Evidence That CYP2C19 Is the Major (*S*)-Mephenytoin 4′-Hydroxylase in Humans†

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ABSTRACT: The present study assesses the role of members of the human CYP2C subfamily in the 4′-hydroxylation of (*S*)-mephenytoin. When recombinant CYP2C proteins were expressed using a yeast cDNA expression system, 2C19 stereospecifically 4′-hydroxylated (*S*)-mephenytoin with a turnover number at least 10 times higher than that of human liver microsomes. 2C9 (both Ile^{359} and Leu^{359} alleles) and 2C18 (Thr^{385} and Met^{385} alleles) metabolized this substrate at a rate 100-fold lower than 2C19, and metabolism by these 2C proteins was not stereospecific for the *S*-enantiomer. 2C8 exhibited very little mephenytoin 4′-hydroxylase activity. In contrast, the Ile^{359} allele of 2C9 had a high turnover number for the hydroxylation of tolbutamide, while the Leu^{359} allele was less active toward this substrate. Immunoblot analysis of 16 human liver donor samples indicated that (*S*)-mephenytoin 4′-hydroxylase activity correlated with the hepatic CYP2C19 content, but it did not correlate with the hepatic content of CYP2C9. Moreover, direct sequencing of the polymerase chain reaction (PCR) products of 2C9 mRNA from six of these human livers through areas of known allelic variations indicated that the identity of the allele of 2C9 (Cys^{144} vs Arg, Tyr^{358} vs Cys, Ile^{359} vs Leu, or Gly^{417} vs Asp) did not appear to influence (*S*)-mephenytoin 4′-hydroxylase activity in these samples. These data indicate that 2C19 is the principal determinant of (*S*)-mephenytoin 4′-hydroxylase activity in human liver.

There are interindividual differences in the metabolism of a number of drugs in humans. Many of these variations have been attributed to polymorphisms in cytochrome P450s.[1,2] A polymorphism has been demonstrated in the 4′-hydroxylation of the *S*-enantiomer of mephenytoin but not in the metabolism of the *R*-enantiomer (Küpfer & Preisig, 1984; Wedlund *et al.*, 1984). Two phenotypes, extensive and poor metabolizers, are present in the human population. Poor metabolizers are detected at low frequencies in Caucasians (2–5%) but at higher frequencies in the Japanese population (~20%) (Nakamura *et al.*, 1985; Jurima *et al.*, 1985). 4′-Hydroxylation of (*S*)-mephenytoin is 3–10-fold higher than that of the *R*-enantiomer in extensive metabolizers, but the ratio is approximately 1 or less in poor metabolizers (Yasumori *et al.*, 1990). Rates of (*S*)-mephenytoin 4′-hydroxylation in liver microsomes are also much higher than those of (*R*)-mephenytoin in extensive metabolizers.

Although (*S*)-mephenytoin appears to be metabolized by a form of cytochrome P450 belonging to the 2C subfamily (Umbenhauer *et al.*, 1987; Ged *et al.*, 1988), there is considerable controversy concerning the identity of the cytochrome(s) responsible for metabolism of this substrate. Yasumori *et al.* (1991) reported that an allelic variant of 2C9 ($Arg^{144}Tyr^{358}Iso^{359}Gly^{417}$) had catalytic activity toward (*S*)-mephenytoin using a cDNA-directed expression system in yeast. However, Srivastava *et al.* (1991) expressed an identical cDNA in yeast and a $Arg^{144}Cys^{358}Iso^{359}Asp^{417}$ variant (2C10 by present nomenclature) but were unable to demonstrate catalytic activity of 2C9 or 2C10 toward (*S*)-mephenytoin. Relling *et al.* (1990) was also unable to demonstrate catalytic activity of an allelic variant of $Cys^{144}Tyr^{358}Ile^{359}Gly^{417}$-2C9 toward (*S*)-mephenytoin using a retroviral cDNA expression system in HepG2 cells. In contrast, all of these 2C9 variants metabolized tolbutamide in the various expression systems (Srivastava *et al.*, 1991; Relling *et al.*, 1990). Therefore the role of 2C9 in the polymorphism in (*S*)-mephenytoin metabolism remains questionable at this time.

Recently, we isolated four full-length cDNAs belonging to the P4502C subfamily (2C8, 2C9, 2C18, and 2C19) including two allelic variants of both 2C9 and 2C18 (Romkes *et al.*, 1991). In the present study, we utilized a yeast cDNA expression system in an attempt to achieve relatively high levels of expression of the recombinant CYP2C proteins. We then compared the ability of these proteins to metabolize (*R*)- and (*S*)-mephenytoin and tolbutamide.

MATERIALS AND METHODS

Materials. Human liver samples were obtained from Dr. Fred Guengerich, Vanderbilt University, Nashville, TN. Restriction endonucleases were purchased from Stratagene Cloning Systems (La Jolla, CA). [α-^{32}P]dCTP (3000 Ci/mmol), [γ^{32}P]ATP (5000 Ci/mmol), and [α-^{35}S]dATP (650

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[1] We have used the recommended P450 gene nomenclature (Nebert *et al.*, 1991) throughout this paper.

[2] Abbreviations: SSC, standard sodium citrate; SDS, sodium dodecyl sulfate; P450, cytochrome P450.

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Ci/mmol) were from Amersham Corporation (Arlington Heights, IL). Nirvanol was obtained from Adrian Kupfer, University of Berne, Berne, Switzerland, and separated into its *R*- and *S*-enantiomers as described by Sobotka *et al.* (1932). Radiolabeled (*S*)- and (*R*)-(*N-methyl*-^{14}C)mephenytoin were then synthesized by DuPont (Wilmington, DE) by methylation of (*R*)- and (*S*)-nirvanol. The radiochemical purity of both isomers was greater than 98% as assessed by HPLC. A single impurity which accounted for less than 2% of the parent compound was not characterized, since it eluted after the metabolites and parent compound. Moreover, the percentage of the impurity remained the same (less than 2%) before and after incubations. All sequencing was done by the dideoxy method using Sequenase Kits (U.S. Biochemical Corporation, Cleveland, OH). The specific activities of the *S*- and *R*-enantiomers were 20.7 and 20.9 mCi/mmol, respectively. All other reagents used are listed below or were of the highest quality available.

Amplification of 2C18 and 2C9 RNA for Direct Sequencing. Total RNA from selected human liver samples was isolated by the single-step method (Chomczynski & Sacchi, 1987) using TRI REAGENT, (Mol. Res. Center, Inc., Cincinnati, OH). RNA (10 μg) was reverse transcribed using 2.5 μM random hexamers as the 3′-primer by incubating for 1 h at 42 °C using 2.5 units/μL of M-MLV reverse transcriptase (BRL, Grand Island, NY) in 10 mM Tris-HCl, pH 8.3, 5 mM KCl, 5 mM $MgCl_2$, 1 unit/μL RNase inhibitor (Promega, Madison, WI), and 1 mM each of dATP, dCTP, dGTP, and dTTP (Perkin Elmer Cetus, Norwalk, CT). The samples were then heated for 5 min at 99 °C to terminate the reverse transcription.

The cDNA was then amplified for a region containing the allelic differences in 2C18 and 2C9 using a nested PCR method. The DNA was amplified in 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) containing 1 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, and 20 pmol of each of the 5′- and 3′-primers in a final reaction volume of 100 μL. The reaction mixture was heated at 94 °C for 5 min before addition of 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus). For PCR of 2C18, the 3′-primer was 5′-TGGCCCTGATAAGGGAGAAT-3′ and the 5′-primers were 5′-ATCCAGAGATACATTGACCTC-3′ (outer) and 5′-CCATGAAGTGACCTGTGATG-3′ (inner). For 2C9, the 3′-primer was 5′-AAAGATGGATAATGCCCCAG-3′ and the 5′-primers were 5′-GAAGGAGATCCGGCGTTTCT-3′ (outer) and 5′-GGCGTTTCTCCCTCATGACG-3′ (inner). The outer amplification was performed for 20 cycles consisting of denaturation at 94 °C for 1 min, annealing at the appropriate temperature for 30 s, and extension at 72 °C for 1 min. After a 50-fold dilution, PCR was carried out similarly with the inner primers for 35 additional cycles.

