0090-9556/97/2506-0762–767$02.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 25, No. 6
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Printed in U.S.A.

INHIBITORY ANTI-PEPTIDE ANTIBODY AGAINST HUMAN CYP3A4

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(Received November 27, 1996; accepted February 10, 1997)

ABSTRACT:

An inhibitory anti-peptide antibody was raised against a 21-amino acid peptide (VKRMKESRLEDTQKHRVDFLQ) corresponding to residues 253–273 of human cytochrome P450 3A4. High titer antibodies were produced by rabbits immunized with this peptide coupled to keyhole limpet hemocyanin, as judged by ELISA. Anti-peptide antibody recognized a single protein band in microsomes prepared from cells expressing recombinant human CYP3A4 in immunoblotting analysis. No immunodetectable proteins were found in microsomes containing other cytochrome P450 isoforms. In addition, the antibody did not recognize CYP3A5, a closely related isoform in the CYP3A family. In human liver microsomes, only one protein band which comigrated with human CYP3A4 was recognized by this antibody and the relative blotting intensity of this protein band correlated significantly with human CYP3A4-catalyzed testosterone 6β-hydroxylase activities (*r* = 0.96). More importantly, this antibody exhibited greater than 90–95% inhibition of testosterone 6β-hydroxylation, while other cytochrome P450-mediated reactions in human liver microsomes were not inhibited. Because of its specificity and inhibitory potency, this anti-peptide antibody should be a valuable tool in evaluating the role of CYP3A in mediating *in vitro* metabolism of therapeutic agents.

Significant progress has been made in recent years in the use of *in vitro* metabolism of therapeutic agents to address the potential *in vivo* induction, inhibition, drug-drug interaction, and individual variability issues (1–5). Central to these studies is the unambiguous identification of specific drug-metabolizing enzyme(s), particularly human cytochrome P450 isoform(s) responsible for the metabolism of drugs. This objective can be achieved by using selective cytochrome P450 inhibitors, antibodies, recombinant cytochrome P450s, and correlation analysis (2).

Polyclonal or monoclonal antibodies produced against purified cytochrome P450 or specific peptide sequence unique to individual cytochrome P450 isoforms have been used widely to study the regulation, structure, and function of cytochrome P450s (6–10). Specific and inhibitory antibodies are extremely useful tools for the identification of specific cytochrome P450 involvement in the *in vitro* metabolism of therapeutic agents. However, because of a limited supply of purified single cytochrome P450s used for immunization, antibodies against specific human cytochrome P450s are not always available for such studies. In addition, antibodies raised against specific cytochrome P450 isolated from human liver tissues potentially could cross react with other cytochrome P450 isoforms (11). The nonspecificity is possibly a result of contamination with structurally related cytochrome P450s which are difficult to separate. Cross reactivity has also been reported even when purified recombinant cytochrome P450 was used as an immunogen (12, 13). This is because some of these antibodies can recognize regions of high sequence homology in related cytochrome P450s. By using a peptide that targets a specific region of the protein as an immunogen, one may overcome these problems and hopefully generate antibodies with less potential for cross reactivity. To this end, we have attempted to generate antibodies against specific peptide sequences for different cytochrome P450 isoforms. Since many of the peptide antibodies reported in the literature are either noninhibitory or only partially inhibitory (14–17), we have focused on the production of highly specific and highly inhibitory antibodies against cytochrome P450s. Partially inhibitory antibodies are not useful for the identification of the specific cytochrome P450s responsible for the metabolism of therapeutic agents since one cannot determine whether partial inhibition is a result of the involvement of additional cytochrome P450 isoforms or the weak inhibitory property of the antibodies. In this paper we report the production of antibodies that are inhibitory against a 21-amino acid peptide corresponding to amino acid 253–273 of human CYP3A4. To our knowledge, this is the first peptide antibody against CYP3A4 that shows greater than 90–95% inhibition on CYP3A4-mediated reactions.

