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MONOCLONAL ANTIBODIES TO RAT LIVER CYTOCHROME P-450 2c/RLM5 THAT REGIOSPECIFICALLY INHIBIT STEROID METABOLISM*

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Abstract—Hybridomas were formed from myeloma cells and spleen cells derived from BALB/c female mice immunized with purified liver microsomal cytochrome P-450 2c/RLM5 (P-450 gene IIC11) isolated from untreated adult male rats. Six hybridoma clones produced monoclonal antibodies (MAbs) of the IgM(κ) type. All the MAbs bound strongly to P-450 2c/RLM5 when measured by radioimmunoassay, and four of the six specifically immunoprecipitated P-450 2c/RLM5 in an Ouchterlony double-immunodiffusion test. These four MAbs also bound but did not immunoprecipitate P-450 RLM3. The MAbs that precipitated P-450 2c/RLM5 neither bound nor precipitated P-450 PB-B (gene IIB1) and P-450 BNF-B (gene IA1) of rats or P-450 LM2 and P-450 LM4 of rabbits. In contrast, mouse polyclonal anti-P-450 2c/RLM5 antibody strongly immunoprecipitated P-450 RLM3 as well as P-450 2c/RLM5 and to a lesser extent P-450 PB-B and P-450 LM2. The MAbs that precipitated P-450 2c/RLM5 also inhibited by more than 90% androstenedione 16α-hydroxylase activity of untreated rat microsomes, but did not inhibit microsomal 6β- or 7α-hydroxylation. In addition, complete inhibition of both androstenedione 16α-hydroxylation and testosterone 16α-hydroxylation was observed in a reconstituted system with P-450 2c/RLM5. Androstenedione 6β-hydroxylation catalyzed by P-450 2c/RLM5 was also inhibited, whereas P-450 3-catalyzed 7α-hydroxylation was not inhibited by the MAbs. P-450 2c/RLM5 catalyzed 2α-, 16α- and 6β-hydroxylation of progesterone in a reconstituted system were also inhibited by the MAb by 60–80%. These MAbs should prove useful for "reaction phenotyping," i.e. for defining the contribution of microsomal P-450 2c/RLM5 to the oxidative metabolism of endogenous steroids and other P-450 substrates in animal and human tissues.

Cytochrome P-450 (P-450)‖ is the key component of the mixed-function oxidases which metabolize numerous drugs, carcinogens and other xenobiotics, as well as endobiotics such as prostaglandins, fatty acids and steroids [1–3]. There is a large multiplicity of P-450 forms, and many of these are induced by the administration of a variety of inducers [1–4]. P-450s are also expressed constitutively and some are developmentally regulated and age dependent [5–7]. The purification and characterization of some individual forms of cytochrome P-450 have clarified their function and role in the metabolism of different xenobiotics and endobiotics. The purification procedures, however, are cumbersome and slow, generally inapplicable to minor P-450 forms, and the reliability of the reconstitution system in which the P-450s are apart from their natural membrane environment is unknown. We have developed a powerful approach to the study of P-450 multiplicity by using monoclonal antibodies (MAbs) that are directed to epitope specific cytochromes P-450. These MAbs can be used as probes for the detection and immunopurification of the epitope specific cytochrome P-450. Futhermore, MAbs that inhibit cytochrome P-450 enzyme activity can be used to "reaction phenotype," i.e. to determine the contribution of the MAb epitope specific P-450 to the total metabolism of a substrate in any tissue preparation. We have prepared a number of MAbs to different P-450s [8–15] and have used them for quantitative detection of P-450s by radioimmunoassay [16–18], P-450 immunopurification [19–22], and reaction phenotyping by inhibition of enzyme activity [23–25]. This paper reports the preparation and characterization of MAbs to cytochrome P-450 2c/RLM5 [26–28], a male specific form of P-450 [5] active in the metabolism of steroid hormones as well as certain xenobiotics. Several of these MAbs to P-450 2c/RLM5 were found to inhibit steroid metabolism regiospecifically and thus will be useful in

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‖ Abbreviations: P-450, cytochrome P-450; specific cytochrome P-450 forms and corresponding genes (where known) are designated as P-450 RLM3 and P-450 2c/RLM5 (gene IIC11) for untreated liver microsomal P-450 form 3 and 5; P-450 PB-B (gene IIB1) and P-450 BNF-B (gene IA1), phenobarbital- and β-naphthoflavone-induced P-450 from rats; P-450 LM2, phenobarbital-inducible liver microsomal P-450 of rabbits; P-450 LM4, β-naphthoflavone inducible liver microsomal cytochrome P-450 of rabbits; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; and CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate.