The PCR products were purified using a Centricon-30, dried, suspended in 40 μL of sterile water, and sequenced using Sequenase Kits and a P^{33}-end labeled sequencing primer. For 2C18, the primer used was 2C18.1184R 5′-TTGTCATTGT-GCAG-3′. Sequencing primers for 2C9 were 2C9.1030F 5′-CACATGCCCTACACA-3′, 2C9.385F 5′-TGACGCTGCG-GAATT-3′, and 2C9.783F 5′-GGACTTTATTGATTG-3′.

Full-length 2C9 cDNA was also amplified by PCR from a human liver (115) with high (*S*)-mephenytoin 4′-hydroxylase activity using the primers 5′-ATGATTCTCTTGTGGTCCT-3′ and 5′-AAAGATGGATAATGCCCCCAG-3′. The PCR reaction was similar to above, except that the primer concentrations were increased 10-fold (0.25 μM). The PCR products were then cloned into the pCR1000 vector using the TA Cloning System (InVitrogen, San Diego, CA) and sequenced to identify the allelic variant present.

Additional Sequences of 2C cDNAs Used in the Expression Studies. Two full-length clones of 2C8 (7b and 7c) isolated in our earlier study (Romkes *et al.*, 1991) were sequenced through the coding region in the present study. The sequences were similar to that of the 2C8(HP1-1) reported by Okino *et al.* (1987); however, both clones had coding changes at position 390 (A → C) (Asn^{130} → Thr) and G → C at position 792 (Met^{264} → Ile) and a change in the noncoding region at 1497-(T → C). These changes were rechecked by the authors and presumably represent a second allelic variant of 2C8 since Tukey and co-workers reverified their original sequence (personal communication). The Thr^{130} and Ile^{264} amino acids found in our 2C8 clones are conserved in the remainder of the human P4502C subfamily (2C9, 2C18, and 2C19) and are therefore consistent with the amino acid substitutions in other members of this subfamily.

Yeast Strains and Media. Saccharomyces cerevisiae 334 (MAT α,pep4-3,prb1-1122,ura3-52,leu 2-3,112,reg1-501,gal1), a protease deficient strain kindly provided by Dr. Ed Perkins (NIEHS), was used as the recipient strain in these studies and propagated nonselectively in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) (Hovland *et al.* 1989). For the selection of Leu^+ transformants, the cells were grown in synthetic complete medium minus leucine (Rose *et al.*, 1990). Plates were made by the addition of 2% agar.

Plasmid Construction. The strategy for cloning the P4502C cDNAs into the yeast vector pAAH5 is described below. The 5′-noncoding sequence of the P4502C cDNAs was eliminated by PCR amplification to optimize expression in yeast cells. The 5′-primer introduced a *Hind*III cloning site and a six A-residue consensus sequence upstream of the ATG codon to promote efficient translation in yeast (Hamilton *et al.*, 1987; Cullin & Pompom 1988). The 3′-primer was positioned between the stop codon and polyadenylation site and introduced a second *Hind*III site. cDNA inserts in the pBluescript vector (0.1 μg) (Romkes *et al.*, 1991) were amplified by PCR as described before except that the reaction contained 3.5 mM $MgCl_2$, 0.25 μM each of the 5′- and 3′-primers, and 1 μL of PerfectMatch (Stratagene, La Jolla, CA). Amplification was performed in sequential cycles, with the first cycle including denaturation for 1 min at 94 °C, annealing at the appropriate temperature for 1 min, and polymerization at 72 °C for 3 min. The remaining 24 cycles consisted of a denaturation step at 94 °C for 1 min and a combined annealing/extension step at 72 °C for 3 min. After the last cycle, all samples were incubated an additional 10 min at 72 °C. The primers used were as follows: 2C8, 5′-GCAAGCTTAAAAAAATG-GAACCTTTTGTGGTCCT-3′ and 5′-GCAAGCTTGCCA-GATGGGCTAGCATTCT-3′; 2C9, 5′-GCAAGCTT-AAAAAAATGGATTCTCTTGTGGTCCT-3′ and 5′-G-CAAGCTTGCCAGGCCATCTGCTCTTCT-3′; 2C19, 5′-GCAAGCTTAAAAAAATGGATTCTCTTGT-GGTCCT-3′ and 5′-GCAAGCTTGCCAGACC-ATCTGTGCTTCT-3′.

The PCR products were cloned into the pCR1000 vector (InVitrogen, San Diego, CA). Recombinant plasmids were isolated from *Escherichia coli* (INVαF′) cells using Qiagen plasmid purification kits, and the PCR products were completely sequenced as described above to verify the fidelity of the PCR reaction. A mutation of Asp^2 → Val was initially introduced inadvertantly in 29c via the primers utilized due to an error in the original sequencing at this position (Romkes *et al.*, 1993). Therefore, the correct 2C18-Asp^2 cDNAs were

cloned into the pAAH5 vector by an alternate strategy. The 3'-end was cut with NdeI, blunted, and ligated to a *Sma*I/*Hind*III adapter. The clone was then partially digested with *Bam*HI which cuts after the initiation ATG as well as internally, and the intact 1700 fragment gel purified. A *Bam*HI/*Hind*III linker was prepared from the oligos 5'-AGCTTAAAAAAATG-3' (upper) and 5'-GATC-CATTTTTTTA-3' (lower), annealed, and ligated to the cDNA fragment to introduce a *Hind*III cloning site and regenerate the ATG codon.

The PCR amplified cDNAs were isolated by *Hind*III digestion and ligated into the pAAH5 yeast expression vector, and the proper orientation was confirmed by restriction analysis and sequencing. The expression vector pAAH5, which contains the yeast ADH1 promoter and terminator regions and the Leu2 selectable marker, was kindly provided by Dr. M. Negishi (NIEHS). The recombinant plasmids were isolated from *E. coli* DH5α cells using Qiagen plasmid purification kits and transformed into yeast as described previously (Faletto *et al.*, 1992) using the lithium acetate method of Ito *et al.* (1983).

Immunoblots and Cytochrome P450 Determinations. Yeast microsomes or whole-cell lysates were prepared from transformed cells isolated at mid-logarithmic phase as described previously (Oeda *et al.*, 1985) with slight modifications (Faletto *et al.*, 1992) and stored at −80 °C in 0.1 M phosphate (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. Protein concentrations were determined by the method of Bradford *et al.* (1976). SDS–polyacrylamide gel electrophoresis and Western blots were performed on yeast microsomes or whole-cell lysates (Faletto *et al.*, 1992) and immunoblots probed with antibody to the appropriate P450 as described (Yeowell *et al.*, 1985). Cytochromes P4502C8 and P4502C9 and NADPH:P450 reductase were purified from human liver microsomes (Raucy & Lasker, 1991) and antibodies to 2C8 and 2C9 prepared in rabbits as previously described (Leo *et al.*, 1989). Specific peptides NH_2-CIDYLPGSHNKIAEN-FA-COOH (amino acids 231–249) for P4502C18 and NH_2-CLAFMESDILEKVK-COOH (amino acids 236–249) for 2C19 were selected from amino regions where these P450s vary from other known 2C subfamily members (Romkes *et al.*, 1991). These peptides were synthesized and conjugated to bovine serum albumin via *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester, and antibodies to the conjugates were raised in rabbits by BIOSYNTHESIS INC. (Denton, TX). *E. coli* lysate (4 mg/mL) was added to the primary peptide antibody in first step of the immunoblot procedure to block nonspecific reactions of these rabbit antibodies to yeast cell-wall proteins. Cytochrome P450 concentrations of microsomes were determined by dithionite-reduced carbon monoxide difference spectra by the method of Omura and Sato (1964) using an extinction coefficient of 91 mM^{-1} cm^{-1}.

Microsomes of human livers were prepared as described by Raucy and Lasker (1991). SDS–polyacrylamide gel electrophoresis and immunoblot analysis were performed as above except that immunoblots were developed using the ECL (enhanced chemiluminescence) Western blotting kit from Amersham (UK). Immunoblots were scanned with a laser densitometer (LKB Instruments).

