MONOCLONAL ANTIBODIES SPECIFIC AND INHIBITORY TO HUMAN CYTOCHROMES P450 2C8, 2C9, AND 2C19

KRISTOPHER W. KRAUSZ, INNA GOLDFARB, JEROEN T. M. BUTERS,1 TIAN J. YANG,2 FRANK J. GONZALEZ, AND HARRY V. GELBOIN

Laboratory of Molecular Carcinogenesis and Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

(Received May 8, 2001; accepted July 20, 2001)

This paper is available online at http://dmd.aspetjournals.org

ABSTRACT:

Hybridomas were isolated that produce 13 monoclonal antibodies (mAbs) that are specific and highly inhibitory to members of the human P450 2C subfamily, 2C8, 2C9, 2C9*2, and 2C19. Many of the mAbs to P450 2C8, 2C9, and 2C19 are specific and exhibit potent inhibitory activity (85–95%). mAb 281-1-1 specifically binds, immunoblots, and strongly inhibits the activity of P450 2C8. mAb 763-15-5 specifically binds and strongly inhibits the activity of P450 2C9. mAb 1-7-4-8 specifically binds and strongly inhibits the activity of P450 2C19. The other mAbs bind and inhibit sets and subsets of the P450 2C family. The single and the combinatorial use of the mAbs can “reaction phenotype”, i.e., determine the metabolic contribution and interindividual variation of a P450 isoform for the metabolism of a drug or nondrug xenobiotic in human liver microsomes. The utility of the mAb-based analytic system was examined with the model substrates Taxol (paclitaxel), diazepam, tolbutamide, diclofenac, mephenytoin, and imipramine. The mAb system can identify drugs metabolized by a common P450 or several P450s and polymorphic P450s. The mAb system identifies drugs or drug metabolic pathways that are catalyzed by a single P450 and thus may be used for in vivo phenotyping. The mAb system can identify whether a particular drug is metabolized by a single P450 that may exhibit polymorphic expression in humans. The mAb system offers large potential for studies of cytochrome P450 function useful in drug discovery and reduces the possibility of adverse drug reactions due to polymorphisms and drug interactions.

The human cytochrome P450s collectively metabolize a multitude of drugs and nondrug xenobiots, including toxins, mutagens, and carcinogens such as aflatoxin, as well as endobiotics such as steroids, prostaglandins, and arachidonic acids (Ioannides, 1996). Drug metabolism in human liver is primarily catalyzed by 12 major microsomal P450 enzymes having different substrate and product specificities and that are heterogeneously distributed in tissues (Ioannides, 1996; Ren dic and Di Carlo, 1997). P450-catalyzed metabolism of drugs and nondrug xenobiots is a key element in drug disposition and may be responsible for certain adverse drug reactions, chemical toxicity, and immunotoxicity (Ioannides, 1996).

The human cytochrome P450 2C subfamily consists of four isoforms: 2C8, 2C9, 2C18, and 2C19. The 2C isoforms are collectively among the most important human enzymes responsible for the metabolism of a wide variety of drugs, including Taxol (paclitaxel)1 (Rahman et al., 1994) phenytoin, tolbutamide, S-warfarin, losartan, S-mephenytoin, and diazepam (Miners and Birkett, 1998).

Monoclonal antibodies are reagents par excellence (Yelton and Scharff, 1981) that have proved to be of great value for the precise identification, measurement, and functional characterization of each P450 isoform (Gelboin, 1993). The mAbs3 are derived from potentially immortal hybridomas and are specific and highly inhibitory to the enzyme activity of the target P450 and thus are powerful reagents for “reaction phenotyping”, i.e., for measuring the metabolic contribution of each of the multiple P450s to a substrates metabolism (Gelboin, 1993).

This article reports the isolation of hybridomas producing monoclonal antibodies that are both specific and highly inhibitory to individual P450 isoforms and isoform sets of the 2C subfamily of human P450s, namely, 2C8, the alleles 2C9*1 and 2C9*2, and 2C18 and 2C19. One of the mAbs is specific to 2C9*2, a single allele of polymorphic 2C9, which differs from the wild-type 2C9*1 by a single amino acid substitution, Arg144Cys (Krausz et al., 2000).

In previous reports, mAbs specific to seven individual major human liver microsome (HLM) P450 isoforms were described, namely, 1A1 (Fujino et al., 1982), 1A2 (Yang et al., 1998b), 2A6 (Sa et al., 1999), 2B6 (Yang et al., 1998a), 2D6 (Gelboin et al., 1997), 2E1 (Gelboin et al., 1996), and 3A4/5 (Gelboin et al., 1995), and to the entire 2C subfamily (Park et al., 1989; Gelboin et al., 1997). The mAbs measured the contribution of each target P450 to the metabolism of a variety of examined drugs (Gelboin et al., 1999; Yang et al., 1999). Many of the mAbs can also be used to measure the tissue P450 protein content by immunoblot analysis. Using the new inhibitory mAbs singly or combinatorially we

1 Current address: Institute for Toxicol-GSF, Oberschleissheim, Germany.
2 Current address: DuPont Pharmaceuticals Co., Newark, Delaware.
3 Abbreviations used are: mAb, monoclonal antibody; HLM, human liver microsome; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; NDZ, nordiazepam; DIM, desipramine; RAF, relative activity factor; pAb, polyclonal antibody.
have examined the contribution of each of the 2C subfamily P450s to the metabolism of the model drugs Taxol, diazepam, tolbutamide, diclofenac, S-mephenytoin, and imipramine.

