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MONOCLONAL ANTIBODIES TO PHENOBARBITAL-INDUCED RAT LIVER CYTOCHROME P-450

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Abstract—Somatic cell hybrids were made between mouse myeloma cells and spleen cells derived from BALB/c female mice immunized with purified phenobarbital-induced rat liver cytochrome P-450 (PB-P-450). Hybridomas were selected in HAT medium, and the monoclonal antibodies (MAbs) produced were screened for binding to the PB-P-450 by radioimmunoassay, for immunoprecipitation of the PB-P-450, and for inhibition of PB-P-450-catalyzed enzyme activity. In two experiments, MAbs of the IgM and IgG1 were produced that bound and, in certain cases, precipitated PB-P-450. None of these MAbs, however, inhibited the PB-P-450-dependent aryl hydrocarbon hydroxylase (AHH) activity. In two other experiments, MAbs to PB-P-450 were produced that bound, precipitated and, in several cases, strongly or completely inhibited the AHH and 7-ethoxycoumarin deethylase (ECD) activities of PB-P-450. These MAbs showed no activity toward the purified 3-methylcholanthrene-induced cytochrome P-450 (MC-P-450), β-naphthoflavone-induced cytochrome P-450 (BNF-P-450) or pregnenolone 16-α-carbonitrile-induced cytochrome P-450 (PCN-P-450) in respect to RIA determined binding, immunoprecipitation, or inhibition of AHH activity. One of the monoclonal antibodies, MAb 2-66-3, inhibited the AHH activity of liver microsomes from PB-treated rats by 43% but did not inhibit the AHH activity of liver microsomes from control, BNF-, or MC-treated rats. The MAb 2-66-3 also inhibited ECD in microsomes from PB-treated rats by 22%. The MAb 2-66-3 showed high cross-reactivity for binding, immunoprecipitation and inhibition of enzyme activity of PB-induced cytochrome P-450 from rabbit liver (PB-P-450$_{LM2}$). Two other MAbs, 4-7-1 and 4-29-5, completely inhibited the AHH of the purified PB-P-450. MAbs to different cytochromes P-450 will be of extraordinary usefulness for a variety of studies including phenotyping of individuals, species, and tissues and for the genetic analysis of P-450s as well as for the direct assay, purification, and structure determination of various cytochromes P-450.

Cytochrome P-450 exists in multiple forms and is a key component of the mixed-function oxidase system. This enzyme system catalyzes the oxidation of drugs and other xenobiotics including mutagens and carcinogens, as well as certain endogenous substrates such as fatty acids, prostaglandins and steroids [1, 2]. Recent studies of highly purified cytochromes P-450 [3–11] have demonstrated a multiplicity of cytochrome P-450 isozymes, which differ in subunit molecular weights, spectral properties, electrophoretic mobilities, substrates and inducer specificities [1, 3–12], immunogenic properties [1, 6, 7, 9, 10, 13, 14], peptide and partial amino acid sequence analysis [1, 6, 10, 11], and product regiospecificities [10, 15–17]. Drugs and xenobiotics are detoxified as well as activated to toxic metabolites, mutagens and carcinogens [18, 19] by the cytochrome P-450 mixed-function oxidases. Specific forms of cytochrome P-450 may direct substrate flow into alternative metabolic pathways and thus regulate the interactions between environmental chemicals and the individual [20–27]. Monoclonal antibodies (MAbs§) are made by hybridomas formed from myeloma cells fused with single spleen cells previously primed for a specific antigenic determinant. Thus, MAbs are pure reagents specific for single epitopes on the antigen, and the hybridomas producing them are potentially immortal [28]. We have reported previously preparations of panels of MAbs to two rabbit cytochromes P-450 [29, 30] and an MC-induced P-450 from rat liver [31]. The latter MAb identifies a type of P-450 present in the tissues of humans [32] as well as those from mouse, guinea pig

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§ Abbreviations: MAb, monoclonal antibody; BP, benzo[*a*]pyrene; PB, phenobarbital; MC, 3-methylcholanthrene; BNF, β-naphthoflavone; PCN, pregnenolone-16-α-carbonitrile; PB-P-450, MC-P-450, BNF-P-450, and PCN-P-450, liver microsomal cytochrome P-450 of rats treated with PB, MC, BNF, or PCN; P-450$_{LM2}$ and P-450$_{LM4}$, different forms of cytochrome P-450 from rabbit liver; HL-450, cytochrome P-450 from human liver; HAT medium, Dulbecco's modified Eagle's medium with 25 mM glucose and 4 mM glutamine, supplemented with 10% fetal calf serum, 10% horse serum, 50 μg gentamicin per ml, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine; RIA, radioimmunoassay; 3-OH-BP and 9-OH-BP, 3-hydroxy and 9-hydroxy benzo[*a*]pyrene; PBS, phosphate-buffered saline; NBS, hybrid cells from myeloma cells and spleen from unimmunized normal BALB/c mice; AHH, aryl hydrocarbon hydroxylase; ECD, 7-ethoxycoumarin deethylase; and HPLC, high-pressure liquid chromatography.

and hamster (T. Fujino, D. West, A. K. Radkowsky, S. S. Park, and H. V. Gelboin, manuscript in preparation). In this study we report the preparation and properties of MAbs to a phenobarbital-induced P-450 of rat liver. Several of the MAbs immunoprecipitate and strongly inhibit the AHH and ECD activities of PB-P-450. One of the latter monoclonal antibodies also inhibits the enzyme activity of cytochrome $P\text{-}450_{LM2}$, the predominant form in liver from rabbits treated with phenobarbital [4]. In addition, we have prepared panels of other MAbs that either bind and immunoprecipitate rat liver PB-P-450, or only bind the rat liver PB-P-450. Thus, we have now accumulated panels of MAbs to different forms of cytochrome P-450. We believe that these MAbs will be of extraordinary usefulness in the purification of cytochromes P-450 as well as in studies of the multiplicity, substrate and product specificity, and the genetics of cytochrome P-450.

MATERIALS AND METHODS

Preparation of cytochrome P-450 and microsomes. The liver microsomal cytochromes P-450 from rats treated with phenobarbital (PB-P-450), 3-methylcholanthrene (MC-P-450), and β-naphthoflavone (BNF-P-450) were purified as described [5, 10]. The "B_2" fractions were used as antigens to prepare the MAbs as well as for immunoprecipitation and enzyme assays. NADPH-cytochrome P-450 reductase was prepared as described [5].