Purification of Cytochromes from Recombinant Yeast Microsomes. Recombinant yeast microsomes were prepared from a 10–12-L culture, and recombinant P450s were purified by aminooctylsepharose chromatography as described by Iwasaki *et al.* (1991). The Emulgen was then removed from protein by adsorption of the protein to a 4 g hydroxylapatite column (Hypatite C, Clarkson Chemical Company, Williamsport, PA) and equilibrated with 10 mM potassium phosphate buffer (pH 7.2), 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT, and the column was washed with the same buffer until the absorbance at 280 nm returned to zero. The P450 was then eluted with 400 mM potassium phosphate (pH 7.2), 20% glycerol, 0.5% cholate, 0.1 mM EDTA, and 0.1 mM DTT and dialyzed overnight against 100 mM potassium phosphate buffer (pH 7.4), 20% glycerol, and 0.1 mM EDTA. Absolute and CO difference spectra of purified P450s were determined in the same buffer but containing 0.2% Emulgen and 0.5% cholate.

Tolbutamide Hydroxylase Assays. Tolbutamide hydroxylase activity was measured according to Knodell *et al.* (1987) with several modifications. Yeast microsomes (1 mg of protein) were preincubated with 300 pmol hamster P450 reductase in 0.2 mL of the incubation buffer (below) for 3 min at 37 °C. The reaction was then placed on ice and incubated in 0.2 mL of 50 mM HEPES buffer (pH 7.4) containing 1.5 mM $MgCl_2$ and 0.1 mM EDTA in a final volume of 1 mL and 1 mM sodium tolbutamide. The reaction was initiated with 0.5 mM NADPH. Human liver microsomes (0.22 mg of protein) were incubated without reductase. Incubations with reconstituted recombinant P450s contained 50 pmol purified P450 enzyme, 150 pmol P450 reductase, and 15 μg of dilauroylphosphatidylcholine and were performed in 100 mM potassium phosphate buffer (pH 7.4). Reactions were terminated after 60 min at 37 °C by the addition of 50 μL of 4 N HCl, followed by extraction with 3 mL of water-saturated ethyl acetate. The ethyl acetate extracts were dried under nitrogen at 40 °C, the residue was resolubilized in 200 μL of methanol, and 4-hydroxytolbutamide was then assayed using HPLC by injecting 50 μL of the solubilized extract onto a μBONDAPAK C_{18} column (4.6 × 300 mm) using 0.05% phosphoric acid, pH 2.6, acetonitrile (6:4, v/v) as the mobile phase with a flow rate of 1 mL/min. The column eluate was monitored at 230 nm, and rates of product formation were determined from standard curves prepared by adding varying amounts of 4-hydroxytolbutamide to incubations conducted without NADPH. Preliminary experiments confirmed that 4-hydroxytolbutamide formation by human liver microsomes (30–120 pmol of P450) was linear for up to 90 min. Samples were analyzed in triplicate.

Mephenytoin 4'-Hydroxylase Assay. Mephenytoin 4'-hydroxylase activity was measured by a modifications of the radiometric HPLC assay described by Shimada *et al.* (1986) as described below. Purified or recombinant yeast microsomes (10–50 pmol) were preincubated with dilauroylphosphatidylcholine (15 μg per 50 pmol of P450), P450 reductase (500 units per 50 mol of P450), and human cytochrome b_5 (2:1 molar ratio when added). The reconstituted mixture was preincubated for 5 min at 37 °C and then placed on ice. A final concentration of 0.4 mM radiolabeled (*S*)- or (*R*)-mephenytoin (20.7 and 20.9 mCi/mM) was added to 50 mM HEPES buffer (pH 7.4) containing 0.1 mM EDTA and 1.5 mM $MgCl_2$ for recombinant 2C proteins. The mixture was then incubated at 37 °C with shaking for 3 min, and the reaction was started with the addition of 2 mM NADPH and terminated after 30 min with an equal volume of methanol. Cytochrome b_5 was not included in all CYP2C18 reactions, since it had no effect or produced a slight inhibition on the activity of this CYP protein. Reaction volumes were generally 0.25 mL except when the volume of recombinant purified cytochrome or yeast microsomes was greater than 50 μL. In these cases, the volume was increased to 0.5 mL to limit the

volume of glycerol from the purified preparation to <4% of the final volume. Incubations with human microsomes did not contain exogenous P450 reductase or cytochrome b_5, and they were carried out in 0.1 M phosphate buffer (pH 7.4) instead of HEPES buffer. Initial experiments showed that (*S*)-mephenytoin hydroxylase activity of human liver microsomes was linear for at least 60 min and from 0.05 through 0.2 mg of microsomal protein and that of the *R*-enantiomer was linear through 1 mg of microsomal protein.

At the end of the incubation period, the reactions were terminated with an equal volume of methanol. The incubation mixture was centrifuged at 10000*g* for 10 min and an aliquot analyzed using assayed directly using HPLC without extraction. Samples with particularly low activity were concentrated by lyophilization and redissolved in a small volume of methanol/water (1:1) before assay. The HPLC system consisted of a reverse-phase C18 (10 μm) Versapak, 300-mm × 4.1-mm column (Altech Associates, Deerfield, IL) using an isocratic solvent system consisting of methanol/water (45:55) with a flow rate of 1 mL/min for 25 min. Detection of radioactive peaks was accomplished using an on-line Flow-One radiochemical detector (Radiomatic Instruments Co., Tampa, FL). Detection of the unlabeled 4′-hydroxymephenytoin authentic standard was performed using an on-line multiwavelength UV detector at both 211 and 230 nm.

Statistical Analyses. Tolbutamide hydroxylase and mephenytoin hydroxylase activities of microsomes prepared from different recombinant yeasts were compared by analysis of variance and by Fisher's least significant difference test (Carmer & Swanson, 1973).

RESULTS

Expression of P4502C cDNAs in Yeast. Western blot analysis confirmed the expression of the recombinant human CYP2C proteins in the recombinant yeast (Figure 1). Antibodies to 2C8 and 2C9 recognized polypeptide bands of approximately 50 000 D (2C8) and 55 000 D (2C9) which corresponded in mobility to that of the recombinant proteins purified from yeast microsomes. These mobilities corresponded to that of the corresponding 2C8 and 2C9 proteins purified from human liver (not shown). 2C19 was recognized by antibodies to both 2C9 and the 2C19 peptide. This protein corresponded in mobility (<50 000 D) to the lowest of three bands in Western blots of human liver microsomes probed with antibody to human 2C9. The mobility of 2C18 was intermediate between that of 2C8 and 2C19. Antibodies to 2C18 and 2C19 peptides were specific for their antigens; however, antibody to 2C9 cross-reacted strongly with 2C19 and weakly with 2C8 and 2C18.

CO difference spectral analysis indicated that the recombinant P4502C proteins were expressed variably in transformed yeast cells. CYP2C8 was expressed at levels as high as 160–250 pmol/mg of protein in some yeast microsomal preparations. 2C18, 65 (2C9), and 25 (2C9) were expressed at levels of 20–60 pmol/mg of microsomal protein. Initially, 11a (2C19) was expressed extremely poorly, and the CO difference spectrum of the recombinant 2C19 yeast was indistinguishable from that of control yeast (<7 pmol/mg of protein). However, after repeated transfections and selection, we achieved expression of 2C19 at ~17 pmol/mg of protein. All of the CYP2C proteins were low-spin hemoproteins. CYP2C18 appeared to be somewhat unstable in yeast microsomes with a large proportion (~$^1/_3$ to $^1/_2$) of the P450 being converted to P420 in the presence of dithionite and carbon monoxide.