The isolation of the mAbs to the P450 2C isoforms satisfies our goal of obtaining a library of hybridomas producing specific inhibitory mAbs to each of the major forms of human liver P450s responsible for drug and nondrug xenobiotic and endobiotic metabolism.*

Materials and Methods

Chemicals, Tolbutamide, diclofenac, diazepam, phenanthrene, and NADPH were obtained from Sigma (St. Louis, MO). Taxol, 6o-hydroxytaxol, S-mephenytoin, 4'-hydroxymephenytoin, bufuralol, 1'-hydroxybufuralol, hydroxyltolbutamide, and 4'-hydroxydiclofenac were obtained from GENTEST (Woburn, MA). Imipramine and desipramine were obtained from ICN Bio-technicals (Aurora, OH). All reagents were of analytical grade. Nordiazepam was a gift from Dr. Shen Yang (DuPont Pharmaceuticals, Newark, DE).

**P450 Expression and Hybridoma Production.** P450 2C9 recombinant baculovirus was previously constructed (Grogan et al., 1995). cDNA P450 2C9 recombinant baculovirus was previously constructed (Grogan et al., 1995). cDNA P450 2C9 recombinant baculovirus was previously constructed (Grogan et al., 1995). Three days postinfection, cells were harvested, the P450 difference spectrum measured, and the cells were stored at −80°C. The P450 was extracted from the cells, as previously described (Gelboin et al., 1998), yielding 268.0 nmol of P450 2C8, 22.5 nmol of P450 2C9, and 60.5 nmol of P450 2C19 each from 1 l of individual cell culture preparations.

The extracted, expressed P450 was used as the immunogen. BALB/c mice were immunized with 30 to 50 µg of extracted P450 2C8, 2C9, or 2C19 emulsified in 0.2 ml of adjuvant once a week for 3 weeks, sacrificed, and the spleen cells isolated. The complete immunization technique and hybridization of spleen cells with myeloma cells (NS-1) has been detailed (Gelboin et al., 1988). Ninety-six-well plates were coated with 1.5 pmol of P450 enzyme. Culture fluid and/or diluted ascites was used as the primary mAb, with an alkaline phosphatase-conjugated anti-mouse IgG (Fc specific) (Jack-son Immunoresearch, West Grove, PA) used as a secondary antibody. Color-
Immunometric assays were measured at 405 nm wavelength with an Emax plate reader by using SOFTmax version 2.31 software (Molecular Devices, Sunnyvale, CA) as detailed (Krausz et al., 2000). All ELISA data are presented as mean ± SEM and have been background corrected and normalized to zero. Wells with an optical density reading >0.6 with specific immunogen and an optical density <0.1 with wild-type antigen were investigated further. All resulting specific hybridomas were cloned at least three times. SDS-polyacrylamide gel electrophoresis gels and immunoblots were performed as previously described (Gelboin et al., 1996). Relative intensities of immunostained blots were determined using a Molecular Dynamics model 300B computing densitometer. Quantified values (volume in pixels) of bands were background subtracted and rated as follows: −, (<51) no binding/background; +, (51–150) weak binding; ++, (151–250) strong binding; and ++++, (>250) very strong binding.

mAb isotyping was performed using radial immunodiffusion kits purchased from The Binding Site (San Diego, CA). All mAbs described in this article are mouse IgG1, with the exception of mAb 1-68-11, which is a mouse IgM.

**Metabolic Incubations.** Metabolism and immunoinhibition studies were performed using cDNA-expressed P450 enzymes and HLMs. Metabolic incubations were generally conducted as follows: 0.057 to 30 μl (1.2–1000 μg) of ascites protein containing mAb was preincubated with 30 to 50 pmol of expressed P450 enzyme or 150 to 300 pmol of P450 in HLMs in 0.5 ml of buffer (100 mM Tris, pH 7.5) at 37 °C for 5 min. Substrate (in 10 μl of methanol), 1 mM NADPH, and additional buffer were added to a final volume of 1.0 ml. The reactions were allowed to proceed for 60 min with the tolbutamide, and S-mephenytoin; 15 min with diclofenac; and 30 min with phenanthrene, diazepam, imipramine, and bufuralol; and stopped with 1 ml of acetone. The metabolites were extracted with 8 ml of dichloromethane and subjected to HPLC separation conditions for diclofenac (50 μM) to 4-hydroxydiclofenac (Crespi and Penman, 1997), tolbutamide (200 μM) to hydroxytolbutamide (Relling et al., 1990), S-mephenytoin (150 μM) to 4-hydroxymephenytoin (Heyn et al., 1996), diazepam (150 μM) to nordiazepam (Yang et al., 1998c), imipramine (200 μM) to desipramine and 2-hydroxyimipramine (Yang et al., 1999), Taxol (150 μM) to 6a-hydroxytaxol (Rahman et al., 1994), phenanthrene (200 μM) to 9,10-diol and 9-phenol (Yang et al., 1999), and bufuralol (50 μM) to 1’-hydroxybufuralol (Gelboin et al., 1997), as previously described. HPLC was performed using a Hewlett Packard model 1050 Series system equipped with an autosampler, a quaternary solvent delivery system, and a diode array detector controlled by the Hewlett Packard Chemstations software. Metabolite retention times were compared with authentic standards (when available) and metabolite peaks were quantitated based on ratios with internal standards. Control incubations contained a mAb.
against hen egg white lysozyme (mAb HyHel-9) to assess for nonspecific reactions. Percentage of inhibition was calculated based on activity in the presence of the specific mAb relative to the activity with the control mAb. Percentage of inhibition equals 100 minus percentage of control. Data are the mean of duplicate incubations by using expressed enzymes and duplicate incubations by using HLMs.