Media and cells. Dulbecco's modified Eagle's medium, fetal calf serum and horse serum were purchased from the Grand Island Biological Co, Grand Island, NY. The myeloma cell lines NS-1 and X63Ag8.653, which were azaguanine resistant and nonproducers of immunoglobin, were obtained from Dr. John D. Minna and from the Human Genetic Mutant Cell Repository, Institute for Medical Research, New Jersey. They were grown in Dulbecco's modified Eagle's medium with 25 mM glucose and 4 mM glutamine, and supplemented with 10% fetal calf serum, 10% horse serum and 50 μg gentamicin per ml (complete medium). Mouse spleen-myeloma hybridomas were grown in the complete medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT medium) [33]. Dulbecco's modified Eagle's medium with 5.6 mM glucose plus 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, pH 7.4, was used as washing medium in the preparations of the hybridomas [30, 31].

Immunization of mice and preparation of mouse sera. Ten-week old female BALB/c mice were immunized by intraperitioneal injections every week for 4 weeks with 10 μg of purified PB-P-450 emulsified in 0.2 ml of Freund's complete adjuvant (Miles). The following week they were further immunized by an i.v. injection of 10 μg of PB-P-450 in Dulbecco's phosphate-buffered saline, pH 7.4 (PBS). Three days after the i.v. injection, blood samples were obtained from the tails of mice and tested for antibody production. After confirmation of antibody production, the mice were kept 1 month without immunization, followed by a second series of immunizations with an i.p. injection of 10 μg PB-P-450 in complete adjuvant and an i.v. injection in PBS. Three days after the i.v. booster injection, five mice were killed in a bag containing dry ice, and the spleen cells were isolated. Blood was collected by heart puncture to obtain sera from the immunized mice. The blood was stored in a 15-ml conical centrifuge tube at room temperature for 1 hr and then at 4° overnight and was spun at 1000 *g* for 30 min to obtain the clear serum. The sera were stored at −90°.

Production of hybridoma cells. The fusion of the myeloma cells with spleen cells was carried out essentially as described [28, 30, 31], except that polyethylene glycol 1000 was used for the fusion [34] and X63Ag8.653 [35] or SP2/OAg14 [36] was used for the fusions in addition to NS-1 [37] which we used in the previous reports [29–31].

Preparation of MAbs in culture fluids and mouse peritoneal ascites fluid. Hybrid cells producing antibodies were grown in flasks containing complete medium plus hypoxanthine and thymidine (HT medium) and then transferred to serum-free medium. The culture fluids were cleared by centrifugation and filtration through a 0.45 μm filter and concentration with Amicon filter 15. Alternatively, cells were collected by centrifugation, resuspended in PBS, and inoculated into female BALB/c mice i.p. (5×10^6 cells in 0.2 ml of PBS); ascites fluids were collected with a syringe or by puncturing the abdomen after 2–3 weeks and were clarified as described above for serum.

Measurement of protein concentration. Protein concentrations in culture fluids and in ascites fluid were measured by the method of Lowry *et al.* [38].

Radioimmunoassay. ^{125}I-labeled anti-mouse IgG [specific for heavy and light chain or ^{125}I-labeled $F(ab')_2$ fragments of anti-mouse IgG (50 μCi/μg protein per 0.5 ml; Amersham] was used for the detection of MAbs and in studies of binding to PB-P-450. [^{35}S]Methionine rat anti-mouse IgG(k) MAbs labeled *in vitro* were specific to the k chain, were produced by hybridoma (cell line 187.1) between MPC-11 (IgG2b,k) primed rat spleen cells and nonproducing myeloma cells (X63Ag8.653), and were used in some binding studies [39]. The details of the solid phase RIA are described in previous reports [30, 31].

Double-immunodiffusion analysis. The Ouchterlony double-immunodiffusion technique was utilized to measure the presence and nature of the antibodies in the mouse sera, culture fluids, and ascites fluids. Various antigen or antibody solutions (20 μl samples) were placed in disc gel wells (Cappel), incubated at room temperature, and observed for precipitin bands, which appeared in 2–3 days. For some precipitin bands the disc gels were removed from the plates and placed in 0.85% saline for 2 days, in deionized water for 1 day, and stained for 30 min with 0.3% Coomassie blue in 30% methanol and 10% acetic acid solution. The gels were destained until the precipitin bands were clearly visible above background.

Measurement of the effect of MAbs on cytochrome P-450 enzyme activity. AHH activity was determined by measuring the amount of BP conversion to phenolic products equivalent to 3-OH-BP [40]. The reaction mixture in the reconstituted mixed-function

oxidase system contained 250 μl of 0.2 M Tris–HCl (pH 7.6), 30 μl dilauroylglyceryl-3-phosphorylcholine (1 mg/ml), 30 μl NADPH (0.17 mg), 30 μl of 0.1 M $MgCl_2$, and 10 μl of 2 mM BP and 10 μg NADPH–cytochrome P-450 reductase in a total volume of 1 ml. For analysis of antibody inhibition of enzyme activity, about 10 pmoles of the cytochrome P-450 in 80 μl PBS was preincubated with 420 μl of antibody fluids for 15 min at room temperature, and the mixture was assayed for AHH activity at 37° for 20 min. Microsomes were assayed in the presence or absence of antibody as described above in a 1.0-ml reaction mixture containing 100 μl of 0.5 M Tris–HCl (pH 7.6), 100 μl of NADPH (0.5 mg), 30 μl of 0.1 M $MgCl_2$, and 50 μl of 2 mM BP. The cytochromes P-450_{LM2} and P-450_{LM4} were supplied by Dr. M. Coon (University of Michigan).

The same procedure used in the AHH assay was used to measure the enzyme-catalyzed formation of BP metabolites by HPLC [15], except that the substrate was 50 nmoles of [7, 10-^{14}C]BP as previously described [30, 31]. Cytochrome P-450-catalyzed 7-ethoxycoumarin deethylation (ECD) was measured by the methods of Greenlee and Poland [41].

RESULTS

Identification and classification of MAbs. Four series of hybridizations were carried out with spleen cells from BALB/c female mice immunized with purified PB-P-450 and the myeloma cells X63Ag8.653 [35], SP2/OAg14 [36], or NS-1 [37]. Mice with immune serum that bound to PB-P-450 at levels two to three times higher than normal serum (Table 1) were used as the donors of spleen cells for the hybridization.