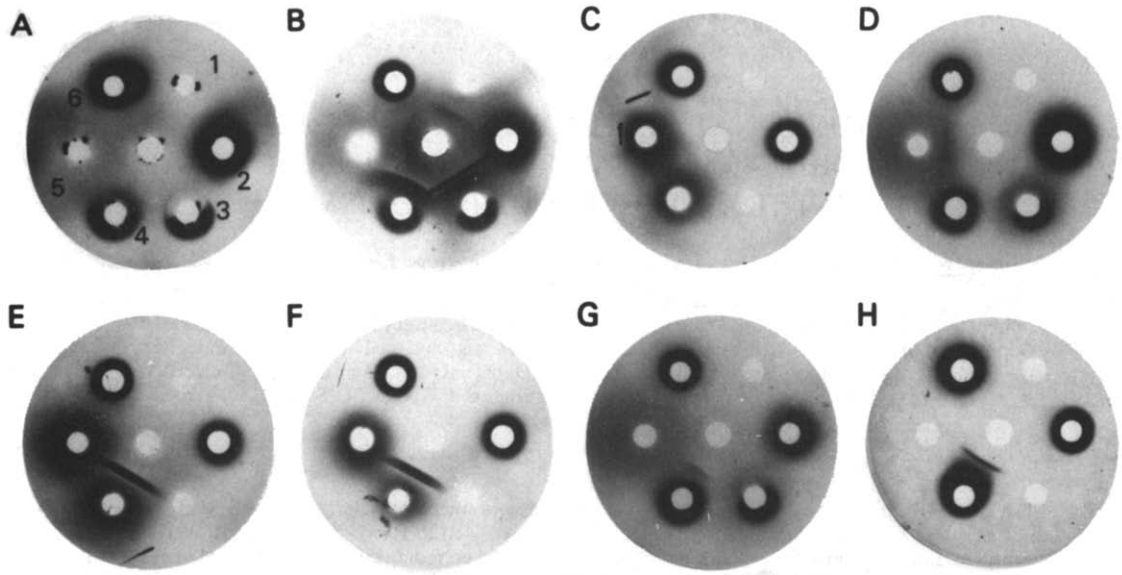

Fig. 1. Double-immunodiffusion analysis of the specificity of monoclonal and polyclonal antibodies for purified cytochrome P-450 2c/RLM5. Immunodiffusion plates were incubated for 5 days at room temperature, stained with Coomassie blue, and destained in glacial acetic acid/methyl alcohol/H_2O (by vol., 1:3:6) solution. Center wells of panels A–H, respectively, contained 20 μl of non-immunized normal mouse serum, serum polyclonal antibody to cytochrome P-450 2c/RLM5, ascites fluids of myeloma P3x63 (43.0 mg/ml), serum-free culture fluid concentrates (CFS^-) of nonspecific MAb NBS 1-48-5 (4.4 mg/ml), specific MAbs to P-450 2c/RLM5, MAb 1-68-2 (1.85 mg/ml), MAb 1-68-11 (1.75 mg/ml), and MAb 1-68-14 (1.95 mg/ml), and culture fluid concentrate containing MAb 1-126-1 (50 mg/ml). Outer wells, 1–6 of panels A–H, respectively, contained 20 μl of the following purified cytochromes P-450: P-450 PB-B (0.36 mg/ml), P-450 BNF-B (1.5 mg/ml), P-450 RLM3 (0.91 mg/ml), P-450 2c/RLM5 (0.73 mg/ml) of rats, and P-450 LM2 (1.6 mg/ml) and P-450 LM4 (1.28 mg/ml) of rabbits.

defining the specificity and contribution of P-450 2c/RLM5 to steroid metabolism in animal tissues.

MATERIALS AND METHODS

Preparation of microsomes and cytochrome P-450. Microsomes were prepared from the livers of adult male rats (230–260 g). Cytochromes P-450 RLM3 and P-450 RLM5 were purified from Emulgen 911 solubilized microsomes as previously described [26]. Cytochrome P-450 2c, which is equivalent to cytochrome P-450 RLM5 [29], was prepared as previously reported and used in the reconstitution experiments described in this report [5, 28]. The designation cytochrome P-450 2c/RLM5 is used here when referring to these equivalent forms. Rat cytochrome P-450 PB-B and P-450 BNF-B were supplied by Dr F. P. Guengerich and rabbit cytochrome P-450 LM2 and P-450 LM4 were supplied by Dr M. Coon.