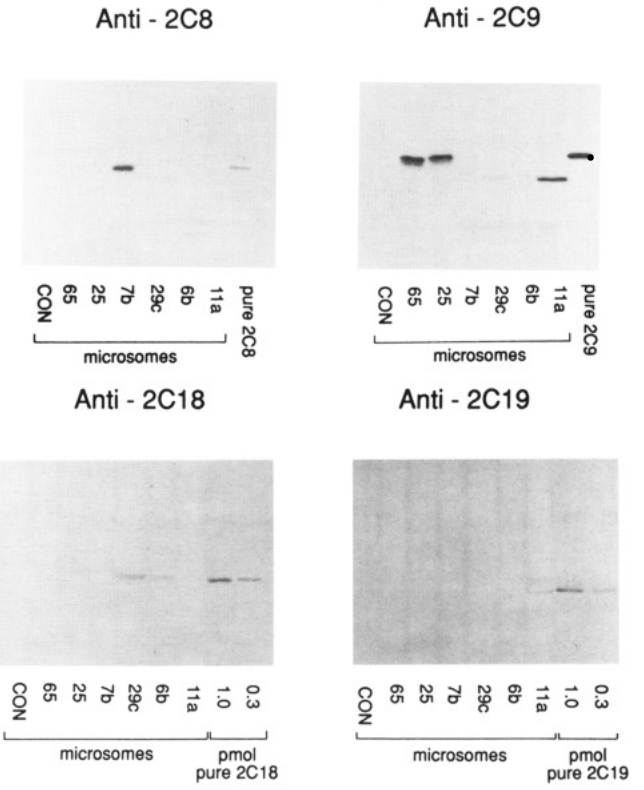

FIGURE 1: Western blots of recombinant yeast microsomes expressing the P4502C cDNAs indicated. Yeast microsomes (50 μg protein) expressing recombinant 2C9 (clones 65 and 25), 2C18 (6b and 29c), 2C8 (7b) and 2C19 (11a) were electrophoresed on 7.5% SDS–polyacrylamide gels and immunoblotted as described in Methods using antibody to 2C8 (HLx) or 2C9 purified from human liver or using antibody to unique peptides in 2C18 or 2C19 as the primary antibodies. Control yeast microsomes (CON) contained no recombinant proteins. Pure cytochrome P450s were partially purified from recombinant 7b (2C8), 65 (2C9), 29c (2C18), or 11a (2C19) as described in Methods.

None of the other recombinant CYP2C proteins showed this lack of stability.

Optimization of Tolbutamide and (S)-Mephenytoin Hydroxylase Assays. Preliminary studies indicated that exogenous P450 reductase (500 units/50 pmol of P450) stimulated metabolism of tolbutamide by recombinant 2C9 in yeast microsomes >10-fold and stimulated (*S*)-mephenytoin hydroxylase activity approximately 2-fold. Activity of the recombinant 2C proteins was linear with amount of P450 for 30 min through at least 20 pmol P450 for 2C19 (Figure 2) and 50 pmol for the other CYP2C forms (not shown). Cytochrome b_5 stimulated (*S*)-mephenytoin hydroxylase activity of both 2C9 and 2C19 in yeast microsomes and the optimal ratio of b_5 to P450 was approximately 2:1, but it generally had no effect or produced a slight inhibition of mephenytoin hydroxylase activity of 2C18 (Figure 3). This difference is consistent with the fact that all of the CYP2C proteins except 2C18 contain a Ser at position 127 which is a recognition site for cAMP protein kinase (^{125}Arg-Arg-Phe-Ser^{128}) (Müller *et al.*, 1985), and this sequence is also thought to be part of a b^5 binding site (Jansson *et al.*, 1987; Juvonen *et al.*, 1992). 2C18 contains Cys at position 127.

Mephenytoin 4′-hydroxylase activity of recombinant yeast microsomes was consistently higher in HEPES than phosphate buffer, while activity of human liver microsomes was ~2-fold higher in phosphate buffer (pH 7.4). Therefore, recombinant proteins were subsequently assayed in HEPES buffer with exogenous reductase and cytochrome b_5 except for 2C18 which

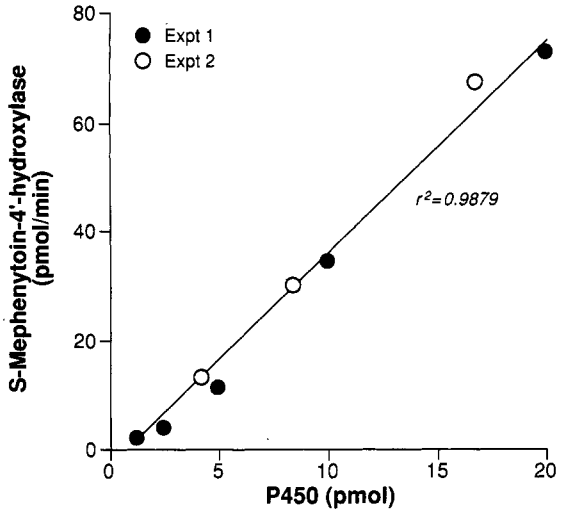

FIGURE 2: Linearity of (S)-mephenytoin 4'-hydroxylase activity with amount of recombinant 2C19 yeast microsomes. Incubations were for 30 min and contained 10 000 units of reductase per nmol. Open and closed circles represent individual points of two separate experiments.

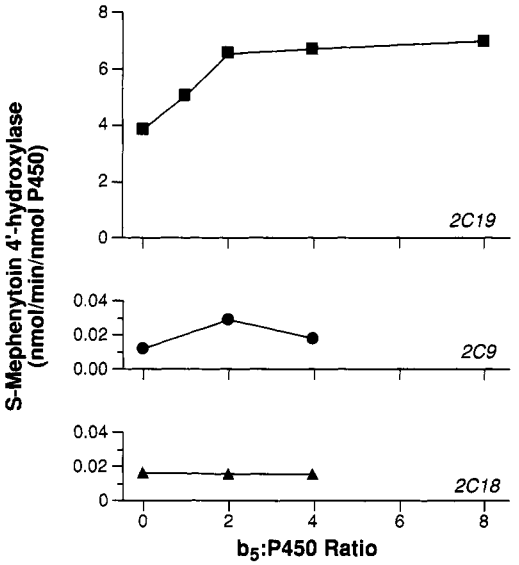

FIGURE 3: Effects of cytochrome b_5 on mephenytoin hydroxylase activity of recombinant yeast microsomes. For 2C9 (65) and 2C18 (29c), the incubation contained 50 pmol of P450 and 500 units of reductase. For 2C19, the incubation contained 5 pmol and P450 and 100 units of reductase. Incubation time was 60 min.

was tested both with and without cytochrome b_5. Human liver microsomal activities were assayed in phosphate buffer.

Mephenytoin Hydroxylase Activity of Recombinant Human 2C Proteins. (S)-Mephenytoin 4'-hydroxylase activities of yeast microsomes containing recombinant human CYP2C proteins were compared under optimized conditions described above. HPLC profiles of the metabolites of (S)-mephenytoin produced by human liver microsomes (high metabolizer) and recombinant human CYP2C proteins are shown in Figure 4 and the results summarized in Table 1. Recombinant 2C19 4'-hydroxylated (S)-mephenytoin at a rate of ~5 nmol min^{-1} $nmol^{-1}$ P450 which was 1 order of magnitude higher than the rate of 4'-hydroxylation in human liver microsomes (Table 1 and Figure 4). The retention time (5–6 min) of the 4'-hydroxymephenytoin metabolite was identical to that of the authentic unlabeled standard. 2C19 also produced small quantities of two unknown metabolites eluted at 3–4 and 7–8 min. These unknown metabolites were also produced by liver microsomes,

