

Monoclonal Antibodies That Inhibit Enzyme Activity of 3-Methylcholanthrene-induced Cytochrome P-450

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ABSTRACT

Somatic cell hybrids were made between mouse myeloma cells and spleen cells derived from BALB/c mice immunized with liver microsomal cytochrome P-450 purified from rats treated with 3-methylcholanthrene (MC-P-450). Thirty-seven independent hybrid clones among 66 tested produced monoclonal antibodies to the MC-P-450 as measured by radioimmunoassay. More than 10 of the monoclonal antibodies formed were positive for MC-P-450 with respect to protein binding measured by radioimmunoassay, precipitation of the enzyme caused by antibody binding and enzyme aggregation, and inhibition of enzymatic activity. Analysis by gel electrophoresis indicated that a single microsomal protein band interacted with the antibody and that this band comigrated with MC-P-450. These monoclonal antibodies interacted with the major form of cytochrome P-450 from β -naphthoflavone-induced rats as well as with MC-P-450 but did not bind, precipitate, or inhibit the activity of the major form of cytochrome P-450 from phenobarbital-treated rats. The monoclonal antibodies inhibited 7-ethoxycoumarin deethylase and benzo(a)pyrene hydroxylation activity of the purified MC-P-450 with varying degrees, up to 90%, the latter as measured by the aryl hydrocarbon hydroxylase assay for phenol production. Analysis of benzo(a)pyrene metabolism by high-pressure liquid chromatography indicated that the monoclonal antibodies inhibited the enzyme activity of the purified MC-P-450 at all of the positions at which oxidation occurs. The monoclonal antibodies also inhibited both aryl hydrocarbon hydroxylase and 7-ethoxycoumarin deethylase of liver microsomes from 3-methylcholanthrene-treated rats by 70%, indicating that these activities are functions affected by antibody binding to a common or identical antigenic site on cytochrome(s) P-450 which account for 70% of the total activity in these microsomes. Microsomes from control or phenobarbital-treated rats were unaffected, suggesting that their enzyme activity is a function of a cytochrome P-450 other than that sensitive to the MC-P-450 directed antibody and that the latter P-450 is absent in these microsomes. High-pressure liquid chromatographic analysis of antibody inhibition of benzo(a)pyrene metabolism by microsomes from 3-methylcholanthrene-treated rats showed an inhibition of phenol and diol formation that ranged from 46 to 72%. 1,6-Quinone production was not affected, which suggests that this metabolite was formed by another cytochrome P-450 isozyme or nonenzymatically. The purity, specificity, and potential immortality of their hybridoma cell source will make the monoclonal antibodies extraordinarily useful for the study of substrate and inducer

specificity and in the identification and quantitative assay of multiple forms of the cytochrome P-450 and the determination of their content and function in different tissues, species, and individuals.

INTRODUCTION

The polycyclic aromatic hydrocarbons are a major class of environmental carcinogens to which humans are exposed. A prototype and most common hydrocarbon of this class is BP³ (1). The biological activities of BP, *i.e.*, toxicity, mutagenicity, covalent binding to DNA, and transformation, require metabolic activation (10, 11, 19, 41). A metabolic pathway leading to the BP diol-epoxides is a major pathway of carcinogen activation (19, 41, 42), whereas many of the other pathways leading to intermediate phenols, dihydrodiols, and quinones which are subsequently converted to water-soluble conjugates of glutathione, uridine diphosphoglucuronic acid, and sulfates are believed to be routes of detoxification. The mixed-function oxidases (aryl hydrocarbon hydroxylases) containing cytochrome P-450 are the initial enzyme systems that metabolize BP as well as other carcinogens, drugs, and endogenous substrates such as steroids (2, 8, 10, 12, 42). Several different forms of cytochrome P-450 have been isolated and characterized (3, 27). In rats, the different forms may be induced by various xenobiotics. Some of these forms have been purified and exhibit different biological, immunological, and kinetic properties (15, 17, 20, 32, 35-37, 40), as well as substrate and product stereospecificity for BP (5).

We have previously reported the isolation of monoclonal antibodies to rabbit liver microsomal cytochrome P-450_{LM2} and P-450_{LM4} which demonstrated distinctive specificity in immunoprecipitation and inhibition of the activities of each form of enzyme (31). In this study, we have used the somatic cell hybridoma system to prepare monoclonal antibodies to the major cytochrome P-450 from liver microsomes of rats treated with MC. These monoclonal antibodies are highly specific and bind and precipitate as well as inhibit several enzyme activities of the cytochrome P-450 isolated from rats treated with MC or β -naphthoflavone (identical with 7,8-benzoflavone). These monoclonal antibodies did not interact with or inhibit the activity

³ The abbreviations used are: BP, benzo(a)pyrene; P-450_{LM2} and P-450_{LM4}, different forms of cytochrome P-450 from rabbit liver; MC, 3-methylcholanthrene; PB-P-450, cytochrome P-450 from phenobarbital-treated rats; MC-P-450, cytochrome P-450 isolated from the liver of 3-methylcholanthrene-treated rats; BNF-P-450, cytochrome P-450 from β -naphthoflavone-treated rats; HAT, Dulbecco's modified Eagle's medium with 25 mM glucose and 4 mM glutamine, supplemented with 10% fetal calf serum, 10% horse serum, 50 μ g gentamicin per ml, 100 μ M hypoxanthine, 0.4 μ M aminopterin, and 16 μ M thymidine; RIA, radioimmunoassay; 3-OH-BP, 3-hydroxybenzo(a)pyrene; AHH, aryl hydrocarbon (benzo(a)pyrene) hydroxylase; HPLC, high-pressure liquid chromatography; NBS, hybrid cells formed from RGN5-1 myeloma cells and spleen cells from unimmunized mice; 1-OH-BP, 1-hydroxybenzo(a)pyrene; 7-OH-BP, 7-hydroxybenzo(a)pyrene; 9-OH, 9-hydroxybenzo(a)pyrene; PBS, phosphate-buffered saline.

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of the major cytochromes P-450 of rats treated with phenobarbital or with microsomal preparations from control or phenobarbital-treated rats. The specificity of monoclonal antibodies should be useful for studies on the mechanism of enzyme action such as enzyme-substrate and enzyme-inducer specificity, as well as in the determination of the content, function, genetics, and regulation of the different forms of cytochrome P-450 in different tissues, species, and individuals.

MATERIALS AND METHODS

Preparation of Cytochrome P-450 and Microsomes. Three liver microsomal cytochrome P-450s from rats treated with phenobarbital (PB-P-450), 3-methylcholanthrene (MC-P-450), and β -naphthoflavone (BNF-P-450) were purified as described elsewhere (16). The "B₂" fractions were used in this study. NADPH-cytochrome P-450 reductase was prepared as described (16).

Media and Cells. Dulbecco's modified Eagle's medium, fetal calf serum, and horse serum were purchased from Grand Island Biological Co. The myeloma cell line RGNS-1, which was azaguanine resistant and a nonproducer of immunoglobulin, was obtained from Dr. John D. Minna and grown in Dulbecco's modified Eagle's medium with 25 mM glucose and 4 mM glutamine supplemented with 10% fetal calf serum, 10% horse serum, and 50 μ g gentamicin per ml (complete medium). Mouse spleen-myeloma hybridomas were grown in HAT medium (26). Dulbecco's modified Eagle's medium with 5.6 mM glucose plus 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, was used as washing medium in the preparation of the hybridomas.

