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Monoclonal Antibodies Inhibitory to Rat Hepatic Cytochromes P-450: P-450 Form Specificities and Use as Probes for Cytochrome P-450-Dependent Steroid Hydroxylations

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SUMMARY

Cytochrome P-450 (P-450) form specificities were established for a total of nine monoclonal antibodies (MAbs) raised to four distinct rat hepatic P-450 enzymes (P-450s 2c, PB-2a, PB-4, and BNF-B), using a combination of enzyme-linked immunosorbent analysis, dot immunoblotting, Western blotting, Ouchterlony immunodiffusion, and immunoinhibition analyses. Four of the MAbs were fully (≥85%) inhibitory toward the corresponding immunoreactive P-450s when assayed in purified, reconstituted enzyme systems, while two of the MAbs were partially inhibitory, with a maximum of 50 or 80% inhibition achieved in the presence of saturating MAb. Inhibitory MAbs reactive with P-450s 2c, 3, and PB-4, respectively, were used to demonstrate that the formation of multiple hydroxytestosterone metabolites by each of the respective purified P-450 enzymes is reflective of their inherent catalytic specificities and not due to the presence of immunochemically distinguishable P-450 enzyme contaminants. P-450 form-specific contributions to rat hepatic microsomal steroid hormone hydroxylase activities were then assessed using the inhibitory MAbs as probes. MAb-reactive P-450 2c was shown to be the major (≥85%) catalyst of microsomal testosterone and androstenedione 16α-hydroxylation in both untreated and β-naphthoflavone-induced rats. However, this P-450 form catalyzed only ~30% of hepatic microsomal steroid 16α-hydroxylase activity in phenobarbital-induced adult males, and ≤10% of steroid 16α-hydroxylase activity in (phenobarbital-induced immature males or adult females, where the balance of 16α-hydroxylase activity is catalyzed by MAb-reactive P-450 PB-4. Although MAb-reactive P-450 PB-4 catalyzed the majority (≥90%) of microsomal androstenedione 16β-hydroxylation in phenobarbital-induced rats, this P-450 enzyme did not contribute to the low level 16β-hydroxylase activity of uninduced liver samples. Finally, MAb-reactive P-450 3 catalyzed at least 85% of microsomal androstenedione 7α-hydroxylation, independent of the age, sex, or induction status of the animals used as source of liver microsomes. These findings demonstrate the usefulness of MAbs as probes for the contributions of individual P-450 enzymes to the metabolism of steroid hormones susceptible to hydroxylation at multiple sites.

The multiple enzymes (forms) of mammalian liver P-450 catalyze oxidative metabolism of structurally diverse lipophilic agents, including many drugs, carcinogens, and other foreign chemicals, as well as endogenous compounds such as steroid hormones, fatty acids, and cholesterol. The broad capacity of hepatic tissue for P-450-dependent xenobiotic metabolism can be modulated by exposure of individuals to various foreign compounds, many of which induce or, alternatively, suppress the levels of individual P-450 forms (1). MAbs have been developed to several of the major xenobiotic-inducible P-450s that have been purified from rat or rabbit liver. These antibodies have proven useful for the detection, immunopurification, and partial sequence analysis of immunochemically related P-450 enzymes found in hepatic and extrahepatic tissues, as well as for the identification of orthologous P-450 enzymes found in other species (e.g., Refs. 2–4). MAbs raised to a constitutive rabbit hepatic P-450 have, in addition, aided in the identification and cDNA cloning of previously unknown P-450 forms (5, 6).

P-450 enzyme multiplicity and overlapping substrate specificity have greatly complicated efforts at understanding the precise roles of individual P-450 forms in the metabolism, activation, and detoxification of different drugs and environ-

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ABBREVIATIONS: P-450, cytochrome P-450; MAb, monoclonal antibody; OH, hydroxy; PB, phenobarbital; PCN, pregnenolone 16α-carbonitrile; ELISA, enzyme-linked immunosorbent analysis; UT, untreated; BNF, β-naphthoflavone; ISF, isosafrole; PBS, phosphate-buffered saline: 10 mM KPi (pH 7.4), 0.9% NaCl (w/v); BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; KPi, potassium phosphate buffer.

mental agents. Studies using inhibitory MAbs have therefore been undertaken to assess the contributions made by individual P-450 enzymes to drug metabolism and carcinogen bioactivation catalyzed by liver microsomal preparations (7). MAb studies have also been carried out to define the roles played by individual P-450s in the metabolism of endogenous compounds, such as steroid hormones (e.g., Refs. 8 and 9), as they may relate to physiological variations in hormone metabolism in human populations (10). Studies carried out in our laboratory (11, 12) and by others (13, 14) have established that several purified rat hepatic P-450s metabolize steroid hormones at multiple sites but with significantly higher regio- and stereoselectivities than are observed using xenobiotic substrates. Although some metabolites (e.g., 6β-OH-testosterone) can be formed by more than one purified P-450, other metabolites appear to be uniquely associated with a single P-450 enzyme (e.g., 2α-OH-testosterone with P-450 2c,[1] 7α-OH-testosterone with P-450 3, and 16β-OH-androstenedione with P-450 PB-4) (15). These and other findings have suggested that hydroxy steroid metabolites can be used as P-450 enzyme-specific monitors of microsomal monooxygenase activity (16). In the current study, P-450 form specificities and inhibitory activities are defined for a series of nine MAbs raised to four individual rat hepatic P-450 forms. These MAbs are then used to evaluate whether the hydroxylation of steroid hormones at multiple sites by highly purified P-450 enzymes is a reflection of their inherent catalytic specificities. Finally, the MAbs are used as probes to extend our previous findings on the P-450 enzyme-specific contributions to androgen metabolism catalyzed by liver microsomes isolated from rats differing in age, sex, and induction status.

Materials and Methods

P-450 enzymes and nomenclature. Rat hepatic P-450 enzymes PB-1, PB-2a, 2c, 2d, 3, PB-4, and PB-5, numbered according to their relative order of elution from DEAE-cellulose, were purified to apparent protein homogeneity from Sprague-Dawley rats (Charles River Breeding Laboratory); detergent was removed on hydroxylapatite as described previously (11, 12). Designations given to apparently equivalent P-450 preparations studied by other investigators include the following: P-450 PB-1 (gene IIC6) = PB-C; P-450 PB-2a (gene IIIA1) = p, PCN-E; P-450 2c (gene II C11) = h, UT-A, RLM5, male; P-450 2d = i, UT-I, female; P-450 3 (gene IIA1) = a, UT-F; P-450 PB-4 (gene IIB1) = b, PB-B; P-450 PB-5 (gene IIB2) = e, PB-D (12, 14, 15, 17–20). In addition, the major polycyclic hydrocarbon-inducible forms are designated P-450 BNF-B (gene IA1) (= P-450c) and P-450 ISF-G (gene IA2) = (P-450d) according to the system of Guengerich *et al.* (18). The constitutive form P-450g, designated according to the system of Ryan *et al.* (17) and characterized as described in Ref. 21, was kindly provided by Dr. J. A. Goldstein, National Institute of Environmental Health Sciences.

P-450 PB-2a used in the current study was isolated from adult male rats induced with troleandomycin (Pfizer, Inc.; four daily ip injections of 500 mg of drug/kg rat; drug suspended at 125 mg/ml corn oil) using essentially the same method reported previously for enzyme isolated from PB-induced rats (12). Both of these preparations probably correspond to the dexamethasone/PB/PCN-inducible P-450 form(s) termed P-450p, PCN-E, or PCN-1 (18, 19, 22). The term P-450 2a is used to designate the constitutive, male-specific P-450-catalyzing microsomal steroid hormone 6β-hydroxylation (16). This P-450 has also been termed PCN-2 and is ~88% homologous to the inducible forms(s) represented by PB-2a (22).