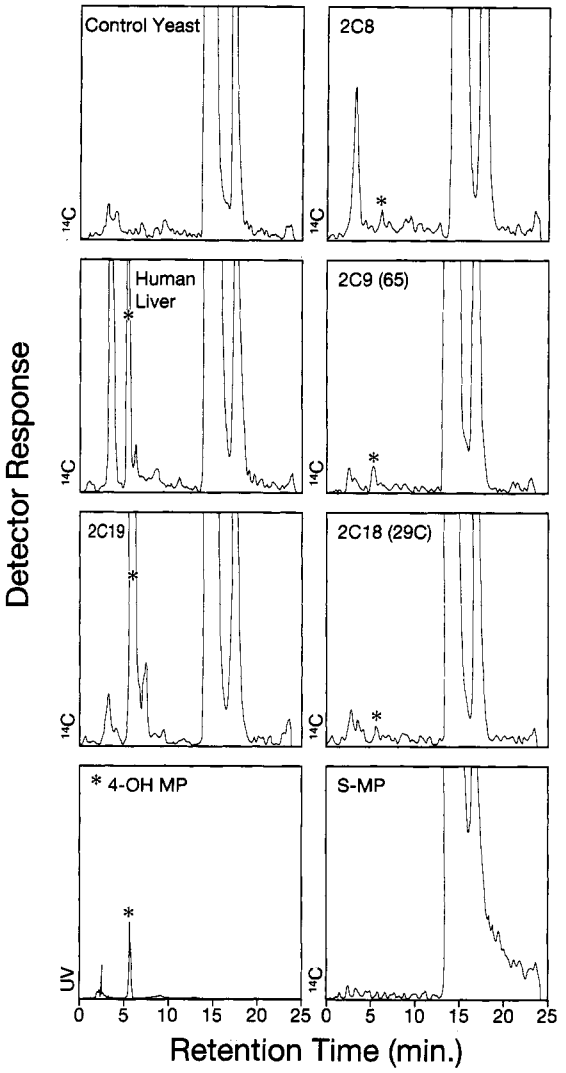

FIGURE 4: Representative HPLC radiochromatograms of metabolites formed after incubation of human liver microsomes (HBI6) and recombinant human CYP2C proteins with (S)-[*N-methyl*-^{14}C]-mephenytoin. Incubation conditions are described in Table 1.

and the metabolite with the shorter retention time was the principal metabolite produced by 2C8. Parent (S)-mephenytoin eluted at 14–15 min followed by the unknown impurity which eluted at 16–17 min. Similar retention times were observed for (R)-mephenytoin and its metabolites (not shown).

The rate of 4'-hydroxymephenytoin formation by 2C19 was at least 100-fold higher than that of 2C9 [both alleles, 2C18 (both alleles), and 2C8 (Table 1)] (0.04 versus 4.6 nmol min^{-1} $nmol^{-1}$). It was also ~20 times higher than that of human liver microsomes. The rate of 4'-hydroxylation of (S)-mephenytoin by 2C8 appeared to be consistently lower than that of 2C9. The 4'-hydroxylation of mephenytoin by 2C19 was stereospecific; the rate of (S)-hyroxylation was at least 30-fold higher than that of (R)-hydroxylation (Table 1). In contrast, the 4'-hydroxylation of mephenytoin by the other human CYP2C proteins did not appear to be stereospecific.

Recombinant CYP2C proteins were purified from yeast microsomes and their ability to 4'-hydroxylate the S- and R-enantiomers of mephenytoin were also examined in a reconstituted system (Table 2). 2C19 had similar turnover numbers for (S)-mephenytoin 4'-hydroxylation in the reconstituted system and in recombinant yeast microsomes fortified with reductase. This turnover number was at least 10 times

Table 1: (*S*)-Mephenytoin 4′-Hydroxylase Activities in Recombinant Human CYP2C Yeast Microsomes[a]

microsomes	mephenytoin 4′-hydroxylase activity (nmol min^{-1} nmol^{-1} P450)		
	S	*R*	*R*/*S* ratio
controls	0.028 ± 0.001	0.024 ± 0.003	0.9
2C9-Ile^{359} (65)	0.043 ± 0.000	0.041 ± 0.005	0.9
2C9-Leu^{359} (25)	0.031 ± 0.009	0.040 ± 0.01	1.3
2C8	0.037 ± 0.001	0.016 ± 0.001	0.4
2C18-Thr^{385} (29c) + b5	0.042 ± 0.004	0.054 ± 0.003[b]	1.3
2C18-Thr^{385}(29c), no b5	0.034 ± 0.008	–	
2C18-Met^{385} (6b)	0.023 ± 0.004	0.019 ± 0.005	0.9
2C19 (11a)	4.6 ± 0.3[b,c,e]	0.14 ± 0.02[b]	0.03
human liver microsomes HBI 6	0.283 ± 0.037[b,d,e]	0.117[b,d]	0.4

[a] (*S*)-Mephenytoin hydroxylase assayed as described in Methods. Reaction mixtures contained 10 pmol of recombinant CYP2C19 or 50 pmol of other recombinant CYP2C yeast microsomes, 500 units of purified P450 reductase, and 15 μg phospholipid per 50 pmol of P450, and 0.4 mM radioactive substrate in 0.1 M HEPES buffer (pH 7.4). Unless otherwise stated recombinant yeast microsomes were also reconstituted with a 2:1 molar ratio of cytochrome b_5. Reactions were incubated at 37 °C for 30 min with 1 mM NADPH. Control reactions contained the same reaction mixture and were incubated similarly with an equivalent amount of control yeast microsomal protein (1 mg). Specific content of P450 of the recombinant yeast microsomes ranged from 35–48 pmol/mg except for 2C8 (191 pmol/mg) and 2C19 (17 pmol/mg). Control liver reactions contained 0.1 mg of microsomal protein but were not fortified with reductase cytochrome b_5, or phospholipid and were incubated with 0.1 M phosphate buffer (pH 7.4). Values represent the means ± SE [b] Activity significantly higher than that of control yeast microsomes, $P < 0.05$. Analysis of variance and Fisher's least significant difference test. [c] 2C19 activity significantly higher than activities of all other recombinant CYP2C proteins or human liver microsomes, $P < 0.05$. [d] Human liver microsomes significantly higher than recombinant microsomes except 2C19, $P < 0.05$. [e] Significant difference between (*S*)- and (*R*)-mephenytoin hydroxylase activities, $P < 0.05$.

Table 2: Mephenytoin 4′-Hydroxylase and Tolbutamide Hydroxylase Activities of Purified Recombinant Human P450s from the 2C Subfamily[a]

P4502C (clone)	mephenytoin 4′-hydroxylase activity (nmol min^{-1} nmol^{-1} P450)		*R*/*S* ratio	tolbutamide hydroxylase activity (pmol min^{-1} nmol^{-1} P450)
	S	*R*		
2C19	6.17 ± 0.24[b–d]	0.19 ± 0.04[b]	0.03	ND
2C9-Ile^{359} (65)	0.081 ± 0.006[b]	0.063 ± 0.003[b]	0.77	122 + 29[b,c]
2C9-Leu^{359} (25)	ND	ND		10 + 2
2C18-Asp^{2}Thr^{385} (29c–1a)	0.116 ± 0.010[b]	0.147 ± 0.025[b]	1.3	ND
2C18-Val^{2}Thr^{385} (29c)	0.019 ± 0.001	0.073 ± 0.009[b,d]	2.7	102 ± 2[b,e]
2C18-Asp^{2}Met^{385} (6b)	0.103 ± 0.016[b]	0.107 ± 0.005[d]	1.0	ND
2C8	0.057 ± 0.009[b,d]	0.023 ± 0.004	0.4	12 ± 4
2C8 purified from human liver	0.032 + 0.003	0.051 + 0.030	1.6	ND
2C9 purified from human liver	0.033 ± 0.001	0.051 ± 0.007[b,d]	1.6	109 ± 16 (390, 2,840)[b,e,f]
human liver microsomes HBI6	0.46 ± 0.02[b]	0.28 ± 0.01	0.6	ND
human liver microsomes UC8936				408 ± 21

[a] Recombinant P450s were purified from yeast microsomes and assays performed as described in Methods. 2C9 were was purified from human liver (Raucy & Lasker, 1991). Assays were performed in triplicate and values represent means ± SE. Blank reactions (containing all components except the P450) were subtracted (22 ± 5) from tolbutamide hydroxylase values. Blank reactions for the (*S*)-mephenytoin assay were not subtracted since no distinct peaks with the exact retention times of 4′-hydroxymephenytoin were observed; however, background radioactivity was in the range of ~0.025 ± 0.01 nmol min^{-1} nmol^{-1}. ND = not determined. [b] Increased over blank values, $P < 0.05$. [c] Mephenytoin hydroxylase activity of 2C19 significantly greater than all other values, $P < 0.05$. [d] (*S*)-Mephenytoin hydroxylase activity of significantly different from (*R*)-mephenytoin values, $P < 0.05$. [e] Tolbutamide hydroxylase activity of 65 and 29c was significantly greater than 25 or 2C8 ($P < 0.0001$). [f] Tolbutamide hydroxylase activity of two other 2C9 preparations derived from different human livers in parentheses.