Inhibition of phenanthrene metabolism to 9,10-diol was used to test for inhibitory cross-reactivity of the mAbs with expressed P450 1A1, 1A2, 2A6, 2B6, 2D6, 2E1, 3A4, 3A5; inhibition of phenanthrene metabolism to the 9-phenol was assayed for cross-reactivity with P450 2A6; and inhibition of bufuralol metabolism to 1H-11032-11032-hydroxybufuralol was assayed for cross-reactivity with P450 2D6. Inhibition of diazepam conversion to nordiazepam (NDZ) was also used to test the inhibitory cross-reactivity of the mAbs among the P450 2C isoforms.

**Isolation and Screening of Hybridomas and Monoclonal Antibodies.**

mAbs to P450 2C8.

mAbs from 830 hybridoma clones produced 16 mAbs that bound to 2C8. Three of the 16 hybridoma clones produced mAbs that bound specifically and strongly to only 2C8. The three mAbs were 281-1-1, 13-1-3, and 5-1-5, which were both specific and strong inhibitors (90%) of expressed 2C8 enzyme activity. The three mAbs also immunoblot 2C8. Of the three mAbs the mAb 281-1-1 was further characterized (see Results).

mAbs specific to individual P450 2C9, 2C19, and to isoform sets of 2C subfamily. To screen for mAbs to 2C9, mAbs produced from 1088 hybridomas were screened and 68 clones were identified that bound to 2C9. Six of the 68

### TABLE 5
Specificity of Immunoblotting activity of monoclonal antibodies to expressed isoforms of the P450 2C subfamily

<table>
<thead>
<tr>
<th>mAb/P450</th>
<th>2C8</th>
<th>2C9*1</th>
<th>2C9*2</th>
<th>2C9*3</th>
<th>2C18</th>
<th>2C19</th>
<th>1A1, 1A2, 2A6, 2B6, 2D6, 2E1, 3A4, 3A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb 292-2-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>mAb 763-15-5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>mAb 763-15-20</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>mAb 592-2-5</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>mAb 327-8-3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>mAb 5-7-5</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>mAb 1-68-11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Binding intensity was rated as follows: -, no binding; +, weak binding; ++, strong binding; ++++, very strong binding. Qualitative values are described under Materials and Methods.

**A**

Diazepam Metabolism
- P450 2C9*1
- P450 2C9*2
- P450 2C19

Phenanthrene Metabolism
- P450 2C9*1
- P450 2C9*2
- P450 2C18
- P450 2C19

**B**

A, specificity of mAb 763-15-5 inhibition of P450 2C9. B, mAbs anti-2C19 inhibition of metabolism. C, inhibitory activity of purified mAb 1-7-4-8 IgG. D, specificity of mAb 1-7-4-8 to P450 2C19. mAb was added as indicated to 25 to 40 pmol of expressed P450 and preincubated for 5 min at 37°C. The reaction was initiated by addition of DZ (200 µM) or phenanthrene (150 µM) and NADPH (1 mM) and allowed to proceed for 30 min. N-Demethylation of DZ and 9,10-diol formation of phenanthrene were measured. Data are the mean of duplicate incubations.

**C**

Diazepam Metabolism
- P450 2C9*1
- P450 2C9*2
- P450 2C19

Phenanthrene Metabolism
- P450 2C9*1
- P450 2C9*2
- P450 2C19

**D**

A, specificity of mAb 1-7-4-8 inhibition of P450 2C9, 2C18, and 2C19 metabolism of diazepam (DZ) and phenanthrene.

**FIG. 2.** Inhibition of expressed P450 2C9, 2C18, and 2C19 metabolism of diazepam (DZ) and phenanthrene.
Results

The specificity of mAb 281-1-1, mAb 13-1-3, and mAb 5-1-5 was determined with expressed P450 2C8 as measured by ELISA binding (Table 1). The three mAbs do not bind to any of the other isoforms of the 2C subfamily 2C9*1, 2C9*2, 2C9*3, 2C18, and 2C19 nor to the other isoforms of P450s listed, namely, 1A1, 1A2, 2A6, 2B6, 2D6, 2E1, 3A4, and 3A5. mAb 281-1-1, mAb 13-1-3, and mAb 5-1-5 are also completely specific to 2C8 for immunoblotting activity and do not cross-react with any of the other 13 P450 isoforms examined (Table 2). Thus, the three mAbs can be used to specifically detect and measure the amount of 2C8 protein in human tissues. Table 3 shows the ELISA analysis of a panel of seven mAbs to cDNA-expressed isoforms of the P450 2C subfamily, namely, 2C8, the three alleles 2C9*1, 2C9*2, and 2C9*3, and 2C18 and 2C19 and reveals their binding specificity. The mAbs to isoforms of the 2C subfamily do not bind to any of the eight non-2C P450s listed above. mAb 763-15-5 binds strongly to each of the three alleles of 2C9 and does not cross-react significantly with any of the other 2C isoforms, 2C8, 2C18, and 2C19, or the eight non-2C P450 isoforms. The mAb 763-15-5 is also a specific and potent inhibitor of 2C9 enzyme activity (Fig. 2) and is the mAb of choice for investigating 2C9 function. The mAb 763-15-20 is not inhibitory, binds weakly to the three 2C9 alleles and 2C8 but not to 2C18 or 2C19 nor to the non-2C family P450s, but yields strong and specific immunoblots with 2C8 and 2C9 (Fig. 1). mAb 592-2-5 binds to the three 2C9 alleles equally well, less to the minor 2C18, but not to 2C8, 2C19, or other P450s. mAb 327-8-3 shows specificity of binding similar to mAb 592-2-5. mAb 5-7-5 binds all the 2C isoforms except 2C8 and is thus useful for combinatorial analyses with mAb 592-2-5 for the detection of 2C19. The binding of an mAb to different isoforms indicates the presence of a common epitope to the various target isoforms (see Results; Tables 1-7).