Monoclonal antibodies (MAbs). Mouse MAbs previously raised against rat hepatic P-450 BNF-B (23), P-450 PB-4 (24), and P-450 PB-2a (25) were used in the current study (see Table 1, under Results and Discussion, for designations given to individual MAbs). Preparation of an MAb against rat hepatic P-450 2c (MAb D1; see Table 1) was carried out using similar methods and will be described in greater detail elsewhere.[2] Ascites fluids prepared in BALB/c mice (23) were clarified and then used as source of MAbs for all of the experiments reported in this study.

Immunochemical analyses. MAbs were assayed for their P-450 form specificities using the ELISA assay described previously (12). These ELISAs and the dot immunoblotting assay described below were carried out under conditions of saturating MAb (generally 2–20 μg/ml) and with P-450 antigens bound to the microtiter plates or nitrocellulose sheets under conditions that gave signals within the linear range of P-450 reactivity. Dot immunoblotting on nitrocellulose was performed as follows. Purified and detergent-free P-450 enzymes (25 ng in 2 μl) were spotted onto nitrocellulose and the nitrocellulose was allowed to dry. The nitrocellulose sheet was soaked at room temperature for 2–16 hr in PBS containing 20 mg/ml BSA, mounted in a Deca-Probe apparatus (Hoefer Scientific, San Francisco, CA), and then incubated with PBS containing 10 mg/ml BSA and 20 μg of ascites protein/ml for 3 hr with gentle rocking (2 ml per lane, each incubated with a different MAb). The nitrocellulose sheet was then removed from the Deca-Probe and washed sequentially with: (a) PBS containing 10 mg/ml BSA, (b) PBS, (c) PBS containing 0.5% Tween 20, (d) PBS, and (e) PBS (5 min each wash with 40–50 ml of buffer). The nitrocellulose was then incubated with PBS containing 10 mg/ml BSA and second antibody (goat anti-mouse IgG, horseradish peroxidase conjugate, 1:1000 dilution; Bio-Rad Laboratories) for 1 hr, followed by the five sequential washes detailed above. Peroxidase staining was accomplished by incubating the nitrocellulose sheet for 15–60 min in PBS containing 0.18 mg/ml 4-chloro-1-naphthol and 6% (v/v) methanol, with H_2O_2 added to 0.03% just before use.

Ouchterlony immunodiffusion analyses were performed on microscope slides in 0.9% agarose (Seakem, medium EEO) containing 1 M glycine/NaOH, 10 mM KPi (pH 7.4), 0.5% NaCl, 0.1% sodium azide, 0.2 mM EDTA, and in the absence of non-ionic detergent (26) for 48–72 hr in a humid environment. MAbs were applied to the center well at 0.6 mg/ml and purified P-450 forms to each of five surrounding wells at 1.5 nmol of P-450/ml (4 μl of sample applied/well). Following immunodiffusion, slides were soaked in 0.3 M NaCl for 48 hr, dried overnight by blotting with filter paper, and then stained for 15–45 min in 0.1% Coomassie Blue R-250 dissolved in aqueous 10% acetic acid/45% ethanol. Slides were then destained in aqueous 10% acetic acid/25% ethanol and photographed. Slides used for these experiments were precoated with a layer of 0.2% agarose and then oven-dried in order to facilitate adherance of the 0.9% agarose gel to the microscope slide during staining and destaining.

Western blotting. Liver microsomes (5–30 μg of protein) or purified P-450 forms (50–200 ng) were electrophoresed on standard Laemlli SDS-polyacrylamide gels (10%). Electrophoretic transfer to 0.45-μm nitrocellulose sheets (Vangard International, Neptune, NJ) was then carried out in a Hoefer Transphor apparatus at 120 V for 60 min in 96 mM glycine, 12.5 mM Tris base (pH 8.4), 0.015% SDS (w/v), 20% methanol (v/v) (buffer precooled to 4°). Nonspecific protein-binding sites were blocked by incubating the blots in PBS containing 0.3% (w/v) Tween 20 (solution A) for 30 min at 37°, followed by three 10-min incubations in PBS containing 0.05% (w/v) Tween 20 (solution B). Blots were then incubated overnight at 20–22° in Seal-a-Meal bags containing 10–15 ml of antibody solution (20 μg of ascites protein/ml of solution B) for a 14 × 14 cm blot. Blots were then washed three times with solution B (5 min/wash) and incubated for 60 min at room temperature with second antibody as already described for the dot immunoblotting analyses. Blots were washed twice with solution B,

[1] The nomenclature used in this study to identify individual rat hepatic P-450 enzymes is described under Materials and Methods.

[2] S. S. Park et al., manuscript in preparation.

once with solution A, then twice again with solution B followed by reaction with the peroxidase substrate as described above.

Enzyme assays. Microsomal assays were performed using 40 μg of microsomal protein/0.4 ml of reaction mix containing 0.1 M KPi (pH 7.4), 0.1 mM EDTA. Assay samples were warmed to 37° and the reactions were then initiated by addition of NADPH to 1 mM. Purified P-450 enzymes (10–20 pmol) were mixed with purified P-450 reductase (30–60 pmol; Ref. 27) and dilauroylphosphatidyl choline (1–2 μg) in a volume of 0.05 ml, reconstituted for 10 min at 22°, and then diluted to 0.375 ml with 0.1 M KPi (pH 7.4), 0.1 mM EDTA. Reactions were initiated by addition of NADPH to 0.3 mM. Immunoinhibitions were carried out by preincubating complete reaction mixtures *minus* NADPH with MAb for 45 min at 22°. Samples were then warmed to 37° and the monooxygenase reactions were initiated by addition of NADPH. Oxidative metabolism of 7-ethoxycoumarin (27) and pentoxyresorufin (28) was measured fluorimetrically as described in the indicated references. Hydroxylation of testosterone and androstenedione was assayed using radiolabeled steroid substrates with product analysis by thin layer chromatography (11, 12). Measured catalytic activities were generally reproducible to within ~15% from one experiment to the next. Patterns of MAb inhibition presented in Figs. 3–5 and Tables 3 and 4 (see Results and Discussion) were each representative of two to three independent determinations. Although these experiments were carried out using clarified ascites, very similar results were obtained using purified MAbs (not shown), establishing that the observed inhibitions are not due to nonspecific effects of the ascites protein.

Results and Discussion

P-450 form specificities of MAbs. The P-450 form specificities of nine anti-P-450 MAbs were assessed by using both ELISA and dot immunoblotting methods on a total of 10