higher than that of human liver microsomes, and it was 50–100 times higher than that of recombinant 2C9, 2C18, or 2C8. The turnover number of recombinant 2C19 was ~100 times higher than the activity of a preparation of 2C9 purified from human liver. 4′-Hydroxylation of mephenytoin by 2C19 was stereospecific for the *S*-enantiomer, while metabolism by 2C9 was not stereospecific. Somewhat surprisingly, 2C18 appeared to be stereoselective for the *R*-enantiomer of mephenytoin. The turnover number of 2C19 for (*S*)-mephenytoin 4′-hydroxylase was also ~30 times higher than the turnover numbers reported for a preparation of 2C9 purified from human liver $P450_{MP}$ purified from human liver by Svristava *et al.* (1991) (0.21 nmol min^{-1} nmol^{-1} P450).

In contrast to its poor catalytic activity toward (*S*)-mephenytoin, 2C9 appeared to be the principal tolbutamide hydroxylase (Tables 2 and 3). Although the turnover numbers for hydroxylation of tolbutamide by the purified recombinant 2C9 were somewhat lower than expected, recombinant yeast microsomes assayed in the presence of exogenous reductase gave turnover numbers which were consistent with those of 2C9 purified from human liver. The Ile^{359} allele of 2C9 had a 3-fold higher turnover number for tolbutamide than the Leu^{359} allele when activity of the recombinant microsomes was adjusted for P450 content (Table 3). 2C19 also appeared to metabolize tolbutamide at a rate comparable to that of 2C9, although this rate was difficult to estimate due to the low specific content of P450 in the recombinant 2C19 yeast clone available at the time of these assays. The two alleles of 2C18 exhibited lower tolbutamide hydroxylase activity than 2C9 in recombinant yeast microsomes.

Comparisons of Immunoblot Analysis of CYP2C Proteins in Human Livers with Liver Microsomal (S)-Mephenytoin 4′-Hydroxylase Activities. Microsomes from 16 human liver donor samples were analyzed for (*S*)- and (*R*)-mephenytoin 4′-hydroxylase activities and were analyzed for CYP2C proteins by Western blot analysis (Figure 5) using an antibody to 2C8 and a polyclonal antibody to 2C9 which recognized several proteins in human microsomes including recombinant 2C18 and 2C19. Unfortunately, both 2C18 and 2C19 have mobilities similar to that of the low molecular weight band recognized in human microsomes by most antibodies to 2C9. However, an antibody to a 2C19 peptide was specific for 2C19. We were not successful in detecting 2C18 in human liver samples using a peptide antibody to 2C18 which was not very

Table 3: Tolbutamide Hydroxylase Activities of Recombinant Human CYP2C Yeast Microsomes[a]

microsomes	P450 content (pmol/mg)	tolbutamide hydroxylase activity (nmol min^{-1} mg^{-1}) protein	P450
control yeast	<5	0.3 ± 0.01	
2C9-Ile^{359} (65)	55	169.8 ± 7.4[b,c]	3.4 ± 0.15
2C9-Leu^{359} (25)	20	14.8 ± 0.3[b,d]	0.99 ± 0.02
2C8	80	8.5 ± 0.2[b]	0.11 ± 0.003
2C18-Asp^{2}Thr^{385} (29c–1a)	53	9.3 ± 0.7[b]	0.19 ± 0.02
2C18-Asp^{2}Met^{385} (6b–9)	34	11.1 ± 1.2[b]	0.37 ± 0.04
2C19 (11a–3)	≤7	18.4 ± 2.4[b,e]	ND
UC8936 human liver microsomes	227	116 ± 0.8[b]	2.3 ± 0.02

[a] Tolbutamide hydroxylase activities measured as described in Methods. Reaction mixtures contained 1 mg of yeast microsomal protein or 0.2 mg of UC8936 human liver microsomal protein (50 pmol of P450). Purified P450 reductase (1000 units) was included in reactions with yeast microsomes but not human microsomes. Values were the means ± SE. ND = not calculated due to low specific content of 2C19 in yeast in this experiment. [b] Significantly higher than control yeast microsomes ($P < 0.05$). Pairwise comparisons using Fisher's least significant difference test. [c] Clone 65 significantly higher than all other clones ($P < 0.0001$). [d] Clone 25 significantly greater than 2C8 ($P < 0.0005$). [e] Clone 11a significantly higher than 2C8 ($P < 0.0001$).

sensitive (~5 pmol detection limit), indicating that this protein is expressed poorly in human liver (<50 pmol/mg).

Initial comparisons suggested that 2C19 content of liver microsomes was consistent with their (S)-mephenytoin 4'-hydroxylase activities (Figure 5). In particular, samples 129 and 130 had extremely low (S)-mephenytoin hydroxylase values and low S/R ratios, and 2C19 appeared to be essentially absent in these microsomal samples. When immunoblots were scanned by laser densitometer, 2C19 content of the 16 human liver microsomes correlated significantly with (S)-mephenytoin 4'-hydroxylase activity ($r = 0.718$, $P < 0.005$) (Figure 6), but the content of 2C9 did not correlate with this catalytic activity ($r = 0.49$, $P > 0.05$; not shown). There was also a significant correlation between 2C8 content and (S)-mephenytoin 4'-hydroxylase activity ($r = 0.82$, $P < 0.0001$). However, this correlation is probably fortuitous, since this CYP protein does not appear to metabolize this substrate either in recombinant form or when purified from human liver. Alternatively, it may reflect a common mechanism of regulation for some of the CYP2C proteins.

Sequences of 2C9 and 2C18 mRNAs in Livers with High or Low (S)-Mephenytoin 4'-Hydroxylase Activities. 2C18 and 2C9 mRNAs from six of the above livers were amplified by PCR and directly sequenced through areas of known allelic variation to determine whether there was a relationship between (S)-mephenytoin 4'-hydroxylase activity and the presence of a particular allelic variant (Table 4). When the total 2C18 PCR products were sequenced, the two individuals with the highest (S)-mephenytoin hydroxylase activity were homozygous for Thr^{385}(ACG). Of the two individuals with the lowest activity, one was homozygous for Met^{385} and one was heterozygous for Thr/Met^{385} (AC/TG). Two individuals with intermediate activity were also homozygous for Thr^{385}. Similarly, when 2C9 mRNA from these same individuals was amplified and sequenced through known alleleic variations, sample 108 [low (S)-mephenytoin 4'-hydroxylase activity] was heterozygous at C/T^{430} (coding for Cys/Arg^{144}), while the other five individuals were homozygous for C^{430} (Arg^{144}). Sequencing samples through bases 1072–1077, all samples except for 106 (high activity) read ^{1072}TACATT^{1077}, coding for Tyr^{358}Ile^{359}. Sample 106 read TACA/CTT indicating that it was heterozygous for Ile/Leu^{359}. These results do not suggest that there is any relationship between (S)-mephenytoin 4'-hydroxylase activity and the identity of an allelic variant of 2C18 or 2C9 in human liver.

Srivastava *et al.* (1991) reported that amino acid sequencing of tryptic peptides of P450$_{MP}$ purified from liver 115 (which had a high turnover number for the 4'-hydroxylation of (S)-mephenytoin) yield a sequence essentially identical to that of 2C9 cDNAs sequenced to date except for the presence of a glycine at position 276 instead of histidine. In the present study, direct sequencing of the PCR products of 2C9 mRNA from the six livers (including 115) through bases 826–828 indicated that all of these livers were homozygous for His^{276}, not glycine. Moreover, a full-length cDNA for 2C9 amplified by PCR from mRNA from liver 115 was also completely sequenced and found to be identical to our clone 65 allelic variant (Arg^{144}His^{276}Tyr^{358}Ile^{359}Gly^{417}) (Romkes *et al.*, 1991).