Another mAb resulting from immunization with 2C9*2 was mAb 292-2-3. The details of its isolation have been previously described (Krausz et al., 2000) and its further characterization are described under Results. Previously, mAb 1-68-11 was made with purified rat liver 2c/RLM5 (Park et al., 1989), which was subsequently found to inhibit all four of the human 2C isoforms: 2C8, 2C9, 2C18, and 2C19 (Gelboin et al., 1997). The screening for mAbs to 2C19. The screening for binding of mAbs produced from 763 hybridoma clones detected the binding to 2C19 mAbs from 15 clones. Three of the latter mAbs strongly and specifically bound and inhibited 2C19 metabolic activity. These are mAb 1-7-4-8, mAb 1-5-1-3, and mAb 1-1-11. Of the latter mAb 1-7-4-8 was characterized more fully (see Results).

### Table 1

<table>
<thead>
<tr>
<th>P450</th>
<th>2C0</th>
<th>2C0*1</th>
<th>2C0*2</th>
<th>2C18</th>
<th>2C19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Activity</td>
<td>3.52</td>
<td>0.32</td>
<td>0.38</td>
<td>1.15</td>
<td>14.4</td>
</tr>
<tr>
<td>mAbs anti-2C8</td>
<td>281-1-1</td>
<td>3.52</td>
<td>0.32</td>
<td>0.38</td>
<td>1.15</td>
</tr>
<tr>
<td>mAbs anti-2C9</td>
<td>292-2-3</td>
<td>281-1-1</td>
<td>3.52</td>
<td>0.32</td>
<td>0.38</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Specificity of mAbs anti-2C8, 2C9, and 2C19 for inhibition of diazepam N-demethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb/P450</td>
</tr>
<tr>
<td>Control Activity</td>
</tr>
<tr>
<td>mAbs anti-2C8</td>
</tr>
<tr>
<td>mAbs anti-2C9</td>
</tr>
<tr>
<td>mAbs anti-2C19</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Specificity of mAbs anti-2C8, 2C9, and 2C19 for inhibition of diazepam N-demethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb/P450</td>
</tr>
<tr>
<td>Control Activity</td>
</tr>
<tr>
<td>mAbs anti-2C8</td>
</tr>
<tr>
<td>mAbs anti-2C9</td>
</tr>
<tr>
<td>mAbs anti-2C19</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Specificity of mAbs anti-2C8, 2C9, and 2C19 for inhibition of diazepam N-demethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb/P450</td>
</tr>
<tr>
<td>Control Activity</td>
</tr>
<tr>
<td>mAbs anti-2C8</td>
</tr>
<tr>
<td>mAbs anti-2C9</td>
</tr>
<tr>
<td>mAbs anti-2C19</td>
</tr>
</tbody>
</table>

### Table 5

<table>
<thead>
<tr>
<th>Specificity of mAbs anti-2C8, 2C9, and 2C19 for inhibition of diazepam N-demethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb/P450</td>
</tr>
<tr>
<td>Control Activity</td>
</tr>
<tr>
<td>mAbs anti-2C8</td>
</tr>
<tr>
<td>mAbs anti-2C9</td>
</tr>
<tr>
<td>mAbs anti-2C19</td>
</tr>
</tbody>
</table>

### Table 6

<table>
<thead>
<tr>
<th>Specificity of inhibitory activity of mAbs anti-P450 2C8, 2C9, and 2C19 for phenanthrene metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formation of 9-10 diol was measured except for 2A6, where 9-phenol was measured, and 2D6, where bufurolol was used as the substrate and 1-OH bufurolol was measured.</td>
</tr>
</tbody>
</table>

### Table 7

<table>
<thead>
<tr>
<th>Specificity of mAbs anti-2C8, 2C9, and 2C19 for inhibition of diazepam N-demethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb/P450</td>
</tr>
<tr>
<td>Control Activity</td>
</tr>
<tr>
<td>mAbs anti-2C8</td>
</tr>
<tr>
<td>mAbs anti-2C9</td>
</tr>
<tr>
<td>mAbs anti-2C19</td>
</tr>
</tbody>
</table>
of the 2C family and thus detects all of the isoforms of the entire 2C family and has been previously reported (Gelboin et al., 1997).