Materials and Methods

Materials. Testosterone, 6β-hydroxytestosterone, phenacetin, tolbutamide, chlorzoxazone, corticosterone, glucose 6-phosphate, NADP, and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Acetaminophen was obtained from Aldrich Chemical Co. (Milwaukee, WI). Methylhydroxytolbutamide and 6-hydroxychlorzoxazone were obtained from Research Biochemical International (Natick, MA). Bufuralol and 1′-hydroxybufuralol were purchased from Gentest Corp. (Woburn, MA). All other reagents and solvents were of high analytical grade supplied by Fisher Scientific (Fair Lawn, NJ).

Human liver microsomal preparations were kindly provided by Dr. Judy Raucy (Agouron Institute, La Jolla, CA). Microsomes were prepared as described elsewhere (18). The pyrophosphate-washed microsomes were resuspended at a protein concentration of 10–15 mg/ml in 10 mM potassium phosphate buffer, pH 7.4 containing 0.25 M sucrose, and frozen at −80°C until used. Protein concentrations and P450 contents were determined using the bicinchoninic acid procedure (19) and according to Omura and Sato (20), respectively. Microsomes from cells containing human cytochrome P450 were obtained from Gentest Corp. (Woburn, MA). Antibody against purified CYP3A4 was kindly provided by Dr. Jerome Lasker (Mount Sinai Medical Center, New York, NY).

Peptide Synthesis and Conjugation to Carrier Protein. The peptide (VKRMKESRLEDTQKHRVDFLQ) was synthesized on an Applied Biosystems 430 A peptide synthesizer using solid phase chemistry by AnaSpec, Inc. (San Jose, CA). A cysteine residue was introduced to the *N*-terminus for use in

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conjugation to the carrier protein. The peptide was coupled to keyhole limpet hemocyanin (KLH)[1] using *N*-succinimidyl bromoacetate as a cross-linking reagent (21).

Antibody Production and Purification. The immunization was performed at HRP Inc. (Denver, PA) according to the method of Vaitukaitis *et al.* (22). All animal protocols in this study were conducted in accordance with Merck Institutional Animal Care and Use Committee Guidelines. Rabbits were initially immunized with 100 μg of the peptide conjugated to KLH in complete Freund's adjuvant by intranodal injection and then by sc injection with 250 μg in Freund's incomplete adjuvant on day 20. Rabbits were continuously boosted at 3-week intervals with 125 μg of antigen in Freund's incomplete adjuvant. Sera were collected 10 days after each injection. IgG fractions were purified from rabbit sera by caprylic acid precipitation and ammonium sulfate fractionation (23). The titers of the antisera were determined by ELISA as described (24).

Western Blots. Human liver microsomes (15 μg) or microsomes from cells containing recombinant human cytochrome P450 (50 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis according to Laemmli (25) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) using the method of Towbin *et al.* (26). The nitrocellulose sheets were blocked with nonfat dry milk, incubated with antibody and then treated with ^{125}I-Protein A (Amersham Corp.). The immunoblotting intensity was quantified by the pdi Imaging Densitometer and Quantity One Software (pdi Inc., Huntington Station, NY).

Immunoinhibition. Immunoinhibition was conducted by preincubating microsomes for 30 min at room temperature with various amounts of rabbit pre-immune IgG or anti-peptide IgG. Reaction was started by addition of buffer, NADPH-generating system and substrate as described for the following enzyme assays.

Enzyme Assays. Testosterone 6β-hydroxylation was determined as described (27). Microsomal samples were incubated with 100 μM testosterone in 100 mM potassium phosphate buffer (pH 7.4) with 1 mM EDTA, 6 mM $MgCl_2$, and an NADPH-generating system consisting of 10 mM glucose 6-phosphate, 1 mM NADP and 0.35 units glucose 6-phosphate dehydrogenase in a total volume of 0.5 ml. Reactions were performed at 37°C for 10 min with 0.34 mg of human liver microsomes containing 0.1 nmol cytochrome P450, and at 37°C for 20 min with 0.25 mg of microsomes prepared from human B-lymphoblast cells. After reactions were stopped by adding 5 ml of CH_2Cl_2, the samples were spiked with 20 μl of 1 mM corticosterone as internal standard, vortexed and centrifuged at 3,000*g* for 10 min. The organic layer was removed and evaporated to dryness under nitrogen stream. Samples were dissolved in 0.2 ml of methanol and analyzed by HPLC. Phenacetin *O*-deethylation (28), tolbutamide methylhydroxylation (29), bufuralol 1′-hydroxylation (30), and chlorzoxazone 6-hydroxylation (31) were determined for CYP1A2, CYP2C9/10, CYP2D6, and CYP2E1-mediated reactions, respectively. The substrate concentrations and incubation time used for each assay were 100 μM phenacetin for 20 min, 200 μM tolbutamide for 60 min, 100 μM bufuralol for 10 min, or 500 μM chlorzoxazone for 20 min. Reactions were quenched by adding 0.05 ml of 85% H_3PO_4. Samples were centrifuged at 14,000 × *g* for 10 min, and the supernatants were directly injected for HPLC analysis.