The first series of hybridizations was done with 1×10^7 NS-1 and 1×10^8 spleen cells derived from mice immunized with PB-P-450. The growth of hybrid cells in the HAT medium was observed in 38–48 wells of two 24-well plates (Costar). When the culture fluids were tested by RIA, 9 of 37 wells were positive for antibody production for PB-P-450. The positive hybrid cells were cloned and tested again for antibody production by RIA. Six clones were positive among 47 clones which were derived from 7 positive wells. Four of these are shown in Table 1. They all were of the IgM type. None of these MAbs immunoprecipitated or inhibited the AHH enzyme activity of PB-P-450.

Table 1. Characteristics of monoclonal antibodies to liver microsomal cytochrome P-450 of rats treated with phenobarbital*

Expt.	Antibodies		Binding to PB-P-450 (RIA: cpm)	Ig subclass†	Double-immunodiffusion P-450		
					PB	MC	BNF
Normal mouse serum			3,285		–	–	–
Anti-PB-P-450 mouse serum			7,140		+	–	–
I.	NS-1 (myeloma cells)		1,233	–	–	–	–
	PB-P-450	1-48-9p3*‡	8,711	μ	–	–	–
		1-48-13p3	6,315	μ	–	–	–
		1-48-15p3	6,688	μ	–	–	–
		1-33-2p3	8,133	μ	–	–	–
II.	P3X63p70 (myeloma cells)		2,856	γ1	–	–	–
	PB-P-450	2-8-1p3	18,001	γ1	–	–	–
		2-66-3p5	20,097	γ1	+	–	–
III.	NBS	1-48-5p28§	2,100	γ2a	–	–	–
	PB-P-450	3-10-2p4	16,301	γ1	+	–	–
		3-17-1p4	18,048	γ1	–	–	–
		3-18-5p4	9,748	γ1	–	–	–
		3-23-5p4	17,750	μ	+	–	–
		3-47-4p4	12,973	γ1	+	–	–
IV.	P3X63p70 (myeloma cells)		811	γ1	–	–	–
	NBS	1-14-1-3p5	589	γ1	–	–	–
	PB-P-450	4-2-12p4	9,052	γ2a	+	–	–
		4-7-1p4	21,936	γ2a	+	–	–
		4-22-6p4	3,969	γ1	+	–	–
		4-25-4p4	7,499	γ1	+	–	–
		4-27-4p4	5,071	γ1	+	–	–
		4-29-5p4	10,409	γ2b	+	–	–
		4-30-3p4	2,365	γ1	–	–	–
		4-47-2p4	7,812	γ1	+	–	–
	MC-P-450	1-7-1p6	1,750	γ1	–	+	+

* Culture fluids were used as the source of monoclonal antibodies for the binding to PB-P-450 and immunoglobulin classification. Ascites fluids were used for double-immunodiffusion analyses. Normal unimmunized and the immune serum were used for binding in RIA with 10^{-2} dilutions.

† We adopted the nomenclature of Fahey *et al.* [42, 43] for mouse immunoglobulin classification.

‡ First number indicates experiment number; second, well number; third, clone number; and p, number of passages.

§ Control normal spleen cell hybrid.

The second series of hybridizations was done with the myeloma cell line X63Ag8.653. After immunization and hybridization, cell growth was observed after 2–3 weeks in 36 of 96 wells. Production of antibodies that bound PB-P-450 were observed in only two wells. After these hybrid cells were cloned, all 52 hybrid clones derived from the two wells produced MAbs to PB-P-450. Two of the clones are shown in Table 1. The 2-8-1p3 showed binding to PB-P-450 by RIA but did not precipitate or inhibit the PB-P-450 extensively. Another clone, 2-66-3p5, exhibited both binding to and immunoprecipitation of the PB-P-450 as well as strong inhibition of the PB-P-450-dependent AHH activity.

The third series of hybridizations was done with SP2/OAg14 myeloma cells and spleen cells from immunized mice. Growth was observed in 50 wells, 10 of which were positive for MAb production. Properties of five clones are shown in Table 1; all were positive by RIA and three of the five precipitated the PB-P-450. None, however, inhibited enzyme activity, and four of the MAbs of this series were of the IgG1 type and one was of IgM type.

The fourth series of hybridizations was done with SP2/OAg14 myeloma cells and spleen cells from immunized mice. Growth was observed in 80 wells and 11 were positive for PB-P-450 specific MAb production as tested by RIA. The properties of eight independent hybrid clones derived from the eight wells are shown in Table 1. The MAbs from seven of these hybridomas precipitated PB-P-450 but did not precipitate MC-P-450 or BNF-P-450. MAbs from four of the positive hybridomas inhibited enzyme activity. Of the eight hybridomas, five produced IgG1, two produced IgG2a and one produced IgG2b immunoglobulins.

Figure 1 shows the double-immunodiffusion analyses for the determination of subclass of the monoclonal antibodies, MAb PB-P-450 2-8-1p3 and MAb PB-P-450 2-66-3p3, present in serum-free culture fluids (Fig. 1, B and C). These give rise to precipitin reactions only with rabbit anti-mouse IgG1. The culture fluid of the parent myeloma cell, X63Ag8.653, showed no reaction with any of the rabbit anti-mouse immunoglobulin sera tested: anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b, anti-mouse IgG_3, anti-mouse IgM or anti-mouse IgA (Fig. 1A). Thus, the IgG produced in the culture fluids was the sole class of immunoglobulins expressed by the immunoglobulin genes derived from the spleen cells. Also, no immunoglobulin secretion was observed with NS-1 [29–31] and SP2/OAg14 (data not shown).

Specificity of MAbs. The anti PB-P-450 mouse serum and MAbs 2-66-3p5, 3-10-2p4, 3-23-5p4 and 3-47-4p4 clearly showed precipitin reactions with PB-P-450 in the Ochterlony double-immunodiffusion analyses. The PB MAb did not precipitate MC-P-450, BNF-P-450, $P\text{-}450_{LM4}$ or HL-P-450 (from human liver) (Table 1, Fig. 2C), but 2-66-3p5 and 3-23-5p4 also showed strong precipitin reactions with phenobarbital-induced cytochrome $P\text{-}450_{LM2}$ from rabbit liver, indicating that both the rat and rabbit PB-induced cytochrome P-450 isozymes share a common antigenic determinant(s). In contrast to the MAb 2-66-3p5, the monoclonal antibody to MC-P-450 1-7-1p6 [31] was specific for MC-P-450 and BNF-P-450, which are believed to be identical [5] (Fig. 2D). Although positive by RIA, the other MAb raised to PB-P-450 (PB-P-450 2-8-1p3, Fig. 2B) did not immunoprecipitate the PB-P-450 or inhibit its enzyme activity extensively. Non-specific MAb, NBS 1-14-1p4 (IgG1 producer), did not react with any of the cytochrome P-450 isozymes (Fig. 2A). The strong binding of nonprecipitating MAb, PB-P-450 2-8-1p3, is shown in Fig. 3; it is specific to PB-P-450 (Fig. 3A) and not to MC-P-450 (Fig. 3B).