Immunization of mice and production of hybridomas. Female Balb/c mice were immunized by i.p. injection weekly for 4 weeks with 10 μg P-450 2c/RLM5 emulsified in 0.2 ml Freund's complete adjuvent (Miles). To enhance the immune response, the mice were injected i.p. with the antigen in Dulbecco's phosphate-buffered saline (PBS, pH 7.4) 3–4 days before they were killed. Five mice exhibiting immune sera that bound to P-450 2c/RLM5 at levels ten to fifteen times higher than normal sera (Table 1; determined after a 100-fold dilution of each serum) were used as the donors of spleen cells for the hybridization. The spleen cells were removed and hybridized with myeloma SP2/0 cells using polyethylene glycol 1500 (50%; Boehringer–Mannheim). Hybridomas were selected in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine calf serum, 10% horse serum, 10 μM hypoxanthine (H), 0.4 μM aminopterin (A) and 16 μM thymidine (T) (Hat medium), and screened for MAb producing cells by RIA. The hybrid cells were cloned three times to ensure their monoclonality. The final clones were grown in medium (HT medium) in which only aminopterin was depleted. These cells were used for i.p. inoculation of Balb/c female mice to obtain ascites fluid containing the desired MAbs or were grown in serum-free culture medium to obtain serum-free culture concentrates as the source of MAbs. Details of these procedures were described previously [10, 11]. Where indicated, IgMs were purified to homogeneity from the ascites fluid as described [32].

Radioimmunoassay (RIA). RIA was adopted for the screening of MAb producing hybridomas as well as for the determination of MAbs binding specificities. Hybridoma culture fluids, ascites fluid or purified MAbs were incubated at room temperature for 2 hr in microtiter plates (Costar) which were precoated with different P-450s (10 μg/well) overnight at 4°. The wells were then filled with 3% bovine serum albumin/2% sodium azide/PBS and incubated for 30 min. The microtiter wells were washed three

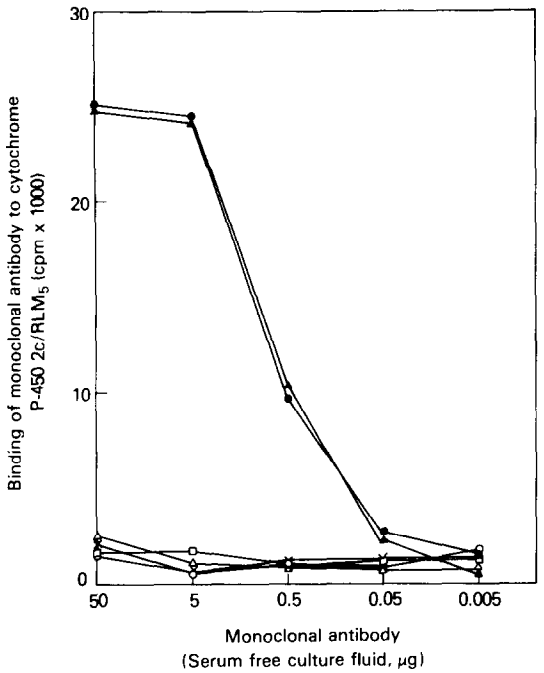

Fig. 2. Binding specificity (RIA) of MAb 1-68-14 for cytochrome P-450 2c/RLM5 and other forms of cytochrome P-450. Cytochromes P-450 2c/RLM5 (●), P-450 RLM3 (▲), P-450 PB-B (×), P-450 BNF-B (□), P-450 LM2 (○), and P-450 LM4 (△) were placed in 96-well microtiter plates (10 μg/well) and incubated at 4° overnight. Unbound P-450s were removed by washing; MAbs were added to each well at the indicated concentrations and then incubated at room temperature for 2 hr. Bound MAbs were then measured by the binding of ^{35}S-methionine labeled rat anti-mouse IgG1(κ) in the solid phase RIA.