HPLC Analysis. The HPLC used was a Shimadzu SCL 10A system controller consisting of two LC 10AS pumps, a SIL 10A automatic sample injector, SPD10A UV-VIS spectrophotometric detector, and RF10A spectrofluorometric detector. Aliquots of 50 μl samples from the testosterone 6β-hydroxylation incubation were injected onto a Zorbax ODS C18 column (4.6 mm × 250 mm, 5 μm, Sigma-Aldrich, Milwaukee, WI). Substrate and metabolite were eluted from the column with methanol (7.5% tetrahydrofuran) : H_2O. (7.5% tetrahydrofuran) by a linear gradient from 35% to 60% in 35 min at a flow rate of 1 ml/min and monitored at 254 nm. The retention times for 6β-hydroxytestosterone, corticosterone, and testosterone were 8.9, 17.5, and 25.2 min, respectively. Chromatographic analyses were carried out for phenacetin *O*-deethylation, tolbutamide methylhydroxylation, bufuralol 1′-hydroxylation and chlorzoxazone 6-hydroxylation on a Zorbax SB C8 column (4.6 mm × 75 mm, 3.5 μm) at a flow rate of 2 ml/min by a linear gradient elution with the mobile phase which consisted of buffer A (10 mM of ammonium acetate and 0.1% trifluoracetic acid in H_2O) and buffer B (10 mM ammonium acetate and 0.1% trifluoroacetoc acid in 90% acetonitrile and 10% methanol). The per cent of buffer B in the gradient, run time, detection wavelength, and the retention times of substrate and its metabolite in these cytochrome P450-mediated reactions are as follows: phenacetin *O*-deethylation (5–40%, 12 min, 254 nm, acetaminophen 2.5 min and phenacetin 8.2 min); tolbutamide methylhydroxylation (10–65%, 10 min, 230 nm, 3-methyltolbutamide 5.3 min, and tolbutamide 7.9 min); bufuralol 1′-hydroxylation (15–45%, 10 min, excitation 252 nm and emission 302 nm by spectrofluorometer, 1′-hydroxybufuralol 3.8 min, and bufuralol 8.1 min); and chlorzoxazone 6-hydroxylation (8–65%, 10 min, 287 nm, 6-hydroxychlorxazone 4.1 min, and chlorzoxazone 6.4 min).

TABLE 1

Sequences aligment between CYP101, CYP102 and some major human cytochrome P450s[a]

Isoform	Sequences	Residues
Predicted Structure	----G---> <- H	
CYP101	LIPIIEQRRQKPGT----DAIS	205–222
CYP102	VDKIIADRKASGEQSD--DLLT	217–236
CYP3A4	VKRMKESRLEDTQKHRV-DFLQ	253–273
CYP1A2	LQKTVQEHYQDFDKNSVRDITG	264–285
CYP2C9	ILEKVKEHQESMDMNNPQDFID	244–265
CYP2D6	DELLTEHRMTWDPAQPPRDLTE	252–273
CYP2E1	VSERVKEHHQSLDPNCPRDLTD	246–267

[a] Sequence aligment of human cytochrome P450s near the loop region between helices G and H of CYP101 and CYP102 as proposed by Lewis (32).