The high specificity and strong binding of mAb 1-7-4-8, mAb 1-1-11, and mAb 1-5-1-3 to 2C19 are shown in Table 4. They do not bind 2C8 nor the other five 2C P450 isoforms or the non-2C P450s. The three anti-2C19 mAbs show a low level of binding to the 2C9 alleles, but all three mAbs are completely specific for the inhibition of 2C19 metabolic activity at a low 4-fold wide range of mAb concentrations. Seven mAbs exhibit immunoblotting activity (Table 5); however, mAb 292-2-3, mAb 763-15-5, and mAb 1-68-11, all of which bind to different 2C isoforms, do not exhibit any immunoblotting activity. The mAb 763-15-20 immunoblots all three alleles of 2C9 as well as 2C8, but does not immunoblot 2C18, 2C19, or the other eight non-2C family P450s. The combinatory use of mAb 763-15-20 and mAb 5-7-5 measures total protein content of the entire 2C family. mAb 592-2-5 and mAb 327-8-3 exhibit identical positive immunoblotting activity for the three 2C9 alleles and 2C18. mAb 5-7-5 immunoblots all the 2C isoforms except 2C8. Either mAb 592-2-5 or mAb 327-8-3 may be used combinatorially with mAb 5-7-5 to determine 2C19 protein content because mAb 5-7-5 immunoblots 2C19 as well as the three alleles of 2C9 and 2C18. Figure 1 shows the specificity of immunoblotting activity and utility of mAb 763-15-20, mAb 281-1-1, and mAb 592-2-5. Thus, mAb 281-1-1 specifically immunoblots P450 2C8 only, mAb 763-15-20 immunoblots both 2C8 and all three alleles of 2C9 and does not immunoblot 2C18 or 2C19, and mAb 592-2-5 immunoblots all three alleles and 2C18. Thus, the amount of P450 2C8, 2C9, and 2C18 can be determined by the single or combinatorial use of the three mAbs. The specificity of the inhibitory activity of mAb 763-15-5 for 2C9-catalyzed diazepam and phenanthrene metabolism is shown in Fig. 2A. At low levels of 2 to 8 μl of ascites fluid, mAb 763-15-5 inhibits 2C9-catalyzed metabolism by 85 to 90% with no cross-reactivity with other P450s. High concentrations of mAb 763-15-5 inhibited 2C18 phenanthrene and diazepam metabolism slightly (up to 25%). The potent inhibitory activity of mAb 1-7-4-8, mAb 1-5-1-3, and mAb 1-1-11-1 to 2C19-catalyzed metabolism of diazepam is shown in Fig. 2B. mAb 1-7-4-8 is an extremely strong inhibitor of 2C19 enzyme activity when added even at a low level of 0.5 μl of ascites fluid (>95%). The inhibitory activity of the purified IgG of mAb 1-7-4-8 is shown in Fig. 2C. The mAb 1-7-4-8 shows slight inhibitory cross-reactivity to 2C9, less than 20% when added at a very high level of 6.0 μl of ascites fluid. (Fig. 2D; Tables 6 and 7). Similar cross-reaction was observed with mAb 1-11-1 and mAb 1-5-1-3. Results showing the specificity of mAb inhibition of phenanthrene hydroxylation and diazepam N-demethylation are shown in Tables 6 and 7, respectively. The mAbs specific for 2C8, namely, mAb 28-1-1, mAb 13-1-3, and mAb 5-1-5 inhibit phenanthrene metabolism (Table 6) of the expressed human 2C8 by 83 to 91% and diazepam N-demethylation to NDZ by 91% (Table 7). None of the three mAbs exhibit any cross-reactive inhibition of metabolism catalyzed by any of the other members of the 2C family, namely, 2C9*1, 2C9*2, 2C18, and 2C19 or nine non-2C family P450 isoforms. Tables 6 and 7 also show the specificity of inhibitory activity of eight other mAbs to different isoforms of the 2C family. mAb 763-15-5 is specific for the inhibition of wild-type 2C9*1 and 2C9*2. mAb 592-2-5 inhibits the same two alleles of 2C9 and 2C18, and mAb 5-7-5 inhibits 2C9*1, 2C9*2, 2C18, and 2C19. The mAb 292-2-3 inhibits 2C9*2 and no other 2C9 allele or other P450. mAb 1-68-11 is a very powerful inhibitor of both phenanthrene and diazepam metabolism catalyzed by each of the four 2C isoforms 2C8, 2C9, 2C18, and 2C19 (Gelboin et al., 1997). Three mAbs, mAb 1-7-4-8, mAb 1-1-11, and mAb 1-5-1-3 are strong inhibitors of 2C19-catalyzed phenanthrene and diazepam metabolism.

![Image](312x227 to 557x730)

**Fig. 3.** mAb anti-2C8 inhibition of taxol conversion to 6α-hydroxytaxol in expressed P450 and human liver microsomes.

A. Inhibition of expressed P450 2C8. B. Inhibitory activity of purified mAb 281-1-1 IgG. C. Inhibition of HLMs. mAbs were added at indicated amounts to 40 pmol of expressed P450 2C8 or 200 pmol of P450 in HLMs and preincubated for 5 min at 37°C. The reaction was initiated by addition of taxol (50 μM) and NADPH (1 mM) and allowed to proceed for 30 min. The metabolite was assayed by HPLC as described under Materials and Methods. Data are the mean of duplicate incubations.

Early studies revealed that P450 2C8 catalyzes Taxol conversion to 6α-hydroxytaxol (Rahman et al., 1994). The inhibition curves revealed that expressed 2C8-catalyzed Taxol conversion to the 6α-OH metabolite is inhibited by mAb 281-1-1, mAb 5-1-5, and mAb 13-1-3 (Fig. 3A). The inhibitory activity of the purified IgG of mAb 281-1-1 is shown in Fig. 3B.