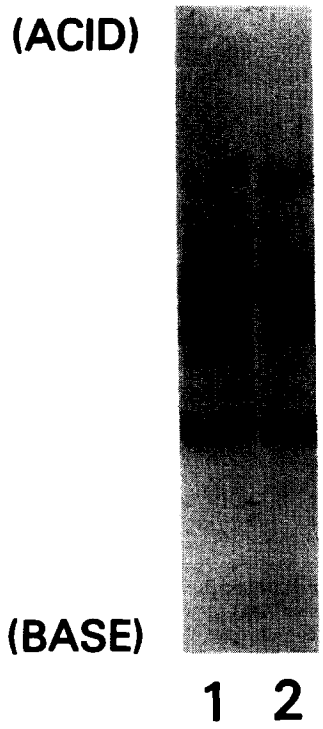

Fig. 3. Isoelectric focusing analysis of purified MAbs 1-68-11 and 1-68-14. The two purified IgMs were electrophoresed in isoelectric focusing gels, and then protein bands were visualized by Coomassie blue staining.

times with PBS and incubated overnight at 4° with ^{35}S-methionine-labeled rat anti-mouse κ chain [11]. The binding of MAbs to P-450s was detected by the radioactively labeled second antibody remaining bound to the MAbs after washing five times with PBS.

Double-immunodiffusion analysis. The Ouchterlony double-immunodiffusion assay was utilized to determine the presence and nature of antibodies in the mouse sera, culture fluids, and ascites fluids. Various antigens or antibody solutions (20-μl samples) were placed in 1% agarose disc gel wells, incubated at room temperature, and observed for precipitin bands, which appeared after different incubation periods. For a clear detection of precipitin bands, the disc gel was removed from the plate and stained as previously described [11].

Effect of MAbs on enzyme activities of liver microsomes and reconstituted P-450 systems. Hydroxylation of androstenedione and progesterone catalyzed by either microsomes or reconstituted cytochrome P-450 systems were assayed using radiolabeled steroid substrates with product analysis by thin-layer chromatography as previously described [5, 28]. The reconstituted system containing cytochrome P-450 2c/RLM5 (12 pmol), NADPH–cytochrome P-450 reductase (35 pmol) and dilauroylphosphatidylcholine (1 μg) was preincubated with MAbs in buffer containing 10 nmol of ^{14}C-labeled steroid in a total volume of 0.05 ml for 4 min at 20–22°. Samples were then diluted with 0.1 M KP_i (pH 7.4), 0.1 mM EDTA, warmed to 37° for 4 min, and 0.3 mM NADPH was added to initiate the reaction (final volume, 0.4 ml). The catalytic activities in the absence of monoclonal antibodies (control) corresponded to the following values (expressed as nmol of androstenedione (A) or progesterone (P) metabolite formed/min/nmol P-450): for P-450 2c/RLM5 in Fig. 4A: 16α-OH-A = 10.2, 6β-OH-A = 1.18, and secondary metabolite of A = 1.90; for P-450 2c/RLM5 in Fig. 4B: 16α-OH-A = 6.1 and 6 β-OH-A = 0.7; for P-450 3 in Fig. 4B: 7α-OH-A = 5.9; for P-450 2c/RLM5 in Fig. 5A and 5B: 2α-OH-P = 8.4, 16α-OH-P = 4.4, and 6β-OH-P = 0.5. For microsomal assays, rat liver microsomes (40 μg) were preincubated with MAbs for 45 min at 20–22° in the presence of 10 nmol ^{14}C-labeled steroid in a total volume of 0.05 ml. Buffer and NADPH (1 mM) were then added, and steroid hydroxylation was assayed as in the reconstituted system.

Isoelectric focusing. Isoelectric focusing of purified MAbs was performed in a thin polyacrylamide gel (ampholyte, pH range 3.5 to 9.5) containing 10 M urea and 2% CHAPS detergent at 800 V for 4 hr at 37°. The gel was fixed with 4.4% (w/v) sulfosalicylic acid, 14.7% (w/v) trichloroacetic acid and then stained with Coomassie Blue G250 (Sigma Chemical Co., St Louis, MO) to visualize the protein bands.

Table 1. Characteristics of monoclonal antibodies to rat cytochrome P-450 2c/RLM5

Source of antibodies	Ig subclass	Binding to P-450 2c/RLM5 (RIA, cpm)	Immunoprecipitation by double-immunodiffusion					
			P-450 form					
			PB-B	BNF-B	RLM3	2c/RLM5	LM2	LM4
Normal mouse serum		845	–	–	–	–	–	–
Mouse serum antibody to P-450 2c/RLM5		8,450–10,055	+	–	++++	+++	+	–
Monoclonal antibodies*								
Nonspecific								
Myeloma P3x63	IgG1(κ)	920	–	–	–	–	–	–
NBS 1-48-5p25	IgG2a(κ)		–	–	–	–	–	–
Made to P-450 2c/RLM5								
1-68-2p3	IgM(κ)	5,420	–	–	–	+++	–	–
1-68-11p3	IgM(κ)	5,750	–	–	–	+++	–	–
1-68-14p3	IgM(κ)	5,990	–	–	–	+	–	–
1-86-4p3	IgM(κ)	4,700	–	–	–	–	–	–
1-96-1p3	IgM(κ)	2,440	–	–	–	–	–	–
1-126-1p3	IgM(κ)	4,545	–	–	–	+++	–	–