Immunization of Mice and Preparation of Mouse Sera. Eleven-week-old female BALB/c mice were immunized by i.p. injections every week for a period of 4 weeks with 12 μ g of purified MC-P-450 emulsified in 0.2 ml of Freund's complete adjuvant. The following week, they further immunized by an i.v. injection of 12 μ g of MC-P-450 in Dulbecco's phosphate-buffered saline, pH 7.4. Three days after the i.v. injection, 5 mice were sacrificed in a bag containing dry ice, and the spleens 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 \times 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 (24), except that polyethylene glycol 1000 was used for the fusion (7). The spleens from 5 immunized mice were cut into small pieces, and the cells were dissociated by squeezing the tissue through a sterile mesh (80 mesh) placed over a 50-ml glass centrifuge tube and washing the cells with 10 ml of washing medium. The tube containing the dissociated cells was kept in ice for 15 min, and 8 ml of the upper cell suspension were layered on the top of 2 ml fetal calf serum in a 15-ml plastic tube and centrifuged for 10 min at 1500 rpm. The cell pellet was resuspended in 2 ml of 155 mM NH₄Cl and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.2), and kept in a 37° water bath for 2 min with shaking to break RBC. The NH₄Cl-treated cells were transferred to a 15-ml conical plastic centrifuge tube containing 2 ml fetal calf serum and spun at 1500 rpm for 10 min. The cell pellets were washed twice and resuspended in 5 ml of washing medium. For the process of hybridization, 1 \times 10⁷ RGNS-1 cells and 1 \times 10⁶ spleen cells were combined in a 50-ml plastic conical centrifuge tube and spun at 1500 rpm for 15 min. The cell pellets were loosened; 1 ml of 50% polyethylene glycol in washing medium, neutralized with 7.5% sodium bicarbonate, was added dropwise to the mixture of cells during a 1-min period; and the mixture was diluted gradually with 1 and 20 ml of washing medium during periods of 1 and 4 min, respectively. The polyethylene glycol-treated cells were collected by centrifugation at 1000 rpm for 10 min, suspended in 100 ml of complete medium, dispensed in 2 ml in each of 48 wells of 2 Costar plates, and incubated

in the CO₂ incubator at 37° overnight. The following day, 1-ml aliquots of the supernatant fluids were removed from each well and replaced with the HAT medium. This procedure was repeated for 3 days with fresh HAT medium added daily. Subsequently, the HAT medium was changed every 2 to 3 days. After 2 to 3 weeks, the wells were scored for the growth of the hybridoma cells by the change of medium color from red to yellow and by microscopic observation. Antibody production into the culture media was measured by RIA. The cells in the antibody-containing wells were selected, and cells were diluted and cloned into a 96-well microtiter plate (Costar) by distributing a 0.2-ml cell suspension into a single well with a probability of 0.4 cell/well. Of 96 wells, approximately 1 to 20 showed growth of a single colony which were picked and transferred to 24-well plates for further growth and determination of antibody presence. Occasionally, 2 colonies appeared after cloning and these were discarded. Monoclonal antibodies were obtained either as supernatant cell culture fluid or as ascites and were periodically checked and subclones prepared when the titer was diminished. A typical experiment indicating the number of wells showing cell growth and the cloning efficiency is described in "Results." The monoclonality and stability of one of the hybridoma clones, MC-P-450 1-7-1, also was tested further by passaging the cells 8 times, followed by recloning, further passaging and testing for P-450 binding, and inhibition of enzyme activity.

Preparation of Monoclonal Antibodies in Mouse Peritoneal Ascites Fluid. Hybrid cells producing antibodies were grown in flasks containing HAT or hypoxanthine and thymidine medium and collected by centrifugation, resuspended in PBS, and inoculated into female BALB/c mice i.p. (5 \times 10⁶ cells in 0.2 ml of PBS). After 2 to 3 weeks, the ascites fluids were collected with a syringe or by puncturing the abdomen and were clarified as described above for serum.

RIA. ¹²⁵I-labeled anti-mouse IgG (specific for heavy and light chain) or ¹²⁵I-labeled F(ab')₂ fragments of anti-mouse IgG (50 μ Ci/ μ g protein per 0.5 ml; Amersham) was used for the detection of monoclonal antibodies which were bound to MC-P-450. The radioactivity was arranged to be 50,000 to 100,000 cpm/sample. The solid-phase RIA was carried out with modifications described previously (6, 18). The wells of a microtiter plate (flexible, polyvinyl chloride; Dynatech) were coated with MC-P-450 by incubating with 100 μ l of the purified cytochrome P-450 in PBS (0.1 mg/ml) for 2 hr at 37°. The remaining nonspecific sites were covered with bovine serum albumin by replacing the cytochrome P-450 solution with PBS containing 3% bovine albumin and 2% sodium azide and incubating for 30 min. The wells were washed with PBS 3 times, and the cytochrome P-450 bound to the wells was incubated for 2 hr with the culture fluids of monoclonal antibodies. The monoclonal antibodies bound to enzymes were then incubated with ¹²⁵I-labeled rabbit anti-mouse IgG or ¹²⁵I-labeled F(ab')₂ overnight and washed 5 times with PBS. The plastic wells were dried and separated by cutting with a hot wire. The individual wells were placed in plastic vials, and the radioactivity was measured in a γ counter.

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- μ l samples) were placed in disc gel wells (Cappel), incubated at room temperature, and observed for precipitin bands, which usually appeared after 2 to 3 days.

Electrophoresis of Cytochrome P-450 and Binding to Monoclonal Antibodies. Purified and solubilized microsomes were electrophoresed in 7.5% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulfate as described (25), with the exception that pyronin Y was used as the tracking dye. The resolved proteins were electrophoretically transferred to nitrocellulose sheets (0.45 μ m; Millipore Corp.) (38) and detected after sequential treatment of the nitrocellulose sheets with hybridoma anti-cytochrome P-450, rabbit anti-mouse IgG (Cappel), horseradish peroxidase/antiperoxidase complex (Miles Laboratories), and 3,3'-diaminobenzidine/H₂O₂ (13). The dilution of hybridoma fluid was 1/10, and the dilution of rabbit anti-mouse IgG was 1/20. Other

materials were diluted to the same extent as described previously (13). Nitrocellulose sheets were stained for protein with Amido black as described elsewhere (33).

Measurement of Cytochrome P-450 Enzyme Activity. AHH activity was determined by measuring the amount of BP conversion to phenolic products equivalent to 3-OH-BP (28). The reaction mixture in the reconstituted mixed-function oxidase system contained 250 μ l 0.2 M Tris-HCl (pH 7.6), 30 μ l dilauroylglyceryl-3-phosphorylcholine (1 mg/ml), 30 μ l NADPH (0.17 mg), 30 μ l 0.1 M MgCl₂ and 10 μ l 2 mM BP in a total volume of 1 ml. For analysis of antibody inhibition of enzyme activity, 0.5 μ g of the cytochrome P-450 in 80 μ l PBS was incubated with 420 μ l antibody fluids for 15 min at room temperature, and then the mixture was analyzed for AHH activity at 37° for 20 min. Microsomes, either intact or solubilized with Emulgen 913, were assayed in the presence or absence of antibody, as described above, in a 1.0-ml reaction mixture containing 90 μ l 0.5 M Tris-HCl (pH 7.6), 100 μ l NADPH (0.5 mg), 30 μ l 0.1 M MgCl₂, and 50 μ l 2 mM BP. Fifteen μ l of NADPH-cytochrome P-450 reductase (0.2 μ g) were added when purified cytochrome P-450 or solubilized microsomes were assayed in the reaction mixture. Cytochrome P-450-catalyzed BP metabolism was also measured by HPLC as described previously (5).

The same procedure as in the AHH assay was used to measure the enzyme-catalyzed formation of BP metabolites, except that the substrate was 50 nmol of [7,10-¹⁴C]BP dissolved in 0.025 ml of 10% tetrahydrofuran and 90% methanol. The reaction was stopped by the addition of 1.0 ml of acetone at 4°, and the mixture was extracted twice with 2.0 ml of ethyl acetate. HPLC was performed with a Spectra-Physics Model 3500 liquid chromatograph with a DuPont Zorbax octadecyltrimethoxysilane column (6.2 mm inner diameter x 0.25 m), with monitoring at 254 nm. The column was eluted at a rate of 0.8 ml/min with a linear gradient from 60% methanol in water to 100% methanol at a sweep time of 45 min for BP metabolites. Twenty-drop fractions were collected. For the analysis of BP metabolites, a mixture of synthetic BP phenols, diols, and quinones was cochromatographed with the samples. Cytochrome P-450-catalyzed 7-ethoxycoumarin de-ethylation was measured as described (14).

RESULTS

Mouse Serum Antibodies. Mice immunized for either 3 or 4 consecutive weeks with purified cytochrome P-450 from MC-treated rats produced antibodies which precipitated the MC-P-450 detected by Ouchterlony immunodiffusion (Fig. 1). Precipitin bands were formed between anti-MC-P-450 mouse sera and MC-P-450 and also between anti-MC-P-450 mouse sera and BNF-P-450. No precipitin bands, however, were observed between the anti-MC-P-450 serum and PB-P-450 or between the serum from unimmunized mice and MC-P-450.