Figure 3C shows the inhibitory effects of mAbs 281-1-1 and 13-1-3.
on Taxol metabolism in HLMs. These highly specific and potent mAbs inhibit the enzyme activity of both the expressed 2C8 and HLM-catalyzed 6α-OH formation by 85 to 90%, which indicates that in human liver microsomes, more than 85% of 6α-OH Taxol formation is catalyzed by P450 2C8. Figure 4 shows the role of 2C8 in Taxol conversion to the 6α-OH in eight samples of HLMs. The addition of mAb 281-1-1 inhibits 6α-OH Taxol formation by 80 to 90% in each of the HLM samples, indicating the predominant and singular role of 2C8 in 6α-OH Taxol formation and the very low degree of interindividual variation for the contribution of 2C8. The small variation in 2C8 contribution is in contrast to the large interindividual variation in total basal activity of about 2-fold (0.24–0.49 nmol/min/nmol of P450). In a previous report with mAb-based analysis in multiple HLMs, P450 3A4 was found to be the major catalyst.
Materials and Methods

P450 in 1 ml of phosphate buffer containing 1 mM NADPH. The reaction was incubated for 1 h at 37°C. Hydroxylolubutamide was assayed by HPLC as described under Materials and Methods. Data are the mean of duplicate incubations.

of 3-OH Taxol formation (Gelboin et al., 1995). Thus, the mAbs are useful for determining the role of a P450 isoform for the metabolism of a drug through different metabolic pathways.

mAb-based combinatorial analyses of diazepam metabolism in HLMs with mAbs to 2B6, 3A4, 2C8, 2C9, and 2C19 is shown in Fig. 5. A previous report revealed the validity of the combinatorial system (Yang et al., 1999), and in a previous application showed that 3A4 and 2B6 were the major contributors to diazepam N-demethylation to NDZ in HLMs (Yang et al., 1999). In Fig. 5 the mAbs anti-2B6 and anti-3A4 were combined to remove the dominant 2B6 and 3A4 activity from the HLMs so that the role of 2C8, 2C9, and 2C19 would be better assessed for NDZ formation in multiple HLM. In Fig. 5, mAbs anti-3A4 and anti-2B6 were added together and the combined contribution of activity of 3A4 and 2B6 showed large interindividual variation among nine HLMs ranging from 20% in HLM 79 to 90% in HLM 74. The 2C9 contribution was also highly variable, greater than 30% in four HLMs (77, 78, 79, and 81) and showed a lesser contribution, from 5 to 25% in four other HLMs (74, 75, 76, and 80). 2C8 was found to significantly contribute to NDZ formation, ranging variably from 2 to 35%, and 2C19 does not contribute to NDZ formation (Fig. 5).

Tolbutamide is among the major drugs known to be metabolized by 2C9 (Miners and Birkett, 1998). Figure 6 shows the tolbutamide 6-hydroxylation activity of 14 expressed P450 isoforms. The 2C9*1 showed the highest activity, 2C9*2 about half as much activity, and 2C19 about one-fourth the activity. Figure 7, A and B, shows the mAb inhibition curves of tolbutamide hydroxylation catalyzed by expressed P450 2C9*1 (Fig. 7A) and the allele 2C9*2 (Fig. 7B). mAb 763-15-5 was the most potent monoclonal antibody, inhibiting 2C9 activity by more than 90% upon the addition of 4.0 μl of the mAb 763-15-5 ascites fluid. The other three mAbs shown in Fig. 7A are somewhat less inhibitory to 2C9 than the mAb 763-15-5. Figure 7 shows that mAb 292-2-3 is specific for the 2C9*2 allele, inhibiting its activity by greater than 90% and neither inhibiting the wild-type 2C9*1 nor the 2C9*3 alleles (data not shown for 2C9*3). Combinatorial analyses determined the contribution of P450s 2C8, 2C9, and 2C19 to tolbutamide hydroxylation in eight HLMs (Fig. 8). 2C9 was the primary enzyme contributing 75 to 90% of hydroxylase activity. The interindividual variation in the role of 2C9 was very minimal, whereas the basal control activity showed much larger variation, about 3-fold, ranging from 0.132 to 0.368. The relative activities exhibited by the expressed 2C8, 2C9, and 2C19 are considerably different than the enzyme activities of each of the P450 isoforms when measured by combinatorial analysis with the mAbs in HLMs. This is especially true of the 2C8 and 2C19 contribution. The expressed 2C8 shows very minimal enzyme activity, whereas the mAb-based HLM analysis shows a variable but potentially significant contribution from both 2C8 and 2C19. Expressed 2C19 exhibited a 5-fold greater activity than the expressed 2C8. Thus, the relative activities of the expressed P450s do not accurately reflect their metabolic contribution in HLMs as measured by mAb-based analyses.

mAb 763-15-5, specific for 2C9, added singly to eight HLMs inhibited diclofenac activity 85 to 90% and thus was the predominant contributor to diclofenac hydroxylation, with little interindividual variation (Fig. 10). However, an additional 2 to 5% inhibition was observed with the potent 2C family inhibitor mAb 1-68-11 added separately, which indicates a minor involvement of another 2C subfamily isoform. The interindividual difference in total basal activity of the eight HLM samples was much larger, about 3-fold. Figure 11 shows the activity of 14 expressed human P450s for the metabolic conversion of S-mephenytoin to its 4-hydroxy metabolite. S-Mephenytoin has been used as a model substrate for the in vivo phenotyping of 2C19 (Streetman et al., 2000). The 2C19 was the predominant expressed isoform exhibiting hydrolyase activity that was 15-fold greater than the activity of 2C9*1. Figure 12 shows also that 2C19 is the dominant enzyme metabolizing mephenytoin in seven HLMs, and anti-2C19 added singly inhibits its metabolism by 90 to 98%. However, the combinatorial addition of anti-2C9 and anti-2C19 showed, in contrast to the relative activities of the expressed enzymes, that 2C9 may have a significant role in mephenytoin metabolism, contributing about 10 to 20% of activity in the seven HLMs. The indication that 2C9 exhibits a minor role for mephenytoin metabolism suggests that mephenytoin metabolism may not be an absolute indicator for in vivo 2C19 phenotyping.