Results

Selection of Peptide. Table 1 shows the sequence alignment of five major human cytochrome P450s in the loop region between helices G and H of CYP101 and CYP102 as proposed by Lewis (32). Peptide (VKRMKESRLEDTQKHRVDFLQ) corresponding to amino acid 253–273 of human CYP3A4 was selected based on low sequence homology among the major human cytochrome P450s, high surface probability, and hydrophilicity. Fig. 1 shows the hydrophilicity and surface probability of human CYP3A4 and the 21-amino acid peptide. The hydrophilicity was calculated according to the algorithm of Hopp and Woods (33) by averaging over a window of seven residues. The surface probability was calculated according to a formula of Emini *et al.* (34). In addition, this 21-amino acid sequence in CYP3A4 was aligned with a 20-amino acid sequence in CYP2D6 (254–273) which was successfully used to produce inhibitory antibodies (35).

Production of Antibody. As shown in fig. 2, high titer antibody (1:204,800) was produced in immunized rabbit as judged by ELISA. Antiserum collected from one of the rabbits strongly inhibited testosterone 6β-hydroxylation by human liver microsomes as well as recombinant CYP3A4. The production of inhibitory antibodies by this rabbit is still maintained with repeated immunizations at 3-week intervals. The IgG was purified and characterized for its specificity in immunoblotting and immunoinhibition.

Specificity of Antibody. As shown in fig. 3A, when a panel of microsomes prepared from human B-lymphoblastoid cells that expressed specific human cytochrome P450 isoforms were used for western blot analysis, only cytochrome P450 in microsomes containing human CYP3A4 was recognized by this anti-peptide antibody. No immunodetactable bands were observed in other microsomes containing CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1, and CYP2F1. In addition, this peptide antibody recognized a single protein band in human liver microsomes (fig. 3B) that

[1] Abbreviations used are: CYP, cytochrome P450; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet haemocyanin; LKM-1 autoantibodies, liver-kidney microsomal-1 autoantibodies.

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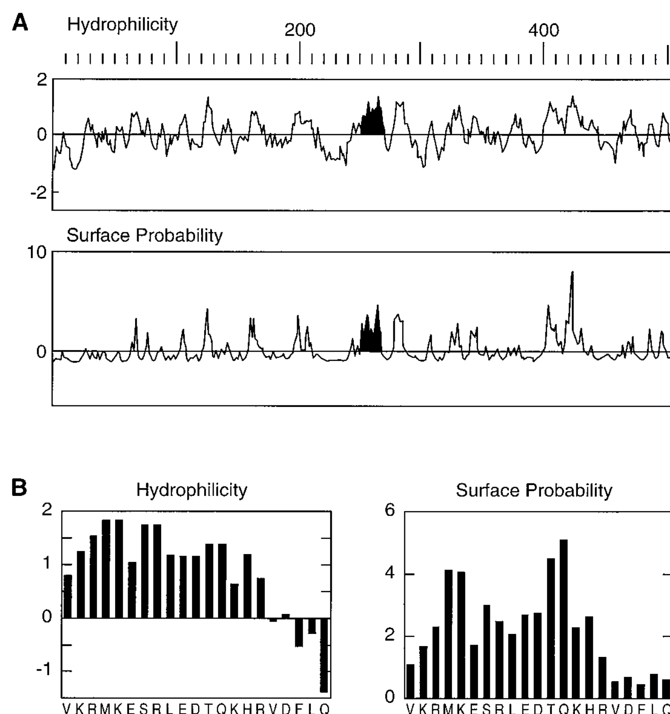

FIG. 1. *Hydrophilicity and surface probability of human CYP3A4 and the 21-amino acid peptide.*

The hydrophilicity and surface probability of human CYP3A4 (panel A) and the 21-amino acid sequence (panel B) were calculated as described (33,34). Shaded area indicates the amino acid sequences selected for antibody production.

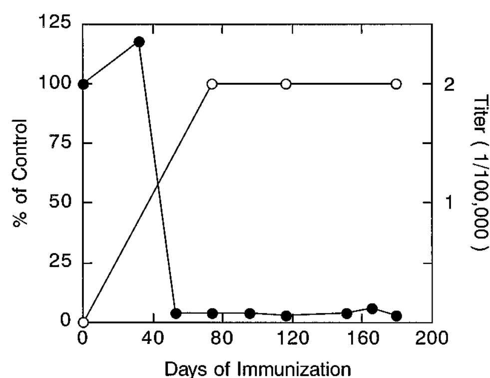

FIG. 2. *Effect of anti-peptide antisera on testosterone 6β-hydroxylation.*

Human liver microsomes containing 0.13 nmol of cytochrome P450 were preincubated with 0.2 ml sera collected during the course of immunization. Testosterone 6β-hydroxylase activity (●) was measured as described in *Materials and Methods*. The control activity (with preimmune serum) was 2.88 nmol/min/nmol P450. The titers (○) of the antisera were determined by ELISA.