Identification and Classification of Monoclonal Antibodies. Five mice immunized for 4 consecutive weeks with MC-P-450 were sacrificed, and spleens were removed for dissociation of spleen cells. Hybrid cells were formed upon fusion of 10⁷ myeloma cells with 10⁸ spleen cells derived from the mice immunized with MC-P-450. The growth of hybrid cells prepared as described above occurred in 76 of 96 wells containing the selective HAT medium (26). Of the 76 wells containing hybrid cells, all were positive for mouse immunoglobulin production when tested with ¹²⁵I-labeled F(ab')₂ of anti-mouse IgG (heavy and light chain specific). The hybrid cells producing antibodies in the primary screening were cloned into plates containing 96 wells. After 2 to 4 weeks, formation of single clones was observed in usually about 1 to 20 wells. On rare occasions, 2 colonies were observed which were discarded. The single hybridoma clones were transferred to Costar plates, each

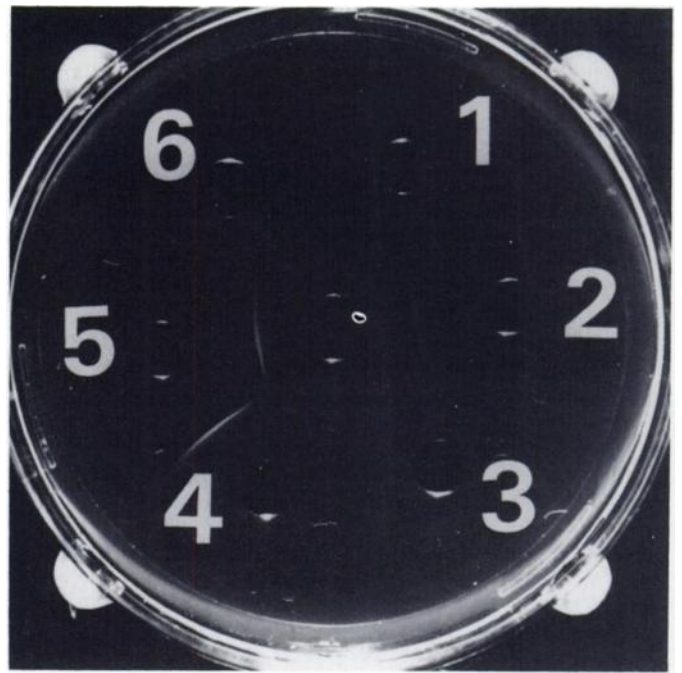


Fig. 1. Double immunodiffusion analysis of the sera of mice immunized with purified rat liver microsomal cytochrome P-450 from rats treated with MC. Wells were filled with 20 μ l of the following samples. Center, MC-induced rat cytochrome P-450 (0.30 mg/ml). Well 1, phenobarbital-induced rat cytochrome P-450 (0.34 mg/ml); Wells 2 and 5, sera from mice immunized with MC-induced cytochrome P-450 for 3 and 4 weeks; Wells 3 and 6, unimmunized mouse serum; Well 4, β -naphthoflavone-induced cytochrome P-450 (0.60 mg/ml).

containing 24 wells, for massive growth. The production of antibody by these clones in the culture fluid was examined by RIA. Of the 66 independent hybrid clones examined, 37 were positive for antibody production as indicated by RIA. The supernatant culture fluid of the control parent myeloma cells and the remaining 29 hybrid cell clones did not contain any mouse immunoglobulin that bound to rat MC-P-450 by RIA. The stability of one of the clones, 1-7-1, was examined by passaging 8 times and recloning twice, followed by additional passaging. Of 175 subclones, 162, or 93%, were positive for binding as measured by RIA. Five of these subclones are shown in Table 1. Thus, 93% of the cells from this clone maintained their monoclonal antibody production during prolonged passaging.

Table 1 shows the data of the RIA for 12 of the clones. These showed radioactivity ranging from about 2 to 7 times that of the control hybrid cells. The cytochrome MC-P-450 antibody-producing hybridomas, 1-2-10p12, 1-7-1p2, 1-8-1p2, 1-25-3p7, 1-28-1p2, 1-31-2p2, 1-32-1p2, 1-36-1p12, 1-43-1p12, 1-56-2p12, 1-56-5p12, and 1-56-6p12 were further grown in the growth and HAT medium described in "Materials and Methods," and the culture fluids were concentrated 25-fold. The concentrates were examined by double immunodiffusion analysis. In no case was more than one detectable precipitin band observed between any of the culture fluids and rabbit anti-mouse immunoglobulin sera, anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA. This result is consistent with the monoclonal nature of these antibodies. Table 1 shows that with 3 of the clones tested, the presence of the detergent Emulgen prevents the formation of the precipitin bands. The precipitation of the MC-P-450 by the monoclonal antibodies was probably

Table 1
 Characteristics of monoclonal antibodies to liver microsomal cytochrome P-450 isolated from rats treated with MC

Culture fluid was used as the source of monoclonal antibodies for the binding to immunoglobulin MC-P-450 complex and immunoglobulin classification. Both the culture fluids and ascites were used for double immunodiffusion analyses.

Source of antibodies	Binding to immunoglobulin MC-P-450 (cpm)	Immunoglobulin subclass	Double immunodiffusion P-450		
			Phenobarbital	MC	β -Naphthoflavone
Normal mouse serum			-	-	-
Anti-MC-P-450 mouse serum			-	+++	+++
Anti-MC-P-450 mouse serum + Emulgen	1			+++	
Myeloma \times unimmunized mouse spleen cell hybrid, NBS 1-3-1p7 ^a	1270	IgG2b	-	-	-
Myeloma \times immunized mouse spleen cell hybrids					
MCP-450 1-2-10p10	4503	IgG1	-	++	++
1-7-1p2	8134	IgG1	-	+++	+++
1-7-1p2 + Emulgen				-	
1-7-1p8c2-43p3	6860				
1-7-1p8c2-48p3	6587				
1-7-1p8c2-73p3	7461				
1-7-1p8c2-105p3	9387				
1-7-1p8c2-160p3	7586				
1-8-1p2	7674	IgG1	-	+++	+++
1-8-1p2 + Emulgen				-	
1-25-3p2	2870	IgG1	-	+	+
1-28-1p2	3430	IgM	-	++	++
1-31-2p2	5609	IgG1	-	+++	+++
1-31-2p2 + Emulgen				-	
1-32-1p2	5803	IgG1	-	+	+
1-36-1p12	3397	IgG2a	-	++	++
1-43-1p12	4346	IgM	-	-	-
1-56-2p12	4816	IgG1	-	+++	+++
1-56-5p12	4700	IgG1	-	+++	+++
1-56-6p12	4639	IgG1	-	+++	+++

^a p, passage number; c, number of times cloned.

due to binding by the antibody coupled with enzyme aggregation, since precipitation did not occur in the presence of detergent. This observation reflects the monoclonality of the antibodies, since the same detergent concentration did not interfere with the double immunodiffusion precipitation of the MC-P-450 by the polyclonal anti-MC-P-450 mouse serum. The precipitin bands between the rabbit anti-mouse immunoglobulin sera and the culture fluids from 3 of the clones are shown in Fig. 2, and the results with all 12 clones are summarized in Table 1. Fig. 2A shows that the parent myeloma cells, RGNS-1p2, did not secrete detectable levels of immunoglobulin and no reaction was observed with any of the anti-IgGs added, *i.e.*, anti-IgG, anti-IgM, or anti-IgA. The hybridoma clones MC-P-450 1-7-1p2 (Fig. 2B) and MC-P-450 1-8-1p2 (Fig. 2C) produced IgG1 subclass immunoglobulins, and MC-P-450 1-28-1p2 produced IgM (Fig. 2D). MC-P-450 1-2-10p10, MC-P-450 1-25-3p2, MC-P-450 1-31-2p2, MC-P-450 1-32-1p2, MC-P-450 1-56-2, MC-P-450 1-56-5, and MC-P-450 1-56-6 also produced IgG1. MC-P-450 1-36-1p12 produced IgG2a and MC-P-450 1-43-1p12 produced IgM (Table 1).