DISCUSSION

There has been considerable controversy concerning the role of P4502C9 to the 4'-hydroxylation of (S)-mephenytoin and in the genetic polymorphism observed in the metabolism of this substrate in humans. Nearly full-length cDNAs for 2C9 and the closely related 2C10 were first isolated by Umbenhauer *et al.* (1987) using antibody to a purified human P450(P450$_{MP-1}$) which metabolized (S)-mephenytoin. However, when the full-length cDNA for 2C10 was constructed from the known N-terminal sequence of P450$_{MP-1}$ and expressed in yeast, recombinant 2C10 did not 4'-hydroxylate (S)-mephenytoin (Brian *et al.*, 1989). A full-length cDNA for 2C9 (Tyr^{358}Ile^{359}Gly^{417}) constructed by site-directed mutagenesis from 2C10 (Cys^{358}Ile^{359}Asp^{417}) also failed to metabolize (S)-mephenytoin when expressed in yeast (Srivastava *et al.*, 1991). In contrast, studies by Yasumori *et al.* (1989, 1991) indicated that an identical cDNA expressed in a slightly different yeast expression system 4'-hydroxylated (S)-mephenytoin preferentially over (R)-mephenytoin. However, the turnover numbers for the 4'-hydroxylation of (S)-mephenytoin by recombinant 2C9 in yeast microsomes (0.015 nmol min^{-1} nmol^{-1}) were approximately 20-fold lower than values normally obtained with human liver microsomes (Brian *et al.*, 1989; Meier & Meyer, 1987), although they were stimulated 10-fold by the addition of cytochrome b_5 and lipid to the yeast microsomes. Relling *et al.* (1990) reported that an allelic variant of 2C9, which was similar to that used by Yasumori *et al.* (1989) but contained a Cys^{144} instead of Arg^{144}, exhibited (R)-mephenytoin but not (S)-mephenytoin 4'-hydroxylase activity when expressed in HepG2 cells using a retroviral expression vector. Using a COS-1 cell cDNA expression system, we earlier reported that recombinant 2C18 metabolized racemic mephenytoin, but we could not direct metabolism of this substrate in cells transfected with 2C9 or 2C19 (Romkes *et al.*, 1991). However, COS-1 cells express low amounts of recombinant P450 proteins, and the rate of metabolism of this substrate was near the limits of detection.

Therefore, in the present study, we utilized a yeast cDNA expression system to achieve much higher levels of expression of the recombinant CYP2C proteins and a highly sensitive radiometric assay to enable us to compare the ability of the human CYP2C subfamily to 4'-hydroxylate both (S)- and (R)-mephenytoin. Our present data clearly show that CYP2C19 stereospecifically hydroxylates (S)-mephenytoin at the 4'-position at a rate which is at least 10 times higher than the rate in human liver microsomes. A low rate of 4'-hydroxylation of mephenytoin could be detected with both recombinant 2C9 and 2C18, but the hydroxylation by these

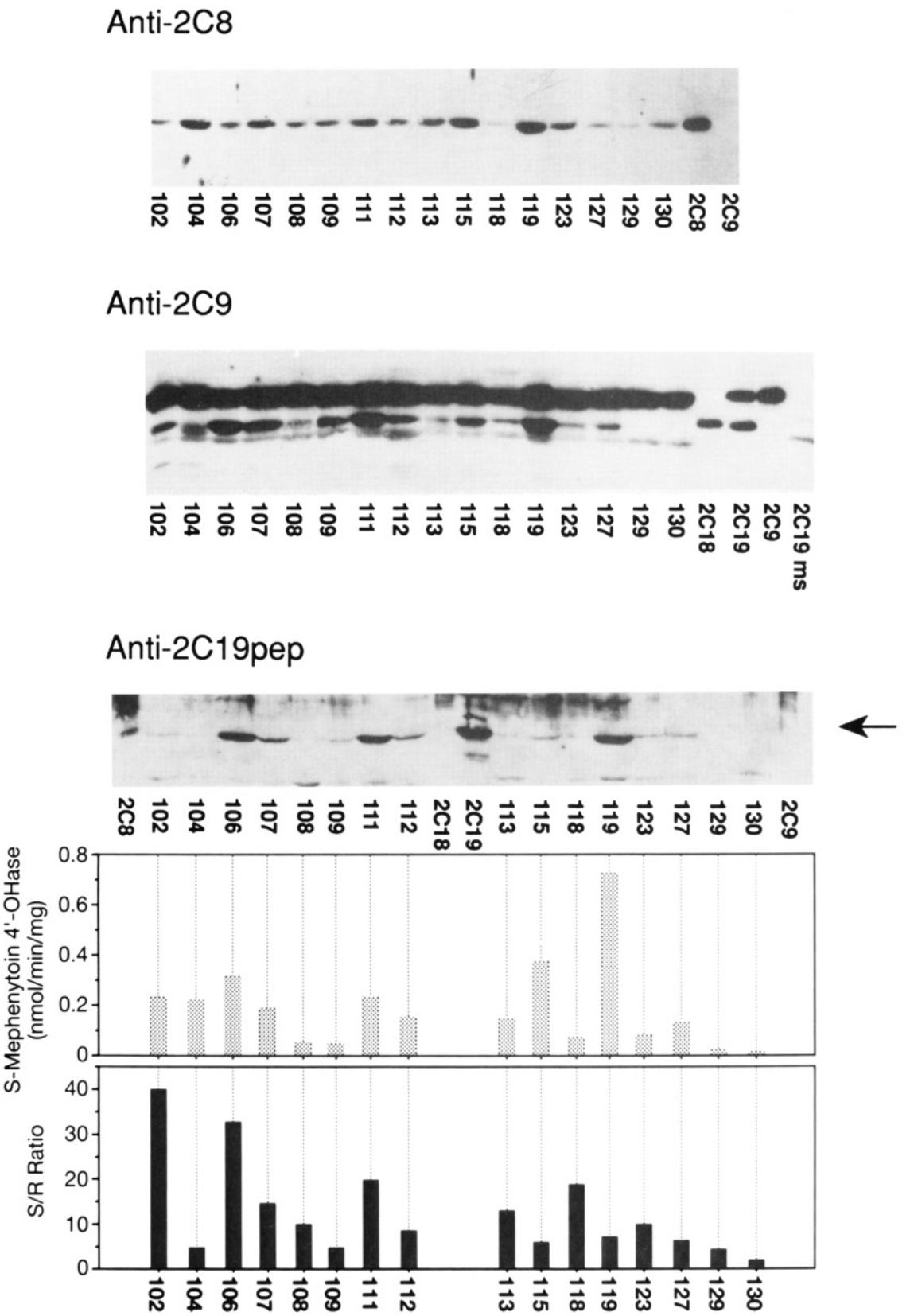

FIGURE 5: Western blot analysis of CYP2C proteins, (*S*)-mephenytoin 4′-hydroxylase activity, and ratios of *S*/*R* mephenytoin hydroxylase activities of 16 human liver samples. 2C8, 2C9, and 2C18 represent recombinant purified proteins. For the 2C8 immunoblot, lanes contained 20 μg of human liver microsomal protein or 1 pmol of purified recombinant 2C8 or 1.25 pmol purified recombinant 2C9. For the 2C9 immunoblot, lanes contained 100 μg of protein or purified recombinant 2C9 (2.5 pmol), 2C18 (3 pmol) or 2C19 (2 pmol). The top band in the 2C19 lane is a nonspecific reaction to a yeast protein which was not seen when the blot was developed for less time. For the *anti*-2C19 peptide, lanes contained 100 μg protein or 5 pmol of the recombinant proteins. Liver microsomal (*S*)-mephenytoin 4′-hydroxylase, and *R*/*S* ratios of 16 human liver microsomes (bottom 2 panels).

cytochromes was not stereospecific for the *S*-enantiomer. In fact, 2C18 appeared to preferentially metabolize the *R*-enantiomer.

