyme expression, consistent with the results found here.

The absence of immunodetectable CYP1A1 and CYP1B1 is consistent with the low level of expression of these P450 enzymes in human liver [38, 66, 67], these forms normally being expressed in extrahepatic tissues and even then, in the case of CYP1A1, only after exposure to inducing agents [68, 69]. Although detection of CYP1A1 mRNA has been reported in human liver [70] none was detected by reverse transcriptase polymerase chain reaction in any of our samples (Prof. O. Pelkonen and Dr. H. Raunio, personal communication). Also, only very low levels of CYP1B1 mRNA expression have been detected in human liver [67].

None of the antibodies produced any noticeable binding to proteins other than the target P450 enzymes in human liver microsomal fraction. Each of the peptides used for immunisation were unique to the target P450 enzyme, although, as discussed above, some P450 enzymes contained quite similar sequences. However, other than orthologous P450 enzymes from other species no other proteins in the SWISSPROT database contained these sequences. As demonstrated previously, the specificity of antipeptide antibodies depends on whether the peptide used for immunisation occurs in other proteins. In theory, such binding should be predictable provided that the primary structures of relevant proteins are known. In practice, resultant antibodies have been found to be highly specific.

In conclusion, these results demonstrate the utility of the antipeptide approach for producing specific antibodies against human P450 enzymes. In most cases it was possible to produce highly specific antibodies by targeting the C terminus of the P450 enzymes. This requires the synthesis of only short peptides of 4–5 residues for use as immunogens. The approach was successful even when the C termini of related P450 enzymes were very similar (CYP3A4 and CYP3A5). Nevertheless, the C termini of some P450 enzymes were too similar (CYP2A6 and CYP2B6), or indeed identical (CYP2C forms), for form specific antibodies to be produced in this way, but for such cases it is possible to target other regions of the apoprotein to produce selectively binding antibodies (CYP2A6 and CYP2B6). Antibodies targeted against such regions require larger peptides, of about 7-12 residues, as immunogens and some consideration of the physicochemical properties of putative target regions [14].

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