Specificity of Monoclonal Antibodies. Twelve hybrid clones producing high-binding antibodies were selected as independent hybrid clones representing different wells and were grown in the presence of serum, harvested, transferred to serum-free medium, and incubated at 37° for 48 to 72 hr to obtain mouse immunoglobulins free of bovine and horse immunoglobulins usually present in the growth medium. The culture fluids were clarified by centrifugation and were concentrated 25 to 50

times with Amicon filters for double immunodiffusion analyses and enzyme assays. All MC-P-450 hybrid clones produced monoclonal antibodies detected by RIA and identified as mouse immunoglobulins (Table 1; Fig. 2). Upon double immunodiffusion analyses, 3 clones, MC-P-450 1-7-1p2, MC-P-450 1-8-1p2, and MC-P-450 1-31-2p2, showed a precipitin band with MC-P-450 after incubation at room temperature for 3 days. These same clones did not show a precipitin band with MC-P-450 in the presence of detergent. Three other clones, MC-P-450 1-25-3p2, MC-P-450 1-28-1p2, and MC-P-450 1-32-1p2, required 6 days of incubation before a precipitin band was formed with MC-P-450 (Fig. 3B). These monoclonal antibodies also precipitated BNF-P-450 (Fig. 3C), but no precipitation was observed between any of these monoclonal antibodies and PB-P-450 during prolonged incubations (Fig. 3A). Thus, the monoclonal antibodies are directed to common antigenic sites on the MC-P-450 and BNF-P-450, which are lacking in the PB-P-450. The hybrid cells producing the monoclonal antibodies were also grown in mice *i.p.*, and ascites fluids were collected for double immunodiffusion analyses. As shown in Fig. 4, B and C, monoclonal antibodies, MC-P-450 1-7-1p5 and MC-P-450 1-8-1p5, precipitated MC-P-450 and BNF-P-450 but not PB-P-450. These monoclonal antibodies also cross-reacted with rabbit cytochrome P-450_{LM4} but not with rabbit cytochrome P-450_{LM2} or with human liver cytochrome P-450.⁴ The mono-

⁴ S. S. Park, T. Fujino, M. Miller, D. West, F. P. Guengerich, and H. V. Gelboin, manuscript in preparation.

clonal antibodies (IgG2a) produced by the nonspecific hybrid made between myeloma and unimmunized mice spleen cells did not precipitate any of the cytochromes P-450 (Fig. 4A). The double immunodiffusion analyses for other hybrid clones are shown on Table 1. Only MC-P-450 1-43-1p12 failed to show precipitation with MC-P-450. The specificity of the monoclonal antibodies to MC-P-450 was also demonstrated in the binding of the monoclonal antibodies to MC-P-450, as determined by the use of ^{125}I -F(ab')₂ of anti-mouse IgG in a solid-phase RIA. Chart 1 shows that the monoclonal antibodies to

MC-P-450 bind to MC-P-450 and to BNF-P-450 but do not significantly bind to PB-P-450 over a wide concentration range of antibody.

Selective Binding of Monoclonal Antibodies to Microsomal Proteins and Cytochrome P-450. The data shown in Fig. 5 indicate that, when microsomal proteins are resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets, the antibodies produced by clones MC-P-450 1-7-1p5 and MC-P-450 1-31-2p5 recognized only a single band in the resolved microsomal electro-

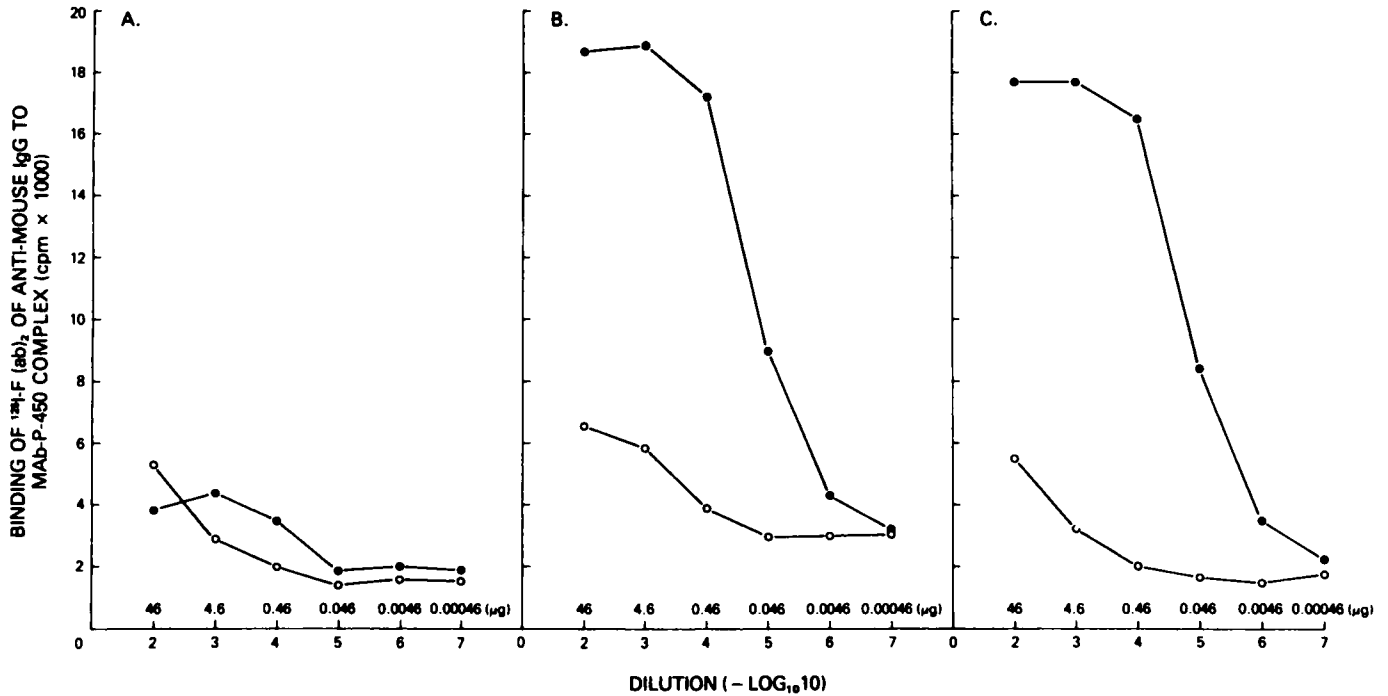


Chart 1. Analysis of the specificity of monoclonal antibodies to MC-induced rat liver cytochrome P-450 by solid-phase RIA. The concentration of monoclonal antibodies (ascites) produced by nonspecific hybridoma, NBS 1-48-5p22 (O, IgG2a producer), and by MC-P-450 1-7-1p5 (●, IgG1 producer), were adjusted to 46 mg/ml and subjected to solid-phase RIA with ^{125}I -F(ab')₂ of anti-mouse IgG (specific activity, 31 $\mu\text{Ci}/\mu\text{g}$). Rat liver microsomal cytochrome P-450 (0.1 mg/ml; 100 μl /well) coated on microtiter wells: A, phenobarbital-induced cytochrome P-450; B, MC-induced cytochrome P-450; and C, β -naphthoflavone-induced cytochrome P-450. See the legend to Fig. 4 for the ascites preparations.

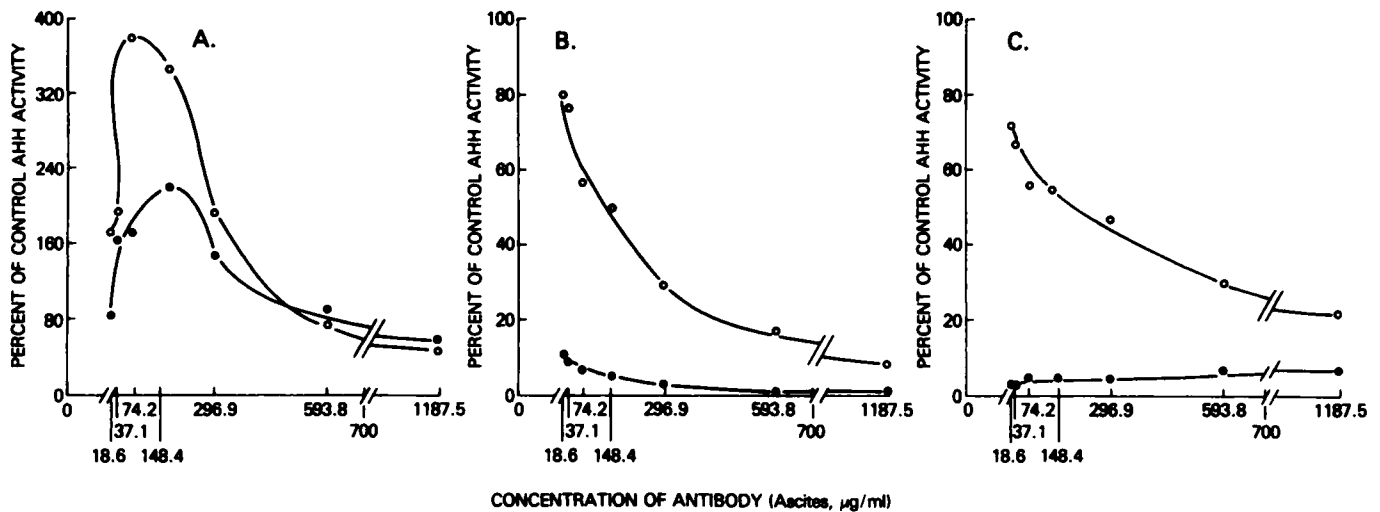


Chart 2. Effect of monoclonal antibodies to MC-induced rat liver microsomal cytochrome P-450 on AHH activity. A 420- μl sample of each concentration of ascites by the nonspecific hybrid, NBS 1-3-1p7 (O, IgG2b producer), or by specific hybrid, MC-P-450 1-7-1p5 (●, IgG1 producer), was incubated with cytochrome P-450 in 80 μl PBS at room temperature for 15 min and assayed for AHH activity in a reconstituted system. A, B, and C, 10.25, 9.25, and 10.32 pmol of cytochrome P-450 from rats treated with phenobarbital, MC, or β -naphthoflavone were used in the AHH assay, respectively. See the legend to Fig. 4 for the ascites preparation.