MAb inhibition of P-450 enzyme activity (purified cytochrome P-450). The effects of MAbs on enzyme activities were measured with MAbs contained in serum-free culture fluid concentrates or in the ascites fluid from mice injected with hybridomas intraperitoneally. The results with serum-free culture fluid concentrates for eight hybrids are shown in Table 2A. The AHH activity of a reconstituted mixed-function oxidase system containing purified PB-P-450 was inhibited more than 90% by four MAbs. The effects of ascites fluid containing MAbs

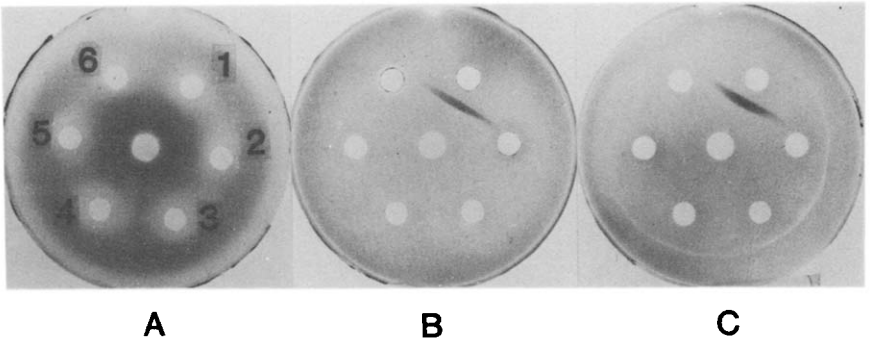

Fig. 1. Double-immunodiffusion analysis of Ig secretion and monoclonal antibodies to purified PB-P-450. Center wells of A, B, and C contained 20 μl of concentrates (25 times) from serum-free culture fluids of parent X63Ag8.653p3 myeloma (non-producer) and hybridomas PB-P-450 2-8-1p3 and PB-P-450 2-66-3p3. Outer wells 1, 2, 3, 4, 5, and 6 contained 20 μl of rabbit anti-mouse IgG1, rabbit anti-mouse IgG2a, rabbit anti-mouse 1gG2b, rabbit anti-mouse IgG3, goat anti-mouse IgM and rabbit anti-mouse IgA.

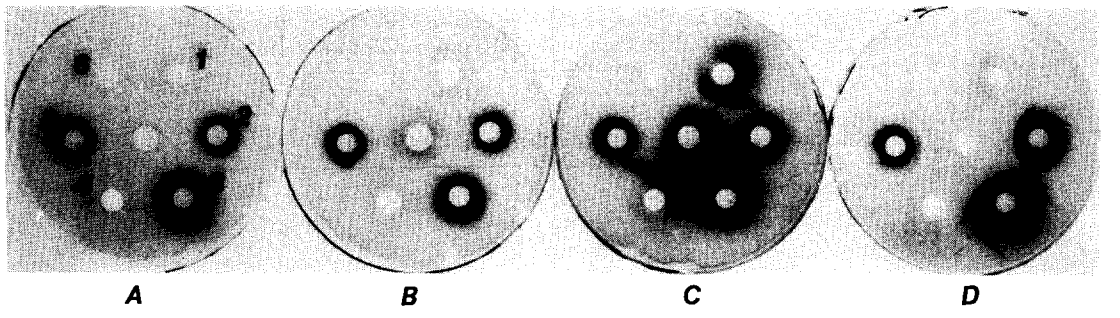

Fig. 2. Double-immunodiffusion analysis of the specificity of monoclonal antibodies to purified PB-P-450. The plates were incubated for 3 days at room temperature. (A) Center, non-specific ascites fluid of monoclonal antibody from hybrid NBS 1-14-1p4 (X63Ag8.653 × unimmunized mice spleen cells, IgG1 producer). Key: (1) PB-P-450 (0.39 mg/ml), (2) MC-P-450 (0.28 mg/ml), (3) BNF-P-450 (0.55 mg/ml), (4) P-450$_{LM2}$ (0.79 mg/ml), (5) P-450$_{LM4}$ (0.62 mg/ml), and (6) HL-P-450 (0.064 mg/ml). (B, C and D) Centers, ascites fluid of monoclonal antibodies from hybrids PB-P-450 2-8-1p3, PB-P-450 2-66-3p5, and MC-P-450 1-7-1p6; 1–6 same as in (A).

NBS 1-48-5p23 (control normal spleen cell hybrid), PB-P-450 2-8-1p3 and PB-P-450 2-66-3p5 on the AHH activity of four different cytochrome P-450 isozymes are shown in Table 2B. With the MAb present in 50 μg of ascites fluid, PB-P-450 2-8-1p3 inhibited only 32% of PB-P-450 AHH activity. Non-specific control MAb NBS 1-48-5p23 enhanced the PB-P-450 AHH activity by 46%. We have observed this effect of control MAb on the purified P-450s. The reason for the enhancement of AHH activity by non-specific MAbs is not known. It is possible that the non-specific protein enhanced the association of components of the AHH system. The highly inhibitory PB-P-450-specific MAb PB-P-450 2-66-3p5 as well as the MAb PB-2-8-1p3 had no effect on AHH activity of the major forms of cytochromes P-450 from rats treated with MC, BNF or PCN. The effects of various concentrations of MAbs on the AHH activity of different forms of the P-450 isozymes are shown in Fig. 4. AHH activity of PB-P-450 was inhibited by 95% with as little as 4 μg of 2-66-3p5 (Fig. 4A). Figure 4 also shows that this MAb had no significant effects on the AHH activities of purified MC-P-450 (Fig. 4B), BNF-P-450 (Fig. 4C) or PCN-P-450 (Fig. 4D). The MAbs, 2-66-3p5, 2-8-1p3, 4-7-1p3 and 4-29-5p3, also inhibited ECD activity of PB-P-450 by 43–99% (Table 2B).