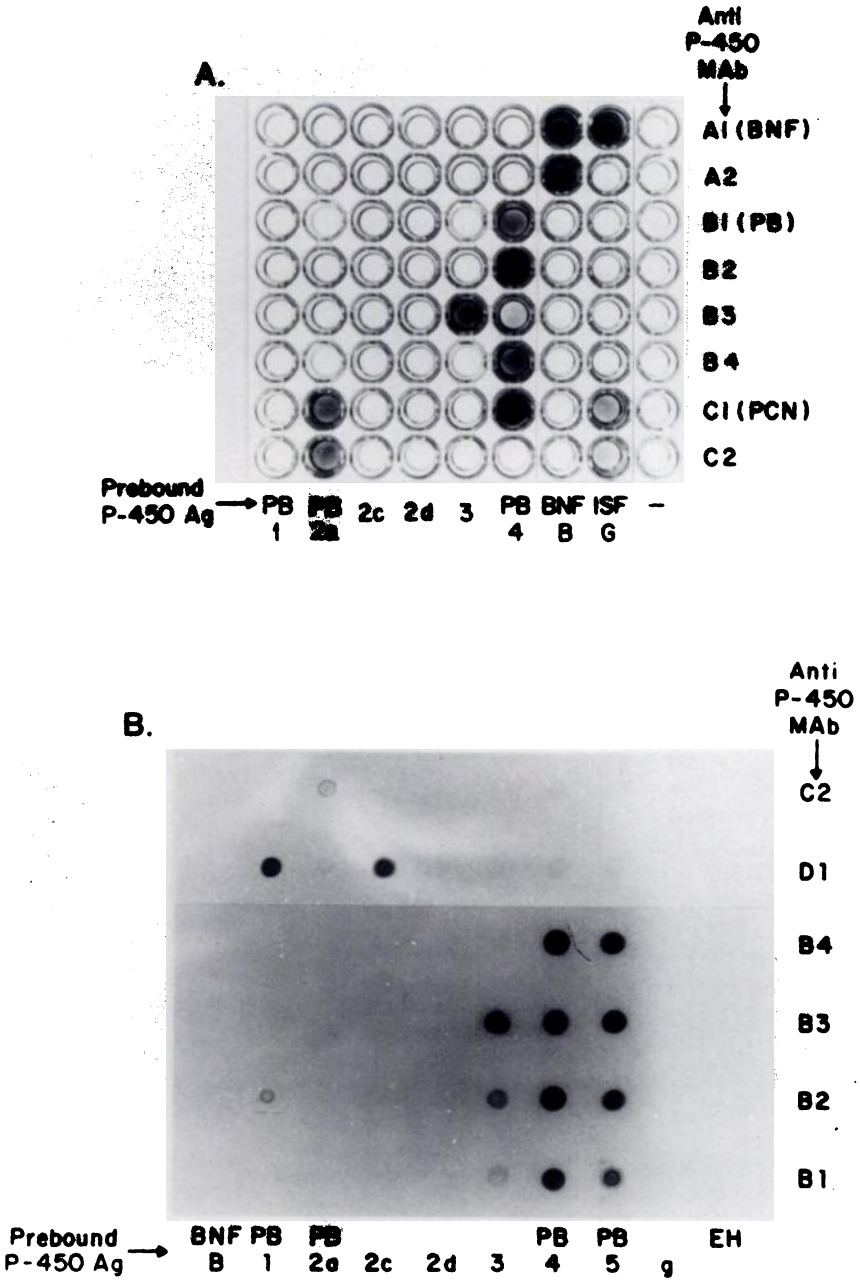

Fig. 1. P-450 form specificities of MAbs determined by ELISA (A) and by dot immunoblotting analysis (B). *A.* Purified P-450 antigens were bound to the wells of the microtiter plate along each column, after which excess nonspecific binding sites were saturated with BSA and the indicated MAbs were incubated across each row. Shown is a photograph of the plate after binding of second antibody (goat anti-mouse, horseradish peroxidase conjugate) and reaction with a peroxidase substrate as described previously (12). A_{415} values for the MAb-reactive P-450s ranged from 1.0 to 1.75 for the experiment shown, with the exception of MAb B2/P-450 PB-4, which yielded an A_{415} = 0.60. A_{415} values for the nonreactive P-450s ranged from ~5% to 20% of these values (mean = 11%). In contrast, cross-reactive P-450 forms bound an amount of MAb that ranged from 80% to 190% of the amount bound by the homologous P-450 form, perhaps reflecting relative strengths of the MAb-antigen interactions. [Thus, for MAb C1, A_{415}(P-450 ISF-G)/A_{415}(P-450 PB-2a) = 0.8 and for MAb B3, A_{415}(P-450 3)/A_{415}(P-450 PB-4) = 1.9] . No peroxidase product was observed in the absence of P-450 antigen ($A_{415} \leq$ 0.05; last column on the *right*) or in the absence of MAb, second antibody, or peroxidase substrate (not shown). *B.* Immunoblotting on nitrocellulose was performed as described under Materials and Methods. Shown is a photograph of the blot after reaction with peroxidase substrate. Purified P-450s (25 ng) were applied along each column and individual MAbs were incubated across each row as indicated. Reaction with the peroxidase substrate was allowed to proceed for 60 min to facilitate detection of minor cross-reactivities (e.g., reaction of P-450 PB-1 with MAb B2 and of P-450 3 with MAb B1). *g*, P-450g; *EH*, epoxide hydrase.

TABLE 1
Characterization of anti-P-450 MAbs

MAb	Clone designation	P-450 immunogen	Cross-reactive P-450 forms[a]	Inhibitory activity[b]
A1	1-7-1	BNF-B	ISF-G	+++
A2	1-31-2	BNF-B	–	–[c]
B1	2-8-1	PB-4	PB-5	+
B2	2-66-3	PB-4	PB-5,(3)[d]	++
B3	4-7-1	PB-4	3,PB-5	+++
B4	4-29-5	PB-4	PB-5	+++
C1	2-3-2	PB-2a	2a,PB-4, PB-5,(ISF-G)[e]	–[f]
C2	2-13-1	PB-2a	2a	–[f]
D1	1-68-11	2c	PB-1	+++

[a] Listed are the P-450 forms exhibiting a significant degree of cross-reactivity with each MAb, as determined by ELISA and dot immunoblot analysis. In most cases these cross-reactivities were confirmed by Ouchterlony immunodiffusion analysis and Western blotting, and for the inhibitory antibodies, by their cross-inhibitory activity. Cross-reactivity of MAbs C1 and C2 with P-450 2a was determined by Western blotting (see the text).

[b] Inhibition of 7-ethoxycoumarin *O*-deethylation measured in purified, reconstituted systems. –, no significant inhibition (±10–20%); + or ++, partial inhibition in the presence of saturating MAb; +++, complete (≥85%) inhibition of catalytic activity.

[c] In an earlier study from one of our laboratories this same clone was reported to be inhibitory (23). The reason for the discrepancy between those findings and our current observations is not known.

[d] Cross-reactivity of MAb B2 with P-450 3, detectable by dot immunoblotting (Fig. 1B) but not by ELISA (Fig. 1A), was noticeably weaker than its cross-reactivity with P-450 PB-5 (Fig. 1B).

[e] Crossreactivity with P-450 ISF-G detected by ELISA (Fig. 1A) but not by dot immunoblotting (data not shown).

[f] See text footnote 4.

distinct rat hepatic P-450 forms (Fig. 1, Table 1). Included in these analyses were MAbs A1 and A2, raised against P-450 BNF-B; MAbs B1–B4, raised against P-450 PB-4; MAbs C1 and C2, raised against P-450 PB-2a; and MAb D1, raised against P-450 2c. MAb A2 bound specifically to the P-450 immunogen (i.e., to P-450 BNF-B), while MAb A1 bound, in addition, the structurally related P-450 ISF-G; these findings are consistent with previous studies using these same MAbs for immunopurification of 3-methylcholanthrene-inducible microsomal P-450s (29). MAbs B1, B2, and B4 each reacted with P-450 PB-4 and the highly homologous P-450 PB-5, with no major cross-reactivities with seven other highly purified P-450s observed. By contrast, MAb B3 reacted well with P-450 3 in addition to PB-4 and PB-5. Minor cross-reactivities between MAb B1 and P-450 3, as well as between MAb B2 and P-450s 3 and PB-1, were also seen (Fig. 1B). MAb C2 bound to purified P-450 PB-2a, while MAb C1 reacted with P-450s PB-4, PB-5, and ISF-G in addition to P-450 PB-2a. MAb D1 bound both P-450 PB-1 and the immunizing P-450 2c, as demonstrated by dot immunoblotting (Fig. 1B). Interestingly, MAb D1 did not recognize P-450 2d, which we have previously shown to be immunochemically related to both P-450 2c and P-450 PB-1 (12). Repeated attempts to demonstrate interactions between MAb D1 and P-450 2c or P-450 PB-1 using ELISA methods were unsuccessful. Although none of the MAbs gave a positive reaction with either P-450 2d or P-450g, these two P-450 preparations were fully reactive with polyclonal anti-P-450 antibodies under comparable assay conditions (data not shown).