Previous studies with the inhibitory mAbs reported that 2D6 and the 2C family isoforms were dominant contributors to imipramine 2-hydroxylation and that 1A2 was the major P450-catalyzing DIM formation. However, the 2C family and to a lesser extent 3A4 also contributed to DIM formation (Yang et al., 1999). These studies were extended with eight HLMs by using the newly developed mAbs to the 2C family isoforms to study the role of each 2C isoform. In two of the HLMs, the 2C8 contributed 20 and 33%, respectively, to the 2-hydroxylation and in two HLMs, 2C9 and 2C19, contributed low but significant activity to DIM formation. These results with a few HLM may suggest large interindividual differences in the role of the different 2C P450s for imipramine hydroxylation and DIM formation (K. Krausz and H. Gelboin, unpublished results).

Discussion

This study reports the isolation and characterization of 13 monoclonal antibodies derived from individual hybridomas that target the major isoforms of the human liver P450 2C subfamily, 2C8, 2C9, 2C19, and the 2C9*2 allele. These mAbs were tested for specificity of binding by ELISA, immunoblotting, and inhibitory activity toward the major human liver P450s (Tables 1–7). Eight of the 13 mAbs are

![Fig. 6. Tolbutamide metabolism by cDNA-expressed human P450s.](image-url)
specific to a single P450 isoform and five of the mAbs target more than one P450. The latter identify P450 isoforms that share a common mAb-directed epitope. The amount of mAb inhibition of a drug's metabolism with saturating levels of substrate in HLMs determines the maximum contribution of the target P450 to the metabolism of the drug substrate. Thus, the mAbs are useful for reaction phenotyping. Additionally, the mAbs determine interindividual variability in the role of a P450 (Gelboin et al., 1997). The mAbs can be used either singly or combinatorially to assess the role of the target P450. We reported previously the isolation, characterization, and reaction phenotyping of drug metabolism in HLMs with mAbs specific and inhibitory to P450 1A1, 1A2, 2A6, 2B6, 2C9*2, 2D6, 2E1, and 3A4/5, and an mAb that inhibits the entire 2C subfamily. Here, we report the efficacy of the newly isolated mAbs for measuring the metabolic

**Fig. 7.** Inhibition of P450 2C9 tolbutamide metabolism.

A, inhibition of 2C9*1 metabolism. B, inhibition of P450 2C9*2 metabolism. Tolbutamide (200 μM) was incubated with 35 to 40 pmol of cDNA-expressed P450 and with the mAb indicated in 1 ml of phosphate buffer containing 1 mM NADPH. The reaction was incubated for 60 min at 37°C. Hydroxytolbutamide was assayed by HPLC as described under Materials and Methods. Data are the mean of duplicate incubations.
contribution of each of the 2C subfamily isoforms for the metabolism in HLMs of six model drug substrates, namely, Taxol, tolbutamide, diazepam, diclofenac, S-mephenytoin, and imipramine. The specific-}

ity of the mAbs was determined by their inhibition of each of the major expressed human P450s. The mAbs to the P450 2C isoforms were extensively characterized. The most highly characterized mAbs are mAb 281-1-1 specific to 2C8, mAb 763-15-5 specific to 2C9, mAb 1-7-4-8 specific to 2C19, and mAb 292-2-3 specific to 2C9*2 allele (Krausz et al., 2000). Each of the latter mAbs inhibits their target P450 by 85 to 95%.

The study found that 80 to 90% of Taxol conversion to the 6-OH metabolite was catalyzed by 2C8 with little interindividual variability, although there was more than a 2-fold difference in basal Taxol 6-hydroxylation in the eight HLMs. Previous studies using an mAb to P450 3A4/5 showed that 72 to 90% of Taxol conversion to the 3-OH metabolite was catalyzed entirely by 3A4/5, with little or no contribution from other P450s (Yang et al., 1999). This study examined diazepam N-demethylation to NDZ in HLMs by using mAb anti-2B6, 3A4, 2C8, 2C9, and 2C19. In previous studies mAbs anti-3A4/5, anti-2B6, and anti-2C subfamily showed that the 3A4, 2B6, and 2C subfamily were the major contributors to NDZ formation with large interindividual differences among the HLMs. Here, we determined the role of individual P450s and found that in addition to the major role of 3A4/5 and 2B6, 2C9 contributed 16 to 50% and 2C8, 10 to 40% of the metabolic activity forming NDZ. In one of the eight HLM samples all the activity was due to the combined 3A4 and 2B6. The other eight HLMs showed large interindividual differences in the contribution of the combined 3A4 and 2B6 as well as activity by 2C9 and 2C8. No contribution of P450 2C19 was observed.

P450 2C9 was shown to be a major catalyst for tolbutamide hydroxylation (Relling et al., 1990). 2C19 was also found to be active for tolbutamide hydroxylation by HLMs (Wester et al., 2000). The expressed 2C9*1 and 2C9*2 showed the highest activity for tolbutamide hydroxylation, 2C19 only one-fourth the activity, and 2C8 less than 5% of the 2C9 activity (Fig. 6). mAb-based analyses showed that tolbutamide hydroxylation was primarily a function of 2C9, which contributed about 75% of its metabolism with little interindividual differences. 2C19 and 2C8 each contributed lesser amounts, ranging from 5 to 10% (Fig. 8). The relative activities of the expressed P450s are in contrast to the results obtained with the mAb-based HLM analysis, which shows high activity for the 2C9 but substantially more activity for the 2C8 and less for 2C19 than the relative values shown by the expressed P450s.