co-migrated with recombinant human CYP3A4. In liver microsome UC9402, no immunoreactive band was detected, consistent with the observation that this particular microsomal preparation had very little detectable testosterone 6β-hydroxylase activity. The quantity of immunoblotted protein in human liver microsomes correlated significantly with testosterone 6β-hydroxylase activity (fig. 4).

The specificity of this anti-peptide antibody was also evaluated against CYP3A5, a closely related isoform found in human liver. In this 21-amino acid sequence, CYP3A5 differs from CYP3A4 by only five amino acids. When examined by immunoblotting, this antibody did not recognize CYP3A5 (fig. 5A) whereas an antibody prepared against purified CYP3A4 recognized both CYP3A4 and CYP3A5 (fig. 5B). Thus, the specificity of this anti-peptide antibody is quite remarkable. Consistent with this observed specificity, CYP3A5-catalyzed testosterone 6β-hydoxylation was not inhibited by this antibody (data not shown).

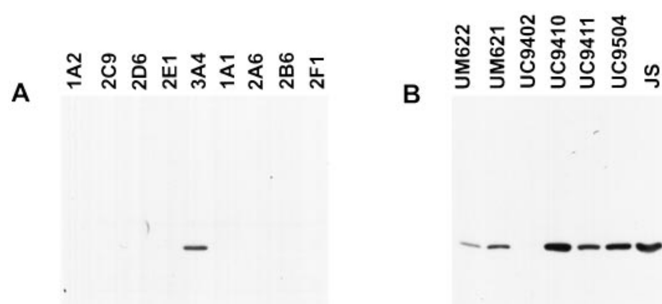

FIG. 3. *Western Blot detection of cytochrome P450 in microsomal samples.*

Microsomes prepared from human B-lymphoblastoid cells which expressed specific human cytochrome P450 as indicated were immunoblotted with anti-peptide antibody to determine the specificity of antibody (panel A). The levels of immunodetectable CYP3A4 in human liver microsomes (panel B) with anti-peptide antibody were determined and quantified as described in *Material and Methods.*

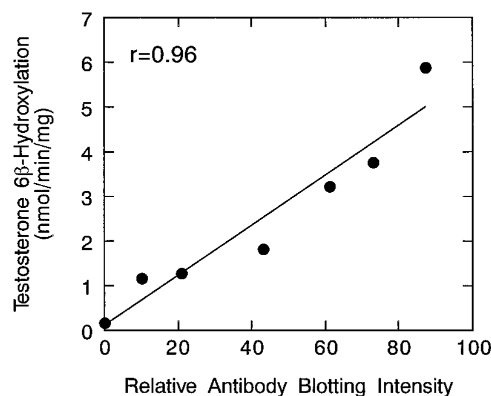

FIG. 4. *Correlation of human CYP3A4 immunoblotting intensity with testosterone 6β-hydroxylation.*

Human CYP3A4-catalyzed testosterone 6β-hydroxylase activity was determined in seven human liver microsomes. The relative blotting intensity of CYP3A4 in human liver microsomes with anti-peptide antibody was measured as shown in fig. 3. The coefficients of correlation between testosterone 6β-hydroxylation and the blotting intensity were determined by linear regression analysis.

Effect of the Anti-peptide Antibody against CYP-Mediated Reactions. Purified IgG strongly inhibited testosterone 6β-hydroxylation, a CYP3A4-catalyzed reaction, in human liver microsomes (fig. 6A) and microsome prepared from human B-lymphoblast cells expressing recombinant human CYP3A4 and P450 reductase (fig. 6B). Greater than 90% of the activities were inhibited at an IgG to cytochrome P450 ratio of 2.5 in human liver microsomes. In contrast, the purified IgG showed little or no inhibition toward other human cytochrome P450-mediated reactions, namely, phenacetin *O*-deethylation, tolbutamide methylhydroxylation, bufuralol 1′-hydroxylation, and chlorzoxazone 6-hydroxylation (fig. 7).