* Serum-free culture fluids were used as the source of MAbs for mouse immunoglobulin subclass typing and double immunodiffusion, and serum plus culture fluids were used for binding to cytochrome P-450 2c/RLM5 in RIA. In solid phase RIA, cytochrome P-450 2c/RLM5 (10 μg) was precoated with monoclonal antibodies, and their binding was quantitated with ^{35}S-methionine-labeled rat anti-mouse IgG1(κ) (57,160 cpm/18 μg/well) as described in Materials and Methods.

RESULTS

Identification and characterization of MAbs. Hybridization of spleen cells (1×10^8) from immunized mice with myeloma cells (1×10^7) yielded hybrid cells in 129 wells. Nineteen of these wells contained antibodies to P-450 2c/RLM5 that were RIA-positive at levels two to six times higher than the nonspecific MAbs [IgG1(κ)] produced by the P3×63 myeloma cells. The characteristics of six clones originating from four of the 19 wells and producing MAbs to P-450 2c/RLM5 after three consecutive clonings are shown in Table 1. The nomenclature used for the hybrid clones and mouse immunoglobin (Ig) classification has been described in a previous paper [11]. The first number of the hybrid clone indicates the experiment number; second, well number; third, clone number; and p, number of passages.

Specificity of MAbs. Six MAbs were tested by immunoprecipitin and binding reactions (RIA) for their specificity towards the constitutive P-450 forms RLM3 and 2c/RLM5 and the major xenobiotic-inducible rat and rabbit P-450 forms designated PB-B, BNF-B, LM2 and LM4 respectively [30, 31]. As shown in Table 1 and Fig. 1, normal mouse serum gave no reaction with the six purified cytochromes P-450 (Fig. 1A). Polyclonal antibodies to P-450 2c/RLM5 showed strong reactions with both P-450 RLM3 and P-450 2c/RLM5 and to a lesser extent with P-450 PB-B and P-450 LM2. Precipitin reactions were not observed with P-450 BNF-B or P-450 LM4 (Fig. 1B). The MAb contained in serum-free culture fluids, MAbs 1-68-2 (Fig. 1E), 1-68-11 (Fig. 1F), 1-68-14) (Fig. 1G) and 1-126-1 (Fig. 1H), all showed positive reactions with P-450 2c/RLM5 but no reaction with P-450 RLM3. The four MAbs to P-450 2c/RLM5 did not cross-react with any of the other four P-450 preparations: rat P-450s PB-B and BNF-B, and rabbit P-450s LM2 and LM4 (Table 1, Fig. 1E, Fig. 1F, Fig. 1G, Fig. 1H). Nonspecific MAbs P3x63 (Fig. 1C) and NBS 1-48-5 (Fig. 1D) also did not exhibit reactions with any of the six P-450 preparations.

The specificity of one of the MAbs to P-450 2c/RLM5, MAb 1-68-14, was examined further by indirect solid phase RIA (Fig. 2). The binding specificity was similar to that observed for P-450 RLM3, another constitutive form of rat P-450. The results indicate that P-450 RLM3 and P-450 RLM5 share a common epitope recognized by MAb 1-68-14. The immunoprecipitin reaction, however, by MAb 1-68-14 and three other MAbs to P-450 2c/RLM5 was strong with 2c/RLM5 but negative with P-450 RLM3. Thus, although the two P-450s share a common epitope detected by MAb 1-68-14, they have other unique structural features which cause them to respond differently in immunoprecipitin reactions.

Since MAb 1-68-11 and MAb 1-68-14 originated from the same hybridization well and behaved similarly in the above analyses, we purified them to apparent homogeneity. For further study they were electrofocussed in polyacrylamide gels in the presence of 10 M urea and 2% CHAPS. This revealed indistinguishable patterns of MAb peptide bands (Fig. 3), suggesting that these two MAbs may be identical or very similar.