phoretogram. This band migrated to the same position as highly purified MC-P-450, which was also recognized by the antibodies. No primary interaction between the antibodies and PB-P-450 was observed. The nonspecific monoclonal antibody, NBS 1-48-5p22 did not yield visible bands with any of the microsomal proteins.

Monoclonal Antibody Inhibition of P-450 Enzyme Activity (Purified Cytochromes P-450). We previously observed that the supernatant culture fluid or ascites fluids of myeloma control cells nonspecifically inhibit the AHH activity of the purified rabbit cytochrome P-450_{LM2} in a reconstituted system (31). This nonspecific inhibition was also observed with the ascites fluid from the unimmunized mouse spleen cell hybridoma, NBS 1-3-1p7, which is the nonspecific IgG2b producer. Chart 2 shows this inhibitory effect at high concentrations of proteins (>0.5 mg/ml) of the ascites fluid either from the control unimmunized spleen cell hybridoma or from the antibody-producing hybridomas. At high dilutions and low protein concentrations (<0.3 mg/ml), the control ascites fluid from the unimmunized hybridoma enhanced PB-P-450 AHH activity (Chart 2A). We do not understand the mechanism of this stimulation. Upon dilution of the fluids containing the monoclonal antibodies, the inhibitions were relieved to 80% of control AHH activity (Chart 2, B and C). At the low protein concentrations, the ascites from the specific IgG1 producer, MC-P-450 1-7-1p5, inhibited the AHH activity of MC-P-450 and BNF-P-450 (Chart 2, B and C) by 90 to 97%. The same ascites fluid did not inhibit PB-P-450 AHH activity at any of the concentrations tested (Chart 2A). Table 2 shows the specificity of the monoclonal antibodies produced by hybridomas MC-P-450 1-7-1p5, MC-P-450 1-8-1p5, and MC-P-450 1-31-2p5 for inhibition of AHH and 7-ethoxycoumarin deethylase activity of highly purified cytochromes P-450. Each of the monoclonal antibodies inhibited the AHH activity of MC-P-450 and BNF-P-450 by 90 to 99% at a concentration of 200 µg ascites fluid protein per ml. These also inhibited the 7-ethoxycoumarin deethylase activity of MC-P-450 and much less affected the 7-ethoxycoumarin deethylase activity of PB-P-450. Each of the monoclonal antibodies exhibited only negligible effects on PB-P-450 AHH activity. At high concentrations, the control monoclonal antibodies of NBS 1-3-48p7-22 inhibited the AHH activity of MC-P-450 and BNF-P-450 53 and 48%, respectively. Similar to the results in Chart 2, this inhibition was lost at high dilutions of the nonspecific antibody but was retained with the specific monoclonal antibody. In the separate experiment (Table 2, Experiment A-2), the control monoclonal antibodies of NBS 1-48-5p23 did not affect the AHH activity of MC-P-450 and greatly enhanced PB-450 activity. The inhibition by other monoclonal antibodies on the AHH activities of MC-P-450 varied from 7 to 98%, while the AHH activities of PB-P-450 were enhanced in the presence of monoclonal antibodies. Table 2, Experiment 3, shows the stability of the monoclonal antibodies during prolonged passaging of the hybridoma cells followed by subcloning. Thus, the supernatant fluid from 5 subclones of 1-7-1p12 and the parent population all inhibited MC-P-450 by about 70 to 80%.

Monoclonal Antibody Inhibition of Microsomal AHH and 7-Ethoxycoumarin Deethylase Activity. Table 3 shows the effects of control hybridoma antibody and 3 MC-P-450 monoclonal antibodies on the enzyme activity of liver microsomes from control, phenobarbital-induced, and MC-induced rats. Control (NBS) fluids enhanced the AHH activity and had no

Table 2
Effects of monoclonal antibodies to purified MC-P-450 on AHH and 7-ethoxycoumarin Deethylase of purified cytochrome P-450s from rats treated with MC (MC-P-450), β-naphthoflavone (BNF-P-450), and Phenobarbital (PB-P-450)

Monoclonal antibodies ^a	AHH (pmol 3-OH-BP/nmol P-450/min)		
	MC-P-450	BNF-P-450	PB-P-450
Experiment A-1			
None	2498 (100) ^b	1094 (100)	381 (100)
NBS 1-3-48p7-22 ^c	1177 (47)	570 (52)	401 (106)
MC-P-450 1-7-1p5	97 (4)	44 (4)	318 (84)
MC-P-450 1-8-1p5	256 (10)	100 (9)	317 (83)
MC-P-450 1-31-12p5	116 (5)	10 (1)	360 (95)
Experiment A-2			
None	2866 (100)		94 (100)
NBS 1-48-5p23	3255 (116)		256 (272)
MC-P-450 1-2-10p12	2000 (70)		231 (245)
1-7-1p12	331 (12)		282 (299)
1-8-1p5	282 (10)		176 (186)
1-25-3p10	2348 (82)		222 (235)
1-28-1p10	3056 (107)		304 (322)
1-31-p5	209 (8)		181 (192)
1-32-1p2	2661 (93)		263 (280)
1-36-1p12	2535 (88)		261 (277)
1-43-1p12	2208 (77)		213 (226)
1-56-2p12	1088 (38)		252 (267)
1-56-5p12	889 (31)		187 (198)
1-56-6-12	975 (34)		214 (227)
Experiment 3^d			
None	2792 (100)		
NBS 1-48-5p21	2199 (79)		
MC-P-450 1-7-1p12	658 (24)		
1-7-1p8c2-43p3	613 (22)		
1-7-1p8c2-48p3	786 (28)		
1-7-1p8c2-73p3	912 (33)		
1-7-1p8c2-105p3	644 (23)		
1-7-1p8c2-160p3	623 (22)		
Monoclonal antibodies	Ethoxycoumarin deethylase (pmol 7-hydroxycoumarin/nmol P-450/min)		
	MC-P-450	PB-P-450	
Experiment B			
None	152 (100)	18 (100)	
NBS 1-48-5p23	110 (72)	8 (44)	
MC-P-450 1-7-1p5-12	9 (6)	6 (33)	

^a Different rat liver microsomal cytochrome P-450s (9.3 to 14.9 pmol) were incubated with 200 µg ascites fluid protein in PBS at room temperature for 15 min and assayed for AHH and ethoxycoumarin deethylase activities in the reconstituted systems.

^b Numbers in parentheses, percentage of values with no antibody added. These should also be compared to the NBS controls.

^c p, passage number; c, number of times cloned.

^d This experiment was done with serum-free culture fluids (200 µg protein) as the antibody source.

effect on 7-ethoxycoumarin deethylase activity. All 3 monoclonal antibodies to MC-P-450 inhibited both enzyme activities by about 67 to 76%. In both cases, the amount of antibody used was greatly in excess of saturating levels. Thus, both of these activities are equally susceptible to binding by antibody to a single antigenic site. This may be interpreted to indicate that both activities are functions of a single enzyme. The monoclonal antibody binding would thus be equally effective in blocking both enzyme activities. An alternative hypothesis is that there are different proteins with common antigenic sites responsible for the 2 enzyme activities. In either case, the data suggest that these enzymes are responsible for about 70% of the enzyme activities in liver microsomes from MC-treated rats. With liver microsomes from either control or phenobarbital-treated rats, each of the enzyme activities were stimulated by about 11 to 60% by either the NBS control or MC-P-450 antibodies. Thus, these microsomes do not appear to contain the same antigenic sites present in MC microsomes which,

Table 3

Effect of monoclonal antibodies to liver microsomal cytochrome P-450 of rats treated with MC on AHH and 7-ethoxycoumarin deethylase activities of the microsomes of rats treated with phenobarbital or MC

Different sources of rat microsomes were incubated with 200 µg ascites fluid protein for AHH and 400 µg for 7-ethoxycoumarin deethylase in PBS at room temperature for 15 min and assayed for the enzyme activities using 100 nmol of BP or 500 nmol of 7-ethoxycoumarin as substrate in a final volume of 1 ml at 37° for 10 min.