One of the MAbs to rat liver PB-P-450, the MAb

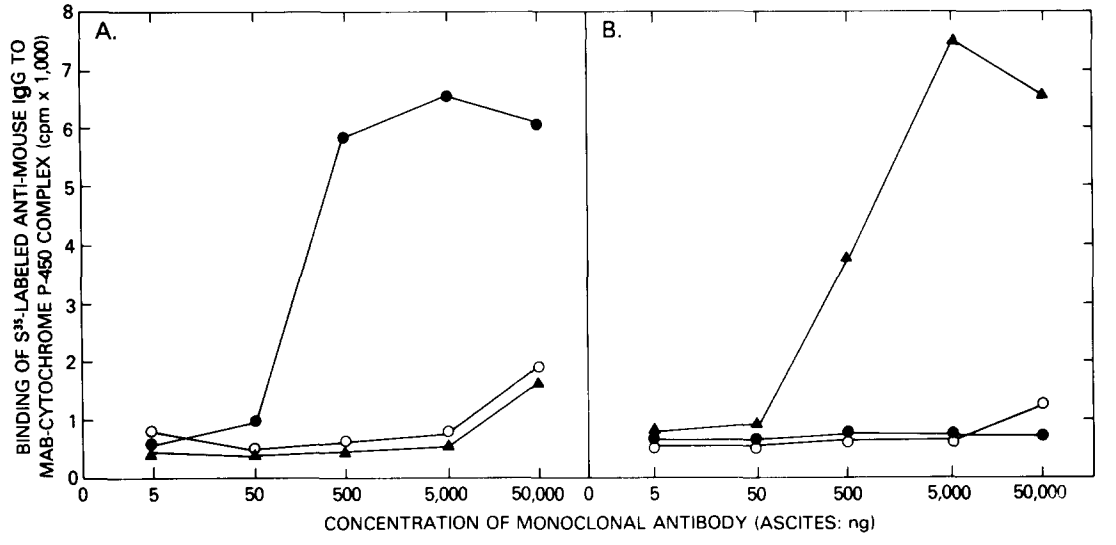

Fig. 3. Specificity in binding of monoclonal antibodies to P-450 isozymes. Cytochrome P-450 of rats treated with phenobarbital (PB-P-450, panel A) or 3-methylcholanthrene (MC-P-450, panel B) was precoated on 96 microtiter well plates, incubated with ascites of NBS 1-48-5p23 (non-specific hybrid, IgG2a producer; —○—), PB-P-450 2-8-1p3 (specific to PB-P-450, IgG1 producer; —●—) or MC-P-450 1-7-1p12 (specific to MC-P-450, IgG1 producer; —▲—). The bound monoclonal antibodies were detected with [^{35}]S-methionine-labeled rat anti-mouse IgG(k) (45,756 cpm/2 μl/13 μg/well) in the solid phase radioimmunoassay as described in Materials and Methods.

Table 2A. Effect of monoclonal antibodies to PB-P-450 on the aryl hydrocarbon hydroxylase activity (AHH) of purified PB-P-450*

Source of monoclonal antibodies	AHH 3-OH-BP (pmoles/nmoles P-450/min)	% Control
None (control)	95.2	100
NBS 1-48-5p23	171.0	180
PB-P-450 4-2-12p4	4.0	4
4-7-1p3	<1.0	<1
4-22-6p3	9.8	10
4-25-4p4	189.0	199
4-27-4p4	204.0	214
4-29-5p4	<1.0	<1
4-30-3p4	93.3	100
4-47-4p4	300.0	315

* Purified cytochrome P-450 (PB-P-45, 10 pmoles) and monoclonal antibodies (200 μg serum-free culture concentrates) were preincubated in PBS at room temperature for 15 min and subjected to AHH assay at 37° for 20 min using benzo[*a*]pyrene (100 nmoles) as the substrate.

2-66-3p5, bound strongly to rabbit liver microsomal cytochrome P-450_{LM2}. We tested the effect of MAb 2-66-3p3 on the AHH activity of rabbit liver cytochrome P-450_{LM2} and P-450_{LM4} (Table 3). The AHH activity of PB-P-450_{LM2} was inhibited 75% by ascites fluid containing 50 μg and 97% by 100 μg of the ascites fluid (protein weight) containing the MAb 2-66-3p3. This MAb had no significant effect on the AHH activity of PB-P-450_{LM4}. MAb 2-8-1p3 had essentially no inhibitory effect on the AHH activities of cytochrome P-450_{LM2} compared with the non-specific control monoclonal antibody, NBS 1-14-1p4 (IgG1 producer), and rather enhanced the AHH activity of P-450_{LM4}. The data indicate that rat liver PB-P-450 and rabbit PB-P-50_{LM2} share a common antigenic determinant which is recognized by PB-2-66-3p3. The binding of the enzymes to the MAb interferes with the catalytic activity of the cytochrome P-450. Previously, we reported that MAbs to rabbit liver cytochrome P-450_{LM2} are specific to cytochrome P-450_{LM2} and do not cross-react with the other rabbit isozymes measured by binding or by inhibition of enzyme activity [29, 30]. As shown in Fig. 5, the MAb to cytochrome P-450_{LM2} also inhibited 68% of the rat PB-P-450 AHH activity. In contrast, the non-specific MAb, NBS 1-48-5p23 (γ2a), enhanced the rat PB-P-450 AHH activity by 46%. Thus, it is apparent that the catalytic sites of rat liver cytochrome PB-P-450 [5, 10, 25] and PB-induced rabbit liver cytochrome P-450_{LM2} [4] are related in that both are sensitive to inhibition by the MAbs to either enzyme.

MAb inhibition of microsomal AHH and 7-ethoxycoumarin. Table 4 shows that control MAb (NBS) producing different types of immunoglobulins and five different MAbs of different Ig type showed either a non-specific stimulation or no effect on the AHH activity of the microsomes from control, PB-, or MC-treated rats. The MAb PB-2-66-3p5 inhibited the PB microsomes by 43% and did not affect the control or MC microsomes.

Table 4 also shows the effects of the MAbs to PB-P-450 and MC-P-450 on ethoxycoumarin deethylase (ECD) activity of phenobarbital-induced rat microsomes. MAb 2-66-3p10 inhibited 22% of ECD of these microsomes.

Control MAb NBS and MAb to MC-P-450, MAb 1-7-1p6 had no effect on the enzyme activities for AHH and ECD of microsomes from PB-treated rats.