Several anti-P-450 MAbs have been shown to precipitate purified P-450s upon immunodiffusion in the absence of non-ionic detergent, perhaps due to the tendency of the P-450s to self-aggregate (30). Ouchterlony immunodiffusion analyses were therefore performed in order to provide an independent means of assessing the P-450 form specificities of the nine MAbs. The results obtained generally confirmed the specificities indicated by ELISA and immunoblotting. Thus, for example, MAb D1 immunoprecipitated P-450s PB-1 and 2c but not seven other P-450 forms (Fig. 2A and B), MAb B2 immunoprecipitated P-450s PB-4 and PB-5 (Fig. 2C), and MAb B3 immunoprecipitated P-450 3 (Fig. 2D).

Western blotting analyses were carried out to further characterize the P-450 form specificities of the MAbs and, in addition, to assess their possible cross-reactivity with unknown microsomal P-450s that might resolve from the parent P-450 antigens upon electrophoresis in SDS-polyacrylamide gels. MAbs A1, A2, B1, B2, B4, C1, and C2 were found to give good signals on Western blots, in each case confirming the major antigenic specificities summarized in Table 1 (data not shown). Thus, Western blots of BNF- or ISF-induced liver microsomes probed with MAbs A1 and A2 revealed either a single band (MAb A2) or a doublet of bands (MAb A1) that was undetectable in uninduced or in PB- or dexamethasone-induced liver microsomes. These bands corresponded in electrophoretic mo-

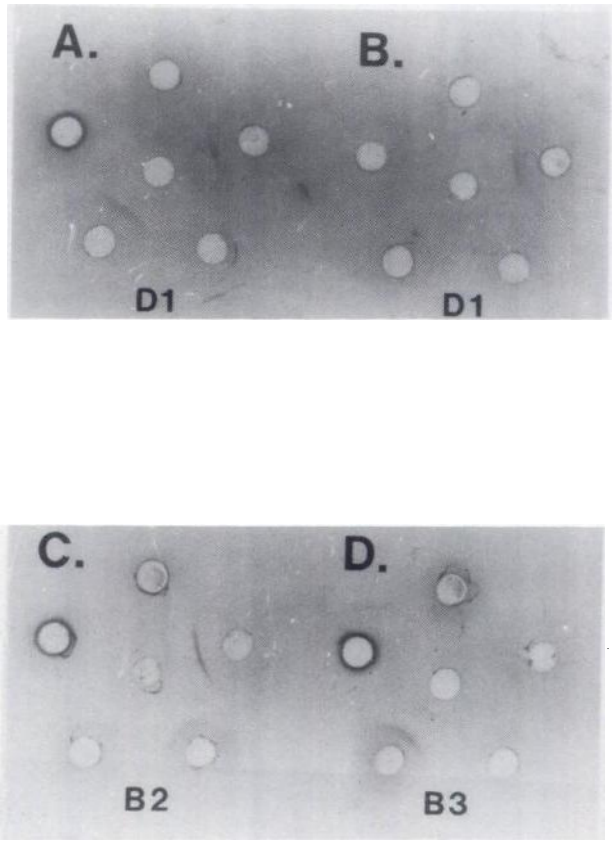

Fig. 2. Ouchterlony immunodiffusion analysis of MAb-P-450 interactions. A. MAb D1 (center well) was immunodiffused versus the following P-450 forms applied to the outer wells (P-450 forms are listed *clockwise*, beginning at the apex of the pentagon pattern): P-450s BNF-B, 2c, 2d, PB-1, and PB-2a. B. MAb D1 versus P-450s PB-4, 2c, g, 3, and PB-5. C. MAb B2 versus P-450s BNF-B, PB-5, PB-4, 3, and PB-2a. D. MAb B3 versus P-450s BNF-B, PB-5, PB-4, 3, and PB-2a. Clear precipitin bands are formed by MAb D1 with P-450s 2c and PB-1, by MAb B2 with P-450 PB-5, and by MAb B3 with P-450 3. A more diffuse precipitin pattern was formed by MAb B2 with PB-4 (C) as well as by MAb B3 with PB-4 and with PB-5 (D) under these conditions by immunodiffusion.

bility to P-450s BNF-B and ISF-G. Similarly, MAbs B2 and B4 reacted specifically with the PB-inducible microsomal P-450s PB-4 and PB-5. MAb C2 reacted with the inducible P-450 PB-2a (or closely related forms) expressed in liver microsomes isolated from rats induced with either PB, dexamethasone, troleandomycin, or PCN, whereas MAb C1 reacted, in addition, with P-450s PB-4 and PB-5 in the case of PB-induced microsomes. Moreover, both MAbs C1 and C2 also reacted on the Western blots with a protein having the same electrophoretic mobility as PB-2a that was expressed constitutively in adult male (but not female) microsomes, i.e., with P-450 2a. Western blotting signals were either weak or undetectable with MAbs B3 and D1, suggesting that they have much lower binding affinity for SDS-denatured P-450s (probed on the Western blots) as compared to the undenatured cytochromes (probed on the dot immunoblots).[3]

Inhibitory potency of MAbs. Each of the nine MAbs was assayed for its ability to inhibit 7-ethoxycoumarin metabolism catalyzed by the corresponding P-450 immunogen in a purified, reconstituted system. MAbs A1, B3, B4, and D1 were shown to be fully (≥85%) inhibitory, whereas MAbs A2, C1, and C2 were not inhibitory, even at the highest concentrations tested (200 μg of MAb/12 pmol of P-450) (Table 1 and data not shown).[4] In the case of MAbs B1 and B2, partial inhibition of P-450 PB-4 activity was observed in the presence of apparently saturating levels of MAb. Kinetic analyses (Table 2) established that the partial (~80%) inhibition of P-450 PB-4 activity by MAb B2 reflected both a decrease in V_{max} and an increase in K_m. By contrast, the 50% inhibition of P-450 PB-4 by MAb B1 was accompanied by a 50% reduction in V_{max} with no change in K_m. An analogous V_{max} effect was observed when MAb B4 was present at subsaturating concentrations (Table 2, experiment 2). These findings suggest that the MAb B1-P-450 PB-4 complex retains partial enzyme activity. Alternatively, MAb B1 may distinguish between P-450 PB-4 and a closely related P-450 form (perhaps an allozyme; e.g., Ref. 31) which contributes about 50% of the observed catalytic activity of the P-450 PB-4 preparations used in these studies.