Source of micro- somes (inducer)	Antibodies	AHH		7-Ethoxycoumarin deethylase	
		pmol 3- OH-BP/ mg/min	% of control	pmol 7- hydroxy- cou- marin/ mg/min	% of control
Experiment 1					
Uninduced	None	262	100	740	100
	NBS 1-48-5p22 ^a	367	140	815	111
	MC-P-450 1-7-1p5	384	147	953	129
	MC-P-450 1-8-1p5	365	139	984	133
	MC-P-450 1-31-2p5	394	151	953	115
Phenobarbital	None	384	100	961	100
	NBS 1-48-5p22	600	156	1266	132
	MC-P-450 1-7-1p5	615	160	1164	121
	MC-P-450 1-8-1p5	590	153	1156	120
	MC-P-450 1-31-2p5	593	154	1188	124
MC	None	858	100	5359	100
	NBS 1-48-5p22	1170	136	5280	98
	MC-P-450 1-7-1p5	287	33	1556	29
	MC-P-450 1-8-1p5	208	24	1634	30
	MC-P-450 1-31-2p5	270	31	1496	28
Experiment 2					
MC	None	6342	100		
	NBS 1-48-5p21	10120	160		
	MC-P-450 1-7-1p12	1399	22		
	1-7-1p8c2-43p3	1190	19		
	1-7-1p8c2-48p3	1295	20		
	1-7-1p8c2-73p3	1064	17		
	1-7-1p8c2-105p3	1372	22		
	1-7-1p8c2-160p3	1386	22		

^a p, passage number; c, number of times cloned.

Table 4

Effect of monoclonal antibodies to liver microsomal cytochrome P-450 of rats treated with MC on BP metabolism by MC-induced cytochrome P-450

MC-induced rat cytochrome P-450 (18.5 pmol) was incubated with 296.9 µg monoclonal antibody (NBS 1-48-5p22, nonspecific, or MC-P-450 1-7-1p5, specific to MC-P-450) in 0.5 ml PBS at room temperature for 15 min and transferred to a reaction mixture for AHH assay or HPLC in final volume of 1 ml. Twenty-four nmol [7,10-¹⁴C]BP was used as substrate for HPLC.

BP metabolites	Specific activity (pmol/nmol P-450/min)				
	MC-P-450 (control)	MC-P-450 + NBS 1- 48-5p22 ^a	% of control	MC-P- 450 + MC-P- 450 1-7- 1p5	% of control
Unknown	480	219	45	<1	0
9,10-Diol	267	147	55	<1	0
4,5-Diol	443	93	58	<1	0
7,8-Diol		165			
9-OH-BP	617	394	63	15	2
7-OH-BP	350	209	59	<1	0
1-OH-BP	404	267	66	16	4
3-OH-BP	1161	779	67	42	4
6,12-Quinone	60	22	38	<1	0
Total	4720	3226	68	124	4

^a p, passage number.

when antibody bound, result in enzyme inhibition. Table 3 shows the inhibitory effect of 1-7-1p12 and 5 of its subclones. Monoclonal antibodies from all 5 subclones and the parent

clone inhibited the AHH of a high titer MC-induced microsome by about 80%. These results demonstrate the stability of the hybridoma clone for AHH inhibition of monoclonal antibody production.

HPLC Analyses of BP Metabolism by a Purified Cytochrome P-450 System and Microsomes (Inhibition by Monoclonal Antibodies). Table 4 shows the distribution of metabolites formed from BP by a reconstituted mixed-function oxidase system containing MC-P-450 and NADPH cytochrome P-450 reductase. Four phenols formed are the 1-OH-BP, 3-OH-BP, 7-OH-BP, and 9-OH-BP; one quinone, the 6,12-quinone; 3 dihydrodiols, the 4,5-diol, 7,8-diol, and 9,10-diol; and an unknown metabolite. The diols are formed from epoxide intermediates through the action of epoxide hydrolase, which is present in small amounts in the cytochrome P-450 preparation. The addition of control NBS 1-48-5p22 ascites fluid causes a nonspecific inhibition of individual metabolite formation, ranging from about 32 to 62%. The monoclonal antibody MC-P-450 1-7-1p5 at protein concentration identical to that of control antibody causes a large inhibition of the formation of all metabolites. In the presence of the monoclonal antibody, the unknown metabolite, the 3 dihydrodiols, the 7-OH-BP, and the 6,12-quinone are all not detectable, and the formation of the remaining metabolites is inhibited by more than 96%. The monoclonal antibody thus binds to a site required for the formation of each of the metabolites, and no metabolites are formed when this antigenic determinant is bound by antibody.

Table 5

Effect of monoclonal antibodies to liver microsomal cytochrome P-450 of rats treated with MC on BP metabolism by MC-induced microsomes

MC-induced rat microsomes (50 µg) were incubated with 200 µg monoclonal antibodies (NBS 1-48-5p22, nonspecific, or MC-P-450 1-7-1p5, specific to MC-P-450) in 0.5 ml at room temperature for 15 min and transferred to a reaction mixture for AHH assay or HPLC in a final volume of 1 ml. Fifty nmol [$^7,10\text{-}^{14}\text{C}$]BP were used as a substrate in the assays involving HPLC.

BP metabolites	Specific activity (pmol/mg protein/min)				
	MC micro- somes (control)	MC micro- somes + NBS 1-48- 5p22 ^a	% of control	MC micro- somes + MC P-450 1-7-1p5	% of control
9,10-Diol	4,788	6,348	132	2,600	54
4,5-Diol	1,995	2,390	119	648	32
7,8-Diol	2,936	3,474	118	1,372	46
Fraction 1	133	140		88	
9-OH-BP	1,653	1,437	86	468	28
7-OH-BP	2,798	347	12	1,004	35
1-OH-BP	1,548	440	28	<1	0
3-OH-BP	12,546	13,690	109	5,242	41
1,6-Quinone	134	489	365	385	288
3,6-Quinone	60	50	84	18	31
6,12-Quinone	79	147	186	3	4
Total	28,745	33,112	115	11,831	41

^a p, passage number.

Thus, it appears that each of the metabolites formed is produced at the same active site on the enzyme. HPLC analysis of the metabolism of BP with microsomes from MC-induced rats is shown in Table 5. The metabolite peaks formed are qualitatively similar to those formed with the purified reconstituted system, except that several additional peaks appear. These are: Fraction 1, believed to be a mixture of the 4-OH and 4,5-epoxide, and the additional quinones, 1,6-quinone and 3,6-quinone. Nonspecific NBS 1-48-5p22 antibody enhances the formation of all the metabolites except the 1-OH-BP and 7-OH-BP, the formation of which is inhibited. The monoclonal antibody MC-P-450 1-7-1p5 causes inhibition of total metabolite formation by about 59%, an amount similar to the inhibition of AHH activity. The inhibition of formation of each metabolite ranges from about 46 to 72%, except for the 1-OH-BP and 6,12-quinone, which appear to be inhibited to a much greater extent. These metabolites, however, are separated with difficulty, and it is possible that they are contaminants of the adjacent peaks in these preparations. There are some differences in the relative inhibition of various metabolite formation, which may reflect the relative affinity of the antibody for different minor forms of the cytochrome P-450 that may be present in the microsomes. Specific monoclonal antibodies inhibited 59% of the total metabolites in the mixed-function oxidase system containing MC-induced microsomes. A most interesting finding is the lack of inhibition, in fact the stimulation, of 1,6-quinone formation in the presence of the MC-P-450 monoclonal antibody. This suggests that oxidation at position 6 from which the quinone may be derived is either catalyzed by a different, a non-P-450 mechanism, or subsequent to primary oxidation at other positions of the BP.