Effects of MAbs on steroid metabolism. The effects of MAb 1-68-11 and MAb 1-68-14 on steroid metabolism are shown in Tables 2 and 3 and Figs 4 and 5. MAb 1-68-11 specifically inhibited liver microsomal androstenedione 16α-hydroxylase activity almost completely (~90%) at a level of 0.05 mg purified

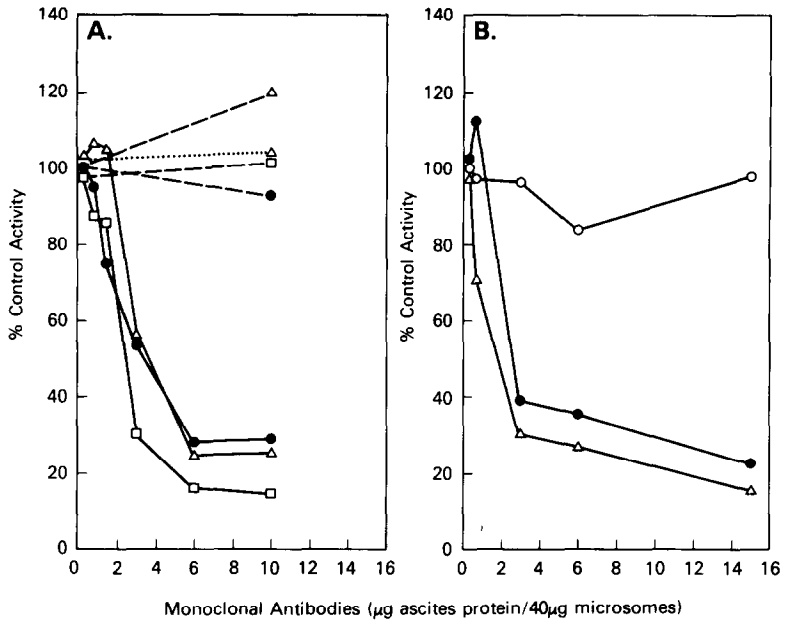

Fig. 4. MAb inhibition of androstenedione metabolism catalyzed by purified, reconstituted P-450 2c/RLM5. P-450 2c/RLM5 was reconstituted as described under Materials and Methods, and then preincubated with MAb 4-29-5 (. . . . .), MAb 1-7-1 (---) or MAb 1-68-11 (—) (panel A) and MAb 1-68-14 (—) (panel B) in the presence of ^{14}C-labeled androstenedione. The hydroxylation products of androstenedione at the 6β- (●)- and 16α- (△)-positions, as well as an unidentified polar compound, probably corresponding to a secondary metabolite (-□-), were analyzed by thin-layer chromatography as reported under Materials and Methods. These MAbs did not inhibit androstenedione 7α-hydroxylation catalyzed by reconstituted P-450 3 (panel B, -○-). The secondary metabolite was not quantitated in the experiment shown in panel B. See Materials and Methods for control activity values.

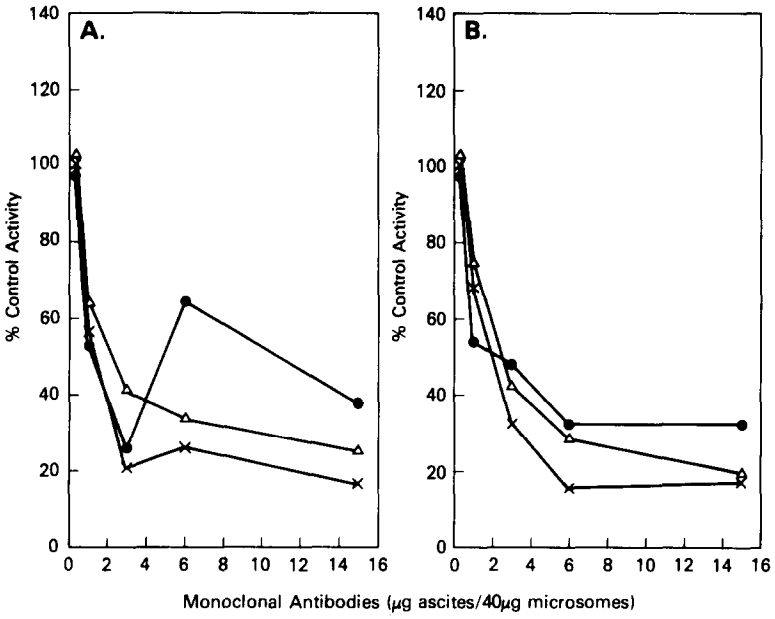

Fig. 5. MAb inhibition of progesterone metabolism in the reconstituted systems. Purified and reconstituted P-450 2c/RLM5 was preincubated with MAb 1-68-11 (panel A) or MAb 1-68-14 (panel B) in the presence of ^{14}C-labeled progesterone. The hydroxylation products of progesterone, 2α-hydroxy (×)-, 6β-hydroxy (●)- and 16α-hydroxy (△) progesterone were analyzed by thin-layer chromatography. See Materials and Methods for control activity values.