MAbs A1, B3, and D1 were also shown to be inhibitory to P-450-dependent microsomal 7-ethoxycoumarin metabolism, indicating that the corresponding P-450 epitopes are exposed and not buried within the lipid bilayer. These MAbs could be used as effective probes to titrate the respective contributions of P-450s 3 + PB-4 + PB-5 (MAb B3), P-450s 2c + PB-1 (MAb D1), and P-450s BNF-B + ISF-G (MAb A1) to microsomal 7-ethoxycoumarin *O*-deethylation (Fig. 3). The absence of a significant contribution of P-450s 2c + PB-1 to 7-ethoxycoumarin metabolism in BNF-induced microsomes (Fig. 3C) probably reflects the suppression by β-naphthoflavone of P-450s 2c and PB-1 (12, 32), coupled with its 6-fold induction of microsomal 7-ethoxycoumarin metabolism (Fig. 3, legend). Interestingly, MAb A1, although >90% inhibitory to purified P-450s BNF-B and ISF-G in reconstituted systems (Table 1), only inhibited ~50–60% of 7-ethoxycoumarin metabolism in BNF-induced liver microsomes (Fig. 3C). This value corresponds to inhibition of only 60–70% of the BNF-induced activity, suggesting that BNF-inducible P-450s unreactive toward MAb A1 may contribute significantly to the microsomal metabolism of 7-ethoxycoumarin. Alternatively, a substantial portion of mi-

TABLE 2

Kinetic analysis of MAb inhibition of reconstituted P-450 PB-4 activity

MAbs were preincubated with purified and reconstituted P-450 PB-4, and 7-ethoxycoumarin metabolism was then assayed as described under Materials and Methods. MAb A1, which does not bind P-450 PB-4, was used to assess the effects of nonspecific ascites protein. Kinetic parameters were determined at concentrations of 7-ethoxycoumarin ranging from 67 μM to 1 mM (limit of substrate solubility) using analytical and statistical methods described previously (42). Experiment 1 was performed under conditions where MAbs B1 and B2 exhibited their maximal inhibitory potency (~50% and 80%, respectively, at 1 mM substrate), and experiment 2 was performed using MAb B4 under conditions where it was only ~30% inhibitory. Complete (>95%) inhibition by MAb B4 was achievable at concentrations above ~40 μg of ascites protein/ml.

MAb		7-Ethoxycoumarin *O*-deethylation	
		Apparent K_m	V_{max}
	μg ascites protein/ml	*mM*	*nmol of product/min–nmol P-450*
Experiment 1			
A1	125	0.24 ± 0.03	19.5 ± 1.8
B1	125	0.24 ± 0.04	10.2 ± 1.5
B2	125	0.34 ± 0.05	6.6 ± 0.8
Experiment 2			
		0.26 ± 0.02	21.2 ± 1.2
A1	10	0.29 ± 0.02	22.0 ± 2.1
B4	10	0.30 ± 0.03	15.5 ± 1.3

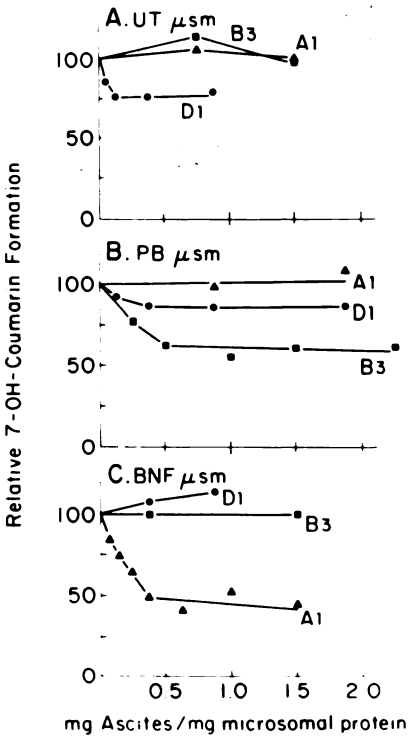

Fig. 3. P-450 form-specific contributions to hepatic microsomal 7-ethoxycoumarin *O*-deethylation determined by MAb inhibition. Liver microsomes prepared from untreated adult male rats (*UT μsm*, A) or from adult males induced with PB (*PB μsm*, B), or BNF (*BNF μsm*, C) were preincubated with the indicated MAbs, and the metabolism of 7-ethoxycoumarin was then assayed as described under Materials and Methods. MAb B4 inhibited microsomal 7-ethoxycoumarin metabolism in a manner similar to that of MAb B3 (not shown). Uninhibited catalytic rates corresponded to 0.8, 2.4, and 5.1 nmol of 7-hydroxycoumarin formed/mg of microsomal protein.

[3] Loss of Western blot reactivity upon heating in SDS has recently been observed for both purified and microsomal P-450s (41).

[4] In the case of MAbs C1 and C2, the absence of inhibitory activity was confirmed using partially purified preparations of P-450 2a and P-450 PB-2a that were active at androstenedione 6β-hydroxylation. MAb C1 was also shown to be noninhibitory toward P-450 PB-4-catalyzed 7-ethoxycoumarin *O*-deethylation.

crosomal P-450 BNF-B or P-450 ISF-G might not be readily accessible to the MAb.

MAb inhibition of steroid hormone hydroxylations catalyzed by purified P-450s. Rat hepatic P-450 enzymes purified to apparent homogeneity hydroxylate steroid hormones at multiple sites. The inhibitory MAbs described above were therefore used to probe for the presence of P-450 enzyme contaminants that might contribute to formation of one or more of the observed hydroxy steroid metabolites (Fig. 4). MAb B3 effected a complete (>95%) and specific inhibition of the P-450 PB-4-dependent conversion of testosterone to each of its principal oxidation products, 16α-OH-testosterone, 16β-OH-testosterone, and androstenedione (Fig. 4A), with similar results obtained when using MAb B4 (Fig. 4C). In control experiments, neither MAb A1 (Fig. 4A) nor MAb D1 (data not shown) inhibited these activities and, in addition, MAb B3 did not inhibit the formation of 16α-OH-testosterone, 2α-OH-testosterone, or androstenedione catalyzed by purified and reconstituted P-450 2c (not shown). MAb B3, which is cross-reactive with P-450 3 (Table 1), could also inhibit by at least 85% the P-450 3-catalyzed formation of 7α-OH-testosterone, the major testosterone metabolite of this P-450, as well as 6α-OH-testosterone, a minor metabolite coresponding to only ~5% of the total P-450 3 product (Fig. 4B). Somewhat higher levels of MAb B3 were, however, required to inhibit P-450 3 as compared to P-450 PB-4 (cf. Fig. 4A). In a similar fashion MAb D1 inhibited by at least 85% each of the testosterone oxidation activities associated with purified P-450 2c (Fig. 4D), as well as the androstenedione hydroxylase activities of this cytochrome (16α-OH- and 6β-OH-androstenedione formation; data not shown). These findings provide good evidence that the formation of multiple hydroxy steroid metabolites by the puri-