DISCUSSION

The cytochromes P-450 contained in the mixed-function oxidases are the primary enzymes responsible for the metab-

olism of many drugs, carcinogens, and xenobiotics, as well as some endogenous steroids (2, 8, 10). In this role, they are responsible for both the detoxification of those compounds and their activation to toxic, mutagenic, or carcinogenic forms. Metabolic pathway choice may reflect the distribution of the different forms of the cytochrome P-450 in a given tissue, organ, species, or individual, since the various cytochromes P-450 display stereoselectivity for both substrate and product formation (5, 27). Furthermore, exposure of the organism to specific inducers, which may be of environmental or dietary origin, may change dramatically the content and distribution of the cytochromes P-450. Thus, specific forms of the enzyme can be induced by various classes of inducers in a variety of tissues of the whole animal or in cell culture. Thus, understanding the genetic regulation and distribution of these enzymes may give us information useful for determining individual responses to drugs and environmental pollutants and carcinogen susceptibility (8, 9, 23).

Ordinary polyclonal rabbit-produced antibodies have been very useful in studying cytochrome P-450 substrate specificity, homogeneity, multiplicity, and species cross-reactivity (4, 20, 30, 34-37, 40). These studies have been nicely reviewed by Lu and West (27). The polyclonality of rabbit antibodies, even to absolutely pure antigens, represents antibody heterogeneity, in that each contributing antibody in the preparation may be directed to different antigenic sites. This also contributes to variation in different antibody preparations. They are thus limited in their usefulness for precise quantitative as well as some qualitative studies. Their polyclonality and thus variation in different preparations may be the cause of the discrepancy between 2 laboratories in their estimate of the content of phenobarbital- and MC-inducible forms in rat liver (30, 36, 37). Monoclonal antibodies are absolutely pure and directed to only a single antigenic site (43). These features, and the potential immortality of their hybridoma cell source, make them reagents *par excellence* and extraordinarily superior to ordinary polyclonal antibodies (43) for many purposes. Monoclonal antibodies absolutely specific to different antigenic sites on the P-450 enzymes may be very useful tools for determining the genetic composition of individuals and the role of the enzyme in specific drug and carcinogen oxidations. The present study shows that the monoclonal antibodies can distinguish different forms of rat liver microsomal cytochrome P-450. The MC-P-450 monoclonal antibodies bind, precipitate, and inhibit both the MC-P-450 and BNF-P-450 enzyme activity but not that of PB-P-450. Thus, the results suggest that the P-450 induced by MC and the P-450 induced by β -naphthoflavone are identical, at least with respect to the antigenic determinants governing monoclonal antibody recognition. The PB-P-450 lacks this antigenic determinant. The monoclonal antibodies to MC-induced cytochrome P-450 inhibited the hydroxylation of BP as well as deethylation of 7-ethoxycoumarin. The parallel inhibitory activity toward both enzymatic activities suggests that there is a common antigenic site necessary for both the hydroxylation of BP and the deethylation of 7-ethoxycoumarin, and these enzymatic activities are functions of the same enzyme protein or enzyme proteins with a common antigenic determinant. In the study of BP metabolism with HPLC, we observed that addition of monoclonal antibodies to the reconstituted cytochrome P-450 system inhibited the formation of metabolites almost completely. Thus, all sites of BP oxidation

are completely blocked by the antibody interaction with the MC-P-450. With liver microsomes, however, the monoclonal antibody inhibition studies suggest that one metabolite, the 1,6-quinone, is derived by a mechanism different than that for the other metabolites of BP. The presence of multiple forms of cytochrome P-450 has been reported from several laboratories (3, 15, 20, 27, 32). Addition of the monoclonal antibody to microsomes from MC-treated rats blocked both AHH and 7-ethoxycoumarin deethylase activity of microsomes from MC-treated rats by almost 70% and BP metabolism measured with HPLC by about 60%. These monoclonal antibodies had no inhibitory effect on any of the above activities in microsomes from either untreated rats or those pretreated with phenobarbital. Thus, the enzyme containing the antibody-binding antigenic site, *i.e.*, the MC-P-450, is responsible for at least 70% of total AHH and 7-ethoxycoumarin deethylase enzyme activity in microsomes from MC-pretreated rats. The same enzyme form is not present in significant amounts in liver microsomes from either control rats or phenobarbital-treated rats. Analysis of BP metabolism in microsomes shows that there is some redistribution of metabolite ratio in the presence of the monoclonal antibodies to MC-P-450 which may reflect a redistribution of active cytochrome P-450 forms. Among 12 hybridomas forming monoclonal antibodies to MC-P-450, 9 were of the IgG1 type, one was of the IgG2a type, and 2 of the 12 were of the IgM types. The differences in inhibitory effect on mixed-function oxidase activities between the monomeric and pentameric form of immunoglobulins are yet to be studied. The monoclonal antibodies should be useful in a variety of ways for examining binding sites and determining the multiplicity of forms in different tissues. They will also facilitate determining the tissue, organ, species, and individual differences in cytochromes P-450 content. There are several reports of individual differences in humans in the inducibility and activity of AHH (9, 22, 23, 29). The monoclonal antibodies should prove useful in clarifying the genetic basis for these differences and help define biochemical individuality in cytochrome P-450 type.

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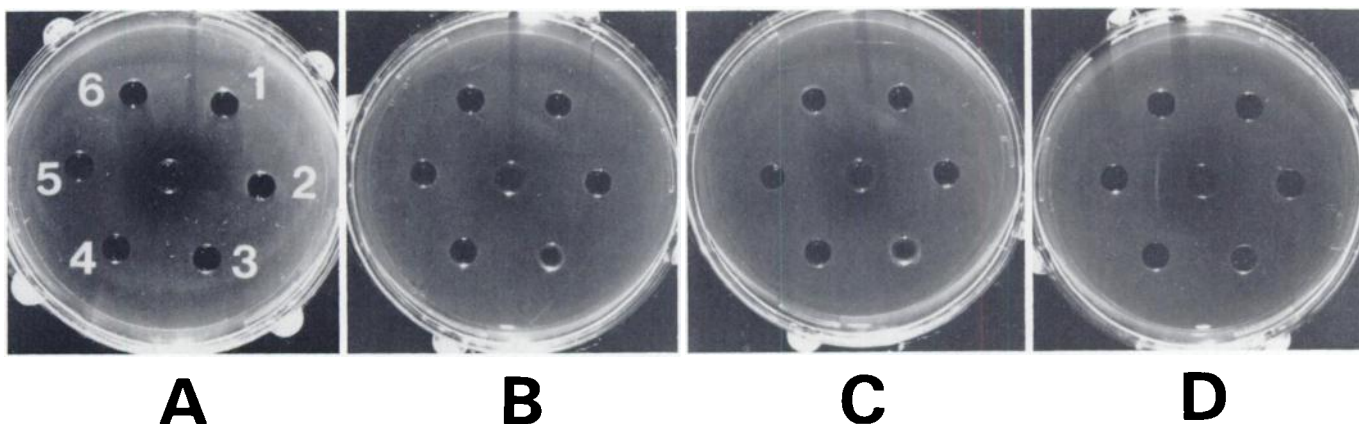


Fig. 2. Double immunodiffusion analysis of immunoglobulin secretion and monoclonal antibodies to purified MC-induced rat cytochrome P-450. Center wells of A, B, C, and D were filled with 20 μ l of culture fluids (25 times concentrated) from parent myeloma RGNS-1-p73 (nonproducer), hybridomas, MC-P-450 1-7-1p2, MC-P-450 1-8-1p2, and MC-P-450 1-28-1p2. Outer wells of 1 to 6 were filled with 20 μ l serum of rabbit anti-mouse IgG1, rabbit anti-mouse IgG2a, rabbit anti-mouse IgG2b, rabbit anti-mouse IgG3, goat anti-mouse IgM, or rabbit anti-mouse IgA, respectively.