HPLC analysis of BP metabolism by a purified cytochrome P-450 system. Table 5 shows the effect of MAb to PB-P-450 on BP metabolism in a reconstituted mixed-function oxidase system containing PB-P-450 and NADPH–cytochrome P-450 reductase. The metabolites formed from BP separated into two phenol peaks containing predominantly 3-OH-BP and 9-OH-BP, three quinone peaks (1,6-, 3,6- and 6,12-quinones), a 4,5-diol peak, and an unknown metabolite. The 4,5-diol could be formed from epoxide intermediates through the action of epoxide hydrolase, which was present in small amounts in the cytochrome P-450 preparations. The MAb to PB-P-450, 2-66-3p5, at a concentration of 50 μg protein (ascites fluid) inhibited metabolite formation by 86–100%. In the presence of the MAb, more than 90% of quinone formation was inhibited and no 4,5-diol formation was observed. Formation of the 3-OH-BP and 9-OH-BP peaks was inhibited by 93 and 86% respectively. The total of the metabolites formed in the presence of the MAb to PB-P-450 was only 7% of the total metabolites formed in the absence of the MAb. We frequently observed non-specific inhibition of AHH activity of cytochrome P-450 by bovine serum albumin or MAbs produced by normal spleen cell hybrids [30]. As shown in Table 5, the addition of control NBS 1-48-5p23 ascites caused some non-specific inhibition in the formation of individual metabolites, ranging from 9 to 68%. This result indicated that the MAb was bound to a site of the enzyme required for the formation of each of the intermediate metabolites, and little or no metabolite was formed when the antigenic determinants were bound by the MAbs.

Table 2B. Effect of monoclonal antibodies to PB-P-450 on aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase (ECD) activities of different forms of liver microsomal cytochrome P-450 derived from rats treated with phenobarbital (PB), 3-methylcholanthrene (MC), β-naphthoflavone (BNF) or pregnenolone-16-α-carbonitrile (PCN)*

Purified enzymes (pmoles)		Source of monoclonal antibodies	AHH 3-OH-BP (pmoles/nmole P-450/min)	% Control
PB-P-450 (11.2)		None (control)	139	100
		NBS 1-48-5p23	203	146
		PB-P-450 2-66-3p5	4	3
		2-8-1p3	94	68
PB-P-450 (11.2)		None (control)	34	100
		NBS 1-48-5p23	43	125
		PB-P-450 4-7-1p3	0	0
		4-29-5p3	0	0
MC-P-450 (10.4)		None (control)	655	100
		NBS 1-48-5p23	705	108
		PB-P-450 2-66-3p5	773	118
		2-8-1p3	596	91
BNF-P-450 (11.0)		None (control)	620	100
		NBS 1-48-5p23	616	99
		PB-P-450 2-66-3p5	552	89
		2-8-1p3	1396	225
PCN-P-450 (12.8)		None (control)	7	100
		NBS 1-48-5p23	10	133
		PB-P-450 2-66-3p5	9	117
		2-8-1p3	12	157
			ECD 7-OH-C (pmoles/nmole P-450/min)	% Control
PB-P-450 (11.2)	A.	None (control)	5699	100
		NBS 1-48-5p23	5294	93
		PB-P-450 2-66-3p5	679	12
		PB-P-450 2-8-1p3	3242	57
	B.	None (control)	263	100
		NBS 1-48-5p23	221	84
		PB-P-450 4-7-1p3	4	2
		4-29-5p3	4	1

* Cytochrome P-450 and monoclonal antibodies (50 μg ascites for AHH and ECD) were preincubated in PBS at room temperature for 15 min and subjected to AHH and ECD assays at 37° for 20 min using benzo[*a*]pyrene (100 nmoles) and 7-ethoxycoumarin (500 nmoles) as the substrates.

DISCUSSION

Cytochromes P-450 are heme proteins which are the key components of the mixed-function oxidases [44]. Cytochromes P-450 incorporate atmospheric oxygen into exogenous substrates as well as into endogenous steroids, prostaglandins, and fatty acids [45]. The mixed-function oxidases are responsible for detoxification and for the generation of toxic, mutagenic and carcinogenic metabolites [2, 18, 19]. Multiple forms of cytochrome P-450 have been isolated and described (see Refs. 1 and 2). A primary research goal is to identify the types of cytochromes P-450 that are responsible for carcinogen activation as well as specific drug metabolism, and which lead to either toxicity or detoxification. Thus, the isolation and characterization of different cytochromes P-450 are important steps in understanding the genetics and function of cytochromes P-450 in biological systems. The development of hybridoma technology by Köhler and Milstein [28] permits the production and isolation of MAbs to specific antigenic determinants. These MAbs can be useful in distinguishing and purifying different forms of cytochrome P-450 [29–32]. We have reported previously the preparation of both enzyme-inhibiting and non-enzyme-inhibiting MAbs to two rabbit liver cytochromes P-450, the P-450_{LM2} and the P-450_{LM4} [29, 30], and to an MC-induced P-450 from rat liver [31]. In this paper we report the preparation and properties of panels of MAbs to a phenobarbital-induced rat liver cytochrome P-450.

In four separate experiments, hybridomas were formed between myeloma cells and spleen cells of