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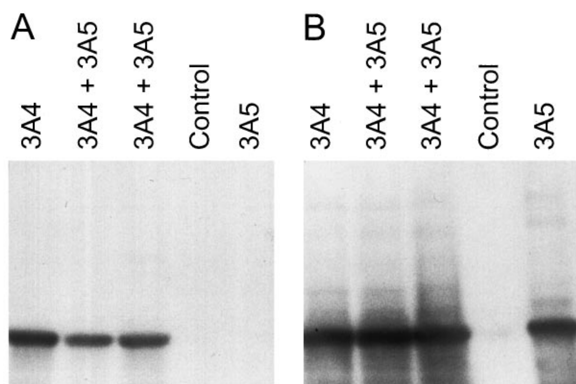

FIG. 5. *Western Blot detection of CYP3A4 and CYP3A5 in microsomes prepared from baculovirus infected insect cells.*

Microsomes prepared from control insect cells or cells containing expressed CYP3A4 and CYP3A5 were immunoblotted with anti-peptide antibody (panel A) or antibody against purified CYP3A4 (panel B).

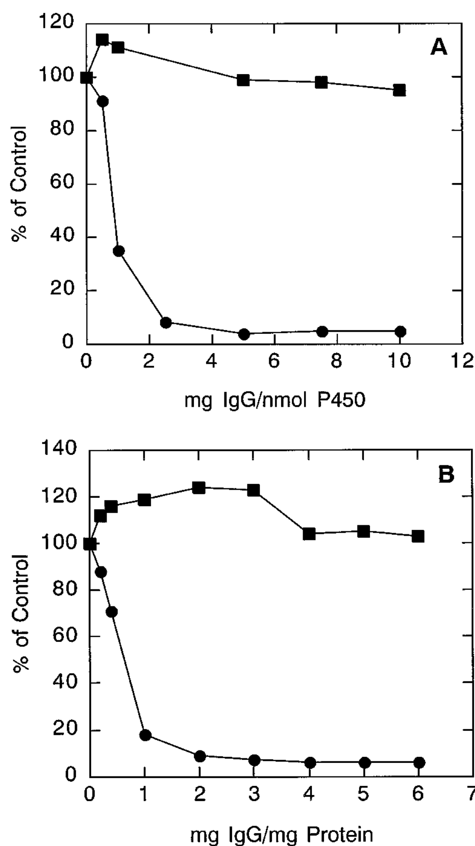

FIG. 6. *Inhibition of testosterone 6β-hydroxylation by anti-peptide IgG.*

Human liver microsomes containing 0.1 nmol cytochrome P450 (A) or microsomes (0.25 mg) prepared from human B-lymphoblastoid cells expressing human CYP3A4 and P450 reductase (B) were preincubated with 0.05–1.5 mg of preimmune IgG (■) or anti-peptide IgG (●). The control activities of testosterone 6β-hydroxylation were 3.15 nmol/min/nmol P450 for human liver microsome and 1.08 nmol/min/mg protein for microsome from cells expressing human CYP3A4 and P450 reductase.

Discussion

To select a peptide to produce inhibitory antibody with high specificity for a single isoform of cytochrome P450, many criteria need to be considered. These include structural characteristics of the peptide, degree of sequence homology of the targeted peptide compared with the sequence of other isoforms, and the location of the regions in the enzyme involved in substrate binding and recognition. Because three-dimensional structures of mammalian cytochrome P450s are not available, the proposed substrate recognition sites have been located by using amino acid sequence alignment with bacterial cytochrome P450s (32, 36, 37). Many cytochrome P450 epitopes in various regions of the target cytochrome P450s have been identified by the preparation of antibodies against selected peptides (9, 13, 38–41). For example, Leeders *et al.* (42) identified a minimum antibody-binding sequence in CYP3A1 which located in the K-helix of the protein by epitope mapping studies.