Mephenytoin metabolism of expressed 2C19 is 20-fold greater than the activity of expressed 2C9 (Fig. 11). The mAb-based analysis, however, shows that 2C9 may contribute significantly (10 to 20%) to mephenytoin metabolism (Fig. 12). Thus, although 2C19 is the predominant P450 for mephenytoin metabolism, the 2C9 can also participate. mAb 763-15-5 did not inhibit the expressed 2C19 mephenytoin hydroxylation, even at high mAb levels (data not shown), demonstrating that the 10 to 20% inhibition observed with mAb 763-15-5 in HLMs is specific for P450 2C9 and not due to cross-reactivity with 2C19. This is important because mephenytoin has been used as an in vivo marker for 2C19 (Streetman et al., 2000).
Understanding P450-directed metabolism is important for studying the potential toxicity of a drug or drug-drug interactions and identifying drugs metabolized by polymorphic P450 isoforms. An important method for characterizing P450s has been the use of cDNA expression systems. By using a variety of vectors such as vaccinia (Battula et al., 1987), baculovirus (Grogan et al., 1995), and bacteria and yeast (Gonzalez and Korzekwa, 1995), pure cytochromes P450s are obtained and characterized for substrate and product specificity and kinetic character. The baculovirus system yields amounts of P450s adequate for use as immunogens for monoclonal antibody production (Gelboin et al., 1998). The expression systems alone, however, yield information that does not adequately portray a P450s metabolic function in HLMs. Different expression systems may yield different amounts of P450s, e.g., 3A4 varies considerably for testosterone hydroxylation in different expression systems (Crespi and Penman, 1997). In addition, interindividual P450 content and catalytic activity in human liver microsomes was found to vary about 10-fold (Shimada et al., 1994). Interindividual differences in metabolism and P450 protein content, factors such as NADPH P450 oxidoreductase, cytochrome b5, and the membrane lipid environment may significantly influence the metabolic role of a P450 in human liver (Remmel and Burchell, 1995). By using selective substrates, relative activity factors (RAFs), derived from the ratios of the catalytic activity in HLMs to that of cDNA-expressed enzymes, have been suggested for defining a P450s metabolic function in HLMs (Crespi, 1995). RAFs have been used for scaling from cDNA-expressed P450s to human liver and compared with values by using immunoquantified P450 liver protein. By using these alternative methods with substrates specifically metabolized by each P450 isoform, the two methods gave similar results for 2C19, 2D6, and 3A4, but the RAFs for 1A2 and 2B6 were 5- to 10-fold and 6-fold higher using quantified P450 protein levels (Venkatakrishnan et al., 2000). Thus, the RAF method does not seem sufficiently reliable for determining the role of a P450 in HLMs.

Polyclonal antibodies (pAbs) have been used for defining P450 metabolic function. pAbs are heterogeneous reagents derived from a single immunization and commonly exhibit considerable cross-reactivity (Harlow and Lane, 1988) and thus cannot be standardized for universal or interlaboratory use. Sequential or parallel
immunizations with identical immunogens result in pAbs containing different heterogeneous populations of antibodies, and thus are nonidentical reagents lacking the immortal and stable properties of hybridomas producing identical epitope-specific mAbs (Yelton and Scharff, 1981). A number of different approaches have used both classically purified P450s (Shimada et al., 1994) and small P450-specific polypeptides as immunogens for the production of pAbs. In one study, the pAb obtained by immunization with classically purified P450 2C9 required back absorption with 2C9 to obtain the desired antibody to P450 2C19 (Wester et al., 2000). In another study, pAbs to the major human P450s were made with synthetic peptides of small regions of the desired P450s. The resultant pAbs immunoblotted the major P450s in HLMs and can immunoquantify P450 isoform levels (Edwards et al., 1998). However, they are not inhibitory and of no value for reaction phenotyping. The polypeptide immunogens commonly produce pAbs that are not enzyme inhibitory. However, some polypeptide immunogens have resulted in enzyme inhibitory pAbs, e.g., pAbs to P450 3A4 (Wang and Lu, 1997) and to 2C19 (Schulz-Utermoehl et al., 2000) have been reported, although the latter pAb exhibited some cross-reactivity with P450 2C8.

There has been an undisciplined proliferation in the use of chemical inhibitors for reaction phenotyping. The chemical inhibitors are aptly termed "selective" rather than "specific" and commonly the selectivity is undefined. Several studies have demonstrated a highly variable cross-reactivity of chemical inhibitors (Rendic and Di Carlo, 1997; Sai et al., 2000). Chemical inhibitors lacking defined specificity are not suitable for determining the metabolic role of a P450 for drug metabolism. The mAbs can be used as standards for determining chemical inhibitor specificity and potency (Sai et al., 2000).

The mAb analysis of P450-based metabolism is simple, precise, quantitative, and comprehensive, and a new dimension of P450...
methodology. mAb-based reaction phenotyping in HLMs coupled with characterization of the patients phenotype will yield vital information to the clinician for drug therapeutics and drug discovery, and reduce the incidence of adverse drug reactions and drug-related mortality.

Acknowledgments. We thank Tara Millington for excellent assistance in the preparation of this manuscript.

References


Edwards RJ, Adams DA, Watts PA, Davies DS, and Boobis AR (1998) Development of a methodology. mAb-based reaction phenotyping in HLMs coupled with characterization of the patients phenotype will yield vital information to the clinician for drug therapeutics and drug discovery, and reduce the incidence of adverse drug reactions and drug-related mortality.

Acknowledgments. We thank Tara Millington for excellent assistance in the preparation of this manuscript.

References