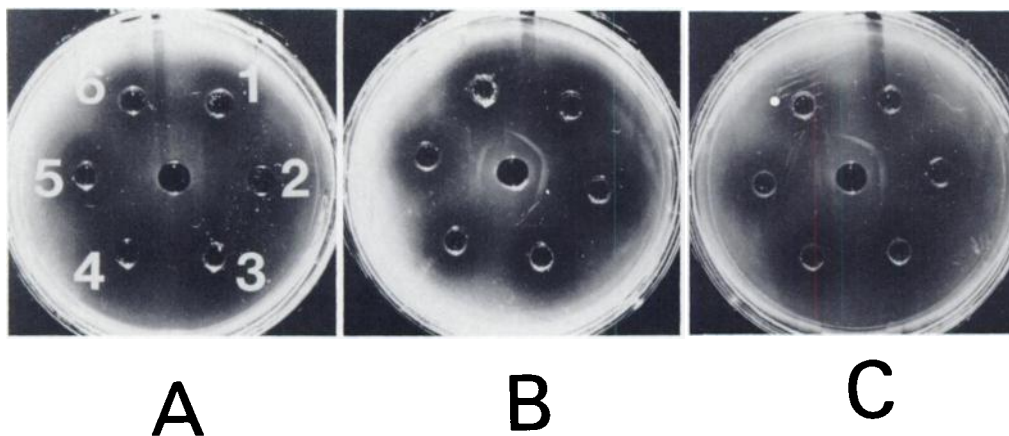


Fig. 3. Double immunodiffusion analysis of monoclonal antibodies in culture fluids (25 times concentrated) produced by hybridomas made between myeloma and spleen cells immunized with MC-induced rat liver cytochrome P-450. Center, A. Phenobarbital-induced rat liver cytochrome P-450 (0.34 mg/ml). Outer wells of 1 to 6, monoclonal antibodies produced by hybridomas MC-P-450 1-7-1p2, MC-P-450 1-8-1p2, MC-P-450 1-28-1p2, MC-P-450 1-25-3p2, MC-P-450 1-31-2p2, and MC-P-450 1-32-1p2. Center, B. MC-induced rat liver cytochrome P-450 (0.3 mg/ml). Center, C. β -naphthoflavone-induced rat liver cytochrome P-450 (0.3 mg/ml). Wells 1 to 6, same as A. Wells were filled with 20 μ l of above samples, and plates were incubated at room temperature for 3 days.

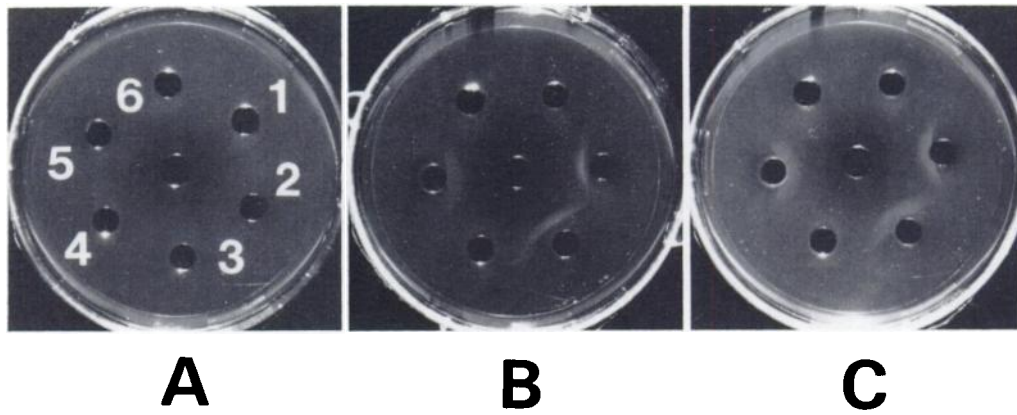


Fig. 4. Double immunodiffusion analysis of cross-reactivity of MC-P-450 monoclonal antibodies from ascites fluids with rat, rabbit, and human cytochromes P-450. Cells (5×10^8) of nonspecific hybridoma made between myeloma and unimmunized mouse spleen cells, and specific hybridomas made between myeloma and spleen cells of mice immunized with MC-induced rat cytochrome P-450, were grown in BALB/c mice i.p., and ascites were collected as described in "Materials and Methods." Center, A. monoclonal antibody produced by nonspecific hybridoma, NBS 1-48-5p23 (IgG2a producer). Well 1, phenobarbital-induced rat liver cytochrome P-450 (0.3 mg/ml); well 2, MC-induced rat liver cytochrome P-450 (0.34 mg/ml); well 3, β -naphthoflavone-induced rat liver cytochrome P-450 (0.30 mg/ml); wells 4 and 5, phenobarbital-induced rabbit liver cytochrome P-450_{LM2} (0.32 mg/ml) and P-450_{LM4} (0.34 mg/ml); well 6, human liver cytochrome P-450 (0.26 mg/ml) (39). Centers, B and C. monoclonal antibodies to MC-induced rat liver cytochrome P-450 produced by hybridomas (IgG1 producers), MC-P-450 1-7-1p5 and MC-P-450 1-8-1p5. Wells 1 to 6, same as A. Wells were filled with 20 μ l of the above samples.

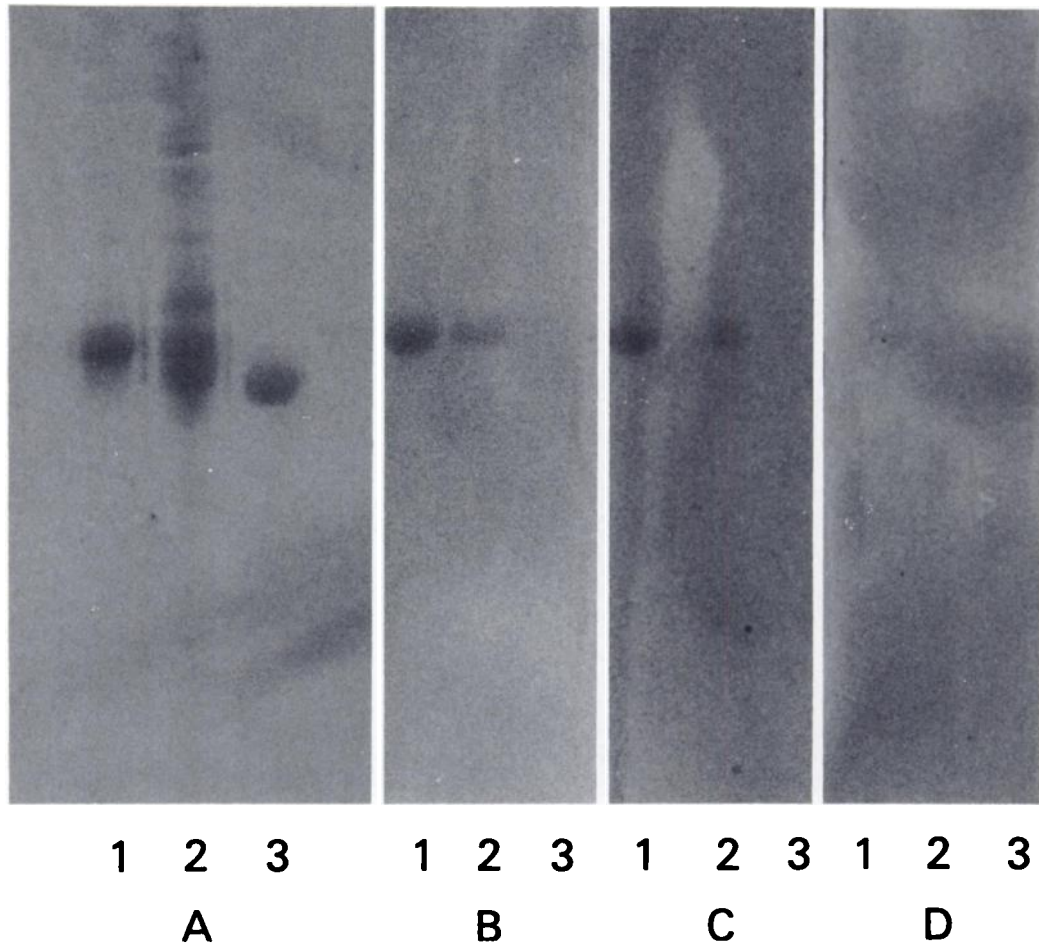


Fig. 5. Electrophoresis of rat liver cytochrome P-450 and microsomes and visualization of resolved bands with antibodies. In each section, Well 1 contained 2 μ g of purified MC-induced cytochrome; Well 2 contained 10 μ g of MC-induced rat liver microsomes; and Well 3 contained 2 μ g of purified phenobarbital-induced cytochrome P-450. Electrophoresis, transfer to nitrocellulose, and staining were carried out as described under "Materials and Methods." Gel A was stained with Amido black; Gels B and C were treated with hybridoma ascites fluids plus the appropriate antibodies and related reagents; Gel B was treated with monoclonal antibodies of clone MC-P-450 1-31-2p5; Gel C was treated with monoclonal antibodies of clone MC-P-450 1-7-1p5; and Gel D was treated with control antibodies (NBS 1-48-5p22).