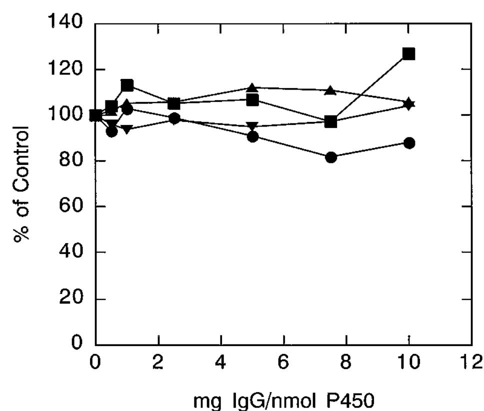

FIG. 7. *Effect of anti-peptide antibody on CYP-mediated reactions.*

CYP1A2, CYP2C9/10, CYP2D6, and CYP2E1-mediated reactions were determined after 30 min preincubation of human liver microsomes containing 0.1 nmol cytochrome P450 with 0.05–1 mg of preimmune IgG or antipeptide IgG. The control activities were 2.39, 0.19, 3.46, and 7.35 nmol/min/nmol P450 for phenacetin O-deethylation (●), tolbutamide methylhydroxylation (■), bufuralol 1′-hydroxylation (▲), and chlorzoxazone 6-hydroxylation (▼), respectively. There were no significant inhibitions on all reactions when preimmune serum was used in the incubations.

The peptide used in this study is hydrophilic, with high surface probability and located in the loop region between G helix and H helix which is near to but not in any substrate recognition sites proposed previously. Manns *et al.* (43, 44) reported the presence of inhibitory antibodies against CYP2D6 in certain individuals and identified a 33-amino acid sequence in this region containing the epitope(s) recognized by these autoantibodies. In addition, the common epitope of LKM-1 autoantibodies was identified as a 8-amino acid peptide (DPAQPPRD) corresponding to the residues 263–270 of CYP2D6 (44). Furthermore, inhibitory peptide antibodies against human CYP2D6 were produced previously by selecting a 20-amino acid peptide (residues 254–273) in this region as immunogen (35).

When rabbits were immunized with the same KLH-conjugated peptide, high titer antibodies against CYP3A4 were produced in all three rabbits as judged by ELISA. However, antibodies produced by two rabbits were not inhibitory. Thus, only the highly inhibitory antisera were purified and characterized in this study. The reason for this animal dependent variation in inhibitory property of antibodies is unknown. In a previous study (35), we also noted that all rabbits produced high titer antibodies against a peptide (residues 254–273) of CYP2D6 and antibodies have variable degree of inhibitions against CYP2D6-mediated dextromethophan O-demethylation in human liver microsomes. Such observed variable results are probably not just limited to the use of peptide as immunogen. Other investigators have reported that antibodies against purified, recombinant human cytochrome P450s exhibit different degrees of inhibition on metabolism

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(12). Soucek *et al.* (13) reported that polyclonal antibodies raised against recombinant human P450s varied in specificity, depending upon the individual rabbits used. More studies are needed to investigate the individual variability on the production of inhibitory antibodies in animals. Before we can consistently generate inhibitory antibodies in all animals, it is important to screen each animal individually to determine the inhibitory potency of the antibody on metabolism.

On the basis of the results from western blots and inhibition studies, the anti-peptide antibody can bind to both native and denatured forms of human CYP3A4. The binding of this antibody to CYP3A4, but not other cytochrome P450 isoforms including the closely related CYP3A5, confirms that the amino acid sequence in this loop region is unique for CYP3A4. Production of inhibitory antibodies against CYP3A4 peptide provides a valuable tool for evaluating the role of this important cytochrome P450 in mediating *in vitro* metabolism of new therapeutic agents. In addition, noninhibitory antibodies can be used for cytochrome P450 epitope investigation, gene expression and regulation, tissue localization, and many other studies. In conclusion, the amino acid sequence near the loop region between G helix and H helix seems to be an excellent target for the design of inhibitory antibodies against various forms of cytochrome P450.

Acknowledgments. The authors thank Drs. Judy Raucy and Jerome Lasker for providing human liver microsomes and antibody against purified CYP3A4; Deborah Newton, Rosa Sanchez, and Brian Dean for technical advice on HPLC analysis; Madhusudana Sayyaparaju for assistance on the use of Imaging Densitometer; and Terry Rafferty for preparation of the manuscript.

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