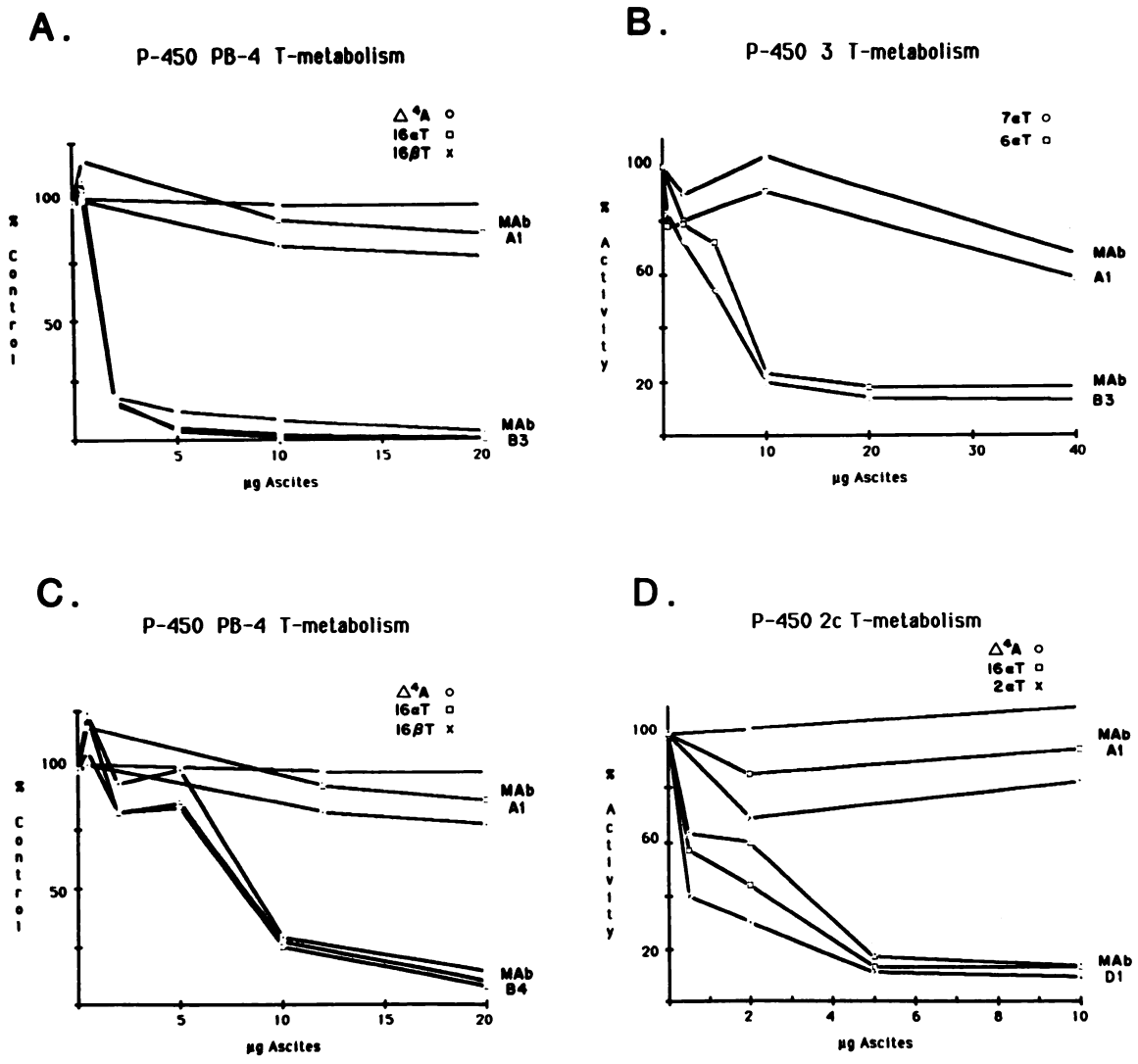

Fig. 4. MAb inhibition of multiple testosterone hydroxylase activities catalyzed by purified P-450 enzymes. Purified P-450s 2c, 3, and PB-4 were reconstituted with lipid and P-450 reductase, and preincubated with the indicated MAbs; then, testosterone hydroxylase activities were assayed as described under Materials and Methods. Shown are the effects of the indicated MAbs on the formation of each of the testosterone metabolites of P-450 PB-4 (A and C), P-450 3 (B), and P-450 2c (D). In the control experiments shown, no inhibition was observed when MAb A1 was used in place of the three specific antibodies. Uninhibited catalytic activities corresponded to the following values (expressed as nmol of hydroxytestosterone metabolite formed/min-nmol P-450): for P-450 PB-4 (A and C): 16α-OH-T = 6.2, 16β-OH-T = 4.2, and androstenedione = 3.7; for P-450 3 (B): 7α-OH-T = 9.0 and 6α-OH-T = 0.5, for P-450 2c (D): 16α-OH-T = 3.4, 2α-OH-T = 3.5, and A = 1.2. *T*, testosterone; *A*, androstenedione.

fied enzyme preparations is a true reflection of the inherent catalytic specificities of the corresponding P-450 forms.[5] An analogous conclusion has recently been drawn for the pregnenolone 17α-hydroxylase and C17-C20 lyase activities of adrenocorticol microsomal P-450$_{17\alpha}$ using a different experimental approach (33).

P-450 form-specific contributions to liver microsomal steroid hormone hydroxylations. We have previously demonstrated, using polyclonal antibodies, that immunoreactive P-450 2c is the principle androstenedione 16α-hydroxylase of untreated and polycyclic-induced adult male rat liver (12), immunoreactive P-450 3 is the major androstenedione 7α-hydroxylase and, in the case of PB-induced animals, immunoreactive P-450 PB-4 is the major androstenedione 16β-hydroxylase (16). These observations were confirmed and extended in a series of immunoinhibition experiments using MAb B4 to probe for PB-4 (+PB-5)-dependent microsomal activities, MAb B3 to probe for P-450 3 (+PB-4/PB-5)-dependent activities, and MAb D1 to probe for P-450 2c (+PB-1)-dependent activities.[6] The results of these experiments, carried out using liver microsomes isolated from adult and immature male and adult female rats (untreated, PB-induced or BNF-induced), can be summarized as follows.

(a) *Microsomal androstenedione 16β-hydroxylase activity.* MAbs B3 and B4 inhibited by about 90% the P-450 PB-4-dependent androstenedione 16β-hydroxylase activity of liver microsomes isolated from PB-induced immature male, adult male, and adult female rats (Table 3). In each case up to about 10% of the 16β-hydroxylase activity was resistant to inhibition (even in the presence of MAb levels that were 2-fold higher than the levels reported in Table 3), suggesting that a subpopulation of microsomal PB-4 may be inaccessible to the MAbs employed, possibly owing to the orientation of the P-450 towards the inside of the microsomal vesicles or to its interaction with P-450 reductase (cf. Ref. 34). The low level androstenedione 16β-hydroxylase activity exhibited by uninduced liver microsomes is *not*, however, catalyzed by immunoreactive P-450 PB-4 (Table 3, column 1), indicating that the induction of PB-4-dependent microsomal activity by PB is significantly greater than the 30-fold increase in 16β-hydroxylase activity exhibited by the PB microsomes. This conclusion is consistent with the 100-fold induction by PB of P-450 PB-4-dependent pentoxyresorufin *O*-dealkylase activity reported by Lubet *et al.* (28) and confirmed in the present study.[7]

(b) *Microsomal androstenedione and testosterone 16α-hydroxylase activities.* MAb D1 inhibited in a selective and near-quantitative fashion (~85–90%) androstenedione 16α-hydroxylation catalyzed by untreated or BNF-induced liver microsomes (Fig. 5A and Table 3). In contrast, this MAb inhibited only about 30% of steroid 16α-hydroxylase activity in PB-induced adult male liver microsomes and 10% or less in the PB-induced immature males and adult females [independent of whether testosterone (Fig. 5B) or androstenedione (Table 3) was used as substrate]; these observations are consistent with the previously reported adult male-specific expression of P-450 2c (12, 35). Complementary results were obtained using MAbs B3 and B4; androstenedione and testosterone 16α-hydroxylations were not susceptible to inhibition by these antibodies in the uninduced or β-naphthoflavone-induced microsomes [which express very low levels of P-450 PB-4 (e.g., Ref. 16) and its associated androstenedione 16β-hydroxylase activity (Table 3)] but were 60–70% inhibitable in PB-induced adult male liver microsomes and about 90% inhibitable in either immature males or adult females induced with PB. Thus, liver microsomal androstenedione and testosterone 16α-hydroxylase activities *do* provide a reliable monitor of P-450 2c catalytic levels in untreated or in BNF-induced adult male rats, but *not* in the case of the PB-induced animals, where immunoreactive P-450 PB-4 contributes significantly to steroid 16α-hydroxylation. The absence of a significant alteration in the overall rate of androstenedione 16α-hydroxylation upon PB treatment of adult male rats (Table 3, column 1 versus column 2) is thus a reflection of a suppression of P-450 2c-dependent 16α-hydroxylation which is fully compensated for by induction of P-450

TABLE 3

MAb Inhibition of microsomal androstenedione hydroxylation

Liver microsomes (40 μg of protein) prepared from adult male or female rats that were either untreated (UT) or induced with PB or BNF, as indicated, were preincubated with MAbs B3, B4 (100 μg of ascites), or D1 (25 μg of ascites), and the four indicated androstenedione hydroxylase activities were determined as described under Materials and Methods. Data shown were all obtained in the same experiment and are expressed as specific activities (nmol of metabolite/min-mg microsomal protein; italic values) or as per cent relative to uninhibited control samples.