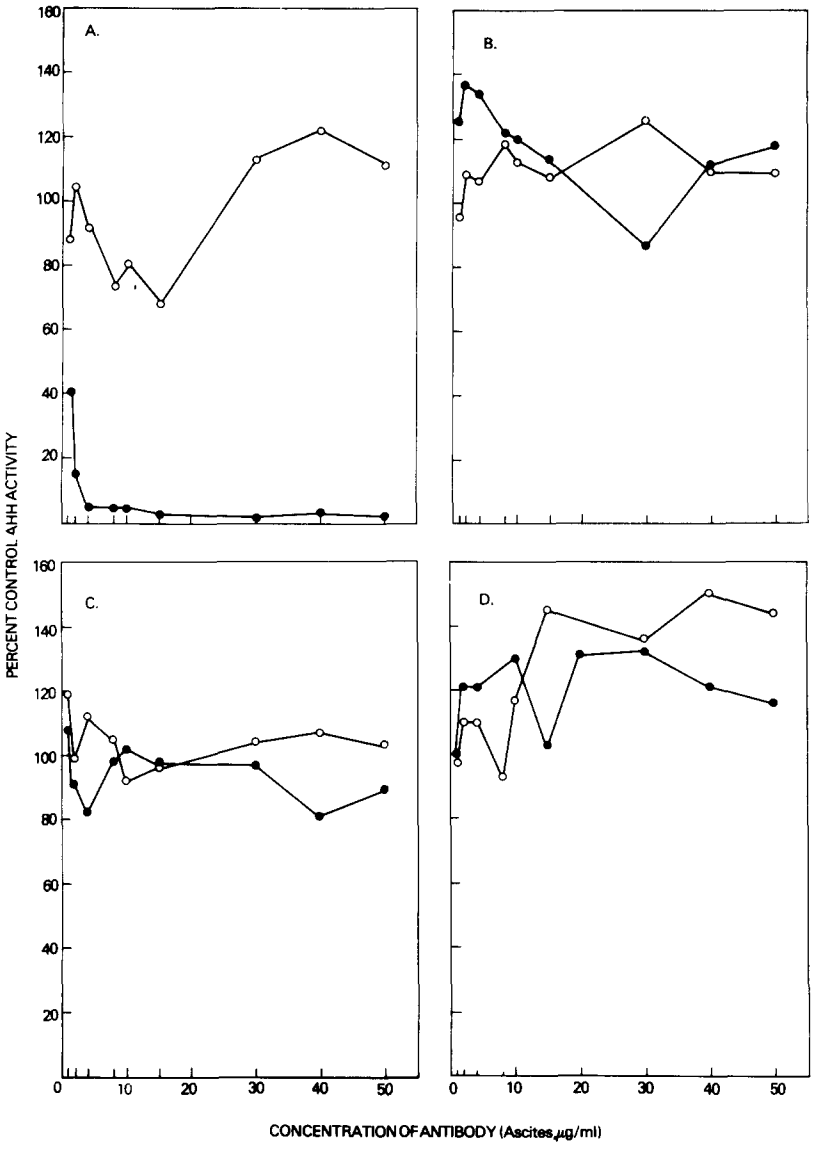

Fig. 4. Effect of monoclonal antibodies to PB-induced rat liver microsomal cytochrome P-450 on the AHH activity of purified cytochrome P-450 isozymes. A 420-μl sample of each concentration of protein in ascites fluid from the non-specific hybrid, NBS 1-48-5p23 (—○—, IgG2a producer), or from PB-P-450 2-66-3p5 (—●—, IgG1 producer), was incubated with cytochrome P-450 in 80 μl PBS at room temperature for 15 min and assayed for AHH activity in a reconstituted system containing 10 μg NADPH–cytochrome P-450 reductase. (A, B, C, and D) 10 pmoles of cytochrome P-450 from rats treated with PB, MC, BNF, or PCN was used as an enzyme source in AHH assay.

BALB/c mice immunized with purified liver microsomal cytochrome P-450 from rats treated with phenobarbital. Nineteen independent hybrid clones were capable of binding PB-P-450, as measured by RIA. Of the nineteen only eleven immunoprecipitated the PB-P-450 and of the latter eleven, only five strongly inhibited enzyme activity. Thus, it appears that the different panels of MAbs we obtained were directed against different antigenic determinants of the PB-P-450 molecule. The MAb 2-66-3p5 strongly inhibited the AHH activity of PB-P-450 but did not inhibit the AHH of purified MC-P-450, BNF-P-450 or PCN-P-450 isolated from rat liver. The AHH activity of PB-induced microsomes was also inhibited by the MAb 2-66-3p5. We also observed that the MAb PB-P-450 2-66-3p5 immunoprecipitated and inhibited the AHH activity of rabbit liver PB-induced cytochrome $P\text{-}450_{LM2}$ (Fig. 2C). PB-P-450-catalyzed deethylation of 7-ethoxycoumarin was also almost completely inhibited by MAbs 2-66-3p5, 4-7-1p3 and 4-29-5p3, indicating that the hydroxylation of BP and the deethylation were catalyzed at an active site of PB-P-450 which was interfered with when the MAb was bound to the enzyme. Thomas *et al.* [13] and

Table 3. Effect of monoclonal antibodies to rat liver PB-P-450 on the AHH activity of rabbit liver cytochrome P-450_{LM2} and P-450_{LM4}*

Cytochrome P-450	Source of monoclonal antibodies (Ig class)	Ascites protein (μg)	AHH (pmoles 3-OH-BP/nmole P-450/min)	% Control
LM2			497	100
	NBS 1-14-1p4	50	260	52
	(γ1)	100	262	53
	PB-P-4502-8-1p3	50	392	79
	(γ1)	100	300	60
	PB-P-4502-66-3p3	50	125	25
	(γ1)	100	16	3
LM4			1.5	100
	NBS 1-14-1p4	50	1.5	100
		100	1.9	127
	PB-P-4502-8-1p3	50	1.8	120
		100	2.4	160
	PB-P-4502-66-3p3	50	1.8	120
		100	1.3	87

* Rabbit liver microsomal cytochrome P-450_{LM2} or cytochrome P-450_{LM4} (142.3 pmoles) was preincubated with each ascites in a final volume of 0.5 ml PBS at room temperature for 15 min and subjected to AHH assay in a reconstituted system containing 216 pmoles NADPH-cytochrome P-450 reductase at 37° for 20 min. The purified P-450_{LM2} and P-450_{LM4} were gifts of Dr. M. Coon.

Guengerich *et al.* [10] have observed that rabbit polyclonal antibodies raised against rat PB-P-450 precipitate rabbit liver cytochrome P-450_{LM2} [13, 25]. In the Ochterlony double-imunodiffusion analysis described by Thomas *et al.* [13], there was also a significant cross-reaction between rabbit anti-PB-P-450 and rat liver cytochrome P-448 from 3-methylcholanthrene-treated rats. We did not observe any cross-reaction of the rat MC-induced P-448 with the MAb 2-66-3p5. This may have been due to the much higher degree of specificity of the MAb. We have reported on the specificity of MAbs to rabbit liver cytochrome P-450_{LM2} [29, 30]. In this study we also tested the cross-reactivity of the MAb to the rabbit

Table 4. Effect of monoclonal antibodies on AHH and 7-ethoxycoumarin deethylase (ECD) activities of liver microsomes of untreated rats and rats treated with phenobarbital (PB) or 3-methycholanthrene (MC)*

Source of monoclonal antibodies	Ig class	AHH (3-OH-BP pmoles/mg/min)					
		Untreated	% Control	PB	% Control	MC	% Control
None (control)		81	100	156	100	2357	100
NBS 1-14-1p3	γ1	161	200	213	137	3436	146
NBS 1-48-3p5	μ	173	214	185	119	3104	132
NBS 1-48-5p23	γ2a	180	222	187	120	3371	143
PB-P-450 1-33-2p3	μ	170	210	193	124	3277	139
PB-P-450 1-48-9p3	μ	170	209	206	132	3087	131
PB-P-450 1-48-15p3	μ	154	190	198	127	3017	128
PB-P-450 2-8-1p3	γ1	157	194	198	127	3440	146
PB-P-450 2-66-3p5	γ1	145	180	89	57	2739	116
PB-P-450_{LM2} 1-26-11p35	γ1	156	192	184	118	3225	137
MC-P-450 1-7-1p6	γ1	163	202	211	135	505	21
		ECD (7-OH-C pmoles/mg/min)					
				PB	% Control		
None (control)				4960	100		
NBS 1-48-5p28	γ2a			5010	101		
PB-P-450 2-66-3p10	γ1			3890	78		
MC-P-450 1-7-1p6	γ1			5090	103		

* Microsomes (10 μg) and ascites (200 μg for AHH and 100 μg for ECD) were preincubated in a final volume of 0.5 ml PBS at room temperature for 15 min and subjected to AHH assay at 37° for 10 min with shaking.