In the present study, the rate of 4′-hydroxylation of mephenytoin by 2C19 is ~100 times higher than that of either 2C9 or 2C18, and 2C8 appears to be even less active. The

Table 4: Alleles in Human Livers with Varying (S)-Mephenytoin 4′-Hydroxylase Phenotypes

phenotype	S-MPOHase nmol min^{-1} mg^{-1}	liver donor	2C18 allele	2C9 allele
high	0.286	106	Thr^{385}	Arg^{144} His^{276} Tyr^{358} Ile/Leu^{359}
high	0.351	115	Thr^{385}	Arg^{144} His^{276} Tyr^{358} Ile^{359}
intermediate	0.070	118	Thr^{385}	Arg^{144} His^{276} Tyr^{358} Leu^{359}
intermediate	0.081	123	Thr^{385}	Arg^{144} His^{276} Tyr^{358} Ile^{359}
low	0.051	108	Thr/Met^{385}	Arg/Cys^{144} His^{276} Tyr^{358} Ile^{359}
low	0.025	129	Met/Met^{385}	Arg^{144} His^{276} Tyr^{358} Ile^{359}

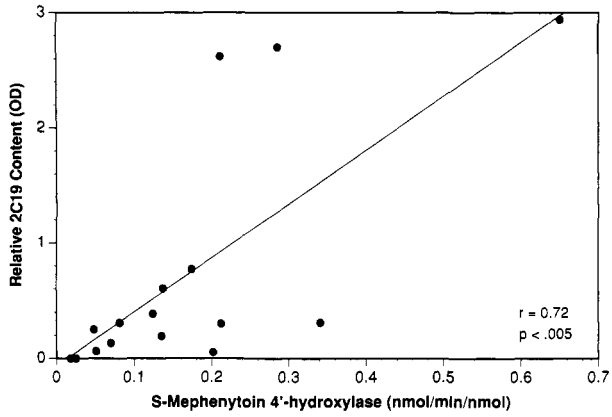

FIGURE 6: Correlation between hepatic 2C19 content (Figure 5) and (S)-mephenytoin hydroxylase activity $r = 0.714$ ($P < 0.005$). Hepatic content of 2C8 also correlated with catalytic activity $r = 0.82$ ($P = 0.001$), but 2C9 did not ($r = 0.42$) (not shown).

present study provides the first example of a human CYP protein which metabolizes (S)-mephenytoin with a turnover number appreciably higher than that of human liver microsomes. The rate is also ~30 higher than that reported for $P450_{MP}$, a cytochrome with (S)-mephenytoin hydroxylase activity which was purified from human liver by Srivastava *et al.*, 1991). There was no substantial difference in the ability of the Ile^{359} or Leu^{359} alleles of 2C9, or the Thr^{385} or Met^{385} alleles of 2C18, to metabolize mephenytoin in the present study. Our 2C9 Ile^{359} variant is identical in the coding region to that isolated by Yasumori *et al.* (1987). The low but detectable rate of 4′-hydroxylation of (S)-mephenytoin by 2C9 which was detected in the present study with high specific activity ^{14}C-labeled (S)-mephenytoin undoubtedly explains the conflicting reports from various laboratories concerning the ability of this cytochrome to metabolize mephenytoin (Yasumori *et al.*, 1991; Srivastava *et al.*, 1991; Relling *et al.*, 1991). However, our data show that the (S)-mephenytoin 4′-hydroxylase activity of 2C19 is approximately 2 orders of magnitude higher than that of either 2C9, 2C18, or 2C8. The difference in the catalytic activity of 2C9 and 2C19 toward (S)-mephenytoin is particularly interesting when one considers the fact that the two proteins are 96% similar in their amino acid structure. However, it has been established that changes in even a single amino acid can affect both turnover numbers as well as substrate specificity of certain P450 proteins (Lindberg & Negishi, 1989).

Some earlier studies suggested that the hepatic content of human form 2 (2C9) correlated ($r = 0.74$, $p < 0.001$) with microsomal (S)-mephenytoin 4′-hydroxylase activity of human liver microsomes (Yasumori *et al.*, 1990; 1991). However, the relative amounts of immunodetectable human form 2 on Western blots did not appear to be completely consistent with the (S)-mephenytoin 4′-hydroxylase activity in extensive and poor metabolizers (Yasumori *et al.*, 1991). Moreover, other laboratories found no correlation between immunodetectable MP-1 (2C9) or MP-2 and microsomal (S)-mephenytoin hydroxylase activity in human liver (Shimada *et al.*, 1986), 2C9 or 2C8 mRNAs and rates of microsomal (S)-mephenytoin 4′-hydroxylase in human liver (Ged *et al.*, 1988), or tolbutamide metabolism (which is presumably mediated by 2C9) and (S)-mephenytoin 4′-hydroxylation Relling *et al.* (1990).

In the present study, the amount of 2C19 in human liver microsomes correlated with the (S)-mephenytoin 4′-hydroxylase activities while the amount of 2C9 did not. These conclusions are consistent with those of Wrighton *et al.* (1993), who recently reported that the liver content of a lower band recognized by a monoclonal antibody to 2C9, which they identified as 2C19, appeared to correlate with the (S)-mephenytoin hydroxylase activity of 14 liver samples. It is not clear whether their monoclonal antibody would also recognize 2C18 which has a similar mobility and shares considerable amino acid homology with 2C19. However, we have come to a similar conclusion with a specific peptide antibody for 2C19. The correlation between 2C19 content of human liver microsomes on immunoblots and liver microsomal (S)-mephenytoin 4′-hydroxylase activity in our samples is consistent with the relative activities of the recombinant 2C proteins.

Several laboratories (Yasumori *et al.*, 1991; Srivastava *et al.*, 1991) have suggested the logical possibility that a variant of 2C9 might be the (S)-mephenytoin 4′-hydroxylase. Svristava *et al.* (1991) isolated a 2C9 protein ($P450_{MP}$) from an individual with high hepatic (S)-mephenytoin 4′-hydroxylase activity and low tolbutamide hydroxylase and a second form ($P450_{TB}$) from an individual with relatively high tolbutamide hydroxylase activity and low (S)-mephenytoin 4′-hydroxylase. The purified proteins appeared to metabolize either tolbutamide ($P450_{TB}$) or (S)-mephenytoin ($P450_{MP}$) but not both. They sequenced some of the tryptic peptides of both proteins. The amino sequences of $P450_{TB}$ (16% sequenced) matched the predicted sequences of cDNAs for 2C9 and 2C10. The amino acid sequence of $P450_{MP}$ showed one mismatch with 2C9 out of 219 residues sequenced (glycine for histidine at 276). However, in the present study, amplification of 2C9 mRNA and direct sequencing of the PCR products from six livers, including 115, indicated that the 2C9 in all of these livers contained His^{276}. Moreover, direct sequencing of the PCR products of 2C18 and 2C9 mRNAs in areas of other known allelic variations did not support the theory that there is any relationship between (S)-mephenytoin 4′-hydroxylase activity of human liver microsomes and the identity of the allelic variants of 2C18 (Thr/Met^{385}) or 2C9 (Arg/Cys^{144}, Tyr/Cys^{358}, Ile/Leu^{359}) in these tissues.

In summary, our results clearly show that 2C19 has an extremely high turnover number for the 4′-hydroxylation of (S)-mephenytoin. The hydroxylation was stereospecific for the S-enantiomer, and the turnover number was approximately 100-fold higher than that of 2C9, 2C18, or 2C8. The hepatic content of 2C19 in 16 liver microsomal samples correlated with their (S)-mephenytoin 4′-hydroxylase activities. In contrast, 2C9 appeared to be the primary tolbutamide hydroxylase, although 2C19 may also contribute to this catalytic activity. Although the Ile^{359} allele of 2C9 had a

higher turnover number for tolbutamide than the ^{359}Leu allele, the identity of the allelic variant of 2C9 or 2C18 did not appear to influence (*S*)-mephenytoin 4′-hydroxylase activity. Our data clearly indicate that 2C19 is the principal determinant of (*S*)-mephenytoin 4′-hydroxylase activity in human liver.

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