	♂, UT	♂, PB	Im♂, PB	♂, BNF	♀, UT	♀, PB
16β-OH-A						
No MAb	*0.34*	*10.0*	*7.34*	*0.14*	*0.15*	*4.69*
+ MAb B3	216	9	6	–[a]	–	9
+ MAb B4	147	14	10	–	–	10
+ MAb D1	84	75[b]	74[b]	–	–	68[b]
16α-OH-A						
No MAb	*1.61*	*1.60*	*1.12*	*0.18*	*0.10*	*0.60*
+ MAb B3	120	33	12	101	110	13
+ MAb B4	102	44	12	90	95	13
+ MAb D1	14	71	92	19	40	86
7α-OH-A						
No MAb	*0.33*	*0.56*	*1.47*	*0.33*	*0.41*	*0.87*
+ MAb B3	30	17	14	19	17	14
+MAb B4	96	118	104	93	112	105
+ MAb D1	93	105	99	98	92	96
6β-OH-A						
No MAb	*1.14*	*4.25*	*3.00*	*0.38*	*0.10*	*0.30*
+ MAb B3	132	159	120	137	–	130
+ MAb B4	122	168	123	98	–	142
+ MAb D1	125	122	110	95	–	123

[a] Extent of inhibition was not determined since the activities were very low, even in the absence of MAb.

[b] This apparent inhibition by MAb D1 ascites of 16β-hydroxylation catalyzed by phenobarbital-induced microsomes was nonspecific and was not observed using purified MAb D1. MAb purification did not affect any of the other inhibitions effected by this or the other MAbs.

[5] Although these MAbs probably do not distinguish between P-450 forms and their allozymic variants (e.g., Ref. 31), the experiments in Fig. 4 suggest that the patterns of hydroxylated metabolites observed with each isozyme do not reflect contamination by more distantly related P-450 enzymes.

[6] Although MAb D1 is cross-reactive with P-450 PB-1, that P-450 does not hydroxylate these steroids at appreciable rates in reconstituted systems (Ref. 42 and unpublished results). These observations, together with data obtained using polyclonal anti-PB-1 antibodies (data not shown), suggest that the major effects of MAb D1 on the microsomal steroid hydroxylase activities examined in this study can be ascribed to its inhibition of microsomal P-450 2c. Similarly, although the possible cross-reactivity of MAb D1 with P-450f (which is in the same gene subfamily as P-450 PB-1) was not evaluated, the expression of P-450f at significant levels in adult female rat liver microsomes (17) indicates that it is unlikely to contribute to the adult male-specific steroid 16α- and 2α-hydroxylase activities inhibited by MAb D1 in these experiments.

[7] MAbs B3 and B4 (but not MAb A1 or MAb D1) inhibited by ~98% the pentoxyresorufin *O*-dealkylase activity of PB-induced adult male rat liver microsomes. However, MAbs B3 and B4 did not inhibit the low level *O*-dealkylase activity of uninduced adult male liver microsomes (cf. insensitivity of uninduced androstenedione 16β-hydroxylase to MAb B3 or B4 inhibition; Table 3).

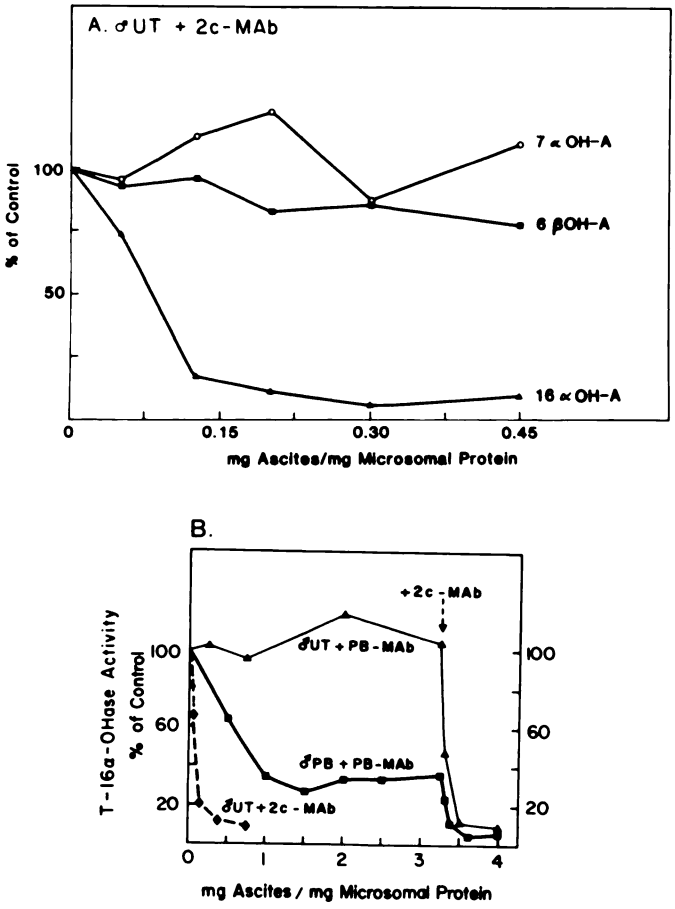

Fig. 5. MAb inhibition of microsomal hydroxylation of androstenedione and testosterone. Liver microsomes prepared from untreated adult male rats (♂ UT) or adult male rats induced with PB (♂ PB) were preincubated with MAb D1 (*2c-MAb*) or MAb B4 (*PB-MAb*), and metabolism of androstenedione (A) or testosterone (B) was determined as described under Materials and Methods. In cases where indicated (B, *dashed arrow*), microsomes were incubated with 3.25 mg of MAb B4/mg of microsomal protein *plus* variable amounts of MAb D1 to give the ratio of total ascites protein to microsomal protein indicated in the figure. Shown in A are the effects of MAb D1 on each of the major androstenedione hydroxylase activities of untreated adult male liver microsomes. Shown in B are the effects of the indicated MAbs on microsomal testosterone 16α-hydroxylation. MAb B4 also inhibited testosterone 16β-hydroxylation (in the PB-induced microsomes only) but had no effect on testosterone 2α-, 6β-, or 7α-hydroxylation, whereas MAb D1 inhibited testosterone 2α-hydroxylation in parallel to 16α-hydroxylation (not shown). Uninhibited catalytic activities (expressed as nmol of product formed/min-mg of microsomal protein) were as follows: in A: 16α-OH-A = 1.9, 6β-OH-A = 1.3, 7α-OH-A = 0.2; in B: 16α-OH-T = 1.1 in the untreated microsomes and 2.0 in the PB-induced microsomes. *T*, testosterone; *A*, androstenedione.

PB-4. Interestingly, the 16β-hydroxy to 16α-hydroxy androstenedione product ratio suggested for microsomal P-450 PB-4 by these experiments (ranging from 7.3 to 9.6)[8] is similar to the ratio observed with purified P-450 PB-4 in reconstituted systems (11, 13). This suggests that the regioselectivity of P-450 PB-4 is not markedly altered during the course of microsome solubilization and enzyme purification.