Table 2. Selective inhibition of microsomal androstenedione 16α-hydroxylase activity by purified MAb 1-68-11 to cytochrome P-450 2c/RLM5

IgM (μg)	Hydroxylase activity (nmol metabolite/min/mg microsomes)					
	16α-OH-A	(%)	6β-OH-A	(%)	7α-OH-A	(%)
0	0.84	100	0.66	100	0.12	100
0.5	0.58	69	0.75	114	0.11	92
1.0	0.17	20	0.76	115	0.10	83
2.0	0.10	12	0.71	108	0.15	125

Liver microsomes (40 μg) isolated from untreated adult male Fisher 344 rats were preincubated with 0.5 to 2.0 μg purified MAb 1-68-11p3 for 45 min at 22–23° in the presence of 10 nmol ^{14}C-labeled androstenedione (50 μl to total volume). Buffer and NADPH (1 mM) were then added, and androstenedione hydroxylation was assayed as described in Materials and Methods.

MAb/mg microsomal protein. The same concentration of MAb had essentially no effect on microsomal androstenedione 6β- and 7α-hydroxylase activities (Table 2). Thus, microsomal androstenedione 16α-hydroxylation was specifically and almost entirely catalyzed by MAb 1-68-11 immunosensitive P-450 forms. These findings are consistent with the previous demonstration by one of our laboratories that P-450 2c/RLM5 is the major form of cytochrome P-450 responsible for microsomal 16α-hydroxylation of androstenedione in uninduced adult male rats [5], with immunoreactive P-450 2a and P-450 3 catalyzing the majority of microsomal androstenedione 6β- and 7α-hydroxylation, respectively [6]. When P-450 2c/RLM5 was reconstituted with NADPH–cytochrome P-450 reductase and dilauroylphosphatidylcholine, MAb 1-68-11 and MAb 1-68-14 markedly inhibited 6β- and 16α-hydroxylation of androstenedione as well as formation of an unknown polar metabolite, presumed to be a secondary product (Fig. 4A). In contrast, androstenedione 7α-hydroxylation catalyzed by purified P-450 3 was not inhibited (Fig. 4B). MAb 1-7-1 which is reactive toward P-450 BNF-B [10] and MAb 4-29-5 directed toward P-450 PB-B [11] had no effect on the P-450 2c/RLM5 catalyzed hydroxylation of androstenedione (Fig. 4A), demonstrating that the MAbs reported here were specific for P-450 2c/RLM5 catalyzed steroid hydroxylation. In a preliminary study, strong inhibition by MAb 1-68-11 of P-450 2c/RLM5 catalyzed 16α-hydroxylation and 2α-hydroxylation of testosterone was observed [33].

We also examined the effects of MAb 1-68-11 and MAb 1-68-14 on P-450 2c/RLM5 catalyzed progesterone metabolism (Fig. 5). The formation of the metabolites, 2α-, 6β- and 16α-hydroxyprogesterone, was inhibited markedly by both MAbs.