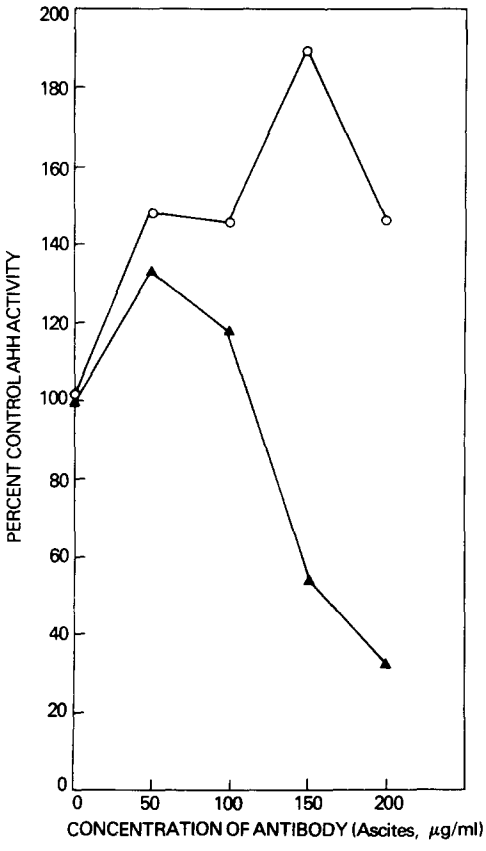

Fig. 5. Inhibitory effect of monoclonal antibody to PB-induced rabbit liver microsomal cytochrome P-450_{LM2} on AHH activity of purified liver cytochrome P-450 of rats treated with phenobarbital. A 420-μl sample of each protein concentration of ascites from non-specific hybrid, NBS 1-48-5p23 (—○—, IgG2a producer), or from specific hybrid, PB-P-450_{LM2} 1-26-11p35 (—▲—, IgG1 producer), was incubated with purified cytochrome P-450 of rats treated with phenobarbital in 80 μl PBS at room temperature for 15 min and assayed for AHH activity in a reconstituted system as indicated in Fig. 4.

P-450_{LM2} on the PB-P-450 of rat liver. The MAb to rabbit cytochrome P-450_{LM2}, 1-26-11p35, showed an inhibitory effect on AHH activity of the purified PB-P-450 (Fig. 5), although we did not observe an inhibitory effect of the MAb on the enzyme activity of microsomes from PB-induced rabbits (Table 4). It may be that the structural configuration of cytochrome P-450 associated with NADPH–cytochrome P-450 reductase and phospholipids in rabbit liver endoplasmic reticulum hinders the MAb from binding the antigenic site. A similarity between the primary structure of rat PB-P-450 and rabbit cytochrome P-450_{LM2} has been reported [46]. Our results indicate that rat liver PB-P-450 and rabbit liver cytochrome P-450_{LM2} share a common antigenic determinant.

We have shown previously that MAbs can be used to measure the contribution of specific types of P-450 to a given P-450-catalyzed reaction including those of human tissues [32]. Various forms of cytochrome P-450 are induced in rats by phenobarbital [10]. In this study we have shown the contribution of the P-450 which is sensitive to MAb 2-66-3p5 to two different P-450 reactions of liver microsomes from PB-treated rats. Our study measured the amount of MAb sensitive P-450 that contributed to total microsomal aryl hydrocarbon hydroxylase and 7-ethycoumarin deethylase. This MAb 2-66-3p5 sensitive P-450 contributed significantly to the two enzyme activities. HPLC analysis of BP metabolism by PB-P-450 in the presence of the MAb 2-66-3p5 indicated that the formation of each metabolite was affected, and little or no metabolite was formed when the PB-P-450 was bound by the MAb 2-66-3p5. The MAbs described here are additions to our library of MAbs to different types of P-450. We have reported recently that monoclonal antibodies can be used to direct sensitive radioimmunoassays for the cytochromes P-450 in tissues that contain the P-450 with the MAb recognized epitope. This type of RIA could utilize MAbs that bind the cytochrome P-450 and do not necessarily inhibit enzyme activity. This

Table 5. Effect of monoclonal antibodies to PB cytochrome P-450 on BP metabolism by PB-induced cytochrome P-450*

BP metabolites	Specific activity (pmoles/nmole P-450/min)		
	PB-P-450 (control)	PB-P-450+ NBS1-48-5p23	PB-P-45+ P-4502-66-3-5p5
4,5-Diol	124	67 (54)	0 (0)
9-OH-BP	611	288 (47)	84 (14)
3-OH-BP	1067	758 (71)	69 (7)
1,6-Quinone	409	372 (91)	9 (2)
3,6-Quinone	109	94 (86)	9 (0)
Fraction 1	270	98 (36)	36 (13)
6,12-Quinone	181	57 (32)	9 (5)
Total	2771	1734 (63)	205 (7)

* PB-induced cytochrome P-450 (148.6 pmoles) was preincubated with 50 μg monoclonal antibodies (NBS 1-48-5p23, non-specific, or PB-P-450 2-66-3p5, specific to PB-P-450) in 0.5 ml PBS at room temperature for 15 min and transferred to a reaction mixture of reconstituted system for AHH or HPLC in a final volume of 1 ml. Twenty nmoles of cold BP for AHH or 50 nmoles [7,10-^{14}C]BP for HPLC was used as a substrate. The numbers in parentheses indicates the percentage of control metabolites.

assay may be very useful in measuring low levels of P-450 for which enzyme assays are of insufficient sensitivity [47].

In another important use, we have reported that Sepharose bound with specific MAbs are highly specific immuno-adsorbents for the specific cytochromes P-450 to which the MAbs are directed. The use of these Sepharose-MAbs adsorbants permits an essentially one-step purification of specific cytochromes P-450 [48]. MAbs will prove extremely useful as tools in phenotyping tissue, species and individual differences in P-450 profile as well as in P-450 purification and isolation and thus lead to a better understanding of P-450 genetics [49, 50] and the role of specific P-450s in the drug and carcinogen sensitivity of different individuals.

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