[8] Calculation assumes that P-450 PB-4 catalyzes 65%, 90%, and 90% of the total androstenedione 16α-hydroxylase activity of PB-induced adult male, immature male, and adule female liver microsomes, respectively, and that P-450 PB-4 catalyzes 100% of androstenedione 16β-hydroxylation in these same microsomes (see Table 3). Thus, microsomal P-450 PB-4 16β/16α ratios of 10.0/(1.60 × 65%) = 9.6, 7.24/(1.12 × 90%) = 7.3, and 4.69/(0.60 × 88%) = 8.7 are suggested by the data in Table 3.

(c) *Microsomal androstenedione 7α-hydroxylase activity.* MAb B3 inhibited androstenedione 7α-hydroxylase activity by up to 85% in all six microsomal samples analyzed (Table 3). A similar degree of inhibition of microsomal testosterone 7α-hydroxylation was also observed (e.g., Table 4). Since these steroid 7α-hydroxylase activities were not inhibited by MAb B4 (or by MAb D1), we deduce that the effect of MAb B3 on steroid 7α-hydroxylation results from its interaction with microsomal P-450 3. That 15–20% of microsomal 7α-hydroxylase activity was resistant to inhibition by saturating MAb is not surprising since a similar degree of residual activity was observed in the experiment using purified and reconstituted P-450 3 (Fig. 4B).

(d) *Microsomal steroid 6β-hydroxylase activity.* None of the MAbs examined was inhibitory toward microsomal 6β-hydroxylation of androstenedione (Table 3) or testosterone (Table 4 and data not shown), consistent with our previous observations that microsomal steroid 6β-hydroxylation is catalyzed by immunoreactive P-450 PB-2a (16). Two of the MAbs actually enhanced 6β-hydroxylation catalyzed by PB-induced microsomes by up to 60–70%. Similar increases in microsomal steroid hydroxylation have been observed in other systems when using polyclonal antibodies [e.g., up to 50% increase in microsomal steroid 7α-hydroxylase activity in the presence of polyclonal anti-P-450 2c; Ref. 12] and may result from a more effective competition by the uninhibited P-450 forms for the rate-limiting microsomal NADPH P-450 reductase. Although purified P-450 BNF-B exhibits good steroid 6β-hydroxylase activity in reconstituted systems, MAb A1 was not inhibitory toward 6β-hydroxylation catalyzed by BNF-induced liver microsomes (which contain significant amounts of the MAb A1-reactive P-450 BNF-B), in agreement with earlier observations (13).

Conclusion. We have characterized the P-450 form specificities of 9 anti-P-450 MAbs using both immunochemical and catalytic approaches. P-450 enzyme cross-reactivities thus detected include ones that have previously been observed using polyclonal antibodies [e.g., cross-reactivity between P-450 BNF-B and P-450 ISF-G (26, 36), between P-450 PB-4 and P-450 PB-5 (27, 37), and between P-450 PB-1 and P-450 2c (12)] as well as cross-reactivities not exhibited by polyclonal antibodies [e.g., cross-reactivity between P-450 PB-4 and P-450 3, as well as between P-450 PB-2a and P-450s PB-4, PB-5, and ISF-G]. Since these latter cross-reactivities have been detected using relatively small numbers of MAbs, they may reflect the presence of a limited number of shared epitopes, rather than extensive sequence homology. Similar conclusions can be drawn from studies identifying individual anti-P-450 PB-4 MAbs that cross-react with either P-450 3 or P450f, in addition to P-450 PB-5 (38).

TABLE 4

Effects of inhibitory MAbs on testosterone hydroxylation catalyzed by BNF-induced rat liver microsomes

MAbs were preincubated with BNF-induced adult male rat liver microsomes (15 mg of ascites protein/mg of microsomal protein), and testosterone (T) metabolism was then determined as described under Materials and Methods. Data are presented as specific activities (nmol of testosterone metabolite/min-mg microsomal protein; italic values) or as per cent of control samples.

	Testosterone metabolite			
	6βOH-T	7αOH-T	16βOH-T	2αOH-T
No MAb	*0.67*	*0.37*	*0.24*	*0.29*
+ MAb A1	94	87	83	106
+ MAb B3	110	14	99	95
+ MAb B4	111	87	85	121

Four inhibitory MAbs of defined P-450 form specificity were used to assess the P-450 enzyme contributions to hepatic microsomal metabolism of androstenedione and testosterone. The results obtained were consistent with the regio- and stereoselectivities exhibited by the corresponding purified P-450 enzymes in reconstituted systems when taking into consideration the relative P-450 enzyme levels present in the microsomal preparations examined. For example, immunoreactive P-450 2c was shown to catalyze the majority (>85%) of microsomal steroid 16α-hydroxylase activity in both uninduced and in β-naphthoflavone-induced adult male rats, which contain significant levels of P-450 2c, whereas immunoreactive P-450 PB-4 catalyzed ≥90% of microsomal steroid 16α-hydroxylase activity in phenobarbital-induced immature male and adult female rats, which contain substantial levels of P-450 PB-4 but only trace amounts of P-450 2c (16). In PB-induced adult male rats, which contain severalfold higher levels of P-450 PB-4 as compared to P-450 2c, 60–70% of microsomal steroid 16α-hydroxylation could be attributed to P-450 PB-4, with the balance catalyzed by P-450 2c, consistent with the findings of Reik *et al.* (38). Similarly, major contributions by P-450s 3 and PB-4 to microsomal steroid 7α- and 16β-hydroxylase activities, respectively, could be demonstrated.

Although these findings are all consistent with the catalytic specificities and relative microsomal levels of the individual P-450 enzymes under study, results based on antibody inhibition experiments such as those reported here are only as reliable as the specificities of the MAbs employed. Potential cross-reactivities with uncharacterized P-450 forms, or with closely related isozymes, need to be considered. For example, recent studies (39) provide evidence for a P-450 form closely related to P-450 3 whose potential reactivity with MAb B3 cannot yet be established. Moreover, although the specificity of MAb D1 was evaluated using several members of the P-450 IIC gene subfamily (i.e., P-450s PB-1, 2c, 2d), other members of this subfamily (e.g., P-450f) were unavailable for analysis. Finally, in the case of P-450 PB-4, antibodies (both polyclonal and monoclonal) are highly cross-reactive with P-450 PB-5 (e.g., Table 1, and Refs. 27 and 37). Therefore, the catalytic contributions of these two closely related P-450 forms (97% identical in primary structure) cannot be adequately resolved using available methodologies. Although one might presume that the contribution by P-450 PB-5 to microsomal androstenedione 16β-hydroxylation is minimal on the basis of the much lower activity exhibited by purified PB-5 as compared to purified PB-4 in reconstituted enzyme systems (27, 37), at least one rat P-450 form appears unstable to purification (P-450 PB-2a; Refs. 16 and 40), indicating that such a presumption might not be warranted.

None of the anti-P-450 PB-2a MAbs examined were inhibitory, precluding further studies on the contributions of this or closely related (22) P-450 enzymes to microsomal steroid 6β-hydroxylation. Inhibitory MAb to P-450 BNF-B was used, however, to demonstrate that P-450 BNF-B does not catalyze significant 6β-hydroxylation in liver microsomes, even though it exhibits good 6β-hydroxylase activity in reconstituted systems (13). Consistent with this observation, β-naphthoflavone does not induce, but rather, suppresses microsomal 6β-hydroxylation (Table 3), as well as immunoreactive microsomal P-450 PB-2a (16). Thus microsomal P-450 BNF-B exhibits a markedly lower turnover number for steroid 6β-hydroxylation than does the purified cytochrome in reconstituted enzyme systems. We have recently made similar observations with respect to the steroid 6β-hydroxylase activity of P-450g (21).

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