DISCUSSION

Cytochrome P-450 2c/RLM5 is a constitutive, male-specific P-450 form that metabolizes a variety of steroid hormones [29]. This P-450 has been purified to homogeneity and shown to be distinct from other constitutive forms active in steroid hormone metabolism [5, 27, 34, 35]. We have prepared MAbs to different forms of P-450 to study the multiplicity of P-450. Inhibitory MAbs [8–15] have been used for "reaction phenotyping," i.e. determining the contribution of specific P-450s to a reaction occurring in the presence of multiple P-450s such as in microsomes [23–25]. The non-inhibitory as well as inhibitory MAbs have been used for the quantitative analysis of P-450s by RIA [16–18], immunoblotting [13–15], and simple immunopurification [19–22] of the P-450s. Cytochrome P-450 2c/RLM5 catalyzed metabolism of steroid hormones was specifically inhibited by MAbs prepared to P-450 2c/RLM5 but not by others made to P-450 PB-B or P-450 BNF-B. MAbs to P-450 2c/RLM5 were not inhibitory to P-450 PB-B or to P-450 3 catalyzed steroid hormone metabolism [35], indicating that the MAbs reported here do not recognize these other P-450 forms. The possible reaction of these antibodies with other members of P-450 subfamily IIC in addition to P-450 RLM3 (Fig. 2) and P-450 PB-1 (IIC6) [35] remains to be determined. Although microsomal androstenedione 6β-hydroxylation was not very inhibited by the MAbs examined in this study, hydroxylation of that same site catalyzed by purified P-450 2c/RLM5 was inhibited markedly. This result is consistent with the previous demonstration that immunoreactive P-450 2a (and not P-450 2c/RLM5) is the major P-450 catalyst of microsomal androstenedione 6β-hydroxylation [6]. The use of this MAb in this study was a good example which proves that an inhibitory MAb can determine the role of a given P-450 in microsomal metabolism of a substrate. The 16α-hydroxylation reactions of androstenedione and testosterone were inhibited almost completely in both microsomal and reconstituted systems, indicating that P-450 2c/RLM5 is the major P-450 catalyst of these reactions in liver microsomes (Tables 2 and 3), in agreement with earlier reports [5, 33]. This finding demonstrates the unique usefulness of MAbs for determining individual P-450 contribution to microsomal metabolism. It was reported previously that purified P-450 RLM3 forms 6β- and 7α-testosterone and 6β- and 16α-hydroxyprogesterone as major products and 15β-hydroxyprogesterone as a minor product. In contrast, P-450 2c/RLM5 forms 2α- and 16α-hydroxytestosterone and 2α- and 16α-hydroxyprogesterone as major products and 6β-

Table 3. Effect of anti-P-450 MAbs on P-450 2c/RLM5-catalyzed androgen 16α-hydroxylation

Source of MAbs	Antigen	MAb (μg)	Hydroxylase activity (nmol metabolite/min/nmol P-450) 16α-Testosterone	%	16α-androstenedione	%
None			3.43	100	10.2	100
MAb 1-7-1p4	P-450 BNF-B	10	3.24	94	12.5	123
MAb 4-29-5p4	P-450 PB-B	10	ND*		10.2	100
MAb 1-68-11p3	P-450 2c/RLM5	2	1.50	44	6.0	59
		10	0.46	13	2.7	26
		25	0.17	5	0.5	5

P-450 2c/RLM5 (12 pmol) was reconstituted with NADPH–cytochrome P-450 reductase (35 pmol) and dilauroyl phosphatidylcholine (1 μg), and then preincubated with the indicated MAbs in buffer containing 10 nmol of ^{14}C-labeled steroid substrate in a total volume of 60 μl for 45 min at 20–22°. The 16α-hydroxylation reactions of testosterone and of androstenedione were then determined as described under Materials and Methods.

* Not determined.

hydroxytestosterone and 6β-progesterone as minor products [5, 27, 28, 34]. In the presence of MAbs to 2c/RLM5, the formation of 2α- and 16α-hydroxytestosterone [33] and 6β- and 16α-hydroxyprogesterone (Fig. 5) was strongly inhibited. However, microsomal 7α-hydroxylation of testosterone, which is not catalyzed by P-450 2c/RLM5, was not affected. This MAb inhibition demonstrates again the high specificity of P-450 2c/RLM5.

Four MAbs to P-450 2c/RLM5 clearly distinguished rat P-450 2c/RLM5 from P-450 RLM3 by immunoprecipitin formation but not by RIA. However, the MAbs clearly distinguished P-450 2c/RLM5 from rat P-450 PB-B and P-450 BNF-B, and from rabbit P-450 LM2 and LM4 using both immunoprecipitin reaction and RIA. In contrast, polyclonal antibodies to P-450 2c/RLM5 not only failed to distinguish P-450 2c/RLM5 from P-450 RLM3 but also showed some cross-reactivity with rat P-450 PB-B and rabbit P-450 LM2. Thus, as is well established, MAbs are far more specific than polyclonal antibodies, although the MAbs were not able to distinguish P-450 2c/RLM5 from RLM3 by RIA, indicating that these two P-450 forms share at least one common epitope. Structural features other than at the epitope site are clearly unique to the two forms of P-450 and may be the cause of differences in immunoprecipitation. Immunological cross-reactivities amongst constitutive rat P-450 forms have been observed previously [5, 7, 36]. Other laboratories have reported non-inhibitory MAbs to steroid hormone metabolizing P-450 [37, 38]. The MAbs reported in this paper can be used for determining the contribution of immunoreactive cytochrome P-450 2c/RLM5 to position specific metabolism of steroids in any tissues.

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